Bioorganic & Medicinal Chemistry Letters 19 (2009) 2668–2673

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of novel benzene 1,3-dicarboxylic acid inhibitors of bacterial MurD and MurE ligases by structure-based virtual screening approach

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ARTICLE INFO

Article history:
Received 16 February 2009
Revised 25 March 2009
Accepted 27 March 2009
Available online 1 April 2009

Keywords:
MurD and MurE enzymes
Virtual screening
Three-dimensional structure-based pharmacophores
Molecular docking
Antibacterial agents
Drug design

ABSTRACT

The peptidoglycan biosynthetic pathway provides an array of potential targets for antibacterial drug design, attractive especially with respect to selective toxicity. Within this pathway, the members of the Mur ligase family are considered as promising emerging targets for novel antibacterial drug design. Based on the available MurD crystal structures co-crystallised with *N*-sulfonyl glutamic acid inhibitors, a virtual screening campaign was performed, combining three-dimensional structure-based pharmacophores and molecular docking calculations. A novel class of glutamic acid surrogates—benzene 1,3-dicarboxylic acid derivatives—were identified and compounds **14** and **16** found to possess dual MurD and MurE inhibitory activity.

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The increasing incidence of bacterial resistance to most of the available antibiotics has rendered the discovery of novel efficacious antibacterial agents imperative, suggesting the consideration of novel, previously unexploited targets.[1,2] An essential component unique to prokaryotic cells—the peptidoglycan—is traditionally a target of choice with respect to selective toxicity.[3–5] The biosynthesis of the peptidoglycan is a complex multistage process divided into an early stage of intracellular assembly of the UDP-Mur*N*Ac-pentapeptide, followed by a translocation step on the outercellular side and final incorporation into the nascent biopolymer.[3–5] Properly constructed peptidoglycan provides rigidity, flexibility and strength that are necessary for bacterial cells to grow and divide, while withstanding high internal osmotic pressure.[6]

The four members of the ADP-forming bacterial ligase family (the synthesis of the peptide bond is coupled with a concurrent hydrolysis of the ATP molecules to ADP and P_i)—MurC, MurD, MurE and MurF—are involved in the intracellular phase of peptidoglycan assembly, catalysing the synthesis of the peptide moiety by consecutive addition of L-Ala, D-Glu, *meso*-A_2pm (or L-Lys) and D-Ala-D-Ala to the UDP-*N*-acetylmuramic acid (UDP-Mur*N*Ac), the starting UDP-precursor.[3,7]

The Mur ligase family is considered a prime example of modular structure in protein architecture, with molecules made up of three domains or modules allowing for molecular recognition of a specific UDP-substrate.[3,7] The three-domain structure of the family comprises an *N*-terminal domain responsible for binding the UDP-substrate, a central domain bearing resemblance to the ATP-binding domains of a number of ATP- or GTP-ases and a C-terminal domain, which is involved in the binding of the incoming amino acid.[7] Structural investigations of the Mur ligase family have resulted in the identification of different protein conformations, making the whole family a complex collection of dynamic protein targets.[7,8] All Mur ligases presumably act through an analogous sequential enzymatic mechanism, as corroborated by structural,[9] biochemical[10] and computational studies.[11] In the proposed mechanism, the bound UDP-precursor **1** initially reacts with the ATP molecule yielding an acyl-phosphate intermediate,[10] which, following addition of the incoming amino acid, affords the tetrahedral reaction intermediate **2**.[9,11] Finally, the dissociation of the phosphate group results in a new UDP-precursor elongated by the condensed amino acid **3**.[9]

The attraction of the Mur ligase family as a collection of emerging drug targets has resulted in the design of several ligase inhibitors.[3,12–17] A frequently used starting point in the design of MurD (UDP-N-acetylmuramoyl-L-alanyl:D-glutamate ligase)

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doi:10.1016/j.bmcl.2009.03.141

enzyme inhibitors has been a hypothetical structure of the MurD tetrahedral reaction intermediate **2**.[3,9,11] (Fig. 1) From this starting point, low-molecular-weight analogues (e.g., **4–6**) of the MurD tetrahedral intermediate structure, have been published, typically classified as phosphinate **5**[18] and *N*-sulfonyl derivatives **4**[19,20] of glutamic acid. (Fig. 1). Surprisingly, several studies have demonstrated that MurD enzyme, despite its intrinsic preference for incorporation of the D-Glu amino acid, can be successfully inhibited by L-Glu-containing inhibitors.[19,20] In recent work, the first crystal structures of the MurD enzyme bound with the *N*-sulfonyl glutamic acid inhibitors **4** have provided the binding mode of this class of inhibitors.[19,20] Binding free energies calculated by the Linear Interaction Energy (LIE) approach revealed non-polar van der Waals interactions as the main driving force responsible for the binding of these inhibitors.[21]

Several phosphinate inhibitors[22] (e.g., compound **5**) and ß-sulfonamidopeptide **6**,[23] all designed as MurD tetrahedral intermediate analogues, have displayed significant inhibitory activity on the subsequent enzyme in the ligase series—MurE (UDP-N-acetylmuramoyl-L-alanyl-D-glutamate:L-lysine ligase)—indicating that the 'product-like' structures of the MurD inhibitors containing the glutamate moiety can also act as substrate-based MurE inhibitors or even dual inhibitors of both ligases.[22,23] Furthermore, structural comparison of the MurD and MurE active sites have revealed several MurE residues, among them Asp209, His359 and Arg389, that all have their corresponding residues in the MurD enzyme (Asp182, Thr321, Lys348), where they play an important role in the binding of D-glutamate, providing further rationalisation for the observed behaviour of several glutamic acid-based compounds (see superposition of MurD/MurE enzyme in the Supplementary data).[24] All these observations underline a strong potential for the MurD tetrahedral intermediate structure in the design of dual or single (product-based or substrate-based) MurD/MurE inhibitors.

In our ongoing efforts to understand the structure and function of the bacterial ligase family[8,11,21] at the atomic level and identify novel promising inhibitors as potential antibacterial agents, we designed an in silico virtual screening (VS) campaign taking the available MurD crystal structures with *N*-sulfonyl-glutamic acid inhibitors with the general structure **4**, mimicking the tetrahedral intermediate,[19,20] as our starting point. The prime objectives of the VS campaign were identification of novel glutamic acid-based inhibitors and screening for novel rigid replacements of the glutamic acid moiety. Considering the experimental observations implying a strong potential of either dual inhibitory MurD/MurE[22] or single MurE activity[23] of the MurD tetrahedral intermediate-based analogues, all selected VS hits were assayed against both MurD/MurE enzymes.

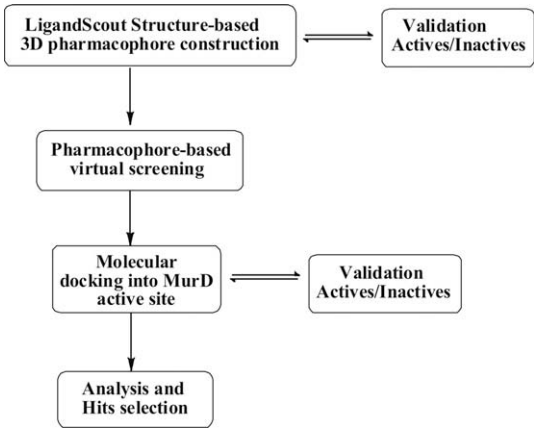


Figure 2. Outline of the virtual screening (VS) workflow used in the identification of novel MurD/MurE ligase inhibitors.

The virtual screening protocol used in our VS campaign is schematically presented in Figure 2. It comprises two screening procedures: initial pharmacophore-based screening, with subsequent refinement of the obtained hits by molecular docking calculations. In the first step of the VS workflow, the available crystal structures of the MurD enzyme (PDB codes: 2JFH, 2JFF, 2UUO, 2UUP)[19,20] with bound *N*-sulfonyl-glutamic acid inhibitors were used in automatic generation of the three-dimensional structure-based pharmacophores using LigandScout software—a powerful structure-based pharmacophore generator.[25]

Aligned 3D structure-based pharmacophores[26] of these ligands are depicted in Figure 3A, and the constructed LigandScout shared-feature pharmacophore used for the subsequent pharmacophore-based virtual screening is shown in Figure 3B. In this pharmacophore model, negative ionisable areas were used to model the negatively charged electrostatic features of the glutamate carboxylic groups, together with two hydrophobic interaction spheres, reflecting the naphthalene and O-substituted lipophilic moieties characteristics of the inhibitors. Exclusion volume spheres were automatically derived from the sterical circumference of the MurD active site

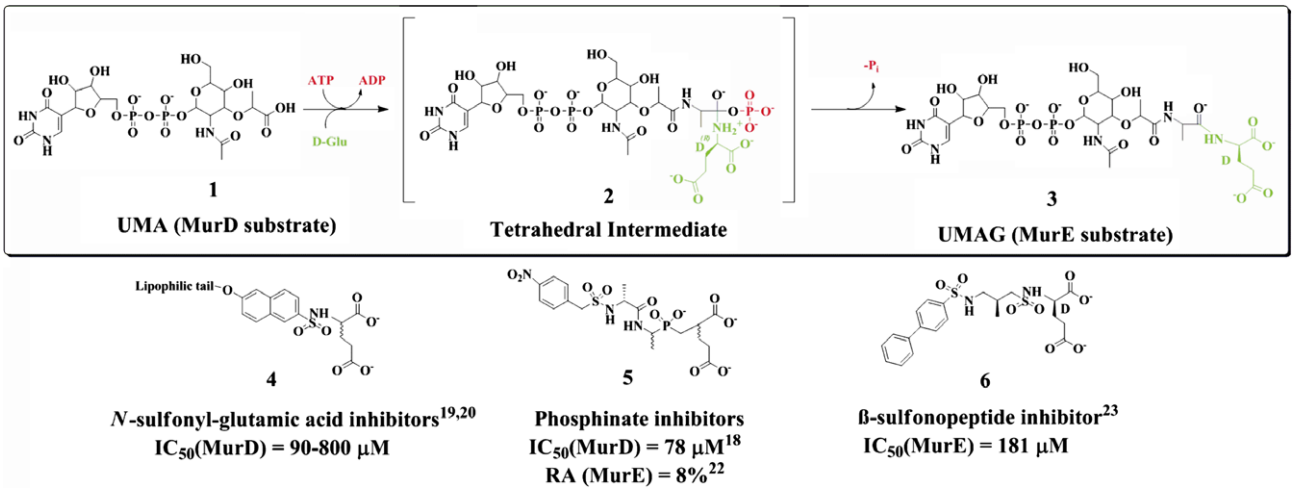


Figure 1. Amide-forming reaction,[9] catalysed by the MurD enzyme and chemical structures of *N*-sulfonyl-[19,20] and phosphinate-[18,22] glutamic acid inhibitors of MurD and MurE[18,19] enzymes designed by considering the structure of the MurD tetrahedral intermediate.

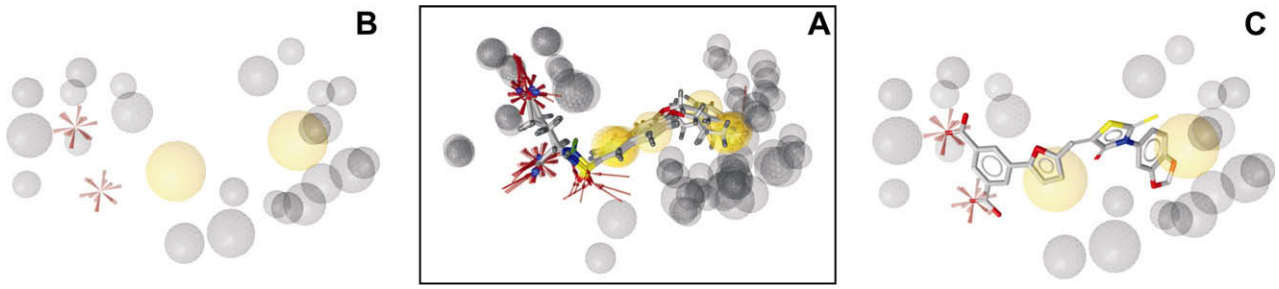


Figure 3. (**A**) Aligned LigandScout[25] 3D structure-based pharmacophores with the corresponding ligands (PDB codes of ligands: 2JFF, 2JFH, 2UUO, 2UUP)[19,20] (**B**) LigandScout 3D structure-based pharmacophore used in Catalyst validation and virtual screening procedure. (**C**) Catalyst-generated conformation of the virtual hit **14** aligned to the LigandScout pharmacophore.

and four additional exclusion volume spheres were created to better account for the spatial constraints of several amino acid side chains (Phe161, Arg37, His183, Ile416) in the MurD active site.

Following recommended practice in VS campaigns, the generated LigandScout pharmacophore was validated for its ability to identify known hit compounds.[27] Pharmacophore modelling tool Catalyst,[28] used in the large scale screening of commercially available compounds, was used for this step. Thirty published MurD compounds[18–20,22,23] were selected for the validation study: 9 active compounds (*N*-sulfonyl-, *N*-carbonyl and phosphinate-glutamic acids) and 21 inactive compounds of the same compound classes. This validation set was screened against the LigandScout pharmacophore, converted into the Catalyst format, using the fast flexible alignment method.[28] It was gratifying to observe that the LigandScout-generated pharmacophore was able to retrieve 8 out of 9 active compounds in the Catalyst environment, producing geometries that were in good agreement with the available structural data. Only 6 out of 21 structurally very similar inactive compounds were recognised by Catalyst. Since the structures of the active and inactive compounds in the validation set were extremely similar, with only subtle structural features distinguishing them (see structures in Tables 1S and 2 of Supplementary data), the construction of a selective pharmacophore was extremely demanding. The constructed pharmacophore used in VS campaign was selective enough to identify virtually all known active compounds and did not result in too many false positives, thus being suitable for large scale screening calculations.[27] Furthermore, the pharmacophore validation results provided confidence in the generated LigandScout pharmacophore model for subsequent screening. (see Supplementary data for details on pharmacophore validation).

Pharmacophore-based VS of a large database of the commercially available compounds (approximately 2 million compounds)[29] was carried out using the Catalyst fast flexible alignment method. The conformation of each hit with the highest FIT value was exported for visual inspection and further analysis. The Catalyst VS screening procedure retrieved 516 hit compounds. Several chemical classes were identified among them: glutamic acid derivatives and, interestingly, compounds bearing the benzene 1,3-dicarboxylate moiety, which neatly fitted the negatively charged pharmacophore features, indicating the identification of a potential novel surrogate for the required dicarboxylic function of the inhibitor. In Figure 3C, the conformation of the Catalyst virtual hit (compound **14**) containing the benzene 1,3-dicarboxylate moiety is displayed, aligned on the LigandScout pharmacophore.

Obtained Catalyst hits were subsequently docked into the MurD enzyme active site using the GOLD molecular docking package.[30] The protein structure 2JFF was used to construct the MurD active site, which was defined around experimental coordinates of the bound inhibitor in the 2JFF structure, resulting in a cavity radius of 13.3 Å. (Fig. 4A) Each molecule was docked 10 times into the binding site by applying the following parameters of the GOLD genetic algorithm (GA) (Population size = 100, Selection pressure = 1.1, No. of Operations = 100,000, No. of Islands = 5, Niche Size = 2, Migrate = 10, Mutate = 95, Crossover = 95). Early termination was allowed if the top 3 solutions were within 1.5 Å of the RMSD value. Two available scoring functions, GOLDscore and CHEMscore, were used.[30] The quality of the generated binding poses, as well as the scoring function assessment (see Supplementary data for details), were validated on a set of 20 glutamic acid inhibitors, (*N*-sulfonyl and *N*-carbonyl derivatives), where the available structural data[19,20] permit a satisfactory validation procedure.[27]

The results of the docking validation procedure showed that both scoring functions yielded ligand binding geometries that were in excellent agreement with the observed binding modes,[19,20] without introducing any constraints to the docking procedure. The set of active compounds was mostly ranked with higher

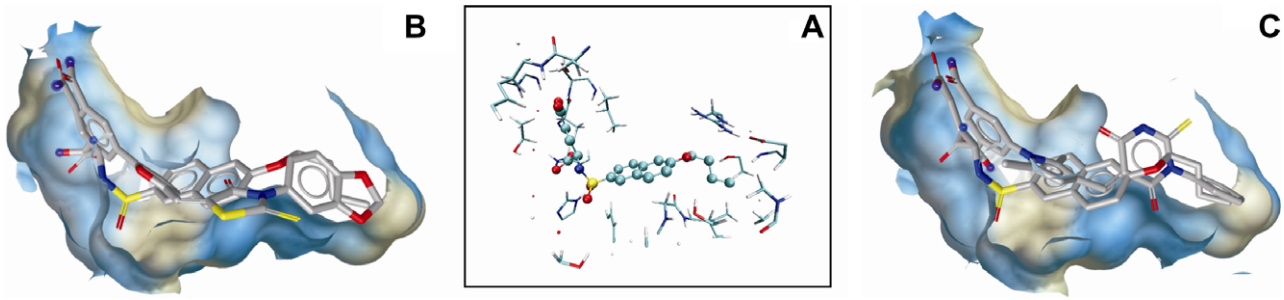


Figure 4. (**A**) Representation of the MurD active site defined around the bound *N*-sulfonyl-glutamic acid inhibitor in a 2JFF crystal structure.[19] (**B**, **C**) GOLD-calculated binding poses of VS hit compounds **14** and **16** docked into the 2JFF binding site. Experimental binding mode of the bound *N*-sulfonyl-glutamic acid inhibitor in 2JFF crystal structure is shown for comparison.

Table 1
Selected hit compounds from the VS campaign with determined values of inhibitory activity on the MurD and MurE enzymes

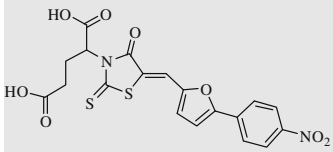
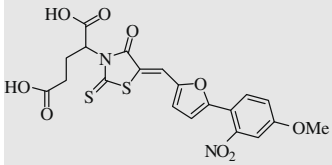
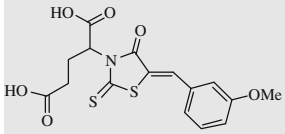
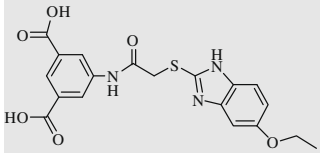
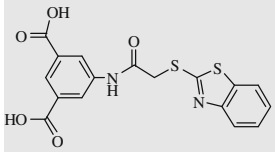
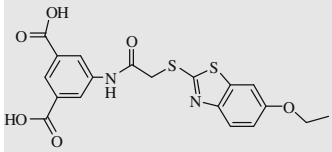
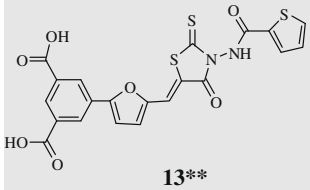
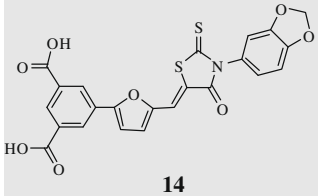
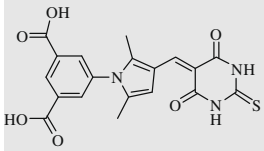
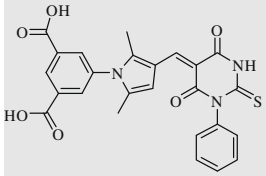
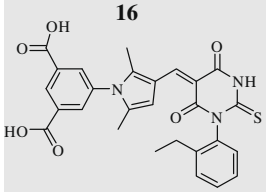
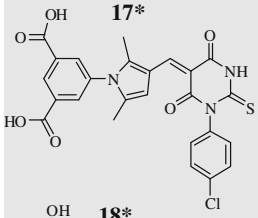
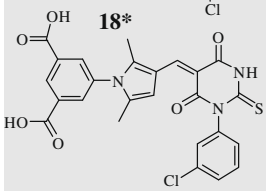
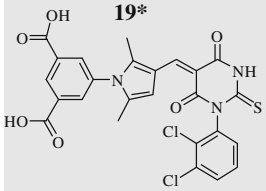
Chemical structure	IC_{50} [μM] or RA (%) MurD	IC_{50} [μM] or RA (%) MurE
 **7***	74%	61 μM
 **8***	87%	70%
 **9***	98%	89%
 **10***	89%	95%
 **11***	97%	94%
 **12***	98%	84%
 **13****	82%	81%
 **14**	270 μM	32 μM

Table 1 (*continued*)

 **15***	99%	76%
 **16**	690 μM	89 μM
 **17***	92%	55%
 **18***	69%	97 μM
 **19***	90%	104 μM
 **20***	83%	79 μM

* Residual activities on both enzymes were measured in the presence of 100 μM of each inhibitor.
** Residual activities on both enzymes were measured in the presence of 250 μM of each inhibitor.

scores; however, some of the inactive representatives were also favourably scored. In general, the performance of the two scoring functions was fairly comparable on this system, and both were therefore utilised in molecular docking experiments to further refine hits obtained in the Catalyst screening (see Supplementary data for details on GOLD validation).

In the following molecular docking of 516 Catalyst hits using GOLD, the obtained binding geometries were visually inspected. The selected compounds **7**–**20** were purchased and assayed in a standard Malachite green assay[31–33] against MurD and MurE enzymes detecting the orthophosphate generated during the reaction (see Supplementary data for the compounds' origin details and vendors quality control procedures). To exclude possible non-specific (promiscuous) inhibition, all compounds were tested in a presence of a detergent (0.005% Triton X-114).[34] Results for the selected assayed hits are presented in Table 1, either as residual

activity (RAs) measured at the presence of a 100 μM (or 250 μM) concentration of each inhibitor or as IC_{50} values.

VS hit compounds **7**–**9** represent glutamic acid derivatives, where glutamic acid is connected directly on the 5-membered 4-oxo-2-thioxothiazolidine—the rhodanine moiety. Interestingly, the structurally comparable 5-benzylidenerhodanine moiety was recently successfully incorporated into novel MurD inhibitors, albeit not being directly bound to the glutamic acid.[35] None of the compounds displayed significant inhibitory activity on the MurD system (RA values of 75–98% at 100 μM concentration of each inhibitor). On the other hand, glutamic acid derivative **7**, where the rhodanine moiety was connected via the methylene bridge with the 4-nitrophenyl-furan, was found to be a MurE inhibitor (IC_{50} = 61 μM), providing another active glutamic acid-based compound, analogous to the identified β-sulfonamidopeptide **6** (IC_{50} = 118 μM).[23]

Several compounds with benzene 1,3-dicarboxylate moiety acting as a rigid replacement of the glutamic acid functionality were tested in the MurD/MurE Malachite green assays. Compounds **10–12** included a more flexible acyclic linker, which connected the dicarboxylate with its hydrophobic structural elements. The measured RA values of 80–100% revealed these compounds were inactive.

Benzene 1,3-dicarboxylate VS hits **13**–**20** were restrained to a more rigid scaffold configuration. Compound **14**, in which the benzene 1,3-dicarboxylate is connected via the furan linker with a substituted rhodanine moiety, was identified as the best hit of the VS campaign exhibiting promising dual inhibition of the MurD (IC_{50} = 270 μM) and MurE enzyme (IC_{50} = 32 μM). The GOLD-calculated bound conformation of **14** in the MurD active site (PDB: 2JFF) is depicted in Figure 4B, with the experimentally determined conformation of the glutamic acid-based inhibitor shown for comparison. According to GOLD calculations, carboxylic functional groups of **14** formed hydrogen bond interactions with the MurD amino acid residues, reproducing the identified H-bond pattern of the *N*-sulfonyl-glutamic acid inhibitors,[19,20] thus additionally demonstrating its potential utilisation as a rigid surrogate of the carboxylic function. One of the carboxylic groups of **14** interacted with Phe422 and Ser415, and the other with Lys348 and Thr321 in the MurD binding site. The furan moiety did not exhibit any specific interactions with the MurD binding site. In addition, the sulfur atom of the rhodanine moiety interacted with backbone nitrogen of Leu13.

VS hit compounds **15**–**20** were fairly analogous to compound **14** with a 2,5-dimethylpyrrol linking moiety connecting the benzene 1,3-dicarboxylate with the substituted 1-phenyl 2-thioxodihydropyrimidine-4,6-diones. Compound **16** was identified as the most promising dual inhibitor of MurD (IC_{50} = 690 μM) and MurE (IC_{50} = 89 μM) enzyme in this compound class. The presence of the 1-phenyl-substitution appeared to be vital for the inhibitory activity, as the non-substituted derivative **15** was found to be a poor inhibitor on both ligases (RAs 99% and 76%, respectively). GOLD-calculated bound conformation of **16** in the 2JFF MurD active site is depicted in Figure 4C. Both carboxylic groups of the benzene 1,3-dicarboxylate were positioned similarly, as in the bound conformation of **14**. The 2-thioxodihydropyrimidine-4,6-dione moiety of **16** was positioned less favourably compared to the rhodanine moiety in compound **14** for formation of hydrogen bonds with the MurD active site, which correlated with the measured lower MurD inhibition. The favourable position of the phenyl moiety, firmly anchored into the MurD uracyl binding pocket,[9] was in agreement with the importance of this moiety for successful MurD inhibition. This structure-activity relationship was comparable with the *N*-sulfonyl-glutamic acid inhibitors, where the presence of the O-substituted lipophilic group in this area of the MurD active site contributed about 1 kcal/mol to the overall binding free energy[21] and was vital for optimal inhibition.[20] VS compounds **17**–**20**, being phenyl-substituted derivatives of **16**, were all found to be MurE inhibitors, with IC_{50} values between 79 μM and 104 μM.

An important issue with several identified compounds (compounds **9** and **13**–**19**) was the configuration of the exocyclic double bond connecting the heterocyclic core with the rest of the molecule. For compound **9** the experimental evidence of the *Z* relative configuration was available. For the compounds **13–20** the configuration was not determined and was treated as a mixture of both conformers. The double bond configurations shown in Table 1 denote the configurations used throughout the virtual screening campaign. Similarly, in the case of the selective glycosyltransferase MurG inhibitors discovered via high-throughput screening, commercially available compounds, incorporating either thioxo dihydropyrimidine-4,6-diones or rhodanines lacked the double configuration determination as well.[36]

Previously, a representative collection of the D-Glu derivatives was tested against a battery of reference bacterial strains (*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa*) and the results are shown in Table 4S of the Supplementary data. No antibacterial activity below MIC = 128 μg/ml could be detected in any of the strains.[39]

In summary, by applying structure-based drug design techniques combining pharmacophore screening and molecular docking calculation, a novel rhodanine-containing glutamic acid-based MurE inhibitor **7** was discovered. Moreover, the VS campaign disclosed a novel class of promising dual inhibitors of MurD and MurE, enzymes **14** and **16**, possessing a benzene 1,3-dicarboxylate moiety acting as a rigid replacement of the glutamic acid function. The two screening procedures were validated by a set of experimental data ensuring the standard quality[27] of the produced pharmacophore and docking models. The disclosed structures represent an attractive alternative to the established glutamic acid moiety strategy[3] and provide novel promising leads that could by further optimization of this presented series (including the optimization of the dicarboxylate moiety) result in the promising novel compounds for the much-needed antibacterial drug design.[37,38]

Acknowledgements

This work was supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia and the European Commission through the EUR-INTAFAR project (LSHM-CT-2004-512138).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.141.

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