Structural Characterization of Diazabicyclooctane β-Lactam “Enhancers” in Complex with Penicillin-Binding Proteins PBP2 and PBP3 of Pseudomonas aeruginosa

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ABSTRACT Multidrug-resistant (MDR) pathogens pose a significant public health threat. A major mechanism of resistance expressed by MDR pathogens is β-lactamase-mediated degradation of β-lactam antibiotics. The diazabicyclooctane (DBO) compounds zidebactam and WCK 5153, recognized as β-lactam “enhancers” due to inhibition of Pseudomonas aeruginosa penicillin-binding protein 2 (PBP2), are also class A and C β-lactamase inhibitors. To structurally probe their mode of PBP2 inhibition as well as investigate why P. aeruginosa PBP2 is less susceptible to inhibition by β-lactam antibiotics compared to the Escherichia coli PBP2, we determined the crystal structure of P. aeruginosa PBP2 in complex with WCK 5153. WCK 5153 forms an inhibitory covalent bond with the catalytic S327 of PBP2. The structure suggests a significant role for the diacetylhydrazide moiety of WCK 5153 in interacting with the aspartate in the S-X-N/D PBP motif. Modeling of zidebactam in the active site of PBP2 reveals a similar binding mode. Both DBOs increase the melting temperature of PBP2, affording their stabilizing interactions. To aid in the design of DBOs that can inhibit multiple PBPs, the ability of three DBOs to interact with P. aeruginosa PBP3 was explored crystallographically. Even though the DBOs show covalent binding to PBP3, they destabilized PBP3. Overall, the studies provide insights into zidebactam and WCK 5153 inhibition of PBP2 compared to their inhibition of PBP3 and the evolutionarily related KPC-2 β-lactamase. These molecular insights into the dual-target DBOs advance our knowledge regarding further DBO optimization efforts to develop novel potent β-lactamase-resistant, non-β-lactam PBP inhibitors.

IMPORTANCE Antibiotic resistance is a significant clinical problem. Developing novel antibiotics that overcome known resistance mechanisms is highly desired. Diazabicyclooctane inhibitors such as zidebactam possess this potential as they readily inactivate penicillin-binding proteins, yet cannot be degraded by β-lactamases. In this study, we characterized the inhibition by diazabicyclooctanes of penicillin-binding proteins PBP2 and PBP3 from Pseudomonas aeruginosa using protein crystallography and biophysical analyses. These structures and analyses help define the antibiotic properties of these inhibitors, explain the decreased

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susceptibility of \( P. \) aeruginosa PBP2 to be inhibited by \( \beta \)-lactam antibiotics, and provide insights that could be used for further antibiotic development.

**KEYWORDS** Pseudomonas aeruginosa, antibiotic resistance, penicillin-binding proteins, structural biology

**RESULTS AND DISCUSSION**

Structural analysis of PBP2. The \( P. \) aeruginosa PBP2 adopts the class B fold of a monofunctional PBP. This fold includes four distinct regions that protrude into the periplasm: (i) an anchor domain, (ii) a linker domain, (iii) a head domain, and (iv) the large C-terminal catalytic transpeptidase (TP) domain which cross-links the nascent...
peptidoglycan strand (this excludes the N-terminal transmembrane anchoring region which is not included in the construct). This PBP2 structure was resolved to 2.87-Å resolution and contains two non-crystallographically related molecules. These monomers are very similar, and our analysis will, therefore, mostly be limited to molecule A (Fig. 1B). Notably, the PBP2 structure lacks the head domain, which could not be modeled in either of the two monomers (Fig. 1B; see also Fig. S1 in the supplemental material). Also, the anchor domain was modeled partially. Electron density for these unmodeled regions was poor, likely due to their inherent disorder or mobility. Structures of homologous PBP2s from Escherichia coli, Helicobacter pylori, and Neisseria gonorrhoeae show a similar fold (14–17); their sequence identities with P. aeruginosa PBP2 are 45%, 32%, and 27%, respectively (Fig. S1 shows their structure-based sequence alignment). Similarly, the head domain also could not be modeled in the N. gonorrhoeae PBP2 structure (PDB ID 5KSH) (17). Furthermore, the head domain in the E. coli and H. pylori structures also does not superimpose well, again indicating that its position can vary (14, 15). The sequence and structure of P. aeruginosa PBP2 are most like those of E. coli PBP2; their superpositioning yields a root mean square deviation (RMSD) of 1.04 Å for 426 Ca atoms, indicating their structural similarity. Figure 1C represents this superposition and also shows the portions of the head domain and the anchor domain that are better resolved in the E. coli PBP2 structure compared to the P. aeruginosa PBP2 structure. The catalytic domain (residues 242 to 646) contains a central β-sheet flanked by several α-helices and an additional small β-sheet. Within this catalytic domain, the highly flexible loop region comprising residues 545 to 560 and the C-terminal residues 623 to 646 are too disordered and could not be modeled (Fig. 1B and Fig. S1). The catalytic serine residue S327 possesses a covalently bound cocrystallized WCK 5153 molecule attached (Fig. 2A).

WCK 5153 occupies the active site of PBP2. The active sites of PBPs possess several conserved motifs, and some of those are shared with homologous serine

FIG 1 Structures of DBO inhibitors and P. aeruginosa PBP2. (A) Chemical structures of DBOs: zidebactam, WCK 5153, WCK 4234, and avibactam. The R1-groups of the DBOs are shaded gray. (B) Co-crystal structure of P. aeruginosa PBP2 in complex with WCK 5153 (the latter is shown in stick representation with cyan-colored carbon atoms). The PBP2 anchor (red), head (blue), linker (green), and catalytic (orange) domains are labeled, as well as most of the secondary structure elements. (C) Superpositioning of the P. aeruginosa PBP2:WCK 5153 complex and E. coli PBP2 structure. The domain color coding for P. aeruginosa PBP2 is the same as in panel B with WCK 5153 depicted in spheres with cyan-colored carbon atoms. E. coli PBP2 is shown in similar but paler colors for its respective domains.
\(\beta\)-lactamases. The nucleophilic serine (S327) is located in the conserved motif S-X-X-K (S327-T328-V329-K330) situated at the beginning of a short \(\alpha_{12}\) helix at the start of the \(\alpha_{12}\) helix (Fig. 2B and Fig. S1). Here, S327 forms a covalent bond with WCK 5153, and the backbone nitrogen interacts with the carbonyl oxygen. In molecule B, the carbonyl oxygen of WCK 5153 is shifted 0.5 Å, such that the backbone nitrogen of T541 now also forms a hydrogen bond interaction (Fig. S2). The covalent bond between S327 and WCK 5153, with the carbonyl oxygen positioned in the oxyanion hole, is a common acyl-enzyme inhibited state for DBOs (14).

Within hydrogen bonding distance of the first motif is the second conserved active site motif, S-X-N/D (S384-N385-D386), located between the \(\alpha_{13}\) and \(\alpha_{14}\) helices. D386 hydrogen bonds with the nitrogen(s) of the diacylhydrazide moiety of WCK 5153 in both molecules A and B (Fig. 2B and Fig. S2). In molecule B, the nitrogen atom of the diacylhydrazide moiety closest to the piperidine ring is slightly rotated and makes a water-mediated interaction (Fig. S2); this water molecule also hydrogen bonds with the backbone carbonyl oxygen of I450. The third PBP motif, K-T/S-G-T (K538-S539-G540-T541), is in the \(\beta_{18}\) strand; the side chain of S539 forms a hydrogen bond with the sulfate of WCK 5153, and T541 interacts with the carbonyl oxygen.

Additional active site residues include W367, which interacts with the hydrophobic face of the DBO piperidine ring of the WCK 5153 scaffold. Also, the N-terminal end of the \(\alpha_{21}\) helix, via the backbone nitrogen of S592 as well as its side chain, forms hydrogen bonds with the sulfate of WCK 5153 (Fig. 2B and Fig. S2). This \(\alpha_{21}\) helix provides a helix dipole-sulfate interaction; such interactions are also observed in unrelated proteins (18, 19).

On the other side of the active site, I450 forms a 4.1-Å van der Waals interaction with the carbon atom of the diacylhydrazide moiety that is closest to the R1-pyrrolidine ring. Additionally, the nitrogen of the Q452 side chain interacts with that same end of the diacylhydrazide moiety, but with its oxygen atom. Overall, the diacylhydrazide moiety of WCK 5153 is significantly involved in active-site interactions; this likely explains the preference of WCK 5153 (and similar zidebactam) over WCK 4234 and avibactam to inhibit \textit{P. aeruginosa} PB2, culminating in their more potent antibacterial properties.

The terminal R1-group pyrrolidine ring of WCK 5153 makes relatively few interactions; one of its carbon atoms establishes a 3.9-Å hydrophobic van der Waals interaction with the main chain C carbon atom of N366 (Fig. 2B and Fig. S2). The tertiary
carbon of this pyrrolidine ring forms a 3.7-Å van der Waals interaction with the hydroxyl oxygen atom of Y390. This apolar-polar interaction would typically constitute an unfavorable interaction of burying a hydrogen bond donor (i.e., the Y390 hydroxyl). However, this is likely not an unfavorable interaction as the Y390 hydroxyl group already forms a weak 3.4- to 3.5-Å hydrogen-bond interaction with the backbone oxygen of R365. Finally, R369 makes a weak 3.5-Å interaction with WCK 5153 (only in molecule A; Fig. 2B and Fig. S2).

Comparison with avibactam-bound *E. coli* PBP2 structure. The *P. aeruginosa* PBP2 structure is similar to the *E. coli* PBP2 complex (Fig. 3). Also, avibactam bound to *E. coli* PBP2 makes similar active site interactions as WCK 5153 with *P. aeruginosa* PBP2. These ligand interaction similarities include the covalent bond with the catalytic serine, and the sulfate of avibactam hydrogen bonding with S545, which is similar to WCK 5153 interacting with the equivalent S539 in *P. aeruginosa* PBP2 (Fig. 2B). The sulfate moiety in avibactam also makes a hydrogen bond with *E. coli* PBP2 T547; this interaction is not observed with the equivalent T541 in the WCK 5153 *P. aeruginosa* complex as the latter side chain is oriented differently. Besides this minor difference, there is one other significant conformational difference near the sulfate binding region which involves the N terminus of helix a21 (Fig. 3). This first turn of the *P. aeruginosa* PBP2 N-terminal α-helix is “unwound” in the *E. coli* PBP2 structure and is repositioned away from the active-site region (Fig. 3). This particular α-helical turn (comprising *P. aeruginosa* PBP2 residues 591 to 594) provides interactions with the sulfate moiety of WCK 5153 via the hydrogen bond(s) involving residue S592 (Fig. 2 and 3 and Fig. S2). These interactions are not present with the sulfate moiety of avibactam when bound to *E. coli* PBP2 due to this conformational difference (Fig. 3). There are steric and other reasons for this conformational difference in *E. coli* PBP2 (*P. aeruginosa* residue numbering in parentheses): the larger Y533 (V527), the smaller S269 (R266), helix-breaking proline P598 (G593), and this helix-loop region also being one residue shorter in *E. coli* PBP2 (Fig. 3 and Fig. S1).

This DBO sulfate binding region of PBPs is also the region that recognizes the carboxyl moiety in β-lactam antibiotics. The above-noted conformational differences between *E. coli* PBP2 and *P. aeruginosa* PBP2 could be, in part, the basis for why the latter PBP is more difficult to inhibit by β-lactam antibiotics: the IC₅₀ values for ceftobiprole, ceftazidime, cefepime, and imipenem are between 8 and 15 times greater for *P. aeruginosa* PBP2 than for *E. coli* PBP2 (20). Other β-lactams have similar IC₅₀ differences (21).
Comparison with WCK 5153 bound KPC-2 β-lactamase structure. With the WCK 5153 bound PBP2 structure solved, a comparison is made to the same DBO when bound to KPC-2 β-lactamase (11). Superpositioning of the WCK 5153 bound structures of *P. aeruginosa* PBP2 and KPC-2 reveals several secondary-structure elements that align well. These elements include PBP2 β-strands β18, β19, and β20, the catalytic S327-containing α12 helix, the α13 and α14 helices, and the loop that connects these last two helices (Fig. 4). KPC-2 contains the same three conserved motifs as described above for PBPs. Residues in these motifs include the catalytic serine (S70) and the conserved active site lysine residues (K234 and K73 in KPC-2, which are analogous to K538 and K330, respectively, in PBP2). The active sites even contain a conserved tryptophan that provides hydrophobic interactions with the DBO, although it is rotated in a different position in KPC-2 (residues 68 to 77, 127 to 135, 231 to 236, and 244 to 248, respectively), yielding an RMSD of 0.87 Å for 30 Cα atoms.

Although KPC-2 also contains the conserved motif S-X-N/D, KPC-2 differs in that it possesses an asparagine at position 132, whereas PBP2 contains an aspartate at the structurally equivalent position 386 (Fig. 4). This difference is very relevant to WCK 5153 binding; in PBP2, the nitrogens of the diacylhydrazide donate hydrogen bonds to D386, whereas this interaction is not possible in KPC-2 since it has N132 at this position (the hydrogen bond accepting oxygen is now a hydrogen bond donating amide NH). This results in a key conformational difference in WCK 5153, which involves the diacylhydrazide oxygen near the label 4 in Fig. 4. In PBP2, the dihedral of the bond involving this diacylhydrazide oxygen atom orients the adjacent nitrogen to hydrogen bond with D386. In KPC-2 this equivalent residue is N132, which causes this diacylhydrazide moiety to reorient such that now the adjacent oxygen of the diacylhydrazide moiety interacts with N132. This conformational change suggests that WCK 5153 demonstrates structural plasticity in that the diacylhydrazide moiety can flip conformation to accommodate binding to both N132-containing KPC-2 and the D386-containing *P. aeruginosa* PBP2. The rest of WCK 5153 adopts a somewhat similar position and conformation in the active site in KPC-2, although the piperidine ring and attached sulfate moiety are shifted. This shift is perhaps due to the different orientations in the tryptophan (W105 in KPC-2; note that this residue can also adopt different conformations in KPC-2 [22]). Additionally, the end of the β18 strand also harbors a shift when comparing PBP2 and KPC-2 (KPC-2 residue T237, which is equivalent to T541 in PBP2).
residues are also close to the piperidine ring of the DBO. Overall, these DBO shifts cause the sulfate moiety in KPC-2 to make hydrogen bonds with S130, T235, and T237.

In contrast, the sulfate moiety of WCK 5153 in the PBP2 structure is shifted (more outward) toward the end of the \( \alpha21 \) helix, forming two hydrogen-bond interactions with the N terminus of this \( \alpha21 \) helix and one hydrogen bond with S539 (equivalent to T235 in KPC-2; Fig. 2B and Fig. 4). Both the PBP2 \( \alpha20 \) and \( \alpha21 \) helices also have corresponding helices in KPC-2, but those helices are shifted (Fig. 4, purple arrows). Remarkably, WCK 5153 is still able to make different yet substantial active site interactions involving its sulfate moiety despite these noted active site differences with KPC-2. As in PBP2, the pyrrolidine moiety of WCK 5153 is making few interactions in KPC-2 (Fig. 2B and Fig. 4) (11).

**DSF analysis of WCK 5153 and zidebactam binding to PBP2.** To probe the effect of WCK 5153 and zidebactam binding on the thermal stability of *P. aeruginosa* PBP2, we carried out differential scanning fluorimetry (DSF)/thermal shift assay experiments. Both DBOs significantly increased the melting temperature (\( T_m \)) of PBP2, which increased from 46.1 ± 0.1°C, when in the absence of ligands, to 52.1 ± 0.1°C and 51.5 ± 0.1°C in the presence of 600 μM WCK 5153 or zidebactam, respectively (Fig. 5A). These values are almost identical at much lower DBO concentrations, 52.2 ± 0.0°C and 51.4 ± 0.1°C in the presence of 9.4 μM WCK 5153 or zidebactam, respectively, indicating their high affinity. These thermal shifts indicate that the binding of either of these DBOs stabilizes PBP2, contributing to their effective inhibition, which translates into their standalone antibacterial activity. These two DBO compounds differ only in the R1-group, where WCK 5153 contains a five-membered pyrrolidine ring and zidebactam a six-membered piperidine ring (Fig. 1A). The previously reported IC\(_{50}\) values of WCK 5153 and zidebactam for PBP2 are 0.14 and 0.26 μg/ml, respectively (13); these values follow the same trend in that WCK 5153 binding leads to a stronger PBP2 stabilization than for zidebactam binding. Nevertheless, it is important to note there is no clear correlation between change in protein stability and affinity of a ligand; the latter is dependent on the rates of the inhibition reaction steps whereas the former is dependent on the combined enthalpic and entropic effects when affecting the thermal stability of the covalent protein-inhibitor complex (23).

To complement the thermal shift data, we modeled zidebactam in the PBP2 active site using the WCK 5153 complex (Fig. 5B) as a starting model. The zidebactam modeling demonstrated a very similar binding mode as WCK 5153, which is not surprising due to their close chemical similarity. The R1-group piperidine ring is also quite solvent exposed, like the corresponding pyrrolidine ring in WCK 5153. This observation perhaps could be a potential reason for WCK 5153’s slightly higher PBP2 affinity (13) and increased PBP2 stability (Fig. 5B): this ring is overall relatively hydrophobic, except for the secondary amine, is larger, and exists in chair conformation in zidebactam versus the envelope form of pyrrolidine ring in WCK 5153. A larger, more hydrophobic solvent-exposed ring could be energetically less favorable for PBP2 binding and stabilization for zidebactam. Note that compared with zidebactam, WCK 5153 also yields lower MIC values against *P. aeruginosa*, both with and without a partnered \( \beta \)-lactam (11).

**Probing DBO binding to *P. aeruginosa* PBP3.** Zidebactam and WCK 5153 do not inhibit *P. aeruginosa* PBP3 activity, probably because of their weak affinities (IC\(_{50}\) values for zidebactam and WCK 5153 are >4 μg/ml for PBP3 as determined using Bocillin-based competition assays with PBP-containing membrane preparations [13]). Comparison of previously reported *P. aeruginosa* PBP3 structures (24–28) and PBP2 structure (resolved in this study) revealed similarities (the active sites of *P. aeruginosa* PBP2 and PBP3 have 6/13 active site residues that are identical); hence, we hypothesized that DBO molecules can also bind to PBP3. Such insights could be useful for optimizing future DBOs that can drive activity by inhibiting multiple PBPs. Hence, we first grew *P. aeruginosa* apo-PBP3 crystals and subsequently incubated three DBOs (zidebactam, avibactam, and WCK 4234) with these apo-PBP3 crystals, and their structures were solved.

PBP3 shares a similar catalytic TP domain with PBP2, yet with a few differences. In
PBP2, helices \(a_{10}\) and \(a_{11}\) in the TP domain are replaced in PBP3 by a short loop. Also, PBP3 has a somewhat smaller linker domain compared with PBP2 as the secondary structure elements \(a_{5}\) and \(\eta_4\) present in PBP2 are deleted and replaced with a short loop. *P. aeruginosa* PBP3 structures with three DBO compounds, avibactam, WCK 4234, and zidebactam, are resolved at 2.39, 2.08, and 2.50 Å, respectively. A representative PBP3 DBO complex with WCK 4234 structure is the highest resolution, and the best-resolved structure of the three is shown (Fig. 6). The overall structures of the DBO complexes are all very similar: the zidebactam and avibactam complexes superimpose onto the WCK 4234 PBP3 complex with RMSD values of 0.55 and 0.49 Å, respectively.

The PBP3 active site S-X-X-K motif (S294-T295-V296-K297) is located at the end of the \(a_2\) helix. The S-X-N/D motif (S349-S350-N351) is located between helices \(a_4\) and \(a_5\), and the third motif, K-T/S-G-T (K484-S485-G486-T487), is part of the \(\beta_3\) strand (Fig. 6 and 7). In all these three structures, several flexible loop segments (residue ranges 197 to 207, 490 to 500, and 528 to 534) could not be modeled; in particular, the last loop located at the beginning of \(a_{11}\) helix (containing residues Y532 and F533) is...
known to undergo various conformational changes during ligand binding and catalysis.

**DBO interactions in PBP3 active sites.** Zidebactam, WCK 4234, and avibactam are covalently bound to the catalytic S294 (Fig. 7). The carbonyl oxygen of each of the DBOs is situated in the oxyanion hole formed by the backbone nitrogens of S294 and T487 (Fig. 7). The sulfate moiety hydrogen bonds with S485 and, depending on the DBO, also with either S349, K484, or T487 (Fig. 7). The R1-group moieties of the DBOs were well defined: for WCK 4234, its nitrile moiety, and for avibactam, its amide moiety. Both these moieties form hydrogen bonds with N351 (Fig. 7B and F). In the zidebactam complex, N351 interacts with the diacylhydrazide oxygen atom closest to the piperidine scaffold of the DBO (Fig. 7D). This zidebactam oxygen also makes indirect active site interactions via hydrogen bonding to a water molecule (wat1). For zidebactam, electron density was present for the diacylhydrazide moiety and the terminal

**FIG 6** Crystal structure of *P. aeruginosa* PBP3 in complex with WCK 4234. The noncatalytic N-terminal domain and catalytic TP domain are colored cyan and gray, respectively. Bound WCK 4234 is shown in spheres with green carbon atoms. Secondary structure elements near the active site are labeled (numbering as in reference 24).
piperidine moiety of the R1-group of zidebactam, but mainly for the tertiary carbon atom (Fig. 7C). This tertiary carbon atom of the R1-group piperidine establishes hydrophobic interactions with Y409 (3.8-Å distance) and is at a larger distance from Y407 (4.8 Å). The carbon atom of the diacylhydrazide moiety connected to this tertiary carbon atom of the R1-group piperidine also makes hydrophobic interactions with Y409 (4.3 Å). Overall, the hydrogen bond interaction with N351 is conserved in all three DBO complexes, yet the moiety making this interaction varies depending on the R1-group of the DBO.

**Comparison of PBP3:zidebactam complex with PBP2:WCK 5153 complex.** The zidebactam PBP3 structure was compared against WCK 5153 complexed to PBP2.
Their carbonyl oxygen atoms are both situated in their respective PBP oxyanion holes. In contrast, the main DBO piperidine ring and attached sulfate moiety are shifted such that the sulfate moiety in the PBP2 complex is positioned 1.9 Å further away from the catalytic serine. These shifts are perhaps due to the sulfate of WCK 5153 needing to reach the N-terminal part of the $\alpha_{21}$ helix in PBP2 to make hydrogen bonding and helix-dipole interactions (Fig. 2B and Fig. 8). Additionally, the larger W367 residue in PBP2, which is the smaller V333 in PBP3, might contribute to this difference.

The PBP2 and PBP3 active sites contain some notable differences. In the S-X-D/N motif in PBP3, this last residue is N351, whereas in PBP2 this residue is D386. Despite this difference, these residues both interact with the diacylhydrazide of the DBOs. The DBOs can accomplish this by reorienting the diacylhydrazide moiety in PBP3 analogous to when bound to the N132-containing KPC-2 (see above). A second important active site difference is that PBP2 contains a large hydrophobic W367 that interacts with the hydrophobic face of the main piperidine ring of WCK 5153, whereas this residue is V333 in PBP3. This interaction could, in part, explain the WCK 5153 (and similar zidebactam) higher affinity for PBP2 than for PBP3. The terminal 5- or 6-membered ring of the R1-groups of WCK 5153 and zidebactam, respectively, makes only limited interactions as described above.

**Comparison of PBP3:DBO with KPC-2:DBO complexes.** Similar to the PBP2:WCK 5153 complex above, we superimposed the zidebactam-bound complexes of PBP3 and KPC-2 β-lactamase, a DBO complex structure determined previously (11) (Fig. S3). The superposition shows that the main DBO piperidine ring and attached sulfate moiety superimpose quite well (sulfate moieties are at a distance of 0.6 Å). Both PBP3 and KPC-2 share an asparagine at the S-X-N/D motif. The diacylhydrazide moiety of zidebactam is therefore oriented in a similar fashion in both structures with the oxygen interacting with the nitrogen atom of N351 in PBP2 and N132 in KPC-2 (Fig. S3). An active site difference involves KPC-2 having a large aromatic tryptophan at position 105 corresponding to the equivalent, yet smaller, V333 in PBP3; this makes the KPC-2
active site somewhat like PBP2 although the conformations of their respective tryptophans are different (Fig. 4 and 8 and Fig. S3).

An additional difference compared to the active site of KPC-2 is in the β-strand that contains the K-T/S-G-T motif. The last residue of this motif (T487 in PBP3 and T237 in KPC-2) is bent more inward toward the active site crevice for KPC-2 compared with PBP3 (Fig. S3). The terminal piperidine of the R1-group of zidebactam in both structures makes only minimal interactions. An additional difference encompasses the Ω-loop in KPC-2, which is absent in the PBP3 and other PBPs. This Ω-loop contains E166 and N170 needed for decacylation of bound ligands, which allows the β-lactamase to hydrolyze β-lactam substrates. Finally, the two helices that shifted when comparing P. aeruginosa PBP2 and E. coli PBP2 (Fig. 4) are also repositioned in KPC-2 compared with P. aeruginosa PBP3 (Fig. S3, purple arrows).

Regarding the other DBOs, the hydrogen bonds of the nitrile and amide moiety of WCK 4234 and avibactam, respectively, with N351 when bound to PBP3 are conserved in KPC-2 via their interaction with N132 (11).

Comparison with other PBP3 inhibitor complexes. Several conformational changes often observed in β-lactam binding to P. aeruginosa PBP3 (24–28) are not observed in the DBO complexes. In most previously determined PBP3 complexes, an aromatic wall is formed with residues Y532, F533, and also Y503 moving close to the hydrophobic parts of the ligand (24). Figure S4A shows this for the carbenicillin PBP3 complex. The F533 side chain often approaches the hydrophobic V333 residue on the other side of the active site to form a hydrophobic bridge (24). The phenyl moiety of carbenicillin (b) and its dimethyl groups (c) attract these aromatic wall residues. In the ceftazidime complex, the dimethyl groups of the 2-carboxypropan-2-yl moiety (b) likely also contribute to the hydrophobic attraction of forming the aromatic wall (Fig. S4B). Note that Y532 and F533 could be resolved only in the higher-resolution WCK 4234 PBP3 complex; these residues are too disordered to be modeled in the zidebactam and avibactam PBP3 complexes. A possible explanation for why the DBO inhibitor complexes do not show this aromatic wall conformation is as follows. The DBO inhibitors have only two hydrophobic carbon atoms that are part of the main DBO piperidine ring that are solvent exposed; this is likely not enough for the aromatic wall to make these hydrophobic interactions as seen in other PBP3:inhibitor complexes.

A second conformational change often observed in PBP3:inhibitor complexes involves the bending of the β-strands β3 and β4 toward the active site. These β-strand shifts allow hydrogen bonding interactions with the amide moiety (a) of ligands like carbenicillin and ceftazidime (24, 25) (Fig. S4A and B). In the latter case, these shifts also allow the formation of hydrogen bonding interactions with aminothiazole moieties in inhibitors such as ceftazidime (d in Fig. S4B). This amide moiety is also a key moiety in the d-Ala-d-Ala end of the peptidoglycan substrate of PBPs. Such a β-lactam-induced conformational change is also observed in N. gonorrhoeae PBP2 (16).

Third, PBP inhibitors that have an aminothiazole ring, such as ceftazidime, are accommodated in the active site by having Y409 swing out to create a pocket and also to provide stacking interactions with this aminothiazole moiety (d in Fig. S4B) (24, 25).

The PBP3:DBO protein conformation is structurally most similar to that of the apo-PBP3 structure (Fig. S4C) as well as the meropenem bound PBP3 structure (Fig. S4D). The β3 and β4 strands are in the same position in all these structures. This similarity is likely a consequence of either an absence of an amide moiety or the situation that, when present in WCK 4234, its amide is not correctly positioned for such a β3 strand interaction. Despite this similarity, the meropenem bound structure does show a partial formation of the aromatic wall via the shift of only F533 interacting with the hydrophobic parts of the sulfur-containing substituent of meropenem (c) (Fig. S4D). Note that even though the tested DBOs bind to P. aeruginosa PBP3, the binding is not of sufficiently high affinity to be able to contribute in antibacterial activity. Avibactam and WCK 4234 also do not possess activity against P. aeruginosa.

**DSF experiments of DBO binding to PBP3.** In contrast to the increase in thermal stability observed with PBP2, zidebactam and WCK 5153, each at 1 mM, lowered the
apo-PBP3 $T_m$ of 42.8 ± 0.3°C to 41.9 ± 0.1°C (Fig. 9). WCK 4234 binding decreased the $T_m$ even more significantly, to 37.0°C. Avibactam at 1.0 and 0.5 mM also decreased the $T_m$ of PBP3 to 35.5 ± 0.4 and 35.9 ± 0.1°C, respectively (Fig. 9). As a positive control for comparison, ceftazidime binding to PBP3 led to a large increase in $T_m$ as previously published (29). Experiments were done in duplicate, and a representative curve is shown for each experiment. (B) Avibactam binding to PBP3. Data are plotted similarly as in Fig. 5. Avibactam was dissolved in water. Experiments were done in triplicate, and a representative curve is shown for each experiment.

The decreasing effects on the $T_m$ of PBP3 by DBO binding are likely due to the absence of hydrophobic moieties on the piperidine ring DBO scaffolds or R1-groups that attract the aromatic wall to interact with the ligand. For example, carbenicillin binding also leads to a substantial increase in the $T_m$ of PBP3 by 13.2°C (25). The decrease in $T_m$ of avibactam and WCK 4234 binding to PBP3 is similar to that obtained for meropenem and imipenem with a reduction in $T_m$ of 4 and 6°C, respectively (24). Meropenem also does not induce the full aromatic wall interactions (Fig. S4D), and imipenem has a very different conformation of this Y532/F533 region (24). That zidebactam/WCK 5153 binding led to a minor negative effect on $T_m$ compared with the more destabilizing effects of WCK 4234 and avibactam can be explained by analyzing the similarities and differences of their binding modes. First, the similarities entail that all three DBOs form a hydrogen bond with N351, and their DBO sulfate active site interactions are also quite similar (Fig. 7). The one key difference that could explain the $T_m$ differences is that zidebactam makes additional stabilizing hydrophobic interactions via its diacylhydrazide moiety and adjacent tertiary carbon atom of the piperidine moiety with mainly Y409
Figure 7. We anticipate that the corresponding similar pyrrolidine ring in WCK 5153 is making similar interactions. The much smaller DBOs WCK 4234 and avibactam cannot make such stabilizing hydrophobic interactions. Note that, as mentioned above, there is no clear correlation between change in protein stability and affinity of a ligand. For example, meropenem and ceftazidime have very different, even opposite effects on the $T_m$ of PBP3, yet their 50% effective concentration (EC50) values of PBP3 binding are nearly identical (24).

The DSF results aid in understanding important differences of DBO binding to PBP2 and PBP3. For P. aeruginosa PBP2 binding of either WCK 5153 or zidebactam caused an upward $T_m$ shift of 5 to 6°C with DBO concentrations as low as 9.4 μM, indicating effective inhibition of PBP2. However, these DBOs did not show a stabilizing effect when binding to PBP3. Although it is not a necessity to have a stabilizing effect for efficient PBP3 binding, most potent PBP3 inhibitors do achieve that.

**Conclusion.** In summary, we present co-crystallized and modeled structures of WCK 5153 and zidebactam with *P. aeruginosa* PBP2, respectively. The PBP2 structure represents the first atomic-level analysis for this *P. aeruginosa* PBP and provides possible explanations for this PBP’s decreased susceptibility to be inhibited by β-lactam antibiotics compared to its *E. coli* equivalent PBP. For comparison, we also determined crystal structures of DBOs zidebactam, WCK 4234, and avibactam in complex with *P. aeruginosa* PBP3 to explain the DBOs’ weaker affinity for this PBP. We show that in addition to the covalent bond, WCK 5153 binds PBP2 via (a) interactions of the carbonyl oxygen with the oxyanion hole, (b) interactions with SS39 and the C-terminal end of helix α21 by the sulfate moiety forming a helix-dipole interaction, (c) interactions by the diacylhydrazide moiety with D386 and Q452, (d) hydrophobic interactions of the pyrrolidine ring, and (e) interactions of the hydrophobic part of the piperidine ring with W367. Upon binding, the DBOs stabilize PBP2, yet destabilize PBP3, as measured via thermal shift assays. Structural comparisons indicate that a strong contributor to WCK 5153 and zidebactam preferentially binding to PBP2 and KPC-2 is the presence of a tryptophan residue to provide hydrophobic interactions; this residue is nonaromatic in PBP3. For zidebactam and WCK 5153, our results show that the diacylhydrazide moiety can adopt different orientations to interact as either a hydrogen acceptor with asparagine (in PBP3 and KPC-2) or a donor with aspartate (in PBP2) in the conserved S-X-N/D motif. This diacylhydrazide moiety of zidebactam and WCK 5153 is relatively more involved in active site interactions than DBOs with smaller side chains like nitrile in WCK 4234 and amide in avibactam. These diacylhydrazide interactions combined with the modest hydrophobic interactions of the R1-group piperidine/pyrrolidine likely explain the preference of zidebactam and WCK 5153 over WCK 4234 and avibactam to inhibit *P. aeruginosa* PBP2 effectively and exhibit potent antibacterial properties.

These molecular insights and the ability of DBOs to be “dual-target” inhibitors could be utilized for optimization efforts to develop novel potent β-lactamase-resistant non-β-lactam DBOs with the ability to bind and functionally inhibit multiple PBPs.

**MATERIALS AND METHODS**

Protein expression, purification, and crystallization. (i) PBP2. *P. aeruginosa* PBP2 (39 to 646 aa) lacking the N-terminal membrane anchoring region (residues 1 to 38) was subcloned into the NdeI cleavage site of the pET28a vector to add an N-terminal histidine tag. The PBP2 plasmid was transformed into LOBSTR RIL competent cells (Kerafast). Six- to 8-liter cultures were grown in terrific broth medium with kanamycin 50 μg/ml and induced at 0.6 to 0.8 OD600 using 50 μM IPTG while shaking for 14 to 16 h at 18°C. After pelleting by centrifugation, the cells were suspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, and 10 mM imidazole). The lysis buffer was supplemented with one EDTA-free protease inhibitor tablet (Sigma-Aldrich) and 1,000 units of Benzonase (ACROBiosystems) and 1 mM MgCl2. Cells were lysed using an Avestin Emulsiflex B-15 apparatus at 4°C, and the cell suspension was centrifuged at 30,597 × g to remove cell debris. The supernatant was incubated with freshly washed Ni-NTA beads and rocked for 2 h at 4°C. The Ni-NTA beads were washed with 50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, 25 mM imidazole buffer and then eluted with the same buffer containing 250 mM imidazole. The elution fractions were dialyzed overnight against 3 liters of 50 mM Tris, pH 8.0, 300 mM NaCl, and 10% glycerol buffer. After centrifugation at 13,000 rpm to remove possible precipitation, a second round of His-tag purification was carried out using a 5-mI Ni-NTA
TABLE 1 Data collection and refinement statistics for crystal structures of PBP2 and PBP3 DBO complexes

| Data collection   | PBP2 WCK 5153 | PBP3 WCK 4234 | PBP3 avibactam | PBP3 zidebactam |
|-------------------|---------------|---------------|----------------|----------------|
| Wavelength (Å)    | 0.979331      | 0.97928       | 0.97928        | 0.97946        |
| Resolution range (Å) | 2.87–29.67 (2.87–3.00) | 2.09–29.25 (2.09–2.14) | 2.39–29.58 (2.39–2.45) | 2.50–25.0 (2.50–2.59) |
| Space group       | P2,2,2        | P2,2,2        | P2,2,2         | P2,2,2         |
| Unit cell (Å³)    | 90.8 (86.1)   | 99.4 (94.3)   | 99.3 (93.4)    | 99.9 (100.0)   |
| Completeness (%)  | 90.0 (87.0)   | 91.9 (96.4)   | 91.9 (96.4)    | 91.9 (96.4)    |
| No. of unique reflections | 23,998 (3,298) | 27,862 (1,915) | 19,459 (1,323) | 17,225 (1,711) |
| Total no. of observations | 81,726         | 183,701       | 122,435        | 207,103        |
| Multiplicity      | 3.4 (3.3)     | 6.6 (6.2)     | 6.3 (6.2)      | 12.0 (12.8)    |
| Mean I/σ(I)       | 5.2 (2.0)     | 8.2 (1.7)     | 10.9 (2.3)     | 22.3 (4.2)     |
| Mean CC(1/2) (%)  | 97.8 (76.5)   | 99.4 (65.8)   | 99.3 (73.3)    | 99.7 (94.2)    |
| Rmerge (%)        | 0.158 (0.514) | 0.134 (0.920) | 0.196 (0.885)  | 0.182 (1.21)   |

Refinement

| Resolution range refinement (Å) | 2.87–29.67 | 2.09–29.25 | 2.39–29.58 | 2.50–24.94 |
| Rwork (%)                      | 0.2580     | 0.1951     | 0.1930     | 0.1996     |
| Rfree (%)                      | 0.3165     | 0.2421     | 0.2577     | 0.2719     |
| Ligands                        | 2 ligands, 1 chloride ion | 1 ligand | 1 ligand | 1 ligand |
| No. of water molecules         | 20         | 84         | 114        | 71         |
| RMSD bond lengths (Å)          | 0.003      | 0.007      | 0.008      | 0.007      |
| RMSD bond angles (°)           | 0.724      | 0.896      | 1.004      | 1.372      |
| B-factors protein (Å²)         | 42.8       | 37.9       | 35.0       | 50.2       |
| B-factors ligands (Å²)         | 44.6       | 39.1       | 40.2       | 74.8       |
| Ramachandran favored (%)       | 95.6       | 96.8       | 96.0       | 95.4       |
| Ramachandran outliers (%)      | 0.57       | 0.43       | 0.0        | 0.22       |

Values in parentheses are for the highest-resolution shell.

HisTrap column (GE Life Sciences). After loading the PBP2 protein, the HisTrap column was washed with 50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, and 20 mM imidazole buffer. The protein was eluted using an imidazole gradient of 20 to 250 mM. The purity of the fractions was checked using SDS-PAGE gel analysis, and the corresponding fractions were pooled and dialyzed against the above-described dialysis buffer. WCK 5153 was subsequently added at a 1:50 protein/compound molar ratio, and the inhibited PBP2 was concentrated to 2.9 mg/ml prior to cocrystallization. The cocrystallization trials with DBO compounds resulted in crystallization hits in MCGS crystallization screens (Anatrace) using the sitting drop method at 20°C. The best crystals were grown in 0.1 M HEPES, pH 7.5, 0.2 M ammonium acetate, and 25% polyethylene glycol (PEG) 3350. The crystal was cryoprotected using perfluoropolyether (Hampton Research) prior to freezing the crystal in liquid nitrogen for data collection.

(ii) PBP3. The P. aeruginosa PBP3 protein expression and purification were carried out as described earlier (24, 29, 30). PBP3 crystals were grown via the sitting drop method using previously published conditions: 30% polyethylene glycol 4000, 0.2 M MgCl₂, 0.1 M Tris, pH 8.5 (24). Apo-PBP3 crystals were used for soaking the individual DBO inhibitors in mother liquor as follows. Crystals were soaked with 2.5 mM zidebactam for 23 h, or with 3 mM either WCK 4234 or avibactam for 1 h prior to freezing the crystals in liquid nitrogen.

Data collection and refinement. (i) PBP2. A needle-shaped crystal of the cocrystallized PBP2 and WCK 5153 complex was used for data collection at the microfocus FMX 17-ID-2 beamline at NSLS-II, and diffraction data were processed using XDS (31) to 2.87-Å resolution (Table 1). The PBP2 structure was solved via molecular replacement using PHASER (32) with the TP domain of E. coli PBP2 structure (14) (PDB ID 6G9F) as a search model. The solution yielded two molecules in the asymmetric unit. The crystallographic refinement was carried out using REFMAC (33) and PHENIX (34), and model building was done using COOT (35). Nocystallographic symmetry restraints were applied during refinement in PHENIX. Refinement parameter files for WCK 5153 were generated using eLBOW (36). The final structure yielded an R-factor/Rmerge of 25.9/31.7% and contained two molecules in the asymmetric unit, each containing a covalently bound WCK 5153. In addition, there are 20 water molecules and one chloride ion. PBP2 chain A contains residues 60 to 87, 162 to 222, 238 to 544, and 561 to 622; chain B contains residues 63 to 86, 164 to 183, 189 to 219, 240 to 544, and 562 to 621. This refined PBP2 structure encompasses most of the construct and includes the anchor, linker, and TP domains. However, the head domain (residues 88 to 161) is not included as it could not be modeled confidently for either monomer due to poor electron density likely due to disorder. The two non-crystallographically related PBP2 molecules are very similar to each other (0.69-Å root mean square deviation for 440 Ca atoms); the 2 monomers in the asymmetric unit do not represent a dimer as no relevant interfaces were observed using PISA (37).

(ii) PBP3. Avibactam- and WCK 4234-soaked PBP3 crystals were used for data collection at the AMX 17-ID-1 beamline at NSLS-II and processed using XDS (Table 1). Data for the avibactam and WCK 4234 complexes were diffracted to 2.39- and 2.09-Å resolution, respectively. Data for the zidebactam-soaked PBP3 crystal were collected at SSRl beamline 12-2, and the data were processed to 2.5-Å resolution using HKL-3000 (38) (Table 1). The three DBO PBP3 complex structures were solved by molecular
replacement using PHASER with the PBP3-imipenem complex protein coordinates as the search model (PDB ID 3PBO) (24). COOT was used for model building, and REFMAC and PHENIX were used for crystallographic refinement. The WCK 4234 complex contains PBP3 residues 56 to 196, 204 to 272, 276 to 467, 471 to 490, 503 to 527, and 532 to 559. The avibactam complex contains PBP3 residues 57 to 190, 213 to 489, 502 to 527, and 536 to 560. The zidebactam complex contains PBP3 residues 57 to 191, 211 to 489, 501 to 527, and 535 to 559. The refinement parameter files for the DBO ligands were generated using eLBOW. The refined R-factor/R_free values are listed in Table 1. The refined structures yielded >94% of the residues in the favorable region of the Ramachandran plot as calculated using MolProbity (39) (Table 1).

Molecular figures were generated using PyMOL (www.pymol.org). Coordinates and structure factors of the *P. aeruginosa* PBP2 WCK 5153 complex and the PBP3 complexes with WCK 4234, avibactam, and zidebactam were deposited in PDB.

**DSF experiments.** The DSF thermal shift assay was conducted on a CFX96-C1000-Touch thermocycler (Bio-Rad) similarly to what we previously described (29, 30). The reactions were carried out in the buffer containing 50 mM HEPES, pH 8.0, 300 mM NaCl, 10% glycerol at a sample volume of 30 μl, with 1.4 μM PBP2 either with or without WCK 5153 and zidebactam compounds dissolved in dimethyl sulfoxide (DMSO). Equivalent DMSO controls were also included, and reactions were run in duplicates. All the reaction mixtures included SYPRO orange dye (Fisher Scientific) at 10× concentration, and the thermal scan was conducted between 25 and 70°C with 0.2°C/min intervals.

For PBP3 (3 μM), the DSF experiments were conducted for all four DBO compounds (zidebactam, WCK 5153, WCK 4234, and avibactam) at the indicated concentrations. The first three DBOs were dissolved in DMSO, and DSF experiments were carried out in duplicate. Avibactam was dissolved in water, and DSF experiments with avibactam-containing mixtures were carried out in triplicate. Ceftazidime (at 200 μM) was used as a positive control similarly to what was previously described (29, 30). The reaction buffer contained 14 mM Tris, pH 8.0, 280 mM NaCl, 7% glycerol, and 10× SYPRO orange.

**Data availability.** Coordinates and structure factors of the *P. aeruginosa* PBP2 WCK 5153 complex and the PBP3 complexes with WCK 4234, avibactam, and zidebactam were deposited in the Worldwide Protein Data Bank (wwPDB) with accession numbers 7KIS, 7KIT, 7NIW, and 7NIW, respectively.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 2.5 MB.

**FIG S2**, TIF file, 2.5 MB.

**FIG S3**, TIF file, 1.6 MB.

**FIG S4**, TIF file, 2.5 MB.

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