Novel Inhibitors of MurA, an Antibacterial Target Enzyme

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Introduction

The bacterial cell wall represents an attractive target site for antibiotic research as it is a fundamental structure for bacterial survival. The enzyme MurA (UDP-*N*-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7.) accomplishes an initial step in the cytoplasmic biosynthesis of peptidoglycan precursor molecules. It catalyzes the transfer reaction of phosphoenolypyruvate (PEP) to the 3' hydroxyl group of UDP-*N*-acetylglucosamine (UNAG) generating enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG) and inorganic phosphate (Fig.1). The rate of phosphate anion liberation can be quantified using a colorimetric malachite green based assay to evaluate the activity of inhibitory substances on the MurA enzyme (Fig.3).

The MurA enzyme consists of two globular domains with a flexible, superficial loop from Pro112 to Pro121 (numbering for the *E. coli* enzyme) that hosts a cysteine residue (Cys115 in *E.coli*) (Fig.2). The broad-spectrum antibiotic fosfomycin - to date still the only known MurA inhibitor with clinical relevance - acts as an analogue of the substrate PEP by irreversible alkylation of the Cys115 thiol group [1].The MurA-dependent metabolites are of vital importance for bacteria, and the enzyme is therefore in the focus of several drug development projects.
In order to establish a basis for rational drug design, a compound collection containing structurally diverse substances was screened for inhibitory activity on the MurA enzyme.

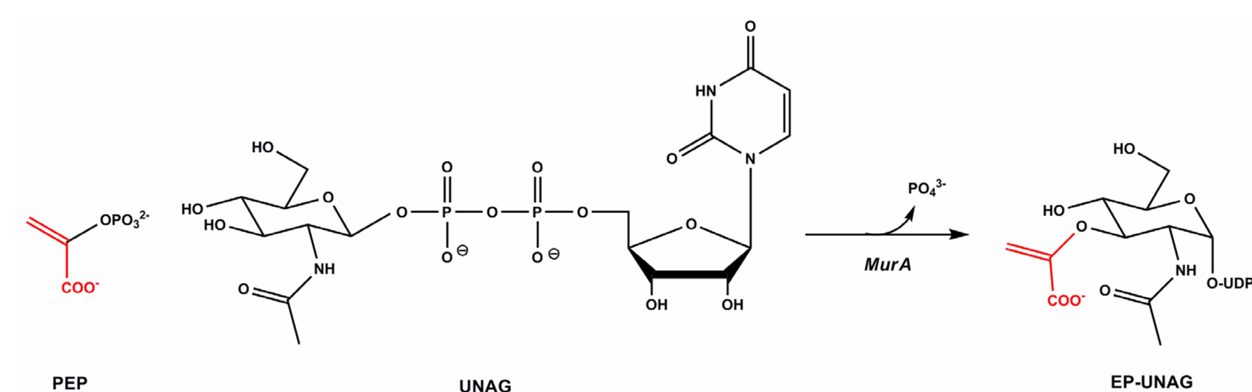

Fig. 1. MurA catalyzes the formation of enolpyruvyl-UNAG (EP-UNAG) from phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UNAG).

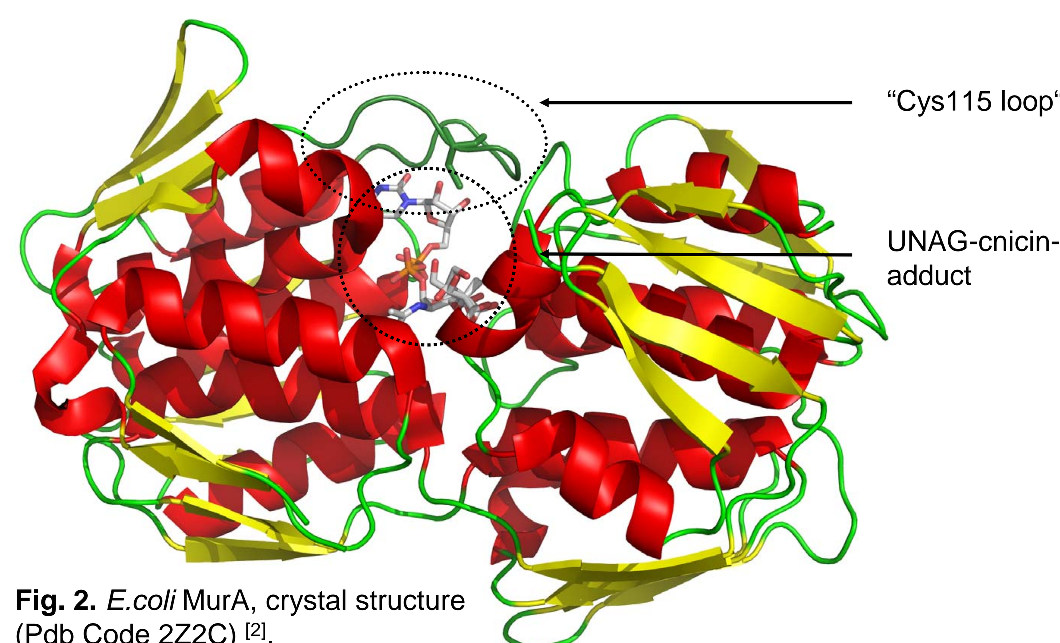

Fig. 2. *E.coli* MurA, crystal structure (Pdb Code 2Z2C) [2].

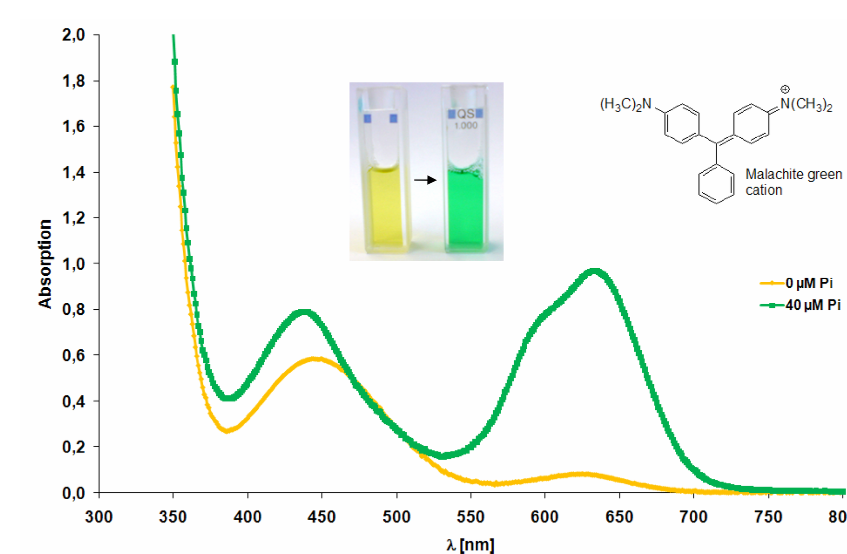

Fig. 3. Absorption shift of the ammonium molybdate/ malachite green-mixture at 620 nm upon addition of inorganic phosphate, visually indicated by a sharp color change from yellow to green.

Screening Results

controls

IC50 [µM] (± sd) *E.coli* WT MurA, 12 nM	Fosfomycin **1**	Cnicin **2**	Artabsin **3**	R = H **4** R = COC_3H_7 **5**	Aesculin **6**
	0.118	16.7	6.00 (± 1.43)	36.74 (± 2.70) 23.84 (± 2.17)	24.41 (± 4.48)

7	**8**	**9**	**10**
16.23	0.82	8.52 (± 0.94)	11.31 (± 2.51)

promiscuous inhibitors

Danthron **11**	**12**	Carbidopa **13**	Caffeoyl-malic-acid **14**
14.41 (± 2.24)	0.4 (± 0.17)	0.043	62 %_1µM

Fig. 4. Chemical structures of compounds with inhibitory activity on MurA – a selection of screening results.

Inhibition of the Cys115Asp mutant

The *E.coli* Cys115Asp mutant is catalytically active but resistant to the antibacterial agent fosfomycin as it lacks the nucleophile Cys115 and is therefore not reactive towards electrophilic attack by epoxides. In order to determine the relevance of Cys115 in the inhibition of MurA by an inhibitory molecule the assay is adapted on the *E.coli* C115D mutant employing the same conditions.

Table 1. Inhibition of MurA and C115D MurA in the presence of UNAG.

Compound	IC50 [µM] (± sd) *E.coli* WT MurA, 12 nM	Inhibition at 25 µM *E.coli* C115D MurA, 12 nM
1	0.118	n.i.
2	16.7	n.i.
7	16.23	n.i.
13	0.043	39%

Pre-incubation of inhibitors with the enzyme and substrate UNAG for 10 min.
(n.i., no inhibition)

A compound displaying no inhibitory effect on the Cys115Asp mutant of *E.coli* MurA at the highest tested concentration would strengthen the idea of a covalent linkage between the Cys thiol group and a functional group as inhibitory species, *i.e.* the bromo-ketone group for compound **7**.
Carbidopa (**13**) additionally modulates the activity of the mutant and of enzymes other than MurA emphasizing its classification as a relatively "promiscuous" enzyme inhibitor.

Fosfomycin (**1**) and cnicin (**2**) were measured as references. In the case of the sesquiterpene lactone cnicin which is a non-covalent suicide inhibitor of MurA, the inhibitory activity is achieved by a reactive ester side chain mimicking the substrate PEP [2,3].

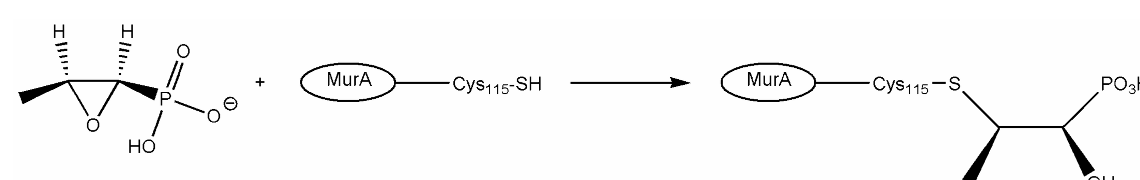

Fig. 5. Inactivation of MurA by fosfomycin. Fosfomycin targets the MurA enzyme through alkylation of the Cys115 thiol group.

Time-dependent inhibition

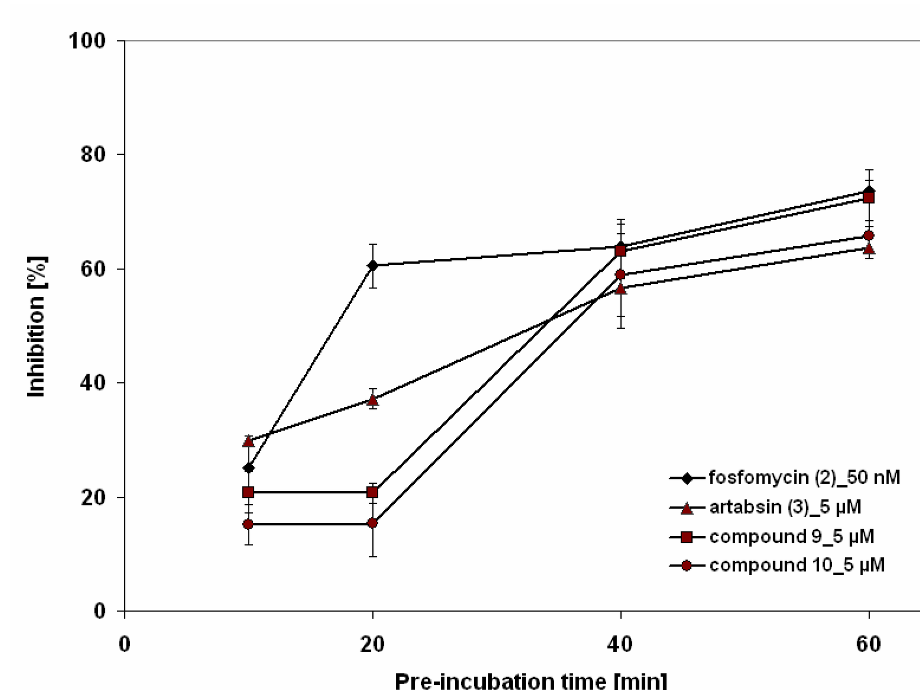

Fig. 6. Time-dependent inhibition of MurA by selected screening hits. The inhibitory effect increases with pre-incubation time indicating an irreversible inhibition or slow and tight binding mode.

Promiscuous screening hits

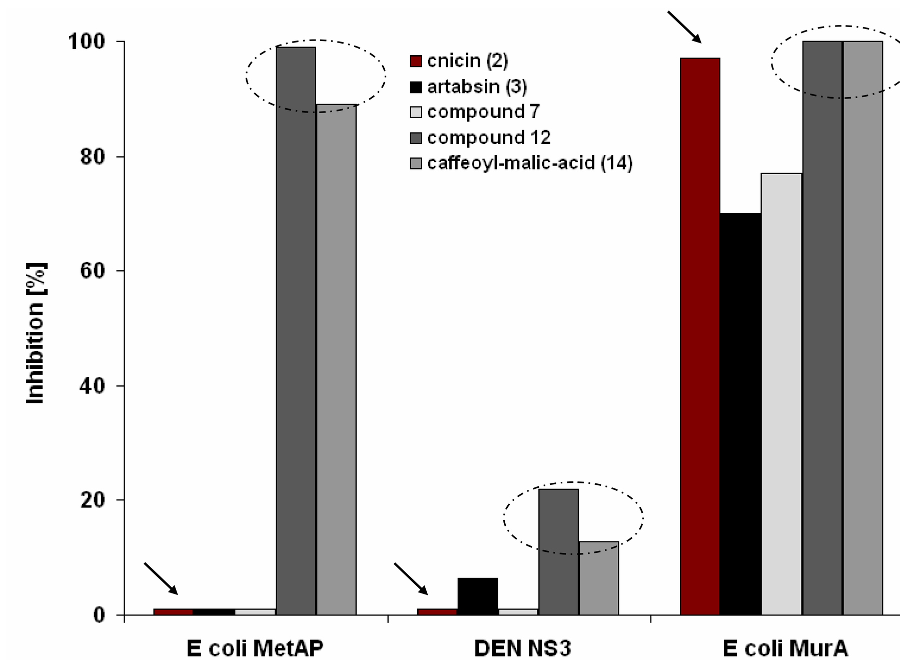

Fig.7. Effect of screening hits on unrelated target enzymes demonstrating nonspecific inhibitory acitivity (dashed lines). In contrast, the selective inhibition of MurA by cnicin (**2**, cf. arrows), artabsin (**3**) and compound **7** is shown.
Inhibitor concentrations 25 µM for Methionine Aminopeptidase (MetAP) and MurA, 50 µM for Dengue Protease (DEN NS3).

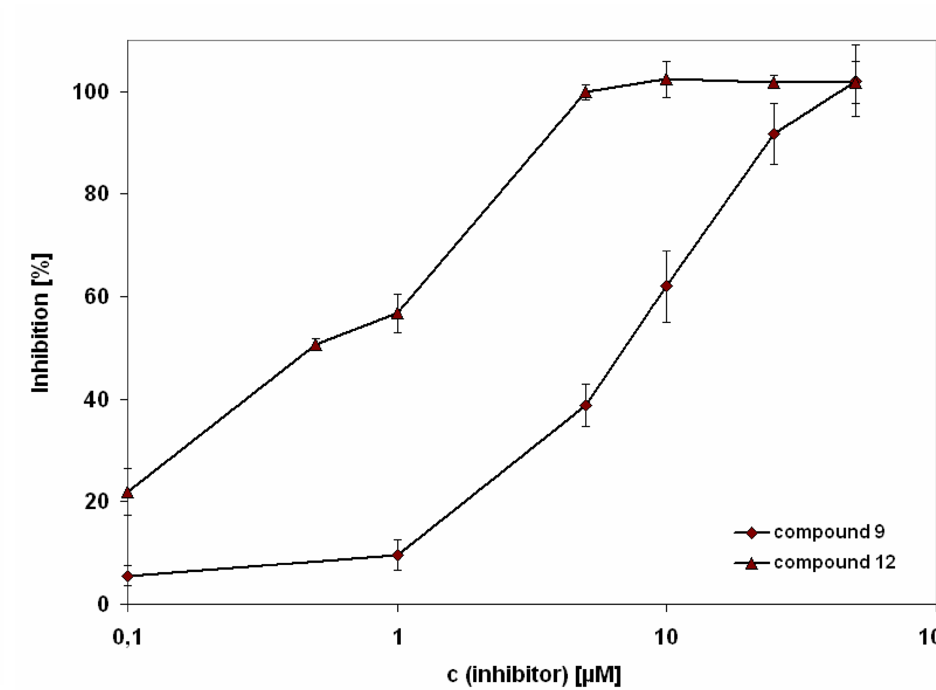

Fig. 8. Representative dose-response curves showing inhibition of MurA. Molecule **9** contains structural requirements for a covalent binding mode, sustained by the results of the time-dependent inhibition study. Unlike other promiscuous inhibitors (especially those forming aggregates), compound **12** does not exhibit an unusually steep concentration-effect-curve. Analogous observations were made for other compounds, such as carbidopa (**13**) and several natural products such as caffeoyl-malic-acid (**14**).

Promiscuous inhibitors that emerged from our screening campaign are typically planar and rigid with phenolic groups. It is assumed that such compounds self-assemble into aggregates in a concentration-dependent manner. The enzyme-sequestering effect is supposed to be promoted either through adsorption of enzyme molecules to the surface of the aggregates or through absorption into the interior of such particles [4].

Conclusion & Outlook

A common technique for new candidate lead identification in drug discovery is the screening of a compound library.
Promiscuous inhibition is a widespread phenomenon in compound libraries. From the screening campaign of approximately 500 synthetic and natural compounds emerged around 30 molecules with modest to high inhibitory activity on MurA. On subsequent examination, one third of these screening hits appeared non-specific and comprised redox-active or polyphenolic substances. However, several promising hits could be identified in this screening campaign for MurA inhibitors.

It is remarkable that these novel molecules exhibit unusual binding kinetics, suggestive of a covalent or non-covalent suicide mechanism as in fosfomycin or cnicin, respectively. Further work will be aimed at elucidating the structure-activity relationships of these classes of compounds and additional structure-based (X-ray crystallographic) studies.
Our current synthetic efforts are also directed at modified nucleosides as potent inhibitors of MurA and other enzymes in this biological pathway, building on the previous expertise with the non-covalent suicide inhibitor, the UNAG-cnicin-adduct [2,3]. Such compounds will have the potential to be multi-target inhibitors, thereby interrupting the biochemical pathway at multiple points, and will be less prone to the development of drug-resistance.

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Acknowledgements

We are grateful to M. Altmeyer, K.H. Heinonen and C. Steuer for providing data on the MetAP enzyme and NS3 serine protease.

This work is supported by the Bundesministerium für Wirtschaft and the Deutsche Forschungsgemeinschaft.