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Chemical validation of DegS as a target for the development of antibiotics with a novel mode of action

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Abstract: *Despite the availability of hundreds of antibiotic drugs, infectious diseases continue to remain one of the most notorious health issues. In addition, the disparity between the spreading of multidrug resistant pathogens and the development of novel classes of antibiotics exemplify an important unmet medical need that can only be addressed by identifying novel targets. Here we demonstrate by the development of the first in vivo active DegS inhibitors based on a pyrazolo[1,5-a]-1,3,5-triazine scaffold that DegS and the cell envelope stress response pathway σE represent a target for generating antibiotics with a novel mode-of-action. Moreover, DegS inhibition is synergistic to well-established membrane perturbating antibiotics thereby opening promising avenues for rational antibiotic drug design.*

The persistent rise in resistances to current antibiotic therapies requires the development of novel antibiotics.[1] Despite the availability of hundreds of antibiotic drugs, these chemotherapies explore only a very limited number of molecular mechanisms,[2] resulting in the unmet medical need to identify and validate mechanistically novel strategies and targets for developing alternative antibiotics.

The bacterial cell envelope stress response pathway σE is essential for bacterial survival under optimal growth but also under stress conditions such as infection of a human host.[3] Its impairment may thus represent an alternative approach for generating antibiotics with a novel mode-of-action. In this pathway, DegS, a S1 serine protease of the **H**igh **t**emperature **r**equirement **A** (HtrA) protease family characterized by the presence of at least one PDZ domain located C-terminal to the protease domain,[4] serves as the molecular stress sensor.[5] To this end, DegS binds the hydrophobic C-termini of misfolded or mislocalized outer membrane proteins via its PDZ domain.[5-6] This binding event triggers a reversible and allosteric activation of the DegS protease domain, allowing DegS to perform the rate-limiting event of stress response, i.e. cleavage of the transmembrane protein and anti-sigma factor "regulator of sigma E", RseA. Further downstream processing then results in transcriptional activation of the stress response.[5] Although the underlying activation mechanism seems to be conserved in other HtrA proteases,[4, 7] its elaboration i.e. a pronounced substrate selectivity for RseA turns DegS into a unique protease that offers the opportunity to develop selective inhibitors.

Proteases are well-established drugable targets.[8] After many years of limited research activity, the potential of proteases as targets for developing antibiotics has recently been rediscovered.[9] For example, inhibitors for bacterial proteases such as ClpXP, the bacterial proteasome or intramembrane proteases such as rhomboids were generated recently.[10] Although small molecule modulators of DegS have already been reported,[11] these cannot be used *in vivo*. Therefore, more suitable DegS inhibitors are required to chemically validate inhibition of the bacterial cell envelope stress pathway as a new strategy for antibiotic development.

To identify suitable DegS inhibitors, we performed an Alpha-screen-based high-throughput screen (HTS) comprising 185.000 chemical compounds, 75 nM DegS, 18 nM of the periplasmic fragment of RseA (later denoted as RseA) as the substrate, and 37.5 nM Boc-FFF-OH as an activating peptide mimicking the C-termini of misfolded hydrophobic proteins (Supporting Fig. 1). 26 compounds displayed 30% DegS inhibition of DegS at 100 µM and were thus chosen as primary hits. Two subsequent concentration-dependent counter screens, i.e. a second RseA digestion assay (10 µM DegS, 30 µM RseA,

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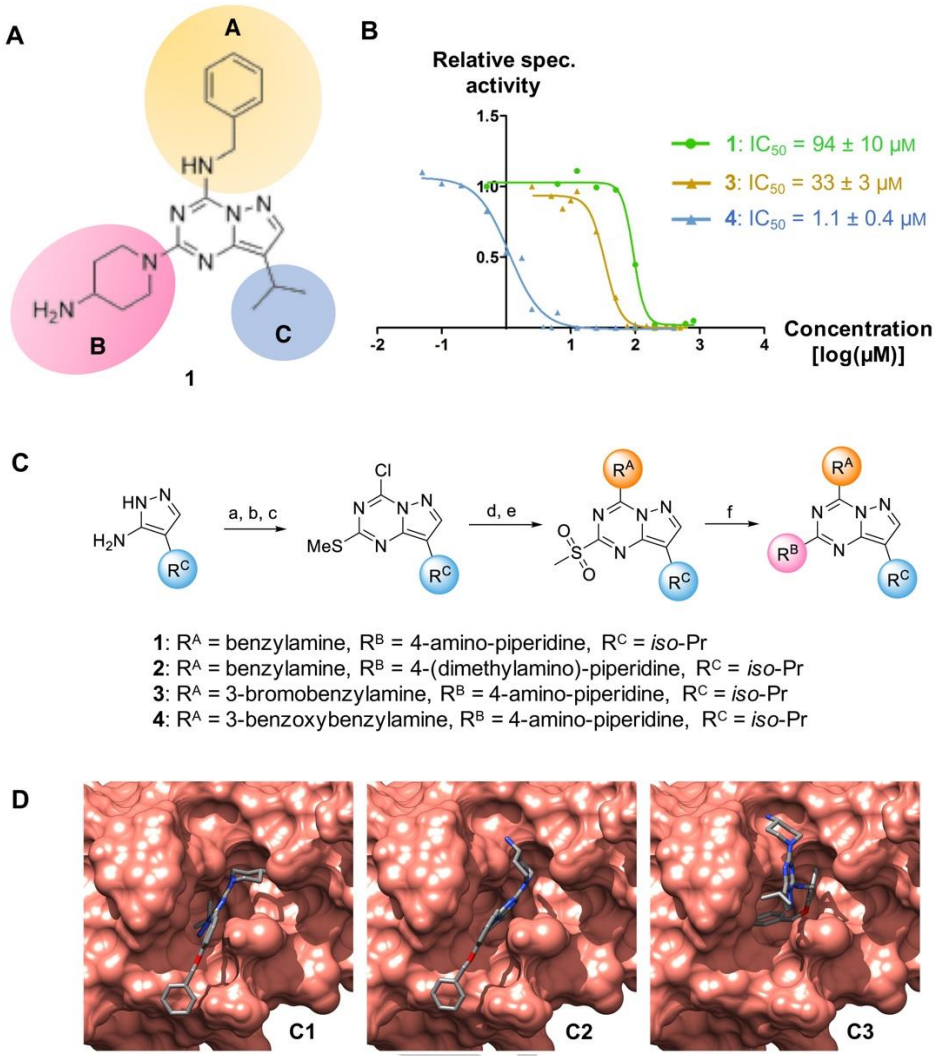


Figure 1. Suitably modified pyrazolo[1,5-a]-1,3,5-triazines are DegS inhibitors. **A**) Chemical structure of the HTS hit compound **1**. **B**) Dose-inhibition curves and corresponding IC_{50} values for indicated pyrazolo[1,5-a]-1,3,5-triazines obtained from gel-based digestion experiments. **C**) Chemical synthesis route employed for generating modified pyrazolo[1,5-a]-1,3,5-triazines for structure-activity relationships. a) i) ethoxycarbonyl isothiocyanate (1 eq.), dichloromethane, 0 °C to rt, 2 h, ii) K_2CO_3 (1.2 eq.), 90 °C, 2 h, b) methyl iodide (1-2 eq.), 2 N NaOH/EtOH (1:2), 0 °C to rt, o/n, c) *N*, *N*-diethyl aniline (3 eq.), $POCl_3$, 90 °C, 2 – 18 h, d) amine 'A' (3 eq.), di-*iso*propyl ethyl amine (5-20 eq.), acetonitrile, rt, 17 – 82 h, e) *meta*-chlorperbenzoic acid (2 – 6 eq.), dichloromethane, rt, 1 – 2.5 h, f) amine 'B' (3 eq.), *N*-methyl-2-pyrrolidone, 120 °C, 18 – 20 h. **D**) The docking of compounds **1**, **2** and **4** into the PDZ domain of DegS leads to three main binding poses (C1 – C3) for **1** and **4** filling the binding pockets of the PDZ domain; for **2**, only C2 and C3 can be realized. These binding poses are here exemplarily shown for compound **4**.

50 µM Boc-FFF-OH) and a colorimetric digestion assay (2.5 µM DegS, 500 µM of the DegS substrate VFNTLPMMGKASPV-pNA) then resulted in the identification of the pyrazolo[1,5-a]-1,3,5-triazine analogue **1** as the most promising hit (Fig. 1A).

Subsequently, the gel-based RseA degradation assay comprising 5 µM DegS, 10 µM RseA and 50 µM of the activating peptide Boc-FFF-OH was used to determine inhibition of DegS by selected compounds. In this assay, **1** displayed concentration-dependent DegS inhibition with an IC_{50} value of 94 ± 10 µM (Fig. 1B and Supporting Fig. 2). To initially test selectivity and potential off target activity, a panel of S1 serine proteases, i.e. chymotrypsin, trypsin and elastase as well as human HTRA1, HTRA2 and HTRA3 were investigated. Moderate to no inhibition of these proteases indicated a promising selectivity (Supporting Fig. 3). Furthermore, **1** was inactive vs. a DegS mutant lacking the PDZ domain ($DegS_{\Delta PDZ}$) suggesting a desirable allosteric mode of inhibition (Supporting Fig. 4).[12] The observed selectivity allowed us to test whether **1** is able to inhibit DegS activity in living bacteria. To this end, we used our previously established *E. coli*-based DegS activity reporter strain.[11] In this engineered strain, DegS activity correlates to beta-galactosidase expression which is measured via hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside in whole cells. As **1** completely inhibited reporter and thus DegS activity, we conclude that the inhibitor is reaching the periplasm

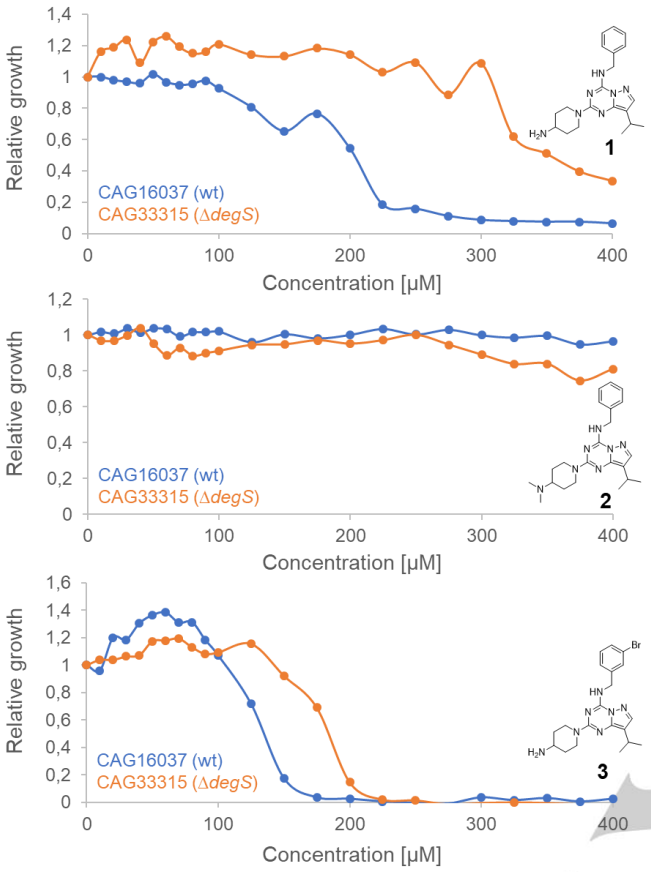
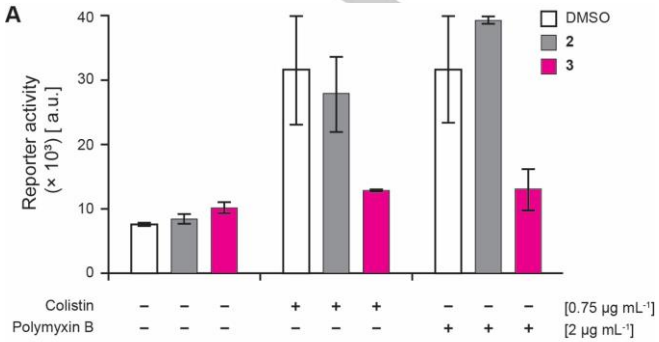


Figure 2. Pyrazolo[1,5-a]-1,3,5-triazine-based DegS inhibitors show DegS-dependent growth inhibitory properties. *E. coli* WT strain CAG16037 and the *ΔdegS* knockout strain CAG33315[13] were grown in presence of the indicated concentrations of **1** as well as the in presence of the inactive compound **2** and the DegS inhibitor **3**. Growth curves were normalized to the corresponding DMSO control. DegS inhibitors show *degS*-dependent growth effects while the inactive derivative **2** does has no effects.

by a nucleophilic substitution via an addition/elimination mechanism to introduce the derivatives at the 'B region' then yielded the final pyrazolo[1,5-a]-1,3,5-triazines. This synthesis route was used to synthesize 31 different derivatives (Supporting Information). Together with further 29 compounds from a chemical library, these were subsequently tested for their DegS inhibitory potential, thereby revealing structural determinants for inhibition.



B

Compound	MIC [µM]	MIC [µM] + Colistin *	FIC **
1	> 200	50	0.25, synergistic
2	> 200	>50	n. d.
3	> 200	25	0.19, synergistic
4	50	3.12	0.19, synergistic

* in presence of 1/8 MIC Colistin; ** FIC = Fractional inhibitory concentration index

Figure 3. Pyrazolo[1,5-a]-1,3,5-triazine-based DegS inhibitors inhibit outer membrane stress response and act synergistically to outer membrane stress inducing antibiotics. **A**) An *in vivo* screen with a DegS activity reporter strain demonstrates that active DegS inhibitors such as **3** impair outer membrane stress response (that was triggered by addition of the outer membrane perturbing agents Colistin (Col) and Polymyxin nanopeptide B (PmBN)) while the inactive control compound **2** does not. **B**) Measurements of minimal inhibitory concentrations for *E. coli* growth in absence or presence of the outer membrane perturbing chemotherapeutic Colistin that was used at a concentration corresponding to 1/8 of its MIC demonstrates synergism between compounds.

(Supporting Fig. 5). As DegS is widely conserved in bacteria, we determined minimal inhibitory concentrations (MIC) for **1** by measuring growth of various Gram positive and Gram negative bacteria. For most bacterial strains, MICs ranged between 100-600 µM (Supporting Table 1). These data suggest that DegS is essential in these strains even under optimal growth conditions, i.e. in rich media. Altogether, our results indicate that **1** may indeed represent a valuable starting point for DegS inhibitor development, although **1** displays overall only moderate DegS inhibitory potency *in vitro* and *in vivo*.

To obtain more potent compounds, we started to synthesize pyrazolo[1,5-a]-1,3,5-triazine derivatives. **1** features three "main" substitution sites (labelled as A, B and C in Fig. 1A). To allow structural variation at all three positions, our synthetic route started from 2-amino pyrazole derivatives that were reacted with ethoxycarbonyl isothiocyanate (Fig. 1C). Methylation of the thiourea moiety with methyl iodide, followed by phosphoroxy trichloride-mediated chlorination yielded the intermediates for introduction of the different 'A region' substitutes. Oxidation via *meta*-chloroperbenzoic acid, followed

Most notably, all structural changes at the 'B' position of **1**, including minimal changes such as the introduction of additional methyl groups at the aminopiperidine moiety as done in derivative **2** led to inactive compounds with IC_{50} > 400 µM, indicating that this region of the inhibitor is interacting with a distinct binding pocket (Supporting Fig. 6 and Supporting Table 2). In contrast, modifications at the 'C' position were better tolerated, although none of the synthesized compounds displayed much improved inhibitory potential (Supporting Table 2). The introduction of *meta*-substituted benzyl amine derivatives at the 'A' position led to significantly better inhibitors, while substitutions at the *ortho*- or *para*-position or non-benzyl amine derivatives did not increase or even result in inactive derivatives (Supporting Table 2). Insertion of hydrophobic residues led to the best inhibitory improvements. For example, a 3-bromo benzyl amine in **3** resulted in an IC_{50} of 33 ± 3 µM. The best inhibitory potential was observed for a 3-benzoxybenzyl amine in **4** that inhibited DegS with an IC_{50} of 1.1 ± 0.4 µM and thus was almost 100-times more potent than the parent compound **1** (Fig. 1B). These studies therefore demonstrated that pyrazolo[1,5-a]-

1,3,5-triazines represent a promising DegS inhibitor class with defined structure-activity relationships and potent inhibitory potential.

To rationalize the structure-activity relationship of the pyrazolo[1,5-a]-1,3,5-triazines at the molecular level, we explored the binding of **1** (parent compound with intermediate activity) and **4** (best binder) to DegS. To validate our model, we also used the inactive compound **2** as a negative control. The first step was to perform docking calculations of these compounds to the PDZ domain of DegS. Binding to the protease domain was discarded since the parent compound **1** is not able to inhibit $DegS_{\Delta PDZ}$ (Supporting Fig. 4). We thus identified three main binding poses (C1, C2 and C3) of **1**, **2** and **4** to the PDZ domain of DegS (Fig 1D). The analysis of C1 and C2 allows rationalizing why chemical modifications of positions 'B' and 'C' failed to improve the IC_{50} of the compounds since in these binding poses both positions are placed in PDZ binding pockets that are limited in space. Next, we performed molecular dynamics (MD) simulations of the three docked compounds with C1 and C2 as starting positions. The MD simulations then showed that both conformations lead to protein-ligand complexes that were conserved during the simulations (see Supporting Information for further discussion). In C1 and C2, all compounds also engage in interactions with loop L3 as indicated by the dynamic network analysis (Supporting Fig. 7-9).[13] To initially test this model, we reasoned that binding of **1** to the PDZ domain of DegS could be contested by allosteric activators. We therefore examined inhibition of DegS by **1** in the presence of two peptides that are known allosteric activators i.e. DNRLGLVYQF and DNRLGLVYWF, the affinity of which is 53 µM and 3.2 µM, respectively.[6] Both peptides prevent inhibition of DegS, suggesting competitive binding to the PDZ domain and thus demonstrating feasibility of the proposed binding mode (Supporting Fig. 10).

We continued to evaluate this class of inhibitors by measuring DegS target engagement in living *E. coli* cells using **1** as the starting compound as well as **3** as an inhibitor with better inhibitor potential (note that the most active compound **4** did not display sufficient water solubility at higher concentrations to perform these assays) and the inactive compound **2** as a negative control. To this end, we investigated the impact of the three compounds on the growth of the DegS WT *E. coli* strain CAG16037 as well as the *ΔdegS* knockout strain CAG33315 (note that strain CAG33315 must contain so far unknown suppressor mutations allowing it to grow in the absence of *degS*) (Fig. 2). In these assays, the DegS inhibitors **1** and, in accordance with its higher inhibitory potential, to a larger extent **3** displayed dose-dependent growth defects; more importantly, stronger effects were observed for the WT than for the *ΔdegS* knockout strain. In contrast, the inactive control compound **2** was insensitive to the different genetic backgrounds. These results demonstrate that the employed compounds target and inhibit DegS in living bacteria, leading to differential DegS-dependent growth defects.

These results encouraged us to further test in vivo target engagement. Accordingly, we modified the DegS activity reporter strain by employing the outer membrane perturbating antibiotics Colistin (Col) and Polymyxin B nonapeptide (PmBN) for DegS activation. While Col is a highly potent membrane-disrupting and bactericidal agent, PmBN is considerably less antibacterial, lacks bactericidal activity and mainly acts to disorganize and permeabilize the outer membrane. In accordance with our findings with **1**, these assays revealed that also the more potent DegS inhibitor **3** completely impaired DegS reporter activity at 30 µM while the inactive control compound **2** had no effects (Fig. 3A). Accordingly, these results not only demonstrate that **3** targets DegS *in vivo* but also indicates that DegS inhibitors may act synergistically with outer membrane perturbing antibiotics. Moreover, our chemical approach supports previous genetic evidence indicating that DegS is essential even under optimal growth conditions.[13]

Given the well-established implications of DegS in cell envelope protein stress response, we tested the synergy of DegS inhibition by pyrazolo[1,5-a]-1,3,5-triazines with the outer membrane perturbing and last resort antibiotic Col. To this end, we first determined the minimal inhibitory concentration of Col for *E. coli* ATCC25922 as 0.25 µg/mL (Supporting Table 3). Subsequently, the corresponding MIC for various pyrazolo[1,5-a]-1,3,5-triazines either in absence or in presence of 0.03 µg/mL Col (corresponding to 1/8 MIC of Col) was determined (Fig. 3B). From these values, we calculated the fractional inhibitory concentration (FIC) index as a measure of synergy between two compounds. For all active DegS inhibitors, a FIC index ≤ 0.5 was determined, indicating synergy. In addition, we tested synergy for further Gram negative bacteria. While the combination of **1** and colistin was synergistic for *Acinetobacter baumanni*, *Enterobacter cloacae* and *Klebsiella pneumoniae*, no synergy was detected for *Pseudomonas aeruginosa* (Supporting Table 4); as **1** alone had no effect on *Pseudomonas aeruginosa* but on the three other strains (Supporting Table 1), this finding further supports the suggested synergy mechanism. Moreover, the synergy between the DegS inhibitor and membrane-perturbing antibiotic is selective. We have tested the combination of **1** with other antibiotic classes with different mechanisms of action and could not see any increase in antibiotic susceptibility (Supporting Table 5). Overall, these studies demonstrate that impairment of the outer membrane stress response via DegS acts synergistically in combination with established antibiotics inducing outer membrane stress.

In summary, we have developed the first non-covalent small molecule inhibitors of DegS. These inhibitors are based on a pyrazolo[1,5-a]-1,3,5-triazine scaffold and display distinct structure-activity relationships. These compounds also inhibit DegS in living bacteria, thus allowing for the first time to demonstrate that *in vivo* target engagement induces growth inhibitory effects, in particular under stress conditions. In fact, chemotherapeutical induction of outer membrane stress is synergistic to DegS inhibition. Together, these findings validate DegS and the σE stress pathway as new targets for the development of antibiotics with a novel mode-of-action, thus opening promising avenues for rational antibiotic drug design.

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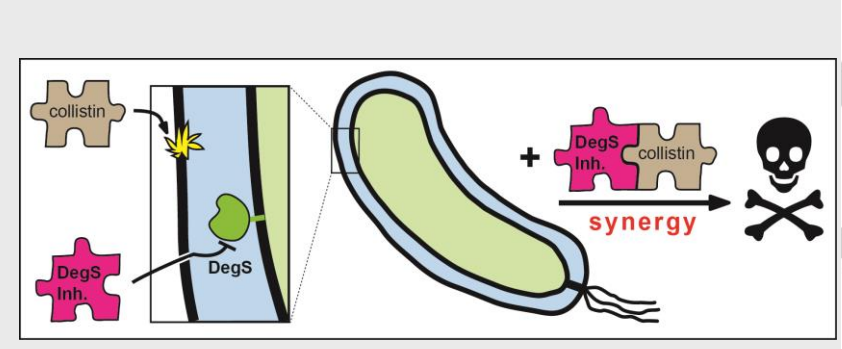

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