Discovery of Novel MurA Inhibitors

Huijong Han[1], Todd Funke[1], Melanie Priestman[1,2] and Ernst Schönbrunn[1]

[1] Department of Medicinal Chemistry, University of Kansas, Lawrence, KS 66045 USA

[2] Albert Einstein College of Medicine, Yeshiva University, Bronx, NY 10461 USA

Abstract

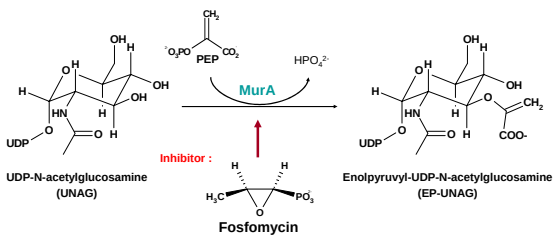
Purpose. To develop novel antibacterial agents by targeting MurA, the enzyme catalyzing the first committed step toward bacterial cell wall biosynthesis.

Methods. High-throughput screening (HTS) was performed at the University of Kansas HTS facility, utilizing a library containing over 100,000 compounds. MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to UDP-N-acetylglucosamine, producing enolpyruvyl-UDP-N-acetylglucosamine and inorganic phosphate. Malachite green was used to detect the amount of inorganic phosphate produced in a 30 minute reaction, and the change in absorbance at 650 nm was measured using a SpectraMax 340PC 380 Absorbance Platereader. Compounds displaying IC50 values lower than 20 μM were thoroughly characterized by inhibition kinetics and fluorescence studies. Co-crystallization of the lead compounds with MurA was attempted.

Results. A total of 84 new MurA inhibitors with IC50 values between 1.0 and 25 mM were discovered and 8 different scaffolds were identified. To date, complete kinetic characterization of 4 of the lead compounds revealed competitive and mixed-competitive inhibition vs. UDP-N-acetylglucosamine, the first substrate of the MurA reaction. Fluorescence studies indicate that these compounds are not active-site directed, but exert their mode of action through reversible binding to a large loop in MurA, thereby obstructing the conformational changes that occur during catalysis. Co-crystallization trials with MurA are currently underway.

Conclusions. To date, the only known potent and selective inhibitor of MurA is the natural product fosfomycin, the active component of the antibiotic Monurol. The rising incidence of fosfomycin resistant pathogenic bacteria requires the development of novel MurA inhibitors with potential antibiotic activity against a broad range of bacterial infections. Using HTS, we have identified 8 lead compounds with different scaffolds that inhibit *Enterobacter cloacae* MurA in the low micromolar range. The data gained from the characterization of the molecular mode of action of these novel inhibitors should provide a thorough understanding of how this medicinally important enzyme can be effectively targeted by small molecules.

Background



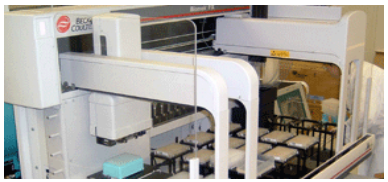
- MurA catalyzes the first step in the biosynthesis of bacterial cell wall
- Fosfomycin is the only effective antibiotic targeting MurA
- Many pathogenic bacteria are resistant to fosfomycin

Mechanisms for Fosfomycin Resistance

1. Mutation of Cys115 to Asp
2. Impaired fosfomycin transporters
3. Inactivation by fosfomycin resistance protein (FosA)

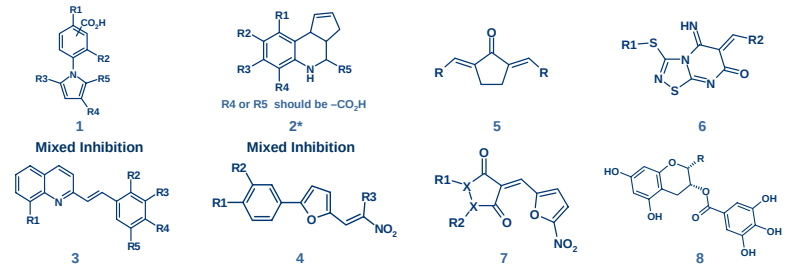
→ **Discovery of new potent inhibitors is needed !**

High Throughput Screening (HTS)



- **100,000 compounds were tested**
- **8 new MurA inhibitor scaffolds with IC_{50} <30 μM**
- **The scaffolds were characterized by steady state kinetics and ANS fluorescence assay**

Structures of eight lead scaffolds



Kinetic Characterization for HTS lead compound 2-2

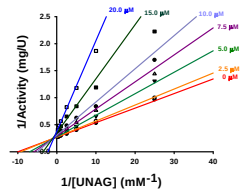


Fig 1. Lineweaver-Burk presentation showed mixed inhibition for 2-2

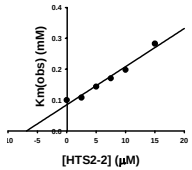


Fig 2. Replot of observed K_m from fig 1 as a function of inhibitor concentration. K_i for 2-2 is 6.83±0.89 μM

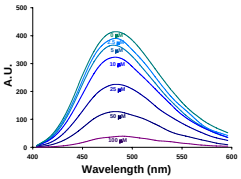


Fig 3. Fluorescence scans from 400-600 nm, with excitation at 366 nm. Inhibition of MurA by compound 2-2 assayed with 200 μM ANS, 125 μg MurA, with increasing concentration of compound 2-2, as labeled.

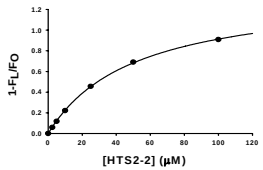


Fig 4. Replot of compound 2-2 ANS fluorescence data, fit to Michaelis-Menten equation. K_d for 2-2 is 50.9±1.7 μM

Table 1. K_i and K_d values for the representative compound from each scaffolds

Inhibitor	K_i (μM)	K_d (μM)
1-1	6.5±0.6	62.4±11.5
2-2	6.8±0.9	50.9±1.7
3-1	41.5±0.5	10.7±1.3
4-1	1.5±0.2	N/A*

(* Compound 4-1 has intrinsic fluorescence and cannot be studied using ANS assay.)

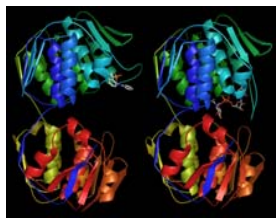


Fig 5. Crystal structure of MurA with ANS (left) and MurA with UNAG (right). ANS binds to the loop region on MurA. When UNAG binds to MurA, the conformation of loop changed forming closed form of MurA

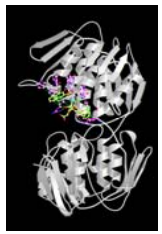


Fig 6. Crystal structure of MurA with T6361 (Aventis). This compound inhibits MurA by suspending the induced fit mechanism of MurA

→ **Kinetic characterization shows that compound 2-2 may inhibit MurA by suspending the induced fit mechanism in a manner similar to T6361 (Fig 6.). Crystallization and kinetic studies in different conditions and mutant enzymes (W71 and K248) are underway.**

Role of Arg120 and Cys115 in the MurA reaction

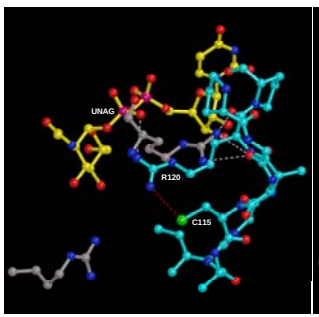


Fig 7. Crystal structure of wt MurA with UNAG (yellow). ARG120 is hydrogen bonded with UNAG.

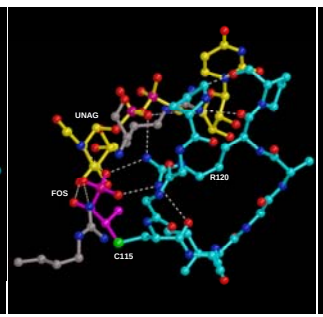


Fig 8. Crystal structure of wt MurA with UNAG (yellow) and fosfomycin (magenta). Fosfomycin covalently attaches to CYS115.

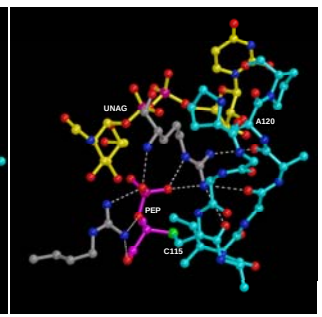


Fig 9. Crystal structure of Arg120Ala MurA with UNAG (yellow) and PEP (magenta). PEP is covalently bound to CYS115.

Arg120 is a strictly conserved residue in MurA. Co-crystallization of Arg120Ala MurA with both substrates revealed PEP covalently bound to Cys115 (Fig 9). It appears that this mutant enzyme induces the thioketal formation between PEP and Cys115. Further studies are underway to elucidate if catalysis proceeds via a covalent PEP-thioketal intermediate.

Conclusion

1. **All scaffolds from HTS are reversible inhibitors.**
2. **All compounds appear to bind to the loop containing Cys115.**
3. **The compounds do not induce the open-closed transition of MurA.**
4. **The mutation of active site residues such as Arg120 helps to identify reaction intermediates.**
5. **The combination of HTS and structural studies should enable the discovery of novel potent and selective inhibitors of MurA.**

References

1. Schönbrunn, E.; Sack, S.; Eschenburg, S.; Perrakis, A.; Krekel, F.; Amrhein, N.; Mandelkow, E. *Structure* **1996,** *4*, 1065-1075.
2. Skarzynski, T.; Mistry, A.; Wonacott, A.; Hutchinson, S.E.; Kelly, V.A.; Duncan, K. *Structure* **1996,** *4*, 1465-1474.
3. Schönbrunn, E.; Svergun, D.I.; Amrhein, N.; Koch, M.H. *Eur. J. Biochem.* **1998,** *253*, 406-412.
4. Kim, D. H.; Lees, W. J.; Kempsell, K. E.; Lane, W. S.; Duncan, K.; Walsh, C. T. Biochemistry **1996**, *35(15)*, 4923-8.
5. Schönbrunn, E.; Eschenburg, S.; Luger, K.; Kabsch, W.; Amrhein, N. *PNAS* **2000**, *97(12)*, 6345-6349.
6. Eschenburg, S.; Priestman, M. A.; Abdul-Latif, F. A.; Delachaume, C.; Fassy, F.; Schönbrunn, E. *J. Biol. Chem*. **2005**, *280(14)*, 14070-14075.

Funding: NIH RO3 AI065678-01