


**Weidel, E., Negri, M., Empting, M., Hinsberger, S., Hartmann, R.W.**

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# Composing Compound Libraries for Hit Discovery – Rationality-Driven Preselection or Random Choice by Structural Diversity?

*Elisabeth Weidel[†], Matthias Negri[†#], Martin Empting[†], Stefan Hinsberger[†], Rolf W. Hartmann[†¶]**

[†]Department Drug Design and Optimization, Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Campus C2.3, 66123 Saarbrücken, Germany

[¶]Department of Pharmaceutical and Medicinal Chemistry, Saarland University, Campus C2.3, 66123 Saarbrücken, Germany

[#]Current address: Boehringer Ingelheim Pharma GmbH & Co. KG, Research Networking Germany, Birkendorfer Strasse 65, 88397 Biberach an der Riss, Germany

* Corresponding author

Tel: +49-681-302-70304

Fax +49-681-302-70308

E-mail: rwh@mx.uni-saarland.de

## ABSTRACT

In order to identify new scaffolds for drug discovery, SPR is frequently used to screen structurally diverse libraries. Usually, hit rates are low and identification processes are time consuming. Hence, approaches which improve hit rates and, thus, reduce the library size are required. In this work, we studied three often used strategies for their applicability to identify inhibitors of PqsD. In two of them, target-specific aspects like inhibition of a homologous protein or predicted binding determined by virtual screening were used for compound preselection. Finally, a fragment library, covering a large chemical space, was screened and served as comparison. Indeed, higher hit rates were observed for methods employing preselected libraries indicating that target-oriented compound selection provides a time-effective alternative.

### Defined Key Terms

**SPR**: Surface plasmon resonance spectroscopy - biophysical method to measure biomolecular interactions in real-time. One of the interactants is immobilized on a sensor chip, while the other is dissolved and passed over the immobilized partner as a solution. If an interaction occurs a change in response is observed due to the mass dependency of the SPR signal [1].

**PqsD**: Protein in *Pseudomonas aeruginosa*, which catalyzes a key step in the biosynthesis of HHQ (2-heptyl-4-quinolone). HHQ is the biosynthetic precursor of PQS (2-heptyl-3-hydroxy-4(1H)-quinolone). Both molecules are signal molecules and ligands of the receptor PqsR, which is involved in virulence factor production and biofilm formation [2-5].

**FabH**: *ß*-Ketoacyl-acyl carrier protein (ACP) synthase III (FabH) is a key enzyme in the biosynthesis of bacterial fatty acids. FabH acts as initiator of the fatty acid chain elongation cycle and is involved in the regulation of the entire biosynthetic pathway [6-9].

**Fragments**: are considered as substructures of drug-like substances, which are likely to match to a given binding site. Due to their small molecular weight, they show higher ligand efficiencies and the small and simple molecule structures open up good opportunities for further optimization [10, 11].

**Pharmacophore**: an ensemble of structural features of the ligand site, which are recognized by the target and are responsible for the biological activity of the molecule [12].

**Ligand efficiency**: (LE) is a parameter to assess the potential of a primary hit and is frequently used to select the most promising candidates, to be optimized into lead compounds. The LE can be calculated by conversion of the $K_d$ value into the free binding energy at 300K, which is then divided by the number of heavy atoms (non-hydrogen atoms) [11].

## INTRODUCTION

The need for new anti-infective agents is rising due to the emerging problem of multidrug-resistant pathogens and the increasing variety within bacterial strains [13]. Despite this, the approval of new anti-infectives has been continuously declining. Bacterial infections are getting more and more difficult to cure, due to the lack of active agents [14-18]. The few novel antibiotics on the market are mostly derived from known drugs. Due to their intrinsic similarity to the parent molecule, these compounds are highly susceptible to cross resistance. To really improve antibacterial therapy new targets and novel scaffolds are needed to develop drugs which are less susceptible for the existent resistance mechanisms [19-22].

Nowadays, there is an increasing tendency to perform screenings with biophysical methods like surface plasmon resonance spectroscopy (SPR) to identify suitable molecular starting points for drug development campaigns. To this end, usually huge randomly composed libraries are screened to cover a large chemical space [23, 24]. Although, these biophysical measurements require comparatively little costs, the process is very time consuming and provides only low hit rates [25, 26]. Libraries, whose compounds were not only randomly chosen, but also rationally selected with regard to target-associated characteristics, would probably lead to higher hit rates and would hence be more efficient. This conclusion is also obtained by Valler and Green, who compared diversity screening with focused screening [27]. For targets, where a crystal structure is available, validated docking algorithms can be applied to select promising compounds [28]. The hit rate of such focused screenings is usually between 1 – 10% [29] and is one hundredfold higher than those obtained in random screenings. However, the focused methods are not accurate enough to simply identify a handful of compounds to be tested. To compensate for these deficits, a broader selection of molecules within a defined level of docking score has to be subjected to experimental examination. Furthermore, focused screenings possess the risk of excluding favorable structural motifs from employed libraries by choice of unsuitable selection criteria. In the worst case scenario, all compounds have to be tested like in the random screening approach to identify hits with adequate potency [27].

In this work, we investigate the applicability of three different strategies to the efficient identification of compounds inhibiting the anti-biofilm target PqsD. This bacterial protein is an essential enzyme involved in 4-hydroxy-2-alkylquinolone (HAQ)-based quorum sensing (QS) used by the opportunistic human pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* causes life-threatening infections and is difficult to eradicate using available anti-biotic treatments [30]. We have shown that a blockade of PqsD-mediated signal molecule synthesis leads to disruption of HAQ-based QS and attenuation of biofilm formation [31]. Hence, using this promising target to identify novel molecular scaffolds as suitable quorum sensing

inhibitors (QSI) might pave the way towards innovative anti-infective agents. With the aim to investigate the effectiveness of different screening approaches, we followed three commonly used strategies differing in the manner how the employed compound libraries were composed (Figure 1). In strategy A we took advantage of the high homology between PqsD and the *ß*-Ketoacyl-acyl carrier protein (ACP) synthase III. Hence, seven described FabH inhibitors were selected as starting points for the development of PqsD inhibitors (Figure 2A) [32-37]. After synthesis of additional derivatives compounds **A1** – **A12** were tested for *in vitro* activity. In strategy B, we assembled a structurally diverse library of reasonable size containing fragment-like compounds. In the last approach the library selection was based on a virtual prescreening of 880 ligands using the crystal structure of PqsD in complex with the natural substrate as well as some proposed PqsD:inhibitor complexes reported previously [38-40]. In every approach the compounds were first tested by SPR for their binding and afterwards in an enzymatic assay for their PqsD inhibition. The most potent inhibitors were further evaluated by SPR-based binding site investigations.

**Figure 1.** Schematic overview of the different approaches for the identification of novel PqsD inhibitors: **A**. Me-Too approach[38, 41]. **B**. Fragment Library Screening. **C**. Virtual Screening.

## MATERIALS & METHODS

**Chemistry.** General instructions, synthetic methods applied and spectroscopic data for compounds used in approach A are provided in the SI S13. All tested compounds have >95% chemical purity as measured by HPLC.

**Compound libraries.** The compound libraries of approach B and C were obtained by different vendors. None of these libraries contained compounds which were selected in regard of known PqsD inhibitors to provide an unbiased screening scenario. For the fragment screening 500 compounds were purchased from Maybridge (Loughborough, UK). In the virtual screening part the library was consistent of 179 compounds from Villapharma (Murcia, Spain), 214 from Asinex (www.asinex.com) and 487 from AnalytiCon (Berlin, Germany).

**Expression and purification of recombinant PqsD wild type and mutants in E. coli.** *E. coli* BL21 (λDE3) bacteria were transformed with plasmid harboring PqsD (pET28b(+)/*pqsD*) [52]. Overexpression, purification and storage of the $His_6$-tagged PqsD was performed as described by Storz *et al. [31]*.

**Assay procedure for *in vitro* PqsD inhibition.** The assay was performed monitoring the enzyme activity by measuring the HHQ formation as described by Storz *et al. [31]* with some modifications: the flow rate was 750 µL/min and an Accucore RP-MS column, 150 x 2.1 mm, 2.6 µm (Thermo Scientific, Waltham, Massachusetts, USA), was applied. All reactions were performed in sextuplicate.

**Surface Plasmon Resonance Experiments.**

All surface plasmon resonance experiments were performed in instrument running buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, 5% DMSO (v/v), 0.05% P20 (v/v)) using a Reichert SR7500DC biosensor (Buffalo, USA) equipped with research-grade CMD-500M sensor chips provided by XanTec Bioanalytics (Düsseldorf, Germany) at 18°C. Scrubber 2 software (Version 2.0c, 2008, BioLogic Software) was used for proceeding and analyzing the data. Changes in refractive index due to DMSO dependent solvent effects were corrected by use of a calibration curve (seven solutions, 4.25% - 5.75% DMSO in buffer solutions).

**Immobilization of $H_6$-PqsD and PqsD-mutants.** Immobilization of $H_6$-PqsD (38.6kDA, >90% pure based on SDS-PAGE) was performed analogous to the method described by Henn *et al. [53]* at 18°C. The mutants were immobilized analogous to wild-type PqsD.

**SPR Screening.** For the screening all compounds were tested at 100 µM. Each analyte was injected twice on two different sensor chips. To collect the binding response the analyte was dissolved in running buffer and injected for 120 sec association and 300 sec dissociation. For the measurements in approach B and C a positive control as well as a negative control was injected every $13^{th}$ injection to check the surface stability. The screening reproducibility was calculated according to the equation of Zhang *et al. [54]*. The calculated Z' factors were in the range between 0.8 and 0.97, indicating these runs as reproducible. Compounds were ranked according to the obtained binding responses in the equilibrium. (Immobilization levels: Me-Too: 5742 RU; fragment screening: 4410 RU and 6011 RU; virtual screening: 3901 RU and 4980 RU)

**ACoA preincubation studies.** The ACoA preincubation studies were performed as previously described [39]. Once the baseline is stable again the compounds were injected twice and the responses at equilibrium were compared to those obtained with the untreated surface. For **A1**, **A9** and **B91** concentrations of 100µM were used. Since a strong binding signal was observed for compound **A12** in the first screening, the concentration was decreased to 20 µM. The converse procedure was used for **C29** and **C62.** Since the originally obtained responses were quite low (~ 3 RU), the concentrations were increased to 250 µM. The compounds were injected for 120 s or 180 s association times and 300 s dissociation time. The observed

binding signals were compared to the one obtained before incubation with ACoA. (Immobilization levels: 3579 RU)

**SPR based binding studies on $H_6$-PqsD and PqsD-mutants.** The binding studies were performed at a constant flow rate of 30 µL/min using the same concentrations as in the ACoA preincubation studies. The analytes were injected twice for 120 sec association and 300 sec dissociation time. The binding studies on PqsD mutants were performed analogous to the wild type studies. Responses were obtained at equilibrium and compared to the wild-type signals. (Immobilization levels: S317F: 8357RU; C112A: 4151RU; H257F: 6897RU)

**Virtual Screening.** An *in house* database of 950 compounds acquired from three commercial vendors (Asinex, AnalytiCon, Villapharma) was used for virtual screening. The molecular docking studies were performed by means of GOLDv5.0 [55, 56] within Molecular Operating Environment (MOE) [57]. The CHEMPLP scoring function was used to score the docking poses, whereas a pharmacophore-model (see below) was used for initial placement and filtering. 30 runs per molecule were performed. Default GOLD parameters were used with carbons, halogens, and non-polar sulfur atoms matching hydrophobic regions. The 3D-structure of PqsD PDB-id 3H77 was used for docking: residue 112 (anthranilate-Cys112) of both chains A and B was mutated back into wild-type Cys112 and geometry optimization of the enzyme was performed using the LigX module of MOE. The active site was determined including all residues within 9 Å of the co-crystallized ACoA molecule of 3H77. All compounds with a CHEMPLP value > 60 (in total 102 compounds) were selected for biological evaluation using the PqsD assay.

**Pharmacophore Model.** All three existing structures of PqsD (PDB-id 3H76, 3H77, 3H78) were superimposed and the co-crystallized ligands (3H77: ACoA and anthranilate covalently bound to Cys112; 3H78: both anthranilic acid molecules) plus a 2-benzamidobenzoic acid in binding mode 2 [39] were used to generate a pharmacophore model within MOE. 25 features were defined (SI S14) and a partial match criterium of >8 was set. Feature F1 (anthranilate-Cys) was set as essential feature in order to force placements within the L-shaped active-site channel of PqsD.

## RESULTS AND DISCUSSION

### Strategy A: 'Me too' Approach

In drug discovery the term 'me too' is used to describe the development of compounds, which are derived from known active agents. In this investigation the term was used in a slightly different context, as no PqsD inhibitors were used as a starting point for the development. Instead of this, we took advantage of

the close homology between the target protein PqsD and FabH. Comparing available crystal structures of both proteins (PDB IDs 3H76 and 3IL9 [38, 41]) reveals that they share a nearly identical 3D fold with an overall root mean square deviation (RMSD) of only 1.3 Å, almost 40% sequence identity and conserved active site residues. Therefore, compounds active against FabH (inter alia **A1**, **A3**, **A5**) were supposed to bind PqsD as well rendering them as suitable starting points for the development of QSIs [32-37]. Additionally, some structurally similar derivatives (**A2**, **A4**, **A6**, **A7**, **A8**, **A9**, **A10**, **A11**, **A12**) were also evaluated (Figure 2A).

The 12 compounds were tested for their ability to bind to PqsD by SPR. Binding responses for almost all compounds were obtained, except for **A6** (SI S1). **A11** showed a much higher response than the other compounds, indicating a strong binding affinity to PqsD. To investigate whether the heights of the responses correlate with the inhibitory activities, every compound was tested for its inhibition of PqsD in a functional *in vitro* assay. Nine out of the twelve compounds were able to inhibit PqsD, six of them were identified as strong PqsD inhibitors (>80% inhibition at 50 μM). The comparison of the binding signals with the inhibition values revealed a rough correlation (Figure 2B). An exception was the finding that the highest response was achieved with **A11**, however, in the inhibition assay this compound was less active than **A1**, which gave a much lower SPR response in return. However, a reasonable correlation between SPR response and inhibition was observed within structurally similar compounds. This trend was most prominent in the class of the benzamidobenzoic acids. Here, SPR responses and inhibitory activities concurrently increased in the order from **A9** over **A12**, **A10** to **A11**. Such a tendency was also observed in the other compound classes, where always the compound with the highest response exhibited the best inhibition (**A2–A4** and **A5–A7**).

The lack of over all correlation between binding and inhibition, when comparing all compounds **A1**-**A12**, might be the result of different binding sites for individual compounds. In detail, measured inhibitory potencies do not only depend on affinity of molecules to the target but also on their ability to disrupt the substrate-enzyme interaction and catalytic conversion to the product. Compounds of comparable affinity but different binding sites (e.g. fully, partially or not at all blocking the substrate binding site, the latter including allosteric inhibitors) are likely to display differing activities in substrate-dependent enzyme assays. Hence, elucidation of the binding site is of utmost interest, as this provides an additional ranking criteria for the choice of the best compound to be optimized (*vide infra* in section “Binding Studies”).

**Figure 2A**. Structures of the described FabH inhibitors and synthesized derivatives [32-37] **B**. Inhibition values and binding signals of compounds **A1** – **A12**

In total, five strong PqsD inhibitors were identified, indicating that the 'me too' approach is not only applicable to ligand structures as usually used in literature, but also to the target itself as shown here.

**Strategy B: Fragment screening**

SPR screenings are routinely conducted to identify new scaffolds for ligands. Usually, very large libraries are tested and, compared to that, only a small number of binding compounds are obtained. Hence, low hit rates of 1 to 4% are commonly associated with such 'non-rational' high- or medium-throughput screenings of diverse fragment libraries [23, 42]. To mimic this strategy and to provide a basis for comparison between the different approaches, we employed a commercially available fragment library of considerable size for screening strategy B. Here no target-specific selection criteria were applied. Instead, the compounds were chosen to achieve high structural diversity and drug-likeness. The 500 molecules covered a large chemical space and all of them fulfilled the 'rule of three' for fragment-like structures. To investigate the whole library two SPR screening experiments each using a new sensor chip with freshly immobilized target protein were conducted. To provide comparability between both runs, compound **A11** was included as a reference and all signals were normalized to the response of this positive control (calculations are shown in SI S2). In Figure 3 the obtained binding signals of the 500 compounds are presented. As illustrated in this plot a huge number of binders were identified.

For more than 60% of the tested fragments binding was observed. Furthermore, three compounds showed unspecific binding as their SPR responses exceeded the calculated maximum responses ($R_{max}$) [43]. The high number of binders was unexpected and can be possibly traced back to the very simple scaffolds, which can undergo weak interactions with eventually several sites. Since the SPR methodology we have used is very sensitive, these weak interactions could be detected and may explain the high rate of 312 positives. The large number of PqsD-binding fragments made a stringent selection of candidates for functional evaluation in our secondary assay necessary. We chose 40% normalized SPR response (in reference to **A11**) as a cut-off value and thus selected the 22 most promising compounds. They were tested at 100 µM (in contrast to the compounds of strategy A which were tested at 50 µM) to compensate their small molecular size and to ensure that even low inhibitory activities are detected [10]. However, only three compounds exhibited a moderate inhibition (>50% at 100 µM) (Figure 3C, SI S3), all other PqsD binders within this set of compounds were inactive.

**Figure 3.** SPR-based identification of PqsD inhibitors using a fragment library (strategy B). **A.** SPR responses of fragments screened at 100 µM using preselection strategy B are illustrated as percent binding (normalized to the SPR response of reference **A11**). Compounds with a percentage of more than 40% are primary hits (red points), whereas a

percentage less than 40% represents moderate binders (black points) and less than 7% of SPR response (normalized to positive control) characterize non binders (black points). Unspecific binders (exceeding their Rmax) are illustrated as green points. **B.** The binders are represented by the left column and separated in moderate binders (black), hit compounds (red) and unspecific binders (green). The black column on the right illustrates the percentage of compounds not binding to PqsD. **C.** Hit compounds identified via strategy B: structures, inhibition values at 100 µM, and SPR responses at 100 µM.

Nevertheless, two compounds showed a strong inhibition of PqsD and are, thus, interesting starting points for future investigations.

To examine whether the primary selection criterion based on high SPR response was valid, a collection of eight representative non-binders as well as three unspecific binders were tested for PqsD inhibition (SI S4). As expected, non-binders generally did not show an effect on PqsD-mediated HHQ synthesis with one exception where a weak inhibition (18%) was observed. Two of the unspecific binders (**B379** and **B252**) exhibited a strong PqsD inhibition of >50%. The SPR response of **B252** did only slightly exceed $R_{max}$ (5 RU), but in comparison to all other compounds the obtained binding signal was significantly higher (around 15 RU). Such strong interactions are unusual for small molecules and maybe caused by formation of aggregates. For further investigation, the compound was tested in a concentration-dependent manner. In the lower concentration range (0.5 - 62.5 µM), the compound behaved like a binder with a 1:1 stoichiometry. But above 62.5 µM the increase in response was remarkably high. Here, $R_{max}$ was exceeded by more than a factor of three (SI S5). This observation is indicative of promiscuous binding as described by Giannetti *et al. [43]* and, thus, the compound was excluded from further development. These results corroborate the strategy of using SPR data to sieve out unspecific and non-binding compounds. Three inhibitors were detected, two of them showed a high activity and could serve as starting points for further optimization. Concerning the initial number of 22 primary positives, a hit rate of 13% was obtained, indicating that this approach is significantly less effective than strategy A (hit rate of 82%). Additionally, the cutoff of 40% had to be set rather arbitrarily to reduce the number of compounds for the enzyme assay. Hence, additional functional inhibitors might be missed due to this selection criterion.

**Strategy C: Virtual Screening**

In the third approach, a library based on *in silico* studies was employed to follow a rationality-driven compound preselection methodology. Therefore, a 3D pharmacophore model was built using available crystal structures of PqsD in complex with the natural substrate ACoA (PDB ID 3H77) and without ligand (PDB ID: 3H76) [38]. Additionally, features of recently reported docking poses proposed for PqsD

inhibitors described by us were included in this model [39, 40]. Approximately 900 compounds were screened and ranked according to their binding score (Figure 4A). The best 102 (binding score >60) were selected for PqsD binding experiments via SPR.

Almost 60% of the compounds showed binding (Figure 4B and 4C). Hence, although a structure-guided prescreening was applied a rather high percentage of 40% of compounds did not bind to PqsD. However, it is not unusual that *in silico* generated pharmacophore models and chosen ranking criteria need further refinement to increase predictive power.

To select compounds for the functional PqsD assay, we set the SPR cutoff to 30% resulting in 18 test candidates. 12 compounds showed inhibitory activity, six of them acted as potent inhibitors (>50% at 50 µM). The determined hit rate of 67% is similar to that obtained in strategy A, confirming the assumption that screenings with rationality-driven compound selection facilitate efficient and straightforward inhibitor identification. A closer look at the structures of the six most potent molecules found via this strategy revealed that four of them possess a common 2-aminopyrimidine motif (**C68**, **C69**, **C90** and **C174**, Figure 4D).

**Figure 4.** SPR-based identification of PqsD inhibitors of a compound library composed by virtual screening (strategy C). **A.** Binding score of the library compounds. Red points represent selected compounds for SPR evaluation (binding score > 60) **B.** SPR responses of compounds screened at 100 µM using preselection strategy C are illustrated as percent binding (normalized to the SPR response of reference **A11)**. Compounds with a percentage of more than 40% are primary hits (red points), whereas a percentage less than 40% represents moderate binders (black points) and less than 7% of SPR response (normalized to positive control) characterize non binders (black points). Unspecific binders (exceeding their Rmax) are illustrated as green points. **C.** The binders are represented by the left column and separated in moderate binders (black), hit compounds (red) and unspecific binders (green). The black column on the right illustrates the percentage of compounds not binding to PqsD. **D**. Hit structures identifies by strategy C and inhibition values

Hence, we concluded that this structural feature might be favorable for PqsD inhibition. This observation led us reevaluate both our *in silico* preselection methodology as well as the subsequent SPR ranking for possible false negatives. Using the 2-aminopyrimidine core as search query we identified additional 115 compounds sharing this scaffold within the initial library used for virtual screening. Of these, 12 had been sieved out in the second step by SPR as they showed either no binding (4 compounds) or only weak binding (8 compounds). As expected, the non-binders were essentially inactive in our functional enzyme assay. However, six out of the eight compounds with weak SPR response showed an inhibition above 20% at 50 µM (Table 1). Nevertheless, these values were generally lower than those of our best hits identified by SPR and justified their exclusion from further testing.

**Table 1.** Comparison of inhibition values between SPR identified primary hits and SPR discarded compounds (N=non binders, W= weak binders)

| Primary Hits | | Discarded Compounds | |
|---|---|---|---|
| **Cmpd.** | **Inhibition** | **Cmpd.** | **Inhibition** |
| **C26** | 24% | **N1** | <10% |
| **C31** | 12% | **N2** | <10% |
| **C43** | 36% | **N3** | <10% |
| **C68** | 86% | **N4** | <10% |
| **C69** | 96% | **W1** | 21% |
| **C78** | <10% | **W2** | 12% |
| **C90** | 53% | **W3** | 30% |
| **C100** | <10% | **W4** | 32% |
| **C116** | 15% | **W5** | 32% |
| **C174** | 51% | **W6** | 23% |
| **C176** | <10% | **W7** | 43% |
| **C190** | 18% | **W8** | <10% |
| **C194** | <10% | | |
| **C234** | 26% | | |
| **C242** | <10% | | |
| **C330** | 73% | | |
| **C368** | <10% | | |
| **C542** | 80% | | |

We identified a rather high number of 103 additional compounds bearing a 2-aminopyrimidine moiety for functional analysis within the array of compounds deselected by virtual screening. Nearly 80% of them exhibited no or only weak inhibition (< 50%) (SI S6) leading to the conclusion that discarding them was a correct decision. However, nine compounds exhibited a PqsD inhibition of over 80% demonstrating the limitations of the *in silico* method which largely depends on optimally chosen parameters within the scoring functions and used force fields.

Relying solely on the unrefined virtual prescreening method, some of the most potent 2-aminopyrimidine derivatives (**C25**, **C41**, **C54**, **C103**, **C111**, and **C151**) would have been missed out. However, through a critical inspection of the initial hits we were able to identify the aforementioned motif as a beneficial core structure compensating the shortcomings of the applied procedure. Hence, strategy C as well as the employed pharmacophore-based *in silico* method provided a valuable tool to handle large compound libraries by significantly reducing the number of compounds to be tested in the lab. Additionally, a good ratio of SPR binders to functional hits has been achieved (*vide infra* in SPR evaluation section) while additional inhibitors were found through structural similarity considerations. In total, we identified 30

compounds which strongly inhibit PqsD (SI S7). Compounds possessing the 2-aminopyrimidine core may be used to derive an early SAR, guiding further development.

**SPR Evaluation**

To examine if compound screening by SPR is a suitable strategy for identification of novel PqsD inhibitors, the correlation between SPR binding and protein inhibition was investigated. Therefore, all of the additionally discovered 2-aminopyrimidine derivatives formerly discarded by virtual screening were also tested by SPR and in the enzyme assay (SI S8 and S9). This comparison revealed that approximately 80% of the compounds showed either binding and inhibition (55%) or no binding and no inhibition (25%). In contrast, for 10% of the compounds binding was observed, but PqsD was not inhibited. Reasons for that could be only week interactions not sufficient to compete with the substrates or binding to a site not involved in catalytic reaction like the protein surface. The remaining 10% reduced HHQ formation, but did not show binding to PqsD. Perhaps, the parameters used in the SPR experiments were not suitable for the detection of these interactions [24]. The low inhibition values (most of them between 12 - 27%) demonstrated that no potent molecule was excluded.

The obtained results indicate that in the reported case it is reasonable to neglect compounds showing no SPR response, because they will most likely be inactive. Overall, these results reveal a good correlation between binding and inhibitory activity, demonstrating that SPR is a suitable prescreening method to detect possible PqsD inhibitors.

## BINDING STUDIES

Using strategies A, B, and C, 37 novel PqsD inhibitors with $IC_{50}$ values lower than 50 µM have been identified (SI S10). Among them 10 compounds with potencies in the single-digit micromolar range were found. The most promising inhibitors with $IC_{50}$ values below 5 µM are shown in Table 2. Interestingly, their SPR responses were quite different using the standard protocol and no clear correlation between this binding experiment and inhibitory potency was observed. Indeed, these compounds possess different molecular scaffolds and varying physicochemical properties. Hence, different binding modes and/or binding sites are possible. To investigate this more precisely, we applied a specially designed SPR experiment using PqsD with a covalently blocked substrate binding site. These experiments should facilitate the selection of the most suitable compounds for further optimization.

**Table 2.** Inhibition values and binding responses at 100 µM of the most promising inhibitors.

| Cmpd. | $IC_{50}$ [µM] | Response [RU] |
|---|---|---|
| **A1** | 0.2 ± 0.1 | 11.6 ± 3.5 |
| **A12** | 4.4 ± 0.6 | 91.4 ± 2.9 |
| **B91** | 4.7 ± 1.3 | 13.8 ± 0.9 |
| **C25** | 2.6 ± 0.3 | 3.2 ± 0.2 |
| **C54** | 4.3 ± 0.1 | 2.9 ± 0.2 |

**Blockade of the ACoA binding site**

In 2009, Bera *et al.* published the crystal structure of PqsD in complex with its natural ligand clearly showing a covalent enzyme-thioester adduct, which was formed at the active site Cys112 after addition of the primary substrate anthraniloyl-CoA. This finding can be exploited for binding site investigations as described by Weidel *et al.* (Figure 5A) [38, 39]. For example, inhibitors **A1** and **B91** were not able to bind to immobilized PqsD with a blocked active site (Figure 5B). According to the available X-ray structures, no significant conformational changes occur upon formation of the covalent anthraniloyl-enzyme adduct (compare 3H76 and 3H77) [38]. Hence, we conclude that these two compounds have binding sites directly at or near the active site residues. On the contrary, SPR responses of **A11**, and **C25** did not depend on ACoA pretreatment suggesting an alternative binding site. The signal difference observed for **C54** pointed to a possible overlap of the binding site with the blocked area.

**Figure 5.** SPR-based binding site investigation of novel PqsD inhibitors identified through strategies A, B, and C. **A.** Schematic depiction of the SPR method applied for binding site investigation. **B**. Binding of selected compounds to PqsD. Left: binding before ACoA injection (equivalent to step 3 in **A**); right: binding after formation of the covalent anthranilate-PqsD complex (equivalent to step 9 in **A**). The injection order was as follows: **A1**, **A11**, **B91**, **C25**, and **C54**.

**Binding studies with PqsD mutants**

In addition to the experiments on the anthranilate-based blockade of the PqsD active site, we performed binding studies with single-amino acid mutants as described by Weidel *et al. [39]*. Therefore, active site residues Cys112 and His257 were individually replaced by alanine through site-directed mutagenesis to remove either the thiol- or the imidazole functionality as possible interaction partners. Furthermore, we generated a third PqsD variant by exchanging Ser317 by a sterically more demanding phenylalanine to reduce the size of the active site pocket.

In case of compounds **A1** and **B91**, all aforementioned protein modifications led to a complete loss of SPR response (SI S11). Both observations are in accordance with the results obtained in the ACoA experiment characterizing inhibitors **A1** and **B91** as active site blockers. Regarding **C54**, a decrease in the signal was observed, when the active site was blocked by ACoA treatment, which could be explained by overlapping regions of interaction. Experiments using the mutated enzyme variants corroborated this finding as each of the resulting signals were reduced by ~13 RU compared to the PqsD wild type. **C25** and **A11** were not influenced in their binding by the bound anthranilate. However, the mutations led to a decrease of binding signals, which might seem contradictory at first glance. In a recent publication, we describe a similar phenomenon, the binding mode of a compound structurally similar to **A11** had been investigated [39]. We concluded that this class of compounds acts as a channel blocker interacting with the upper section of the substrate funnel. According to our proposed binding mode, some of the substituents may reach into the active site of PqsD explaining observed effects of amino acid exchanges. Due to the structural similarity the same binding mode for **A11** is very likely and may also apply for **C25**.

In summary, the binding of all inhibitors in this set of compounds was either directly affected by ACoA pretreatment (**A1**, **B91**, and **C54**) or by the performed amino acid replacements (**A1**, **A11**, **B91**, **C25**, and **C54**).

**A1** seemed to be the most suitable compound to be optimized as it was the most potent inhibitor binding deep in the substrate pocket directly at the ACoA binding site. However, its molecular structure containing a hydrochinon system raised some concerns about possible chemo reactivity and/or stability. Indeed, a visual inspection of a 10 mM DMSO stock solution of **A1** at ambient temperature revealed a rapid color shift from colorless to red in the course of one day indicating a possible compound decomposition. An investigation using LC-MS confirmed the appearance of an oxidized degradation product (SI S12). As this oxidized species was not active anymore, **A1** was excluded from further considerations to be used for optimization. Finally, we were able to identify four promising functional hits as scaffolds for the development of potent PqsD inhibitors providing at least two different binding modes.

## Future Perspective

In this work, three different approaches were pursued and evaluated for their suitability to identify novel PqsD inhibitors. The target protein PqsD from *P. aeruginosa* served as a model system allowing for the parallel conduction of a ’me too’ approach, a fragment screening and a virtual screening by molecular docking. These methodologies were employed to provide preselection criteria for the composition of the individual libraries being either rationality-driven (‘me too’ and virtual screening) or based on random collection aiming for structural diversity. In each strategy, primary positives were identified via SPR

binding experiments followed by functional evaluation in an enzyme assay. These experiments led to the identification of novel promising hits for future optimization.

In general, our 'me too' approach exploiting knowledge about the target PqsD and its structural homologue FabH was quite successful. Due to the high similarity of these enzymes, inhibitors of PqsD were readily identified starting from a rather small selection of only 12 compounds. This demonstrates the effectiveness of structure-guided considerations prior to the library screening step. However, to apply this approach knowledge about homologous protein(s) and existing inhibitors thereof are imperative. Due to the intrinsic requirement of a dual- or even multi-target-hitting scaffold, considerable attention has to be paid on target selectivity within the subsequent compound optimization process. Since FabH inhibition is reported to have only a minor impact on *P. aeruginosa* viability [44, 45], we belief that unwanted off-target effects may not occur in the reported system. Finally, we would like to emphasize that despite of a high hit rate of 75%, only one candidate for optimization was obtained at the end of this investigation. The other eight functional hits where dropped either due to their insufficient potency or due to chemical instability. The described hit compound **A11** is structurally similar to an already published class of compounds [39] which proved to be ineffective on the cellular level (unpublished data). Hence, a lot of effort might be necessary to achieve the desired activity on *P. aeruginosa* via further optimization steps.

The second approach was based on the screening of a fragment library, which had been composed with an emphasis on structural diversity. This strategy has the advantage that no information about the target structure is required [46]. Thus, it can be readily applied to screen against any given protein or enzyme of interest when a functional assay is available to evaluate primary positives. We decided to screen a fragment library, because the simplicity of the structures leads to a higher probability of matching to the target-binding site [47]. Additionally, if a compound of fragment-like size shows binding at reasonable concentrations, it should readily possess an optimal interaction geometry. Thus, the identified fragment binders are promising candidates for optimization, which is reflected by high ligand efficiency values [11]. The target affinity necessary for a lead compound can be achieved by subsequent application of fragment linking or fragment growing strategies [48-50].

In this approach, three inhibitors with a moderate potency were detected among the 500 compounds investigated. As expected, a lot of the PqsD-binding fragments discovered by SPR were not able to inhibit the enzyme. It is very likely that these compounds interact with the target at a site remote from the active center. As no target-oriented preselection has been conducted to increase the probability of functional binders, the observation of a high number of such 'false primary positives' is an intrinsic drawback of this strategy. Noteworthy, we applied a library of fragment-like structures in this approach. Due to the low molecular weight of these compounds, high inhibitory potencies of identified hits are not very likely [10]. Hence, it was all the more surprising to discover a fragment with an $IC_{50}$ value in the single-digit

micromolar range. Indeed, it cannot be excluded that some additional PqsD inhibitors have been dropped due to our selected SPR cutoff value. To reduce the effort and time consumption associated with the functional evaluation in the enzyme assay to a reasonable level, such a selection criterion is nonetheless necessary. But, regarding the size of the library screened and the amount of primary actives obtained, the final yield of only one potent fragment was quite disappointing. To reduce the number of nonfunctional PqsD binders in a future approach an additional measurement of all candidates against immobilized enzyme with an active site blocked either by anthranilate or even a rationally designed covalent inhibitor should be considered [51]. This would allow for the direct detection of active site binding fragments. Of course, this methodology requires knowledge and available experimental data on the target and is, thus, not a generic option. Finally, the best inhibitor **B91** identified by strategy B was not considered for further optimization, because more potent inhibitors were discovered in the other approaches.

Strategy C included a compound selection based on a predicted binding score calculated by pharmacophore-guided molecular docking. To perform this kind of screening a protein structure has to be available as well as information on the binding modes of the substrate or identified inhibitors. The established *in silico* prescreening method exhibited an acceptable predictive efficiency, as approximately 60% of the computationally selected compounds showed binding to PqsD. Testing the best binders in the functional assay revealed a rate of 67% functional hits, which is similar to the value achieved by strategy A. Through inspection of the best hits, the 2-aminopyrimidine core was identified as a common scaffold. The evaluation of additional compounds from the parent library consisting of this scaffold in our enzyme assay, led to the identification of 30 potent inhibitors. Hence, future SAR investigations can be conducted in a straightforward manner rendering this compound class the most suitable starting point for further optimization steps. In future, the predictive power of the applied *in silico* method can be improved by use of obtained hit structures from all strategies to rescore the docking results and refine the used parameters. This should enable to further enhance the hit rate of this model for future screenings of broader virtual libraries.

In conclusion, our study underlines the potential benefits from implementing rationality-driven preselection methodologies within the screening-based hit discovery process leading to a more favorable hit-to-effort ratio (Table 3). In our experimental setup, screening strategies A and C using compound libraries assembled via target-guided considerations demonstrated higher efficacies than strategy B which was solely based on structural diversity. As mentioned before, the *P. aeruginosa* Protein PqsD served as model system and thus conclusions drawn for this particular protein may not always be applicable to other targets. However, we have already reported on different classes of PqsD inhibitors [31, 39, 51] and could show that it was possible to address this enzyme by small molecule inhibitors in cell-based assays. Hence, the employed target is in principle a druggable one supporting the notion that conclusions presented herein

could be exploited for screening campaigns aiming on other targets. Thus, we encourage researchers to include and further develop similar knowledge-based preselection methodologies for future hit finding endeavors.

**Table 3.** Comparison of the three screening strategies: Overview different steps leading to final hit identification

| Strategy | Library size | Preselection criteria | Preselection | SPR binder | Primary positives | Functional hits | Hit rate[#] |
|---|---|---|---|---|---|---|---|
| **A** | * | homology | 12 | 11 | 11 | 9 | 82% |
| **B** | 1500 | structural diversity | 500 | 315 | 22 | 3 | 13% |
| **C** | 880 | binding score | 102 | 61 | 18 | 12 | 67% |

*not determined, dependent on available inhibitors of homologous enzyme
[#] hitrate calculated as the ratio of functional hits to primary positives

## Executive Summary

## Background

- The development of new antibiotics with novel modes of action is an indispensable task as the existing antibiotics are becoming increasingly inactive due to resistance development.
- Biophysical screening methods like SPR are frequently used to identify novel bioactive scaffolds
- Libraries consistent of a large number of randomly selected compounds are often screened, however, this strategy leads to low hit rates
- If compounds were selected with regard to target specific properties the hit rate could be increased

## Types of Screening

### Me-Too:

- Benefit: Exhibited a high hit rate and is easily to perform, because inhibitors are available. Only a small number of compounds have to be tested, because almost all molecules were active.
- Limitations: Existence of a homologous protein and described inhibitors. Selectivity could be an issue in the subsequent steps of compound development

### Fragment Screening:

- Benefit: To perform this screening no requirements on the target site are needed like in approach A or C
- Limitations: High number of false positives through weak and unspecific interactions. Leads to low hit rates

**Virtual Screening**

- Benefit: Exhibited a high hit rate and SAR information was obtained.
- Limitations: 3-D protein structure and binding modes of inhibitors or substrates have to be available, which is not always the case.

## Conclusion

- Inhibitors were identified in every approach, but the most potent ones in A and C.
- SPR screenings using libraries, which were composed based on considerations of target specific aspects led to higher hit rates and thus were less time consuming
- In strategy C the most promising candidate for optimization was obtained
- Correlation between binding and inhibition was found indicating SPR as a suitable screening method


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