Distinctive Binding of Avibactam to Penicillin-Binding Proteins of Gram-Negative and Gram-Positive Bacteria

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Avibactam is a novel non-β-lactam β-lactamase inhibitor that covalently acylates a variety of β-lactamases, causing inhibition. Although avibactam presents limited antibacterial activity, its acylation ability toward bacterial penicillin-binding proteins (PBPs) was investigated. Staphylococcus aureus was of particular interest due to the reported β-lactamase activity of PBP4. The binding of avibactam to PBPs was measured by adding increasing concentrations to membrane preparations of a variety of Gram-positive and Gram-negative bacteria prior to addition of the fluorescent reagent Bocillin FL. Relative binding (measured here as the 50% inhibitory concentration [IC50]) to PBPs was estimated by quantification of fluorescence after gel electrophoresis. Avibactam was found to selectively bind to some PBPs. In Escherichia coli, Pseudomonas aeruginosa, Haemophilus influenzae, and S. aureus, avibactam primarily bound to PBP2, with IC50’s of 0.92, 1.1, 3.0, and 51 μg/ml, respectively, whereas binding to PBP3 was observed in Streptococcus pneumoniae (IC50, 8.1 μg/ml). Interestingly, avibactam was able to significantly enhance labeling of S. aureus PBP4 by Bocillin FL. In PBP competition assays with S. aureus, where avibactam was used at a fixed concentration in combination with varied amounts of ceftazidime, the apparent IC50 of ceftazidime was found to be very similar to that determined for ceftazidime when used alone. In conclusion, avibactam is able to covalently bind to some bacterial PBPs. Identification of these PBP targets may allow the development of new diazabicyclooctane derivatives with improved affinity for PBPs or new combination therapies that act on multiple PBP targets.
marker Bocillin FL (24). Of particular interest was S. aureus PBP4, which appears to display some β-lactamase activity (25).

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MATERIALS AND METHODS

Antibiotics. Avibactam was provided by AstraZeneca Pharmaceuticals (Wilmington, NC, USA). Cefazidime was obtained from Lab Express International (Fairfield, NJ, USA). Oxacillin and amdinocillin were from Sigma-Aldrich Canada (Oakville, ON, Canada). PBP was labeled using the fluorescent reporter molecule Bocillin FL (Invitrogen-Molecular Probes, Eugene, OR, USA).

Antibiotic susceptibility testing. Susceptibility testing was performed using the broth microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (27). MICs were determined in cation-adjusted Mueller-Hinton broth (CAMHB; Becton Dickinson Canada Inc., Mississauga, ON, Canada). MICs for S. pneumoniae were determined in CAMHB supplemented with 2.5% lysed horse blood.

Bacterial strains and growth conditions. E. coli K-12 (250HT11) and P. aeruginosa 1771 (391HT2) were grown in brain heart infusion (BHI) broth at 35°C. For bacterial membrane preparation, an overnight bacterial culture was diluted in fresh medium and grown to an A600 of ~1.5. H. influenzae strain Rd was grown in BHI broth supplemented with Haemophilus test medium supplement (Oxoid, Nepean, ON, Canada), which includes hemin and β-NAD (both at 15 μg/ml) to reach an A600 of ~0.6 to 0.7 at 35°C. S. aureus ATCC 29213 was used as a typical methicillin-susceptible strain and was grown to reach an A600 of ~0.6 to 0.7 in BHI broth at 35°C. S. pneumoniae R6 is a typical penicillin-susceptible strain (28). It was grown on blood agar plates at 37°C in 5% CO2. E. coli, S. aureus, and S. pneumoniae were disrupted by a French press instrument, and the bacterial lysates were fixed in BHI at 37°C before addition of 3 μg/ml of protease inhibitor cocktail (Sigma-Aldrich), DNase (6 μg/ml), and RNase (6 μg/ml). For S. pneumoniae and H. influenzae, lysozyme (400 μg/ml) was also added. After 30 min of treatment, cells were disrupted by a French press instrument, and the bacterial lysates were centrifuged at 6,000 × g for 30 min at 4°C to remove unbroken cells. The supernatant was then centrifuged at 150,000 × g for 40 min at 4°C using a fixed-angle rotor to collect the membranes. The membranes were suspended in a minimal volume of buffer (typically 500 μl) and stored at ~80°C. The protein concentrations in the membrane preparations were estimated by using the Micro bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL) with bovine serum albumin as a standard.

PBPs binding and competition assays. The relative binding of test compounds for bacterial PBPs was determined in a competition assay with the fluorescent reporter molecule Bocillin FL. An aliquot of bacterial membrane preparation was used for E. coli, P. aeruginosa, and S. aureus (10 to 20 μg of membrane proteins), S. pneumoniae (30 to 40 μg of membrane proteins), or H. influenzae (100 to 120 μg of membrane proteins) in each assay mixture. The amount of membrane preparation used for each species was selected so that the PBP banding patterns obtained on gels would reflect those previously published in the literature. In the sets of assay mixtures (~7 μl of membrane preparation in a 15-μl final volume), increasing concentrations of each test compound were first added for 10 min at 30°C before Bocillin FL was added at a final concentration of 100 μM for an additional 20 min of incubation. Thereafter, electrophoresis loading buffer, containing sodium dodecyl sulfate (SDS) and fresh β-mercaptoethanol as a reducing reagent, was added to the mixture before heating for 3 min at 95°C. Samples were spun for 2 min in a microcentrifuge before loading for electrophoresis.

SDS-PAGE, PBP detection, and IC50 determinations. Proteins from the PBP assay mixtures were separated by electrophoresis using an SDS-polyacrylamide discontinuous gel system (5% stacking and 10% separating gels). After electrophoresis, the gels were quickly rinsed in water and fixed in a 50% methanol–7% acetic acid solution for 30 min. Gels were scanned to collect the images of the PBP profiles by using either a Molecular Imager FX Pro instrument (Bio-Rad Laboratories, Mississauga, ON, Canada) or a Typhoon 9400 scanner (GE Healthcare) (excitation at 488 nm and emission at 520 to 530 nm). The PBP image was quantified using Quantity One 1-D analysis software (version 4.6.6; Bio-Rad Laboratories, Richmond, CA). Each individual labeled PBP band was selected, and its volume (surface × intensity) was measured. The relative binding or IC50, i.e., the concentration of test compound (in micrograms per milliliter) that was needed to reduce by 50% the binding of Bocillin FL to individual PBPs was then determined by plotting the PBP band volumes versus compound concentrations. The 100% binding of Bocillin FL was represented by the PBPs labeled with Bocillin FL but with no drug or avibactam added to the mixture. For drug combination experiments with S. aureus, the 100% value was represented by the PBPs labeled with Bocillin FL in the presence of avibactam at 4 μg/ml. The IC50 for each PBP of interest was calculated from at least eight test molecule concentrations (E. coli, P. aeruginosa) or three independent PBP binding assays using six different concentrations in each experiment (H. influenzae, S. aureus, S. pneumoniae).

RESULTS AND DISCUSSION

Figure 1 shows the pattern of avibactam binding to E. coli and S. aureus PBPs. Relative binding to PBPs (IC50s) are reported in Table 1 for those species and other Gram-negative and -positive bacteria. Avibactam binds moderately but very selectively to a few PBPs, notably to E. coli PBP2. Our results and IC50s for PBP2 (Table 1) confirmed the very recently reported E. coli PBP binding data for avibactam and another diazabicyclooctane derivative, namely, OP0595 (20). These results were very similar to those observed for amdinocillin (Table 1), as previously reported by Spratt and Pardee (29). Such selective PBP2 binding was also reported for the β-lactamase inhibitor clavulanic acid (30). The E. coli PBP binding profile we observed and the binding of
the reference compounds amdinocillin and ceftazidime (Table 1) are consistent with published data (31, 32). PBP2 is essential for cell elongation and rod shape maintenance (33), and its inhibition causes rounding of the cells as reported after exposure of *E. coli* cells to amdinocillin (29) and OP0595 (22).

The *H. influenzae* PBP banding profile generated by the fluorescent penicillin Bocillin FL appeared as previously reported for strain Rd (34, 35) and for strain ATCC 49766 (36) (data not shown). As for *E. coli*, the principal *H. influenzae* PBP target of avibactam was PBP2 (Table 1). Concerning *P. aeruginosa*, avibactam preferentially bound to PBP2 but also to PBP3, and even more moderately to PBP4. The relative binding levels of amdinocillin and ceftazidime for *P. aeruginosa* PBP2 and PBP3, respectively, were similar to those previously reported (32) and were higher than those measured for avibactam (Table 1).

The PBP banding profile of *S. aureus* is shown in Fig. 1b. The principal PBP target of avibactam is PBP2 and to some extent PBP3 (Table 1). Using avibactam concentrations of 4 μg/ml and above, PBP4 became strongly labeled by the reporter molecule Bocillin FL (Fig. 1b). Accordingly, the addition of avibactam at a fixed concentration of 4 μg/ml allowed detection of oxacillin binding to PBP4; in other words, the binding of increasing concentrations of oxacillin to PBP4 were revealed by the reduction of PBP4 labeling by Bocillin FL (Fig. 2a). The same phenomenon was also observed with ceftazidime, although its affinity for PBP4 was much lower (Fig. 2b). The apparent IC\(_{50}\) of the combined agents (avibactam and ceftazidime) for each of the PBPs of *S. aureus* were found to be very similar to those determined for ceftazidime used alone (Fig. 2c and Table 1). The exception was PBP4, which was only sufficiently labeled by Bocillin FL in the presence of avibactam.

The strong labeling of *S. aureus* PBP4 by Bocillin FL in the presence of avibactam is intriguing, especially as this phenomenon was concentration dependent (Fig. 1b) and not observed for any other PBP of the other bacterial species tested. This phenomenon cannot only be due to the increased availability of Bocillin FL for PBP4 when a competing β-lactam binds to other PBP targets, since, for example, addition of ceftazidime (0.03 to 1,024 μg/ml) still did not increase labeling of PBP4 by Bocillin FL (Fig. 2c). Published crystal structures of PBP4–β-lactam complexes provide evidence of the β-lactamase activity of *S. aureus* PBP4 (25). Accordingly, PBP4 often has been difficult to detect by use of various β-lactam–based reporter molecules (34, 37). The observed binding of Bocillin FL to *S. aureus* PBP4 in the presence of avibactam may thus tentatively be explained by an inhibition of the PBP4 β-lactamase activity by avibactam. Some β-lactamases have been shown to release intact avibactam after being acylated (5), and hence avibactam can be qualified as a slowly reversible non-β-lactam inhibitor. In the PBP assay, a combined effect of avibactam binding to *S. aureus* PBP2 and PBP3 as well as inhibition of PBP4 β-lactamase activity may increase available amounts of Bocillin FL for PBP4 labeling. The putative inhibition of PBP4 β-lactamase activity by avibactam and the possibility of an alternate binding site for avibactam on PBP4, however, remain to be demonstrated biochemically.

*S. aureus* PBP4 activity determines the level of peptidoglycan cross-linking and together with PBP2 participates in methicillin-resistant *S. aureus* (MRSA) PBP2a function (38). Depending on the genetic background of the studied strains, PBP4 is either considered nonessential (39) or an important resistance determinant of community-acquired MRSA (CA-MRSA) (40). To that effect, the anti-PBP4 β-lactam cefoxitin has been shown to enhance oxacillin activity against CA-MRSA but not against hospital-acquired MRSA (40). It would be interesting to see if avibactam could help in tackling specific MRSA strains by helping binding of β-lactams to PBP4.

The structure of *S. aureus* PBP4 has many similarities to that of *E. coli* PBP5 (25). However, the Gram-positive functional homolog of *E. coli* PBP2 is more difficult to identify, especially in cocci that lack cylindrical elongation (41). While the class B-type PBP2 seems to be the preferred target of avibactam in *E. coli*, the class C PBP3 is the predominant target of avibactam in *S. pneumoniae* (Table 1). The *S. pneumoniae* PBP banding profile generated with Bocillin FL appeared as previously reported for strain R6

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**TABLE 1** Binding of test molecules to PBPs of Gram-negative and Gram-positive bacteria

| Species          | Compound | MIC (μg/ml) | Relative binding (IC\(_{50}\) [μg/ml]) for PBP² |
|------------------|----------|------------|-----------------------------------------------|
|                  |          | 1 | 1a | 1b | 1a/b | 2 | 2X | 2a/b | 3 | 3a/b | 4 | 5 | 5/6 |
| **E. coli**      | AVI      | 8 | >13 | >13 | 0.92 | >13 | >13 | >13 | >13 | >13 | >13 | >13 | >13 |
|                  | MEC      | 0.06 | >13 | >13 | 0.02 | >13 | >13 | >13 | >13 | >13 | >13 | >13 | >13 |
|                  | CAZ      | 0.5 | 15  | 8.0 | >25  | 0.20 | >25 | >25 | >25 | >25 | >25 | >25 | >25 |
| **P. aeruginosa**| AVI      | >128 | >13 | >13 | 1.1  | 1.8  | 11  | >13 | >13 | >13 | >13 | >13 | >13 |
|                  | MEC      | — | >13 | >13 | 0.21 | >13 | >13 | >13 | >13 | >13 | >13 | >13 | >13 |
|                  | CAZ      | 4 | 0.19 | 3.9 | >25  | 0.04 | 1.6 | >25 | >25 | >25 | >25 | >25 | >25 |
| **H. influenzae**| AVI      | 64 | >32 | >32 | 3.0  | >32 | >32 | >32 | >32 | >32 | >32 | >32 | >32 |
| **S. pneumoniae**| AVI      | 256 | >256 | >256 | 51.0 | 156 | >256 | >256 | >256 | >256 | >256 | >256 | >256 |
|                  | CAZ      | 16 | 1.6  | >8  | >8  | >8  | >8  | >8  | >8  | >8  | >8  | >8  | >8  |
|                  | CAZ + AVI | 16  | 1.7  | 0.9  | 9.2  | 96  | 96  | 96  | 96  | 96  | 96  | 96  | 96  |

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a AVI, avibactam; MEC, amdinocillin; CAZ, ceftazidime.

b A > sign preceding a value indicates that the IC\(_{50}\) was greater than the highest dose tested.

—, the MIC was not measured.

d The avibactam concentration was fixed at 4 μg/ml, while ceftazidime concentrations were varied in 2-fold increments.

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by Paik et al. (28) (data not shown). The class C type 5 PBP group includes not only *S. pneumoniae* PBP3 but also *E. coli* PBP5 and *S. aureus* PBP4 (33).

In conclusion, the non-β-lactam β-lactamase inhibitor avibactam is able to covalently bind to some bacterial PBPs, notably to *E. coli* and *H. influenzae* PBP2, to PBP2 and 3 of *P. aeruginosa* and *S. aureus*, and to PBP3 of *S. pneumoniae*, which may explain its moderate antibacterial activity against some bacterial strains and species. In addition to the ability of avibactam to inhibit several β-lactamase types, identification of those PBP targets may allow the development of new derivatives with improved affinity for PBPs or new combination therapies that act on multiple PBP targets. The possible inhibition of *S. aureus* PBP4 β-lactamase activity by avibactam also warrants further investigation.

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