

# Pharmacodynamic Profiling of a Siderophore-Conjugated Monocarbam in *Pseudomonas aeruginosa*: Assessing the Risk for Resistance and Attenuated Efficacy

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The objective of this study was to investigate the risk of attenuated efficacy due to adaptive resistance for the siderophore-conjugated monocarbam SMC-3176 in *Pseudomonas aeruginosa* by using a pharmacokinetic/pharmacodynamic (PK/PD) approach. MICs were determined in cation-adjusted Mueller-Hinton broth (MHB) and in Chelex-treated, dialyzed MHB (CDMHB). Spontaneous resistance was assessed at 2× to 16× the MIC and the resulting mutants sequenced. Efficacy was evaluated in a neutropenic mouse thigh model at 3.13 to 400 mg/kg of body weight every 3 h for 24 h and analyzed for association with free time above the MIC ( $fT > MIC$ ). To closer emulate the conditions of the *in vivo* model, we developed a novel assay testing activity mouse whole blood (WB). All mutations were found in genes related to iron uptake: *piuA*, *piuC*, *pirR*, *fecl1*, and *pvdS*. Against four *P. aeruginosa* isolates, SMC-3176 displayed predictable efficacy corresponding to the  $fT > MIC$  using the MIC in CDMHB ( $R^2 = 0.968$  to  $0.985$ ), with stasis to 2-log kill achieved at 59.4 to 81.1%. Efficacy did not translate for *P. aeruginosa* isolate JJ 4-36, as the *in vivo* responses were inconsistent with  $fT > MIC$  exposures and implied a threshold concentration that was greater than the MIC. The results of the mouse WB assay indicated that efficacy was not predictable using the MIC for JJ 4-36 and four additional isolates, against which *in vivo* failures of another siderophore-conjugated  $\beta$ -lactam were previously reported. SMC-3176 carries a risk of attenuated efficacy in *P. aeruginosa* due to rapid adaptive resistance preventing entry via the siderophore-mediated iron uptake systems. Substantial *in vivo* testing is warranted for compounds using the siderophore approach to thoroughly screen for this *in vitro-in vivo* disconnect in *P. aeruginosa*.

There has been a renewal of discovery and development efforts of siderophore-conjugated antibiotics intended for the treatment of Gram-negative infections, including those caused by the ever-challenging *Pseudomonas aeruginosa* (1–7). Coupling antibiotics to siderophores is an attractive strategy, as passage across the bacterium's poorly permeable outer membrane is facilitated via the iron uptake pathways (Trojan horse concept) (8, 9). However, a large number of siderophore systems are available for iron transport in *P. aeruginosa*, as well as complex regulatory mechanisms to govern their expression (10). Consequently, *P. aeruginosa* has the ability to adapt its methods for iron acquisition and potentially prevent conjugated agents from utilizing this mode of entry.

Such adaptive behavior was first reported with the siderophore-conjugated monobactam MB-1, for which *in vivo* failures (i.e., bacterial regrowth instead of reduction) were observed to be in discordance with the *in vitro* activity indicated by standard MIC testing (11). The attenuation in efficacy was determined not to be a result of insufficient drug exposure, as measured according to the pharmacokinetic/pharmacodynamic (PK/PD) index of  $\beta$ -lactams. Antimicrobial pharmacodynamics has long been reliably expressed as a function of the MIC, which is linked to the pharmacokinetics to constitute an exposure-response relationship (e.g., area under the concentration-time curve [AUC]-to-MIC ratio, peak concentration [ $C_{max}$ ]-to-MIC ratio, or the percentage of time above the MIC) (12); however, concern has now been raised over the predictability of the *in vitro* MIC for similar siderophore conjugates.

Our primary objective was to assess for differentiation in the

risk of attenuated efficacy in *P. aeruginosa* for a new hydroxypyridinone siderophore-conjugated monocyclic  $\beta$ -lactam, SMC-3176 (a monocarbam) (Fig. 1). We sought to accomplish this by in-depth PK/PD profiling and characterization of resistance mechanisms using a collection of clinically relevant isolates. While a body of *in vivo* work across numerous *P. aeruginosa* isolates was performed for MB-1, PK/PD information at the isolate level was

Received 7 April 2015 Returned for modification 24 June 2015

Accepted 29 September 2015

Accepted manuscript posted online 5 October 2015

Citation Kim A, Kutschke A, Ehmann DE, Patey SA, Crandon JL, Gorseth E, Miller AA, McLaughlin RE, Blinn CM, Chen A, Nayar AS, Dangel B, Tsai AS, Rooney MT, Murphy-Benenato KE, Eakin AE, Nicolau DP. 2015. Pharmacodynamic profiling of a siderophore-conjugated monocarbam in *Pseudomonas aeruginosa*: assessing the risk for resistance and attenuated efficacy. Antimicrob Agents Chemother 59:7743–7752. doi:10.1128/AAC.00831-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00831-15>.

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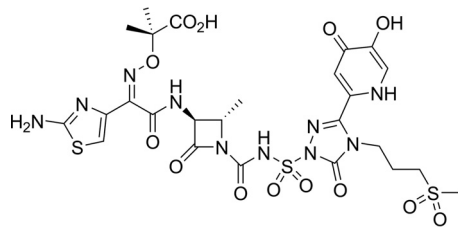


FIG 1 Chemical structure of SMC-3176.

limited to single assessments, given the nature of the study design (11). We opted for a dose-ranging design to obtain estimates spanning the range of drug exposures and *in vivo* responses for each individual isolate. Correspondingly, alternative *in vitro* assays (as opposed to the standard broth microdilution MIC test in standard medium) were also explored in an attempt to bridge both sides of the possible *in vitro-in vivo* disconnect. These involved manipulating test conditions (e.g., using various medium types [broth, agar, whole blood, or supplementing with an iron chelator]) to more closely resemble *in vivo* iron-limited conditions, in the presence of which the siderophore uptake systems are activated.

(This work was presented in part at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, 5 to 9 September 2014, Washington, DC, abstracts A-684 [13] and F-1566 [14]).

## MATERIALS AND METHODS

**Antimicrobial agents.** SMC-3176 and comparator siderophore-conjugated  $\beta$ -lactams (MB-1 and MC-8) were synthesized at AstraZeneca (see Methods S1 in the supplemental material). MC-8 is related to the siderophore-conjugated monocarbam MC-1 and was selected for testing because of its improved hydrolytic stability over MC-1 (3). For non-siderophore-conjugated reference compounds, analytical-grade versions of aztreonam, ceftazidime, meropenem, and colistin sulfate (as the representative non- $\beta$ -lactam agent) were used. All compounds were formulated according to published literature or as supported by internal stability data for *in vitro* and/or *in vivo* use.

**Bacterial isolates.** Clinical isolates of *P. aeruginosa* were obtained from the culture collections at AstraZeneca, Hartford Hospital (Hartford, CT), or the International Health Management Associates, Inc. (Schaumburg, IL) for use in all studies.

All isolates were grown and tested in cation-adjusted Mueller-Hinton broth (MHB), unless otherwise indicated. Select *P. aeruginosa* isolates ( $n = 12$ ) for key *in vitro* and *in vivo* experiments were profiled using the Illumina whole-genome sequencing (WGS) platform for  $\beta$ -lactamases, the *ampC* regulon, and siderophore-uptake machinery, as shown in Table 1 (see Methods S2 in the supplemental material).

**Susceptibility testing.** MICs were determined in MHB (Becton, Dickinson and Company, Franklin Lakes, NJ) by broth microdilution, according to CLSI guidelines (15, 16). Against a subset of 10 isolates available for *in vivo* testing, MICs were additionally determined by broth microdilution in medium treated with an iron chelator by using Chelex-treated, dialyzed MHB (CDMHB) (11). CDMHB was prepared, as previously described, on a large-batch scale (see Methods S3 in the supplemental material). For isolates evaluated in the animal efficacy studies,  $\geq 10$  MIC replicates in MHB and  $\geq 5$  MIC replicates in CDMHB were performed to obtain the modal MIC. Modal MICs were used for reporting and in PK/PD analyses of SMC-3176 efficacy.

**Resistance assessment.** Spontaneous resistance studies for SMC-3176 were conducted against five *P. aeruginosa* isolates (PAO1, ARC3483, ARC3502, ARC3506, and ARC3514), which were chosen based on  $\beta$ -lactamase content. The MICs were first determined in MHB agar (Becton, Dickinson and Company, Franklin Lakes, NJ), as per CLSI guidelines (15, 16). Spontaneous resistance was then assessed by plating  $1 \times 10^9$  CFU in triplicate on compound-containing MHB agar plates at concentrations of up to  $16\times$  the MIC. The plates inoculated with  $100 \mu\text{l}$  of bacterial suspension were incubated at  $37^\circ\text{C}$  for 24 h, after which the colonies were counted. The resistance frequency was calculated as the ratio of average CFU in the presence of compound versus that in the absence of compound. Resistance of the isolated mutants was verified by MIC determinations in MHB after passaging twice on nonselective medium. WGS was performed using the Illumina MiSeq V2 instrument (Illumina, Inc., San Diego, CA) to determine the genetic basis of resistance (see Methods S2 in the supplemental material).

Isogenic mutants constructed in the background of PAO1 were also assessed by MIC determinations in MHB. The following deletion isolates were constructed by Microbiotix, Inc. (Worcester, MA) using a modified version of splicing by overlap extension (SOE) PCR:  $\Delta\text{piuA}$ ,  $\Delta\text{pirA}$ ,  $\Delta\text{piuA} + \Delta\text{pirA}$ , and  $\Delta\text{tonB1}$  as the comparator for disruption of multiple siderophore systems. An additional deletion isolate,  $\Delta\text{piuC}$ , was constructed at AstraZeneca (see Methods S4 in the supplemental material).

**Animal infection model.** Female ICR mice weighing approximately 20 g (Charles River Laboratories, Inc., Wilmington, MA) or 25 g (Harlan Sprague Dawley, Inc., Indianapolis, IN) were prepared for the neutropenic mouse thigh infection model using *P. aeruginosa* isolates (17). The

TABLE 1 Key *P. aeruginosa* isolates studied in resistance selection and animal efficacy experiments<sup>a</sup>

| Isolate   | Acquired $\beta$ -lactamase <sup>b</sup> | Amp regulon <sup>c</sup>    | Iron related <sup>d</sup>   |
|---|--|-----------------------------|-----------------------------|
| ARC545 (PAO1)   | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| ARC3483 (PAO1 $\Delta\text{ampC} \Delta\text{poxB}$ ) | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| ARC3502   | VIM-1                                    | No predicted LOF alteration | No predicted LOF alteration |
| ARC3506   | VEB-1, OXA-10                            | No predicted LOF alteration | No predicted LOF alteration |
| ARC3514   | KPC-2                                    | No predicted LOF alteration | No predicted LOF alteration |
| ARC4659   | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| AZ 8-18   | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| AZ 32-13  | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| JJ 4-36   | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| JJ 5-35   | None                                     | No predicted LOF alteration | No predicted LOF alteration |
| JJ 8-16   | None                                     | E163* in AmpD               | No predicted LOF alteration |
| JJ 11-54  | None                                     | No predicted LOF alteration | No predicted LOF alteration |

<sup>a</sup> See Table S5 in the supplemental material for a detailed description of the genotypes. LOF, loss of function.

<sup>b</sup> All isolates contained chromosomal *ampC* and *poxB* genes.

<sup>c</sup> Amp regulon includes *ampD*, *ampR*, and *dacB*.

<sup>d</sup> Siderophore-related genes were *piuA*, *piuC*, *pirA*, *pirR*, *fecI*, *pvdA*, *pvdS*, and *fpvA*.

animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care, and the studies were executed under protocols approved by the Institutional Animal Care and Use Committees at AstraZeneca and Hartford Hospital. The mice were rendered neutropenic by intraperitoneal injections of cyclophosphamide at 150 mg/kg of body weight on day -4 and 100 mg/kg on day -1 prior to inoculation. Thigh infection was produced by intramuscular injection of the inoculum, prepared from a fresh subculture, to target  $1 \times 10^6$  CFU/thigh. Treatment was initiated at 2 h postinoculation, and all doses were administered subcutaneously.

**In vivo efficacy and PK/PD.** The mice were prepared as described for the neutropenic thigh infection model for all pharmacokinetic and efficacy studies. The pharmacokinetics of SMC-3176 was evaluated following single doses of 6.25, 25, and 100 mg/kg. Blood samples were collected from 6 mice per time point for 8 time points per dose at 0.08, 0.25, 0.5, 1, 1.5, 2, 3, and 4 h postdose. Plasma samples were separated after centrifugation in the presence of EDTA and stored at  $-20^\circ\text{C}$  until bioanalysis. The concentrations of SMC-3176 in mouse plasma were measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS), for which the assay was linear over a range of 0.005 to 10  $\mu\text{g/ml}$  ( $R^2 = 0.995$ ), and quality control accuracy was  $100\% \pm 15\%$ . In brief, plasma samples were extracted in acetonitrile containing 10% trichloroacetic acid and 250 ng/ml carbutamide as an internal standard, centrifuged, and loaded onto a Polaris C18 analytical column (20 by 2.1 mm, 3  $\mu\text{m}$  pore size) (Agilent Technologies, Santa Clara, CA). The high-performance liquid chromatography (HPLC) system consisted of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile), which was increased in a linear fashion from 5% to 95% over 2 min at a flow rate of 0.8 ml/min. The samples were analyzed using the Applied Biosystems API4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) tuned to the most intense mass transition using multiple-reaction monitoring mode. Pharmacokinetic parameters in the neutropenic thigh-infected mouse were determined from single-dose data for each dose level by compartmental analysis (Phoenix WinNonlin 6.3; Certara, St. Louis, MO), with model selection based on the visual inspection of fit, Akaike's information criterion, and correlation coefficient value ( $R^2 \geq 0.920$ ). Plasma exposures following multiple-dose administration in the efficacy studies were simulated by mean parameters and verified to correspond between observed and simulated concentrations. To reflect free (i.e., unbound, microbiologically active) drug, simulated plasma concentration-time data were corrected for SMC-3176 protein binding in mouse plasma (55.6%).

SMC-3176 was examined for *in vivo* efficacy using the neutropenic mouse thigh infection model against five *P. aeruginosa* isolates (PAO1, ARC3502, ARC3506, ARC4659, and JJ 4-36) chosen with consideration as to  $\beta$ -lactamase content. Multiple doses ranging from 25 to 3,200 mg/kg/day were divided and administered every 3 h (q3h) for 3 mice per dose group. Growth control animals received vehicle solution in the same manner as SMC-3176-treated groups. Mice were euthanized by  $\text{CO}_2$  exposure, followed by cervical dislocation just prior to antibiotic initiation (pretreatment) and 24 h after treatment initiation (vehicle and treatment groups). For certain isolates, the vehicle control animals were sacrificed at an earlier time point prior to expiry due to the aggressively virulent features of the pseudomonal infection. Infected thigh muscles were aseptically removed, individually homogenized, and plated in serial dilutions on Trypticase soy agar for bacterial counts. Efficacy was assessed by the bacterial density in thigh tissue and was defined as the change in  $\log_{10}$  CFU per thigh or per gram of thigh for antibiotic-treated groups versus the pretreatment group. The relationship between efficacy and the PK/PD index of interest for SMC-3176, the free time above the MIC ( $fT > \text{MIC}$ ), was analyzed by the inhibitory sigmoid maximum effect ( $E_{\text{max}}$ ) model (Phoenix WinNonlin 6.3; Certara, St. Louis, MO), and magnitudes of the exposure index associated with 80% of the maximal effect ( $\text{EI}_{80}$ ), net bacteriostasis, and 1- and 2-log reductions were determined. Thigh tissue samples from the vehicle control group and select treatment groups of

SMC-3176 were additionally cultured on  $8\times$  the MIC MHB agar plates to assess for potentially resistant colonies.

**CDMHB agarose growth assay.** In a follow-up to the SMC-3176 efficacy results, an *in vitro* CDMHB agarose growth assay was employed, as previously described (11). Bacterial isolates were first grown in Luria broth (LB) (Sigma-Aldrich, St. Louis, MO) to mid-log phase and then plated at  $5 \times 10^6$  CFU on 100-mm diameter CDMHB agarose plates (poured using CDMHB and 1.5% UltraPure agarose [Life Technologies, Grand Island, NY]) containing SMC-3176 at 0 to 32  $\mu\text{g/ml}$  in doubling dilutions for a target bacterial density of  $9.1 \times 10^4$  CFU/ $\text{cm}^2$  per plate. CDMHB agarose plates inoculated with 100  $\mu\text{l}$  of bacterial suspension were incubated at  $37^\circ\text{C}$  for 40 h and then inspected visually under UV light to determine the highest SMC-3176 concentration that supported bacterial growth (as opposed to the lowest concentration that inhibited growth). A minimum of two independent tests were performed for each isolate to assess the reproducibility of the assay.

**Pyoverdine quantification.** *P. aeruginosa* isolates evaluated in the animal efficacy model were also profiled for pyoverdine expression. Bacterial suspensions were prepared in LB by overnight incubation at  $37^\circ\text{C}$  and diluted in 25 ml of MHB or CDMHB to target absorbance at 600 nm ( $A_{600}$ ) of 0.1. *P. aeruginosa* cultures in MHB or CDMHB were then grown at  $37^\circ\text{C}$  with shaking for 3 h (over log-phase growth), during which 1-ml samples were collected for bacterial growth by  $A_{600}$  and for pyoverdine quantification. Following centrifugation of the samples, the pyoverdine content of the separated supernatant was determined by measuring absorbance at the characteristic color wavelength of pyoverdine, 405 nm (18). Pyoverdine concentrations were calculated based on a linear standard curve generated with pyoverdine purchased from Sigma-Aldrich (St. Louis, MO) and normalized to bacterial cell density, measured as milligrams per milliliter per  $A_{600}$ .

**Whole-blood MIC assay.** An alternative growth inhibition assay was developed using mouse whole blood (WB) to more closely emulate the animal efficacy model. Ninety-six-well plates containing 2  $\mu\text{l}$  of  $1 \times 10^6$  CFU/ml inoculum, 2  $\mu\text{l}$  of serially diluted drug, and 26  $\mu\text{l}$  of either freshly harvested, heparinized, and pooled mouse WB or LB medium (as control) per well were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  with shaking for 6 h. Next, 170  $\mu\text{l}$  of distilled, deionized water was added to each well and incubated for another 5 min at room temperature to allow for red blood cell lysis. Lastly, 3  $\mu\text{l}$  from each well was spotted onto LB agar in 86 by 128-mm microplate trays and incubated overnight at  $37^\circ\text{C}$ . The WB MIC was the lowest drug concentration at which no bacterial growth was observed following the final overnight incubation. A minimum of three independent tests were performed for each isolate to assess the reproducibility of the assay.

## RESULTS

**In vitro activity by MIC.** Against 100 contemporary, clinical isolates of *P. aeruginosa*, SMC-3176 displayed  $\text{MIC}_{50/90\text{s}}$  of 0.12/1 mg/liter by standard broth microdilution in MHB. Conversely, the  $\text{MIC}_{50/90\text{s}}$  for aztreonam, ceftazidime, and meropenem were 16/ $>64$ , 32/ $>64$ , and 8/ $>64$  mg/liter, respectively. SMC-3176 was unaffected by a variety of  $\beta$ -lactamases in a characterized panel of 20 clinical *P. aeruginosa* isolates, as potent activity was maintained against isolates containing extended-spectrum  $\beta$ -lactamases, carbapenemases, and metallo- $\beta$ -lactamases (Table 2).

Against a subset of *P. aeruginosa* isolates fit for *in vivo* testing ( $n = 10$ ), SMC-3176 MICs were within one dilution between determinations in standard MHB (0.12 to 0.5 mg/liter) and that in the iron-chelated medium, CDMHB (0.06 to 0.5 mg/liter), except for one isolate, ARC4659. A significant improvement in susceptibility was exhibited for SMC-3176 with this clinical isolate, from an elevated MIC of 8 mg/liter in MHB to a lower MIC of 0.5 mg/liter in CDMHB. Similar reductions in MIC were observed for the siderophore-conjugated comparators MC-8 and MB-1 (MIC in MHB, 8 to 16 mg/liter; MIC in CDMHB, 0.06 to 0.5 mg/liter).

TABLE 2 *In vitro* activity of SMC-3176 against clinical isolates of *P. aeruginosa* ( $n = 20$ ) containing various  $\beta$ -lactamases

| Isolate    | Acquired $\beta$ -lactamase <sup>a</sup> | MIC in MHB (mg/liter) |           |             |           |
|------------|--|-----------------------|-----------|-------------|-----------|
|            |  | SMC-3176              | Aztreonam | Ceftazidime | Meropenem |
| ATCC 27853 | None                                     | 0.5                   | 4         | 2           | 1         |
| ARC3502    | VIM-1                                    | 0.12                  | 64        | >64         | >64       |
| ARC3504    | SHV-2                                    | 0.06                  | 64        | 32          | 4         |
| ARC3505    | PER-1, OXA-10                            | 0.5                   | >64       | >64         | 4         |
| ARC3506    | VEB-1, OXA-10                            | 0.12                  | >64       | >64         | 8         |
| ARC3509    | None                                     | 0.06                  | >64       | >64         | 16        |
| ARC3511    | KPC-2                                    | 0.06                  | >64       | 64          | 32        |
| ARC3514    | KPC-2                                    | 0.06                  | >64       | >64         | >64       |
| ARC3606    | TEM-24                                   | 0.06                  | 16        | 64          | 4         |
| ARC3607    | OXA-2                                    | 4                     | 64        | >64         | 8         |
| ARC3611    | None                                     | 0.5                   | 32        | 32          | 4         |
| ARC3612    | None                                     | 0.06                  | 16        | 64          | 16        |
| ARC3727    | VIM-2, OXA-4                             | 0.25                  | 16        | 32          | >64       |
| ARC3729    | VEB-1, OXA-10                            | 0.25                  | >64       | >64         | 16        |
| ARC3756    | IMP-18, VIM-2, OXA-2                     | 0.25                  | 16        | >64         | >64       |
| ARC3916    | GES-1, OXA-2                             | 0.12                  | 16        | >64         | 16        |
| ARC3925    | VIM-2                                    | 0.12                  | 32        | 32          | >64       |
| ARC4060    | PER-1, OXA-10                            | 0.06                  | >64       | >64         | 16        |
| ARC4063    | VEB-1                                    | 0.5                   | >64       | >64         | 32        |
| ARC4068    | IMP-15, OXA-2                            | 0.016                 | 32        | >64         | >64       |

<sup>a</sup> All isolates contained chromosomal *ampC* and *poxB* genes.

Accordingly, an *in vivo* efficacy experiment with ARC4659 was arranged to investigate whether efficacy could be achieved despite the nonsusceptible SMC-3176 MIC by standard MHB methods. Of note, we were unable to identify features in the siderophore-related genes of ARC4659 that could account for the large MIC difference across medium types (see Table S5 in the supplemental material).

**Mechanisms of resistance.** The spontaneous resistance frequencies for SMC-3176 were  $5.5 \times 10^{-8}$ ,  $9.4 \times 10^{-8}$ ,  $9.9 \times 10^{-8}$ ,

$5.1 \times 10^{-7}$ , and  $2.0 \times 10^{-7}$  at  $4\times$  the MIC in PAO1, ARC3483, ARC3502, ARC3506, and ARC3514, respectively. At  $8\times$  the MIC, the frequencies were  $8.4 \times 10^{-9}$ ,  $7.9 \times 10^{-9}$ ,  $1.1 \times 10^{-8}$ ,  $1.5 \times 10^{-7}$ , and  $3.0 \times 10^{-8}$ , respectively. The SMC-3176 MICs for stably resistant isolates were 16- to 128-fold higher than those of the parental isolates, as presented in Table 3. All SMC-3176 mutants displayed cross-resistance to siderophore-conjugated comparators, while the MICs were unchanged for siderophore-free reference  $\beta$ -lactams (Table 3).

TABLE 3 Characterization and susceptibilities of mutants generated from *in vitro* spontaneous resistance studies for SMC-3176 in *P. aeruginosa*

| Parent/mutant<br>(selection concn [ $\times$ MIC]) | Mutation <sup>a</sup>                         | MIC in MHB (mg/liter) |      |      |           |             |           |
|--|---|-----------------------|------|------|-----------|-------------|-----------|
|  |   | SMC-3176              | MB-1 | MC-8 | Aztreonam | Ceftazidime | Meropenem |
| PAO1   |   | 0.25                  | 0.5  | 0.5  | 4         | 2           | 1         |
| A1-PAO1 (4)  | $\Delta$ [PiuC(PA4515)...PA4521] <sup>b</sup> | 4                     | 4    | 16   | 4         | 1           | 2         |
| A2-PAO1 (8)  | <i>pfecI</i> (-44 T→C) <sup>c</sup>           | 32                    | 8    | 16   | 8         | 2           | 2         |
| ARC3483  |   | 0.25                  | 0.5  | 0.5  | 2         | 1           | 0.5       |
| B1-ARC3483 (2)                                     | PiuC [Q177fs]                                 | 8                     | 16   | 16   | 2         | 0.5         | 0.5       |
| ARC3502  |   | 0.25                  | 0.25 | 0.25 | 64        | >64         | >64       |
| C1-ARC3502 (4)                                     | PiuA [Q186fs]                                 | 8                     | 4    | 4    | 32        | >64         | >64       |
| C2-ARC3502 (4)                                     | <i>pfecI</i> (-41 C→A) <sup>c</sup>           | 4                     | 4    | 8    | 32        | >64         | >64       |
| C3-ARC3502 (8)                                     | PiuA [Q604fs]                                 | 8                     | 8    | 4    | >64       | >64         | >64       |
| ARC3506  |   | 0.25                  | 1    | 0.25 | >64       | >64         | 4         |
| D2-ARC3506 (8)                                     | <i>pfecI</i> (-45 C→T) <sup>c</sup>           | 32                    | 32   | 32   | >64       | >64         | 16        |
| ARC3514  |   | 0.13                  | 0.13 | 0.13 | >64       | >64         | >64       |
| E1-ARC3514 (4)                                     | PiuC [W175*]                                  | 1                     | 8    | 2    | >64       | >64         | >64       |
| E2-ARC3514 (8)                                     | <i>ppvdS</i> (-88 T→A) <sup>c</sup>           | 2                     | >64  | 8    | >64       | >64         | >64       |
| E3-ARC3514 (16)                                    | PirR [G132fs]                                 | 1                     | 2    | 1    | >64       | >64         | >64       |

<sup>a</sup> Mutations noted by amino acid changes in coding regions or base pair changes in gene promoter. fs, frameshift; p, promoter.

<sup>b</sup> Indicates a 8,725-bp deletion (5056096..5064820 relative to PAO1) encompassing PiuC (PA4515) through PA4521 and the terminal 14 amino acids of AmpD [W174fs].

<sup>c</sup> Point mutation in promoter at predicted Fur binding site.



TABLE 4 Susceptibility of isogenic *P. aeruginosa* isolates containing knockout mutations in genes related to siderophore receptors

| Mutation                                    | MIC in MHB (mg/liter) |      |      |           |             |           |
|---|-----------------------|------|------|-----------|-------------|-----------|
|   | SMC-3176              | MB-1 | MC-8 | Aztreonam | Ceftazidime | Meropenem |
| (PAO1, parent)                              | 0.25                  | 0.5  | 0.5  | 4         | 2           | 1         |
| $\Delta$ <i>piuA</i>                        | 4                     | 16   | 8    | 16        | 4           | 2         |
| $\Delta$ <i>piuC</i>                        | 16                    | 32   | 8    | 16        | 4           | 1         |
| $\Delta$ <i>pirA</i>                        | 0.5                   | 0.5  | 1    | 16        | 4           | 2         |
| $\Delta$ <i>piuA</i> + $\Delta$ <i>pirA</i> | 32                    | 64   | >64  | 8         | 4           | 2         |
| $\Delta$ <i>tonB1</i> <sup>a</sup>          | >64                   | >64  | >64  | 8         | 2           | 2         |

<sup>a</sup> MIC determined in medium supplemented with 160  $\mu$ M Fe(SO<sub>4</sub>).

Ten resistant mutants were profiled by WGS, and all contained changes in genes associated with iron uptake (Table 3). For five mutants, a variety of genetic changes were seen in the *piuA* and *piuC* genes, including insertions, deletions, and frameshifts all likely to result in a loss of function of the encoded proteins. PiuA is an outer membrane siderophore receptor with similarity to the Fiu catechol receptor, while PiuC is a predicted Fe(II)-dependent oxygenase with its gene located directly adjacent to PiuA (19, 20). The predicted effect of a *piuA* or *piuC* mutation is decreased expression of the PiuA receptor, which is one of two receptors that hydroxypyridinone-based siderophore conjugates are believed to exploit for entry, with the other being PirA (20, 21). A frameshift mutation was also found in the gene encoding PirR, which is a new resistance mechanism to be reported for a siderophore-conjugated  $\beta$ -lactam. PirR is the response regulator of a two-component regulatory system predicted to activate expression of the PirA receptor, and a mutation in PirR is expected to result in decreased expression of PirA (10).

Three isolates contained mutations in the predicted Fur-binding region of the *fecI* promoter (*pfecI*) at positions of -41, -44, and -45 nucleotides before the start of the gene. The *fecI* gene is an extracytoplasmic function (ECF) sigma-70 factor that acts as a transcriptional activator of the ferric citrate siderophore system (22). Mutations at this *pfecI* location have been reported for BAL30072-resistant isolates, and it was shown that by derepressing the *fecIRA* operon, a mutation in the Fur-binding promoter of *fecI* led to increased expression of the ferric citrate siderophore receptor FecA (20). Transcriptional analysis of our C2-ARC3502 *pfecI* mutant yielded the same result of elevated *fecA* expression relative to the parent (data not shown).

In addition to *pfecI*, another instance of a promoter mutation at a Fur repressor site was found upstream of *ppvS*, which is an ECF sigma-70 factor that controls the expression of synthesis genes for pyoverdine (23). The predicted effect of a *ppvS* mutation is the derepression of pyoverdine synthesis, which would lead to enhanced expression of the cognate siderophore receptor for pyoverdine, FpvA. Both promoter mutations (*pfecI* and *ppvS*) are expected to result in increased expression of siderophore receptors (FecA and FpvA), which would outcompete the availability of PiuA and PirA and thus decrease SMC-3176 entry (20).

Isogenic isolates with knockout mutations in implicated siderophore receptor genes were used to confirm that mutations from selection studies were indeed the cause of SMC-3176 resistance (Table 4). Single *piuA* and *piuC* deletions conferred large decreases in susceptibility for SMC-3176 and other siderophore-conjugated  $\beta$ -lactams. The *pirA* deletion conferred no susceptibility change on its own; however, in conjunction with the *piuA*

deletion, it resulted in a loss of activity that was greater than that of  $\Delta$ *piuA* alone (11).

**In vivo efficacy and PK/PD.** A one-compartment model with first-order input and elimination was used to characterize the pharmacokinetics of SMC-3176 in the neutropenic thigh-infected mouse, with the following final parameters: volume of distribution (V), 899 ml/kg; clearance (CL), 2006 ml/h/kg; and elimination half-life ( $t_{1/2}$ ), 0.30 h. SMC-3176 displayed linear pharmacokinetics over the range of doses studied at 6.25 mg/kg ( $C_{max}$ , 10.5  $\mu$ g/ml; time to  $C_{max}$  [ $T_{max}$ ], 0.13 h; AUC, 5.19  $\mu$ g  $\cdot$  h/ml), 25 mg/kg ( $C_{max}$ , 29.3  $\mu$ g/ml;  $T_{max}$ , 0.10 h; AUC, 14.6  $\mu$ g  $\cdot$  h/ml), and 100 mg/kg ( $C_{max}$ , 62.6  $\mu$ g/ml;  $T_{max}$ , 0.12 h; AUC, 39.8  $\mu$ g  $\cdot$  h/ml) (see Fig. S6 to S8 in the supplemental material, respectively). The mean  $\pm$  standard deviation (SD) starting bacterial density in the model was  $5.9 \pm 0.3$ ,  $6.6 \pm 0.2$ ,  $6.4 \pm 0.3$ ,  $5.5 \pm 0.4$ , and  $5.8 \pm 0.1$  log<sub>10</sub> CFU per thigh or gram of thigh for PAO1, ARC3502, ARC3506, ARC4659, and JJ 4-36, respectively. Bacterial growth was approximately 2 to 3 log, respectively, progressing to  $7.6 \pm 0.3$ ,  $8.5 \pm 0.3$ ,  $8.1 \pm 0.7$ ,  $8.2 \pm 0.3$ , and  $9.5 \pm 0.2$  log<sub>10</sub> CFU per thigh or gram of thigh in vehicle controls by 24 h.

Efficacy was demonstrated in a dose-dependent fashion against PAO1 and clinical isolates ARC3502 and ARC3506, with a maxi-

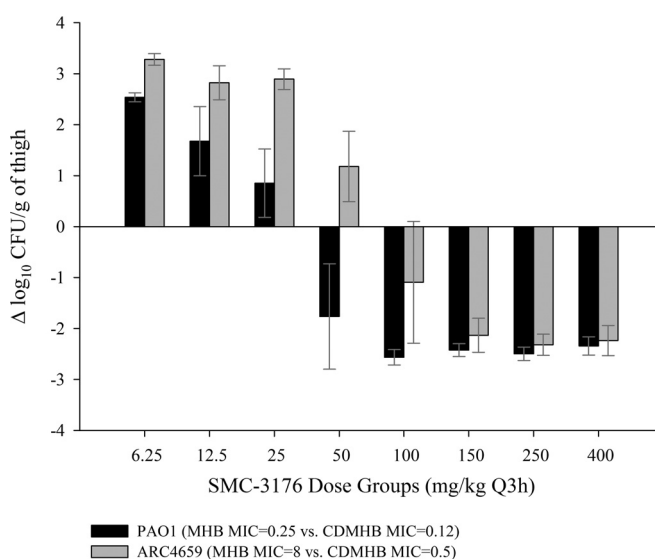
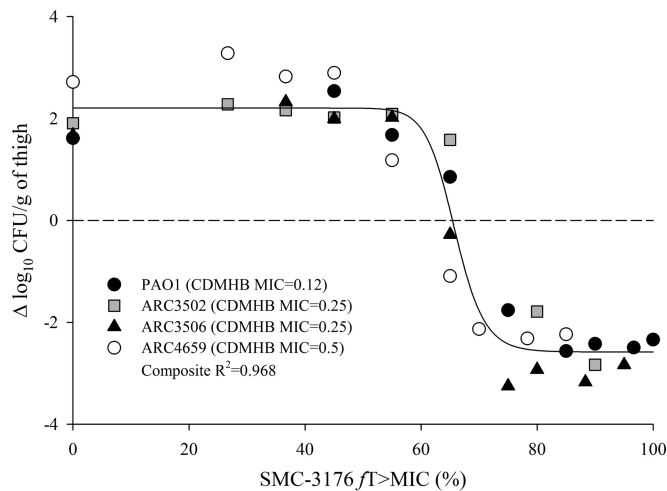


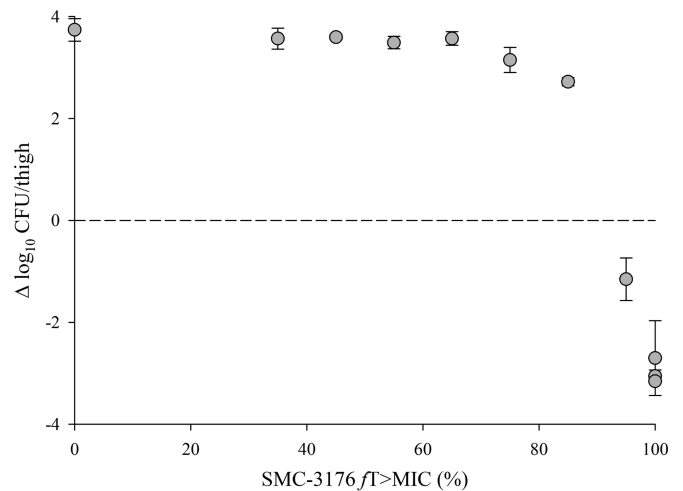
FIG 2 Efficacy defined by the change in bacterial burden for SMC-3176 against representative *P. aeruginosa* isolates PAO1 and ARC4659 in the neutropenic mouse thigh model. Each bar represents the mean  $\pm$  SD from six data points.



**FIG 3** Composite relationship between  $fT > MIC$  and *in vivo* efficacy for SMC-3176 against four *P. aeruginosa* isolates in the neutropenic mouse thigh model. Each symbol represents mean value from six data points.

mal reduction in bacterial load of approximately 3 log relative to that at the start of treatment. SMC-3176 was also efficacious against ARC4659 despite the elevated, nonsusceptible MIC in MHB of 8 mg/liter. Rather, efficacy was more appropriately described by the MIC in CDMHB of 0.5 mg/liter, as *in vivo* responses against ARC4659 paralleled those against PAO1, for which the MICs in MHB and CDMHB were 0.25 and 0.12 mg/liter, respectively (Fig. 2). Using the MIC in CDMHB, *in vivo* efficacy for SMC-3176 was predictable against the four isolates in association with the  $fT > MIC$  ( $R^2 = 0.968$  to  $0.985$ ) (Fig. 3). SMC-3176 exposures ranged from 59.4 to 81.1%  $fT > MIC$  for stasis to 2-log kill effects, consistent with those reported for historical  $\beta$ -lactams, such as aztreonam or cephalosporins (Table 5) (12, 24).

Against isolate JJ 4-36, however, *in vivo* responses for SMC-3176 were not predictable by MIC, be it in MHB (0.12 mg/liter) or CDMHB (0.06 mg/liter). While a maximal effect of 3-log kill was observed, it was achieved only at 100%  $fT > MIC$ , with consistent bacterial regrowth occurring over the 60 to 80%  $fT > MIC$  range (Fig. 4). This lack of predictable efficacy by the MIC was similarly reported for MB-1 against the same JJ 4-36 and four other *P. aeruginosa* isolates (AZ 32-13, JJ 5-35, JJ 8-16, and JJ 11-54) using a single humanized dosing regimen in a neutropenic mouse thigh model (11). With *P. aeruginosa* JJ 4-36, there appeared to be a threshold concentration greater than the MIC in MHB or CDMHB at which bacterial reduction was still achievable, if dosed high enough. From the multiple pharmacodynamic point estimates obtained, it was possible to back-extrapolate the *in vivo*



**FIG 4** Lack of correlation between  $fT > MIC$  and *in vivo* response for SMC-3176 against *P. aeruginosa* JJ 4-36 (CDMHB MIC, 0.06 mg/liter) in the neutropenic mouse thigh model. Each symbol represents mean value  $\pm$  SD from six data points.

threshold based on the PK/PD behavior of established  $\beta$ -lactams; this was estimated to be 0.5 mg/liter for SMC-3176. Likewise, when reexamined using our dose-ranging approach, *in vivo* responses for MB-1 failed to correlate by MIC (in MHB, 1 mg/liter; in CDMHB, 0.25 mg/liter) against JJ 4-36 and implied a higher threshold of 4 or 8 mg/liter (see Fig. S9 in the supplemental material).

**Exploratory *in vitro* assays.** The CDMHB agarose growth assay was previously developed and reported to be highly correlated with *in vivo* outcomes for MB-1 (11). It should be emphasized that this assay is different and separate from the MIC determinations in CDMHB based on the experimental methods (e.g., agarose plates versus broth microtiter trays) and the final endpoint (i.e., the highest concentration at which bacterial growth was sustained versus the lowest concentration that inhibited growth). For SMC-3176, however, the assay was neither reproducible nor reliable for predicting the *in vivo* responses against the five *P. aeruginosa* isolates studied. The results for the maximum concentration with growth on CDMHB plates were varied upon repeat testing in PAO1 (0.12 and 1 mg/liter), ARC3502 (0.5 and 4 mg/liter), and ARC3506 (1 and 8 mg/liter). Values were more reproducible with ARC4659 (2 and 2 mg/liter) and JJ 4-36 (0.5, 0.5, and 1 mg/liter), and yet, there was no consistent trend associated with *in vivo* responses that held true across isolates. Although validated for MB-1 by the authors of that study (11) and by our own laboratory, the utility of the assay appeared to be compound specific and did not extend to SMC-3176.

**TABLE 5**  $fT > MIC$  associated with various efficacy endpoints for SMC-3176 against *P. aeruginosa* in the neutropenic mouse thigh model

| Isolate | MIC (mg/liter) in: |       | % $fT > MIC$ (% CV) by CDMHB MIC at <sup>a</sup> : |                |                |                  |
|---------|--------------------|-------|--|----------------|----------------|------------------|
|         | MHB                | CDMHB | Stasis   | 1-log kill     | 2-log kill     | EI <sub>80</sub> |
| PAO1    | 0.25               | 0.12  | 68.0 (1.1)   | 71.3 (1.3)     | 76.6 (2.0)     | 76.5 (2.3)       |
| ARC3502 | 0.12               | 0.25  | 72.9 (1.2)   | 76.5 (1.1)     | 81.1 (1.2)     | 84.1 (2.3)       |
| ARC3506 | 0.12               | 0.25  | 63.4 (1.2)   | 66.9 (1.2)     | 71.3 (1.9)     | 74.3 (3.1)       |
| ARC4659 | 8                  | 0.5   | 59.4 (1.2)   | 63.5 (1.5)     | 71.4 (2.5)     | 70.3 (3.3)       |
| JJ 4-36 | 0.12               | 0.06  | Indeterminable                                     | Indeterminable | Indeterminable | Indeterminable   |

<sup>a</sup> Indeterminable indicates that exposures for efficacy could not be appropriately determined against isolate JJ 4-36, due to the lack of a well-corresponding PK/PD relationship.

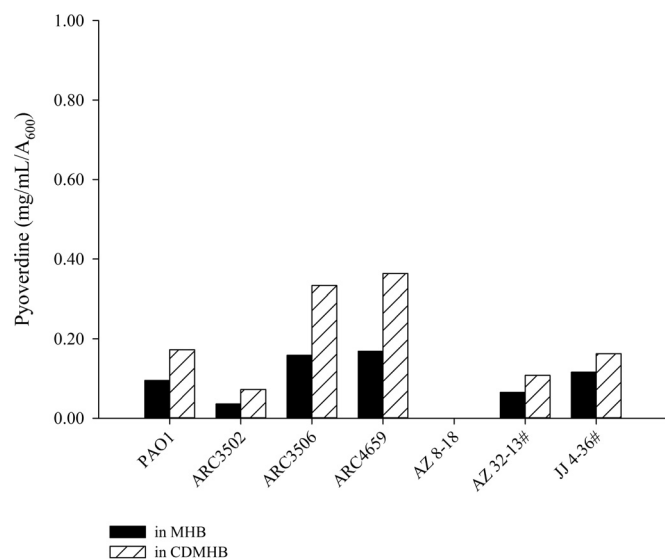


FIG 5 Pyoverdine production from *P. aeruginosa* isolates in MHB and CDMHB liquid cultures during log-phase growth at 3 h. #, isolates against which *in vivo* failures were previously reported for siderophore-conjugated monobactam MB-1 (11).

In the course of conducting the CDMHB growth assay, increased pyoverdine expression was detected, as evidenced by the fluorescent green haze surrounding colonies recovered on CDMHB agarose plates. Inability of the siderophore conjugate to outcompete pyoverdine, the major native siderophore of *P. aeruginosa* with high iron affinity, has been implicated as a possible cause of attenuated efficacy (11). Thus, an attempt was made to effectively quantify pyoverdine from isolates evaluated for efficacy in search of an *in vivo* correlation. When measured upon growth in liquid cultures, pyoverdine production was modestly higher in CDMHB than in MHB (Fig. 5). The amount of CDMHB-induced pyoverdine production, however, did not vary widely among isolates, despite contrasting outcomes in the animal efficacy model. Notably, *P. aeruginosa* JJ 4-36 (in CDMHB, 0.162 mg/ml/A<sub>600</sub>) did not produce more pyoverdine than the reference isogenic isolate, PAO1 (in CDMHB, 0.173 mg/ml/A<sub>600</sub>).

Having been unsuccessful in uncovering an informative *in vitro* tool for SMC-3176, the mouse WB MIC assay was retrospec-

tively developed in a final attempt to identify isolates prone to adaptive resistance, like JJ 4-36. First, the results of the WB MIC assay were highly reproducible for SMC-3176; values were within 1 to 2 doubling dilutions upon repeat testing in all 10 isolates studied (Table 6). The MICs determined in LB under CO<sub>2</sub> served as points of reference and were well matched to standard MICs in MHB. Second, the assay results were consistently in alignment with the *in vivo* responses for SMC-3176 across isolates. When interpreted based on the shift in MIC (and not the numerical value itself) from LB to mouse WB under CO<sub>2</sub>, significant increases of  $\geq 16$ -fold were observed with JJ 4-36 and four other *P. aeruginosa* isolates against which *in vivo* failures were reported for MB-1. Minor to moderate shifts in MICs were seen in isolates with predictable *in vivo* efficacy, most notably including ARC4659, which demonstrated a decreasing trend in MIC, akin to the MIC reduction observed between the MHB and CDMHB media. This behavior of a  $\geq 16$ -fold MIC increase in mouse WB was unique to siderophore-conjugated compounds, including MB-1, as the MICs were overall comparable to those generated by standard MHB methods for aztreonam and colistin sulfate (Table 7). The MICs for meropenem were not as comparable, but no significant shifts in the upward direction were observed with any isolate.

## DISCUSSION

In the face of the intrinsic impermeability and the many existing resistance mechanisms behind the multidrug-resistant nature of *P. aeruginosa* (from an array of  $\beta$ -lactamases to efflux pump systems and loss of outer membrane porin proteins) (25, 26), siderophore-antibiotic conjugates appear to be a promising endeavor during a dearth of truly novel agents. Screening cascades in early antibacterial discovery are typically launched with *in vitro* susceptibility testing of candidate compounds, and while standard MIC tests provided exquisitely potent values for SMC-3176, a report of a competitor siderophore conjugate with unanticipated loss of *in vivo* efficacy (11) served to caution investigators. Here, we determined the resistance mechanisms and PK/PD profile of the siderophore-conjugated monocarbam SMC-3176 in *P. aeruginosa* and further explored *in vitro* tools (broth microdilution MIC in MHB versus in CDMHB, CDMHB agarose growth assay, quantification of pyoverdine, and mouse WB MIC assay) to address the disconnect between *in vitro* measures of antibacterial activity and *in vivo* efficacy.

TABLE 6 Correlation between results of the mouse whole-blood assay and *in vivo* response for SMC-3176 against *P. aeruginosa*

| Isolate               | MIC (mg/liter) in: |       |                        | MIC shift in mouse WB assay <sup>a</sup> | <i>In vivo</i> response  |                               |
|-----------------------|--------------------|-------|------------------------|--|--------------------------|-------------------------------|
|                       | MHB                | CDMHB | LB CO <sub>2</sub>     |  |                          |                               |
| PAO1                  | 0.25               | 0.12  | 0.25, 0.25, 0.25, 0.25 | 0.25, 0.25, 0.5, 0.5                     | 2-fold increase          | Efficacy predicted by MIC     |
| ARC3502               | 0.12               | 0.25  | 0.25, 0.25, 0.25, 0.5  | 1, 2, 2, 4                               | 8-fold increase          | Efficacy predicted by MIC     |
| ARC3506               | 0.12               | 0.25  | 0.25, 0.25, 0.5        | 2, 2, 2                                  | 8-fold increase          | Efficacy predicted by MIC     |
| ARC4659               | 8                  | 0.5   | 8, 8, 8, 16            | 2, 2, 2, 2                               | 4-fold decrease          | Efficacy predicted by MIC     |
| AZ 8-18               | 0.5                | 0.25  | 0.12, 0.25, 0.25       | 0.5, 0.5, 0.5                            | 2-fold increase          | Not tested                    |
| AZ 32-13 <sup>b</sup> | 0.5                | 0.5   | 0.5, 0.5, 0.5          | 8, 16, 16                                | $\geq 16$ -fold increase | Not tested                    |
| JJ 4-36 <sup>b</sup>  | 0.12               | 0.06  | 0.06, 0.12, 0.12, 0.12 | 2, 2, 2, 4                               | $\geq 16$ -fold increase | Efficacy not predicted by MIC |
| JJ 5-35 <sup>b</sup>  | 0.5                | 0.12  | 0.25, 0.25, 0.5        | 4, 8, 16                                 | $\geq 16$ -fold increase | Not tested                    |
| JJ 8-16 <sup>b</sup>  | 0.12               | 0.06  | 0.25, 0.25, 0.5, 0.5   | 4, 8, 8, 16                              | $\geq 16$ -fold increase | Not tested                    |
| JJ 11-54 <sup>b</sup> | 0.5                | 0.25  | 0.25, 0.5, 0.5, 0.5    | 4, 8, 8, 8                               | $\geq 16$ -fold increase | Not tested                    |

<sup>a</sup> Assay results interpreted by the fold increase between the modal or central MIC in LB CO<sub>2</sub> and the modal or central MIC in mouse WB CO<sub>2</sub> from three or more independent tests.

<sup>b</sup> Isolates against which *in vivo* failures were previously reported for siderophore-conjugated monobactam MB-1 (11).

TABLE 7 Activities of siderophore-free reference compounds against *P. aeruginosa* in the mouse whole-blood assay

| Isolate  | MIC (mg/liter) in: |                    |                                       |           |                    |                                       |                  |                    |                                       |
|----------|--------------------|--------------------|---------------------------------------|-----------|--------------------|---------------------------------------|------------------|--------------------|---------------------------------------|
|          | Aztreonam          |                    |                                       | Meropenem |                    |                                       | Colistin sulfate |                    |                                       |
|          | MHB                | LB CO <sub>2</sub> | Mouse WB CO <sub>2</sub> <sup>a</sup> | MHB       | LB CO <sub>2</sub> | Mouse WB CO <sub>2</sub> <sup>a</sup> | MHB              | LB CO <sub>2</sub> | Mouse WB CO <sub>2</sub> <sup>a</sup> |
| PAO1     | 4                  | 4                  | 2                                     | 0.25      | 0.12               | 0.03                                  | 0.5              | 1                  | 2                                     |
| ARC3502  | 64                 | 16                 | 8                                     | >64       | 8                  | 8                                     | 0.5              | 0.5                | 2                                     |
| ARC3506  | >64                | 256                | 128                                   | 8         | 4                  | 4                                     | 0.5              | 0.5                | 2                                     |
| ARC4659  | 16                 | 16                 | 16                                    | 0.5       | 0.03               | 0.03                                  | 0.5              | 0.5                | 1                                     |
| AZ 8-18  | 8                  | 16                 | 16                                    | 8         | 4                  | 4                                     | 0.5              | 1                  | 1                                     |
| AZ 32-13 | 16                 | 64                 | 64                                    | 2         | 0.5                | 0.06                                  | 0.5              | 0.5                | 2                                     |
| JJ 4-36  | 32                 | 64                 | 32                                    | 32        | 8                  | 4                                     | 1                | 2                  | 2                                     |
| JJ 5-35  | 32                 | 64                 | 64                                    | 32        | 8                  | 16                                    | 0.5              | 1                  | 1                                     |
| JJ 8-16  | 16                 | 64                 | 64                                    | 2         | 0.5                | 0.12                                  | 0.5              | 1                  | 2                                     |
| JJ 11-54 | >64                | 64                 | 64                                    | 4         | 0.5                | 0.12                                  | 1                | 2                  | 2                                     |

<sup>a</sup> Assay results interpreted by the fold increase between the modal MIC in LB CO<sub>2</sub> and the modal MIC in mouse WB CO<sub>2</sub> from three independent tests.

The path to resistance against SMC-3176 was multiple (via *piuA*, *piuC*, *pirR*, *fecI*, and *pvdS* genes), and all genes were linked to various components of the siderophore-mediated iron uptake systems in *P. aeruginosa*. It should be noted that no stably resistant colonies were recovered from any of the animal efficacy studies. This finding was indicated as being a distinct feature of the transient, adaptive resistance described for MB-1 by Tomaras and colleagues (11). Display of adaptive resistance (i.e., resistance induced in the presence of drug and reversed in the drug's absence) is not unfamiliar to *P. aeruginosa* and has been encountered with aminoglycosides and polymyxins (27–31). Although largely unnoticed due to the clinical practice of combining aminoglycosides with other antibiotics, adaptive resistance has been documented in time course experiments following multiple doses of drug in animal and human studies of *P. aeruginosa* infection (29, 30). In these observations, the susceptible MIC was reflected in the *in vivo* response that followed only the first-dose exposure to netilmicin in neutropenic thigh-infected mice and tobramycin in cystic fibrosis patients, and bacterial kill was considerably lessened or lacking with subsequent doses. Similar experiments may prove to be informative for siderophore conjugates; however, such studies were not feasible with SMC-3176 due to the substantial amount of compound required to enable multiple pharmacodynamic assessments at various time points following multiple-dose administration.

While a single pharmacodynamic point estimate (either regrowth or reduction) was only possible for MB-1 by the design of the study (11), the complete spectrum of *in vivo* response was profiled for SMC-3176, having tested a wide range of dose regimens against each isolate. This approach exposed that previously concluded treatment failures were not complete, clear-cut failures, but that efficacious bacterial kill could still be achieved *in vivo* if drug exposures surpassed a certain, but yet indeterminable, threshold. Despite our best efforts, it remains unclear as to why isolate JJ 4-36 behaved differently from the four other *P. aeruginosa* isolates tested (PAO1, ARC3502, ARC3506, and ARC4659), such that kill effects were observed only at SMC-3176 exposures exceeding the 60 to 80% *fT*>MIC mark. It would be inappropriate to imply that achieving 100% *fT*>MIC would evade the effects of adaptive resistance. That estimation is being driven by the denominator, i.e., the MIC value, which was incor-

rect and overpredicted the level of SMC-3176 activity against JJ 4-36. Moreover, the 100% *fT*>MIC descriptor does not capture the full extent of drug exposure; different dose regimens may provide the same 100% *fT*>MIC but vary significantly in other parameters, like  $C_{max}$  and AUC. Such was the case with MB-1, for which four regimens that all provided 100% *fT*>MIC (using our dose-ranging approach) produced varied effects from 2-log growth to 2-log kill against JJ 4-36. The PK/PD index for SMC-3176 was reasonably assumed to be time driven (supported by *in vitro* time-kill results for SMC-3176 [data not shown]), as this has been well-established for various drug classes belonging to the  $\beta$ -lactam family (12, 24), and greater emphasis was placed on exploring the potential loss of *in vivo* efficacy.

The issue of adaptive resistance appeared to be isolate specific and was suspected to extend beyond JJ 4-36 to other clinical *P. aeruginosa* isolates. Tomaras and colleagues (11) observed *in vivo* regrowth against their MB-1 humanized dosing regimen in five out of nine isolates, suggesting a probability of encountering similarly adaptive isolates in the clinical setting. Further compounding this concern, unfavorable *in vivo* responses against JJ 4-36 occurred at standard inocula for both MB-1 and SMC-3176. Because the adaptive resistance phenomenon involves a phenotypic transformation and not the actual selection of resistant mutants, it can and did occur at the standard inoculum for SMC-3176, irrespective of the spontaneous mutation frequency. For aminoglycosides, the adaptive phenomenon along with concentration-dependent killing are fortuitously suited to the once-daily dosing regimen (32, 33), in which large dosages are separated by long intervals to take advantage of first-exposure effects while providing a drug-free period to allow reversal of the phenotype to the susceptible state (27–29). Whether a clinical dosing regimen can be engineered that would likewise circumvent adaptive resistance for siderophore-conjugated  $\beta$ -lactams remains to be determined.

Despite our findings that adaptive resistance can be overcome with increased drug exposure, it continues to be a question as to whether the *in vivo* threshold can be elucidated from a few isolates and effectively applied to the broader *P. aeruginosa* population. Several *in vitro* tests were designed in pursuit of a tool that would at best quantitatively predict the *in vivo* response for SMC-3176 across isolates, or at least qualitatively identify problematic adaptive isolates, like JJ 4-36. To start, broth microdilution MICs de-



terminated in iron-chelated MHB (i.e., in CDMHB) translated appropriately to *in vivo* efficacy against the four nonadaptive isolates tested versus values determined in unmodified MHB. Standard MIC methods have been rightly manipulated before for certain agents to more closely mimic the clinical situation as it relates to how the drug exerts its antibacterial activity, such as daptomycin requiring calcium supplementation to broth medium or fosfomycin requiring supplementation of glucose-6-phosphate to agar medium (16, 34, 35). However, simple MIC testing in CDMHB failed to convey the particular *in vivo* readout against JJ 4-36. Subsequently, we moved to adopt the CDMHB agarose growth assay that was conceived with MB-1, which disappointingly did not yield consistent or informative data for SMC-3176. Thereafter, an assay was conducted to quantify the endogenous siderophore, pyoverdine, in nonadaptive versus adaptive isolates, but this measurement also failed to provide insight. Hyperproduction of pyoverdine was visually detectable on CDMHB agarose plates but was not distinguishable among isolates; thus, by quantifying pyoverdine, we had aimed to identify meaningful differences with the increased expression by adaptively resistant isolates. In the end, we settled on the MIC assay in mouse WB (as opposed to another variation of broth medium), which was the only assay to correlate with the *in vivo* response for SMC-3176 across all examined isolates, including JJ 4-36. The utility of the assay was limited to isolate screening only and could not be leveraged for an estimation of SMC-3176's *in vivo* threshold concentration from the WB MIC value. As such, the question remains as to how an infected patient in the clinical setting may be treated with SMC-3176 (and possibly other siderophore conjugates) if there is no reliable *in vitro* susceptibility test to inform the likelihood of its treatment success.

Taken together, this work indicates that SMC-3176 is vulnerable to attenuated efficacy in *P. aeruginosa* due to rapid adaptive resistance against its siderophore-mediated mode of entry. While comprehensive PK/PD profiling led us to discover an *in vivo* threshold at which efficacy can be achieved, identifying such a threshold that can be applied across the pseudomonal population is not feasible with current *in vitro* tools, which includes the qualitative but not quantitative mouse WB assay. Combination therapy with another antipseudomonal agent that uses a non-siderophore-dependent route or with an agent that can potentiate the activity of the siderophore conjugate is a consideration (36, 37). Ultimately, extensive *in vivo* testing against numerous clinical isolates is warranted for siderophore-conjugated compounds to thoroughly screen for the potential disconnect between the *in vitro* MIC and *in vivo* response in *P. aeruginosa*.

## ACKNOWLEDGMENTS

This work was supported by AstraZeneca Pharmaceuticals LP. All authors were financially compensated or employed by AstraZeneca Pharmaceuticals LP at the time of the study and may hold stock/stock options in the company.

Much appreciation is given to John Mueller, Boudewijn de Jonge, Joseph Newman, Thomas Durand-Réville, Sarah McLeod, and Maria Uria-Nickelsen for their valued scientific contributions and to Lena Grosser, Maryann San Martin, Jennifer Scafidi, Krista Farrington, Jianyan Wang, Wenzhan Yang, James Whiteaker, Catherine Gilbert, and Pamela Tessier for their dedicated technical assistance.

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