SPR741, an Antibiotic Adjuvant, Potentiates the \textit{In Vitro} and \textit{In Vivo} Activity of Rifampin against Clinically Relevant Extensively Drug-Resistant \textit{Acinetobacter baumannii}

Daniel V. Zurawski,\textsuperscript{a} Alexandria A. Reinhart,\textsuperscript{a} Yonas A. Alamneh,\textsuperscript{a} Michael J. Pucci,\textsuperscript{b} Yuanzheng Si,\textsuperscript{a} Rania Abu-Taleb,\textsuperscript{a} Jonathan P. Shearer,\textsuperscript{a} Samandra T. Demons,\textsuperscript{a} Stuart D. Tyner,\textsuperscript{a} Troy Lister\textsuperscript{a}

\textsuperscript{a}Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; \textsuperscript{b}Spero Therapeutics, Inc., Cambridge, Massachusetts, USA

\textbf{ABSTRACT} \textit{Acinetobacter baumannii} is responsible for 10% of all nosocomial infections and has >50% mortality rates when causing ventilator-associated pneumonia. In this proof-of-concept study, we evaluated SPR741, an antibiotic adjuvant that permeabilizes the Gram-negative membrane, in combination with rifampin against AB5075, an extensively drug-resistant (XDR) \textit{A. baumannii} strain. In standard \textit{in vitro} assays and in a murine pulmonary model, we found that this drug combination can significantly reduce bacterial burden and promote animal survival despite an aggressive infection.

\textbf{KEYWORDS} \textit{Acinetobacter}, ESKAPE pathogens, animal models, antibacterial, antibiotic adjuvants, antibiotic resistance, antibiotics, mouse model, pulmonary model, virulent strain

\textit{Acinetobacter baumannii} gained notoriety as the bacterial species most frequently isolated from U.S. soldiers from 2004 to 2010, with >3,500 infections associated with war wounds (1–4). Presently, \textit{A. baumannii} is a significant problem worldwide because of extensively drug-resistant (XDR) strains and the aggressive nature of some infections causing ventilator-associated pneumonia (5–7). Recently, the World Health Organization cited a critical need for \textit{A. baumannii} research because of increased drug resistance and lack of treatments (8). To address the immediate need, research strategies such as antibiotic adjuvants, which involve the current antibiotic armamentarium, may provide a faster path to clinical application.

Antibiotic adjuvants are typically small molecules that sensitize a bacterium to clinically approved antibiotics (9). Specific examples with respect to \textit{A. baumannii} include 2-aminoimidazole-based compounds, which disrupt two-component signaling (10), and anthracyclines, which potentiate activity of rifampin and linezolid (11). Here, we evaluated SPR741 (formerly NAB741), a polymyxin-B-derived molecule specifically designed to minimize the nephrotoxicity associated with this antibacterial class (12). SPR741 has reduced positive charge and lacks the highly lipophilic fatty-acid side chain present in polymyxins, which are the two structural features responsible for clinical nephrotoxicity (12, 13). As a proof of concept, we tested a combination of SPR741 and rifampin because colistin and rifampin have proven to be an effective combination in both mouse models and in patients (14–18). Rifampin was also previously tested against AB5075, a highly virulent XDR strain, in a murine pulmonary model of infection (19), which facilitated dosing for this study.

First, the SPR741/rifampin combination was tested \textit{in vitro} against \textit{A. baumannii}. The
MICs for SPR741 and rifampin were determined for AB5075 to be 128 \( \mu g/ml \) and 4.0 \( \mu g/ml \), respectively, using standard CLSI methods in cation-adjusted Mueller-Hinton broth (CAMHB) (20). This combination was assessed with the checkerboard method to determine fractional inhibitory concentration (FIC) (21), where synergistic activity was defined by an FIC of \( \leq 0.5 \) (22). The MIC of rifampin dropped from 4.0 to 0.5 \( \mu g/ml \) in the presence of 2.0 \( \mu g/ml \) SPR741, an 8-fold reduction, thus producing an FIC of 0.14 and indicating synergy. An isobologram was generated from these results (Fig. 1A).

Next, we examined whether this synergy was applicable across the whole species of \( A. \ baumannii \). We analyzed a previously described 28-strain diversity set (19) and determined MICs for all strains. A combination of 4.0 \( \mu g/ml \) of SPR741 and 1.0 \( \mu g/ml \) rifampin inhibited the growth of 96% of the strains, with a minimum 4-fold reduction of most MICs. AB3027 was the exception, as it is significantly resistant to rifampin (MIC \( > 128 \mu g/ml \); Table 1).

To further evaluate activity, time-kill assays (3 biological replicates) were performed with 2.0 \( \mu g/ml \) SPR741 and 1.0 \( \mu g/ml \) rifampin against AB5075 grown in CAHMB as previously described (23). Each drug used alone had little effect on growth. In contrast, the combination resulted in fewer than 10 organisms (limit of detection) on LB plates.
at 2, 4, and 6 h (Fig. 1B), a significant result (two-way ANOVA, \( P = 0.0048 \)). This result confirmed that the combination of SPR741/rifampin had a bactericidal, synergistic effect that should be further tested in vivo.

The methods for the murine pulmonary model of *A. baumannii* infection were previously detailed (19), and were conducted similarly for this study. In pilot experiments, we initially evaluated three doses of SPR741 (40, 60, or 80 mg/kg) once daily (QD) or twice daily (BID), with 5.0 mg/kg or 10.0 mg/kg doses of rifampin also provided QD or BID. Only the BID-treated mice survived over the course of 1 week, suggesting that QD treatment was not sufficient (data not shown). Next, two independent experiments were conducted using 10 mice per group. Four hours after *A. baumannii* inoculation, groups were treated with sterile saline (negative control), 5.0 mg/kg rifampin, 60 mg/kg SPR741 BID, or the combination of SPR741 40 mg/kg or 60 mg/kg SPR741 with 5.0 mg/kg rifampin BID for the next 3 days. The survival rate for 60 mg/kg SPR741 combined with 5.0 mg/kg rifampin BID was 90% (Fig. 2A), a significant success for this aggressive infection model (Mantel-Cox test, \( P = 0.0027 \)). In contrast, untreated animals or mice receiving SPR741 alone succumbed to infection (Fig. 2A). Rifampin alone only provided 50% survival (Fig. 2A).

Separate experiments were then conducted to evaluate bacterial burden via CFU (CFU/g of lung tissue). Mice were sacrificed on day 2 before the untreated control animals succumbed to infection, as previously described (19). These results mirrored the survival results, where the combination of SPR741/rifampin decreased bacterial burden by 6.0 \( \log_{10} \) CFU/g compared to the vehicle-alone control (Mann-Whitney U test, \( P < 0.0001 \)) (Fig. 2B). When comparing the combination of SPR741/rifampin to rifampin treatment alone, a 2.0-\( \log_{10} \) reduction in burden was seen with the addition

| *A. baumannii* strain | MIC for rifampin (g/ml) | MIC for SPR741 (4.0 µg/ml) | Growth in presence of SPR741 (4.0 µg/ml) + rifampin (1.0 µg/ml) |
|-----------------------|-------------------------|-----------------------------|-----------------------------------------------|
| AB967                 | 4                       | <64                         | –                                             |
| AB2828                | 2                       | 256                         | –                                             |
| AB3340                | 2                       | 256                         | –                                             |
| AB3560                | 4                       | 128                         | –                                             |
| AB3638                | 2                       | 256                         | –                                             |
| AB3785                | 4                       | 128                         | –                                             |
| AB3806                | 2                       | 256                         | –                                             |
| AB3927                | >256                    | 256                         | +                                             |
| AB4025                | 4                       | 128                         | –                                             |
| AB4026                | 4                       | >256                        | –                                             |
| AB4027                | 4                       | >256                        | –                                             |
| AB4052                | 4                       | 256                         | –                                             |
| AB4269                | 8                       | >256                        | –                                             |
| AB4448                | 4                       | <64                         | –                                             |
| AB4456                | 4                       | 128                         | –                                             |
| AB4490                | 4                       | 128                         | –                                             |
| AB4498                | 4                       | 256                         | –                                             |
| AB4795                | 2                       | 128                         | –                                             |
| AB4857                | 4                       | 256                         | –                                             |
| AB4878                | 4                       | 256                         | –                                             |
| AB4932                | 16                      | <64                         | –                                             |
| AB4957                | 4                       | 256                         | –                                             |
| AB4991                | 4                       | 128                         | –                                             |
| AB5001                | 4                       | 256                         | –                                             |
| AB5075                | 2                       | 128                         | –                                             |
| AB5197                | 4                       | 256                         | –                                             |
| AB5256                | 4                       | 128                         | –                                             |
| AB5674                | 2                       | 128                         | –                                             |
| AB5711                | 4                       | 128                         | –                                             |

\( ^{a} \)MICs were determined separately in the presence of the combination of SPR741 at 4.0 µg/ml and rifampin at 1.0 µg/ml.

\( ^{b} \)−, no growth; +, growth.
of SPR741, which was also statistically significant (Mann-Whitney U test, $P = 0.0029$) (Fig. 2B).

This investigation is a promising start with regard to in vivo safety and efficacy of SPR741 combinations against Gram-negative pathogens. In pilot experiments, more mice did succumb (80% survival) with 80 mg/kg BID doses of SPR741. The reason for this is unclear, but clearance and complete animal survival are difficult to achieve in this model. AB5075 is highly aggressive and bacteria reach high numbers in lung tissue, followed by dissemination into the bloodstream and colonization of other organs, including heart, spleen, and kidneys (4, 19). With regard to toxicity, previously presented results (P. Shastri and S. Coleman, ASM Microbe, Boston, MA, 16 to 20 June 2016) determined that the 60 mg/kg dose in mice scales to a human dose of approximately 200 to 400 mg. SPR741 demonstrated a no-observed-adverse-effect-level (NOAEL) of $>60$ mg/kg/day in cynomolgus monkeys (S. Coleman, M. Bleavins, T. Lister, M. Vaara, and T.R. Parr, ASM Microbe, Boston, MA, 16 to 20 June 2016), while nephrotoxicity was observed at 12 mg/kg/day with polymyxin B. Spero Therapeutics recently completed SPR741 dosing in healthy volunteers (https://clinicaltrials.gov/ct2/show/NCT03022175, ClinicalTrials registration no. NCT03022175). With these prior results and the data obtained from this proof-of-concept study, more preclinical investigations of SPR741-antibiotic combinations are warranted to evaluate
efficacy against other bacterial species. Furthermore, animal models mimicking other clinical indications and evaluating pharmacokinetics/pharmacodynamics (PK/PD) are also currently being pursued.

ACKNOWLEDGMENTS

We thank the other members of Spero Therapeutics, Inc. and the Wound Infections Department at WRAIR for their support; these studies would not be possible without them.

The research presented here was partially supported by grant PR150337 awarded to M.J.P. (Spero Therapeutics, Inc.) and D.V.Z (WRAIR) from the Peer-Reviewed Medical Research Program.

M.J.P. and T.L. are employees of Spero Therapeutics, Inc.

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense.

Research was conducted under an approved animal protocol (16-BRD-48S) in an AAALACi-accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

REFERENCES

1. Keen EF, III, Murray CK, Robinson BJ, Hospenthal DR, Co EM, Aldous WK. 2010. Changes in the incidences of multidrug-resistant and extensively drug-resistant organisms isolated in a military center. Infect Control Hosp Epidemiol 31:728–732. https://doi.org/10.1086/653617.
2. Yun HC, Murray CK, Roop SA, Hospenthal DR, Gourdine E, Dooley DP. 2006. Bacteria recovered from patients admitted to a deployed U.S. military hospital in Baghdad, Iraq. Mil Med 171:821–825.
3. Yun HC, Branstetter JG, Murray CK. 2008. Osteomyelitis in military personnel wounded in Iraq and Afghanistan. J Trauma 64:5163–5168. https://doi.org/10.1097/TA.0b0131e31816088c.
4. Hobson DW, Schuh JC, Zurawski DW, Wang J, Arbabi S, McVean M, Funk KA. 2016. The first cut is the deepest: the history and development of safe treatments for wound healing and tissue repair. Int J Toxicol 35:491–498. https://doi.org/10.1177/1091581816656804.
5. Wong D, Nielsen TB, Bonomo RA, Pantapalangkoor P, Luna B, Spellberg B. 2017. Clinical and pathophysiological overview of Acinetobacter infections: a century of challenges. Clin Microbiol Rev 30:409–447.
6. Spellberg B, Bonomo RA. 2014. The deadly impact of extreme drug resistance in Acinetobacter baumannii. Crit Care Med 42:1289–1291. https://doi.org/10.1097/CCM.0000000000001818.
7. Potron A, Poirel L, Nordmann P. 2015. Emerging broad-spectrum resistance in Pseudomonas aeruginosa and Acinetobacter baumannii: mechanisms and epidemiology. Int J Antimicrob Agents 45:568–585. https://doi.org/10.1016/j.ijantimicag.2015.03.001.
8. WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. World Health Organization, Geneva, Switzerland. http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/.
9. Wright GD. 2016. Antibiotic adjuvants: rescuing antibiotics from resistance. Trends Microbiol 24:862–871. https://doi.org/10.1016/j.tim.2016.06.009.
10. Thompson RJ, Bobay BG, Stowe SD, Olson AL, Peng L, Su Z, Actis LA, Melander C, Cavanagh J. 2012. Identification of BfmR, a response regulator involved in biofilm development, as a target for a 2-aminodiazole-based antibiotic film agent. Biochemistry 51:9776–9787. https://doi.org/10.1021/bi3015289.
11. Cox G, Koteva K, Wright GD. 2014. An unusual case of anthracoclynes potentiating Gram-positive antibiotics in intrinsically resistant Gram-negative bacteria. J Antimicrob Chemother 69:1844–1855. https://doi.org/10.1093/jac/dku057.
12. Vaara M, Silkanen O, Apajalahti J, Fox J, Frimodt-Moller N, He H, Pout Daly A, Li J, Nation RL, Vaara T. 2010. A novel polymyxin derivative that lacks the fatty acid tail and carries only three positive charges has strong synergism with agents excluded by the intact outer membrane. Antimicrob Agents Chemother 54:3341–3346. https://doi.org/10.1128/AAC.01439-09.
13. Vehman JA, Nguyen MH, Doi Y. 2014. Treatment options for carbapenem-resistant and extensively drug-resistant Acinetobacter baumannii infections. Drugs 74:1315–1333. https://doi.org/10.1007/s40265-014-0267-8.
14. Pachon-Ibanez ME, Docobo-Perez F, Lopez-Rojas R, Domínguez-Herrera J, Jimenez-Mejias ME, Garcia-Curiel A, Pichardo C, Jimenez L, Pachon J. 2010. Efficacy of rifampin and its combinations with imipenem, sulbactam, and colistin in experimental models of infection caused by imipenem-resistant Acinetobacter baumannii. Antimicrob Agents Chemother 54:1165–1172. https://doi.org/10.1128/AAC.00367-09.
15. Song JY, Cheong HJ, Lee J, Sung AK, Kim WJ. 2009. Efficacy of mono-therapy and combined antibiotic therapy for carbapenem-resistant Acinetobacter baumannii pneumonia in an immunosuppressed mouse model. Int J Antimicrob Agents 33:33–39. https://doi.org/10.1016/j.ijantimicag.2008.07.008.
16. Durante-Mangoni E, Signoriello G, Andini R, Mattei A, De Cristoforo M, Murino P, Bassetti M, Malacarne P, Petrosillo N, Galdieri N, Mocavero P, Coricone A, Viscoli C, Zarrilli R, Gallo C, Urti R. 2013. Colistin and rifampicin compared with colistin alone for the treatment of serious infections due to extensively drug-resistant Acinetobacter baumannii: a multicenter, randomized clinical trial. Clin Infect Dis 57:349–358. https://doi.org/10.1093/cid/cit253.
17. Jacobs AC, Thompson MG, Black CC, Kessler JL, Clark LP, McQueary CN, Gancz HY, Corey BW, Moon JK, Si Y, Owen MT, Hallock JD, Kwak YI, Summers A, Li CZ, Rasko DA, Penwell WF, Honnold CL, Wise MC, Waterman PE, Lesho EP, Steward RL, Actis LA, Palys TJ, Craft DW, Zurawski DV. 2014. ABS057, a highly virulent isolate of Acinetobacter baumannii, as a
model strain for the evaluation of pathogenesis and Antimicrobial Treat-
ments. mBio 5:e01076–01014. https://doi.org/10.1128/mBio.01076-14.

20. Clinical and Laboratory Standards Institute. 2015. Performance standards
for antimicrobial susceptibility testing, 26th ed. Approved standard
M100-S26. Clinical and Laboratory Standards Institute, Wayne, PA.

21. Garcia LS. 2010. Synergism testing: broth microdilution checkerboard
and broth macrodilution methods, p 140–162. In Garcia LS (ed), Clinical
microbiology procedures handbook, 3rd ed. ASM Press, Washington, DC.

22. Hall MJ, Middleton RF, Westmacott D. 1983. The fractional inhibitory
concentration (FIC) index as a measure of synergy. J Antimicrob Che-
mother 11:427–433. https://doi.org/10.1093/jac/11.5.427.

23. Joly-Guillou ML, Decré D, Herman JL, Bourdeller E, Bergogne-Bérézin E.
1995. Bactericidal in-vitro activity of beta-lactams and beta-lactamase
inhibitors, alone or associated, against clinical strains of Acinetobacter
baumannii: effect of combination with aminoglycosides. J Antimicrob
Chemother 36:619–629. https://doi.org/10.1093/jac/36.4.619.