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Molecular Interactions of Cephalosporins with the Deep Binding Pocket of the RND Transporter AcrB

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Abstract

The drug/proton antiporter AcrB, part of the major efflux pump AcrABZ-TolC in *Escherichia coli*, is characterized by its impressive ability to transport chemically diverse compounds, conferring a multidrug resistance phenotype. However, the molecular features differentiating between good and poor substrates of the pump have yet to be identified. In this work, we combined molecular docking with molecular dynamics simulations to study the interactions between AcrB and two representative cephalosporins, cefepime and ceftazidime (a good and poor substrate of AcrB, respectively). Our analysis revealed different binding preferences of the two compounds toward the subsites of the large deep binding pocket of AcrB. Cefepime, although less hydrophobic than ceftazidime, showed a higher affinity than ceftazidime for the so-called hydrophobic trap, a region known for binding inhibitors and substrates. This supports the hypothesis that surface complementarity between the molecule and AcrB, more than the intrinsic hydrophobicity of the antibiotic, is a feature required for the interaction within this region. Oppositely, the preference of ceftazidime for binding outside the hydrophobic trap might not be optimal for triggering allosteric conformational changes needed to the transporter to accomplish its function. Altogether, our findings could provide valuable information for the design of new antibiotics less susceptible to the efflux mechanism.

Graphical Abstract

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Supporting Information

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Details on the methods employed for the binding free energy calculations and calculations of surface complementarity of the complexes, physico-chemical properties of CAZ and CEF, graphical representations of the docking poses, and additional information on CAZ and CEF derived from the MD simulations (cluster population, 2D root mean square deviations, solvation, root mean square fluctuations, minimal projection areas) (PDF)

Trajectories for CAZ_{IN1}, CAZ_{OUT1}, CAZ_{OUT2}, CAZ_{OUT3}, CEF_{IN1}, CEF_{IN2}, CEF_{OUT1}, and CEF_{OUT2} (ZIP)

The authors declare no competing financial interest.

INTRODUCTION

The rising of multidrug-resistant (MDR) Gram-negative bacteria and the consequent decrease in the efficacy of antibiotics represent an urgent public health concern.^{1–3} Resistance-nodulation-cell division (RND) efflux pumps play a major role in MDR Gram-negative bacteria, acting as the final export hub of a complex coordinated network of transporters.^{4–8} One of the most studied members of the RND superfamily is represented by the AcrABZ-TolC efflux pump of *Escherichia coli*.^{6,9–15} This complex is constituted by an outer membrane (OM) protein (TolC), a membrane fusion protein (AcrA), a small inner membrane protein (AcrZ), and a homotrimeric H⁺/drug antiporter (AcrB). In particular, the AcrB transporter is responsible for the extrusion from the periplasm or the inner membrane (IM)^{13,16,17} of a vast number of chemically diverse substrates, including antibiotics. Each protomer of AcrB is constituted by a transmembrane (TM) domain (where a proton gradient provides the energy for substrate translocation),¹⁸ a porter domain (responsible for the uptake and first transport of substrates), and a funnel domain (constituted by a channel linked to AcrA/TolC) (Figure 1a). To date, symmetric and asymmetric conformations of AcrB, corresponding to the resting and active states of the transporter, have been identified by X-ray crystallography.^{9,10,12,19–22}

In the asymmetric conformation of AcrB, each protomer is found in a different structural state defined as loose (L), tight (T), and open (O).^{9,14,15} This finding suggested that the efflux of substrates is induced by a functional rotation mechanism,^{9–11} where each protomer rotates functionally (but not physically) through each different state. The validity of this hypothesis has been supported by subsequent experimental^{23,24} and computational studies.^{25–29} The most simple description of this rotation mechanism consists of a substrate entering

from the periplasm and reaching the access pocket (AP),^{12,30} which is located in the vestibule region between PC1 and PC2 subdomains of the monomer in the L state. Following the conformational transition from L to T, the substrate is then pushed within the deep binding pocket (DP), which is located near the funnel domain.^{9–12,30} The lower part of the DP, named “hydrophobic trap” (HP-trap),³¹ is an important recognition site for both efflux inhibitors^{12,32,33} and typical AcrB substrates.^{17,34} The AP and DP are separated by a “switch loop”, which, along with a “bottom-loop”, mediate the transport of substrates toward the DP.^{12,30,35,36} The final conformational change from T to O pushes the substrate through the exit gate (EG) toward TolC.¹¹ After the extrusion of the substrate, the monomer reverts to the initial L state, where it is again available to interact with another substrate.

Understanding the relationship between the physico-chemical properties of compounds and their propensity to be extruded by efflux pumps will have crucial implications for both basic research and drug design. The characteristics of the compounds cocrystallized with AcrB^{9–12,14,18,24,30,31} led to the conclusion that a certain degree of lipophilicity is required for substrates of this transporter.³⁷ However, the link between structural and chemical fingerprints of compounds and the peculiar physico-chemical properties of the multidrug binding sites in this and homologous proteins was investigated more quantitatively only in recent years.^{36,38,39} Computational studies have contributed to this improvement, providing detailed information on these systems at an atomistic level (for a recent review, see ref 40 and references therein). In this work, we used different computational methods to investigate the interaction of two representative cephalosporins, namely, ceftazidime and cefepime (from now on called CAZ and CEF, respectively, shown in Figure 1b) with AcrB. CAZ and CEF, third- and fourth-generation cephalosporins, respectively, show a considerable potency against Gram-negative bacteria and a low affinity for β -lactamases.^{41,42} Nonetheless, cephalosporin-resistant bacteria have already been identified.^{43–45} Although MDR is often attributed to the interplay of different resistance mechanisms,^{46–48} there is evidence that cephalosporins might be recognized and expelled by RND efflux pumps in some Gram-negative bacteria.^{49,50} According to the recent minimum inhibitory concentration (MIC) measures presented by Cooper et al.,⁵¹ CEF is more active than CAZ in the wild-type strain of *E. coli*. Furthermore, the deletion of major efflux transporters resulted in an increase in the activity of CEF in both *E. coli* and *Pseudomonas aeruginosa* (2-fold for the former and 16-fold for the latter), validating CEF as a substrate of these transporters. On the other hand, the activity of CAZ was not found to increase after the deletion of efflux pumps, suggesting only a poor affinity to the RND transporters of these bacterial species. However, the results of MIC studies are strongly influenced by the physico-chemical properties of the molecules under investigation, whose different membrane permeabilities could hinder the entity of the contribution of active efflux to resistance.⁵² In this context, direct efflux and affinity measurements^{53,54} combined with a detailed characterization of the interaction between these compounds and high affinity subregions within AcrB⁴⁰ would be crucial. In an attempt to bridge this lack of information, we performed a comparative computational investigation of these two cephalosporins inside the DP of the T monomer of AcrB (DP_T) in order to rationalize how the intrinsic properties of CAZ and CEF impact their recognition (in terms of preferred binding locations and binding affinities) by AcrB. We focused solely on this specific site due to its crucial position in the entire efflux pathway: indeed, the DP_T is

supposed to be visited during extrusion by all the substrates captured by the efflux system,²⁸ and it is also believed to be the putative recognition site of low-molecular-mass AcrB substrates.¹² In particular, selected docking poses identified within the DP_T were used as initial conformations for microsecond-long molecular dynamics simulations, and their affinity to the pocket was evaluated by employing binding free energy calculations. Note that, following the direction already traced in previous studies,^{17,55} this work aims to obtain a detailed map of compound–transporter interactions in the DP_T, which are assumed to be the key for recognition of low-molecular-mass compounds by AcrB. The complete modeling of the functional rotation process associated with the extrusion of substrates, successfully reported in previous works,^{25–29} is outside the scopes of the present study. The outcomes of this work revealed the dynamic behavior of two representative cephalosporins while inside the AcrB transporter and contributed to the identification of crucial interactions between this class of molecules and the residues lining the DP_T. These new insights could be applied to the rational design of improved antibiotics that could be less affected by the extrusion process or, with the appropriate functionalization, could even hamper it.

COMPUTATIONAL METHODS

Molecular Docking.

We first performed an extensive blind docking campaign using Autodock Vina⁵⁶ by adopting a rectangular search space of size $125\text{\AA} \times 125\text{\AA} \times 110\text{\AA}$ enclosing the whole portion of the protein potentially exposed to the ligands. The flexibility of both docking partners was considered indirectly by employing ensembles of conformations. For each compound, representative conformations obtained from an explicitly solvated molecular dynamics (MD) simulation of 1 μs were used (data available at www.dsf.unica.it/translocation/db; also see Table S1).⁵⁷ For the receptor, conformations including X-ray structures and those derived from available microsecond-long MD simulations were considered.³⁹ The choice of the initial conformations was limited to four poses inside the DP_T for both CAZ and CEF. Starting poses were selected according to the estimated binding affinity and the fraction of contacts made by the molecule with the DP_T; only poses in contact with at least 40% of the total residues lining the deep binding pocket were considered (residues lining these specific regions are listed in Table S2). In addition, the position and orientation of the molecules were also taken into account to evaluate the effect on their behavior inside the DP_T. The selected poses are listed in Table S3, and the corresponding 2D interaction patterns are shown in Figure S2.

MD Simulations.

The initial coordinates of the AcrB/cephalosporin complexes were embedded in a pre-equilibrated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) bilayer patch. The embedding of the complex into the POPE bilayer was performed using the PPM server⁵⁸ and CharmmGUI.⁵⁹ The central pore of AcrB was filled with lipids whose numbers were calculated by dividing the approximate area of the central pore by the standard area per lipid of POPE molecules. Each system was then immersed in a box containing TIP3P water molecules⁶⁰ and an adequate number of K⁺ counterions in order to neutralize the negative net charge of the system. An osmolarity value of 0.15 M was reached by adding an

appropriate number of K^+/Cl^- . The ff14SB versions of the AMBER force field⁶¹ and lipid14⁶² were adopted for AcrB and the POPE bilayer, respectively. The AMBER parameters adopted for CAZ and CEF were taken from ref 57. The systems were then minimized with a combination of the steepest descent and conjugate gradient methods gradually releasing positional restraints applied. The AcrB/cephalosporin complexes were heated from 0 to 310 K in two steps: a 1 ns heating from 0 to 100 K in a canonical ensemble (NVT) followed by a 5 ns heating to reach 310 K in an isothermal–isobaric ensemble (NPT) while applying positional restraints along the Z axis on the phosphorous heads of lipids molecules, allowing the merging of the membrane. Multiple equilibration steps of 500 ps each until the stabilization of the box dimensions were performed in the NPT ensemble. A Langevin thermostat using a collision frequency of 1 ps^{-1} and a Berendsen isotropic barostat⁶³ maintained a constant temperature and an average pressure of 1 atm, respectively. A time step of 2 fs was used during the equilibration protocol. The MD simulations were carried out using the PMEMD module of AMBER 14⁶⁴ with a time step of 4 fs in the NVT ensemble after applying the hydrogen mass repartitioning.⁶⁵ Coordinates were saved every 100 ps. Long-range electrostatic interactions were calculated using the particle mesh Ewald method with a cutoff of 9 Å.⁶⁶ 2D root-mean-square deviations (RMSDs) of the ligands were calculated using the cpptraj module of AMBERTools14 and VMD.⁶⁷ Hydrogen bonds (cutoff distance of 3.5 Å and angle of 120° between donor and acceptor atoms) and hydrophobic contacts (distance <2.5 Å between the centers of mass of the molecule and the residues of the DP_T) were analyzed using a customized tcl script. Only the H-bonds and hydrophobic contacts established, respectively, for more than 5 and 10% of the total simulation time were considered relevant. A cluster analysis using the cpptraj module of AMBERTools14 identified the most populated states sampled during the simulation with a fixed clustering radius of 3.5 Å (Table S3). Only the most populated cluster were further taken into account for evaluating the free energies of binding using the molecular mechanics/generalized Born surface area (MM-GBSA)⁶⁸ approach. The solute conformational entropy difference was evaluated employing the method proposed by Kongsted and Ryde,⁶⁹ which has been proven to provide stable results and improved estimates of the free energy of binding in benchmark cases. The surface complementarity between compounds and DP_T was calculated using the PLATINUM web server.⁷⁰ A more detailed description of MM-GBSA and PLATINUM approaches can be found in the Supporting Information.

RESULTS

Molecular Docking and MD Simulations.

The different chemical features of CAZ and CEF are reflected in the overall spatial distribution and binding affinities of the poses predicted by molecular docking. Indeed, the two compounds possess different substituent groups R1 and R2 bound to a shared cephalosporins core (Figure S1). More specifically, CAZ has a planar and rigid pyridine ring in R1, while CEF has a more flexible pyrrolidine ring. In R2, CAZ and CEF share a 2-amino,4-thiazole ring bound to a C=N—O— group. However, the bulkier and charged R2 in CAZ (2-carboxypropan-2-yloxyiminoacetyl) could further lead to a differentiation in the behavior of the two cephalosporins within the DP_T. The selected docking poses for the two

cephalosporins inside the DP_T can be narrowed to two major clusters: those located inside the HP-trap (from now on labeled as “IN”) and those located outside of the HP-trap (labeled as “OUT”) (Figure S2). These poses were used as starting points for the microsecond-long MD simulations of the AcrB/compound complex to assess the dynamical behavior of CAZ and CEF inside the DP_T at an atomistic level. Figures 2 and 3 display the paths connecting the initial and final positions of the MD trajectories of CAZ and CEF, respectively. In the same figures, we report the final configurations and the occurrence of hydrophobic contacts and H-bonds between the compounds and the residues lining the DP_T as observed for the most populated cluster for each simulation (cluster populations and corresponding molecular interactions are reported in Table S4 and Figure S3, respectively). According to the aforementioned classification, the different IN and OUT trajectories for both cephalosporins are presented as follows.

In CAZ_{IN1}, the ligand was initially docked within the HP-trap (Figure S2 and Movie S1) and then slightly moved toward the EG at ~100 ns (~5 Å), following an upward movement of PHE136. The molecule then remained inside the HP-trap until the end of the simulation, stabilized by durable hydrophobic interactions between its cephalosporin core and the phenylalanines of this region (Figure 2 and Movie S1). In CAZ_{OUT1-3}, the molecules were found on top of the HP-trap with opposite orientations to the direction of extrusion (OUT₁₋₂ vs OUT₃) and rotation around the main molecular axis (OUT₁ vs OUT₃), as shown in Figure S2. Interestingly, in spite of their different initial positions, the molecules achieved comparable final conformations in all three simulations (Movies S2–S4). The stability of the final poses is due to the hydrophobic interactions with residues LEU177 (CAZ_{OUT1}), THR44, THR87 (CAZ_{OUT2}), and ILE277 (CAZ_{OUT3}). Additional H-bonds with SER46 (CAZ_{OUT1}), ARG620 (CAZ_{OUT2}), and ASN274 (CAZ_{OUT3}) contributed to the stabilization of these poses (Figure 2 and Figure S4). In CEF_{IN1}, the molecule was initially bound to the bottom of the HP-trap (Figure S2). At ~100 ns, a slight upward movement of the molecule with its core oriented toward the EG was observed (~5 Å). At variance with CAZ_{IN1}, this position was maintained until the end of the simulation, as confirmed by the stable and prolonged contacts with the hydrophobic residues of this region (Figure 3 and Movie S5). In CEF_{IN2}, the compound was initially docked outside the HP-trap with its core aligned to the direction of extrusion (Figure S2). During the simulation, the molecule moved permanently inside the HP-trap at ~300 ns, as confirmed by the newly established hydrophobic contacts (Figure 3 and Movie S6). In both CEF_{OUT1} and CEF_{OUT2}, the antibiotic was initially sitting on top of the HP-trap with its main axis parallel to the EG (Figure S2). In CEF_{OUT1}, the molecule moved toward LEU117 of the O monomer, where the carboxyl group of the cephalosporin core interacted with LYS163 and GLN67, also part of the O monomer (Figure 3 and Movie S7). On the other hand, in CEF_{OUT2}, the molecule approached the EG without rearranging its orientation in the direction of extrusion and was stabilized by a salt bridge formed by the amino group of R1 with ASP276 (Figure 3 and Movie S8).

To complement the detailed analysis of interactions established during MD simulations, we evaluated the number of water molecules surrounding the two cephalosporins within the first hydration shell (Table S5 and Figure S5). Our results show that CAZ is surrounded by a fairly stable number of water molecules (~22) regardless of the region occupied inside the DP_T. In contrast, CEF trajectories present a clearly distinct number of water molecules

depending on the region occupied: lower (~ 16) inside the HP-trap ($\text{CEF}_{\text{IN1-2}}$; see the right side of the graphs in Figure S5) and higher (~ 22) outside of it (CEF_{OUT2}). The only exception to this trend is represented by CEF_{OUT1} , where, at ~ 400 ns, the molecule assumed values comparable to those observed for $\text{CEF}_{\text{IN1-2}}$. This is due to the formation of new hydrophobic contacts with PHE178, ILE277 and ILE278 (Figure S6), leading to a significant loss of surrounding water molecules from ~ 30 to ~ 20 . Moreover, the number of water-mediated HBs between the antibiotics and the protein appears to be slightly higher for CAZ than CEF (Table S5), which is expected due to its higher number of HB acceptors compared to CEF (10 vs 8) and the higher number of water molecules surrounding the molecule.

For the sake of completeness, additional structural properties of the cephalosporins, namely, root-mean-square fluctuation (RMSF) and minimal projection area (MPA), were evaluated for each MD trajectory and compared to the values measured from simulations of the compounds in explicit water solution⁵⁷ (Figure S7 and Table S6). The results obtained for the MPA of the molecules inside of AcrB did not show any remarkable trend linked to the region occupied inside the DP_T and when compared to the values measured from simulations in a water box. The RMSF values obtained for the atoms of the R2 substituent of each molecule are comparable independently from the region occupied inside the DP_T and always lower than those measured in the solvent. On the other hand, this difference seems to be less pronounced in the case of the R1 substituent, especially in CEF_{OUT1} , where the atomic fluctuations showed even a comparable behavior to those observed for the simulation in explicit water. In addition, we evaluated the modification of relevant structural parameters of the transporter¹¹ following the binding of the cephalosporins. In particular, we focused on the TM region helices TM2 and TM8, which, however, did not reveal significant changes in any of the simulations.

To estimate the affinity of the two cephalosporins for AcrB, we performed binding free energy calculations on the set of structures belonging to the most populated conformational clusters extracted from each simulation (Table 1). The comparison of G' for the two cephalosporins bound to the DP_T reveals how CAZ possesses, on average, slightly larger negative values than CEF, hence indicating a moderately higher affinity for this putative binding region of AcrB. In detail, G' values for CEF appear to be comparable, within the error bars, regardless of the position occupied inside the DP_T , except for CEF_{OUT2} , which shows the lowest binding affinity in the series. On the contrary, CAZ conformations located outside the HP-trap ($\text{CAZ}_{\text{OUT1-3}}$) have a higher affinity than the one inside (CAZ_{IN1}). To complement the results obtained with the MM-GBSA method, we also calculated the changes in the configurational entropy upon binding ($T S_{\text{conf}}$). Despite the (expected) large variances, the estimated total binding affinities (G_b) reveal an interesting trend, namely, G_b is lower (higher) for CAZ (CEF) in simulations where the compound is in strong interaction with the HP-trap.

The individual contributions of the DP_T residues to G' of the most populated clusters for each trajectory are shown in Figure 4. A fairly comparable profile among “IN” simulations is evident when comparing row 1 (CAZ_{IN1}) to rows 5 and 6 ($\text{CEF}_{\text{IN1-2}}$). Indeed, for both compounds, the residues providing the largest contribution to G' include PHE136,

PHE178, PHE610, PHE615, and PHE628, that is, those lining the HP-trap. Outside this site, the most important residues vary according to the specific position occupied by the molecule, although residues SER46, GLN176, LEU177, ILE277, and ARG620 seem to grant a consistent contribution to G' in most cases. Additionally, we calculated the complementarity between the molecule and the DP_T using the PLATINUM web server.⁷⁰ This approach implements the formalism of molecular hydrophobicity potential to characterize the hydrophobic match in terms of the fraction of matching the total surface (SM) and the fraction of matching the hydrophobic surface (SM_L) and hydrophilic surface (SM_H) (Table 1, last three columns). These parameters, defined in the Supporting Information, are positive-defined adimensional quantities ranging from 0 (no match) to 1 (complete match). From these results, CEF showed higher (lower) total and hydrophobic matching than CAZ inside (outside) the HP-trap, while an opposite situation is observed for SM_H in the OUT simulations.

DISCUSSION

In this study, we presented the results of a comparative computational analysis of the molecular interactions established by two congeneric compounds with AcrB, the main RND transporter of *E. coli*. Initially, selected docking poses of the cephalosporins CAZ and CEF inside the DP_T of AcrB were grouped according to their location within (IN) or outside (OUT) the HP-trap. Subsequently, these poses were used as starting conformations to perform all-atom microsecond-long MD simulations.

The MD simulations of CAZ and CEF inside the HP-trap revealed that (i) regardless of the initial location, both compounds maintained, once established, strong interactions with the residues of the HP-trap until the end of the simulation. In addition to the phenylalanines lining this site, the residue Y327 also contributed significantly to the stabilization of both cephalosporins. Opposite from hydrophobic interactions, only a single relevant H-bond was observed between GLY179 and the carboxylic group of the cephalosporin core of CAZ_{IN1} . (ii) CAZ and CEF seem to have preferential orientations when they are inside the HP-trap, favoring the packing of the aromatic ring of pyridine (CAZ) and 2-amino,4-thiazole (CEF) by the phenylalanines of the HP-trap, similar to what has been observed for the thiophene ring of nitrocefin¹⁷ (also a cephalosporin, Figure S8). (iii) CEF (CAZ) has a higher (lower) affinity when partly bound to the HP-trap than when interacting with other subsites (namely, in between this site and the EG) (Table 1). The intrinsic chemical features of CAZ (such as an additional negatively charged group in R2) could potentially explain its reduced affinity for the HP-trap and its orientation opposite to that of CEF and nitrocefin (where the R2 substituent is located inside the HP-trap). The G' contributions to the G_b of two different cephalosporins, namely, nitrocefin and cephalotin, were previously evaluated with the same method employed here.¹⁷ Nitrocefin, found inside the HP-trap, possesses a considerably higher G' (-42.5 ± 3.9 kcal/mol) as compared to those of CAZ and CEF (Table 1); hence, the functionalization of the substituents appears to reduce the affinity for the HP-trap causing less optimal interactions with the phenylalanines of this region (Figure S8). Interestingly, at variance with our previous findings on the interaction of carbapenems with AcrB,⁵⁵ the intrinsic molecular hydrophobicity alone does not provide a rationale explaining the different affinities of the two cephalosporins to the HP-trap (lined mainly by the side

chains of hydrophobic phenylalanines). Indeed, while CAZ is more hydrophobic than CEF, as quantified by the octanol/water partition coefficient ($\log P$ 2.3 vs 1.1; see Table S1), its affinity toward the HP-trap is lower than that of CEF. Therefore, it appears that better shape complementarity within the binding interface between CEF and the HP-trap could lead to a more substantial reduction of the excluded volume than CAZ does. This event is accompanied by an increase in the total volume available for the translational displacement of water molecules located in the system, which is in turn associated with a large gain of configurational entropy of water.^{71–73} This hypothesis has been validated by evaluating the total (SM), hydrophilic (SM_H), and lipophilic (SM_L) surface matching between the molecules and the DP_T using the PLATINUM web server⁷⁰ (Table 1, last three columns). By definition, SM is a quantity that can be correlated to the reduction of the excluded volume upon binding between molecular partners. Indeed, the results obtained showed that, inside the HP-trap, high (low) binding affinities are correlated to both high (low) total and lipophilic surface matching (0.44 and 0.46 for CEF_{IN1-2} vs 0.24 for CAZ_{IN1} in the case of SM, and 0.67 and 0.61 for CEF_{IN1-2} vs 0.23 for CAZ_{IN1} in the case of SM_L). Despite the presence of charged moieties in CAZ and CEF, hydrophilic complementarity plays a secondary role, mainly due to the hydrophobic composition of this portion of the DP_T . Henceforth, the resulting surface complementarity of protein/antibiotic, more than the chemical nature of the compound itself, can be a pivotal factor that explains the different affinity between CAZ and CEF for the HP-trap.

In the simulations labeled as “OUT”, CAZ and CEF occupied the region between the EG and the top of the HP-trap. Both molecules stayed close to their initial docking poses, thus showing limited mobility while being stabilized by multiple and durable hydrophobic contacts and bonds (such as LEU177 in CAZ_{OUT1} and CEF_{OUT1} , T44 and T87 in CAZ_{OUT2} , and L177 in CAZ_{OUT1} and CEF_{OUT1}). On the other hand, the trend of the total free energy of binding G_b outside the HP-trap is opposite to what has been observed for IN trajectories: CAZ, while having its axis aligned to the direction of the extrusion pathway, shows a higher affinity than CEF (about -20 vs -14 kcal/mol), which is found oriented in a direction apparently not suitable for a passage through the EG (Figure 3). In general, the higher affinity of CAZ could be explained by its better orientation and reorganization of the interatomic contacts within the residues of the DP_T from the initial docking pose compared to CEF. On the contrary, the lower affinities of CEF are caused by limited interactions with the residues of the DP_T (CEF_{OUT2}) or consistently high entropic costs (CEF_{OUT1} , Table 1). Notably, while the penalty for CAZ to be located outside the HP-trap is of the same order of magnitude of that associated with the conformation inside it (about -20 kcal/mol in all cases, Table 1), the different entropic costs paid for CEF_{OUT1-2} (-24.6 vs -14.3 kcal/mol) and CEF_{IN1-2} (-19.5 vs -11.6 kcal/mol) could be partly associated with the higher flexibility of the R1 substituent in the two different regions, as shown by the behavior of the atomic RMSF (Figure S7). This different trend in the affinity associated with the OUT simulations is indeed confirmed by slightly higher SM values registered for CAZ than CEF (0.45, 0.46, and 0.47 for CAZ_{OUT1-3} vs 0.37 and 0.39 for CEF_{OUT1-2}). However, due to the more diverse residue composition of these portions of the DP_T , the complementarity between the hydrophilic moieties of the two molecules and AcrB (SM_H) plays a major role

compared to the IN simulations (0.62, 0.66, and 0.63 for CAZ_{OUT1-3} vs 0.39 and 0.53 for CEF_{OUT1-2}).

It is interesting to observe how low-molecular-mass compounds, such as imipenem (molecular weight of ~300 g/mol against ~500 g/mol for CAZ and CEF), showed a considerably higher mobility and flexibility while exploring different areas of the DP_T.⁵⁵ On the contrary, CEF and CAZ maintained stable interactions, which reduced their mobility: based on these observations, one could hypothesize that bulkier and more functionalized compounds could definitively take advantage of the functional rotation mechanism for being properly extruded by the efflux pump.

Additionally, as shown by computational and experimental studies,^{17,34,74} the HP-trap is involved in binding of several substrates and, as such, should not be considered as a site exclusively reserved to the binding of inhibitors. Therefore, it is reasonable to believe that a difference between good and poor substrates of AcrB (which could also behave as an inhibitor) could be in the strength of interaction with this site, in turn regulating the dwelling time of compounds at that region of the transporter.^{13,75} Namely, a good substrate is expected to have a higher affinity toward the DP within the monomer in the T state than in the other states. This affinity should be high enough to promote allosteric conformational changes in the TM region of the monomer but low enough to allow for the substrate to translocate toward the EG during the T → O conformational change induced by the proton flux across the TM region, thus not hampering the occurrence of the functional rotation structural changes. Indeed, the contacts observed between CEF and the residues of the HP-trap (and nearby hydrophobic residues) are present also in the available experimental structures of wild-type AcrB in complex with its substrates doxorubicin (F178, I277, V612, F615),³⁰ minocycline (L177, F178, I277, F615),¹² rhodamine 6G (F136, V139, F178, I277, F610, F615, F628),³³ and puromycin (F136, V139, F178, M573, F615, F617, F628).⁷⁶ Such mechanism would be compatible with the effect of the F610A mutation in AcrB, which led to increased MIC and delayed efflux for the substrates of the pump.^{77,78} Indeed, it was shown that this effect is ascertainable to increased packing of the substrates by the HP-trap^{13,75} (which interacts peripherally with substrates, as confirmed, for example, by the available X-ray structures of AcrB in complex with minocycline and doxorubicin³⁰), corresponding to a sizable increase in the binding affinity; this is likely to cause a delay in the feedback mechanism of the pump (from the TM to the pore region) and/or hampering translocation toward the EG in response to the T → O conformational change. As a result of this increased interaction, normal substrates of the wild-type transporter behave more as inhibitors in the F610A variant of AcrB.

Finally, it is interesting to compare our findings to those regarding the translocation of doxorubicin (a known substrate of AcrB) from the DP to the funnel domain upon induction of the T → O conformational change in AcrB.²⁸ In that case, the loss of contacts between doxorubicin and the HP-trap led to an increased hydration of the substrate, with a constant number of water molecules within the first solvation shell fairly thereafter. While a conformational change in AcrB has not been simulated in this study, it is intriguing that a trend comparable to the one of doxorubicin (increased hydration in the absence of contacts with the HP-trap) has been observed solely for the substrate CEF.

In view of these considerations, the binding preference of CAZ for regions in between the HP-trap and the EG could imply a dwelling time of this compound that is not compatible with that needed to propagate the signal of binding toward the TM region of the pump, considering that only the T to O transition opens the EG. Clearly, as stated previously, other processes could be more relevant in explaining the differences observed in the MIC changes of the two compounds upon deletion of constitutively expressed RND efflux pumps, also considering that it was largely demonstrated that such a genetic modification alters the whole physiology of Gram-negative bacteria.⁴ Nonetheless, our findings provide a reasonable hypothesis that rationalizes the available experimental data only relying on subtle but relevant differences in the detailed interactions of CAZ and CEF with AcrB.

CONCLUSIONS

In this work, we investigated the binding modes of two representative cephalosporins, CEF and CAZ, to the DP_T of the major RND transporter AcrB of *E. coli*. With the aim to rationalize experimental findings, which hint that CEF is a substrate of AcrB and CAZ is not, we identified key molecular determinants of the interactions between these compounds and the DP_T of the transporter. These interactions cannot be described at the molecular level by other means (namely, MIC measurements). The chemical and structural differences among the substituents bound to the cephalosporin core led to different interactions within the subregions identified in the DP_T of AcrB. In particular, CAZ, bulkier, negatively charged, and more hydrophobic than CEF, showed a lower propensity to interact with the HP-trap, supporting the hypothesis that surface complementarity more than the intrinsic hydrophobicity alone is a feature required for the interaction with this region. On the other hand, the chemistry of the two cephalosporins appears to be a less important differentiating factor outside the HP-trap, where the molecules showed similar dynamical profiles although with different binding affinities (higher for CAZ than CEF), mainly caused by the different subregions occupied.

This work contributes to enlighten how the subtle balance among different physico-chemical fingerprints of compounds reflects on their interaction with AcrB and the molecular features should be tuned in order to regulate the strength of such interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Side view of the AcrB transporter. The loose (L), tight (T), and open (O) protomers of AcrB are represented in green, cyan, and gray, respectively. The inset shows the front and top magnification of the porter domain of the T monomer highlighting PN1, PN2, PC1, and PC2 subdomains and the access and deep binding pockets represented as surfaces, colored in red and blue, respectively. (b) 2D chemical structures of ceftazidime (CAZ) and cefepime (CEF).

Figure 2.

(Left column) Trajectories of the center of mass of CAZ along the MD simulations of CAZ_{IN1} and CAZ_{OUT1-3}. Initial and final positions of the drug are represented as red and green spheres, respectively, while the cyan tubes represent the paths between these positions. Protein residues lining the DP_T are displayed as a blue surface. The switch-loop and the bottom-loop are represented as yellow and cyan tubes, respectively. HP-trap and exit gate residues are shown as gray and orange sticks, respectively. (Middle column) Final configurations extracted from the MD simulations. The ligand is represented by sticks colored by the element. Residues of the DP_T and water molecules within 3.5 Å of CAZ are represented by sticks colored by the residue type and as red spheres, respectively. (Right column) Occurrences of hydrophobic contacts (black) and H-bonds (red) between CAZ and

DP_T residues observed for the most populated cluster of each trajectory. The total occurrence of each contact observed for the whole trajectory is represented in gray.

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Figure 4.

Per-residue contributions to the G' for CAZ and CEF in the DP_T for the most populated cluster of each simulation. Only residues contributing more than kT at room temperature are considered. IN residue labels are shown in bold. The cells are colored according to their G' values (in kcal/mol). For each compound, results for poses found inside and outside the HP-trap were grouped together to highlight the differences.

Table 1. Total Free Energy of Binding (G_b) for the Most Populated Cluster Trajectories Identified for Each Simulation^a

trajectory	population (%)	G'	$T S_{conf}$	G_b	SM	SM _L	SM _H
CAZ _{IN1}	94	-32.3 ± 3.6	-22.3 ± 5.2	-10.0 ± 6.3	0.24	0.23	0.33
CAZ _{OUT1}	100	-41.0 ± 5.5	-23.2 ± 7.0	-17.8 ± 8.9	0.45	0.19	0.62
CAZ _{OUT2}	100	-35.2 ± 5.5	-16.8 ± 9.9	-18.4 ± 11.3	0.46	0.10	0.66
CAZ _{OUT3}	64	-39.7 ± 5.1	-17.6 ± 10.7	-22.1 ± 11.9	0.47	0.64	0.63
CEF _{IN1}	91	-38.2 ± 4.2	-19.5 ± 8.0	-18.7 ± 9.0	0.44	0.67	0.16
CEF _{IN2}	72	-34.4 ± 5.4	-11.6 ± 7.0	-22.8 ± 8.8	0.46	0.61	0.22
CEF _{OUT1}	69	-38.9 ± 5.6	-24.6 ± 6.6	-14.3 ± 8.7	0.37	0.31	0.39
CEF _{OUT2}	81	-27.8 ± 3.3	-14.3 ± 9.0	-13.5 ± 9.6	0.39	0.01	0.53

^a G_b is evaluated as a sum of the contributions calculated with the MM-CBSA method (G') and the contribution of the conformational entropy of the molecule ($T S_{conf}$). All values are expressed in kcal/mol. Fractions of total (SM), hydrophobic (SM_L), and hydrophilic (SM_H) surface matching as calculated using the PLATINUM web server are reported in the last three columns. For the analytical definition of SM, SM_L, and SM_H see the Supporting Information.