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Kinetic analysis of the inhibition of the drug efflux protein AcrB using surface plasmon resonance

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ABSTRACT

Multidrug efflux protein complexes such as AcrAB-TolC from *Escherichia coli* are paramount in multidrug resistance in Gram-negative bacteria and are also implicated in other processes such as virulence and biofilm formation. Hence efflux pump inhibition, as a means to reverse antimicrobial resistance in clinically relevant pathogens, has gained increased momentum over the past two decades. Significant advances in the structural and functional analysis of AcrB have informed the selection of efflux pump inhibitors (EPIs). However, an accurate method to determine the kinetics of efflux pump inhibition was lacking. In this study we standardised and optimised surface plasmon resonance (SPR) to probe the binding kinetics of substrates and inhibitors to AcrB. The SPR method was also combined with a fluorescence drug binding method by which affinity of two fluorescent AcrB substrates were determined using the same conditions and controls as for SPR. Comparison of the results from the fluorescent assay to those of the SPR assay showed excellent correlation and provided validation for the methods and conditions used for SPR. The kinetic parameters of substrate (doxorubicin, novobiocin and minocycline) binding to AcrB were subsequently determined. Lastly, the kinetics of inhibition of AcrB were probed for two established inhibitors (phenylalanine arginyl β-naphthylamide and 1-1-naphthylmethyl-piperazine) and three novel EPIs: 4-isobutoxy-2-naphthamide (A2), 4-isopentyloxy-2-naphthamide (A3) and 4-benzyloxy-2-naphthamide (A9) have also been probed. The kinetic data obtained could be correlated with inhibitor efficacy and mechanism of action. This study is the first step in the quantitative analysis of the kinetics of inhibition of the clinically important RND-class of multidrug efflux pumps and will allow the design of improved and more potent inhibitors of drug efflux pumps. This article is part of a Special Issue entitled: Beyond the Structure-Function Horizon of Membrane Proteins edited by Ute Hellmich, Rupak Doshi and Benjamin McIlwain.

1. Introduction

Antibiotic resistance is a global problem that needs urgent attention [53,63,71]. The World Health Organisation has recently listed 12 families of bacteria that pose the greatest threat to human health. All the organisms listed as “priority-1” (critical level) are Gram-negative bacteria [72]. Gram-negative bacteria display high levels of intrinsic resistance due to the presence of an outer membrane that act as a permeability barrier and the expression of an array of drug efflux pumps [3,5,21,24] which lowers the concentration of antibiotics inside the cell to sub-toxic levels. Clinical levels of resistance in Gram-negative bacteria are conferred by transporters that consist of complex, macromolecular, tripartite assemblies that span the double membrane and periplasm of these organisms such as the AcrAB-TolC efflux system from *Escherichia coli* [2,5,9,10,12,21,30,35–38,57]. In these complexes an inner membrane protein (e.g. AcrB) acts together with an outer membrane protein (e.g. TolC) and a periplasmic adaptor protein (e.g. AcrA) to form a highly efficient antibiotic efflux system [11,14,16,45,65,67]. The inner membrane protein is from the resistance nodulation division (RND) and is the component responsible for drug recognition and binding [17,25,32,33,39,54]. Therefore, compounds which can directly block the drug binding to AcrB by binding with higher affinity or allosterically trap the IMP in an inactive conformation, are attractive forms of efflux pump inhibitors (EPIs) [13,42,44,64].

The first structures of AcrB in an asymmetric homotrimeric conformation [25,47] not only provided the first structural information on these intractable membrane proteins, but also allowed the deduction of a possible transport mechanism. These structures revealed a functional rotation mechanism where the three monomers cycle through three different conformations during the efflux process designated the loose/access, tight/binding and open/extrusion stages [25,47]. In the ensuing

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years significant advances have been made in the structural and functional determination of AcrB [15,17,31,39,74]. The binding site of AcrB was revealed to be large with two major substrate-binding pockets located along the substrate translocation pathway. The access binding pocket is located closer to the periplasmic bulk in the access/loose monomer of the transport cycle. The deep binding pocket is located much deeper within the substrate transport pathway, and is wide open in the binding/tight monomer of the transport cycle [42–44]. The two binding pockets are separated by a flexible glycine-rich loop. Conformational flexibility of this loop (termed the switch loop) is crucial to allow the conformational changes that drive antibiotic efflux [8,18,28].

These structural and biochemical data was followed by the first structures of inhibitor-bound AcrB which constitutes a significant advance in the field. The structure of the pyranopyrimidine inhibitor D13-9001 bound to AcrB [29] and MX2319 bound to AcrBper (periplasmic domain of AcrB) [50] suggested tight binding of these inhibitors to the narrow hydrophobic pit lined by several phenylalanine residues which prevents the functional rotation of AcrB necessary for drug extrusion and prevent the efflux of antibiotics [29]. EPIs such as phenylalanine arginyl β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) are shown to straddle the top of the switch loop in molecular dynamics simulation studies [59]. This interaction with the switch loop, in turn, controls the movement of substrates in the deep binding pocket and was the proposed mechanism of action of these two EPIs [42]. PAβN and NMP act at concentrations of ≥ 50 μM [6,23]. A derivative of MX2319, MBX3132 binds AcrB with nanomolar affinity as a result of a tight interaction between this compound and residues from the hydrophobic trap [50]. Cryo-electron microscopy of the full tripartite complex confirmed that this inhibitor prevents the functional rotation and traps AcrB predominantly in the symmetric tight/binding conformation [67]. The idea that a (too) high affinity of compounds for the deep binding pocket could prevent the conformational changes necessary for RND-transporters to cycle through the functional rotation was first suggested by Vargiu et al. [58] to interpret MIC changes due to mutations within this pocket. Structural studies are invaluable in our understanding of the interaction of inhibitors with efflux proteins. However, an inhibitor-bound structure provides a snapshot of the process only at one specific time. Computational techniques are a great resource to address mechanistic knowledge gaps, as they can pinpoint functional dynamics of biological systems and has been used to great effect to compare binding of a range of substrates and inhibitors to AcrB. However, MD simulation studies need to be complemented with experimental data.

Nakashima et al. [29] measured the binding affinity of D13-9001 to purified AcrB, MexB, and MexY using isothermal titration calorimetry (ITC). K_d values of 1.15 μM and 3.5 μM respectively for AcrB and MexB were obtained. The biggest limitation of ITC is the large amount of purified protein needed for each substrate that is analysed, which makes this method impracticable for the routine analysis of membrane proteins. Progress on the measurement of kinetic constants was further hampered by the huge contribution of non-specific binding of the lipophilic drugs to these hydrophobic proteins to the total binding observed. The voluminous binding cavity and redundancy in active site residues also meant it is not possible to provide a binding-negative mutant of these proteins to correct for non-specific binding. We included the galactose permease protein GalP, an integral sugar binding protein of similar hydrophobicity to AcrB [62] as control for non-specific drug binding. The kinetics of substrate and inhibitor binding to AcrB were subsequently probed using surface plasmon resonance (SPR). SPR is a very powerful biophysical tool for drug discovery which allows real time monitoring of binding events as well as directly measure affinity and kinetic constants of biomolecular interactions [41]. The major advantage of SPR over other techniques is it does not require particular labelling (e.g. fluorescence or radioactive) to analyse molecules. Moreover, the amount of ligand required is much less than is required in other techniques (such as ITC) which are particularly beneficial for membrane-proteins such as AcrB [41,56]. The method is also scalable to high throughput format.

2. Methods

2.1. Plasmids used

Expression plasmid coding for AcrB with 8-His tags at C-terminal (pAcrB) and expression plasmid coding for GalP with 6-His tags at C-terminal (pGalP) both containing kanamycin resistant marker were used for expression of respective proteins [40,70]. Kanamycin was used at 25 μg/mL.

2.2. Preparation of inside out vesicles (ISO) vesicles

Cells were always freshly transformed with plasmid before use. A single transformant was inoculated and grown overnight at 37 °C in LB broth containing kanamycin. To ensure high level expression, the cultures were incubated at 18 °C until an OD_{660} of around 0.2 before gene transcription was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Growth was continued with shaking at 200 rpm overnight to allow expression of the target protein [48]. The cells were harvested by centrifugation (15 min, 6500*g*, 4 °C) and resuspended in 0.1 M potassium phosphate (pH 7.0). DNAse (10 μg/mL) and $MgSO_4$ (10 mM) were added and the suspension was incubated for 15 min at RT before being passed twice through a cell disruptor (Constant Systems with Thermoflex Temperature control) at 20 kPsi. The suspension was incubated at room temperature for 15 min to allow the DNAse to act. A low speed centrifugation (10 min, 10,000*g*, 4 °C) was performed to remove cell debris (pellet), and the supernatant was subjected to high-speed centrifugation using a 50.2 Ti rotor (40,000*g*, 40 min, 4 °C) to collect the inside out vesicles (ISO vesicles). The pellet was resuspended in 50 mM potassium phosphate buffer pH 7.0 containing 10% glycerol to a protein concentration of approximately 50 mg/mL and stored at − 80 °C. The protein concentration of the inside-out membrane vesicles was determined by the D_C Protein Assay (Bio-Rad Laboratories) with BSA (0 to 1.5 mg/mL) as a standard.

2.3. Purification of his-tagged proteins

GalP protein was overexpressed in *E. coli* C41 (DE3) cells and purified by affinity chromatography according to established protocols [62,68]. Purification of AcrB was essentially the same as previously reported for MexB [48] with some modifications as indicated. The ISO vesicles prepared from cells expressing required proteins were allowed to solubilise (20 mM Tris pH 8.0, 10% glycerol, 300 mM NaCl, 1.5% DDM and 10 mM imidazole pH 8.0) for an hour at room temperature through gentle shaking. Unsolubilised protein was removed by ultracentrifugation (150,000 × *g*, 1 h, 4 °C). Ni–NTA (Ni^{2+}–nitrilotriacetate) resin (Bio-Rad) was equilibrated by washing with 20 resin volumes of deionized water, gravity sedimentation on ice and resuspended with 5 resin volumes of wash buffer A (20 mM Tris pH 8.0, 10% glycerol, 300 mM NaCl, 0.05% DDM and 30 mM imidazole pH 8.0). The supernatant from the ultracentrifugation was added to the Ni-NTA resin and the protein was allowed to bind to the resin by gentle shaking for 1 h at 4 °C. The resin was transferred to a 2 mL polystyrene mini-column (BioRad laboratories) and the unbound fraction was allowed to drain away. The resin in the column was washed with 30 resin volumes of wash buffer A and subsequently with 30 resin volumes of wash buffer B (20 mM K-HEPES pH 7.5, 10% glycerol, 300 mM NaCl, 0.05% DDM and 50 mM imidazole pH 8.0). Five resin volumes of elution buffer (20 mM K-HEPES pH 7.5, 10% glycerol, 300 mM NaCl, 0.5% DDM and 400 mM imidazole pH 8.0) were added to displace the His-tagged protein from the resin. The first 0.5 resin volume of eluant was discarded. The next 3–4 resin volumes of eluant were collected as the purified His-tagged protein. Total purified protein was determined using a microvolume spectrophotometer (Denovix).