Fragment-Based Discovery of Novel *E.coli* DXR Inhibitors Targeting the MEP Pathway

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Introduction

The methylerythritol phosphate ~~pathway~~ (MEP) pathway for the production of isoprenoid precursors is prevalent in most gram-negative bacteria as well as in apicomplexan parasites while being absent in mammalian cells. As the MEP pathway is crucial for pathogen survival (1), the pathway enzymes represent promising targets for antiinfectives.

1-deoxy-D-xylulose 5-phosphate reductoisomerase (**DXR**) is a key enzyme in the MEP pathway. The enzyme catalyzes the conversion of 1-deoxy-D-xylulose-5-phosphate (DXP) and NADPH to 2-C-methyl-D-erythritol 4-phosphate (MEP) and $NADP^+$ (Fig. 1) (1,2).

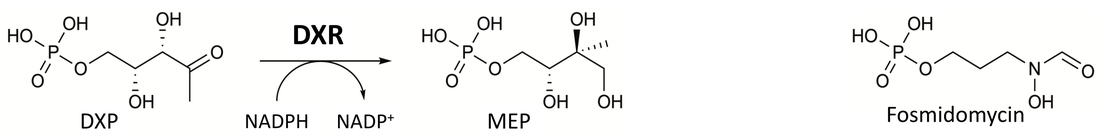

Fig. 1: Reaction catalyzed by DXR (left) and its inhibitor Fosmidomycin (right).

Fosmidomycin, a substrate analogue, is a potent known DXR inhibitor. Its poor pharmacokinetic properties prevented it from being successful in clinical trials (2). In this project, we aim to find novel DXR-inhibitors using a **fragment-based** approach.

Assay Principle

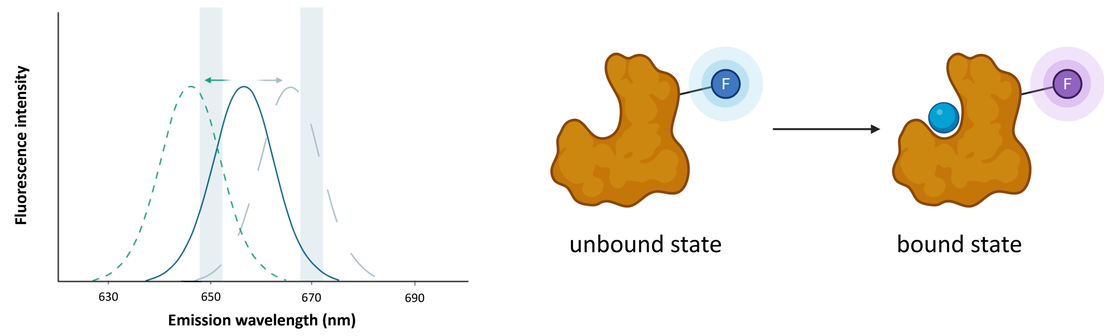

Fig. 2: Spectral shift technology, adapted from Langer et al (3); created in https://BioRender.com.

The **spectral shift** technology requires a fluorescently labelled protein (DXR). Binding interactions of the protein and a ligand change the chemical environment of the fluorophore and thereby lead to a shift in emission wavelength (Fig. 2). By measuring at two wavelengths (650 nm and 670 nm) and determining the ratio of fluorescence intensity, ligand-binding can be quantified. The dissociation constant (K_D) is derived by plotting the ratio against the ligand concentration (Fig. 4).

Fragment Screen

- A fragment library containing ~1000 compounds was screened at 1 mM for *E.coli* DXR binding
  - Good separation between positive and negative controls with Z‘ of the four microtiter plates ranging from 0.70 to 0.82
  - Cut-off was set at Δratio (DMSO) ± 0.015 for each plate (Fig. 5)
  - 113 fragments emerged as hits (10.7 %)
- Hit validation in triplicate at five concentrations resulted in 52 fragments binding at lower concentrations
- 47 compounds were purchased, 23 could be validated as binders in dose-response studies

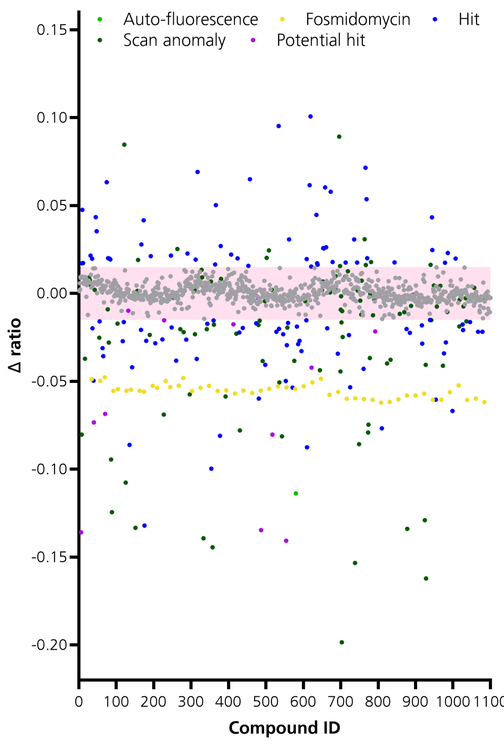

Fig. 3: Results of the fragment screen. Δratio of each compound compared to DMSO control. Area of insignificant signal in pink. Few outliers with auto-fluorescence or quenching.

Hit Confirmation

- Dissociation constants were determined using spectral shift and temperature-related intensity change (TRIC
- Most fragments revealed K_Ds in the low millimolar range (1 to 5 mM)
- The two best fragments demonstrated K_Ds of 145 and 805 µM (Fig. 4)
- A X-ray crystal structure of DXR in complex with one fragment bound to DXR was solved, revealing a novel binding mode

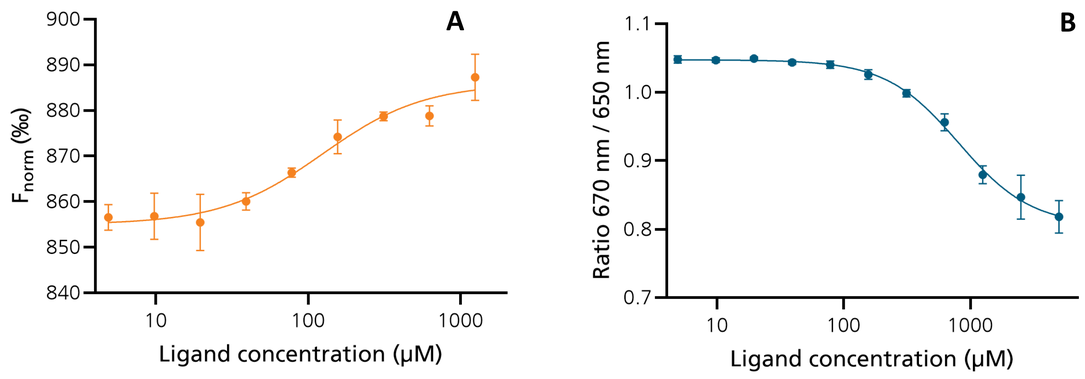

Fig. 4: Dose-response curves of two fragments. (A) Fragment 21 TRIC measurement; K_D = 145 µM. (B) Fragment 15 spectral shift measurement; K_D = 805 µM. Each data point represents the mean ± SD (n=3.)

Functional Assays

- Using a biochemical assay the inhibitory potential of 23 fragments was investigated
  - Fragment 10 showed a ⁄30 % inhibition at 1 mM concentration (Fig. 5)
- A bacterial growth inhibition assay using efflux deficient *E. coli ΔTolC* showed a MIC ₋of 1 mM for fragment 10
- As expected for fragments, the compounds are only active at high concentration (Fig. 5)

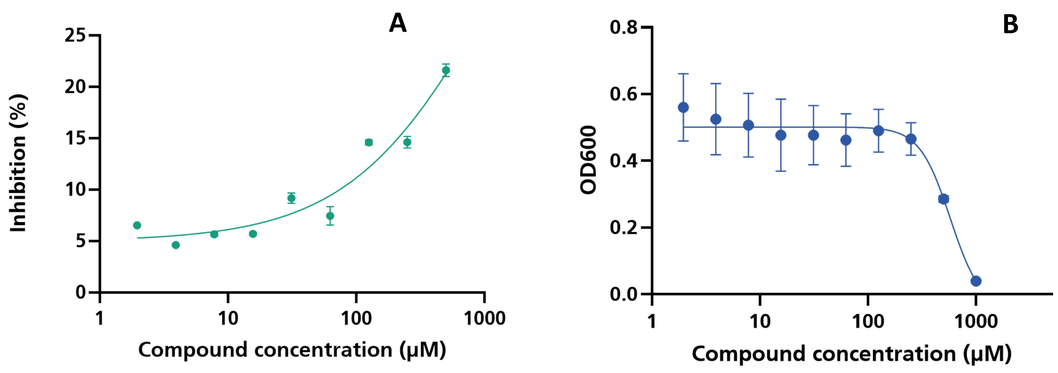

Fig. 5: (A) Dose-response curves of fragment 10. (B) Bacterial growth inhibition assay with fragment 10 using efflux deficient *E. coli*. Each data point represents the mean ± SD (n=3).

Outlook

- Fragment hits are starting points for the development of novel DXR Inhibitors
- Analogue-by-catalogue approach for fragment development

Methods

Protein production: Protein production was done in collaboration with CSSB, Hamburg, using existing protocols. **Protein labeling:** Protein labeling was done following the instructions of the Protein Labeling Kit RED-MALEIMIDE 2nd Generation (NanoTemper Technologies). After equilibration of the B-column, 1 µL dye was diluted in 9 µL labeling buffer and added to 90 µL of 10 µM DXR. This mixture was incubated in the dark at room temperature for 30 min, afterwards excessive dye was removed using the kit’s B-column. **Fragment screen:** Compounds, positive (Fosmidomycin) and negative (DMSO) controls were transferred to 384-well Dianthus-plate (NanoTemper Technologies) using a Beckman Coulter Echo liquid handling system. 20 µL of assay buffer (50 mM Tris pH 7.5, 10 mM $MnCl_2$, 300 mM NaCl, 0.1 % Pluronic, 150 µM NADPH, 10 nM labeled DXR; final DMSO concentration 1 %) was added. The plate was centrifuged (300 rpm, 1 min) and incubated for 20 min at room temperature before measuring on the Dianthus (NanoTemper Technologies). The Dianthus Screening Analysis Software was used for data analysis. **Hit validation:** Measurements were carried out in triplicate at eleven concentrations (5 µM up to 5 mM, depending on the compounds solubility). Procedure was performed as described for the fragment screen. Data analysis was conducted with DataWarrior and GraphPad Prism 10. **Biochemical assay:** Measurements were carried out in triplicate at 10 concentrations. Compounds and controls were transferred to assay plates using a Beckman Coulter Echo liquid handling system. 15 µL enzyme buffer (final concentration: 50 mM MES pH = 6.5, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM DTT, 150 µM NADPH) were added to the plate. After 10 min of incubation, the reaction was started by adding DXP (final concentration 150 µM) and NADPH consumption was measured at 340 nm. **MIC determination:** An internal standard protocol was used to determine the minimum inhibitory concentration. Bacterial growth was monitored by measuring OD600. Measurements were carried out in triplicate at 10 concentrations.

1) Heuston S et al (2012) Isoprenoid biosynthesis in bacterial pathogens. Microbiology 158(6):1389–1401
2) Knak T et al (2022) Over 40 Years of Fosmidomycin Drug Research: A Comprehensive Review and Future Opportunities. Pharmaceuticals (Basel). 2022 Dec 14;15(12):1553
3) Langer A et al (2022) A New Spectral Shift-Based Method to Characterize Molecular Interactions. Assay Drug Dev Technol 20(2):83–94

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