Summary and Outlook

Because of increasing resistance of bacteria against antibiotics new small molecules for disabling or bypassing defence mechanisms of pathogens or for addressing new protein targets are urgently required. It is known that HtrA-proteases play crucial roles in many biologically relevant processes. The stress sensor DegS, a prokaryotic HtrA protease located in the periplasm, has so far not been investigated as a target for chemotherapy development. This is suprising as the stress sensor DegS is the initial factor involved in periplasmatic stress response consisting of DegS-mediated sensing of unfolded or misfolded proteins in the periplasm, resulting in its proteolytic activation. DegS-mediated proteolysis of RseA results in a cascade of reactions that finally leads to the release of σ^E in the cytoplasm that induces transcription of stress genes. Because of its essential functions under stress conditions as well as the fact that DegS is found in many Gram-negative bacteria, DegS might represent a potential target for the development of new antibiotics against infectious disease. This hypothesis was tested in this thesis via the development of DegS inhibitors for biological application.

As a starting point, the LDC in Dortmund had performed prior to the experimental works of this thesis a high-throughput screen for identifying DegS inhibitors. From this screening campaign, they identified 2-(4-aminopiperidin-1-yl)-*N*-benzyl-8-isopropylpyrazolo[1,5-a][1,3,5]triazin-4-amine (**1**) as a potential DegS inhibitor in biochemical and *in vivo* assays. To improve the rather weak inhibition of DegS and its specificity, derivatives of this small molecule structure were synthesized and biochemically tested for DegS inhibition, enabling insights into the underlying structure-activity-relationships of DegS inhibition. The 4-aminopiperidine substituent attached to the pyrazolotriazine thereby turned out to be essential for the inhibitory effect as any modification of this group led to inactive derivatives. The same effect was observed upon modification of the pyrazolotriazine scaffold. In contrast, the isopropyl group was turned out to be modifiable under retention of bioactivity. Substitution by large nonpolar groups improved the inhibitory effect and led to a higher DegS specificity. As a major drawback of LDC compound **1** as well as of structurally related compounds is their off-target inhibition of cyclin dependent kinases. This discovery represents an important step for improving inhibitor specificity. Substitution of isopropyl by an 4-ethoxy phenyl led to an inhibitor 2-(4-

aminopiperidin-1-yl)-*N*-benzyl-8-(4-ethoxyphenyl)pyrazolo[1,5-a][1,3,5]triazin-4-amine (**42**) that displayed only weak inhibitory potential (with IC_{50} values > 30 µM) for tested kinases.

The modification of the benzylamine group allowed to gain insights into the structure-activity relationships at this site of the inhibitor. It turned out that large nonpolar groups improve the inhibition of DegS. Furthermore, it was found that the *meta* position on the benzene ring represents the most effective site for substitutions for improving inhibition. This finding enabled the synthesis of the most potent DegS Inhibitor so far: 2-(4-aminopiperidin-1-yl)-*N*-(3-(benzyloxy)benzyl)-8-isopropylpyrazolo[1,5-a][1,3,5]triazin-4-amine (**17**). For this inhibitor, DegS inhibition rates could be improved from initial 200 µM for inhibitor **1** to 2.5 µM for inhibitor **17**. In the second biochemical assay, an improvement of the IC_{50} value of 150 µM for compound **1** to an IC_{50} value of 51.7 µM for compound **17** was observable. However, while large hydrophobic groups improve the inhibitory effect, they also decrease the pharmacological properties. This requires careful optimization of the binding affinity via increase of hydrophobicity as these inhibitors *in vivo* might be rather inactive, e.g. because their effective concentration in the periplasm in which DegS is located might be rather low. Overall, 27 derivatives of Inhibitor **1** were synthesized and tested. All derivatives with a hydrophobic group in *meta* position attached to the benzene have shown a better inhibitory effect than inhibitor **1** in biochemical assays.

In additional experiments performed by the Suisse-based company BioVersys, it could be shown that colistin and a subset of the synthesized inhibitors display a desirable synergistic behaviour. For further improvement of the inhibitors, it will however be highly desirable to identify the precise binding site which should allow structure-based inhibitor design. Experiments with an azide tagged derivative that may allow photocrosslinking experiments to map the binding site were tried in this thesis but turned out to unsuccessful so far. Accordingly, first attempts to gain a co-crystal structure of inhibitor **17** and DegS were undertaken by collaborators but have not been working out so far within the time frame of this thesis.

An optimization of the synthesis route that may allow to generate structurally more complex benzylamine derivatives via a late-stage introduction at an amino group in *meta* position of the benzene ring turned out to be promising. The modification of the synthesis route enabled a late stage coupling of an acyl chloride to a *meta*-amino derivative. As this approach should also be compatible with the attachment of

carboxylic acids via peptide coupling reagents, this alternative strategy represents a real advance as the unfavourable $POCl_3$ reaction is no longer required in the synthesis of every derivative. Unfortunately, a second optimization approach for derivatization of the group C residues by a Suzuki coupling approach could not be established within this thesis. This limits the derivatisation of group C to those commercially available and $POCl_3$-stable 1H-pryazolo-5-amines. As modifications of this group however turned out to be more DegS specific, the development of an alternative approach in the future would be highly desirable.