JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

THE *IN VITRO* EVALUATION OF *ortho*-/*meta*-/*para*-ALKOXYPHENYLCARBAMIC ACID ESTERS CONTAINING 4-(4-FLUOROPHENYL)PIPERAZIN-1-YL MOIETY AGAINST *MYCOBACTERIUM TUBERCULOSIS* $H_{37}R_a$ STRAIN

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ARTICLE INFO

Received 27. 4. 2014
Revised 13. 5. 2014
Accepted 13. 5. 2014
Published 1. 6. 2014

Regular article

OPEN ACCESS

ABSTRACT

The objective of current paper was to *in vitro* screen the set of *ortho*-/*meta*-/*para*-alkoxyphenylcarbamic acid esters bearing 4-(4--fluorophenyl)piperazin-1-yl moiety for their potency against avirulent *Mycobacterium tuberculosis* $H_{37}R_a$ by applying the micromethod for the determination of the minimum inhibitory concentration (*MIC*). Considered mycobacterial strain was grown in Middlebrook broth, supplemented with Oleic-Albumin-Dextrose-Catalase supplement and mycobactin J (2 µg/mL) as well. The susceptibility of the strain was investigated in a 96-well plate format, the plates were incubated at 37°C for 7 days. Following estimated *MIC* readouts, it was suggested that *meta*-/*para*-alkoxy substitution within lipophilic fragment would be favorable for the activity of currently investigated compounds against given mycobacterium compared to the *ortho*-position of attached alkoxy side chain. From the entire set, *para*-propoxy substituted derivative was considered the most effective with the estimated *MIC*=62.5 µg/mL (0.13 mmol/L).

Keywords: *Mycobacterium tuberculosis* $H_{37}R_a$, *N*-arylpiperazines, alkoxyphenylcarbamates

INTRODUCTION

The long-term methodical research of phenylcarbamic acid derivatives at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava led, among others, to design and synthesis of the compounds which connecting chain consisted of 2-hydroxypropane-1,3-diyl and basic compartment was formed by variously substituted *N*-phenylpiperazin-1-yl moiety (Figure 1). Pharmacological evaluation indicated that these molecules were able to influence the functions of cardiovascular system due to the presence of such structural units. The preliminary structure–activity relationships analyses revealed that the efficiency of given compounds was dependent on (i) isosteric replacement of polar carbamoyloxy group by carboxy moiety, (ii) the position of alkoxycarbonylamino/alkoxy side chain attached to lipophilic phenyl ring and (iii) the selection of the substituent attached to *N*-phenylpiperazin-1-yl fragment as well (**Čelková *et al.*, 1996, 1997; Mlynárová *et al.*, 1996, 2000; Račanská *et al.*, 2010; Seginko *et al.*, 1995**).
The *N*-arylpiperazine moiety was previously identified as so-called privileged structure, i.e. it was defined as a single molecular framework able to provide ligands for diverse receptors (**Evans *et al.*, 1988**) or, in other words, such fragment was capable of binding to multiple receptors with high affinity. Privileged structures represent an ideal source of lead compounds. A single library based upon privileged structures could lead to active substances at a variety of receptors. The exploitation of these molecules should allow medicinal chemist to rapidly discover biologically active compounds across a broad range of therapeutic areas on a reasonable time scale (**Horton *et al.*, 2003**).
In the line with such idea, the phenylcarbamic acid-based compounds which contained *ortho*-/*meta*-/*para*-alkoxyphenylcarbamoyloxy fragment, 2-hydroxypropane-1,3-diyl and 4-(4-fluorophenyl)piperazin-1-yl moieties (Figure 1) were previously *in vitro* tested against various microbial strains. It was found that some of them were regarded as promising agents especially against virulent *Mycobacterium tuberculosis* $H_{37}R_v$ (**Waisser *et al.*, 2007**). From entire inspected series, *para*-butoxy substituted molecule (compound 8 in Table 1) was considered the most active and it has shown the value of minimum inhibitory concentration (*MIC*) 8 µmol/L against mentioned tuberculosis bacteria. Moreover, it was indicated that the efficiency of inspected *ortho*-/*meta*-/*para*-alkoxy substituted derivatives was influenced by their lipohydrophilic properties and by the position of alkoxy substituent attached to phenyl ring as well (**Waisser *et al.*, 2007**).
Following given, the purpose of current research would be to *in vitro* screen the efficiency of concerned alkoxyphenylcarbamic acid esters 1-8 (Figure 1, Table 1) against avirulent *M. tuberculosis* $H_{37}R_a$ which has several characteristics that are different from its virulent sister strain $H_{37}R_v$ (**Zheng *et al.*, 2008**) and to reveal some structural and physicochemical features of these substances which might appear to be essential for their potency maintenance.

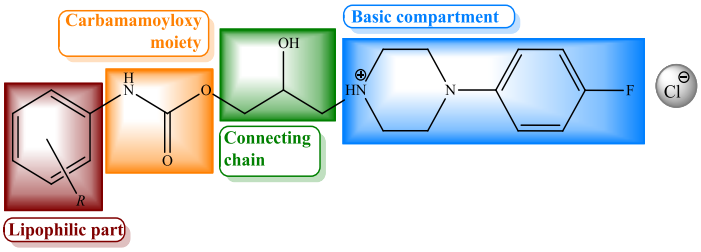

Figure 1 General chemical structure of currently *in vitro* investigated compounds against *M. tuberculosis* $H_{37}R_a$

MATERIAL AND METHODS

The Compounds under the Study

Investigated compounds 1-8 (Table 1), chemically 1-[3-(2-/3-/4-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(4-fluorophenyl)pi- perazinium chlorides (where alkoxy=methoxy to butoxy), were obtained from the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava. Their synthesis and lipohydrophilic characteristics were published previously (**Malík *et al.*, 2004, 2005a,b**).

The *In Vitro* Antimycobacterial Activity Assay

Mycobacterium tuberculosis $H_{37}R_a$ was grown in Middlebrook broth (MB), supplemented with Oleic-Albumin-Dextrose-Catalase supplement (Becton, Dickinson and Company, Cockeysville, USA) and mycobactin J (2 μg/mL) as well. Identification of this isolate was performed using biochemical and molecular protocols. At log phase growth, culture (10 mL) was centrifuged at 15 000 rpm/20 min using a bench top centrifuge Model CR 4-12 (Jouan Inc., Winchester, USA). Following removal of the supernatant, the pellet was washed in fresh Middlebrook 7H9GC broth and re-suspended in fresh supplemented MB (10 mL). The turbidity was adjusted to match McFarland standard No. 1 (3×10^8 cfu) with MB. A further 1 : 20 dilution of the culture was then performed in MB.

The susceptibility of concerned mycobacterial strain was investigated in a 96-well plate format. In these experiments, sterile deionised water (300 μL) was added to all outer-perimeter wells of the plates to minimize evaporation of the medium in the test wells during incubation. Each evaluated compound (100 μL) was incubated with the mycobacterial strain (100 μL). Dilutions of each derivative were prepared in duplicate. For the tested compounds 1-8, the final concentrations were 1 000, 500, 250, 125, 62.5 and 32 μg/mL, respectively. Due to a very limited solubility in distilled water, all substances were firstly dissolved in dimethyl sulfoxide (Sigma-Aldrich, Arklow, Ireland) and subsequently diluted by supplemented MB. The plates were sealed with parafilm and incubated at 37°C for 7 days. Following incubation, a 10% addition of alamarBlue (AbD Serotec, Kidlington, UK) was mixed into each well and readings at 570 nm and 600 nm were taken, initially for background subtraction and subsequently after 24 h re-incubation. The background subtraction is necessary for strongly coloured compounds, where the colour may interfere with the interpretation of any colour change. For non-interfering compounds, a blue colour in the well was interpreted as an absence of growth and a pink colour was scored as growth. The minimum inhibitory concentrations (*MIC*s) were performed visually, i.e. they were initially defined as the lowest concentration which prevented a visual colour change from blue to pink.

The *MIC*s were the lowest concentration of the compound at which no visible bacterial growth was observed. The *MIC* value is routinely and widely used in bacterial assays and is a standard detection limit according to the guidelines of Clinical and Laboratory Standards Institute (**CLSI, 2013**). Isoniazid (INH; Sigma-Aldrich, Munich, Germany), a reference first-line antimycobacterial drug, served as a standard and a negative control as well. The use of such negative control eliminated possible inaccuracies. The results are summarized in μg/mL and mmol/L units as well.

RESULTS AND DISCUSSION

Following general chemical structure of currently *in vitro* investigated compounds 1-8 (Figure 1), their efficiency against *M. tuberculosis* $H_{37}R_a$ could be influenced by: (i) the positional isomerism of alkoxy side chain which was directly attached to lipophilic part (substituent *R* in Table 1), (ii) the increase in the lipophilicity, i.e. by the elongation of such alkoxy group. Possible impacts of given structural aspects were discussed in next sections of the paper.

Current preliminary results outlined that *meta*-alkoxy substituted derivatives, i.e. the compounds 3 and 4 with alkoxy side chain attached to the position 3 of phenyl ring were slightly more effective than corresponding *para*- or *ortho*-alkoxy substituted isomers (the derivatives 5-6 or 1-2, respectively), as shown in Table 1.

Table 1 The *MIC* values of investigated compounds 1-8 against *Mycobacterium tuberculosis* $H_{37}R_a$ obtained from *in vitro* screening

Entry	*R*	log P_{exp}	*MIC*	
			μg/mL	mmol/L
1	2-OCH_3	3.61	>1000	>2.27
2	2-OC_2H_5	3.90	>1000	>2.20
3	3-OCH_3	3.25	>500	>1.14
4	3-OC_2H_5	3.59	>250	>0.55
5	4-OCH_3	3.42	>1000	>2.27
6	4-OC_2H_5	3.28	>500	>1.10
7	4-OC_3H_7	3.12	>62.5	>0.13
8	4-OC_4H_9	3.54	>1000	>2.07
INH	–	–	0.50	3.64×10^{-3}

Legend: INH – isoniazid, log P_{exp} – logarithm of partition coefficient estimated in octan-1-ol–buffer medium, the values were adopted from research papers of **Malík *et al.*** (**2005a,b**)

Previous research (**Malík *et al.*, 2014**) pointed out that the linearity of *para*-alkoxy substituted compounds made the resonance (mesomeric) effect at phenyl ring influencing their electron distribution. 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 the 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 the positive mesomeric one. It was also suggested (**Malík *et al.*, 2014**) that the position of alkoxy side chain and consequential shifts of the electrons could influence possible interactions between such substituted derivatives and effector sites of the mycobacterial cells.

On the other hand, the impact of qualitatively different substitution within basic compartment of those antimycobacterially inspected molecules on the activity should be also taken into the consideration. An integral structural part of previously *in vitro* investigated derivatives was 4-(3-trifluoromethylphenyl)piperazin-1-yl fragment, currently tested compounds contained atom of fluorine which was attached to the position 4´ of the aromatic ring. Despite limited number of the molecules under the study, it might be suggested that given substitution at the position 4´ (i) enabled the linearity of basic part, (ii) involved only slight deactivation of the aromate which could be considered a favorable structural modification.

Briefly, substituents attached to aromatic system exert electronic effects by means of two distinct electronic mechanisms – resonance and inductive impacts. Resonance effects are exerted through the increase or decrease in π electron density within aromatic ring as a result of the π electron overlap between atomic orbital on directly attached ring carbon and an orbital of the substituent (**Dewick, 2006**). Both inductive and resonance influences are somewhat position specific, for fluorine directly attached to sp^2 carbon (aromatic system) is typical electron--withdrawing inductive acting. In *para*-position, given impact is countered by the positive resonance electron-donating one (positive I_π interaction) of this substituent. On the other hand, fluorine atom attached to sp^3 carbon (aliphatic trifluoromethyl group) causes a pronounced electron-withdrawing effects (sigma-withdrawing acting) through a relay of induced dipoles along a chain of bonded atoms (**Hiyama *et al.*, 2000**).

Moreover, Hammet sigma constant for fluorine in *para*-position (σ_p) is 0.06 which represents only slightly higher value than corresponding readout for hydrogen (0.00). For completeness´ sake, the value of mentioned constant of trifluoromethyl group bonded in *meta*-position (σ_m) of the aromate is 0.43 (**Bégué and Bonnet-Delpon, 2008**). The more positive Hammet sigma constant is, the more electron-withdrawing influencing of respective atom/group is observed (**Hiyama *et al.*, 2000**). Following given notable theoretical principle, stronger inductive electron-withdrawing effect for *meta*-trifluoromenthyl group was previously recognized compared to the action of *para*-fluoro substituent (**Bégué and Bonnet-Delpon, 2008; Hiyama *et al.*, 2000**).

Besides, the elongation of alkoxy side chain led to more promising results for *meta*- and *para*-substituted derivatives compared to the *ortho*--substituted ones. Following previously estimated values of logarithm of partition coefficient (log P_{exp}) in the octan-1-ol/buffer system by shake flask method (Table 1), it was observed that *para*-methoxy and *para*-ethoxy derivatives (compounds 5 and 6, respectively) have shown some kind of discrepancy which might be the consequence of dimeric structures creation. Proposed dimers could be formed by the interaction of carbamate functional groups of two monomer units (**Malík *et al.*, 2005b**). As the *MIC*s revealed, the most active substance from entire evaluated set was *para*-propoxy substituted molecule 7 which has shown the *MIC* output of 62.5 μg/mL (0.13 mmol/L). Moreover, as the log P_{exp} readouts documented (Table 1), concerned compound was the least lipophilic structure within evaluated series. In addition, the derivative 7 was more effective than identical positional isomer bearing 4-(3-trifluoromethylphenyl)piperazin-1-yl which has shown *MIC*>500 μg/mL, i.e. >0.97 mmol/L (**Malík *et al.*, 2014**). On the other hand, the substance 7 was regarded as less potent than used standard drug INH with the estimated *MIC*=0.50 μg/mL (3.64 μmol/L).

The *MIC* outputs indicated that the effectiveness of *para*-alkoxy substituted derivatives progressively increased with the increase in the number of carbon atoms of their alkoxyl up to a critical point, which was represented by given molecule 7, beyond which the compound ceased to be active. The observed phenomena has been called the cut-off effect. Scientific teams of Professor Balgavý and Professor Devínsky (**Balgavý and Devínsky, 1996; Devínsky *et al.*, 1990**) extensively reviewed several hypotheses of such effect in biological activities as well as experimental evidences which supported them. Based on the conclusions resulting from the research of above mentioned authors, for investigated compounds 5-8 might be 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 was also proposed (**Balgavý and Devínsky, 1996; Devínsky *et al.*, 1990**) that considered aspect could be a complication in the intercalation of particular compounds into (mostly) lipid bilayer.

Thirdly, following also 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.
All the data obtained will serve as the basis for future design and development of perspective drug candidate which could be regarded as attractive in terms of its potency against given avirulent mycobacterial strain.

CONCLUSION

In the light of structural features and lipophilic properties of previously and currently *in vitro* investigated phenylcarbamic acid-based compounds containing substituted *N*-phenylpiperazin-1-yl moiety could be suggested that for the potency against attenuated *M. tuberculosis* $H_{37}R_a$ strain could be favorable: (i) The linearity, i.e. *para*-alkoxy substitution, of lipophilic part with simultaneous presence of such substituent within basic compartment which could participate in hydrogen bonding either as a hydrogen-bond acceptor or as an inductive activator of a hydrogen bond donor group. (ii) *meta*-Alkoxy substitution and the linearity of basic fragment (i.e. *para*-substituted aromatic ring) combined with the presence of the substituent which rendered the remaining aromatic hydrogen substituents more acidic. The consequence of given structural modification was that the capacity of those compounds to act as hydrogen bridge donors was enhanced. In addition, not only lone electron pairs of fluorine could act as hydrogen bridge acceptors but also electron-rich aromatic π electron system of *N*-phenylpiperazine fragment. (iii) Certain level of the compounds´ lipophilicity which enhancement did not progressively lead to more effective derivatives.

Acknowledgments: The authors are very grateful to Slovak Grant Agency for Science for supporting by the VEGA Grant Project No. 1/0039/12 as well as by the grant project of the Irish Department of Agriculture Fisheries and Food (FIRM): Refs 08RDCIT601 and 08RDCIT617. The authors thank anonymous reviewers for their valuable comments and helpful revision suggestions.

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