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SUSCEPTIBILITY

Apramycin Overcomes the Inherent Lack of Antimicrobial Bactericidal Activity in *Mycobacterium abscessus*

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ABSTRACT Antibiotic therapy of infections caused by the emerging pathogen *Mycobacterium abscessus* is challenging due to the organism's inherent resistance to clinically available antimicrobials. The low bactericidal potency of currently available treatment regimens is of concern and testifies to the poor therapeutic outcomes for pulmonary *M. abscessus* infections. Mechanistically, we demonstrate here that the acetyltransferase Eis2 is responsible for the lack of bactericidal activity of amikacin, the standard aminoglycoside used in combination treatment. In contrast, the aminoglycoside apramycin, with a distinct structure, is not modified by any of the pathogen's innate aminoglycoside resistance mechanisms and is not affected by the multidrug resistance regulator WhiB7. As a consequence, apramycin uniquely shows potent bactericidal activity against *M. abscessus*. This favorable feature of apramycin is reflected in a mouse model of pulmonary *M. abscessus* infection, which demonstrates superior activity, compared with amikacin. These findings encourage the development of apramycin for the treatment of *M. abscessus* infections and suggest that *M. abscessus* eradication in pulmonary disease may be within therapeutic reach.

KEYWORDS apramycin, bactericidal activity, aminoglycoside, *Mycobacterium abscessus*, cystic fibrosis, pulmonary disease, drug resistance, antibiotic treatment

Nontuberculous mycobacteria (NTM) are ubiquitous environmental organisms comprising numerous pathogens that cause chronic pulmonary infections, particularly among patients with preexisting pulmonary diseases, such as cystic fibrosis (CF), bronchiectasis, and chronic obstructive pulmonary diseases (1–4). Of the rapidly growing members of NTM species, *Mycobacterium abscessus* complex (MABSC) has evolved as a major respiratory pathogen in individuals with CF, where it leads to accelerated decline in pulmonary function and can compromise the success of lung transplantation (5–7). Pulmonary infections with *M. abscessus* have become more common, and studies from several countries worldwide have all reported significant increases in the prevalence of *M. abscessus* infections over the past decade (3, 8). It is estimated that 5% to 15% of individuals with CF in Europe and the United States are infected with *M. abscessus* (9–11).

MABSC consists of three subspecies, i.e., *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* (2). Its main threat as a pathogen is not least due to its high innate resistance to antibacterial agents, which affects a broad range of commonly used antibiotics (12). Consequently, limited treatment options for MABSC infections exist, and current recommendations suggest that patients with *M. abscessus* pulmonary disease should receive a multidrug regimen that includes at least three drugs (13–16). Amikacin is considered a cornerstone in the treatment of MABSC infections, particularly infections involving strains that exhibit inducible [*erm*(41)-dependent] macrolide resistance, as most clinical isolates of *M. abscessus* do (17, 18). Antibiotic treatment for a

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TABLE 1 MICs and MBCs of *M. abscessus* strains

Strain	MIC or MBC (mg/L)[a]					
	Amikacin			Apramycin		
	MIC	MBC18	MBC36	MIC	MBC18	MBC36
Clinical isolates						
M. abscessus subsp. *abscessus* 500043/08	1	>32	>32	0.5	2	1
M. abscessus subsp. *abscessus* 500042/08	1	>32	>32	0.5	4	2
M. abscessus subsp. *massiliense* 500044/09	1	>32	>32	0.5	2	1
M. abscessus subsp. *massiliense* 500446/19	1	>32	>32	0.5	4	2
M. abscessus subsp. *bolletii* 179709/08	1	>32	>32	0.5	4	2
M. abscessus subsp. *bolletii* 181739/08	4	>32	>32	0.5	4	2
ATCC type strain and isogenic mutants						
M. abscessus ATCC 19977	1	>32	>32	0.5	2	1
M. abscessus Δ*eis2*	0.25	1	1	0.5	2	1
M. abscessus Δ*aac(2′)*	1	>32	>32	0.5	4	2
M. abscessus Δ*aac(2′)* Δ*eis2*	0.25	2	1	0.5	4	2
M. abscessus Δ*whiB7*	0.25	ND	ND	0.5	ND	ND

[a] MBC18, MBC at 18 h of drug exposure; MBC36, MBC at 36 h of drug exposure; ND, not done.

full 12 months after culture conversion is recommended; however, culture conversion is the exception rather than the rule. Clinical studies of therapeutic outcomes are sparse and, to date, no standardized antibiotic regimens leading to cure rates of >30% to 50% have been reported, with some variation among the subspecies (19–21). In addition to lengthy courses of antimicrobial chemotherapy, surgery may be required to decrease the burden of disease (19).

In general, antibacterial compounds are categorized as bacteriostatic or bactericidal antimicrobials. The bactericidal activity of an antibiotic is particularly relevant for treatment of chronic infections, such as endocarditis, because bacteriostatic activity alone rarely results in resolution of the infection (22). The poor treatment outcomes in pulmonary infections with *M. abscessus* are of concern and may be related to the limited bactericidal activity of available treatment regimens. None of the antibacterials used currently in treatment, not even the most potent drug classes such as aminoglycosides and fluoroquinolones, exhibit bactericidal activity (minimal bactericidal concentration [MBC]/MIC of ≤4) against MABSC (23–25).

RESULTS AND DISCUSSION

We determined the dose- and time-dependent kill curves for amikacin against a panel of clinical isolates representing the three MABSC subspecies (Table 1 and Fig. 1 and 2). MICs for amikacin were in the range of 1 to 4 mg/L, and dose- and time-dependent reductions in CFU were found at drug concentrations above the MIC. However, a

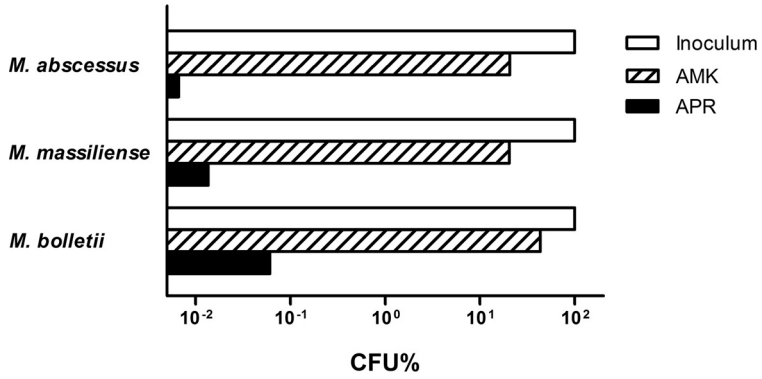

FIG 1 Bactericidal activity of amikacin (AMK) and apramycin (APR) on clinical isolates. Values indicate the percentage of input CFU (x = 100%) following 18 h of incubation in the presence of 4 mg/L amikacin or 4 mg/L apramycin. For details, see Fig. 2.

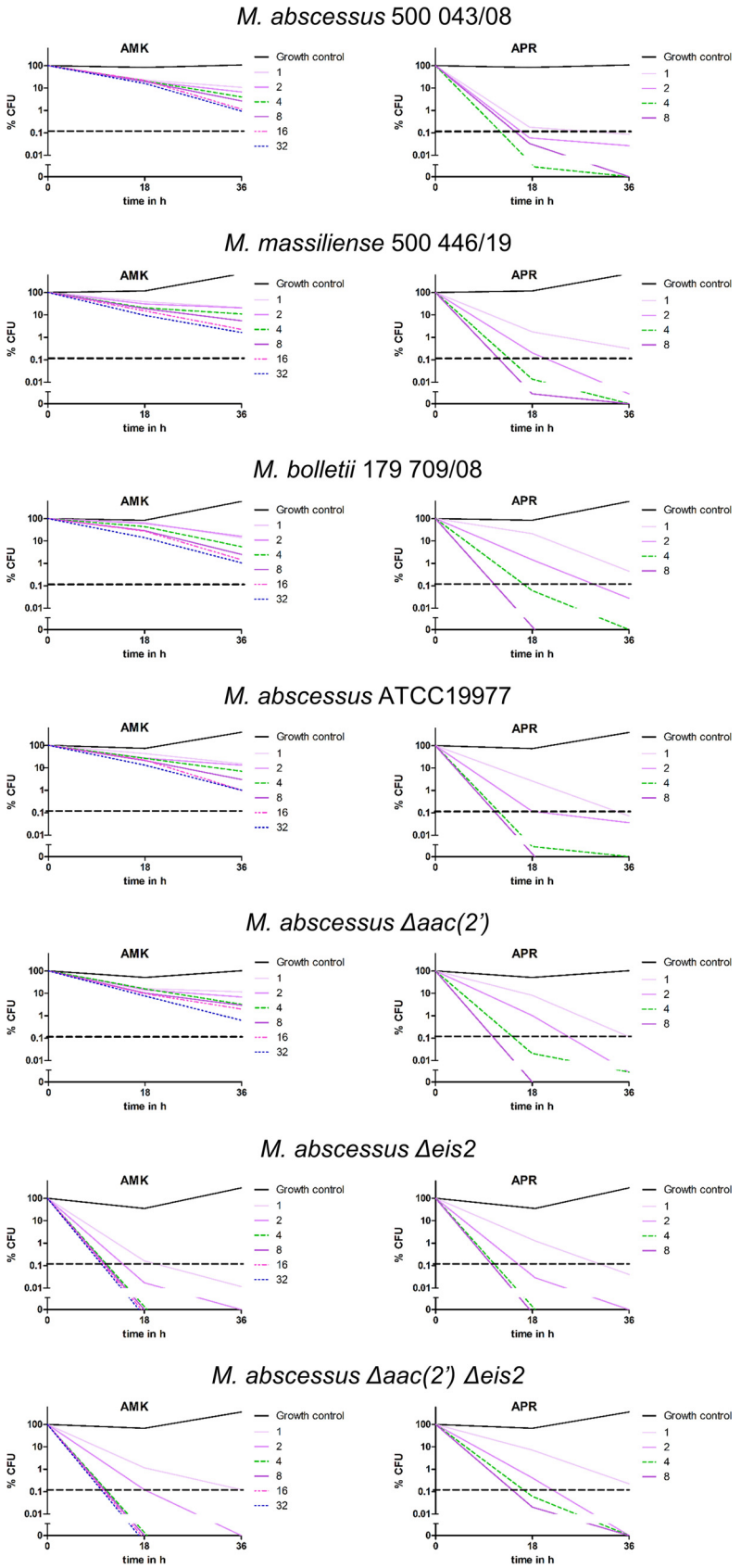

FIG 2 Time-kill curves for amikacin and apramycin against *M. abscessus* strains. *M. abscessus* clinical isolates of the three subspecies, i.e., *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*,

(Continued on next page)

bactericidal effect, defined as 99.9% reduction of the inoculum CFU counts, was not observed even at the highest amikacin concentration tested (32 mg/L). A concentration of 32 mg/L amikacin, however, is already 3-fold higher than the tissue, epithelial lining fluid, and sputum concentrations of about 10 mg/L achieved after intravenous infusion with therapeutic dosing (26, 27).

The genome of *M. abscessus* encodes several drug-modifying enzymes (12, 18). We recently showed that *M. abscessus* aminoglycoside susceptibility is affected by chromosomally encoded aminoglycoside-modifying acetyltransferases (Mabs_4532c and Mabs_4395) (28). Mabs_4532c encodes the promiscuous multiacetyltransferase Eis2 and affects susceptibility to, for example, amikacin and the peptide antibiotic capreomycin, while Mabs_4395 encodes an aminoglycoside 2′-*N*-acetyltransferase [AAC(2′)] that specifically reduces susceptibility to 2′-NH_2-aminoglycosides such as kanamycin B, tobramycin, and gentamicin but spares the 2′-OH aminoglycoside amikacin (28). We hypothesized that *eis2* may prevent the bactericidal activity of amikacin. We determined amikacin MIC and time-kill curves for *M. abscessus* Δ*eis2* and *M. abscessus* Δ*aac(2′)* Δ*eis2* strains. As controls, we used a wild-type (wt) strain and a genetically engineered *M. abscessus* Δ*aac(2′)* strain. As expected, genetic deletion of *aac(2′)* did not affect amikacin MICs, while the amikacin MICs in the Δ*eis2* and Δ*aac(2′)* Δ*eis2* deletion mutants were 4-fold lower than the amikacin MICs in the isogenic parental strain (28) (Table 1). Similarly, amikacin susceptibility in *M. abscessus* was affected by *whiB7*. A strain with *whiB7* deleted showed a 4-fold decreased amikacin MIC (Table 1). The *whiB7* gene encodes a conserved stress response transcription factor that confers broad-range drug resistance in *M. abscessus* by acting through various effector mechanisms, e.g., by regulating genes involved in drug modification (*eis2*), target-modifying genes [*erm*(41)], and drug efflux pumps (29–31). No bactericidal activity of amikacin was observed for the wt strain or the Δ*aac(2′)* deletion mutant. In contrast, a potent bactericidal effect at low drug concentrations (MBC of 1.0 mg/L) was found for the Δ*eis2* mutant and the Δ*aac(2′)* Δ*eis2* double deletion mutant. No difference in the bactericidal activity of amikacin was found between the Δ*eis2* and Δ*aac(2′)* Δ*eis2* deletion strains (Table 1 and Fig. 2). These data demonstrate that *eis2* is necessary and sufficient to specifically abolish the bactericidal activity of amikacin in *M. abscessus*.

Apramycin is an aminoglycoside of unique structure that shows potent MIC activity against *M. abscessus* and exhibits minimal cross-resistance to other aminoglycosides, combined with therapeutic lung exposure and a low toxicity profile (32–35). We determined time- and dose-dependent apramycin kill curves for various MABSC strains, as listed in Table 1. In addition to the genetically engineered deletion mutants of *M. abscessus*, this panel of strains includes clinical isolates representative of the three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*. The apramycin MICs for all strains were 0.5 mg/L, independent of the subspecies or the presence or absence of *eis2*, *aac(2′)*, and *whiB7*. These findings indicate that apramycin is neither a substrate for acetylation by Eis2 or Aac(2′) nor a target for any of the numerous *whiB7*-dependent drug resistance mechanisms. Consequently, the efficacy of apramycin is unlikely to be affected by antagonistic drug interactions, as observed in clarithromycin-amikacin combination treatment due to macrolide-induced upregulation of WhiB7 (31). Most importantly, at doses as low as 1 to 2 mg/L, apramycin exhibited potent bactericidal activity for all strains tested, irrespective of the presence of *eis2* or *aac(2′)* (Table 1 and Fig. 1 and 2). These findings demonstrate that apramycin overcomes the inherent lack of aminoglycoside bactericidal activity in *M. abscessus*.

The potent bactericidal activity of apramycin prompted us to test its activity in an *in vivo M. abscessus* infection model. SCID mice were infected with 10^6 CFU of *M. abscessus*

FIG 2 Legend (Continued)
and *M. abscessus* subsp. *bolletii*, as well as *M. abscessus* ATCC 19977 and its genetically engineered deletion mutants *M. abscessus* Δ*eis2*, *M. abscessus* Δ*aac(2′)*, and *M. abscessus* Δ*aac(2′)* Δ*eis2*, were exposed for 18 h or 36 h to various concentrations (0 and 0.125 to 32 mg/L) of amikacin (AMK) and apramycin (APR). Serial dilutions were spotted and incubated at 37°C for 96 h. Bacteria were counted, and the relative number of CFU, compared to time zero, was plotted. The dashed horizontal lines indicate the 99.9% killing threshold that defines bactericidal activity.

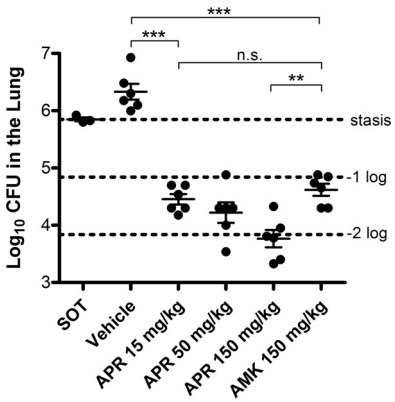

FIG 3 *M. abscessus* CFU counts in the lungs of mice. SCID mice were intravenously infected with *M. abscessus*. One day later, once-daily treatment with either amikacin or different doses of apramycin for 8 consecutive days was started. Mice were euthanized 1 day after receiving the last antibiotic dose. Lungs were homogenized, and extracts were plated on 7H11 agar. A group of three mice was used to calculate the bacterial load at the start of treatment (SOT). One control group ($n = 6$) did not receive antibiotics (vehicle). Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software). *P* values were calculated using one-way ANOVA, and Tukey's multiple-comparison test. n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$.

and rested for 1 day. Then, groups of mice were treated for 8 consecutive days with either a high dose of amikacin (150 mg/kg), three different doses of apramycin (150, 50, and 15 mg/kg), or vehicle control. CFU counts in lung homogenates were determined at day 1 (before the start of treatment) to confirm manifestation and 1 day after administration of the last antibiotic dose (Fig. 3). An amikacin dose of 150 mg/kg reduced CFU counts in the lungs by approximately 1 log unit. A 10-fold lower apramycin dose of 15 mg/kg resulted in a similar CFU reduction. Upon application of higher doses of apramycin, a dose-dependent CFU reduction of up to 2 log units was observed. Thus, apramycin exceeded the efficacy of amikacin by 1 order of magnitude when the drugs were administered at equivalent doses.

In summary, we demonstrate here that apramycin is an antibiotic with bactericidal activity against *M. abscessus*. Its activity is affected neither by *M. abscessus* acetyltransferases nor by the multidrug resistance regulator WhiB7. Our *in vitro* findings translate into potent pathogen reduction in an *in vivo* pulmonary infection model, where apramycin is significantly more potent than amikacin, a drug considered a cornerstone in the treatment of *M. abscessus* infections. These findings warrant the consideration of apramycin for treatment of infections with *M. abscessus* and suggest that apramycin may offer the promising prospect of *M. abscessus* eradication in pulmonary disease. In particular, chronically infected CF patients may benefit from the potent bactericidal activity of this drug candidate.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions. *Mycobacterium abscessus* strains were grown in cation-adjusted Mueller-Hinton (CAMH) broth. Clinical isolates were obtained from the Institute of Medical Microbiology, University of Zurich, and the National Reference Laboratory for Mycobacteria (Zurich, Switzerland). Strains were identified by *rrs* (16S rRNA) gene sequencing and typed to the subspecies level by *rpoB* and *erm*(41) sequencing (36–38). Genetically engineered derivatives of *M. abscessus* ATCC 19977 with gene deletions in *eis2*, *aac(2′)*, or *aac(2′) eis2* have been described previously (28). A targeted deletion mutant of *M. abscessus* deficient in *whiB7* (MAB_ 3508c) was constructed by electroporation of competent cells with plasmid pKH-ΔwhiB7, following the procedure described by Rominski et al. (39). In brief, plasmid pKH-whiB7 is a suicide vector containing approximately 1.5 kbp of the upstream and downstream regions of the target gene, facilitating homologous recombination. The upstream-downstream region is cloned adjacent to an *aac(3)IV* resistance cassette and a DsRed2 marker gene for positive selection and the *M. tuberculosis katG* gene for negative selection (isoniazid susceptibility) (40). Transformants were selected on apramycin-containing plates and identified by red fluorescence. Single crossover transformants were identified by Southern blotting and subjected to isoniazid counterselection. Single colonies were purified, and deletion of the *whiB7* locus was confirmed by Southern blotting.

MIC determinations. Amikacin and apramycin were purchased from Sigma-Aldrich. Antibiotics were dissolved in water according to the manufacturer's recommendations, filter sterilized, aliquoted into stock solutions, and stored at −20°C. MIC determinations were performed according to CLSI guideline M24 (41) and as described (39). Antibiotic stock solutions were prepared in CAMH broth to a concentration of 64 mg/L, and 2-fold serial dilutions in CAMH broth were prepared using sterile 96-well microtiter plates (Greiner Bio-One, Switzerland). A positive growth control lacking antibiotic and a sterile negative control containing CAMH broth only were included in each 96-well microtiter plate. For preparation of the inoculum, three or four colonies from a bacterial strain grown on LB agar were transferred, using a sterile cotton swab, into a tube containing 2 mL of NaCl. In order to achieve a final inoculum titer of 1×10^5 to 5×10^5 CFU/mL for MIC and 1×10^6 to 5×10^6 CFU/mL for MBC (see below), respectively, all bacterial suspensions were adjusted to turbidity equivalent to that of a 0.50 McFarland standard and subsequently diluted in CAMH broth. The final test volume in each well of the microtiter plate was 0.1 mL. The correct titer of each inoculum was checked by assessing CFU counts on LB agar plates. The microdilution plates were capped with adhesive sealing covers and incubated at 37°C for 3 days before the MIC values were assessed by visual inspection. All MIC assays were conducted in triplicate. The MIC was defined as the lowest antibiotic concentration that prevented visible bacterial growth.

Time-kill curves and MBC determinations. At the start of the experiment, the bacterial inoculum was determined by spotting 10-fold serial dilutions of the bacterial suspension from the no-drug control on agar plates and counting CFU. After 18 h and 36 h of incubation, bacterial cells from the MIC plates were resuspended by pipetting prior to spotting of 5-μl aliquots of 10-fold serial dilution series on LB agar plates. The agar plates were incubated for 96 h at 37°C, and CFU were counted. The relative CFU counts were adjusted to the inoculum at time zero. The MBC was defined as the lowest antibiotic concentration that reduced the CFU of the inoculum by ≥99.9%.

***In vivo* infection experiments.** Female SCID mice (Charles River Laboratories), 7 to 9 weeks of age, were infected by intravenous tail vein injection with 1×10^6 CFU/mouse of *M. abscessus* (strain 103, a clinical isolate from a CF patient) (42). Three mice were sacrificed at day 1 postinfection to determine bacterial manifestation prior to the start of treatment. Once-daily antibiotic treatment by subcutaneous injection was started 1 day after infection and continued for 8 consecutive days. The following doses were applied: amikacin, 150 mg/kg/day; apramycin, 150 mg/kg/day, 50 mg/kg/day, or 15 mg/kg/day. Saline served as a vehicle control. Treated mice were sacrificed at day 10 postinfection (including 8 days of antibiotic treatment). Whole lungs were extracted, homogenized in 4.5 mL of 1× phosphate-buffered saline (PBS), and plated in 10-fold serial dilutions on Middlebrook 7H11 agar. Plates were incubated for 7 days at 37°C prior to CFU counting.

The Colorado State University (CSU) animal care program follows the recommendations of the National Research Council *Guide for the Care and Use of Laboratory Animals* (43), the requirements of the Public Health Service (PHS) grants administration manual, and the Animal Welfare Act as amended. CSU files assurances with the DHHS Office of Extramural Research, Office of Laboratory Animal Welfare (OLAW), PHS, and adheres to NIH standards and practices for grantees. The CSU animal welfare assurance number is A3572-01.

Statistical analysis. Bacterial burdens in the untreated control and drug-treated animal organs were analyzed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA). *P* values were calculated using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test. Data are presented using the mean values ($n = 6$) ± the standard errors of the mean (SEMs). Significance was considered for *P* values of <0.05.

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E. C. Böttger and P. Sander conceived the study. D. J. Ordway, S. N. Hobbie, E. C. Böttger, and P. Sander designed the experiments. P. Selchow and A. Petrig conducted *in vitro* experiments. D. Verma and N. Whittel conducted animal experiments. E. C. Böttger and P. Sander wrote the manuscript, with input from all coauthors. The current version of the manuscript was approved by all authors.

S. N. Hobbie and E. C. Böttger are cofounders of and equity holders in Juvabis AG. The other authors have nothing to declare.

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