Research paper

Structure–activity relationships for inhibitors of *Pseudomonas aeruginosa* exoenzyme S ADP-ribosyltransferase activity

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A B S T R A C T

During infection, the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* employs its type III secretion system to translocate the toxin exoenzyme S (ExoS) into the eukaryotic host cell cytoplasm. ExoS is an essential in vivo virulence factor that enables *P. aeruginosa* to avoid phagocytosis and eventually kill the host cell. ExoS elicits its pathogenicity mainly via ADP-ribosyltransferase (ADPRT) activity. We recently identified a new class of ExoS ADPRT inhibitors with *in vitro* IC50 of around 20 μM in an enzymatic assay using a recombinant ExoS ADPRT domain. Herein, we report structure–activity relationships of this compound class by comparing a total of 51 compounds based on a thieno-[2,3-d]pyrimidin-4(3H)-one and 4-oxo-3,4-dihydroquinazoline scaffolds. Improved inhibitors with *in vitro* IC50 values of 6 μM were identified. Importantly, we demonstrated that the most potent inhibitors block ADPRT activity of native full-length ExoS secreted by viable *P. aeruginosa* with an IC50 value of 1.3 μM in an enzymatic assay. This compound class holds promise as starting point for development of novel antibacterial agents.

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1. Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a leading cause of multidrug resistant nosocomial infections, including pneumonia, burn, surgical wound, bloodstream, urinary tract and corneal infections [1,2]. This opportunistic pathogen can cause life-threatening infections particularly in cystic fibrosis patients, burn patients, immunocompromised patients and individuals undergoing chemotherapy. *P. aeruginosa* uses a broad arsenal of virulence factors and strategies, such as type I-VI secretion systems [3], quorum sensing [4] and biofilm formation, to adapt and survive within diverse harsh environmental settings and even under minimal nutritional requirements [5]. Disrupting the function of these virulence factors is a compelling approach to develop novel antibacterial therapeutics [6].

ADP-ribosyl transferases (ADPRTs) constitute one of the most prevalent toxin families utilized by several bacteria to covalently modify and modulate the activity of key host proteins [7]. *P. aeruginosa* upregulates the type III secretion (T3S) system during acute phases of infection to inject effector proteins like exoenzyme S (ExoS), T (ExoT), U (ExoU) and Y (ExoY), into the eukaryotic cytoplasm [8,9]. ExoS and ExoT are bifunctional cytotoxins with 76% amino acid homology that consist of an ADPRT domain at their C-terminal and a GTPase activating domain (GAP) at the N-terminal [10]. Despite the shared functionality between ExoS and ExoT, ExoS remains more promiscuous than its homologue ExoT [10]. ExoS ADPRT recognizes and modifies several unrelated eukaryotic substrates, including low-molecular weight monomeric GTPases e.g. Ras, Rac, Rab, RhoA and Cdc42 [11] and ezrin/radixin/moesin proteins [12]. These modifications lead to malfunction several signal transduction pathways that affect differentiation, growth, and host cell viability by disruption of the actin cytoskeleton and inhibition of DNA synthesis [9,11,13,14]. Previous studies have shown that the ExoS ADPRT domain is associated with the establishment of *P. aeruginosa* infection and killing of host immune cells [14–18]. More importantly, it was recently demonstrated that the ExoS ADPRT is responsible for blocking phagocytosis in a *P. aeruginosa* pneumonia mouse model [18]. These results imply an essential role of ExoS ADPRT activity in pathogenicity and specifically inhibition...
of phagocytosis, while the role of the GAP domain as virulence factor appears to be less pronounced [8,14,16,18]. Therefore, the ExoS ADPRT domain is a putative therapeutic target for the development of anti-virulence therapy or chemical probes to study P. aeruginosa pathogenicity. Although the functions of P. aeruginosa T3S exotoxins have been extensively studied, there are only two reports describing identification of ExoS ADPRT small-molecule inhibitors using either a yeast phenotypic assay [19], or our in vitro enzymatic assay that identified compound 1 and 2 (Fig. 1) as inhibitors of the P. aeruginosa ExoS ADPRT domain [20]. The enzymatic assay is based on recombinant ExoS ADPRT domain and a fluorescent probe, 1N6-etheno-NAD+ (εNAD+) to detect the transferring reaction of the fluorescent moiety, εADP-ribose, to Ras in presence of co-factor 14.3.3 (Fig. 2) [20].

Herein, we established structure–activity relationships (SARs) of the competitive ExoS ADPRT inhibitors 1 (STO1101) and 2 [20] by systematic variation of structural features (Fig. 1) and evaluation of in total 51 compounds in vitro utilizing our enzymatic ADPRT assay. Furthermore, we developed a secondary enzymatic assay using native full-length ExoS expressed and secreted by P. aeruginosa. A subset of the most potent inhibitors were then isolated and verified to inhibit ADPRT activity of native ExoS secreted via the T3S machinery from viable P. aeruginosa.

2. Results and discussion

Based on the results of the pilot screen and hit validation that identified compound 1 and 2 as inhibitors of ExoS ADPRT [20], we carried out a medicinal chemistry program to systematically vary the structures as shown in Fig. 1. All target compounds (Table 1) were synthesized and tested for ExoS ADPRT inhibition (Table 1) in chronological and systematic order employing our previously published assay protocol [20].

2.1. Synthesis

The structural variation based on hit compounds 1 and 2 was divided into four parts (Fig. 1) that included synthesis of building blocks with different substituents, coupling with various linkers, derivatization of the carboxylic acid moiety and modification of the core scaffold.

We first synthesized a number of 2-amino-thiophene-3-carboxamide building blocks 3a–7a by applying the Gewald reaction [21,22] as outlined in Scheme 1, while anthranilamide derivatives 8a–13a were prepared from the corresponding 2-amino benzoic acid [23] (8) or 2-aminobenzonitrile [24] (9–13) as shown in Scheme 2. The 2-aminobenzonitriles with fused five- and six-membered rings were synthesized from the corresponding anilines (see supporting information, S9). Starting from 2-amino-3-carboxamido thiophenes (3a–7a) or anthranilamide derivatives (8a–13a), we developed a general microwave-accelerated two-step one-pot protocol inspired by published procedure [25] to synthesize the target compounds 1, 2, 14–31 and 33–41 (Table 1). The method involved coupling of the aromatic amine with different acid anhydrides or acid chlorides in toluene followed by cyclization upon treatment with aqueous sodium hydroxide to afford the corresponding pyrimidinone products in moderate to good yields over two steps as exemplified in Schemes 1 and 2. Analogue 32 with trans-cyclopropyl linker was however synthesized from the corresponding trans-aldehyde in 33% yield over two steps as shown in Scheme 3 via acid-catalyzed cyclization followed by oxidation and ester hydrolysis under basic condition. Compounds 42 and 43 [26] and 44 and 45 [27] were synthesized according to given reported procedures, while compound 46 was synthesized as shown in Scheme 3 by cyclocondensation of 2-amino-thiophene-3-carboxylate (4b) with formamidine acetate in formamide in 76% yield. Compound 46 was subsequently alkylated to give 47 in 55% overall yield starting from 4b.

The terminal carboxylic acid was derivatized through reduction (48), esterification (49), or amidation (50–52) as outlined in Scheme 4. The tetrazole-based analogue 56 (Scheme 5) was synthesized from the corresponding nitrile-terminated linker (54), which was prepared via Schmidt reaction from the corresponding aldehyde [28] (53). A sequential cyclization upon treatment with LiHMDS (1 M THF) gave 55 in 25% yield. A final nitrile-azole cycloadition gave tetrazole 56 in 15% yield over three steps. The acyclic products 57–58 were isolated after the coupling step in quantitative yield as exemplified in Scheme 1. The dihydropyrimidinone analogue 59 was prepared as shown in Scheme 3 via condensation of 3a with trans-cyclopropyl aldehyde under stoichiometric amount of ammonium chloride [29] to afford a diastereomeric mixture in 68% yield. However, only one diastereoisomer was successfully isolated in pure racemic form. The aminopyrimidine-based analogues 60 and 62 were synthesized as shown in Scheme 3 from the corresponding 2-amino-3-nitrile thiophene (3b) and methyl 3-cyano propionate under basic condition in 13% yield (Scheme 5). Desulfurization of 1 was achieved upon heating with Raney®2800 Ni under basic pH to afford 61 in 45%
Table 1
Structure and activity of analogues synthesized in SAR.

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<th>Structure</th>
<th>Enzymatic assay IC₅₀ (µM)</th>
<th>% Inhibition at 100 µM</th>
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<th>Structure</th>
<th>Enzymatic assay IC₅₀ (µM)</th>
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yield (Scheme 4). The dimethylated compound 63 was synthesized as previously described [20].

**2.2. Structure–activity relationships**

All target compounds (Table 1) were tested at 8–10 different concentrations in an ADPRT enzymatic assay using recombinant ExoS ADPRT domain essentially as described previously [20]. Based on inhibition data IC₅₀ values were calculated (Table 1). For the competitive inhibitors 1 and 2, IC₅₀ values obtained in this study (Table 1) are in agreement with those previously reported (20 and 30 μM and 19 and 25 μM respectively) [20].
2.2.1. Substitution

We have previously shown that expansion of the 5-membered ring in 1 to a 6-membered ring (14), reduced the activity by a factor two [20]. To further explore the role of the ring size, we synthesized a 7-membered and a heterocyclic 6-membered fused system, 15 and 16, respectively, which both showed negligible inhibition (IC\textsubscript{50} > 100 μM). Replacing the fused ring system with a coplanar phenyl ring on position 5 (17) resulted in reduced activity (IC\textsubscript{50} = 56 μM). On the other hand, we turned our attention to compound 2 and synthesized analogues with fused 5- and 6-membered rings on positions 6 and 7 (18 and 19), and 5 and 6 (20 and 21). Unfortunately, none of these analogues showed any improved activity. The 5-membered fused ring systems (18 and 20) were however slightly preferred compared to the corresponding analogues with 6-membered rings (19 and 21), which confirms our previous findings (cf. compounds 1, 14 and 15). A small set of compounds with electron donating and withdrawing groups located on the quinazolinone ring (22 e 25) suggested that a small substituent with a weak electron-withdrawing effect such as fluorne atom (22 and 23) has little influence (IC\textsubscript{50} of 15 μM and 25 μM respectively), while a larger substituent such as NO\textsubscript{2} (24) or OCH\textsubscript{3} (25) resulted in a loss of activity.

Scheme 3. Synthesis of compounds 32, 46, 47, 59, 60 and 62. Reagents and conditions: (a) ethyl 2-formyl-1-cyclopropanecarboxylate (predominantly trans), H\textsubscript{2}SO\textsubscript{4} fuming (10 mol %), THF, μW 65–150 °C, 15 min. (b) NaOH, CH\textsubscript{3}OH/H\textsubscript{2}O (1:1), μW 100 °C, 5 min. (c) NH\textsubscript{2}CH\textsubscript{2}COOH, HCONH\textsubscript{2}, 160 °C, 6 h. (d) CH\textsubscript{3}COOCH\textsubscript{2}CH\textsubscript{2}Br, K\textsubscript{2}CO\textsubscript{3}, acetone, 60 °C 48 h. (e) 2-trans-formyl-1-cyclopropanecarboxylic acid, NH\textsubscript{4}Cl, EtOH, 60 °C, 5–6 h. (f) NCC\textsubscript{3}CH\textsubscript{2}COOCH\textsubscript{3}, NMP, KO\textsubscript{2}Bu, 170 °C, 10 h. (g) LOH, THF/CH\textsubscript{3}OH/H\textsubscript{2}O (3:1:1), μW 65 °C, 15 min.

Scheme 4. Derivatization of carboxylic acid and desulfurization of the hit compound 1: Synthesis of analogues 48–52 and 61. Reagents and conditions: (a) TEA (1 equiv.), TMSCH\textsubscript{2}N\textsubscript{2}, DCM/CH\textsubscript{3}OH, 0 °C to rt. (b) LiAlH\textsubscript{4} (1 M in THF), THF, 0 °C to rt, ovn. (c) Ra/Ni pH 8.0–9.0, DMF/MeOH, 90 °C, 7 h. (d) HATU, NH\textsubscript{4}OH or NH\textsubscript{2}CH\textsubscript{3}, DMF, 0 °C to rt, ovn. (e) NaH, HATU, CH\textsubscript{3}SO\textsubscript{2}NH\textsubscript{2}, DMF, 0 °C to rt, ovn.

Scheme 5. Synthesis of tetrazol-based analogue 56. Reagents and conditions: (a) NaN\textsubscript{3}, TfOH, CH\textsubscript{3}CN, 5 min, rt. (b) SOCl\textsubscript{2}, reflux, 3 h. (c) 3a, pyridine, rt, 0 °C to rt, ovn. (d) LiHMDS (1 M in THF) rt, ovn. (e) NaN\textsubscript{3}, TEA, HCl, PhCH\textsubscript{3}, 100 °C, ovn.
2.2.2. Linker

A longer linker, yet flexible, with 4 carbon atoms (26 and 27) or an ether-linkage (28) was less active with an IC50 values above 50 μM. Removing the linker with the carboxylic acid moiety (46) abrogated the activity. Similar results were obtained when shifting the linker from position 2 to the amide nitrogen located in position 3 (47) (Fig. 1). This indicates the necessity for the carboxylic acid functionality, but also support the hypothesis regarding the need of the amide proton mimicking the nicotinamide molecule since inhibitor 1 is competitive with respect to NAD⁺ [20,30]. Various linkers with aromatic, cyclic and alkenic systems were introduced (29–37, 43–45). Interestingly, a twofold increase in the activity (IC50 of 10 μM) was obtained when a conformation locked linker with a racemic trans-cyclopropane ring (32) was introduced. In comparison, its corresponding cis-analogue (33) showed a 19-fold drop in activity (IC50 of 190 μM). Similar but less significant difference was observed when Z-alkene linker (29, IC50 of 15 μM) was introduced compared to the E-alkene linker (30, IC50 of 70 μM). Aromatic linkers with a furan (44) or a phenyl ring (45) showed the same range of activity (IC50 of 30 μM) as compound 2. Introducing a hydroxyl group and thus a stereogenic center and potential for additional interactions did not improve the activity, but there was a significant difference between the two enantiomers 27 (IC50 of 55 μM) and 28 (IC50 of 191 μM).

Previously, we verified that analogues of 2 with two-carbon atoms linker showed a reduced activity (IC50 of 135 μM) [20]. Interestingly, this effect could be balanced with a three-carbon atom linker 2 (IC50 of 30 μM). To further explore this we synthesized a number of fused ring analogues with two-carbon linker (38–41) and showed that the five-membered fused ring on position 5 and 6 (38, IC50 of 56 μM) could compensate for the shorter linker compared to the non-substituted analogue (IC50 of 135 μM) [20] or analogues with larger ring system (40 and 41, IC50 > 100 μM). These results confirm our finding regarding the importance of the ring size (cf. compounds 1, 14 and 15), and suggest a preference for substitution on position 5 and 6 (38, IC50 of 56 μM) compared to substitutions on position 6 and 7 (39, IC50 of 98 μM).

2.2.3. Carboxylic acid functionality

Earlier, we established that reducing the carboxylic acid moiety to the corresponding alcohol (48) decreased the activity [20]. Here we converted the acid moiety to methyl ester (49), carboxamide (50) or methylcarboxamide (51), which all resulted in a significant loss in activity. Moreover, the carboxamide compounds proved less soluble than their corresponding carboxylic acid analogues, which prevented deduction of reliable IC50 values. Replacing the carboxylic acid with isosteres such as acyl sulfonamide (52) and tetrazole (56) were also proved unsuccessful. Taken together our findings suggest strong preference for the propionic acid residue.

2.2.4. Pyrimidine core scaffold

Opening the pyrimidine ring (57 and 58) resulted in a complete loss of activity (IC50 > 200 μM). Compound 59 in which position 1 and 2 are saturated is an analogue to the most potent inhibitor 32 carrying the trans-cyclopropyl linker. This compound showed at least 13-fold reduction in the activity (IC50 of 138 μM). However, we could isolate only one diastereoisomer in a pure racemic form (59), while the absolute configuration of this analogue remained ambiguous. An analogue based on 4-amino-pyrimidine (60) allowed us to study the locked amide tautomerization of the hit compound 1. As we expected, this modification abolished the activity of the hit compound (IC50 > 200 μM). The same results were observed via simultaneous modification of the amide and the carboxylic acid (62 and 63), which reinforces our hypothesis regarding NAD⁺ mimicking amide moiety [20,30] and the recognition of the propionic acid residue.

2.2.5. Chiral resolution

As a final step, we resolved the racemic mixture of the most potent inhibitor with trans-cyclopropane linker (32) using chiral semi-preparative HPLC (see supporting information S1 and S2). That was achieved by synthesizing the racemic methyl ester in order to improve the solubility of 32 in organic solvents used for the separation and to obtain a good resolution. eventual ester hydrolysis afforded pure trans-enantiomers 64-E1 and 65-E2. Interestingly, one of these enantiomers (64-E1) showed approximately six times more potency compared with its mirror image 65-E2 and was demonstrated to be the most potent inhibitor (Table 2 and Fig. 3A).

2.3. Native ExoS enzymatic assay

The compounds were evaluated in an enzymatic assay using the recombinant ExoS ADPRT domain (20) (Fig. 2). Therefore, it was important to confirm the inhibitory effect of these compounds on native full-length ExoS expressed and secreted by P. aeruginosa. We translated our in vitro enzymatic assay into a real-time assay that directly monitored the enzymatic activity of full-length native ExoS. Secretion of ExoS can be induced in absence of host cells by depletion of calcium from the growth medium. In brief, an overnight culture was diluted and the T3S was induced by incubation at 37 °C for 4 h in calcium depleted media. Subsequently 10 μL of the culture was removed and transferred to a 384-well followed by addition of the substrate viR-Ras, the co-factor 14-3-3ß plate and the compound to be tested. Finally the enzymatic reaction was started by addition of NAD⁺. The enzymatic activity was evaluated using a microplate reader by measuring fluorescence at excitation/emission 302/410. IC50 values were determined kinetically by plotting the relative mean velocity (% of control) during the linear part of the curve verses the concentrations of the compounds. As a negative control a strain, PAKΔexoS, with a deletion of the gene encoding ExoS but expressing ExoT at a normal level was used [31]. This mutant did not show any activity in the enzymatic assay showing that other secreted proteins did not affect the enzymatic assay (data not shown). The most potent inhibitors 1, 2, 29, 32, 64-E1 and 65-E2 were tested in this assay and we found that all inhibitors were capable of blocking full-length ExoS activity with the same overall activity as obtained with the recombinant ADPRT domain (Table 2 and Fig. 3B). Interestingly, all compounds revealed increased in potency against full-length enzyme compared to the recombinant ExoS ADPRT with IC50 down to 1.3 μM for the most potent compound (Table 2). Similar observations have been seen with inhibitors of enzymes of the human poly ADP-ribose polymerase family [32]. These results can be reasoned to depend on many factors including proper folding and conformation of the protein or changes of the enzymatic activity [33,34].

3. Conclusion

To conclude, we previously disclosed the identification of compound 1 and 2 as novel small molecule inhibitors of the P. aeruginosa ExoS ADPRT domain. The compounds demonstrated good in vitro activity and physicochemical properties suitable for a medicinal chemistry program. In this study, we established SARs by synthesizing various derivatives of the hit compound and identified the trans-diastereoisomer 32 containing a cyclopropyl system as the most potent inhibitor in its racemic form with an IC50 of 10 μM. Chiral resolution of the racemic mixture showed that one of the enantiomer 64-E1 is six times more potent.
respectively (Table 2). Finally, we demonstrated that the most potent inhibitors were also capable of blocking the ADPRT activity of native ExoS secreted from viable *P. aeruginosa*. This inhibitor class has potential to be developed further into tools to study and better understand the role of the bacterial ADPRT in pathogenicity of *P. aeruginosa*. Such results will ultimately lead to novel antibacterial agents targeting this challenging Gram-negative pathogen.

### 4. Experimental section

#### 4.1. General chemistry

Chemicals and reagents were purchased from Aldrich, Alfa Aesar, AK Scientific, Matrix Scientific or Apollo Scientific. Organic solvents were dried using the dry solvent system (Glass Contour Solvent Systems, SG Water USA) except PhCH₃ and EtOH, which were dried over activated molecular sieves 3 Å. Microwave reactions were performed in Biotage® Initiator®. Flash chromatography was performed on Biotage® Isolera One using appropriate SNAP Cartridge KP-Sil or SNAP Ultra HP-Sphere 25 μm, and UV absorbance at 254 nm. TLC was performed on Silica gel 60 F254 (Merck) with detection by UV light unless staining solution is mentioned. Preparative HPLC separation were performed on Gilson System HPLC, using a YMC-Actus Triart C18, 12 nm, S-5 μm, 250 × 20.0 mm, with a flow rate of 5 mL/min, detection at 214, 230, or 254 nm and eluent system: (A: 2% CH₃OH in CHCl₃, and B: n-Hexane) 30:70 isocratic system. All target compounds were 95% pure according to HPLC analysis. NMR spectra were recorded at 298 K on Bruker DRX 400 MHz and 600 MHz using the residual peak of the solvent as internal standard. 1H, and DMSO-d₆ (δH 2.50 ppm) or CDCl₃ (δH 7.26 ppm) as internal standard for 1H, and DMSO-d₆ (δH 39.50 ppm) and CDCl₃ (δC 77.16 ppm) as internal standard for 13C. LC-MS were recorded by detecting positive/negative ion (EC/EC') with an electrospray Water Micromass ZQ2000 instrument using XTerra MS C₁₈ (5 μm, 19 × 50 mm column) and H₂O/CH₃CN (0.2% HCOOH) as the eluent system, or Agilent 1290 Infinity II—6130 Quadrupole H₂O/CH₃CN (0.1% HCOOH) as the eluent system. All target compounds were >95% pure according to HPLC analysis.

### Table 2

The half maximal inhibitory concentration of the most potent inhibitors in two independent assays.

<table>
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<td>29 (ME0800)</td>
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<td>15 (13.9–16.2)</td>
<td>4.82 ± 0.02</td>
</tr>
<tr>
<td>32 (ME0805)</td>
<td><img src="image5" alt="Structure" /></td>
<td>11 (9.9–12.6)</td>
<td>4.95 ± 0.02</td>
</tr>
<tr>
<td>64–E1 (E1-ME0966)</td>
<td><img src="image6" alt="Structure" /></td>
<td>6.9 (6.3–7.4)</td>
<td>5.16 ± 0.01</td>
</tr>
<tr>
<td>65–E2 (E2-ME0967)</td>
<td><img src="image7" alt="Structure" /></td>
<td>39 (33.5–45.7)</td>
<td>4.40 ± 0.03</td>
</tr>
</tbody>
</table>

| a IC₅₀ values is based on three independent set of results (n = 3) each of triplicate size (N = 3). |
| b IC₅₀ range calculated with 95% confidence interval (CI). |
| c IC₅₀ values is based on thirteen concentration points and three independent set of results (n = 2) each of triplicate size (N = 3). |
| d nd: not determined, compound was not included in the assay. |
| e Racemic mixture of a pure diastereoisomer. |
| f Pure enantiomer of compound 32, however the absolute configuration could not be determined unambiguously. |
UV−trace, ¹H and ¹³C NMR.

4.1.1. General procedure for the synthesis of 2-amino-carboxamide thiophene by modifying an existing protocol [35]. (Method A)

A microwave vial was charged with cyclopentanone (11.9 mmol, 1 g), cyanoacetamide (1 equiv. 1 g), elemental sulfur (1 equiv. 3 g), and KF-alumina (1.3 equiv. 2.5 g), then capped and dry EtOH (18 mL) was added. The solvent was degassed by bubbling N₂ through the solvent for 5−10 min before it was heated in microwave to 100 °C for 5−10 min. The KF-alumina was filtered off and washed with THF, and the collected solution was added to ice-cold water (250−300 mL). If the product was crashed out from the aqueous solution, it was filtered off to afford 2-amino-3-carboxamide Otherwise, the volatile solvents were evaporated under vacuum and the residual solid was collected. The solid product was dried under high vacuum to afford amino carboxamide in 12−76% yields. The products were characterized by NMR and used as it is without further purification.

4.1.2. General procedure for the synthesis of thieno[2,3-d]pyrimidin-4(3H)-one and quinazolin-4(3H)-one from acid anhydride and acid chloride (Method B)

Aminothiophene-3-carboxamide derivative (2.1 mmol, 400 mg) and the corresponding anhydride/acid chloride (5 equiv. 1.1 g) were suspended in toluene (8.8 mL, 0.25 M of the thiophene derivative) and the mixture was heated in the microwave reactor to 150 °C for 15−30 min. The reaction progress was monitored with LC−MS. After completion, toluene was evaporated under vacuum and the aqueous layer was acidified with HCl (6 M) to pH < 4 and the precipitate was filtered and washed thoroughly with H₂O. The crude solid was dried under high vacuum, dissolved in DMSO and purified by HPLC (A: 0.75% HCOOH in H₂O, B: 0.75% HCOOH in CH₃CN, 10 → 50% B over 25 min) to afford final product. See compound characterization for corresponding yields.

4.1.3. General procedure for the synthesis of thieno[2,3-d] pyrimidin-4(3H)-one from aldehyde linker (Method C)

Synthesis: Method B starting from 3a (55.6% yield, brown solid) and the compound was purified by HPLC (A: 0.2% HCOOH in H₂O, B: 0.2% HCOOH in CH₃CN, 10 → 100% B over 25 min). ¹H NMR (400 MHz, DMSO-d₆): δH 12.56 (s, 1 H), 12.39 (s, 1H), 12.08 (s, 1H), 7.89 (t, J = 9.6 Hz, 1H), 7.68 (t, J = 11.6 Hz, 1H), 2.63 (t, J = 7.3 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.94 (p, J = 7.4 Hz, 2H) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δC 174.03, 160.56, 156.05, 153.08, 148.6, 146.8, 118.9, 114.8, 147.73, 131.2, 131.3, 35.2, 27.1, 21.7 ppm. LC−MS m/z (ES⁺) calcd for C₂₂H₁₄N₂O₃S 443.06; obs 447.20.

4.1.4. 3-(4-oxo-4,5,6,7-tetrahydro-3H-cyclopenta[4,5]thieno[2,3-d]-pyrimidin-2-yl)propanoic acid (1) (ST0101)

Synthesis: Method B starting from 3a (60% yield, amorhous white solid, HPLC purified). ¹H NMR (400 MHz, DMSO-d₆): δH 12.36 (s, 1H), 12.21 (s, 1H), 2.89 (t, J = 7.3 Hz, 2H), 2.83 (t, J = 6.8 Hz, 2H), 2.68 (t, J = 6.8 Hz, 2H), 2.33 (p, J = 7.3 Hz, 2H) ppm. ¹³C NMR (150 MHz, DMSO-d₆): δC 173.4, 168.2, 158.1, 156.3, 139.2, 136.2, 118.1, 30.1, 28.9, 28.7, 28.6, 27.3 ppm. LC−MS m/z (ES⁺) calcd. for C₁₂H₁₂N₂O₃S 265.06 [M + H⁺]; observed 265.06.

4.1.5. 4-(4-oxo-3,4-dihydroquinazolin-2-yl)butanoic acid (2) (ME0569)

Synthesis: This compound was synthesized according to published procedure [36]. ¹H NMR (400 MHz, DMSO-d₆): δH 12.21 (2H, 8.07 (dd, J = 7.9 Hz, 1.3 Hz, 1H), 7.76 (ddd, J = 8.0 Hz, 6.4 Hz, 1.5 Hz, 1H), 7.57 (d, J = 7.9 Hz, 1H), 7.45 (ddd, J = 8.0 Hz, 7.2 Hz, 0.8 Hz, 1H), 2.85 (t, J = 6.8 Hz, 2H), 2.75 (t, J = 6.8 Hz, 2H) ppm. ¹³C NMR (150 MHz, DMSO-d₆): δC 173.4, 161.6, 156.2, 148.6, 134.4, 126.7, 125.9, 125.6, 120.8, 29.8, 29.0 ppm. LC−MS m/z (ES⁺) calcd. for C₁₃H₁₀N₂O₃ [M + H⁺]; 219.07 observed 219.16.

4.1.6. 4-(6,7-difuoro-4-oxo-3,4-dihydroquinazolin-2-yl)butanoic acid (23) (ME0154)

Synthesis: Method B starting from 8a (95% yield, white powder). ¹H NMR (400 MHz, DMSO-d₆): δH 12.39 (s, 1H), 12.08 (s, 1H), 7.89 (t, J = 9.6 Hz, 1H), 7.68 (t, J = 11.6 Hz, 1H), 2.63 (t, J = 7.3 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.94 (p, J = 7.4 Hz, 2H) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δC 174.03, 160.56, 156.05, 153.08, 148.6, 146.8, 118.9, 114.8, 147.73, 131.2, 131.3, 35.2, 27.1, 21.7 ppm. LC−MS m/z (ES⁺) calcd for C₂₁H₁₂F₂N₂O₃ 384.07 [M + H⁺]; observed 381.20.

4.2.1. Native ExoS ADPRT enzymatic assay

The wild type P. aeruginosa strain PAK and the ExoS depleted strain PAKΔexoS [31] were grown over night at 37 °C in Luria broth (LB) on a rotary shaker. The culture was the diluted in fresh LB medium supplemented with 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 20 mM MgCl₂ to an OD₆₀₀ of 0.001 (WPA bioware) and incubated at 37 °C on a rotary shaker. After 4 h of incubation, 10 μl of the bacterial culture was added to 25 μl of reaction mixture (500 nM 14-3-3 (P-3) and 2 μM VHL-Ras in reaction buffer) in a 384 well plate (Black Corning). The compounds were then added in triplicates to the wells (final concentration 400 μM−25 nm, 1% DMSO). The enzymatic reaction was started by adding 5 μl eNAD⁺ (final concentration 25 μM). Each well
had a final volume of 50 μl and contained native ExoS, 500 nM 14-3-3β, 2 μM Vh-Ras and 25 μM eNAD+, 1% DMSO in reaction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 50 mM NaCl, 4 mM MgCl2 and 0.5 mM TCEP). The enzymatic activity was evaluated using SynergyTM H4 Microplate Reader with monochromator, by measuring fluorescence at excitation/emission 302/410. IC50 values were determined kinetically by plotting the relative mean velocity (% of control) during the linear part of the curve versus the concentrations of the compounds. The data analysis was performed using nonlinear regression (curve fit) in GraphPad Prism v.7. Measured Z′ factor for all run was above 0.6.

Author contributions

M.S., C.S., H.S., Å.Fo., and M.EI. designed research and experiments. M.S. designed and performed chemical synthesis as well as biological testing and data analysis of the compounds in the recombinant enzymatic assay. C.S. developed the original recombinant enzymatic assay and performed biological testing for some compounds. M.Eb. performed protein expression and purification. M.S., C.S., and M.EI. wrote the article. All authors edited the manuscript and approved the final version.

Declarations of interest

Conflicts of interest: none.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.11.036.

References