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THE *IN VITRO* EVALUATION OF *ortho*-/*meta*-/*para*-ALKOXYPHENYLCARBAMIC ACID ESTERS BEARING 4-(2-FLUORO-/2-METHYLPHENYL)PIPERAZIN-1-YL MOIETY AGAINST *MYCOBACTERIUM TUBERCULOSIS* $H_{37}R_a$ STRAIN

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ABSTRACT

In a continual research of *ortho*-/*meta*-/*para*-alkoxyphenylcarbamic acid esters as antimycobacterially active compounds, the purpose of current paper was to *in vitro* screen the potency of the molecules containing incorporated substituted *N*-phenylpiperazin-1-yl moiety against attenuated *M. tuberculosis* $H_{37}R_a$ strain which was grown in Middlebrook broth, supplemented with Oleic-Albumin-Dextrose--Catalase supplement and mycobactin J (2 μg/mL) as well. The susceptibility of concerned mycobacterial strain was investigated in a 96-well plate format, the plates were incubated at 37°C for 7 days. According to minimum inhibitory concentrations, as the lowest concentrations which prevented a visual colour change, it was observed that positional *ortho*-/*meta*-/*para*-alkoxy side chain isomerism as well as high lipophilicity did not decisively influenced the effectiveness of inspected compounds. Moreover, qualitatively different substitution within basic compartment in terms of electronic properties and possible hydrophobic interactions did not lead to the improvement in the activity.

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

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by highly virulent bacilli from the *Mycobacterium* (*M.*) strain, remains a major global health problem despite the availability of chemotherapy and bacille Calmette-Guérin vaccine. According to the report of World Health Organization (**WHO, 2013**), such threat causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). It was reported that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths (just under 1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths). Most of these TB cases and deaths occur among men, but the burden of disease among women is also high. In 2012, there were an estimated 2.9 million cases and 410 000 TB deaths among women, as well as an estimated 530 000 cases and 74 000 deaths among children worldwide (**WHO, 2013**). A very brief insight into the history revealed that the *M. tuberculosis* strain H_{37} was originally isolated in 1905 and gained attention for its noted virulence in animal model, a distinctive characteristics used in the classification of „human tuberculosis“ in the early 1900s. According to the ability to establish the infection, in 1934, the H_{37} strain was dissociated into „virulent“ (R_v) strain and its „avirulent“ counterpart (R_a), as reported in the scientific papers of **Steenken *et al.* (1934, 1946)**. The distinguishing characteristics of $H_{37}R_a$ and $H_{37}R_v$ are maintained indefinitely on subculture, suggesting that these two strains differed genetically (**Gonzalo-Asensio *et al.*, 2008; Zheng *et al.*, 2008**). In the last decade, attenuated *M. tuberculosis* $H_{37}R_a$ successively became one of the most commonly used controls for *M. tuberculosis* identification and investigation of its virulence and genetic properties (**Chen *et al.*, 2006; Soto *et al.*, 2002; Wang *et al.*, 2005; Zheng *et al.*, 2008**).

In fact, no new drugs have been developed specifically against mycobacteria since the 1960s. Current drugs have shown a number of toxicity problems, such as marked hepatotoxicity of the first front-line anti-TB drug isoniazid (INH) or pyrazinamide, thus some patients cannot tolerate them (**Primm and Franzblau, 2007; Saukkonen *et al.*, 2006; Younossian *et al.*, 2005**). In addition, some of them (e.g. rifampin) could occasionally cause hepatocellular injury and potentiate hepatotoxicities of other anti-TB medications (**Menzies *et al.*, 2004**).

Synthesis and physicochemical parameters determination of *ortho*-/*meta*-/*para*-alkoxyphenylcarbamic acid esters has been at the heart of the research at the Department of Pharmaceutical Chemistry of Faculty of Pharmacy in Bratislava. Introduced structures were primarily investigated in terms of their very notable local anaesthetic potency by the research groups of professor Čižmárik (**Čižmárik *et al.*, 1976; Čižmárik *et al.*, 1978**) who synthesized and studied especially monobasic esters. In addition, besides these compounds, the research team of professor Csöllei was later focused on preparation and analysis of monobasic and dibasic derivatives as well (**Csöllei *et al.*, 1988; Csöllei *et al.*, 1993**). Concerned molecules consisted of some fundamental parts: lipophilic moiety, polar carbamoyloxy group, (branched) connecting chain and basic fragment. Described model was very similar to the chemical structure of very well-known local anaesthetics, lidocaine (Figure 1), which was discovered from systematic investigations at the Institute of Chemistry at Stockholm University (**Erdtman and Löfgren, 1937; Gordh, 1949**). As referred research papers concluded, the intensity of local anaesthetic efficiency of the basic esters was strongly dependent on the modification of all their essential compartments.

A few years ago, inspecting *ortho*-/*meta*-/*para*-alkoxyphenylcarbamic acid esters as antimycobacterially active agents, it was found out that some of them, which contained *i.a.* substituted *N*-phenylpiperazine fragment, have shown promising activity against *M. tuberculosis* $H_{37}R_v$ (**Waisser *et al.*, 2007**). It was suggested that the presence of sterically bulky substituent (with electron-withdrawing effect) attached to phenyl ring within their basic compartment might be regarded as favorable in terms of the activity against given virulent strain of mycobacteria (**Waisser *et al.*, 2007**). However, none of those alkoxyphenylcarbamic acid-based compounds has been *in vitro* screened for its potency against attenuated *M. tuberculosis* $H_{37}R_a$ strain yet. Following mentioned, the objective of current research would be to *in vitro* investigate and to reveal some structural and physicochemical features of the substances 1-8 (Figure 1, Table 1) which might appear to be essential for the potency maintenance (or even enhancement) of concerned compounds and to contribute to knowledge for further complex structure – antimycobacterial activity relationships study as well.

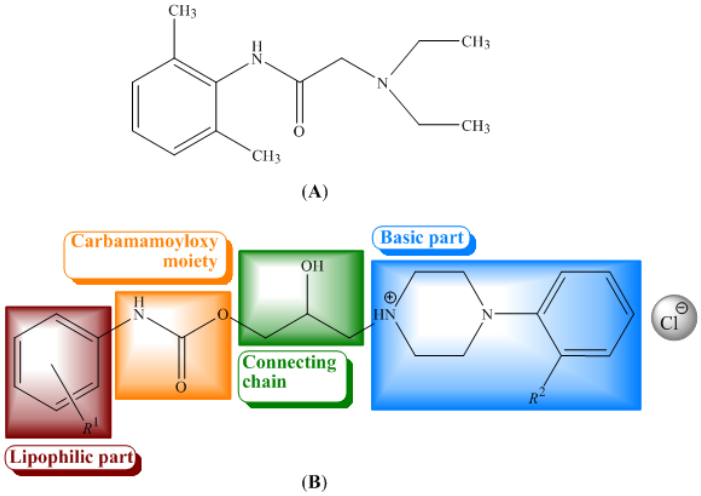

Figure 1 The molecule of lidocaine (**A**) and general chemical structure of currently antimycobacterially investigated compounds (**B**)

MATERIAL AND METHODS

The Compounds under the Study

Investigated compounds labelled as 1-8 (Table 1), chemically 1-[3-(2-/3-/4-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(2-fluoro-/2-methylphenyl)piperazinium chlorides (where alkoxy=methoxy or ethoxy), were obtained from the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava. The molecules 1-8 were prepared and *in vitro* screened as racemates. Their basic physicochemical properties, i.e. solubility profile, acidobasic and lipohydrophilic characteristics as well, were published previously (**Malík *et al.***, **2005**, **2011**).

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 ranged from 1 000 µg/mL to 125 µg/mL. Due to a very limited solubility in distilled water, all substances were firstly dissolved in dimethylsulfoxide 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 initially defined as the lowest concentration which prevented a visual colour change from blue to pink.

The *MIC*s were defined as 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 (Sigma-Aldrich, Munich, Germany) was used as reference first-line antimycobacterial drug. The results are summarized in µg/mL and mmol/L units as well.

RESULTS AND DISCUSSION

Following general chemical structure of the compounds under the study 1-8 (Figure 1), their efficiency against *M. tuberculosis* $H_{37}R_a$ could be influenced by: (i) the position of alkoxy side chain directly attached to lipophilic part (substituent R^1), (ii) the increase in the lipophilicity, i.e. by the elongation of such alkoxy group, (iii) the modification within basic compartment (substituent R^2). Possible impacts of given structural aspects were discussed in next sections of current paper.

As performed *in vitro* experiments clearly revealed, the position of attached alkoxy group was not found to be essential for the activity of tested compounds 1-8 against concerned attenuated mycobacterial strain. The substances containing 4-(2-fluorophenyl)piperazin-1-yl moiety (labelled as 2-F-PhP for further simplification) as well as 3-alkoxy group (the molecules 3 and 4, respectively) were not more effective than those with attached 2-alkoxy side chain (the compounds 1 and 2, respectively). Similarly, when investigating the derivatives with 4-(2-methylphenyl)piperazin-1-yl fragment (2-CH_3-PhP), it was revealed that corresponding positional isomers, with identical number of carbon atoms in alkoxy chain (compound 5 *versus* 7 and 6 *versus* 8, respectively), have shown identical values of *MIC* (Table 1). This finding was considered fist main difference compared to structural requirements which were identified as essential for the activity against the virulent strain $H_{37}R_v$. Previous research also pointed out that the efficiency of *in vitro* screened compounds 1-8 against mentioned strain was dependent on the *ortho-*/*meta-*/*para*-alkoxy side chain isomerism within their molecules and decreased in the line as follows: 3-alkoxy (*meta*-position)=4-alkoxy (*para*) > 2-alkoxy (*ortho*) substituted derivatives (**Waisser *et al.*, 2007**).

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

Entry	R^1	R^2	log P_{exp}	MIC	
				µg/mL	mmol/L
1	2-OCH_3	F	3.41	>500	>1.14
2	2-OC_2H_5	F	3.44	>500	>1.10
3	3-OCH_3	F	3.92	>500	>1.14
4	3-OC_2H_5	F	3.70	>500	>1.10
5	3-OCH_3	CH_3	3.44	>1000	>2.29
6	3-OC_2H_5	CH_3	3.60	>500	>1.11
7	4-OCH_3	CH_3	3.47	>1000	>2.29
8	4-OC_2H_5	CH_3	3.65	>500	>1.11
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.*** (**2005**, **2011**)

According to experimentally estimated values of logarithms of the partition coefficients (log P_{exp}s) which were determined previously in octan-1-ol/buffer medium by classical shake-flask method (**Malík *et al.*, 2005, 2011**) and which ranged in the interval of 3.41-3.92 (Table 1), all the substances *in vitro* tested were highly lipophilic. The elongation of present alkoxy chain from methoxy to ethoxy, i.e. the lipophilicity enhancement, led to symbolic increase in the potency, even only in the group of the substances containing 2-CH_3-PhP fragment (Table 1). Previous investigations of **Waisser *et al.*** (**2003a,b, 2007**) indicated that the *in vitro* activity of the compounds containing ethan--1,2-diyl/propan-1,3-diyl connecting chain and piperidin-1-yl moiety or 2-hydroxypropan-1,3-diyl connecting string and (substituted) *N*-phenylpiperazin-1-yl basic group against *M. tuberculosis* $H_{37}R_v$ became higher with an increase in their hydrophobic properties until a certain level. Supporting given statement, it was clearly documented by the applying linear, parabolic and sigmoidal calculation model as well, that the increase in the activity of basic alkoxyphenylcarbamic acid esters against *M. tuberculosis* $H_{37}R_v$, in connection with the lipophilicity enhancement, was limited due to the observation of cut-off effect (**Waisser *et al.*, 2009**). For the clarification, it was noticed that their efficiency progressively increased with the increase in the number of carbon atoms in their alkoxy moiety up to a critical point beyond which next compound ceased to be active. **Balgavý and Devínsky** (**1996**) extensively reviewed several hypotheses of mentioned phenomenon in biological activities as well as experimental evidences which supported them.

As shown in Figure 1, basic fragment of currently *in vitro* tested molecules 1-8 consisted of variously substituted *N*-phenylpiperazin-1--yl. The presence of fluorine or methyl group attached to the position 2´ of the aromatic part of *N*-phenylpiperazin-1-yl moiety (R^2) could also influence the electronic properties and possible hydrophobic interactions between such substituted derivatives and effector sites of the mycobacterial cells. In more detail, methyl group bonded at aromatic ring of the molecules 5-8 exerted positive inductive *I*-effect and released electron density to phenyl ring. Furthermore, the electron density enhancement of the aromate was also achieved by the hyperconjugation, as a stabilizing interaction between a C–H bond (of methyl group) and an adjacent of σ-bond. On the other hand, halogen atom, which contained the compounds 1-4, possessing lone pair of electrons, withdrawed the electrons inductively (negative inductive *I*-effect) but donated them by mesomeric effect (positive *M*-effect). More detailed information about these influences could be found in the monography (**Hepworth *et al.*, 2002**). As can be seen from the estimated *MIC* readouts (Table 1), considered qualitative change was not reflected in improved

efficiency of currently *in vitro* screened compounds against avirulent *M. tuberculosis* $H_{37}R_a$. In contrast, it should be clearly noticed that applied standard INH was the most effective within entire set of tested compounds and it has shown the value of *MIC*=0.50 μg/mL, as listed in Table 1.

CONCLUSION

In conclusion, the results of current research revealed that positional *ortho*-/*meta*-/*para*-isomerism of alkoxy group directly attached to lipophilic part within substituted phenylcarbamic acid esters as well as their high lipophilicity were not regarded as essential factors for their potency against avirulent *M. tuberculosis* $H_{37}R_a$. Moreover, assuming the presence of basic, variously substituted *N*-phenylpiperazin-1-yl moiety, electronic effects and possible hydrophobic interactions, which were involved by such qualitatively different substitution, were not reflected in improved activity. For further drug development within considered class of the compounds it could be suggested that the presence of two protonated basic centers instead of protonated (substituted) *N*-phenylpiperazin-1-yl fragment would lead to more promising molecules. In addition, it might be not necessary to take into account the integration of aromatic system within their basic part.

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