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* INVESTIGATION OF *ortho*-/*meta*-/*para*-ALKOXYPHENYLCARBAMIC ACID ESTERS CONTAINING SUBSTITUTED *N*-PHENYLPIPERAZIN-1-YL FRAGMENT AGAINST *MYCOBACTERIUM TUBERCULOSIS* $H_{37}R_a$ STRAIN

Ivan Malík[1], Rodney Govender[2], Eva Sedlárová[1], Jozef Csöllei[1,3], Josef Jampílek[3], Aidan Coffey[2], Jim O´Mahony[2], Lukáš Stanzel[1]*

Address(es): Dr. Ivan Malík,
[1]Comenius University in Bratislava, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Odbojárov 10, 832 32 Bratislava, Slovak Republic. Phone number: +421-2-50-117-226.
[2]Cork Institute of Technology, Department of Biological Sciences, Rossa Avenue, Bishopstown, Cork, Ireland.
[3]University of Veterinary and Pharmaceutical Sciences, Faculty of Pharmacy, Department of Chemical Drugs, Palackého 1/3, 612 42 Brno, Czech Republic.

*Corresponding author: malikivan001@gmail.com

ARTICLE INFO

Received 13. 2. 2014
Revised 28. 2. 2014
Accepted 28. 2. 2014
Published 1. 4. 2014

Regular article

OPEN ACCESS

ABSTRACT

The purpose of current paper was to *in vitro* screen the set of *ortho*-/*meta*-/*para*-alkoxyphenylcarbamic acid esters containing 4-(3--trifluoromethylphenyl)piperazin-1-yl moiety for their potency against avirulent *Mycobacterium* (*M.*) *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. According to estimated *MIC* readouts it was concluded that *para*-alkoxy substitution within lipophilic fragment would be favorable for the activity of currently investigated compounds against given mycobacterium. On the other hand, relatively high lipophilicity was not regarded as crucial factor which determined the activity profile of inspected derivatives. Moreover, the importance of the lipohydrophilic properties was considered to be the main difference compared to previous conclusions from *in vitro* screening of these compounds against virulent *M. tuberculosis* $H_{37}R_v$.

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

INTRODUCTION

The best studied virulent laboratory strain of *Mycobacterium (M.) tuberculosis* $H_{37}R_v$ (subscript „v" for virulent) has its avirulent counterpart in *M. tuberculosis* $H_{37}R_a$ (subscript „a" for avirulent). Early scientific papers of **Steenken *et al.* (1934, 1946)** reported that given mycobacterium was originally derived from the classical H_{37} strain and recognized as early as 1934. In general, it was clearly evidenced that various strains of *M. tuberculosis* differed in virulence and immunogenicity in experimental infection models. However, if and how these differences impact on human disease remains still unclear. Studies of the *M. tuberculosis* strain diversity effect in clinical settings have failed to find any consistent patterns (**Coscolla and Gagneux, 2010**). Similarly, the reasons for the decreased virulence of such strain remain not completely understood, however, differences in protein expression were probably an important factor (**Sharma and Tyagi, 2007**). It was shown that considered $H_{37}R_v$ and $H_{37}R_a$ strains were highly similar at protein level. It was suggested that bacterial secretion system and the transmembrane transport system might be important determinants of the ability of both *M. tuberculosis* $H_{37}R_v$ and *M. tuberculosis* $H_{37}R_a$ to cause the disease (**Målen *et al.*, 2011**). In addition, in the last decade, the attenuated *M. tuberculosis* $H_{37}R_a$ has successively became one of the most commonly used controls for *M. tuberculosis* identification and investigation of its virulence properties (**Bifani *et al.*, 2000; Lari *et al.*, 2001; Soto *et al.*, 2002**).

Basic esters of (alkoxy) substituted phenylcarbamic acid have been recognized mainly due to their notable local anaesthetic activity. One of the best known drugs has been heptacainium chloride prepared and tested in the 1970s by Professor Čižmárik and coworkers **(Čižmárik *et al.*, 1976, 1978**). A more detailed summary of the relationships between chemical structure and local anaesthetic activity of such class of the drugs can be found for instance in a review paper of **Pokorná (1998)**. A few years ago, it has been found out that some *ortho*-/*meta*-/- *para*-alkoxyphenylcarbamic acid esters also exhibited promising activities against selected tuberculous as well as potentially pathogenic strains of mycobacteria. The research team of Professor Waisser previously *in vitro* tested the susceptibility of *M. tuberculosis* CNCTC My 331/88 (identical with $H_{37}R_v$), *M. avium* CNCTC My 330/88, *M. kansasii* CNCTC My 235/80 and *M. kansasii* 6509/96, respectively against them (**Waisser *et al.*, 2003a,b**). From structural point of view, concerned derivatives consisted of some fundamental parts: lipophilic moiety, polar carbamoyloxy group, (variously branched) connecting chain and basic fragment. It was reported that the intensity of an antimycobacterial efficiency of these basic esters was strongly dependent on the modification of all their essential structural compartments (**Waisser *et al.*, 2003a,b**).

More detailed insight into the chemical structure of the esters, which contained 2-hydroxypropan-1,3-diyl connecting chain and substituted *N*-phenylpiperazin-1-yl moiety as well, revealed that the presence of the substituent with electron-withdrawing effect attached to phenyl ring within their basic part might be regarded as favorable in terms of the activity against *M. tuberculosis* $H_{37}R_v$ (**Waisser *et al.*, 2007**). On the other hand, only a few of those alkoxyphenylcarbamic acid-based compounds has been *in vitro* screened for the potency against attenuated *M. tuberculosis* $H_{37}R_a$ strain yet (**Malík *et al.*, 2014**). Following mentioned, the objective of current research would be to *in vitro* investigate if the presence of basic 4-(3-trifluoromethylphenyl)piperazin-1-yl moiety within the structure of the substances 1-8 and their relatively high lipophilicity (Figure 1, Table 1) might appear to be essential for their potency maintenance (or even enhancement) and to contribute to knowledge for further complex structure – antimycobacterial activity relationships study as well.

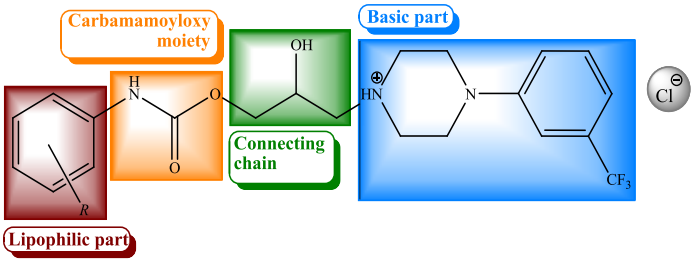

Figure 1 General chemical structure of currently antimycobacterially investigated compounds

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-(3--trifluoromethylphenyl)piperazinium chlorides (where alkoxy=methoxy to propoxy), 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.*, 2005**, **2006**).

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 and 125 µg/mL, respectively. 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 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 the compounds under the study 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*), (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 results outlined that *para*-alkoxy substituted molecules, i.e. the compounds 6-8 with alkoxy side chain attached to the position 4 of phenyl ring were slightly more effective than corresponding *ortho*- or *meta*-alkoxy substituted isomers (the derivatives 1-2 or 3-5, 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	3.57	>250	>0.51
2	2-OC_2H_5	3.60	>1000	>1.98
3	3-OCH_3	3.61	>500	>1.02
4	3-OC_2H_5	3.72	>1000	>1.98
5	3-OC_3H_7	4.03	>500	>0.97
6	4-OCH_3	3.60	>125	>0.26
7	4-OC_2H_5	3.71	>500	>0.99
8	4-OC_3H_7	3.92	>500	>0.97
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**, **2006**)

Based on resonance effect (**Dewick, 2006**), the linearity of currently *in vitro* tested *para*-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. 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.

Previous research pointed out that the efficiency of currently *in vitro* screened compounds 1-8 against the virulent strain $H_{37}R_v$ was dependent on the *ortho*-/*meta*-/*para*-alkoxy side chain isomerism and decreased in the line as follows: 3-alkoxy (*meta*-position)=4-alkoxy (*para*) > 2-alkoxy (*ortho*) substituted derivatives (**Waisser *et al.*, 2007**).

According to experimentally estimated values of logarithms of the partition coefficients (log P_{exp}) which were determined previously in octan-1-ol/buffer medium by classical shake-flask method and which ranged in the interval of 3.57-4.03 (Table 1), all the substances inspected were highly lipophilic (**Malík *et al.*, 2005, 2006**). As the values of *MIC* indicated, the increase in the alkoxy side chain length practically reduced their potential to be more promising antimycobacterially active compounds. This finding was considered another main difference compared to structural requirements which were identified as essential for the activity against the virulent $H_{37}R_v$ strain (**Waisser *et al.*, 2007**).

Following observed *MIC*s, *para*-methoxy substituted substance 6 (Table 1) with its *MIC*=125 µg/mL (0.26 mmol/L) was considered the most active against given bacterial strain. In addition, currently estimated *MIC* outputs were slightly lower than the ones for corresponding *ortho*-/*meta*-/*para*-alkoxy positional isomers *in vitro* screened previously which contained 4-(2-methylphenyl)piperazin-1-yl or 4-(2-methylphenyl)piperazin-1-yl (**Malík *et al.*, 2014**). Inspected derivative 6 was more effective than the molecule bearing *para*-methoxy and 4-(2-methylphenyl)piperazin-1-yl fragments (**Malík *et al.*, 2014**) and which has shown the value of *MIC*>1000 µg/mL (>2.29 mmol/L).

It seemed that outlined modification within chemical structure, i.e. the substitution by strong electron-withdrawing 3-triflurometyl group within *N*-phenylpiperazin-1-yl moiety was slightly more perspective in terms of the activity against attenuated $H_{37}R_a$ strain. The paper documented the decreasing trend of the *MIC*s. It should be also pointed out that (orientative) determined *MIC*s could be regarded as the valuable indicator of given structural change.

In contrast, it should be clearly registered that applied standard INH was the most effective within entire set of currently tested compounds and it has shown the value of *MIC*=0.50 µg/mL (3.64 µmol/L), as listed in Table 1. At this point was interesting to note that previous introduction of homopiperazine moiety instead of ethylenediamine chain within chemical structure of similar derivatives (**Zhang *et al.*, 2009**) led to the improved activity against *M. tuberculosis* $H_{37}R_a$.

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 lipohydrophilic properties of currently *in vitro* investigated substituted phenylcarbamic acid-based compounds, it could be concluded that *para*-alkoxy substitution within their lipophilic fragment would be considered slightly favorable in terms of their potency against avirulent *M. tuberculosis* $H_{37}R_a$. It could be proposed that steric and electronic effects involved by such position of alkoxy side chain could influence possible interactions between tested derivatives and effector sites of the mycobacterial cells. In other words, the distribution and the size of the charge within lipohilic part of evaluated substances seemed to be more important for the activity against given non-tuberculous strain compared to the tuberculous $H_{37}R_v$ one.

Additionally, it was also revealed that the increase in the lipophilicity within the homological series of inspected molecules practically reduced their potential of being more promising antimycobacterially active agents. 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 the molecules with better perspective. In addition, it might be not necessary to take into account the integration of aromatic system within their basic part.

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 the anonymous reviewers for their valuable comments and helpful revision suggestions.

REFERENCES

BIFANI, P., MOGHAZEH, S., SHOPSIN, B., DRISCOLL, J., RAVIKOVITCH, A., KREISWIRTH, B.N. 2000. Molecular characterization of *Mycobacterium tuberculosis* $H_{37}R_v/R_a$ variants: Distinguishing the mycobacterial laboratory strain. *Journal of Clinical Microbiology*, 38(9), 3200-3204.

CLSI. 2013. Clinical and Laboratory Standards Institute. Standards and guidelines regarding clinical and laboratory testing for use within the healthcare and medical testing communities.

COSCOLLA, M., GAGNEUX, S. 2010. Does *M. tuberculosis* genomic diversity explain disease diversity? *Drug Discovery Today. Disease Mechanisms*, 7(1), 43-59.

ČIŽMÁRIK, J., BOROVANSKÝ, A., ŠVEC, P. 1976. Study of local anesthetics. LII. Piperidinoethyl esters of alkoxyphenylcarbamic acids. *Acta Facultatis Pharmaceuticae Universitatis Comenianae*, 29(1), 53-80.

ČIŽMÁRIK, J., BOROVANSKÝ, A., ŠVEC, P. 1978. *N*-[2-(2-Heptyloxyphenylcarbamoyloxy)-ethyl]-piperidinium chloride, heptacaine a new local anaesthetic. *Pharmazie* 33(5), 297-298.

DEWICK, P.M. 2006. Essentials of organic chemistry: for students of pharmacy, medicinal chemistry and biological chemistry. Chichester: John Wiley and Sons, 710 pp. ISBN: 978-0-470-01666-4.

LARI, N., RINDI, L., GARZELLI, C. 2001. Identification of one insertion site of IS*6110* in *Mycobacterium tuberculosis* $H_{37}R_a$ and analysis of the RvD2 deletion in *M. tuberculosis* clinical isolates. *Journal of Medical Microbiology*, 50(9), 805-811.

MALÍK, I., GOVENDER, R., SEDLÁROVÁ, E., CSÖLLEI, J., JAMPÍLEK, J., COFFEY, A., O´MAHONY, J. 2014. 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. *Journal of Microbiology, Biotechnology and Food Sciences*, 3(4), 286-288.

MALÍK, I., SEDLÁROVÁ, E., CSÖLLEI, J., KURFÜRST, P., VANČO, J. 2006. Synthesis, spectral description, and lipophilicity parameters determination of phenylcarbamic acid derivatives with integrated *N*-phenylpiperazine moiety in the structure. *Chemical Papers*, 60(1), 42-47.

MALÍK, I., SEDLÁROVÁ, E., ČIŽMÁRIK, J., ANDRIAMAINTY, F., CSÖLLEI, J. 2005. A study of physicochemical properties of 2-, 3-, 4-alkoxyphenylcarbamic acid derivatives with a substituted *N*-phenylpiperazine moiety in the basic part. *Česká a Slovenská Farmacie*, 54(5), 235-239.

MÅLEN, H., DE SOUSA, G.A., PATHAK, S., SØFTELAND, T., WIKER, H.G. 2011. Comparison of membrane proteins of *Mycobacterium tuberculosis* $H_{37}R_v$ and $H_{37}R_a$ strains. *BioMed Central Microbiology*, 11, doi: 10.1186/1471-2180-11-18.

POKORNÁ, M. 1998. Relationship of the structure and local anaesthetic effect in the group of esters of alkoxy-substituted phenylcarbamic acid. *Česká a Slovenská Farmacie*, 47(1), 14-20.

SHARMA, D., TYAGI, J.S. 2007. The value of comparative genomics in understanding mycobacterial virulence: *Mycobacterium tuberculosis* $H_{37}R_a$ genome sequencing – a worthwhile endeavour. *Journal of Biosciences*, 32(2), 185-189.

SOTO, C.Y., ANDREU, N., GIBERT, I., LUQUIN, M. 2002. Simple and rapid differentiation of *Mycobacterium tuberculosis* $H_{37}R_a$ from *M. tuberculosis* clinical isolates through two cytochemical tests using neutral red and nile blue stains. *Journal of Clinical Microbiology*, 40(8), 3021-3024.

STEENKEN, W., GARDNER, L.U. 1946. History of H_{37} strain of tubercle bacillus. *American Review of Tuberculosis*, 54, 62-66.

STEENKEN, W., OATWAY, W.H., PETROFF, S.A. 1934. Biological studies of the tubercle bacillus. III. Dissociation and pathogenicity of the R and S variants of the human tubercle bacillus (H_{37}). *The Journal of Experimental Medicine*, 60(4), 515-543.

WAISSER, K., DOLEŽAL, R., ČIŽMÁRIK, J., MALÍK, I., KAUSTOVÁ, J. 2007. The potential antituberculotics of the series of 2-hydroxy-3-(4-phenylpiperazin-1-yl)-propylphenylcarbamates. *Folia Pharmaceutica Universitatis Carolinae*, 35-36(1), 45-48.

WAISSER, K., DRAŽKOVÁ, K., ČIŽMÁRIK, J., KAUSTOVÁ, J. 2003a. Antimycobacterial activity of basic ethylesters of alkoxy-substituted phenylcarbamic acids. *Folia Microbiologica*, 48(1), 45-50.

WAISSER, K., DRAŽKOVÁ, K., ČIŽMÁRIK, J., KAUSTOVÁ, J. 2003b. Antimycobacterial activity of piperidinylpropyl esters of alkoxy-substituted phenylcarbamic acids. *Folia Microbiologica*, 48(5), 585-587.

ZHANG, X., HU, Y., CHEN, S., LUO, R., YUE, J., ZHANG, Y., DUAN, W., WANG, H. 2009. Synthesis and evaluation of (*S*,*S*)-*N*,*N*′-bis--[3-(2,2′,6,6′-tetramethylbenzhydryloxy)-2-hydroxypropyl]-ethylendiamine (S2824) analogs with anti-tuberculosis activity. *Bioorganic and Medicinal Chemistry Letters*, 19(21), 6074-6077.