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Mur ligases inhibitors with azastilbene scaffold: Expanding the structure–activity relationship

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ABSTRACT

Antibiotic resistance represents one of the biggest public health challenges in the last few years. Mur ligases (MurC–MurF) are involved in the synthesis of UDP-*N*-acetylmuramyl-pentapeptide, the main building block of bacterial peptidoglycan polymer. They are essential for the survival of bacteria and therefore important antibacterial targets. We report herein the synthesis and structure–activity relationships of Mur ligases inhibitors with an azastilbene scaffold. Several compounds showed promising inhibitory potencies against multiple ligases and one compound also possessed moderate antibacterial activity. These results represent a solid ground for further development and optimization of structurally novel antimicrobial agents to combat the rising bacterial resistance.

Antibiotic resistance represents one of the biggest public health challenges in the last few years. Common infections, such as pneumonia, tuberculosis, gonorrhea are becoming harder or even impossible to treat.[1] Appropriate use of antibacterial agents combined with high hygiene standards are of utmost importance to reduce the spread of resistance.[2,3] Additionally, the development of new antibacterial drugs with previously unaddressed mechanisms of action is necessary. Proposedly, such drugs would have minimized susceptibility to the pre-existing mechanisms of resistance known for currently used antibiotics.[4–6]

Peptidoglycan is an essential component of the bacterial cell wall, composed of interchanging units of *N*-acetylglucosamine and *N*-acetylmuramic acid,[7] and represents a rich source of targets for novel drug development.[8] Mur ligases, namely MurC, MurD, MurE and MurF, are the family of enzymes responsible for the formation of a peptide bond between the UDP-containing substrate and the incoming amino acid, leading to the formation of UDP-*N*-acetylmuramyl-pentapeptide, the main building block of peptidoglycan polymer.[7] Mur ligases are essential for bacterial cell survival and are conserved among different bacteria with no human homologues.[7,8] They consist of a three domain topology and share a similar reaction mechanism. The *N*-terminal domain is responsible for the binding of the UDP-precursor, the central domain for the binding of ATP and the C-terminal domain for the binding of condensing amino acid or dipeptide.[9] These structural and mechanistic similarities in the Mur ligase pathway offer possibilities for inhibition of multiple enzymes simultaneously[10] and represent a promising approach to combat bacterial resistance, since mutations provoking resistance would have to occur concurrently in at least two genes.[11]

In our previous study the Published Kinase Inhibitor Set (PKIS) was assayed against Mur ligases from *Escherichia coli*.[12] Four structurally new scaffolds of ligase inhibitors were identified. Compound **1**, an azastilbene derivative (Fig. 1), was chosen for further mechanistic studies as the most efficient ligand among the four hits according to ligand efficiency parameter (LE (MurD) = 0.21, LE (MurF) = 0.22). Steady-state kinetics and NMR studies were performed on MurD revealing that compound **1** acts as a competitive inhibitor of MurD with respect to D-Glu. The kinetic data were confirmed with NMR measurements, where compound **1** perturbed the signals of the Leu416 methyl groups, indicating the binding of the inhibitor specifically into the D-Glu pocket.[12]

We report here a concise series of compound **1** analogues to further explore the structure–activity relationships of the aza-stilbene class of

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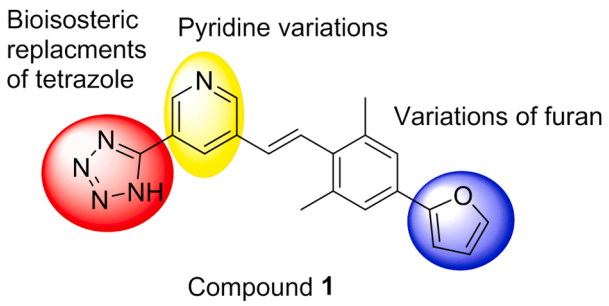


Fig. 1. Schematic representation of structural modifications of compound **1**.

Mur ligase inhibitors. Structural modifications led to improved inhibitory potencies against several Mur ligases. Importantly, moderate antibacterial activity against *Staphylococcus aureus* was also shown for some derivatives.

To gain further understanding of the enzyme-inhibitor interaction, a computational analysis of compound **1** binding to MurD supported by Hybrid docking program (OpenEye Scientific Software[13]) was carried out using the binary complex of MurD with the product UDP-*N*-acetylmuramoyl-L-alanine-D-glutamate (UMAG) (PDB code: 4UAG). The 3D binding modes of compound **1** and UMAG are presented in Fig. 2. Analysis of the docking results revealed two major binding modes for compound **1**, **A** and **B**. Both binding modes adopted conformations in which the tetrazole moiety occupies the D-Glu binding pocket but differs in the orientation of the furan ring. In binding mode **A,** the furan ring was oriented towards the uracil binding pocket, while in binding mode **B** it was oriented towards the glucosamine binding pocket. Both conformations formed several interactions with the surrounding amino acids. These results provide a structural explanation for the inhibitory activity of compound **1** against MurD and give a basic cue for possible inhibitor **1** modification to increase its potency. It is evident from both docking modes that the binding site is not fully occupied by compound **1** and that only tetrazole ring forms several strong H-bond interactions with the enzyme. Pyridine, phenyl and furan moieties are not optimally positioned, they form only a couple of weak interactions with the enzyme and thus leave plenty of possibilities for the optimization of the binding affinity.

The low molecular weight of starting compound **1** and its simplistic structure that tolerates numerous structural variations enabled us to explore the chemical space and to assess the importance of the individual moieties for the inhibition of Mur ligases. Three key fragments were altered systematically to explore the chemical space: the tetrazole moiety, the pyridine central part, and the furan ring (Fig. 1). First,

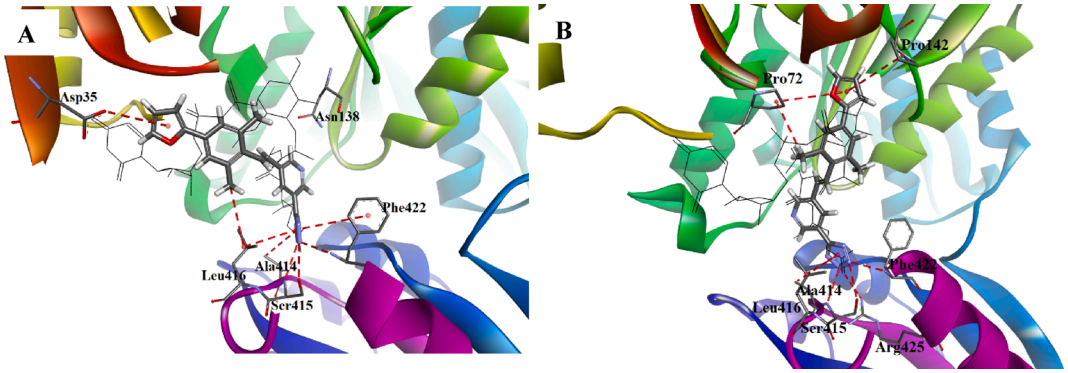
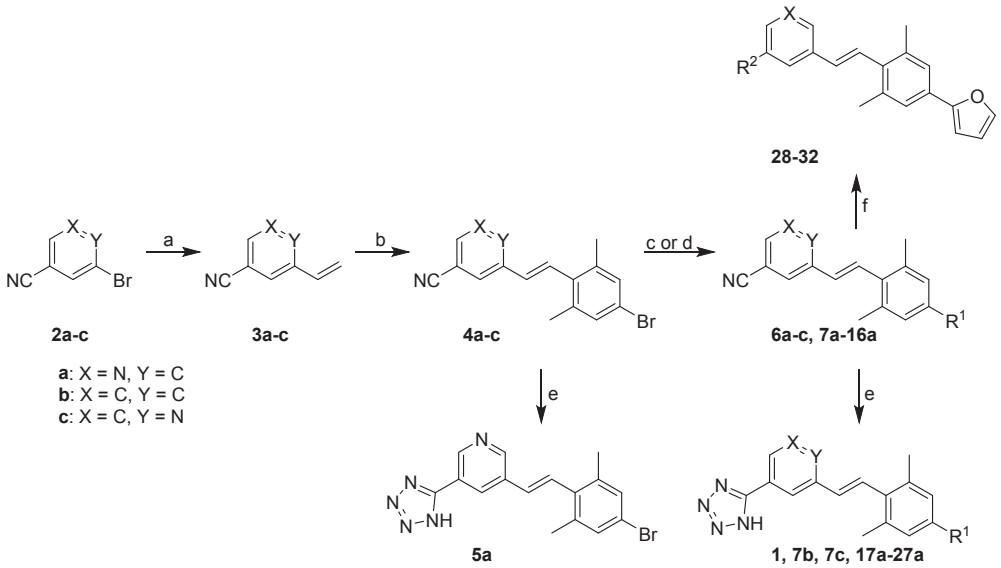
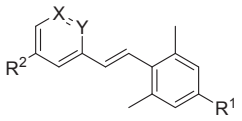


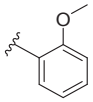
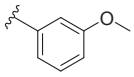
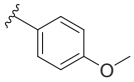
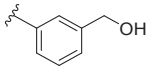
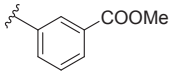
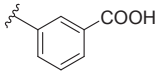
Fig. 2. The predicted binding modes **A** and **B** of compound **1** and UMAG bound to MurD. The amino acids surrounding the ligand are shown in stick presentation and the protein structure is presented in rainbow colours. Interactions with the surrounding amino acids are presented as red dotted lines.



Scheme 1. Reagents and conditions: (a) tributyl(vinyl)tin, LiCl, $Pd(PPh_3)_2Cl_2$, DMF, 70 °C; (b) 5-bromo-2-iodo-1,3-dimethylbenzene, Pd_2dba_3, Et_3N, P(*o*-Tol)$_3$, DMF, 95 °C; (c) corresponding boronic acids, K_2CO_3, $Pd(PPh_3)_4$, H_2O, THF, 100 °C; (d) 2-piperidinone or pyrrolidine, $Pd(OAc)_2$, Xantphos, Cs_2CO_3, dioxane, 100 °C; (e) NH_4Cl, NaN_3, anhydrous DMF, 110 °C; (f) various conditions, procedures described in Supporting Information (SI).

Table 1
% of inhibition and IC_{50} values for the most potent stilbene inhibitors against Mur ligases (MurC, MurD, MurE and MurF) and their antibacterial activities against *E. coli* and *S. aureus*.



Cpd	X	Y	R^1	R^2	MurC		MurD		MurE		MurF		MIC[c] [mM]	
					%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	SA	EC
1	N	C			30%	/	55%	104	51%	79	74%	59	>0.25	>0.25
5a	N	C	-Br		30%	/	46%	131	38%	/	32%	/	0.25	>0.25
6a	N	C		–CN	22%	/	27%	/	35%	/	21%		>0.25	>0.25
17a	N	C			35%	/	42%	104	33%	/	0%	/	0.125	>0.25
18a	N	C			15%	/	23%	/	21%	/	18%	/	>0.25	>0.25
19a	N	C			30%	/	30%	/	37%	/	25%	/	0.25	>0.25
20a	N	C			25%	/	14%	/	23%	/	24%	/	>0.25	>0.25
21a	N	C			10%	/	10%	/	4%	/	0%	/	>0.25	>0.25
22a	N	C			14%	/	9%	/	19%	/	15%	/	>0.25	>0.25
23a	N	C			16%	/	22%	/	18%	/	12%	/	>0.25	>0.25
24a	N	C			15%	/	16%	/	23%	/	16%	/	>0.25	>0.25
25a	N	C			33%	/	26%	/	39%	/	18%	/	>0.25	>0.25
26a	N	C			15%	/	14%	/	30%	/	38%	/	>0.25	>0.25
27a	N	C			22%	/	1%	/	16%	/	29%	/	0.25	>0.25
7b	C	C			5%	/	64%	107	30%	/	21%	/	>0.25	>0.25
7c	C	N			23%	/	73%	56	55%	97	84%	17	>0.25	>0.25
28	N	C			43%	153	70%	64	27%	/	47%	109	>0.25	>0.25
29	N	C			20%	/	40%	168	51%	156	76%	31	>0.25	>0.25
30	N	C			44%	153	45%	126	32%	/	44%	144	0.125	>0.25

(*continued on next page*)

Table 1 (*continued*)

Cpd	X	Y	R^1	R^2	MurC		MurD		MurE		MurF		MIC[c] [mM]	
					%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	%[a]	IC_{50}[b] [μM]	SA	EC
														
31	N	C			42%	151	47%	160	10%	/	40%	201	0.031	0.25
32	N	C			56%	82	70%	85	46%	150	58%	71	>0.25	>0.25

[a] % of inhibition of the enzyme activity at 100 μM of the tested compound. Data are means of two independent experiments. Standard deviations are within 10% of the means.

[b] Concentration of the inhibitor that reduces the activity of enzyme by 50%. The IC_{50} values were determined for the compounds with % of inhibition ≥ 40%.

[c] Minimal concentration of an inhibitor to inhibit the growth of specific bacteria. SA – *Staphylococcus aureus*; EC – *Escherichia coli.*

several different aromatic or heterocyclic rings were introduced to alternate the furan moiety. We then set to investigate the importance of the nitrogen atom and its position in the pyridine core of the molecule. Finally, a variety of compounds were prepared, where the tetrazole fragment was replaced with bioisosteres and different heterocyclic rings. A series of 20 stilbene derivatives was synthesized and evaluated for inhibitory potency against Mur ligases (MurC–MurF) and antibacterial activity against two representative bacterial strains: *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

One of the key features to be considered in the design of inhibitors that act on multiple targets is the degree of structural similarity between the enzymes. Mur ligases C–F reportedly share conserved amino acid regions, structural features and the common kinetic mechanism.[7,14] A common claim found in the literature is that a high degree of similarity between Mur ligases C–F should alleviate the quest for inhibitors that target more than one enzyme.[7,15] However, this has been only partially validated by a small number of dual inhibitors[10] and by our hit compound **1**.[9,16] Importantly, the binding site of compound **1** has only been confirmed by NMR on MurD. One of the questions that arise is whether the increase in activity against one enzyme could lead to a proportional increase in activity against another enzyme. With that in mind, a multiple sequence alignment (MSA) and 3D superposition of UMAG binding sites for all four ligases MurC–F were made to check the similarity among all four enzymes. The results are available among supporting data (Fig. S1, Table S3). The MSA demonstrates that ligases MurC–F show low sequence identity (26%). Furthermore, amino acids that were identified by docking to be relevant for binding are only partially conserved among all four ligases (Table S4). On the other hand, the topologies of UMAG binding sites are similar in Mur ligases with an average RMSD of 4.4 Å. Reasonable topological similarity and low sequence identity indicate that it is theoretically possible on one hand i) to design multiple inhibitors that target all four enzymes, but on the other hand ii) it is uncertain whether an increase in potency against one enzyme is followed by an increase in potency against the other ligase.

Commercially available bromides (5-bromonicotinonitrile (**2a**), 3-bromobenzonitrile (**2b**) and 2-bromoisonicotinonitrile (**2c**)) and tributyl(vinyl)tin were used in a Stille coupling to produce the vinyl intermediates (**3a–c**), followed by a Heck reaction using 5-bromo-2-iodo-1,3-dimethylbenzene to form the corresponding *trans* stilbene derivatives (**4a–c**) (Scheme 1).[17] Then, different boronic acids were used in Suzuki couplings to yield intermediates **6a–c** and **7a–14a**. Additionally, compounds **15a** and **16a** were synthesized under Buchwald-Hartwig cross-coupling conditions using $Pd(OAc)_2$ and Xantphos (4,5-bis (diphenylphosphino)-9,9-dimethylxanthene) as catalysts. Finally, the tetrazole derivatives (**5a**, **7b**, **7c, 17a**–**27a**,) were synthesized using ammonium chloride and sodium azide in DMF. The tetrazole bioisostere of carboxylic acid **28** was synthesized from nitrile **6a** under reflux using basic conditions. The amide derivative **29** was prepared from **6a** by hydrolysis of the nitrile group under mild basic conditions with hydrogen peroxide. 2-Oxazoline (**30**) and 2-imidazoline (**31**) derivatives were obtained using ethanolamine and ethylenediamine, respectively, in the presence of a catalytic amount of sulphur. Condensation of cysteamine and nitrile was a straightforward method for the preparation of a 2-thiazoline derivative **32** (Scheme 1).

First, compounds devoid of tetrazole (**6a**) or the furan fragment (**5a**) were assayed in the Malachite green assay[18] to confirm the importance of both moieties. Both compounds were less potent inhibitors of Mur ligases in comparison to compound **1**, which demonstrates the significance of these two fragments. The first set of compounds (**17a–27a**) with modifications of the furan ring (R^1) generally afforded less potent inhibitors of MurC–MurF. Nonetheless, compound **17a** inhibited MurD ligase in the same range as the starting compound **1**, indicating the importance of the oxygen atom at the *o*-position of the aromatic or heterocyclic ring. Next, compounds **7b** and **7c**, where the central pyridine ring was replaced with phenyl and *o*-substituted pyridine, respectively, showed comparable inhibition of MurD as **1**. However, while compound **7b** was inactive against the other three ligases, **7c** had a comparable inhibitory profile against all Mur ligases as compound **1**. Obviously, the nitrogen atom is important for the interaction with MurE and MurF ligases. Next, replacing the tetrazole part of molecule **1** with bioisosteres and other heterocycles resulted in multiple inhibitors against all four ligases. The compound with the archetypal tetrazole bioisostere - carboxylic acid (**28**), exhibited similar inhibitory potency against MurD and MurF ligases as compound **1**, and it was more potent

Table 2
Calculated ADME descriptors for active compounds with QikProp.

Cpd.	PSA[a]	logP[b]	logS[c]	Pcaco[d]	Oral Absorption (%)
1	80.5	3.5	−5.7	310	92.1
5a	67.4	3.1	−5.1	310	89.7
7b	67.6	4.4	−6.4	565	100
7c	80.5	3.9	−6.0	428	96.7
17a	76.6	4.3	−6.5	310	96.6
28	63.3	4.2	−5.4	140	90.3
29	69.1	3.3	−5.1	469	94.2
30	47.6	4.7	−6.0	3516	100
31	50.4	4.9	−6.6	2359	100
32	63.7	5.6	−6.9	3818	100

Description and recommended values: [a] Van der Waals surface area of polar nitrogen and oxygen in $Å^2$ (7–200), [b] Predicted octanol/water partition coefficient (–2.0 – 6.5), [c] Conformation-independent predicted aqueous solubility (–6.5 – 0.5), [d] Predicted apparent Caco-2 cell permeability in nm/sec (<25 poor, >500 great).

against MurC (**28**, IC_{50} = 153 μM; **1**, % inhibition = 30). Amide derivative (**29**) also displayed similar potency against MurD–MurF ligases as compound **1**. Compounds with isoxazoline (**30**) and imidazoline (**31**) showed very similar potencies against MurC, D and F ligases and the activity was in the same range as for compound **1**. Finally, the majority of compounds inhibited up to three enzymes, but displayed quite various inhibitory potencies in spite of similar topology and amino acid sequences shared by all four Mur ligases. On the other hand, thiazoline **32** possessed balanced inhibitory potencies against all four Mur ligases, thereby acting as a multitarget-directed inhibitor and being thus the most promising compound within the stilbene series (Table 1).

Antibacterial activities against *E. coli* and *S. aureus* were tested following the European Committee for Antibacterial Susceptibility Testing recommendations[19] and Clinical Laboratory Standards Institute protocol.[20] In general, most of the stilbene derivatives showed poor antibacterial activities against both *E. coli* and *S. aureus*. This might be ascribed to their poor penetration into the bacterial cytoplasm or relatively low on target activity. However, compounds **30** and **31** had MIC values of 0.125 and 0.031 mM, respectively, against *S. aureus*, representing an important starting point for further optimization of multiple Mur ligase inhibitors with antibacterial activity.

Finally, important basic physicochemical and ADME properties were calculated for the active compounds (Table 2). The calculated descriptor values for the majority of these fell well within the recommended ranges.[21] All compounds were found to have reasonable PSA and are predicted to have no issues with the oral absorption from GIT (Pcaco, % oral absorption). However, it must be pointed out that the most promising compounds **30**–**32** are not only quite lipophilic (log P = 4.7–5.6) but also poorly soluble (logS < −6). Any future optimisation of the binding affinity should also consider increasing the polarity as well as their solubility.

To conclude, a series of 20 new stilbene derivatives was synthesized and evaluated for the inhibition of four Mur ligases (MurC, D, E and F), which are essential enzymes involved in the biosynthesis of the bacterial cell wall. Five compounds (**7c**, **28**, **29**, **30** and **31**) showed promising inhibitory potencies against three ligases, and compound **32** displayed a balanced inhibition of all four Mur ligases. Importantly, moderate antibacterial activity against *S. aureus* was shown for derivatives **30** and **31**. These results provide a solid ground for further development and optimization of structurally novel antimicrobial agents to combat the rising bacterial resistance.

Author contributions

The manuscript was written through contributions of all of the authors. All of the authors gave their approval to the final version of the manuscript. All of the authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127966.

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