

Research Paper

Antimicrobial effect of *para*-alkoxyphenylcarbamic acid esters containing substituted *N*-phenylpiperazine moiety

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Abstract

In current research, nine basic esters of *para*-alkoxyphenylcarbamic acid with incorporated 4-(4-fluoro-/3-trifluoromethylphenyl)piperazin-1-yl fragment, 6i-6m and 8f-8i, were screened for their *in vitro* antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*, respectively. Taking into account the minimum inhibitory concentration assay (MIC), as the most active against given yeast was evaluated 8i (MIC = 0.20 mg/mL), the most lipophilic structure containing *para*-butoxy and trifluoromethyl substituents. Investigating the efficiency of the compounds bearing only a single atom of fluorine and appropriate *para*-alkoxy side chain against *Candida albicans*, the cut-off effect was observed. From evaluated homological series, the maximum of the effectiveness was noticed for the structure 6 k (MIC = 0.39 mg/mL), containing *para*-propoxy group attached to phenylcarbamoxyloxy fragment, beyond which the compounds ceased to be active. On the contrary, all the tested molecules were against *Staphylococcus aureus* and *Escherichia coli* (MICs > 1.00 mg/mL) practically inactive.

Key words: phenylcarbamates, substituted *N*-phenylpiperazines, *Candida albicans*.

Introduction

Humans and bacteria are coevolving and both sides are engaged in a struggle to maintain the upper hand; the use of technology by our species to modify our environment and improve our quality of life vs. the ability of bacteria to overcome adverse conditions (Woodford, 2003). *Staphylococcus aureus* is a common pathogen associated with serious community and hospital acquired diseases which has been considered a major problem of Public Health for long time (Oliveira *et al.*, 2011). It has caused a wide spectrum of infections ranging from mild impetigo and furuncles, to severe soft-tissue infections, benign local skin infections, necrotizing fasciitis, pneumonia, bloodstream infections, osteomyelitis, endocarditis and other life threatening infections (François *et al.*, 2010; Lucero *et al.*, 2009).

The genus of *Escherichia* includes five species and belongs to the coliform group of bacteria that colonize the intestinal tract of humans. *Escherichia coli* is by far the most common, as well as being the bacterial species most frequently isolated in the clinical microbiology laboratory (Maza *et al.*, 2004). This pathogenic Gram-negative bacterium is classified into the pathotypes, *i.e.* groups of strains that cause a common disease using prevailing and remarkable assortments of virulence factors. It has been the most usual microorganism responsible for sepsis, urinary tract infection, nosocomial pneumonia or wound infections. Moreover, given bacterium has been also regarded as prominent cause of neonatal meningitis and gastroenteritis in developing nations (Barile, 2004; Kaper *et al.*, 2004; Maza *et al.*, 2004; Muhammad *et al.*, 2011; Nataro and Kaper, 1998).

The incidence of infections caused by *Candida* species has been rising for decades (Miyasaka *et al.*, 2008). In

a survey of positive blood cultures performed in the USA, the *Candida* species ranked fifth in terms of overall incidence and represented the fourth most common group of nosocomial pathogens isolated from the intensive care units (Gudlaugsson *et al.*, 2003). A similar incidence of candidemia has been also reported in European hospitals (Gudlaugsson *et al.*, 2003; Yera *et al.*, 2001). Human beings carry the yeast *Candida albicans* and other *Candida* species as a part of their commensal microbiota. However, in hosts predisposed to candidiasis, for example very low birth weight, immunosuppressive, antibiotic, and cytotoxic therapies, *diabetes mellitus*, organ transplant, tumors, immunocompromised patients affected by AIDS and others, these yeasts may act as pathogens. Commensal *Candida* species normally inhabit in the oral, nasal and aural cavity, vaginal canal, rectum, and gastrointestinal tract of host may begin the infectious process (Diaz-Guerra *et al.*, 1997; Hellstein *et al.*, 1993; Miyasaka *et al.*, 2008; Oliveira *et al.*, 2011; Rodrigues *et al.*, 2004).

Although a number of chemotherapeutic agents are nowadays available in therapy, concerned pathogenic microorganisms are developing resistance to them. As more resistant microorganisms continue to emerge in society, the identification of new antimicrobially effective compounds is urgently needed. The objective of the current study is to investigate *in vitro* susceptibility of mentioned clinically significant microbial strains to novel basic esters of *para*-alkoxyphenylcarbamic acid bearing variously substituted basic *N*-phenylpiperazine moiety.

Materials and Methods

Chemistry

The preparation of currently evaluated compounds, labelled as 6i-6m and 8f-8i (Table 1), their spectral charac-

teristics (^1H NMR, ^{13}C NMR, IR, MS, UV/VIS) as well as the elemental analyses data were already published in the literature (Malik *et al.*, 2006; Malik *et al.*, 2004). The determination of fundamental physicochemical parameters of these molecules, *i.e.* solubility profile, surface activity γ , dissociation constant $\text{p}K_a$ and lipophilicity descriptors ($\log P_{\text{exp}}$ estimated by the shake-flask method in the octan-1-ol/buffer medium with $\text{pH} = 7.3$, $\log k'$ from RP-HPLC, R_M from RP-TLC), with appropriate readouts can be found in papers (Malik *et al.*, 2005a, 2005b).

In vitro antimicrobial activity assay

Microorganisms

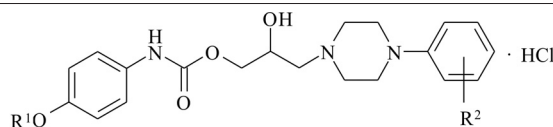
The antimicrobial activity of 6i-6m and 8f-8i was investigated against Gram-positive bacteria *S. aureus* ATCC 6538 (*Micrococcaceae*), Gram-negative bacteria *E. coli* CNCTC 377/79 (*Enterobacteriaceae*) and yeast *C. albicans* CCM 8186 as well. These tested bacterial strains were purchased from American 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 Michalany, Slovak Republic) were used for the cultivation of microorganisms listed in the previous section of this paper. Blood agar was prepared by adding 10% of defibrine sheep's blood to the melted and cooled (50 °C) competent components.

Table 1 - The *in vitro* antimicrobial activity of the alkoxyphenylcarbamic acid-based compounds 6i-8i against selected microbial strains.

Entry	R^1	R^2	MIC (mg/mL)		
			<i>Staphylococcus aureus</i> ATCC 6538	<i>Escherichia coli</i> CNCTC 377/79	<i>Candida albicans</i> CCM 8186
			6i	CH ₃	4'-F
6j	C ₂ H ₅	4'-F	12.50	6.25	1.56
6k	C ₃ H ₇	4'-F	6.25	3.13	0.39
6l	C ₄ H ₉	4'-F	12.50	6.25	0.78
6m	C ₅ H ₁₁	4'-F	12.50	6.25	3.13
8f	CH ₃	3'-CF ₃	6.25	6.25	0.78
8g	C ₂ H ₅	3'-CF ₃	12.50	6.25	0.78
8h	C ₃ H ₇	3'-CF ₃	12.50	12.50	0.39
8i	C ₄ H ₉	3'-CF ₃	12.50	12.50	0.20



Determination of minimum inhibitory concentration (MIC)

The MIC values of investigated compounds were carried out by following the modified procedure described by Mlynarcik *et al.* (1981).

The respective test compounds exhibited very limited solubility in distilled water (Malík *et al.*, 2005a, 2005b) therefore they had to be dissolved in dimethyl sulfoxide (DMSO). Standard suspension of bacteria was prepared from their 24 h 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 48 h cultures cultivated on Sabouraud's agar.

Prepared suspension contained the concentration of 5×10^7 colony forming unit (cfu)/mL of bacteria and 5×10^5 cfu/mL of *Candida*, respectively. The UV/Vis spectrophotometry was used for the determination of the microorganisms concentration, all evaluated suspensions were adjusted to the absorbance value of 0.35 at the wavelength of 540 nm.

Suspension of microorganisms was added in amount of 5 μ L into the solutions of evaluated substances (100 μ L) 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 Michalany, Slovak Republic).

Starting concentration of prepared stock solutions was 50.00 mg of compound *per* mL of DMSO. 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; 0.78; 0.39; 0.20 and 0.10 mg/mL, respectively. In thus diluted final testing medium the antibacterial effect of present DMSO was completely lost.

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 24 h. Upon completion of this process, the volume of 5 μ L of evaluated suspension has been taken from each well by using transferring tool and cultured on a blood agar (*S. aureus* ATCC 6538), Endo agar (*E. coli* CNCTC 377/79) or on Sabouraud's agar (*C. albicans* CCM 8186). Petri dishes were then incubated for 24 h at 37 °C.

The positive control using only an inoculation of microorganisms and the negative control using only DMSO were carried out. The DMSO and nutrient concentration remained stable in each well, only the concentration of inhibitory compound has changed. The MIC was considered to be the lowest concentration of the tested compound which inhibited the visible microbial growth (Mlynarcik *et al.*, 1981). The MIC was dependent on the presence/absence of the culture on used solid media after the transfer of 5 μ L of suspension from each well. The values of MIC were reported in Table 1 in mg/mL units.

Results and Discussion

The efficiency of investigated alkoxyphenylcarbamic acid-based compounds labelled as 6i-6m and 8f-8i, chemically 1-[3-(4-alkoxyphenylcarbamoxy)-2-hydroxypropyl]-4-(4-fluoro-/3-trifluoromethylphenyl)piperazinium chlorides (Table 1), was previously evaluated against tuberculous (Waisser *et al.*, 2007b) and non-tuberculous (Waisser *et al.*, 2007a) strains of mycobacteria.

The estimated values of MIC against *Mycobacterium (M.) tuberculosis* CNCTC My 331/88 of 6i-6m were in the range of 0.004-0.06 mg/mL (related to the interval of 8-125 μ mol/L) and the derivative 6l with MIC = 0.004 mg/mL (8 μ mol/L) was regarded as the most active among them. It became apparent that considered substance was even more effective than concomitantly tested compounds 8f-8i exhibiting the MICs in the interval of 0.008-0.009 mg/mL (16 μ mol/L in all cases). On the other hand, all evaluated molecules were less active (Waisser *et al.*, 2007b) than applied standard isoniazide (INH) which showed MIC = 7.00×10^{-5} mg/mL (0.5 μ mol/L). In addition, all the derivatives from series 8 were completely tested against *M. kansasii* My 235/80 (Waisser *et al.*, 2007a), the determined MICs were in the range of 0.008-0.02 mg/mL (16-32 μ mol/L). Moreover, they exhibited higher efficiency than INH (MIC > 0.03 mg/mL; > 250 μ mol/L). Conclusions from literature (Waisser *et al.*, 2007a, 2007b) pointed out that the MICs of inspected *para*-alkoxyphenylcarbamic acid esters 6i-6m and 8f-8i were comparable with those containing *meta*-alkoxy substituent. Giving those results, the need of form for more extensive profile of an antimicrobial effectiveness of the above compounds has arisen. They were screened for *in vitro* activity against *S. aureus* ATCC 6538 (Gram-positive bacteria, *Micrococcaceae*), *E. coli* CNCTC 377/79 (Gram-negative bacteria, *Enterobacteriaceae*) and *C. albicans* CCM 8186 as a representative species of yeast. The results of current antimicrobial assays are reproduced in Table 1. In general, all the tested compounds showed relatively improved anticandidacidal activity when compared to their antibacterial influencing.

All tested substances were inactive against *S. aureus* ATCC 6538, the most virulent *Staphylococcus* species, with the estimated MICs higher than 1.00 mg/mL regardless to the type of substitution at *N*-phenylpiperazine ring. Identical conclusions have been found in recent antimicrobial evaluation of analogical molecules containing *meta*-alkoxy substituent (Malík *et al.*, 2012). Previously testing piperidinoethyl esters of *ortho*-, *meta*- and *para*-alkoxyphenylcarbamic acid (alkoxy=methoxy to decyloxy), Cizmárik *et al.* (1987) suggested that, except of the steric effects, the lipophilicity of *meta*- and *para*-alkoxy substituted positional isomers (the *ortho*-ones were considered practically inactive) was found as the factor conducive to their activity. More lipophilic molecules with a

suitable position of alkoxy side string displayed relatively higher efficiency. On the contrary, current experimental readouts (Table 1) and the conclusions recently summarized by Malík *et al.* (2012) have pointed out that the positional *meta-/para*-alkoxy isomerism did not seem to be essential factor for the activity of such basic esters against *S. aureus*. More likely there was enzymic splitting of carbamate group in the structure of tested molecules 6i-6m and 8f-8i giving a rise to concrete *para*-alkoxyanilines. In addition, expected antimicrobial activities of each *para*-alkoxyanilines were lower than those with longer alkoxy substituent. These structures probably acted as the actual antimicrobial agents. Given statement was in accordance with conclusions as quoted in papers (Cizmárik and Trupl, 1978; Cizmárik *et al.*, 1987).

From structural point of view, *meta-/para*-position of alkoxy side string appeared to be the crucial factor for the activity maintenance of tested compounds against *E. coli*. The estimated values of MIC related to 6i-6m were higher than 1.00 mg/mL, the structures 8f-8i were not any better off (Table 1). Previously analyzed compounds bearing *meta*-alkoxy substituent and 4-(4-fluorophenyl)piperazin-1-yl, 6d-6g (Malík *et al.*, 2012), were more effective with their MICs in the interval of 0.20-0.78 mg/mL (444-1721 $\mu\text{mol/L}$). In contrast, the molecules 8c-8e with *meta*-alkoxy side string and 4-(3-trifluoromethylphenyl) piperazin-1-yl were regarded as completely inactive (Malík *et al.*, 2012).

The explanation of the reason why the sets 6i-6m and 8f-8i were ineffective could be due to their linearity compared to 6d-6g and 8c-8e, respectively. On the basis of resonance theory (Carey and Sundberg, 2007; Gross and Seybold, 2000), the linearity of the compounds made the resonance (mesomeric) effect at phenyl ring which influenced their electron distribution and lipohydrophilic properties as well. Alkoxy fragments in *para*-position primarily acted through the resonance as electron-donating groups which were able to increase the basicity of nitrogen atom. Given substituents could distribute the negative charge towards amino moiety (part of carbamate group) facilitating its protonation. Nevertheless, described electron-donating resonance effect was countered by the electron-withdrawing inductive effect of these electronegative substituents, however, for *para*-position dominated positive mesomeric action (Dewick, 2006). According to formerly estimated parameters of lipophilicity, *meta*-alkoxy substituted structures were slightly lipophilic than corresponding *para*-alkoxy ones with an equal number of carbon atoms within alkoxy side string. For an illustration, the values of partition coefficient logarithm, $\log P_{\text{exp}}$, of four structures with only a single atom of fluorine, 6d-6g, were in the range of 3.25-3.83, presently evaluated five compounds 6i-6m exhibited the $\log P_{\text{exp}}$ in the interval 3.12-3.78. Similarly, for three molecules bearing trifluoromethyl group, 8c-8e, was determined the $\log P_{\text{exp}}$ value in the range of 3.61-4.03

comparing to four currently tested compounds 8f-8i with their $\log P_{\text{exp}}$ s in the area of 3.60-3.98 (Malík *et al.*, 2005a, 2005b).

The hallmark of Gram-negative bacteria is their cell envelope, which is composed of inner (IM) and outer (OM) membrane separated by a periplasm that contains a thin peptidoglycan (or murein) layer. Although both IM and OM are lipid bilayers that contain proteins, dramatic differences in their structure and composition have been noticed, reflecting their different functions and the dissimilar environments that they contact. The IM is a bilayer composed of phospholipids and proteins. The periplasm comprises approximately 10% of the cell volume and is a highly viscous compartment that is occupied by soluble proteins and the peptidoglycan layer. The periplasm is an oxidizing environment, and it contains enzymes that catalyse the formation of disulphide bonds (Ruiz *et al.*, 2006). The OM is an unusual membrane in terms of its structure and composition. Outer and inner leaflets of OM contain asymmetrically distributed lipids. In outer leaflet are virtually distributed all lipopolysaccharides (LPSs), whereas the inner leaflet has the most of the phospholipids. Due to its physical properties, the OM functions as a selective barrier that prevents the entry of many toxic molecules into the cell, a property that is crucial for bacterial survival in many environments (Ruiz *et al.*, 2006).

As can be deduced from actual experimental data (Table 1) and from literature (Malík *et al.*, 2012), there were several factors which could play an essential role in terms of activity of such phenylcarbamic acid esters against *E. coli*: the lipophilicity, the steric aspects (linearity or non-linearity of molecules) and the electronic interactions induced by substituted *N*-phenylpiperazine moiety.

It could be hypothesized that *meta*-alkoxy substituted positional isomers antimicrobially evaluated by Malík *et al.* (2012) more readily interacted with appropriate reaction sites in OM. In the case of currently analyzed sets 6i-6m and 8f-8i (Table 1) the electron movements, as described above in the text, led to relatively lower level of compounds' lipophilicity which resulted in more complicated internalisation into OM. The subsequent electronic interactions, which have been possibly manifested after the incorporation into the membrane structures through the π -bonds of variously substituted *N*-phenylpiperazine fragment, could represent another contributing factor which affected the efficiency. Suggested hypothesis was also consistent with the conclusions postulated by Mlynacik *et al.* (1991).

The findings from previous research (Malík *et al.*, 2012), dealing with 6d-6g and 8c-8e, have also demonstrated that these lipophilic structures could be promising especially in terms of the potency against *C. albicans*. The evaluated molecules 8c-8e exhibited the MICs in the range of 0.10-0.20 mg/mL (189-399 $\mu\text{mol/L}$), the derivatives 6d-6g were against this yeast considerably less active indi-

cating the MIC readouts in the interval of 0.78-1.56 mg/mL (1621-3552 $\mu\text{mol/L}$).

The efficiency of currently inspected 6i-6m and 8f-8i against *C. albicans* was dependent not only on *para*-alkoxy string length but also on the structural modification of the *N*-phenylpiperazine moiety as confirmed the results in Table 1. In general, the introduction of more lipophilic, electron-withdrawing trifluoromethyl group favoured the compounds 8f-8i compared to those with only a single atom of fluorine (6i-6m).

Anticandidacidal activity of 6i-6m progressively increased with the increase in the number of carbon atoms in their side chain up to a critical point, represented by the derivative 6k with MIC = 0.39 mg/mL (835 $\mu\text{mol/L}$), beyond which the compounds ceased to be active (Table 1). The observed phenomena has been called the cut-off effect. The scientific groups of Balgavý and Devínsky have extensively reviewed several hypotheses of the cut-off effect in biological activities and experimental evidences supporting them (Balgavý and Devínsky, 1996; Devínsky *et al.*, 1990). *C. albicans* has a multilayered cell wall composed of an outer layer of proteins glycosylated with *N*- or *O*-linked mannosyl residues and an inner skeletal layer of α -glucans and chitin (Pinto *et al.*, 2008). Based on the conclusions resulting from the research of above mentioned authors (Balgavý and Devínsky, 1996), for investigated compounds 6i-6m were within this paper suggested several possible reasons for the manifestation of given cut-off phenomena. Firstly, that effect could be caused by a decrease in the achievable compound's concentration at the site of action due to its limited solubility. The drug partition coefficient between the aqueous solution and the site of action increased less rapidly with the increase in side chain length than the aqueous solubility decreased, until a point was reached at which the maximum achievable concentration at the site of action was significantly lower than that required to cause of the biological effect. Secondly, the physical properties in the homologous series could suddenly change at some particular substituent chain length, resulting in the different type of the interaction with the site of action. It has been also proposed that considered aspect could be a complication in the intercalation of particular compounds into (mostly) lipid bilayer. Moreover, mentioned model of action was previously outlined for locally anaesthetically effective cinchocaine homologues (Petter *et al.*, 1970). Thirdly, following earlier experimental observations of Richards *et al.* (1978), degenerate perturbation of the membrane protein structure could be also taken into the consideration. In membrane proteins and at the interfaces there were different sets of hydrophobic sites of different dimensions which could accommodate different types of hydrophobic or amphiphilic molecules.

Relatively higher lipophilicity of 8f-8i enabled their easier internalisation into given eukaryotic pathogen which caused a perturbation of its membranes resulting in the

anticandidacidal effect at relatively lower MIC values which were registered in the range of 0.20-0.78 mg/mL (367-1595 $\mu\text{mol/L}$). Within evaluated set, the most active compound was 8i (Table 1). Present findings were in accordance with conclusions already formulated (Limban *et al.*, 2011; Malík *et al.*, 2012). However, recently investigated *meta*-alkoxy substituted structures were more active than corresponding *para*-positional isomers, probably due to their higher lipophilicity (comparing experimentally estimated $\log P_{\text{exp}}$ values of particular positional isomers with an equal number of carbon atoms in alkoxy side chain).

Going forward, there will be the attempt to *in vitro* investigate antibacterial and anticandidal activity profile of another *meta/para*-alkoxyphenylcarbamic acid derivatives by the testing of new compounds containing electron-donating (methyl) functional group instead of primarily electron-withdrawing (fluoro or trifluoromethyl) ones directly attached to *N*-phenylpiperazine moiety.

Conclusion

Nine novel basic *para*-alkoxyphenylcarbamic acid esters bearing substituted *N*-phenylpiperazine moiety within their basic part were tested against selected Gram-positive (*S. aureus* ATCC 6538), Gram-negative (*E. coli* CNCTC 377/79) bacterial strain and against a yeast (*C. albicans* CCM 8186). Comparing currently and previously estimated results, it was beyond any doubt that the activity against given microorganisms was fundamentally influenced by the position of alkoxy side string attached to the aromatic ring. It became apparent that *para*-alkoxy substituted compounds were practically inactive against both considered bacterial strains due to the resonance mesomeric effect at phenyl ring which was involved by given substitution. The presence of such primarily electron-donating group implied an effect on the electron distribution and also lipohydrophilic properties of the target compounds.

The findings of this research also demonstrated that the activity of the molecules containing 4-(4-fluorophenyl)piperazin-1-yl within their basic part against *C. albicans* increased progressively with the elongation of alkoxy chain up to a critical point, beyond which they ceased to be active. The observed phenomena has been known as the cut-off effect. The intensity of observed inhibitory effect against given yeast reflected the lipophilicity of investigated structures carrying 4-(3-trifluoromethylphenyl)piperazin-1-yl fragment. The most lipophilic compound from whole tested series was evaluated as the most active one.

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