Chapter 5

Small molecule interference of amino acid transport in (non-) pathogenic Gram-positive bacteria

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Abstract

The ATP-binding cassette (ABC) transporter GlnPQ is present in low GC Gram-positive bacteria where it imports glutamine, glutamic acid and asparagine. The transporter has tandem substrate-binding domains (SBDs) fused to the transmembrane domain, and four SBDs are present in the homodimeric functional complex. GlnPQ is required for nitrogen source intake and the system has been implicated in virulence in pathogenic bacteria. GlnPQ lacks homologs in humans and mammals, and is therefore a potential target for drug development. In this study, we identified compounds that inhibit the activity of GlnPQ, using the crystal structures of the SBDs of GlnPQ from Lactococcus lactis for structure-based virtual screening of a drug library database. By combining the virtual screen with a bioactivity assay, we were able to identify 4 lead compounds that affect growth when the target organism is made dependent of GlnPQ. Two of these compounds inhibit GlnPQ via binding to SBD2. Our study offers a strategy to develop drugs against pathogenic Gram-positive bacteria.
1.1 INTRODUCTION

Many (pathogenic) bacteria are amino acid auxotrophs and require glutamate or glutamine for growth. To meet their requirements for these cellular building blocks, the bacteria use amino acid and peptide transporters. Amino acids and peptides are taken up by the cell via ATP- and electrochemical ion gradient-driven transporters ³.

ATP-binding cassette (ABC) transporters form one of the largest superfamilies of proteins, and the systems are abundant in organisms from each kingdom of life ². Most members of the ABC superfamily are involved in the transport of solutes across biological membranes, either as importer or exporter. The most conserved feature of these proteins is the structure of the Nucleotide-Binding Domain (NBD), which provides the energy for transport by the binding and hydrolysis of ATP ³.

The basic unit of ATP-binding cassette (ABC) transporters consists of two transmembrane and two ATP-binding domains, together referred to as the translocator ². Prokaryotic ABC transport systems involved in solute uptake employ an additional protein that captures the ligand and delivers it to the translocator. These so-called substrate-binding proteins (SBPs) are present in the periplasm of Gram-negative bacteria or they can be tethered to the membrane via a lipid or protein anchor (Gram-positive bacteria, Archaea).

In 2002, it was discovered that members within the ABC superfamily can have the SBPs fused to the translocator domains ⁴, most notably the OTCN family involved in the uptake of osmoprotectants, taurine, cyanate and nitrate, and the PAO family which is specific for polar amino acids and opines ⁵. Within the PAO family, one or two domains are linked to the amino-terminal end of the transmembrane subunit, and these are preceded by a signal sequence. Two of these chimeric substrate-binding/transmembrane proteins together with two ATP-binding cassettes form the functional unit for transport, and these systems thus have two or four substrate-binding domains ⁴. ABC transporters with the SBDs fused to the transmembrane domain are most prevalent in Gram-positive bacteria, including Gram-positive pathogens such as Listeria monocytogenes, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Streptococcus pyogenes and others, but they are also found in Gram-negative bacteria. The fusion between transmembrane domain and SBD prevents the receptor from escaping the membrane surface.

Many Gram-positive pathogens are multiple amino acid auxotrophs and dependent on amino acid uptake via transport proteins. In group B. streptococci for example,
the leading cause of neonatal sepsis and meningitis, the GlnPQ transporter is implicated in the regulation of expression of fibronectin adhesins that are necessary for bacterial adhesion to other cells and therefore important for virulence \(^6\). Furthermore, a study on *Streptococcus pneumonia*, the major cause of life-threatening infections like pneumonia, has demonstrated the importance of GlnPQ for bacterial fitness, virulence \(^7\) and even for cell survival \(^8\). In *Lactococcus lactis*, a non-pathogenic Gram-positive bacterium, GlnPQ provides the main route for fast import of glutamine, glutamate and asparagine \(^9\). Disruption of the *glnPQ* gene leads to a loss of glutamine and glutamic acid uptake, and cells do not longer grow on a medium with glutamine or glutamate as the sole source \(^10\). However, the cells grow normally when the missing essential amino acid is supplied in the form of a dipeptide, which is transported via distinct peptide transport systems \(^11\).

Importantly, the GlnPQ-type transporter is not present in humans or other mammals, thus rendering the system a possible drug target. The inhibition of glutamine and glutamate uptake by GlnPQ could thus be a possible route towards treatment of bacterial pathogens. However, no small molecule inhibitors of GlnPQ are currently known.

Recently, the structures of the substrate-binding domains (SBD1 and SBD2) from GlnPQ of *L. lactis* were resolved by X-ray crystallography \(^12\). Liganded structures were solved to a resolution of 1.4 Å for SBD1 and 0.95 Å for SBD2. Furthermore, SBD2 was solved in an unliganded open conformation at 1.5 Å. Although the two SBDs have a similar fold and similar binding pockets, the ligand affinity and specificity of SBD1 and SBD2 differ. For instance, the dissociation constant *K*D for glutamine between SBD1 and SBD2 differs by 100-fold \(^12\).

In this work, we present the potential of GlnPQ as a novel target for drug development. The structures of SBD1 and SBD2 enabled us to perform a structure-based virtual screening (SBVS) \(^13\) for putative inhibitors of GlnPQ. Two distinct molecular docking programs, Autodock-Vina \(^14\) and FlexX \(^15\), were used to make a virtual library of potential inhibitor compounds that target SBD1 and/or SBD2. The two programs were used to virtually place a set of compounds from the available databases into the crystal structure of the target proteins. Further analysis of the hit compounds provided a selection of around 100 compounds, which were experimentally tested in *L. lactis* using a growth inhibition assay. Four compounds selectively inhibited GlnPQ, of which three share the same core structure.
1.2 RESULTS

1.2.1 Reproduction of ligand-conformation by molecular docking

To date, there are no inhibitors of GlnPQ transporters reported in the literature, and therefore it was not possible to directly test and train the virtual selection technique for the enrichment of active compounds. However, a crystal structure of the substrate-binding domain 2 (SBD2) of GlnPQ from *L. lactis* with bound ligand was available. This allowed a comparison of the docking poses of glutamine from molecular docking calculations with the conformation observed in the crystal structure. The molecular docking program FlexX was used to dock glutamine onto crystal structure of SBD2. For this purpose, crystal structures of the closed-liganded SBD2 (0.95 Å) were used as templates for the molecular docking, and all amino acids within a sphere of 6.5 Å radius around the ligand position were targeted. As indicated in Figure 1, the molecular docking program FlexX successfully generates the glutamine pose as found in the crystal structure. The program generates poses that were ranked on the basis of the most favorable binding free energy. The glutamine pose with the best overlap with the crystal structure was within the six top ranked poses.

![Figure 1](image)

Figure 1. (A) Molecular docking of glutamine onto the crystal structure of SBD2 of GlnPQ from *L. lactis*. The glutamine docking-pose was predicted by FlexX. Glutamine pose in the SBDs is shown as sphere representation (colored in grey). (B) Comparison of glutamine pose with glutamine bound in the crystal structure of SBD2. The glutamine pose (grey) almost completely overlaps with the glutamine in the crystal structure (yellow).

1.2.2 Structure-based virtual screening

Two different compound libraries were screened by FlexX and Vina. Library LA contains 972,307 commercially available drug-like structures. Due to limitations arising from commercial licensing, a smaller but structurally more diverse Library
L_B of drug-like compounds was used for FlexX. L_B contains 20,160 compounds. Library L_A was screened with Vina and Library L_B was screened with FlexX. Table 1 shows numbers of successfully screened compounds from FlexX and Vina targeting either SBD1 or SBD2 and the mutual intersections. For example, program FlexX successfully scored 19,581 compounds to SBD1 and 12,780 compounds to SBD2. Intersection (\(\eta\)) is the number of compounds that where successfully screened to both SBD1 and SBD2. The compounds in the intersections could further be taken for consensus scoring. Consensus scoring is a way to improve the selection of active compounds by combining different scoring results in order to obtain higher hit-rates.

<table>
<thead>
<tr>
<th>Program</th>
<th>SBD1</th>
<th>SBD2</th>
<th>(\eta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlexX</td>
<td>19,581</td>
<td>12,780</td>
<td>12,303</td>
</tr>
<tr>
<td>Vina</td>
<td>793,000</td>
<td>764,469</td>
<td>616,016</td>
</tr>
<tr>
<td>(\eta)</td>
<td>14,600</td>
<td>8,462</td>
<td>7,149</td>
</tr>
</tbody>
</table>

\(\eta\) (intersection): is the number of compounds that where successfully screened to both SBD1 and SBD2 (horizontal-wise) or to both FlexX and Vina (vertical-wise).

Based on these initial compound selections we used four different approaches (method M1 to M4) to finally select a set of around 100 compounds to test in the bioactivity assay. The details of these techniques are listed below:

**M1** 16 compounds were selected from the overlap of the best compounds docking to SBD1 or SBD2 according to FlexX and Vina (see Table 1 column A3 and B3). All compounds that scored exceptionally well with both Vina and FlexX at either SBD1 or SBD2.

**M2** 20 compounds were selected by a consensus-score from the FlexX scores according to SBD1 and SBD2 (Table 1 column C1). The best compounds according to the mean of FlexX scores from both SBD1 and SBD2.

**M3** 50 compounds were selected by a consensus-score of FlexX and Vina docking scores according to SBD1 and SBD2 (Table 1 column C3). The best compounds according to the mean of all scores (FlexX/SBD1, FlexX/SBD2, Vina/SBD1, Vina/SBD2).

**M4** 26 compounds were selected by best Vina scores according to SBD1 or SBD2 (Table 1 column A2 and B2). The compounds that scored exceptionally well with Vina on either SBD1 or SBD2.
This selection strategy resulted in a total of 104 compounds. These compounds plus 2 negative controls were purchased and evaluated in a bioactivity assay to identify compounds that specifically inhibit GlnPQ as described in the following paragraph.

1.2.3 Growth-based assay for screening of potential modulators of GlnPQ

To test the in silico screening of the compound library, we developed a bioactivity assay based on the growth of *L. lactis* NZ9000. *L. lactis* requires glutamate (transported in the form of glutamic acid) or glutamine for growth, which are taken up via GlnPQ. Additionally, it can also grow with glutamate- or glutamine-containing peptides (e.g. alanyl-glutamine), which are transported via the di-and tripeptide transporters DtpT and Dpp \(^{11}\). To simplify the analysis of the compounds we created minimal transporters lacking either SBD1 or SBD2 (GlnPQ-SBD2 and GlnPQ-SBD1, respectively) \(^9\).

The growth dependence of *L. lactis* on glutamine and GlnPQ is demonstrated in Figure 2. *L. lactis* NZ9000 with the chromosomal *glnPQ* genes deleted is named GKW9000, and this strain did not grow with glutamine. The *glnPQ* null strain was complemented in trans with *glnPQ* or mutant derivatives. As anticipated, *L. lactis* expressing GlnPQ, GlnPQ-SBD1 or GlnPQ-SBD2 did not grow in chemically-defined medium (CDM) that lack glutamate and glutamine (CDM\(^{**}\)) [see also Figure 3]. In the presence of glutamine or glutamate or alanyl-glutamine, the strains grew albeit with somewhat lower growth rate or and longer lag phase. We note that *L. lactis* expressing GlnPQ-SBD1 required a higher concentration of glutamine than cells expressing GlnPQ wild type or GlnPQ-SBD2 (details are discussed in chapter 4). Yet, GlnPQ with only a single SBD (either SBD1 or SBD2) supports growth of *L. lactis* on glutamine. These data show that GlnPQ is essential for glutamine import and required for high rates of glutamate import. For the initial studies, we used glutamine as GlnPQ-specific substrate to screen for growth inhibition.

In the initial screen *L. lactis* NZ9000 cells were used to test 104 potential inhibitor compounds, selected from the virtual screening. For each compound, we grew *L. lactis* in two CDM\(^{**}\) compositions (details on CDM composition are described in chapter 4). One supplied with glutamine and the other one with glutamine plus alanyl-glutamine. Cell growth in the presence of alanyl-glutamine was used to discriminate between general inhibition by the tested compounds and GlnPQ-specific inhibition.
Figure 2. Growth dependence of *L. lactis* NZ9000. Cells did not grow (□) in CDM that lack glutamine and glutamate (CDM”). Cell grew in CDM” supplied with 1 mM of glutamine (●) or 1 mM of dipeptide alanyl-glutamine (O). Disruption of the *glnPQ* gene in *L. lactis* NZ9000, yielding strain GKW9000, totally abolished cell growth (×). Complementation of strain GKW9000 *in trans* with the *glnPQ* gene restored the growth (■).

Figure 3. Growth of *L. lactis* GKW9000 expressing GlnPQ (A), GlnPQ-SBD1 (B) or GlnPQ-SBD2 (C). Cells were grown in CDM” (O), or in CDM” supplied with 3 mM Na-glutamate (●), 30 mM glutamine (■), or 3 mM alanyl-glutamine (▲). The GlnPQ cartoons are shown above the growth curves. The blue and red filled-circles are glutamine and asparagine, respectively.

The growth phenotypes were used to categorize the compounds into three classes. Class 1 compounds caused growth inhibition in the absence and presence of alanyl-glutamine, which is indicative of non-specific effects. Class 2 inhibited growth in the absence of alanyl-glutamine significantly more than in the presence of the peptide, which is explained as inhibition of glutamine import via GlnPQ.
Class 3 compounds did not affect growth at the concentrations tested and these were left out for further analysis. Out of 104 compounds tested, at least 4 compounds (namely C1, C2, C3, and C4) appeared to selectively inhibit GlnPQ. Three of these potential inhibitors (C1, C2, and C3) share a common “core-structure” (2-methyl-4H, 5H, 6H-cyclopenta [b]-thiophene-3-carboxylicacid) as illustrated in Figure 4.

![Molecular structures of the compounds that inhibit growth of L. lactis in a GlnPQ-dependent manner.](image)

Compound C1, C2, and C3 were identified by method M3, which is the consensus of four docking scores: FlexX scores of SBD1 and SBD2 and Vina scores of SBD1 and SBD2. Furthermore, compounds C1, C2, and C3 were also in the top 50 rank of 12,303 compounds obtained by method M2 (data not shown). On the other hand, compound C4 was found only by method M4, which selects compounds based on the Vina score of the top rank of either SBD1 or SBD2 or both.

The comparison of the FlexX scores (presented as binding free-energy difference, ΔG) of each compound with the average FlexX scores of all compounds is listed in Table 2. The results reveal that C1, C2, and C3 score lower (more negative ΔG) than the average docking score for both SBD1 (-19.3 +/- 4.3 kJ.mol⁻¹) and SBD2 (-7.7 +/- 3.9 kJ.mol⁻¹). On the other hand the Vina scores for SBD1 were much closer to the average value (-29.9 +/- 3.6 kJ.mol⁻¹). The average Vina score for SBD2 was not evaluated, since the distribution deviated from a normal distribution. However, the distribution of Vina scores for SBD2 shows a peak at -17 kJ.mol⁻¹ (see Figure 5). On the basis of this and an approximation of the standard deviation 4 kJ.mol⁻¹ (as observed for the other distributions in Table 2), the Vina score of C4 for SBD2 (-
28.5 kJ.mol\(^{-1}\)) is significantly lower than the peak score of -17 kJ.mol\(^{-1}\). We thus kept C4 for further analysis.

Table 2. \(\Delta G\) (kJ.mol\(^{-1}\)) prediction of compounds (C1-C4) for SBD1 or SBD2.

<table>
<thead>
<tr>
<th>ID</th>
<th>Enamine-ID</th>
<th>SBD1</th>
<th>SBD2</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>T5289708</td>
<td>-29.9</td>
<td>-23.2</td>
<td>FlexX</td>
</tr>
<tr>
<td>C2</td>
<td>Z99597134</td>
<td>-29.2</td>
<td>-23.0</td>
<td>Vina</td>
</tr>
<tr>
<td>C3</td>
<td>T5233657</td>
<td>-32.0</td>
<td>-23.4</td>
<td>FlexX</td>
</tr>
<tr>
<td>C4</td>
<td>T6456644</td>
<td>n.e.</td>
<td>n.e.</td>
<td>Vina</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>-19.3</td>
<td>-7.7</td>
<td>M3</td>
</tr>
<tr>
<td></td>
<td>STD</td>
<td>4.3</td>
<td>3.9</td>
<td>M4</td>
</tr>
</tbody>
</table>

Average and standard deviations (STD) were calculated with respect to compounds from the intersections (Table 1). n.e., not evaluated. The average value is based on all docking scores with the particular scoring function (FlexX or Vina) and the target protein (SBD1 or SBD2). The average value and STD serve to benchmark the significance of the other scores.

Figure 5. Distribution of the predicted binding free-energies of the screened compounds by molecular docking calculation. The results were taken from the intersections in Table 1 according to SBD1 (14,600 compounds) and SBD2 (8,462 compounds). Only compounds with \(\Delta G\) below zero were considered.

Furthermore, the docking poses generated by Vina show that compounds C3 and C4 are placed in the binding pocket of SBD2 and significantly overlap with the position of glutamine in the crystal structure of SBD2 (Figure 6). On the other hand, Vina and FlexX place compounds C1 and C2 on the surface of SBD2, i.e. at the interface between the two lobes of the SBD (Figure 7; panel A). The docking poses generated by FlexX for compounds C1, C2, and C3 are very similar. The aromatic ring systems of the three compounds are placed in a small hydrophobic pocket of SBD2 and the carboxyl group of the compounds is interacting with Arg-333 (Figure 7; panels B, C and D).
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Figure 6. Comparison between glutamine conformation in the crystal structure (A) and the docking pose for compound C3 (B) and C4 (C) in the binding pocket of SBD2. The docking pose of compound C3 and C4 generated by Vina are overlap with the glutamine molecule in the crystal structure of SBD2. The ligands (glutamine, C3, and C4) in the SBD2 binding site are colored in magenta.

Figure 7. The predicted docking pose of compound C1, C2, and C3 in the crystal structure of SBD2 generated by FlexX. (A) Crystal structure of SBD2 with glutamine (magenta) in the binding site and the docking pose of compound C1 at the outer surface of SBD2 (stick representation in yellow). The docking poses of compound C2 and C3 are similar to the docking pose of compound C1; the details of the interactions are shown in panel (B), (C), and (D). The aromatic ring system of C1, C2 and C3 docks in a small hydrophobic pocket of SBD2. The potential interactions between residues of SBD2 and the compounds are shown by yellow dash.
1.2.4 Effect of C1-C4 on the growth characteristic of *L. lactis*

Growth inhibition can be manifested as a longer lag-phase, lower growth rate or /and lower cell yield (biomass formation), *i.e.* the cells may adapt differently to the stress imposed by the tested compounds. Ideally each of these parameters is extracted from growth data, using a mathematical model such as the logistic function or Gompertz. However, in some cases growth did not reach a true exponential phase and the final cell yield was difficult to estimate. To simplify the analysis of the inhibitory effect on cell growth, we combined the three growth parameters into a single factor, termed the "growth characteristic". As previously reported in Hasenbrink et al., the growth characteristic is determined by calculating the area under the growth curves (see Figure 8), thus, taking into account the length of the lag-phase, the rate of growth, and the yield of growth (maximum OD at 600 nm). We used this approach to screen a wide variety of conditions and compounds.

![Figure 8. Illustration of the growth characteristic. Growth of *L. lactis* NZ9000 expressing chromosomal *glnPQ* in the absence or in the presence of 1 mM of compound C3 is shown in panel A and B, respectively. The area under the growth curves from time zero to 16h are shown in raster area and calculated as growth characteristic.](image)

The effect of C1-C4 on the growth of *L. lactis* expressing GlnPQ is shown in Figure 9. With increasing concentration of C1 the growth is inhibited both in the absence (Figure 9; panel A.I) and presence of alanyl-glutamine (Figure 9; panel A.II), but the effect is stronger in the absence of alanyl-glutamine. Similar observations were made for C2, C3 and C4, and these data are presented as growth characteristic (Figure 9; panel B) or growth rate (Figure 9; panel C) as a function of compound concentration. As is evident from the graphs, the growth characteristics and the growth rates decrease as the compound concentration increases; the inhibition is strongest with C1 (Figure 9; panel B.I and C.I, respectively; key symbol in [□]).
Figure 9. Growth inhibition of *L. lactis* NZ9000 by C1-C4. (A) Cells were grown in CDM** supplied with 3 mM glutamine (panel A.I) or with 3 mM glutamine plus 3 mM dipeptide alanyl-glutamine (panel A.II). In both conditions cells were exposed to different concentrations of compound C1; key: grey (0), black (0.25 mM), green (0.5 mM), red (0.75 mM), and blue (1 mM). (B) Growth inhibition by compounds C1 (□), C2 (△), C3 (○), and C4 (▽). Panel B.I and B.II show the cells growth characteristic in CDM** containing glutamine or containing glutamine plus alanyl-glutamine, respectively. (C) Specific growth rate of the corresponding cells and the key symbols are the same as in B.
1.2.5 Inhibition of glutamate transport

Next, we investigated the effect of C1-C4 on growth of *L. lactis* using conditions in which glutamate import required for growth. Although glutamine and glutamate are both taken up via GlnPQ, the specificity of the substrate-binding domains differs. Transport of glutamine occurs with relatively low affinity via SBD1 and high affinity via SBD2. The *K*\(_D\) for glutamate (glutamic acid) binding could not be determined by ITC, but on the basis of uptake experiments glutamate is transported by both SBD1 and SBD2 (chapter 4), and the pH dependence suggests that glutamic acid is the translocated species. Moreover, asparagine is imported via SBD1 and competes with glutamine and glutamate transport when cells are grown in CDM (chapter 4). The difference in specificity and affinity for glutamine explains why *L. lactis* expressing GlnPQ-SBD1 need much higher concentrations of glutamine to grow; asparagine (*K*\(_D\)=0.2 μM) competes strongly with glutamine (*K*\(_D\)=92 μM) when SBD1 is used.

To determine whether C1-C4 affect a specific SBD, we evaluated their effects in cells expressing either GlnPQ-SBD1 or GlnPQ-SBD2 (Figure 10) and using (CDM***) medium containing glutamate (panel A, C, E, G) or alanyl-glutamate (B, D, F, H) as sole source of glutamate and glutamine. In line with the growth inhibition in the presence of glutamine, compound C1 does exert a GlnPQ-specific effect. Similar results were observed for the C1 analog with a cyclopentane (compound C2), or methyl group (compound C3) instead of cyclohexane linked to the core molecule (2-methyl-4H,5H,6H-cyclopenta[b]-thiophene-3-carboxylicacid). We notice that the inhibition of growth diminishes with decreasing size of this functionality. Furthermore, specific growth inhibition was also observed for the unrelated compound C4, albeit that relatively a high concentration was needed. Furthermore, the effect of C1 is exerted primarily on SBD2 as the strain expressing GlnPQ-SBD2 was inhibited more strongly than GlnPQ-SBD1. The effectiveness and specificity of compound C1 and C2 in inhibiting *L. lactis* via SBD2 is evident from the growth characteristics analysis (see also Appendix 1).
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Figure 10. Growth inhibition of *L. lactis* GKW9000 by C1-C4. *L. lactis* GKW9000 expressing GlnPQ (□, ■), GlnPQ-SBD1 (△, ▲), and GlnPQ-SBD2 (○, ●) were grown in a CDM* supplied with 3 mM glutamate (left panels) or 3 mM alanyl-glutamine (right panels). The open and closed symbols represent cells that growth in the absence or in the presence of inhibitor, respectively. Inhibition by 0.1 mM C1 are shown in panels A and B; by 0.25 mM C2 in panels C and D; by 1 mM C3 in panels E and F; and by 1 mM C4 in panels G and H.
1.2.6 In vivo uptake assay

To determine whether or not C1 directly affects amino acid import via GlnPQ, we performed transport assays using \[^{14}\text{C}]\text{glutamine. We found that glutamine uptake via GlnPQ was inhibited by C1 (Figure 11; panel A). In accordance with the growth inhibition, C1 affects transport via SBD2 more strongly than via SBD1 (Figure 11; panel C and B, respectively). We note that the rate of uptake via GlnPQ-SBD1 and GlnPQ-SBD2 is higher than that of cells with GlnPQ, which reflects the higher level of expression of transporters with a single rather than tandem SBDs, as shown previously.}^9

![Figure 11. \[^{14}\text{C}]\text{-glutamine uptake in L. lactis GKW9000 expressing GlnPQ (A), GlnPQ-SBD1 (B) or GlnPQ-SBD2 (C) in the absence (□) or in the presence (■) of 0.25 mM compound C1. The error bars indicate the range of values from at least two measurements.}]

1.3 DISCUSSION

Glutamine and glutamate are major nitrogen sources for biosynthesis, and in addition glutamate serves a major role as osmolyte in the cell\[^9\]. Glutamine can be synthesized from glutamate and ammonium by glutamine synthetase (GlnA)\[^20\] and glutamine can be converted into glutamate by glutaminase\[^21\]. In L. lactis (and other bacteria studied), the concentration of glutamate is much higher than that of glutamine, which reflects the high glutaminase activity\[^18\].
Here, we identified inhibitors of glutamine and glutamate import in *L. lactis*, using a virtual screening of compounds on the basis of the crystal structures of SBD1 and SBD2 of the ABC transporter GlnPQ from *L. lactis*. We identified four compounds (C1, C2, C3, and C4) that inhibit the growth of *L. lactis* more strongly when glutamine is imported as amino acid via GlnPQ than when it is taken up in the form of a dipeptide via one of the peptide transporters. Interestingly, compound C1, C2, and C3 share a similar core structure, and the more hydrophobic the moiety attached to the core, the stronger the inhibition. Compound C1, C2, and C3 are predicted by FlexX to bind at the surface of the protein where they sit in a small hydrophobic pocket. Thus, compound C1, having cyclohexane attached to the core structure, inhibits GlnPQ the strongest, followed by compound C2 (with cyclopentane), and compound C3 (with methyl). Furthermore, by employing strains of *L. lactis* that express GlnPQ with either SBD1 or SBD2, we find that SBD2 is the prime target of the compounds.

The preference of these inhibitors for SBD2 is in agreement with the docking scores. The relative scores of the binding free-energy of compound C1, C2, C3 and C4 against the average score are significantly lower for SBD2 than for SBD1 (for details see Table 2). This is an indication that these compounds are predicted to interact stronger with SBD2 than the majority of the screened compounds. However, looking solely at the individual scores of compound C1, C2, C3, and C4 in FlexX and Vina, SBD1 has lower (more negative) scores than SBD2. This means the interaction of these compounds with SBD1 is predicted to be tighter (higher in affinity) than with SBD2. This is not consistent with what we found in the growth assays. A possible explanation for this apparent contradiction is that we used different conformations of the two proteins. We used the crystal structure of the closed-liganded conformation of SBD2 to virtually dock the screened compounds. Therefore all side chains were optimal for glutamine binding but perhaps not for other compounds that we screened. On the other hand for SBD1, we used the open conformation structure, which may be more favorable for accommodating the screened compounds. It is well known that docking scores strongly depend on optimal target-site conformations. Our results show that docking scores for different target sites have to be interpreted with special care even if they originate from the same program.

The selectivity of compound C1 for SBD2 of GlnPQ is confirmed by the uptake experiments, where we find that inhibition of glutamine uptake via SBD2 is stronger than via SBD1. Furthermore, referring to the predicted pose of compound C1, which is placed on the surface of SBD2 and not in the binding pocket, it is not likely that inhibition occurs via competition with glutamine or glutamate. The
inhibition could be due the transfer of these ligands from the SBD2 to the transmembrane domain or due to conformational changes that affect the affinity of the protein for its natural substrate.

In conclusion: This study provides a strategy and first lead compounds to fight pathogenic bacteria through inhibition of the ABC transporter GlnPQ. By combining structure-based virtual screening and functional assays we are able to enrich four active modulators of amino acid transport. Our growth assay is simple and readily discriminates GlnPQ effectors from specific inhibitors by comparing growth on glutamine (or glutamate) versus alanyl-glutamine. The next step is to improve the compounds by making them more selective for GlnPQ.

1.4 MATERIALS AND METHODS

1.4.1 Strains and growth conditions

*Lactococcus lactis* NZ9000 and *L. lactis* GKW9000 \(^9\) were used to express GlnPQ and variants with either SBD1 (GlnPQ-SBD1) or SBD2 (GlnPQ-SBD2). *L. lactis* cells were cultivated semi-anaerobically at 30°C in a chemically defined medium (CDM; the composition is described in chapter 4) containing 1% (w/v) glucose and 5 \(\mu\)g/ml chloramphenicol. CDM** is CDM without glutamine.

1.4.2 Assay conditions

Pre-cultures were obtained by inoculating 5 ml of medium with a single colony, and growing the cells overnight to A600 ~0.5 in CDM** supplemented with 3 mM alanyl-glutamine. The next day, pre-cultures were diluted to A600 ~ 0.05 in 5 ml fresh CDM** and allowed to grow to A600 ~ 0.5. Next, the cells were harvested and washed twice with 50 mM cold K\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) pH 6.5 and resuspended in the buffer to A600 ~ 0.5. 30 \(\mu\)l of pre-cultures were used to inoculate 300 \(\mu\)l of CDM** supplemented with either of glutamine, glutamate, or alanyl-glutamine plus 0.01% of *L. lactis* NZ9700 \(^{22}\) spent medium (containing the inducer, nisin A), 5 \(\mu\)g/ml chloramphenicol, 1% (w/v) glucose and the test inhibitor compounds. A total of 104 putative inhibitors plus 2 controls (purchased from Enamine Ltd, Ukraine) were screened. The inhibitors were dissolved in 100% (w/v) DMSO and diluted into the growth medium to a final DMSO concentration of 2%. At 2% DMSO, growth of *L. lactis* NZ9000, carrying plasmids with wildtype or mutant derivatives of *glnPQ*, is not affected (see also Appendix 2). Growth was monitored by spectrophotometric determination of the absorbance at 600 nm, using 96 well microtiter plates and an automated microtiterplate reader (Biotek). Growth was
followed for at least 24 hours at 15 min or 30 min intervals and intermittent shaking of the plate.

1.4.3 Data Analysis

The growth characteristic was determined as previously described in \(^{17}\). It was calculated from the area under the curves obtained within at least 16 of hours of growth, following inoculation with 10% of pre-cultures. The specific glutamine or glutamate import-specific effects were calculated by comparing the growth characteristic in the absence and presence of alanyl-glutamine. Additionally, the effect on the growth rate (μ) was determined by fitting the growth curves to a modified logistic model as described in \(^{16}\). Model fitting was performed using Mathematica software \(^{22}\).

1.4.4 In vivo uptake experiments

For glutamine uptake in whole cells, \textit{L. lactis} expressing GlnPQ or mutant derivatives were grown overnight in 5ml CDM** supplemented with 3 mM alanyl-glutamine. The next day, pre-cultures were diluted to A600 ~ 0.05 in 5 ml fresh medium and allowed to grow to A600 ~ 0.5. The cells were induced for 1.5 hours with 0.01% of culture supernatant of the nisin A-producing strain NZ9700\(^{22}\), and harvested by centrifugation for 10 min at 4500 x g. Next, the cells were washed twice with 10 mM Pipes-KOH plus 80 mM KCl pH 6.0 and resuspended in the same buffer to approximately 10 mg/ml of total protein (A600 of 1 corresponds to 0.2 mg of total protein per ml). For uptake assays, the cells were diluted to a final protein concentration of 0.5 mg/ml in 30 mM K-Hepes-Mes-Pipes pH 6.0. Prior to uptake, the cells were temperature-equilibrated and energized in the presence of 10 mM glucose plus 5 mM MgCl\(_2\) at 30°C. After 3 min, the uptake reaction was started by adding 250 μM \[^{14}\text{C}\]-glutamine. At given time intervals, 40 μl samples were taken and diluted into 2 ml ice-cold 100 mM LiCl. The samples were rapidly filtered through 0.45 μm pore-size cellulose nitrate filters (Whatman BA85) and the filter was washed once with ice-cold 100 mM LiCl. The radioactivity on the filters was determined by liquid scintillation counting.

1.4.5 Molecular docking

Molecular docking was used to place a set of compounds from the available database-library into the crystal structure of the target protein. The compounds were docked into the structure of the protein in a variety of positions, conformations, and orientations. The orientation and conformation of a ligand relative to its receptor is called pose. The ligands are ranked based on the best-
scored pose. In this study we used the docking programs Autodock-Vina and FlexX, which are conceptually different in their algorithms. One criterion of the algorithm is how the ligand is treated during the docking simulation. In Autodock Vina the ligand is treated in its entirety, FlexX on the other hand uses an incremental approach to build the ligand starting from a docked "base fragment".

The target sites of GlnPQ were generated with the graphical user interface of FlexX (LeadIT version 2.0), using crystal structures of SBD1 (open conformation) and SBD2 (closed conformation) at resolutions of 1.4 Å and 0.95 Å, respectively. All residues within a sphere of 6.5 Å around the substrate were defined as the target site. Standard parameters were used for weights of the scoring function and the number of intermediate solutions for each fragment. For Autdock-Vina, input files were generated using the AutoDock plug-in for the program PyMOL, using the same crystal structures as used for FlexX. A cubic box with an edge length of 7 Å was centered on the ligand and used as target site.

Two databases for screening of commercially available drug-like compounds were kindly provided by Enamine (http://www.enamine.net). Library LA contained a broad set of 972,307 drug-like compounds, whereas library LB contained a diverse set of 20,160 drug-like compounds. The 3D structures were prepared and protonated with the program Conrina, version 3.48. Tanimoto-coefficients section was calculated using cheminformatics and machine learning software RDKit (http://www.rdkit.org) and default 2048 bit hash Daylight topological fingerprints (Section 0.1.4). The minimum path size was 1 bond and the maximum was 7 bonds.

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REFERENCES


Chapter 5


Appendixes

Appendix 1. Growth inhibition of *L. lactis* GKW9000 expressing GlnPQ (□), GlnPQ-SBD1 (▲), or GlnPQ-SBD2 (●) by C1, C2, C3, or C4 as indicated. The Δgrowth characteristic represents the specific inhibition on GlnPQ. It was calculated as the difference between the growth characteristic of the cells grown in CDM** containing glutamate and of the cells grown in CDM** containing glutamate plus alanyl-glutamine. The Δgrowth characteristic for each strain is plotted against the compound concentrations. Inhibition by compound C1 and C2 are more pronounced on cells expressing GlnPQ-SBD2 (symbol ▲). The Δgrowth characteristic increases as we raise the inhibitor concentration until the maximum is reached, after which the specificity gradually diminishes.

Appendix 2. *L. lactis* growth profile in CDM containing dimethyl sulfoxide (DMSO). Key: without DMSO (■), 1% DMSO (○), 2% DMSO (□), 5% DMSO (▲), or 10% DMSO (●).