Abstract:

Identification and characterization of halogenases acting on nucleoside scaffolds

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Although nucleoside analogs have been intensively exploited for designing new drugs, surprisingly little attention has been paid to their antimicrobial activity. Nucleoside antibiotics are found as diverse groups of secondary metabolites of microbial origin. They include a variety of structural modifications of nucleosides and nucleotides, often leading to intricate molecules. In addition to C-nucleosides or peptidyl nucleosides, halogenated nucleosides have also been described. Enzymatic halogenation is a rapidly developing tool in the synthetic chemist's toolbox. Operating at ambient temperatures in aqueous media, halogenating enzymes allow the regio- and stereoselective installation of halogen atoms. Apart from modulating the physico-chemical properties of molecules and, in consequence, their biological activity, halogen atoms can also serve as chemical linchpins for further derivatization of the molecular scaffold, for example, in chemo-enzymatic cascades. Diverse halogenases have been described so far, however, hardly any halogenases that use nucleosides as the substrate are well characterized. Therefore, the planned project focuses on the identification of nucleoside halogenases (genome mining), the intensive biochemical characterization of the enzymes (including molecular dynamics modeling), and, if necessary, optimization of the enzymes for the synthesis of valuable compounds (bioretrosynthesis). While molecular modeling will be performed in the group of Prof. Dr. Han Sun, genome mining, cloning, expression, and biochemical characterization of the halogenases will be done in the group of Prof. Neubauer. In addition, partners are sought to assist with: i. enzyme engineering (e.g. with Prof. Dr. Matthias Höhne), ii. characterization of the biological activity of the molecules produced (e.g. with the Screening Unit at the Leibniz-Forschungsinstitut für Molekulare Pharmakologie), iii. MS studies (e.g. with Prof. Dr. Juri Rappsilber) or iv. crystallization (e.g. with Dr. Patrick Scheerer).

Extended description version of the project: Identification and characterization of halogenases acting on nucleoside scaffolds

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1. Overall goal of the project

The goal of this project is the identification and characterization of nucleoside halogenases that show the potential to produce highly valuable nucleoside analogs. Due to the importance of halogen substituents (e.g. tuning the hydrophobicity, and formation of halogen bonds), nature has developed a multitude of halogenating enzymes that combine oxidative power with selectivity. The diverse molecular scaffolds of the more than 5000 halogenated natural compounds that have been discovered to date demonstrate the catalytic power of these enzymes. Although diverse halogenases have been described for a wide range of small molecules, nucleoside halogenases are hardly known. Therefore, the proposed project will first focus on the identification of new nucleoside halogenases by genome mining. In a second step, both known and newly identified halogenases will be biochemically characterized with a focus on the substrate spectrum of the enzymes. Molecular dynamics modeling will be performed to best understand the mechanisms of biocatalysis. This will also enable the optimization of the enzymes, with a focus on broadening the substrate spectrum. Finally, the enzymes will be used to produce valuable nucleoside analogs (with a focus on antimicrobial compounds) in an enzymatic process.

2. State of the art

Due to the chemical properties of halogen substituents, they can significantly alter a molecule's property and may impact its bioactivity or pharmacokinetic profile. It is therefore unsurprising that the list of the 200 bestselling small molecule drugs of 2021 contained 87 compounds that carry a halogen atom[1]. This trend is similarly observed in agriculture: 96% of all pesticides launched since 2010 contain a halogen atom[2]. Additionally, carbon-halogen motifs are useful handles for chemical modification, explaining why halogenated species represent common intermediates in synthetic manufacturing routes[3]. It is therefore hardly surprising that chemical halogenation is a well-established technology. Due to existing disadvantages (use of hazardous or toxic chemicals, sometimes poor atom efficiency or selectivity) the development of alternative methods for the selective halogenation of small molecules is of increasing interest. Biocatalytic approaches to obtain halogenated small molecules appear particularly suitable as enzymes are associated with high regio- and stereoselectivity and are operating under mild reaction conditions. Due to the described importance of halogenated compounds, a large number of small molecule halogenases have already been identified, in-depth characterized, or even engineered[1]. However, knowledge of nucleoside/nucleotide halogenases is still comparably limited. The following nucleoside/nucleotide halogenases have been identified so far and characterized to very different degrees:

i. 5´-fluoro-5´-deoxy adenosine synthetase (FDAS). The enzyme was identified from the bacterium *Streptomyces cattleya*[4]. and catalyzes the reaction between S-adenosyl-L-methionine (SAM) and fluorine to form L-methionine and 5´-fluoro-5´-deoxy adenosine. The enzyme is already well characterized: it has been crystallized and its structure was determined[5,6]. Additionally, it has also been shown to work with chlorine as well as fluorine[7].

ii. AdeV, a Fe2+-α-ketoglutarate halogenase that is involved in adechlorin synthesis. Although the biosynthesis of adechlorin (also named 2'-Cl-Pentostatin) had been deciphered since 2017[8], the responsible chlorinase was missing. By re-sequencing the *Actinomadura* genome the group of Zhang found *adeV* whose gene product indeed catalyzed the conversion of 2'-deoxy-adenosine-monophosphate (dAMP) into 2'-Cl-2´-dAMP *in vitro*[9]. Crystal structure and intensive point mutation studies gave insight into the molecular

mechanisms of these enzymes[10]. In-depth studies on the substrate scope of these enzymes are missing so far.

iii. AcmX and AcmY, putative (FAD)-dependent adenosine chlorinases: Although the 2-Cl adenosine nucleoside antibiotics ascamycin and dealanyascamycin were isolated as early as 1984[11], no gene for the chlorination is known to date. Ascamycin and dealanyascamycin are potent broad-spectrum antibiotics[12] and are found in *Streptomyces sp*. JCM9888. Recent studies have revealed the biosynthetic cluster[13], suggesting that AcmX and AcmY might be the responsible FAD-dependent chlorinases.

iv. 4´-fluorinase of *Streptomyces virens* B-24331 and *Streptomyces aureorectus* B-2430: Comparably little is known of the enzyme that is responsible for the fluorination in the biosynthesis of nucleocidin. The biosynthetic cluster has been recently found, and the first enzymes in the early steps of biosynthesis have been characterized[14]. Although gene disruption experiments on *S. virens* revealed genes (namely *nucN*, *nucK*, and *nucO*) that differentially affect total fluoronucleoside biosynthesis[15], the encoding gene for the C-F formation is not yet known.

3. Specific aims and how they may be reached:

To study the nucleoside and nucleotide halogenases, different work packages have to be processed based on the different background information: If the coding regions are not known, they must first be identified.

Work package 1: Genome mining (4´-fluorinase).

- Re-studying the genome sequences of *Streptomyces* strains.
- Cloning and expression of relevant enzyme candidates.
- Identification of 4´-fluorinase activity by HPLC.

Work package 2: Studying the substrate scope of nucleoside and nucleotides halogenases.

- Expression of AdeV, AcmX/AcmY, and 4´-fluorinase.
- Studying the substrate scope of the enzymes by HPLC. Different halogen substituents are tested as well as various nucleoside scaffolds.
- Kinetic and thermodynamic parameters will be determined.

Work package 3: Computational modeling.

- Realistic models of the Enzyme-Substrate Complex will be generated using state-of-art flexible docking algorithms. Existing crystal structures will be used as templates. If not available, structural models will be generated by homology modeling or AlphaFold2.
- Dynamic properties of enzymes in the absence and in the presence of specific substrates will be investigated by classical molecular dynamics simulations, while interaction energies between the enzyme active site and the ligand will be accurately predicted by quantum mechanical using hybrid QM/MM approaches or molecular dynamics based alchemical free energy calculations.

Work package 4: Bioretrosynthesis of halogenated nucleoside or nucleotide analogs.

- Efficient enzymatic pathways will be developed for valuable halogenated nucleosides/nucleotides.
- Based on the substrate scope of the enzymes, derivatives of the natural nucleoside/nucleotide analogs will be produced.
- Biological activity of the new nucleoside/nucleotide analogs will be evaluated. This task may be carried out together with the Screening Unit at the Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin. If necessary, further suitable cooperation partners will be identified during the project.

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