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Original article

Contribution to antimicrobial profile investigation of phenylcarbamic acid derivatives containing substituted *N*-phenylpiperazine fragment

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ABSTRACT

The set of new original, higly lipophilic basic esters of phenylcarbamic acid containing 4-(2-fluoro-/4-fluorophenyl)piperazin-1-yl moiety, labelled as 1–12, was screened for *in vitro* antimicrobial activity against *Escherichia coli, Candida albicans* and *Staphylococcus aureus,* respectively. Following the minimum inhibitory concentration (MIC) assay by microdilution method, all tested molecules were against *S. aureus, E. coli* as well as *C. albicans* practically inactive. The study has revealed that the position of carbamate group have appeared to be the most notable factor which decisively influence the activity of tested compounds in the comparison with the importance of electronic or hydrophobic interactions induced by the substitution at the *N*-phenylpiperazine ring. Additionally, the lipophilicity rising had been regarded as relevant but not ultimate aspect for the effectiveness of these molecules. Finally it can be concluded that mandatory requirement for the activity maintenance against investigated bacterial strains as well as a yeast there had been direct binding of carbamate group and present connecting chain in the molecule of such phenylcarbamic acid derivatives no matter the type of the substituent attached in hydrophilic part.

1. Introduction

Basic esters of alkoxyphenylcarbamic acids are regarded as the drugs exhibiting various biological effects. They are known primarily due to their very significant local anaesthetic activity [1], but they also be considered as important antiulcerous drugs with cytoprotective properties [2, 3]. Furthermore, these molecules exhibit interesting profile from the point of the ability to influence cardiovascular system functions as *β*-adrenolytic, vasodilating and antihypertensive agents [4, 5]. In the last decade Waisser et al. [6, 7, 8] systematically and extensively studied phenylcarbamates in *vitro* against tuberculous *(Mycobacterium tuberculosis)* and some non-tuberculous *(M. avium, M. kansasii)* mycobacterial strains including the QSAR methods. Additionally, predominantly moderate activity of basic alkoxyphenylcarbamates against Gram-positive *Staphylococcus (S.) aureus,* Gram-negative *Escherichia (E.) coli* and a yeast, *Candida (C.) albicans,* was noted from previous research papers [9, 10, 11, 12]. The intensity of abovementioned biological activities had been significantly dependent on the modifications of three fundamental parts of alkoxyphenylcarbamic acids: lipophilic part, connecting chain and basic (usually hydrophilic) fragment as illustrates Fig. 1. Suitable modifications of given primary fragments can lead to corresponding shift of required biological influence. In the most of cases, in evaluated sets of molecules the carbamate (NHCOO) group was directly attached at present connecting chain (Fig. 1). The objective of current research is to evaluate in vitro susceptibility of *Staphylococcus (S.) aureus, Escherichia (E.)* coli and *Candida (C.) albicans* to novel basic esters of phenylcarbamic acids containing variously substituted N-phenylpiperazine moiety and to reveal some structural and physicochemical aspects which could play a significant role from the point of the effectiveness against given microorganisms (Table 1).

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2. Materials and Methods

2.1. Chemicals and Reagents

Currently evaluated compounds labelled as 1–12 (Table 1), chemically, 1-{2-hydroxy-2-{3-/4-[(alkoxycarbonyl)amino]phenyl}ethyl)-4-(2-/4 fluorophenyl)piperazinium chlorides, were purchased from Department of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

2.2. *In vitro* antimicrobial activity assay

Microorganisms

The antimicrobial activity of 1–12 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

For a cultivation of microorganisms, listed in the previous section of this paper, 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 (50^0C) competent components.

Determination of minimum inhibitory concentration (MIC)

The MIC values of investigated compounds were carried out by following the modified procedure previously described in research of Zelenák et al. [13]. The respective test compounds had been dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) due to their very limited solubility in distilled water. 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) per mL of bacteria and 5×10^5 CFU·mL-1 of Candida, respectively. The UV/VIS spectrophotometry was used for the determination of the microorganisms concentration, all evaluated suspensions were adjusted to the absorbance output of 0.35 at the wavelength of 540 nm.

The suspension of microorganisms was added in the amount of 5 μL into the solutions of evaluated compounds (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 (Šarišské Michaľany, Slovak Republic).

Starting concentration of prepared stock solutions was 50.00 mg 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, 0.78, 0.39, 0.20 and 0.10 mg·mL^{-1}, respectively. Antibacterial effect of present DMSO in thus diluted final testing medium 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 μL 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.

Positive control using only an inoculation of microorganisms and negative control using only DMSO were realized parallelly. Both DMSO and nutrient concentrations remained stable in each well, only the concentration of inhibitory compound has changed. All experiments were performed in duplicate. The minimum inhibitory concentration (MIC) was considered to be the lowest concentration of tested compound which inhibited visible microbial growth [13]. 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 1 in mg·mL^{-1} units.

The level of antimicrobial activity of tested compounds has been assessed by the following criteria: the molecules with MIC up to 0.005 mg·mL^{-1} were considered as good active, the derivatives showing MIC in the range of 0.005 – 0.10 mg·mL^{-1} were regarded as active, the interval of 0.10 – 1.00 mg·mL^{-1} was assigned for weakly active compounds and the molecules with MIC above 1.00 mg·mL^{-1} were taken as biologically inactive [14].

3. Results and Discussion

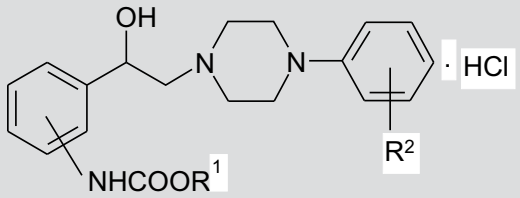
Possible structural features of phenylcarbamic acids derivatives which could influence the activity against tested *S. aureus, E. coli and C. albicans* are: the substitution at lipophilic (aromatic) ring, the position of carbamate group, the type of connecting chain and electronic properties of the substituent in basic fragment of molecule (Fig. 1). Furthermore, another notable factors which have to be taken into the consideration were lipohydrophilic properties of tested structures.

Basic esters of phenylcarbamic acids in which structure had not been carbamate bond directly attached at (substituted) connecting hydrocarbon chain had not been tested against mentioned microorganisms yet. Following currently estimated minimum inhibitory concentration (MIC) values, investigated derivatives containing 3-alkylcarbonylamino moiety, 1–4, have been slightly more efficient against all tested microorganisms than their positional isomers 5–8 as illustrates Table 1. However, from whole analyzed set, the most active molecule 4 has exhibited the MIC values of 3.13 mg·mL-1 (against *E. coli*) and 1.56 mg·mL^{-1} (*against C. albicans*), respectively. As the most efficient substance against S. aureus was evaluated the molecule 2 with MIC = 3.13 mg·mL^{-1}. According the criteria which have been adopted from

paper [14], both given structures have been considered as inactive compounds. The movement of the fluorine substituent from the position 2´ to position 4´ in basic fragment, compounds 9–12, has meant slightly better results, especially against *E. coli* (Table 1). Abovementioned molecules exhibited the MIC values in the range of 1.56–6.25 mg·mL^{-1}. On the other hand, they have also been classified as completely inactive. Following current experimental findings and the results from previous research papers [9, 10, 11, 12] can be assumed that direct linkage of 1-hydroxyethane-1,2-diyl connecting chain and aromatic ring with simultaneous separated binding of carbamate fragment at the aromate has led to significant decrease of the activities against all tested microorganisms. Existing electronic or hydrophobic interactions, induced by the fluorine substitution at the *N*-phenylpiperazine ring, have been considered as only additive factors affecting the efficiency.

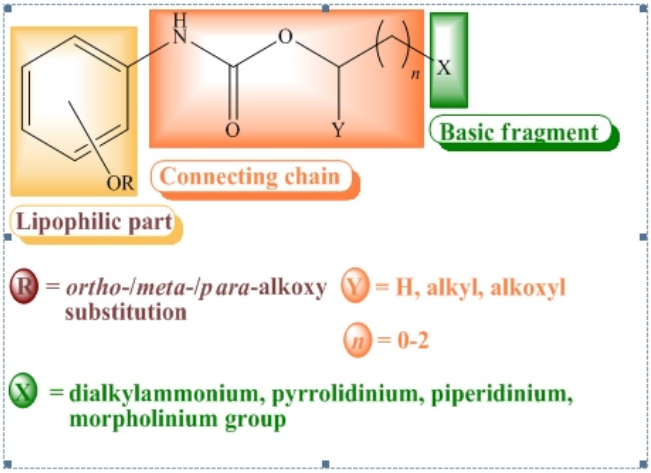
All evaluated structures 1–12 had been regarded as highly lipophilic due to the presence of two aromatic rings as well as the atom of fluorine. Because of that reason they had been dissolved in DMSO instead of distilled water in the testing process. Their lipophilicity enhancement had been in accordance with the increasing of carbon atoms number in alkyl side string. It has been previously reported that in the group of „classical" alkoxyphenylcarbamates [12] or benzoxazole derivatives [15] the lipophilicity parameters have been linearly related to the inhibitory activity against *C. albicans.* Additionally, the proposed mechanism for fungicidal activity of structurally similar aminoanilide local anaesthetics, bupivacaine and lidocaine, has been in the conformity with their lipohydrophilic properties [16]. It has been concluded that the efficiency of bupivacaine was higher than lidocaine because the former structure has been more lipophilic. It has been also suggested that the molecule of bupivacaine has ultimately penetrated the membrane bilayer and it has accommodated in its hydrophobic interior [16]. Following mentioned observations as well as currently experimental results (Table 1), the lipophilicity of evaluated compounds 1–12 has been considered as relevant but not crucial aspect for such activity against *C. albicans*.

Table 1: *In vitro* antimicrobial activity of investigated structures 1–12 against selected microbial strains.



			MIC (mg/mL)		
Entry	**R^1**	**R^2**	***Staphylococcus aureus ATCC 6538***	***Escherichia coli CNCTC 377/79***	***Candida albicans CCM 8186***
1	3-CH_3	2´-F	25.00	25.00	6.25
2	3-C_2H_5	2´-F	3.13	6.25	6.25
3	3-C_3H_7	2´-F	12.50	6.25	6.25
4	3-C_4H_9	2´-F	6.25	3.13	1.56
5	4-C_{H3}	2´-F	25.00	12.50	6.25
6	4-C_2H_5	2´-F	25.00	25.00	6.25
7	4-C_3H_7	2´-F	25.00	25.00	6.25
8	4-C_4H_9	2´-F	25.00	25.00	6.25
9	3-CH_3	4´-F	25.00	6.25	6.25
10	3-C_2H_5	4´-F	6.25	3.13	6.25
11	3-C_3H_7	4´-F	3.13	1.56	1.56
12	3-C_4H_9	4´-F	6.25	3.13	1.56

Fig 1: Chemical structure of basic esters of alkoxyphenylcarbamic acids.



4. Conclusion

The results of this study have pointed that direct binding of carbamate group and connecting chain in the molecules of basic phenylcarbamic acid derivatives has been considered as crucial for the activity maintenance against *S. aureus*, *E. coli* or *C. albicans*. The nature of the substituent attached at present basic *N*-phenylpiperazine moiety and consequent electronic and hydrophobic interactions as well as the lipophilicity could be regarded as substantial but not decisive factors which have positively influenced the activity of such phenylcarbamates against given microorganisms. These experimental findings will be beneficial from the point of further projection and synthesis of new, more perspective antibacterially active compounds.

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