Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 3559–3562

Phosphonate inhibitors of antigen 85C, a crucial enzyme involved in the biosynthesis of the *Mycobacterium tuberculosis* cell wall

Stanislav Gobec,[a,*] Ivan Plantan,[a] Janez Mravljak,[a] Rosalind A. Wilson,[b] Gurdyal S. Besra[b] and Danijel Kikelj[a,*]

[a]*Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia*
[b]*School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

Received 9 January 2004; revised 17 March 2004; accepted 9 April 2004

Abstract—The first phosphonate inhibitors of antigen 85C—a major protein component of the *Mycobacterium tuberculosis* cell wall possessing mycolyltransferase activity were prepared using structure-based design. These potential novel antituberculosis agents, consisting of a phosphonate moiety, hydrophobic alkyl chain and a simple trehalose-mimicking aromatic structure, were designed as tetrahedral transition-state analogue inhibitors of antigen 85C, which catalyzes the key mycolyltransferase reaction involved in cell wall biosynthesis.

1. Introduction

Tuberculosis (TB), an infection caused by *Mycobacterium tuberculosis*, is the leading cause of death from a single infectious agent in the world.[1] Due to pathogenic synergy with HIV and the emergence of drug-resistant and multidrug-resistant TB, the disease has been spreading at a steady rate over the last decade.[2] There are 8 million new TB cases with 3 million deaths per year worldwide.[1] The rapid spread of TB has intensified the need for new and more effective chemotherapeutic agents with novel mechanisms of action to combat the emergence of drug resistance and shorten the duration of chemotherapy.[3]

Since the mycobacterial cell wall envelope is essential for viability and virulence, an understanding of the biochemistry of its formation has become increasingly important in the search for new antimycobacterial drug targets.[4] The mycobacterial cell wall consists of three major components forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex,[5] among which mycolic acids represent the outermost layer. Mycolic acids are high-molecular-weight, α-alkyl-β-hydroxy fatty acids [R_1–CH(OH)–CH(R_2)–COOH] unique to *Mycobacterium* and related genera.[6,7] In the mycobacterial cell wall envelope, they are present as free glycolipids, mainly trehalose monomycolate (TMM) and trehalose dimycolate (TDM, cord factor), and as esters of the terminal pentaarabinofuranosyl units of arabinogalactan.[7–9] Isoniazid and ethambutol, first line chemotherapeutics used in the treatment of TB, inhibit mycobacteria by perturbing the synthesis of mycolic acids and arabinan, respectively, both of which are essential components of the mycobacterial cell wall.[3]

Among the numerous targets[3] available for structure-based anti-TB drug design we focused our attention on antigen 85C, for which a crystal structure has been published recently.[13] The antigen 85 (ag85) complex, composed of three proteins (ag85A, B and C), is a major protein component of the *M. tuberculosis* cell wall. All three proteins contribute to cell wall biosynthesis by catalyzing transfer of mycolic acid from one TMM to another, resulting in TDM and free trehalose (Fig. 1).[10]

A trehalose analogue, 6-azido-6-deoxytrehalose inhibited mycolyltransferase activity of all three members of the ag85 complex in vitro, as well as growth of *Mycobacterium aurum*, indicating the importance of TDM for maintaining the integrity of the *Mycobacterium* cell wall.[10] Furthermore, a *M. tuberculosis* strain lacking a functional ag85C gene showed a 40% decrease in the amount of cell-wall linked mycolic acids.[11] Recently, a

* Corresponding authors. Tel.: +386-1-47-69-500; fax: +386-1-42-58-031; e-mail: gobecs@ffa.uni-lj.si

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.04.052

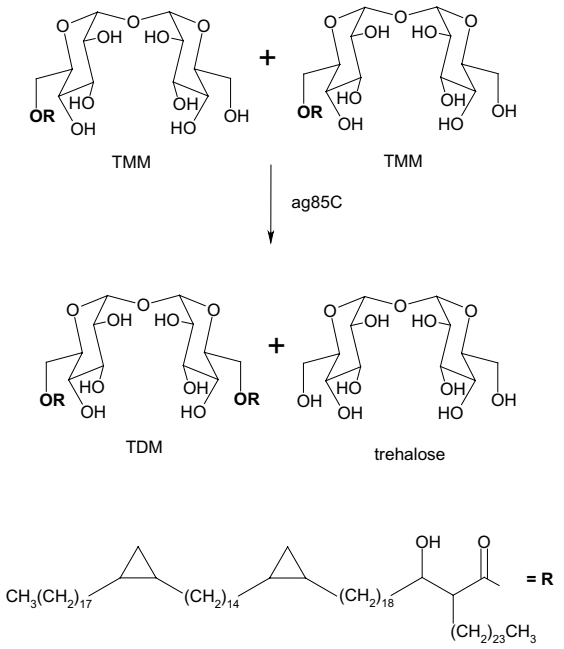


Figure 1. Mycolyltransferase reaction catalyzed by antigen 85C.

series of 6,6′-bis(sulfonamido), *N*,*N*′-dialkylamino and related derivatives of 6,6′-dideoxytrehalose was designed and synthesized to inhibit the ag85 complex. The products were active against *M. tuberculosis* and a panel of clinical isolates of *M. avium*.[12]

Disruption of the TDM structure by inhibition of ag85C mycolyltransferase activity is thus a promising strategy for the development of novel anti-TB agents. The crystal structure of recombinant ag85C from *M. tuberculosis* reveals a typical serine esterase catalytic triad formed by Ser124, Glu228 and His260.[13] A putative picture of the catalytic mechanism of the mycolyl transfer reaction has been proposed.[13] In the first step Ser124 attacks the carboxyl carbon of TMM molecule to give a mycolyl-enzyme intermediate and a free trehalose. In the next step, the 6′-OH group of the second TMM molecule attacks the carboxylate carbon of the acyl-enzyme intermediate, giving TDM. Both steps, acylation and deacylation of the enzyme, proceed via a high-energy tetrahedral transition state.[13] It is well known that properly substituted tetrahedral phosphorus(V) species like phosphonates, phosphonamidates and phosphinates represent good tetrahedral transition-state analogues of both amide and ester bond cleavage or formation. Incorporation of those phosphorus-based transition-state mimetics into the substrate or product analogues generally leads to powerful enzyme inhibitors, especially protease inhibitors.[14]

2. Results and discussion

We wish to present the discovery of a series of new phosphonate inhibitors of ag85C designed to mimic the transition state of its mycolyltransferase reaction. Based on preliminary docking studies[15] in the antigen 85C active site, we decided to use simple alcohols like *N*-(hydroxymethyl)phthalimide, *N*-(2-hydroxyethyl)phthalimide and 3-phenoxybenzyl alcohol to mimic the trehalose of TMM. Alkyl chains of various lengths (C_4 to C_{14}) were selected as hydrophobic groups to partially occupy either the mycolate α-chain binding pocket formed by a 21 Å long channel extending through the core of the ag85C protein, or the mycolic acid β-branch binding shallow on the surface of the protein. Both fragments, trehalose mimetics and mycolic acid side chain mimetics, were linked together by an ethyl phosphonate moiety to give simplified tetrahedral transition-state analogues (Fig. 2). An example of inhibitor (compound **4a**) docked into the antigen 85C active site is presented in Figure 3.

A series of potential inhibitors with different alkyl chain lengths were prepared in order to study the influence of both size and hydrophobicity of target compounds on inhibition of mycolyltransferase activity (Scheme 1). In the first synthetic step alkyl bromides **1a**–**e** were reacted with triethyl phosphite in Michaelis–Arbuzov reaction.[16] The resulting diethylphosphonates **2a**–**e** were partially deprotected with sodium azide[17] to give monoethyl phosphonates **3a**–**e**. After coupling with 3-phenoxybenzyl alcohol using BOP reagent,[18] the target mixed phosphonates **4a**–**e** were obtained. In a similar manner a series of phthalimido derivatives **5a**–**h** was prepared. Finally, two of them (**5d**,**f**) were P-deprotected to give the corresponding phosphonic acid monoesters **6a**–**b**.[19]

The compounds listed in Table 1 were assayed in vitro for their inhibition of recombinant *M. tuberculosis* antigen 85C mycolyltransferase activity.[10] Most of the compounds were very good inhibitors of the ag85C catalyzed mycolyl transfer reaction with IC_{50} values in low micromolar range (<50 μM). Although, according to our design strategy, mixed phosphonates **4a**–**5i** and

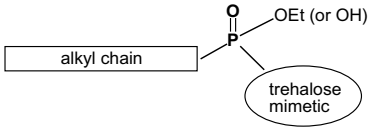


Figure 2. General structure of novel phosphonate inhibitors of antigen 85C.

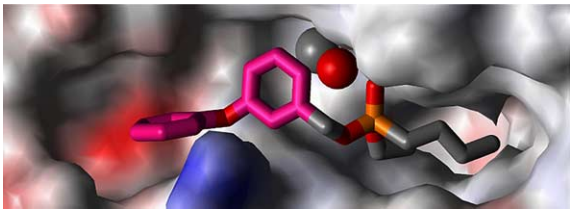
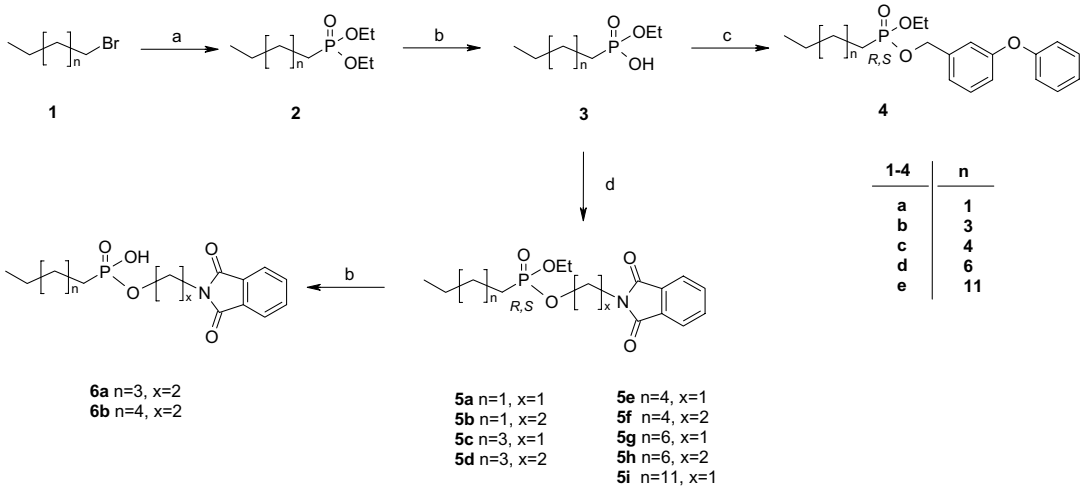


Figure 3. Hypothetical binding mode of inhibitor **4a** in the antigen 85C active site as predicted by AutoDock 3.0. The 3-phenoxybenzyl substituent is located in the trehalose binding pocket and phosphonate moiety is oriented in the vicinity of Ser124. The alkyl chain is accomodated in the mycolate α-chain binding channel extending through the core of ag85C.



Scheme 1. Reagents and conditions: (a) $P(OEt)_3$, 180 °C, 16 h; (b) NaN_3, DMF, 100 °C, 16 h; (c) 3-phenoxybenzyl alcohol, BOP, DIEA, DMF, rt, 12 h; (d) *N*-(hydroxymethyl)phthalimide or *N*-(2-hydroxyethyl)phthalimide, BOP, DIEA, DMF, rt, 12 h.

Table 1. Inhibition of ag85C mycolyltransferase activity[10]

Compound	IC_{50} (μM)
3a	430.72
3b	3.56
3c	1.06
3d	471.31
4a	2.01
4b	42.55
4c	21.03
4d	14.83
4e	862.29
5a	10.0
5b	50.74
5e	1.31
5g	87.09
5h	25.67
5i	40.99
6a	4.39
6b	1.47

phosphonic acids **6** incorporating a trehalose mimetic, were expected to be the most potent inhibitors, to our surprise also simple alkylphosphonic acids **3b** ($IC_{50} = 3.56\,\mu M$) and **3c** ($IC_{50} = 1.06\,\mu M$) were very active. It appears that in the alkylphosphonic acid series **3** medium-size alkyl chains (C_6 or C_7) are preferred over shorter (C_4) or longer (C_9) ones.

In a series of 3-phenoxybenzyl alcohol derivatives the most potent compound was **4a** ($IC_{50} = 2.01\,\mu M$), with the shortest (C_4) alkyl chain. If the length of alkyl chain was increased to C_6, C_7 and C_9, the inhibitory potency of resulting compounds **4b–d** dropped by one order of magnitude. In addition, **4e** with a C_{14} tail was practically inactive against ag85C. Thus, it appears that a combination of 3-phenoxybenzyl alcohol as a trehalose mimetic with *n*-alkylphosphonate moieties results in antigen 85C inhibitors, among which short alkyl chains are optimal for interaction with the enzyme active site.

In a second series of compounds (**5** and **6**), *N*-(hydroxymethyl)phthalimide and *N*-(2-hydroxyethyl)phthalimide were used as a replacement of trehalose. Encouraging inhibitory properties of compounds **5a–i** and **6a–b** (IC_{50} values between 1.31 and 87.09 μM), confirmed a favourable choice of both, trehalose-mimicking moieties and their combination with alkylphosphonate residue supposed to interact with a hydrophobic channel in the antigen 85C core. In contrast to inhibitors **3a–d** and **4a–e** no generalization about the influence of the alkyl chain length on inhibitory activity could be made, although *n*-hexyl and *n*-heptyl side chains (**5e**: $IC_{50} = 1.31\,\mu M$; **6a**: $IC_{50} = 1.31\,\mu M$; **6b**: $IC_{50} = 1.47\,\mu M$) seem to be most favourable. It is interesting to note that for all series of compounds (**3**, **4**, **5** and **6**) poor activity of the long chain analogues was observed. We can speculate that these lipophilic, longer chain analogues bind nonspecifically to the antigen 85C and are thus not available for interacting with the binding site.

3. Conclusion

To conclude, we have designed and synthesized a series of phosphonate inhibitors of mycolyltransferase activity of ag85C, an important *M. tuberculosis* cell wall biosynthetic enzyme. Since ag85 proteins have nearly identical active site residues it can be expected that compounds reported in this communication might have similar efficacy against all three ag85 proteins and thus a promising antimycobacterial activity. Optimization of the reported inhibitors and in vitro determination of their anti-TB activity are in progress and will be reported in due course.

Acknowledgements

The cooperation within this project was stimulated and supported by COST D13 Action and the Medical Research Council (UK). This work was financially supported by the Ministry of Science and Technology of the Republic of Slovenia.

References and notes

1. Bloom, B.; Murray, C. *Science* **1992**, *257*, 1055.
2. Murray, C. J.; Salomon, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13881.
3. Zhang, Y.; Amzel, L. M. *Curr. Drug Targets* **2002**, *3*, 131.
4. Kremer, L.; Besra, G. S. *Exp. Opin. Invest. Drugs* **2002**, *11*, 1033.
5. Brennan, P. J.; Nikaido, H. *Ann. Rev. Biochem.* **1995**, *64*, 29.
6. Barry, C. E., III; Lee, R. E.; Mdluli, K.; Sampson, A. E.; Schroeder, B. G.; Slayden, R. A.; Yuan, Y. *Prog. Lipid Res.* **1998**, *37*, 143.
7. Minkin, D. E. In *The Biology of Mycobacteria*; Ratlage, C., Stanford, J., Eds.; Academic: London, 1992; Vol. 1, pp 95–184.
8. Besra, G. S.; Khoo, K.-H.; McNeil, M. R.; Dell, A.; Morris, H. R.; Brennan, P. J. *Biochemistry* **1995**, *34*, 4257.
9. McNeil, M. R.; Daffe, M.; Brennan, P. J. *J. Biol. Chem.* **1991**, *266*, 6734.
10. Belisle, J. T.; Vissa, V. D.; Sievert, T.; Takayama, K.; Brennan, P. J.; Besra, G. S. *Science* **1997**, *276*, 1420.
11. Jackson, M.; Raynaud, C.; Laneelle, M.-A.; Guilhot, C.; Laurent-Winter, C.; Ensergueix, D.; Gicquel, B.; Daffé, M. *Mol. Microbiol.* **1999**, *31*, 1573.
12. Rose, J. D.; Maddry, J. A.; Comber, R. N.; Suling, W. J.; Wilson, L. N.; Reynolds, R. C. *Carbohydr. Res.* **2002**, *337*, 105.
13. Ronning, D. R.; Klabunde, T.; Besra, G. S.; Vissa, V. D.; Belisle, J. T.; Sacchettini, J. C. *Nat. Struct. Biol.* **2000**, *7*, 141.
14. (a) Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359–1472; (b) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305.
15. Docking calculations were carried out using AutoDock 3.0 with the Lamarckian genetic algorithm as described previously: (a) Morris, G. M.; Goodsell, D. S.; Huey, R.; Olson, A. J. *J. Comput.-Aid. Mol. Des.* **1996**, *10*, 293; (b) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639.
16. Bhattacharya, A. K.; Thyagarajan, G. *Chem. Rev.* **1981**, *81*, 415.
17. Holy, A. *Synthesis* **1998**, 381.
18. Campagne, J.-M.; Coste, J.; Jouin, P. *J. Org. Chem.* **1995**, *60*, 5214.
19. All compounds synthesized in this study gave satisfactory spectroscopic analytical results. Analytical data of some selected compounds; **4a**: Colourless oil; FAB MS m/z 349 $[M+H]^+$, IR (film) ν_{max} 3466, 2959, 1585, 1488, 1256, 1023, 693 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ (ppm) 0.83 (t, 3H, $J = 7.2$ Hz, CH_3CH_2), 1.18 (t, 3H, $J = 7.0$ Hz, CH_3CH_2O), 1.25–1.50 (m, 4H, $CH_3CH_2CH_2$ and $CH_2CH_2CH_2$), 1.65–1.80 (m, 2H, CH_2P), 3.84–4.05 (m, 2H, CH_3CH_2O), 4.99 (dd, 2H, $J = 1.7$ Hz, $J = 8.5$ Hz, $ArCH_2$), 6.95–7.05 (m, 4H, Ar-*H*), 7.12–7.20 (m, 2H, Ar-*H*), 7.35–7.45 (m, 3H, Ar-*H*); ^{31}P NMR (121 MHz, DMSO-d_6) δ (ppm) 36.0. Anal. Calcd for $C_{19}H_{25}O_4P \times 0.8H_2O$: C, 62.92; H, 7.34. Found: C, 62.98; H, 7.43. Compound **5g**: mp = 55–58 °C, FAB MS m/z 396 $[M+H]^+$, IR (film) ν_{max} 2920, 2852, 1778, 1720, 1613, 1466, 1381, 1247, 1192, 1043, 847, 722, 616 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ (ppm) 0.85 (t, $J = 6.8$ Hz, 3H, CH_3CH_2), 1.07–1.34 (m, 15H, CH_3CH_2 and $6CH_2$), 1.35–1.51 (m, 2H, CH_2), 1.68–1.83 (m, 2H, CH_2P), 3.94–4.09 (m, 2H, CH_3CH_2O), 5.46 (d, 2H, $J = 8.7$ Hz, NCH_2O), 7.88–8.02 (m, 4H, Ar-*H*); ^{31}P NMR (121 MHz, DMSO-d_6) δ (ppm) 33.0. Anal. Calcd for $C_{20}H_{30}O_5NP$: C, 60.81; H, 7.60; N, 3.55. Found: C, 60.80; H, 7.73; N, 3.94.