Indonesian J. Pharm. Vol. 27 No. 1 : 53 – 58
ISSN-p : 2338-9427
DOI: 10.14499/indonesianjpharm27iss1pp53

Short Communication

STRUCTURE–ANTIMICROBIAL PROPERTIES STUDY OF SOME DIBASIC PHENYLCARBAMIC ACID ESTERS

Jozef Csöllei[1], Ivan Malík[2*], Marián Bukovský[3]

[1]Dept. of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic
[2]Dept. of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava, Slovak Republic
[3]Dept. of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Comenius University in Bratislava, Slovak Republic

Submitted: 13-10-2015
Revised: 01-11-2015
Accepted: 22-12-2015

*Corresponding author
Ivan Malík

Email:
malikivan001@gmail.com

ABSTRACT

Due to worldwide phenomenon of microbial resistance to commonly used therapeutic agents, antibiotics and antifungals, dibasic di-/trimethylphenylcarbamic acid esters **1**–**3**, a non--traditional series of potential antimicrobials, has been *in vitro* evaluated against chosen gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacterial strains as well as against yeast (*C. albicans*) by the minimum inhibitory concentration (MIC) assay. Taking into consideration chemical structure of tested derivatives, the incorporation of more than one protonated atom of nitrogen into salt forming fragment positively influenced the activity against *E. coli*. On the contrary, the presence of one or more methyl groups instead of 3-alkoxy side chain attached to lipophilic aromatic moiety has not found to be favorable structural feature. In entire set of inspected compounds, the most promising results have been found for the compound **3**, chemically 1-[3-piperidinium-1-yl-2-({[(2,4,6-trimethylphenyl)amino]carbonyl}oxy)propyl]piperidinium dichloride, against *E. coli* with the MIC=1.56mg/mL.

Key words: Dibasic phenylcarbamic acid esters, *Escherichia coli*, hydrogen bonding, lipophilicity

INTRODUCTION

The widespread use of antibiotics has resulted in the emergence of multidrug-resistant bacterial pathogens. The prevalence and rate of antimicrobial resistance among important gram-positive and gram-negative pathogens has been progressive and relentless. Ten years ago, concern centred on gram-positive bacteria, particularly methicillin (oxacillin)--resistant *Staphylococcus aureus* and vancomycin--resistant *Enterococcus* spp. Now, however, clinical microbiologists increasingly agree that multidrug-resistant gram-negative bacteria pose the greatest risk to public health. Classic agents used to treat these pathogens have become outdated. Of the few new drugs available, many have already become targets for bacterial mechanisms of resistance (Kanj and Kanafani, 2011; Li *et al*., 2015; Moran *et al*., 2006; Rodvold and McConeghy, 2014).

It has been reported that the use of local anaesthesia may interfere with microbial assessment of tissue samples or culture specimens taken from patients with local infection because of the anaesthetics´ antimicrobial action. Local anaesthetics (LAs) have been known to bind specifically to the cell membranes of bacteria and to alter their structure and fluidity, to disrupt membrane potential and to inhibit membrane-bound respiratory function (Collura and Letellier, 1990). Lidocaine (lidocainium chloride; Figure 1) is the most studied active agent which has been included in this pharmacotherapeutic class (Begec *et al*., 2007; Gocmen *et al*., 2008; Lu *et al*., 2014). In historical terms, there have been three definitive papers describing, in turn, the chemistry, pharmacology and clinical features of lidocaine – research of Löfgren and Lundquist (1946), Goldberg (1949) and Gordh (1949).

The compounds under current study **1**–**3** have shown structural similarity to the molecule of lidocaine – a typical structural arrangement for LAs. Particularly, the substances **1**–**3** have contained (i) lipophilic alkyl substituted aromatic ring, polar carbamate moiety attached to (ii) branched hydrocarbon connecting chain

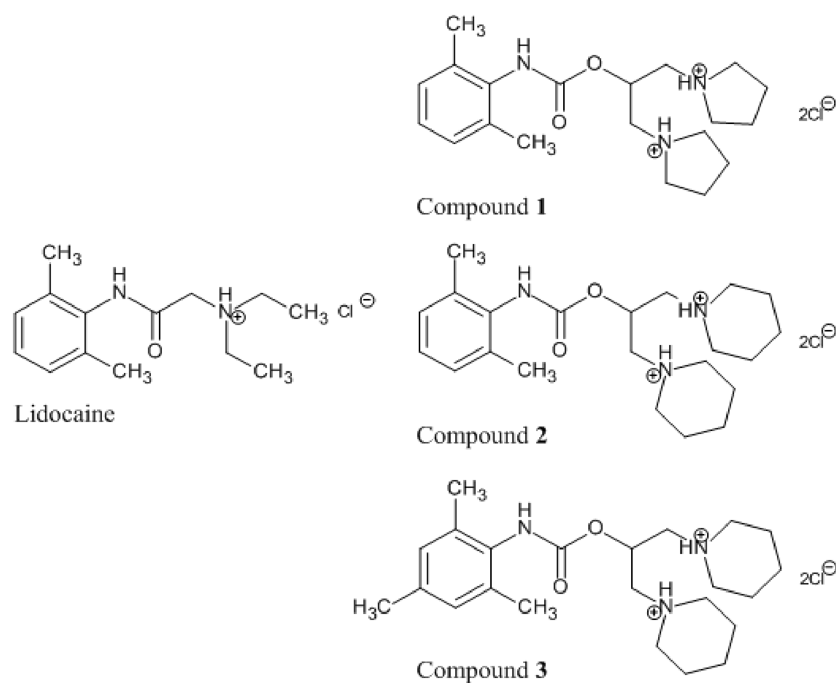

Figure 1. Chemical structure of lidocaine (lidocainium chloride) and dibasic esters of phenylcarbamic acid under the study

and (iii) salt forming fragment containing nitrogen(s) as well, as can be seen in figure 1.

The goal of current research was to *in vitro* investigate the efficiency of given alkylphenylcarbamic acid esters against gram-positive bacteria *Staphylococcus aureus* ATCC 6538 (*Micrococcaceae*), gram-negative bacteria *Escherichia coli* CNCTC 377/79 (*Enterobacteriaceae*) and yeast *Candida albicans* CCM 8186 as well and try to identify some structural features which could be beneficial in terms of their potency.

MATERIALS AND METHODS

The compounds under the study

The evaluated compounds **1**–**3** (Figure 1), chemically 1-[3-pyrrolidinium-1-yl-2-({[(2,6-dimethylphenyl)amino]carbonyl}oxy) propyl]-pyrrolidinium dichloride (**1**), 1-[3-piperidinium-1-yl-2-({[(2,6-dimethylphenyl)amino]carbonyl}oxy)propyl]piperidinium dichloride (**2**) and 1-[3-piperidinium-1-yl-2-({[(2,4,6-trimethyl-phenyl)amino]carbonyl}oxy)propyl]piperidinium dichloride (**3**), were purchased from Department of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic.

The *in vitro* antimicrobial activity assay

Microorganisms

Antimicrobial properties of tested molecules **1**–**3** were investigated against gram-positive bacteria *S. aureus* ATCC 6538 (*Micrococcaceae*), gram-negative bacteria *E. coli* CNCTC 377/79 (*Enterobacteriaceae*) and yeast *C. albicans* CCM 8186 as well. The tested bacterial strains were purchased from American Type Culture Collection (Manassas, United States of America) and Czech National Collection of Type Cultures (Prague, Czech Republic); yeast was obtained from Czech Collection of Microorganisms (Brno, Czech Republic).

Culture media

For a cultivation of the microorganisms, listed in the previous section of this paper, a blood agar, Endo agar and Sabouraud's agar (Imuna, Šarišské Michaľany, Slovak Republic) were used. Blood agar was prepared by adding 10% of defibrine sheep's blood to melted and cooled (50°C) competent components.

Determination of minimum inhibitory concentration (MIC)

The MIC readouts of the moleculs **1**–**3** were carried out by following the procedure previously published in literature (Csöllei *et al.*, 2014).

The respective test compounds were dissolved in distilled water. Standard suspension of bacteria was prepared from their 24h cultures which were cultivated on a blood agar (gram-positive bacteria) and endo agar (gram-negative bacteria). Standard suspension of *Candida* was prepared from its 48h cultures cultivated on Sabouraud's agar.

Prepared suspension contained the concentration of 5×10^7 colony forming units (CFU) *per* mL of bacteria and 5×10^5CFU/mL of *Candida*, respectively. The UV/VIS spectrophotometry was used for the determination of the microorganisms concentration. For the measurements, the UV/VIS range spectrophotometer Jenway, model 6305 (United Kingdom) was carried out. All evaluated suspensions were adjusted to the absorbance value of 0.35 at the wavelength of 540nm.

The suspension of the microorganisms was added in the amount of 5μL into the solutions of inspected compounds (100μL) and to double concentrated peptone broth medium (8%) for bacteria or to Sabouraud's medium (12%) for *Candida*. The peptone broth and Sabouraud's media were purchased from Imuna (Šarišské Michaľany, Slovak Republic).

Starting concentration of prepared stock solutions was 50.00mg of respective compound *per* mL of distilled water. These stock solutions (5%) were then serially diluted by a half and final concentrations were 25.00, 12.50, 6.25, 3.13, 1.56, and 0.78mg/mL, respectively.

Quantitative screening

The quantitative screening was performed using sterile 96-well plastic microtiter plates (with round-bottomed wells) with matching covers. Microorganisms were incubated in each well at 37°C for 24h. Upon completion of this process, the volume of 5μL of evaluated suspension has been taken from each well by using transferring tool and cultured on a blood agar (*S. aureus*), Endo agar (*E. coli*) or on Sabouraud's agar (*C. albicans*), respectively. Petri dishes were then incubated for 24h at 37°C.

The positive control using only an inoculation of microorganisms and the negative control of solvent were realized parallelly. The nutrient concentration remained stable in each well, only the concentration of inhibitory compound changed. All experiments were performed in triplicate.

The MIC was regarded as the lowest concentration of antimicrobial agent required to inhibit the visible growth of microorganism after incubation (Andrews, 2001). The MIC was dependent on the presence/absence of the culture on used solid media after the transfer of 5μL of suspension from each well. Estimated MIC readout was dependent on the presence/absence of the culture on used solid media after the transfer of 5μL of suspension from each well. The MICs are reported in Table I in mg/mL units.

RESULTS AND DISCUSSION

Following chemical structure of currently *in vitro* screened compounds **1**–**3**, their antimicrobial activity has been dependent on: (i) type, number and position of the substituents attached to phenyl ring in lipophilic part, (ii) type and number of protonatable basic centres in salt forming moiety. Possible impacts of given structural aspects were discussed in next sections of the paper.

Current preliminary results have outlined that all the investigated compounds have shown relatively improved activity against gram-negative bacteria when compared to their potency against the gram-positive one or anticandidacidal efficiency, as can be seen in table. I

Previous research of Csöllei *et al.* (2014) has indicated that incorporation of 2-alkoxyphenyl moiety (where alkoxy=butoxy to hexyloxy) instead of current 2,6-dimethyl- or 2,4,6-trimethylphenyl ones has led to inconclusive effect of thus molecules against given microorganisms. On the contrary, the substitution by alkoxy side chain at 3-position has meant notable increasing in the potency of such compounds. The authors have also proposed their possible mechanisms of action.

Table I. The *in vitro* efficiency of inspected compounds **1**–**3** against selected microorganisms

Entry	MIC (mg/mL)		
	S. aureus	***E. coli***	***C. albicans***
1	25.00	25.00	25.00
2	25.00	3.13	25.00
3	25.00	1.56	12.50

The electronic nature, steric hindrance and lipohydrophilic properties have been believed to be important, in order to explain the function of substituents in position 2 and 6 or 2, 4 and 6, respectively (Figure 1). It has been reported that lidocaine has rapidly inhibited the generation of membrane potential in the inverted membrane vesicles of bacteria. It has been suggested that inhibition of membrane potential might contribute to the killing of bacteria, and that outer membrane changes might facilitate access to the inner membrane as a major target of killing (Garlid and Nakashima, 1983; Ohsuka *et al.*, 1994).

Following chemical structures of the molecules under current study, it could be supposed that practically free rotation around the C_{arom}–N bond (Figure 1) could be possible, as reported in previous research (Remko, 1989) which has dealt *ab initio* and quantum-chemical PCILO investigations of tocainide, a structurally very similar molecule due to the presence of 2,6-dimethylphenylaminocarbonyl group. Experimental X-ray values for the torsion angle *a* in the salts of lidocaine (Hanson and Röhrl, 1972; Yoo *et al.*, 1975) have been in the range of 64–71.5°. It could be also possible that, while keeping 2,6-dimethylphenyl moiety, relative position of nitrogen atoms of salt forming groups and „amidic" proton have been different (perhaps not quite optimal) compared to their arrangement in the molecule of lidocaine (Hanson and Banner, 1974).

Furthermore, in the structure of currently tested compounds **1**–**3**, the presence of more polar carbamoyloxy bond instead of the „classical" anilide one has influenced possible linkage of the molecules into chains through N–H · · · O bonding (Gowda *et al.,* 2007). For comprehensiveness, no interaction between methyl groups and NH-moiety has been observed (Gowda *et al.*, 2007; Gowda *et al.*, 2008).

It could be assumed that isosteric replacement of anilide fragment, the presence of two centres of protonation – pyrrolidinium or piperidinium moieties simultaneously with the absence of highly lipophilic alkoxy group attached to phenyl ring, has led to more complicated internalization of inspected derivatives into bacterial outer membrane. Given factors have resulted in relatively higher values of MIC, as can be seen in Table I.

It was stated that the molecules **1**–**3** have been regarded as relatively promising against gram-negative *E. coli.* It could be supposed that pyrrolidinium or piperidinium groups would be the most eligible proton donors in specific (drug–receptor) interactions between exposed phosphoryl and carboxyl groups of highly negatively charged outer face of bacterial outer membrane (Csöllei *et al.*, 2014). Due to the negative charge of phosphate or carboxylate, the hydrogen bond would be fairly strong. That bond type could potentially lead to conformational changes within membrane. It was also interesting taking into account the conformation in which hydrogen atom of the charged „amine" group has been to main backbone of the molecule. For instance, the activity of lidocaine-type molecules, which have contained saturated ring with amine N atom and substitution of a C_{α} atom, have been dependent on thus position: *trans* position to the main backbone has been responsible for antiarrhythmic activity, while *gauche* conformation has promoted local anaesthetic action (Główka and Olczak, 1999; Główka *et al.*, 2005).

Current results have revealed that increasing in the lipophilicity due to incorporation of six-membered piperidinium cycles (compounds **2** and **3**) instead of the five-membered pyrrolidinium ones (**1**) has meant slight improvement in the activity (Table I).

CONCLUSION

Assuming a maintenance of fundamental structural motif of dibasic phenylcarbamic acid esters, for notable efficacy of these compounds against gram-positive and gram-negative bacteria, the presence of lipophilic alkoxy side chain, which has contained more than four carbon atoms, have been regarded as essential. Furthermore, given fragment should be attached to 3-position of phenyl ring. The replacement of thus chain for one or more methyl groups has led to pronounced decrease in the activity, despite structural similarity of these molecules with antimicrobially efficient lidocaine. The conformation in which hydrogen atom of the charged amine group has been to the main backbone could be considered essential.

ACKNOWLEDGEMENTS

The authors dearly thank the anonymous reviewers for their valuable comments and helpful revision suggestions.

REFERENCES

Andrews JM., 2001. Determination of minimum inhibitory concentration. *J. Antimicrob. Chemother.* 48 (Suppl. 1): 5-16.

Begec Z., Gulhas N., Toprak HI., Yetkin G., Kuzucu C., Ersoy, MO., 2007. Comparison of the antibacterial activity of lidocaine 1% *versus* alkalinized lidocaine *in vitro*. *Curr. Ther. Res.* 68: 242-248.

Collura V., Letellier L., 1990. Mechanism of penetration and action of local anesthetics in *Escherichia coli* cells. *Biochim. Biophys. Acta* 1027: 238-244.

Csöllei J., Malík I., Bukovský M., Sedlárová E., 2014. Dibasic esters of *ortho-/meta-*-alkoxyphenylcarbamic acid containing 1-dipropylamino-3-piperidinopropan-1--yl and their antimicrobial activity. *Pol. J. Microbiol.* 63: 231-236.

Garlid KD., Nakashima RA., 1983. Studies of the mechanism of uncoupling by amine local anesthetics. *J. Biol. Chem.* 258: 7974-7980.

Główka ML., Olczak A., 1999. Geometry of lidocaine-like molecules: 2. Crystal structures of 2-benzyl-2-(1-piperidinyl)--*N*-(2,6-dimethylphenyl) acetamide and its hydrochloride hemihydrate. *J. Chem. Crystallogr.* 29: 695-700.

Główka ML., Olczak A., Białasiewicz W., Kwapiszewski W., 2005. Geometry of lidocaine-like molecules: 3. Structure of a potent antiarrhythmic agent: 2-Methyl-2--(1-morpholinyl)-*N*-(2,6-dimethylphenyl) acetamide. *Pol. J. Chem.* 79: 1079-1085.

Gocmen JS., Buyukkocak U., Caglayan O., Aksoy, A., 2008. *In vitro* antibacterial effects of topical local anesthetics. *J. Dermatol. Treat.* 19: 351-353.

Goldberg L., 1949. Studies on local anaesthetics. Pharmacological properties of homologues and isomers of xylocain (alkyl aminoacyl derivatives). *Acta Physiol. Scand.* 18: 1-18.

Gordh T., 1949. Xylocain – a new local analgesic. *Anaesthesia* 4: 21.

Gowda BT., Foro S., Fuess H., 2007. *N*-(2,6--Dimethylphenyl)acetamide. *Acta Cryst.* E63: 3154.

Hanson AW., Banner DW., 1974. 2-Diethylamino-2´,6´-acetoxylidine (lidocaine). *Acta Cryst.* B30: 2486-2488.

Hanson AW., Röhrl M., 1972. The crystal structure of lidocaine hydrochloride monohydrate. *Acta Cryst.* B28: 3567-3571.

Kanj SS., Kanfani ZA., 2011. Current concepts in antimicrobial therapy against resistant gram-negative organisms: Extended--spectrum *β*-lactamase-producing *Enterobacteriaceae*, carbapenem-resistant *Enterobacteriaceae*, and multidrug-resistant *Pseudomonas aeruginosa*. *Mayo Clin. Proc.* 86: 250-259.

Li X-Z., Plésiat P., Nikaido H., 2015. The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria. *Clin. Microbiol. Rev.* 28: 337-418.

Löfgren N., Lundquist B., 1946. Studies on local anaesthetics. *Svensk. Kem. Tidskr.* 58: 206-217.

Lu Ch-W., Lin T-Y., Shieh J-S., Wang M-J., Chiu K-M., 2014. Antimicrobial effect of continuous lidocaine infusion in a *Staphylococcus aureus*-induced wound infection in a mouse model. *Ann. Plast. Surg.* 73: 598-601.

Moran GJ., Krishnadasan A., Gorwitz RJ., Fosheim GE., McDougal LK., Carey RB., Talan DA., 2006. Methicillin--resistant *S. aureus* infections among patients in the emergency department. *N. Engl. J. Med.* 355: 666-674.

Ohsuka S., Ohta M., Masuda K., Arakawa Y., Kaneda T., Kato N., 1994. Lidocaine hydrochloride and acetylsalicylate kill bacteria by disrupting the bacterial membrane potential in different ways. *Microbiol. Immunol.* 38: 429-434.

Remko M., 1989. *Ab initio* and PCILO investigations of the antiarrhythmic tocainide, its cation and hydrochoride. *Chem. Papers* 43: 267-278.

Rodvold KA., McConeghy KW., 2014. Methicillin-resistant *Staphylococcus aureus* therapy: Past, present, and future. *Clin. Infect. Dis.* 58 (Suppl. 1): S20-S27.

Yoo CS., Abola E., Wood MK., Sax M., Pletcher J., 1975. The crystal structure of lidocaine bis-*p*-nitrophenylphosphate. *Acta Cryst.* B31: 1354-1360.