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Research paper

X-ray crystal structures of *Enterococcus faecalis* thymidylate synthase with folate binding site inhibitors

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ABSTRACT

Infections caused by *Enterococcus faecalis* (*Ef*) represent nowadays a relevant health problem. We selected Thymidylate synthase (TS) from this organism as a potential specific target for antibacterial therapy. We have previously demonstrated that species-specific inhibition of the protein can be achieved despite the relatively high structural similarity among bacterial TSs and human TS. We had previously obtained the *Ef*TS crystal structure of the protein in complex with the metabolite 5-formyl-tetrahydrofolate (5-FTHF) suggesting the protein role as metabolite reservoir; however, protein–inhibitors complexes were still missing. In the present work we identified some inhibitors bearing the phthalimidic core from our in-house library and we performed crystallographic screening towards *Ef*TS. We obtained two X-ray crystallographic structures: the first with a weak phthalimidic inhibitor bound in one subunit and 5-hydroxymethylene-6-hydrofolic acid (5-HMHF) in the other subunit; a second X-ray structure complex with methotrexate. The structural information achieved confirm the role of *Ef*TS as an enzyme involved in the folate pool system and provide a structural basis for structure-based drug design.

1. Introduction

Enterococci can be found in soil, food and water and make up a significant portion of the normal gut flora of humans and animals. As for other bacteria of the gut flora, *enterococci* can also cause infectious diseases. Typical enterococcal infections occur in immunosuppressed patients and in hospitalized patients suffering from a wide spectrum of severe illnesses. *Enterococci* nowadays rank second to third in frequency among bacteria isolated from hospitalized patients. They can be often isolated from urinary tract infections, intra-abdominal and pelvic infections, bacteremias, wounds, tissue infections and endocarditis as part of a polymicrobial flora. *Enterococcus faecalis* (*Ef*) and *Enterococcus faecium* are the most prevalent species cultured from humans and they are present in more than 90% of clinical isolates. *Enterococcus faecalis* accounts for 80–90% of clinical strains, while *Enterococcus faecium* for only 5–10%. Some strains of *E. faecalis* and many of *E. faecium* are resistant to multiple antimicrobials [1]. The identification of new antibiotics targeting *Enterococci* infections is challenging since no specific antibacterial agents are known so far. This encourages the starting of active drug discovery programs. Molecules targeting the *Enterococci* folate pathway represent a source of potential and specific drugs able to inhibit the bacterial folate pathway without altering, in principle, the mammalian cells. In our antibacterial discovery programs we have identified some lead compounds bearing a phthalimide moiety targeting bacterial *Lactobacillus casei* (*Lc*) and *Escherichia coli* (*Ec*) thymidylate synthase (TS) and able to discriminate between the bacterial and the host (human) protein. This selectivity led to the discovery of antibacterial agents with a low toxicity [2–10]. TS (EC: 2.1.1.45) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), assisted by the cofactor N^5,N^{10}-methylene tetrahydrofolate (MTHF) [11,12]. TS is the only synthetic source of dTMP in human cells, thus it represents a major target for the design of chemotherapeutic agents [13]. The

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well-known anticancer agents, 5-fluorouracil (the prodrug of 5-fluoro-2′-deoxyuridine monophosphate), methotrexate (MTX) and raltitrexed (Fig. 1), are classical TS inhibitors belonging to the antimetabolite class. In our previous work, compound **α156** (Fig. 1) had been proposed as an antibacterial lead able to face vancomycin-resistant infections caused by gram positive bacteria such as *Staphylococcus aureus*. This molecule also inhibited the *Enterococcus faecalis* clinical isolates and showed a low toxicity towards human cells [7]. In a parallel work we had identified a library of phthalimidic derivatives, 4-methylisoindoline-1,3-dione *N*-derivatives (**I,** Fig. 1) and 4-(aminomethyl)-2-methylisoindoline-1,3-dione derivatives substituted at the amino position (**II**, Fig. 1), that presented inhibitory activity against bacterial TS enzymes. These phthalimidic derivatives inhibited also *Enterococcus faecalis* TS (*Ef*TS), with the best inhibitors showing K_i in the range of 2–8 μM. These compounds exhibited a competitive behavior with respect to the folate cofactor and a significant selectivity towards bacterial TS [8]. *Ef*TS X-ray crystal structure of the native protein had been obtained [14]. According to structural analysis, the protein crystallized in a ternary complex where a folate substrate analogue, 5-formyl-tetrahydrofolate (5-FTHF), originating from the bacterial metabolites pool, co-crystallized with the enzyme. Thus, we can conclude that *Ef*TS could be considered a reservoir for folate substrates in the bacteria folate dependent metabolism. Although no structural information about *Ef*TS-inhibitors complexes are available yet, we could suppose that the target druggability is influenced by the presence of the folate metabolites inside the bacterial cell and that higher affinity inhibitors are necessary to displace 5-FTHF. Aiming to obtain X-ray crystallographic structures to gain additional information on *Ef*TS target druggability and to carry out further structure-based drug design studies, we analyzed our in-house library based on a *N*-substituted phthalimide scaffold bearing a flexible chain linked to the nitrogen in position 2. In details, 56 compounds (Charts 1 and 2) were tested against *Ef*TS, *Ec*TS and human TS (hTS). In the present work, X-ray crystallographic screenings were carried out and one X-ray crystallography complex with a protein inhibitor, (*S*)-**12**, was obtained. In addition, we solved the X-ray crystal structure of *Ef*TS in complex with methotrexate (MTX), a known folate analogue. These studies represent an important starting point for a structure-based drug design aimed at identifying new antibacterial agents against *Enterococcus faecalis* infections.

2. Results and discussion

2.1. Library selection and compound synthesis

With the aim of exploring the chemical space of the phthalimide core, a library of 56 compounds (Charts 1 and 2) was selected from our in-house libraries (for details see experimental section), previously synthesized as intermediates for the preparation of compounds with different pharmacological activities [15–33]. These compounds underwent *in vitro* screening towards *Ef*TS, hTS and *Ec*TS. All the molecules of the new library show an *N*-substituted phthalimide ring (**III**, Fig. 1). The majority of the compounds have an aromatic ring in the side-chain linked to position 2 of the phthalimide moiety. In our previously published library the aromatic moiety in the side-chain was directly linked to the nitrogen in position 2 (**I**, Fig. 1), whereas in the present work almost all the compounds have a spacer between the phthalimide core and the aromatic system of the side-chain, thus being more flexible. Few compounds (**27**–**31, 34, 56**) have a non-aromatic side-chain with a lower steric hindrance. This novel phthalimide library includes several chiral compounds (**1**–**8, 12, 13, 17, 21, 27**–**34, 40**–**43, 50, 51, 56**) and for some of them we explored the biological profile of both the racemic mixture and the single enantiomers.

2.2. Chemistry

Only synthetic procedures followed to obtain compounds not previously characterized in literature are described in this paper. The synthesis of the racemic mixture and of the enantiomers of compound **8** is shown in Scheme 1. Ester (*RS*)-**60** was obtained following Williamson reaction of (*RS*)-bromopropanoate, (*RS*)-**58** with 2,6-dimethylphenol, while (*R*)- and (*S*)-**61** were obtained by Mitsunobu reaction [34,35] of 2,6-dimethylphenol with (*S*)- or (*R*)-methyl lactate [(*S*)- or (*R*)-**59**], respectively. After esters reduction, the obtained alcohols **61** were submitted to Mitsunobu reaction with phthalimide to give the desired compounds. Compound **13** was obtained through hydrolysis of compound **12** (Scheme 2). The synthesis of compounds (*S*)-**17,** (*S*)- and (*R*)-**33** is depicted in Scheme 3. (*S*)- and (*R*)-**33** were obtained by protecting (*S*)- and (*R*)-2-amino-2-phenylethanol, respectively, with phthalic anhydride. Iodination of compound (*S*)-**33** yielded compound (*S*)-**17**. Compounds **20** and **22** were synthesized as reported in Scheme 4. Cyano derivative **18** was converted into the corresponding trimethyltintetrazole derivative **62** by reaction with azido-trimethyltin. The treatment of compound **62** with gaseous HCl gave tetrazole derivative **22**, which was alkylated with methyl iodide to give compound **20**. Compound **24** was obtained by reacting 2-(methoxycarbonyl)benzoic acid with *p*-toluidine in presence of EEDQ as previously described (Scheme 5) [36]. The nitration of (*R*)-**63**, followed by reduction of the nitro group gave (*R*)-**7** and, successively, (*R*)-**43** (Scheme 6), as reported in the literature [26,37,38]. Compounds **25** and **55** were prepared by protecting 4-phenylbutan-1-amine with phthalimide or tetrachlorophthalimide, respectively (Scheme 7). The synthesis of compound **52** is reported in Scheme 8. 3-aminopropanol was protected with phthalic anhydride to give compound **64,** which was submitted to Mitsunobu reaction with 1-naphthol. Compound **65** (Scheme 9) was synthesized by the reaction of 3-aminopropanol with tetrachlorophthalic anhydride and converted into compound **53**. The synthesis of compound **54** is depicted in Scheme 10. Williamson reaction on 3-bromopropanol with thiophenol gave compound **67** which was converted into the thioether **55**. Finally, the synthesis of (*R*)-**1** was obtained via a Mitsunobu reaction starting from (*S*)-**68** (Scheme 11).

2.3. Biological evaluation of the compounds library

The selected 56 compounds were tested against *Ef*TS and hTS enzymes in order to evaluate the inhibitory activity towards TS and the specificity towards the bacterial enzyme (Fig. 2, Table S1, see Supplementary Material). The percentage of inhibition (I %) at 100 μM are reported as heat-map plot in Fig. 2 in which different color codes represent the inhibition range of the compounds. Moreover, the inhibitory activity towards *Escherichia coli* TS (*Ec*TS) was assessed, aiming to explore a larger biological activity profile. Several compounds of the library are chiral, thus they were tested as racemic mixture and/or as homochiral. Thirteen compounds (**4, 7, 11, 18, 20, 23, 35, 39, 40, 45, 47, 52** and **54**) show inhibitory activity towards *Ef*TS (I % = 20–60% at 100 μM). Compounds **18**, (*R*)-**40** and **54**, with K_i of 25, 13 and 24 μM, respectively, turned out to be the best inhibitors of the library. Moreover, they were species-specific inhibitors of *Ef*TS, since they were inactive at 100 μM towards hTS. Few compounds (*RS*)-**1**, (*R*)-**7**, (*R*)-**8**, **9**, **14**, **16**, **24, 25** were able to decrease the *Ec*TS activity by 35–48% at 100 μM and represent the most potent *Ec*TS inhibitors of this set of compounds. In our previous paper [8], we studied the inhibitory activity towards *Ef*TS of more rigid phthalimide derivatives (**6A**, **8A**, **12A**,