Dibasic Esters of ortho-/meta-Alkoxyphenylcarbamic Acid Containing 1-Dipropylamino-3-piperidinopropan-1-yl and Their Antimicrobial Activity

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Abstract

In Europe, the presence of microorganisms that have become resistant to antimicrobials as the most significant disease threat has remained. The aim of the current research was to screen the in vitro susceptibility of Staphylococcus aureus, Escherichia coli and Candida albicans to the series of dibasic esters of ortho-/meta-alkoxyphenylcarbamic acid previously known for their local anaesthetic effectiveness and to contribute for the structure – antimicrobial potency relationships study within that class of the compounds. The antimicrobial activity investigation involved determination of the minimum inhibitory concentration (MIC) by applying the microdilution method; quantitative screening was performed on a blood agar (S. aureus), Endo agar (E. coli) or on Sabouraud’s agar (C. albicans). The activity against all the microorganisms tested was primarily influenced by the position of alkoxy side chain attached to lipophilic aromatic ring and by its length as well. Inspected meta-alkoxy substituted derivatives have shown higher efficiency against all chosen microorganisms than their ortho-alkoxy positional isomers. The most promising results were observed when investigating the activity of meta-alkoxy substituted molecules against E. coli with the estimated MICs in the range of 12–49 µg/ml. Furthermore, such potency was found to be quasi parabolically dependent on alkoxy chain length achieving a maximum for meta-hexyloxy derivative which has shown MIC = 12 µg/ml. Considered compound was also regarded as the most effective against S. aureus with MIC = 98 µg/ml. Evaluating the potency against C. albicans, it was revealed that no molecule within the tested set displayed MIC < 100 µg/ml.

Key words: Escherichia coli, Staphylococcus aureus, alkoxyphenylcarbamic acid

Introduction

The incidence of invasive microbial infections caused by opportunistic pathogens, often characterized by high mortality rates, has been increased over the past two decades (Sharma et al., 2009). The majority of antibiotics currently applied in therapy are connected with drug classes discovered before 1970 (Livermore, 2011). Not surprisingly, the recent expansion of antibacterial and antifungal drug research has occurred because there is an incessant need for developing new compounds to fight life-threatening infections (Ziemska et al., 2013). The rational design of new drugs based on the relatively older molecules, the discovery of the agents with novel modes of action, seeking alternate drug targets or the innovative application of the principles of pharmacokinetics and pharmacodynamics aiming to improve the use of existing drugs could offer promising possibilities in the treatment of bacterial and fungal diseases (Enquist et al., 2012; Livermore, 2011; Perez et al., 2008; Ziemska et al., 2013). Furthermore, many of the currently available drugs are toxic, they also enable recurrence because of being bacteriostatic/fungistatic and not bactericidal/fungicidal (Sharma et al., 2009). In addition, recent years have brought the anthropomorphic problem of antimicrobial resistance which appears to be accelerating, accumulating and worldwide (Okele, 2009; Wise, 2008).

As a consequence of the above, the objective of the current study was to investigate in vitro the susceptibility of some selected clinically significant microbial strains, Staphylococcus aureus, Escherichia coli and Candida albicans, to dibasic esters of ortho-/meta-alkoxyphenylcarbamic acid. The evaluated substances, whose general chemical structure is drawn in Table I, belong to a broad class of compounds which have been previously known particularly due to their significant local anaesthetic activity (Pokorná, 1998). In terms of an...
antimicrobial inspection of such molecules, the first preliminary reference has probably come from the research paper of Mlynarčík and Čižmárik (1976). Their results have shown that some piperidinoethyl esters seemed to be effective inhibitors of microbial growth, in particular Gram-positive bacteria, yeasts and fungi. Those authors also reported that the most active substance against *S. aureus* exhibited MIC = 0.5 μg/ml.

Given those results, the need for more extensive profile of antimicrobial effectiveness of another series of variously substituted alkoxyphenylcarbamic acid-based compounds (Fig. 1) continuously has arisen (Čižmárik et al., 1983; 1986; Malík et al., 2012; Mlynarčík and Čižmárik, 1979). Furthermore, the eventual antimicrobial efficiency of such compounds which have shown local anesthetic activity would be highly welcome in terms of the health status of patients and would supplement primary pharmacological effect as well. Additionally, another advantage might be their inhibitory effect towards microorganisms in a concrete drug form or at site of application (Mlynarčík et al., 1991).

### Experimental

#### Materials and Methods

**Chemistry.** The preparation of currently *in vitro* evaluated compounds labelled as CK-3624-CK-3627 and CK-3634-CK-3637 (Table I), chemically 1-(dipropanylamino-3-piperidinopropan-1-yl)-2-/3-alkoxyphenylcarbamates with alkoxy side chain represented by butoxy-heptyloxy substituent in *ortho* or in *meta*-position, local anesthetic activities as well as acute toxicity indices has already been published (Csöllei et al., 1993).

The determination of some fundamental physicochemical parameters of these molecules, *i.e.* solubility profile, dissociation constant pKₐ and lipophilicity descriptors (log Pₑₓ, estimated by shake-flask method in the octan-1-ol/buffer medium with pH=7.3, log k’ from RP-HPLC, Rₘ from RP-TLC), with appropriate readouts can be found in an earlier paper (Malík et al., 2007).

**In vitro antimicrobial activity assay**

**Microorganisms.** The antimicrobial activity of CK-3624-CK-3627 and CK-3634-CK-3637 was investigated against Gram-positive bacterium *S. aureus* ATCC 6538 (*Micrococcaceae*), Gram-negative bacterium *E. coli* CNCTC 377/79 (*Enterobacteriaceae*) and the yeast *C. albicans* CCM 8186 as well. These 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.** Blood agar, Endo agar and Sabouraud’s agar (Imuna, Šarišské Michaľany, Slovak Republic) were used for cultivation of the microorganisms listed in the previous section of this paper. Blood agar was prepared by adding 10% of defibrinated sheep blood to the melted and cooled (50°C) components.

**Determination of minimum inhibitory concentration (MIC).** The MIC values of investigated compounds were carried out by following the modified procedure described previously (Malík et al., 2012). The respective test compounds were dissolved in distilled water. Standard suspension of bacteria was prepared from their 24 h...
cultures which were cultivated on blood agar (Gram-positive bacteria) and Endo agar (Gram-negative bacteria). Standard suspension of Candida was prepared from 48 h cultures cultivated on Sabouraud’s agar.

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

A suspension of the microorganisms was added in amount of 5 μl into the solutions of evaluated substances (100 μl) 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 (Sarišské Michafany, Slovak Republic).

Starting concentration of prepared stock solutions was 50 000 μg of respective compound per 1 ml of distilled water. These stock solutions (5 %) were then serially diluted twofold and the final concentrations were 25 000, 12 500, 6 250, 3 125, 1 563, 781, 391, 196, 98, 49, 25, 12 and 6 μg/ml, respectively.

**Quantitative screening.** The 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 24 h. Upon completion of this process, the volume of 5 μl of evaluated suspension was taken from each well by using a transferring tool and cultured on blood agar (S. aureus), Endo agar (E. coli) or on Sabouraud’s agar (C. albicans). Petri dishes were then incubated for 24 h at 37°C.

The positive control using only an inoculation of microorganisms and the negative control of solvent were realized parallely. The nutrient concentration remained stable in each well, only the concentration of inhibitory compound changed. All experiments were performed in duplicate. The MIC was considered to be the lowest concentration of the tested compound which inhibited visible microbial growth (Andrews, 2001). The MIC was dependent on the presence/absence of the culture on used solid media after the transfer of 5 μl of suspension from each well. The values of MIC were reported in Table I in μg/ml units.

**Results and Discussion**

From the chemical viewpoint, the common feature of the alkoxyphenylcarbamic acid-based molecules, which have been the objectives of previously published papers (Čižmárik et al., 1983; 1986; Mlynarčík and Čižmárik, 1979), has been the introduction of only one nitrogen atom within their basic part (Fig. 1).

On the contrary, the currently inspected compounds from both series CK-3624-CK-3627 and CK-3634-CK-3637 were considered unique in terms of the presence of a dibasic 1-dipropylamino-3-piperidino-propan-1-yl fragment. That aspect could significantly influence their activity against selected the microorganisms S. aureus, E. coli and C. albicans as well. Besides being clinically significant microbial strains, these microorganisms were chosen for current in vitro evaluation due to the possibility to compare the acquired data related to the substances CK-3624-CK-3627 and CK-3634-CK-3637, respectively, to the results from previous effectiveness determination of monobasic esters of alkoxyphenylcarbamic acid. In the experiments carried out previously, the susceptibility of the same bacterial strains as well as the same yeast to mentioned esters was in vitro screened.

The results of current antimicrobial evaluation are indicated in Table I. In general, all the investigated compounds have shown relatively improved activity against Gram-negative bacteria when compared to their potency against the Gram-positive one or anticondial efficiency.

Assuming the presence of two basic centres within the structure of inspected compounds CK-3624-CK-3627 and CK-3634-CK-3637, respectively, their activity against S. aureus could be influenced by at least these factors: (i) by the position of alkoxy side string (positional isomerism) directly attached to lipophilic aromatic moiety and (ii) by their acidobasic ($pK_a$) values and lipophyphilic ($\log P_{exp}$ data) properties.

Current experimental findings revealed that the steric and the electronic effects, which were connected with the alkoxy side chain positional isomerism, were found to be the crucial factors conducive to the antimicrobial activity of the studies molecules. As indicated, meta-alkoxy substituted compounds, CK-3634-CK-3637, with the substituent attached to the position 3 of the lipophilic phenyl ring, exhibited the MICs against S. aureus in the range of 98–781 μg/ml. In addition, such derivatives were considerably more active than their ortho-positional isomers CK-3624-CK-3627, containing alkoxy substituent attached to the position 2 of the aramote (Table I).

From the chemical viewpoint, the explanation of different level of an antibacterial efficacy could be in the fact that the proximity of ortho-alkoxy side chain to carbamoyloxy group (NHCOO) meant the twist of an aromatic ring plain towards given moiety. Described process then resulted in the planarity violation of considered compounds which led to subsequent conjugation of aromatic ring π-bonds over the amino fragment up to carbonyl.
The outcome of introduced process was different electronic density (charge) on carbonyl moiety which could be one of the possible binding sites to reactive *S. aureus* membrane locations. The transfer of alkoxy chain to meta-position meant the avoidance of mentioned secondary steric phenomenon.

The cell wall of *S. aureus* consists of some fundamental units (Dmitriev et al., 2004; Endl et al., 1983; Schneewind et al., 1995): murein, teichoic acids and wall-associated surface proteins. It was documented (Mlynarčík et al., 1991) that structurally similar alkoxyphenylcarbamic acid esters were bonded to phospholipids and proteins forming the cell wall of *S. aureus* by hydrophobic bonds and after the incorporation into the membrane bilayer by electrostatic bonds. It could be assumed that the compounds under the study interacted with the membrane phospholipids and the planarity maintenance of the aromatic within the structure of the substances CK-3634–CK-3637, i.e., the presence of the areas with negative electrostatic potentials, provided possible electron donor sites. In addition, at physiological pH, phosphate and carboxylate groups of membrane’s phospholipid and lipoprotein domains are negatively charged (Remko and Van Duijn, 1983).

In agreement with the knowledge about the physicochemical properties of currently investigated derivatives, firstly the protonization of aliphatic amine (dipropylamino group) proceeded followed by the protonization of cyclic amine (piperidin-1-yl fragment). At considered acidobasic conditions, the basic moieties of evaluated compounds were partially ionized, as reported in a previous paper by Malik et al. (2007).

Consequently, the interaction of the negatively charged phosphates of the phospholipids with polar proton donor groups could represent one of the possible types of drug-receptor interactions (Remko and Van Duijn, 1983). The higher number of protonated basic (amino) centers within the chemical structure of the inspected compounds, the more likely will be their interaction with negatively charged carboxylate or phosphate fragments.

Furthermore, the lipophilicity of meta-alkoxy substituted molecules CK-3634–CK-3637 was not found as the factor proportionally influencing their potency against *S. aureus*. The hexyloxy derivative CK-3636 was regarded as the most active (MIC = 98 μg/ml) despite the fact that it was not the most lipophilic one from the whole analyzed set. For illustration, previously estimated log $P_{exp}$ readout (Malik et al., 2007) in the partition system octan-1-ol/phosphate buffer medium (pH = 7.3) for CK-3636 was set to 4.00, however, heptyloxy substituted molecule CK-3637 was slightly more lipophilic (log $P_{exp}$ = 4.13).

Anyway, the current statement based on the experiments was not quite completely in line with previous observations of the Čižmárik research team (Čižmárik et al., 1987). Čižmárik and coworkers suggested that more lipophilic piperidinoethyl esters of alkoxyphenylcarbamic acid, with a suitable position of alkoxy side chain, displayed relatively higher efficiency.

In terms of possible further practical applications, the compound CK-3636 would be probably less effective than vancomycin or telavacín (Gould et al., 2011; Hsu et al., 2008; Putnam et al., 2010), well-known drugs currently used in therapeutic practice. Vancomycin has long been regarded as conventional and the most-used option for initial treatment for severe methicillin-resistant *S. aureus* infections.

Current MIC breakpoints define the vancomycin susceptibility lower than 2 μg/ml, as referred in paper of Gould et al. (2011). On the other hand, limited outputs also supported the use of a clinical vancomycin breakpoint of 0.5 μg/ml for broth microdilution (Hsu et al., 2008).

Following the conclusions from an international antimicrobial resistance program (Putnam et al., 2010), telavacín has been considered highly active against *S. aureus* across several geographic regions (Asia-Pacific region, Europe, Latin America, North America) with reported MIC$_{90}$ = 0.12 μg/ml and MIC$_{90}$ = 0.25 μg/ml, respectively.

Similarly, the substance CK-3636 would be probably less effective than cefaroline (Poon et al., 2012), the only US Food and Drug Administration-approved cephalosporin with activity against multidrug-resistant strains of *S. aureus* showing MIC$_{90}$ in the range of 0.5–2.0 μg/ml.

As might be deduced from actual experimental data (Table I), the factors, which would play an essential role in terms of the activity of such dibasic alkoxyphenylcarbamic acid esters against *E. coli*, could correspond to the ones which have been relevant for effectiveness against *S. aureus*: (i) the steric and the electronic aspects primarily induced by the position of alkoxy side chain, (ii) the acidobasic and the lipophilic properties of inspected derivatives and (iii) the electronic interactions evoked mainly by the fragments containing protonated basic centers.

From the structural viewpoint, ortho-/meta-alkoxy side chain isomerism appears to be the decisive factor for the activity maintenance of tested compounds against *E. coli*. In the series of ortho-alkoxy substituted substances, only CK-3624 exhibited relatively acceptable level of the potency (MIC = 781 μg/ml), other structures were weakly active or inactive (Table I).

A completely opposite situation occurred when evaluating the set CK-3634–CK-3637, where these compounds have shown MIC readouts ranging from 12 μg/ml to 49 μg/ml. The efficiency of individual members from a given homological series against the Gram-negative bacteria progressively increased with the number of carbon atom forming alkoxy side chain up to a critical point, which was represented by the derivative
CK-3636 with MIC = 12 μg/ml, beyond which the next homolog was less potent (Table I).

Such observed phenomena were previously defined as the cut-off effect. Balgavý and Devínsky (1996) have extensively reviewed several hypotheses of that aspect in biological activities as well as experimental evidences which supported them.

Previous pharmacological testing has shown that the compound CK-3636 exhibited 60-fold higher index in surface anaesthesia than applied standard cocaine. Furthermore, another standard used, procaine, was 600-fold less potent than CK-3636 in such type of anaesthesia (Csöllei et al., 1993). Similarly, cocaine was regarded as 33-fold less potent and procaine even 60-fold less effective than the considered molecule CK-3636 in infiltrative anaesthesia (Csöllei et al., 1993).

In general, the compounds from the series of CK-3634–CK-3637 were notably more potent than previously in vitro tested corresponding meta-alkoxyphenylcarbamic acid esters with incorporated aliphatic dimethylammonium (Mlynarčík and Čižmárik, 1976), cyclic piperidinium (Mlynarčík and Čižmárik, 1979) or perhydroazepinium (Mlynarčík and Čižmárik, 1976) moiety, respectively (Fig. 1).

Similarly to the structural and physicochemical properties requirements which were essential for the potency of currently screened molecules against S. aureus, except from meta-alkoxy substitution and relatively high lipophilicity, it could be concluded that the presence of more than one protonated atom of nitrogen positively influenced activity against E. coli.

Although a possible mechanism of alkoxyphenylcarbamic acid esters action against E. coli was briefly proposed previously (Malik et al., 2012), the purpose of this research nevertheless was also to provide a more detailed perspective. Based on the above, presumably the electronic effects induced by the protonated nitrogen centres of CK-3634–CK-3637 were primarily responsible for better interaction of these compounds with the Gram-negative outer membrane. It could be supposed that the cationic fragments under consideration would be the most eligible proton donors in specific (drug-receptor) interactions between exposed phosphoryl and carboxyl groups of highly negatively charged outer face. 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. Analogical conclusions also resulted from the paper of Remko and Van Duijnen (1983) wherein the ab initio investigations of local anaesthetic-phospholipid model membrane interactions were studied.

In more detail, cyclic piperidin-1-yl within the basic compartment of the tested compounds could pose a steric constraint for favorable hydrogen bond formed by such group. Following current experimental results and the conclusions from the paper which dealt with structurally similar molecules (Remko et al., 1997), it could be proposed that the protonated form of the compounds CK-3634–CK-3637 would be recognized and bound to the negatively charged carboxylate part of the receptor. In a subsequent step, the interaction between carbonyl group (polar oxygen region, specifically) of the carbamoyloxy moiety and positively charged fragment of the membrane might follow.

Furthermore, according to current results (Table I) it could be also pointed out that the presence of more than one basic centrum of protonation within the structure of concerned compounds would mean a more notable impact on their effectiveness against E. coli than against S. aureus.

In addition, considering the neutral base of the evaluated substances, it could be suggested that they could reach a receptor via hydrophobic pathways and a cationic receptor site would prefer the interaction by means of their polar oxygen areas of carbonyl group (Remko et al., 1997).

The indirect comparison of the efficiency against E. coli between the most perspective compound CK-3636 and a broad spectrum of the comparators which activities were determined within the Tigecycline Evaluation and Surveillance Trial between the years 2004–2009 and which were published in a paper by Andrasevic and Dowzicky (2012), revealed that CK-3636 could be regarded as more promising than amoxicillin/clavulanic acid combination, ampicillin, ceftriaxone or levofloxacin therapy, respectively. On the contrary, tigecycline with its MIC<sub>90</sub> = 0.5 μg/ml or meroxpenem, which has shown MIC<sub>90</sub> ≤ 0.06 μg/ml, could be considered more active.

The activity of the inspected compounds against C. albicans was not dependent only on the position of the alkoxy side chain but also on its length, as the results in Table I indicated. Generally, the introduction of meta-alkoxy group slightly favored the compounds CK-3634–CK-3637 compared to those containing the ortho-alkoxy one.

In the set CK-3624–CK-3627, only the potency of the ortho-hexyloxy derivative (MIC = 781 μg/ml) and the ortho-heptyloxy one (MIC = 391 μg/ml) could be taken into consideration. Other substances within the mentioned series were practically inactive.

Similarly, from the series CK-3634–CK-3637, only those with meta-hexyloxy and meta-heptyloxy side chain appeared to be relatively effective (MIC = 391 μg/ml in both cases).

However, the current experimental observations indicated that the existence of more than one centrum of protonation within the basic compartment leads to a decrease in the activity against C. albicans.

Comparing the currently estimated MICs to the data published in the literature (Mlynarčík and Čižmárik, ...
1976; 1979), the tested compounds were less effective than the previously investigated ortho-/meta-alkoxy positional isomers containing dimethylammonium, piperidinium or perhydroazepinium group, respectively.

Similarly, the introduction of propane-1,3-diyl connecting chain instead of ethane-1,2-diyl or 1-methyl-ethane-1,2-diyl with simultaneous presence of piperidinium moiety (Fig. 1) led to more potent substances (Králová et al., 1995).

It could be suggested that the relatively higher density of positive charge in the basic part of inspected derivatives of both series disabled their smooth internalisation into a given eukaryotic pathogen potentially causing a perturbation of its membranes. That process was reflected in relatively higher MIC values which were estimated in the range of 391–6250 µg/ml (Table I). For the clarification, possible mechanism of action of alkoxyphenylcarbamates against given yeast were suggested in the paper of Malik et al. (2012).

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Literature


