The Benzimidazole SPR719 Shows Promising Concentration-Dependent Activity and Synergy against Nontuberculous Mycobacteria

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ABSTRACT Nontuberculous mycobacterial pulmonary disease (NTM-PD) is emerging worldwide. Currently recommended multidrug treatment regimens yield poor outcomes, and new drugs and regimens are direly needed. SPR719, the active moiety of SPR720, is a new benzimidazole antibiotic with limited data on antimycobacterial activity. We determined MICs and MBCs against 138 clinical and reference strains of *M. avium* complex (MAC), *M. kansasii*, *M. abscessus*, *M. xenopi*, *M. malmoense*, and *M. simiae* and determined synergy with antimycobacterial drugs by checkerboard titrations. To study pharmacodynamics, we performed time-kill kinetics assays of SPR719 alone and in combinations against *M. avium*, *M. kansasii*, and *M. abscessus* and assessed synergy by response surface analysis according to Bliss independence. SPR719 showed potent activity against MAC (MIC₉₀, 2 mg/liter) and *M. kansasii* (MIC₉₀, 0.125 mg/liter) and modest activity against *M. abscessus* (MIC₉₀, 8 mg/liter); its activity is bacteriostatic and concentration-dependent. We recorded a potential for combination therapy with ethambutol against *M. kansasii* and *M. avium* and synergy with clarithromycin against *M. abscessus*. Ethambutol increased the SPR719 kill rate against *M. kansasii* but only prevented SPR719 resistance in *M. avium*. SPR719 is active *in vitro* against NTM; its activity is strongest against *M. kansasii*, followed by MAC and *M. abscessus*. SPR719 shows promise for combination therapy with ethambutol against MAC and *M. kansasii* and synergy with clarithromycin against *M. abscessus*. The parent drug SPR720 could have a role especially in MAC pulmonary disease treatment. Further studies in dynamic models and trials are ongoing to advance clinical development.

KEYWORDS *Mycobacterium*, *Mycobacterium avium*, antibiotic resistance, experimental therapeutics

Nontuberculous mycobacteria (NTM) are increasingly important opportunistic pathogens of humans. In patients with preexisting lung disease or local or systemic impaired immunity, they can cause chronic pulmonary disease (NTM-PD). Depending on underlying disorders, NTM-PD can manifest radiologically as a nodular-bronchiectatic disease or a more severe fibro-cavitary disease, akin to pulmonary tuberculosis (1). Of the 200 NTM species, the *M. avium* complex (MAC) and *M. abscessus* bacteria are the most frequent causative agents of NTM-PD (1).

NTM are intrinsically resistant to most classes of antibiotics; as a result, recommended treatment regimens are complex and toxic combinations of three to five antibiotics, often including intravenous agents. Even with long-term use of these aggressive regimens, cure rates for NTM-PD are as low as 60% for MAC (2) and 45% for *M. abscessus* pulmonary disease (3). New, more effective antibiotics and rational regimens, informed by *in vitro* activity, synergy, and pharmacokinetics/pharmacodynamics science, are direly needed (4).

SPR719 is a novel benzimidazole antibiotic that inhibits the ATPase activity of the

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TABLE 1 MIC range, MIC₅₀, and MIC₉₀ (in mg/liter) of SPR719 against nontuberculous mycobacteria^a

NTM species	MIC range (mg/liter)	MIC ₅₀ (mg/liter)	MIC ₉₀ (mg/liter)
MAC (n = 73)	0.06–4	1	2
<i>M. kansasii</i> (n = 21)	<0.03–0.25	<0.03	0.125
<i>M. abscessus</i> (n = 32)	1 to >32	2	8
<i>M. simiae</i> (n = 4)	2–8	NA	NA
<i>M. malmoense</i> (n = 3)	0.06–0.5	NA	NA
<i>M. xenopi</i> (n = 5)	0.06–0.5	NA	NA

^aMAC, *M. avium* complex; NA, not applicable.

DNA gyrase (encoded by the *gyrB* gene) in mycobacteria (5, 6). It showed low MICs against a wide range of mycobacteria, including both *M. tuberculosis* and NTM (5, 6).

To further substantiate its potential role in NTM-PD treatment, we assessed the activity and pharmacodynamics of SPR719 against the most important causative agents of NTM-PD in an *in vitro* pipeline that includes MIC/MBC tests, checkerboard synergy testing, and time-kill kinetics assays with response surface analysis for further synergy assessments.

RESULTS

Susceptibility and stability testing. The MIC ranges, MIC₅₀ and MIC₉₀ of SPR719 are shown in Table 1; MICs of individual isolates are presented in Table S1 in the supplemental material. SPR719 proved most active against *M. kansasii* (MIC₉₀ of 0.125 μg/ml) and least active against *M. abscessus* isolates (MIC₉₀ of 8 μg/ml; similar for all three subspecies). The MBC/MIC ratio for *M. abscessus* and *M. avium* was ≥8 (bacteriostatic) and for *M. kansasii* was 1 (bactericidal; Table S2). SPR719 proved stable in the microdilution assay; SPR719 is stable in cation-adjusted Mueller-Hinton broth (CAMHB), as MICs against *M. abscessus* after 14 days of preincubation were identical to those after immediate inoculation (Table S3).

Synergy testing. The mean fractional inhibitory concentration indexes (FICI) and ranges are recorded in Table 2; for results of individual isolates, see Table S4. All FICI values were between 0.5 and 4, interpreted as no interaction between combinations of SPR719 and other antimycobacterial drugs. The combination of SPR719 and ethambutol was most promising for MAC and *M. kansasii* isolates (mean FICI of 0.8 and FICI of 0.5 for individual isolates). For *M. abscessus*, the combinations of SPR719 and clarithromycin and tigecycline showed the lowest FICI values (mean FICI of 0.8 and FICI of 0.5 for individual isolates).

Time-kill kinetics. In the time-kill kinetics assay, SPR719 showed concentration-dependent activity against *M. avium* up to 1 × MIC (Fig. 1A). At concentrations ranging from 1 × MIC to 16 × MIC, SPR719 showed similar, 1 log₁₀ CFU, kill rates up to day 10. After 10 days of exposure, outgrowth of *M. avium* was recorded in a concentration-dependent fashion (i.e., higher SPR719 concentrations led to more delay in outgrowth). No colonies grew on agar plates supplemented with 5 × MIC of SPR719, except for the 0.125 × MIC and 0.25 × MIC conditions, which showed a 5 × MIC-tolerant subpopulation of 1% and 5%, respectively, at day 28 (data not shown).

SPR719 also demonstrated concentration-dependent activity against *M. kansasii*, which peaked at the 1 × MIC concentration with 1 log₁₀ CFU kill over 10 days; only at 16 × MIC did the killing effect last until day 28 (Fig. 1B). No tolerant subpopulation was present in any of the conditions over the duration of the experiment.

Against *M. abscessus*, SPR719 also showed concentration-dependent activity, most pronounced in the first 3 days and again in concentrations up to 1 × MIC (Fig. 1C). SPR719 inhibits growth by at least 1 log₁₀ CFU compared to the growth control. Thirty percent of the bacterial population exhibited tolerance to 5 × MIC SPR719 at the start of exposure (Fig. 1D). SPR719 concentrations up to 1 × MIC suppressed these populations; at higher concentrations, this subpopulation was enlarged.

TABLE 2 Fractional inhibitory concentration index (FICI) of SPR719 in combination with established antimycobacterial drugs against *M. abscessus*, MAC, and *M. kansasii*^a

Species	Mean FICI (range) of SPR719 combinations with:						
	CLR	CFZ	EMB	RIF	FOX	TIG	AMK
MAC (n = 10)	1.3 (1 to >2)	1.2 (0.8–1.5)	0.8 (0.5–1.5)	1.2 (0.6–1.5)	NR	NR	1.4 (1–1.5)
<i>M. kansasii</i> (n = 6)	1.5 (1–1.5)	NR	0.8 (0.5–1.5)	1.3 (0.5–1.5)	NR	NR	1.4 (0.6–1.5)
<i>M. abscessus</i> (n = 5)	0.8 (0.5–1)	1.1 (0.8–1.5)	NR	NR	1.4 (1–1.5)	0.8 (0.5–1)	1.5 (1.5)

^aMAC, *M. avium* complex; CLR, clarithromycin; CFZ, clofazimine; RIF, rifampicin; EMB, ethambutol; AMK, amikacin; TIG, tigecycline; FOX, ceftiofur; NR, not relevant to this species.

Time-kill kinetics assays of antibiotic combinations. The time-kill assay of SPR719 and ethambutol against *M. avium* shows that the combination of SPR719 and ethambutol has more effect than SPR719 alone but does not have a stronger killing effect than ethambutol alone (Fig. 2A). Outgrowth of *M. avium*, which occurred after 10 days of SPR719 exposure, was suppressed by the addition of ethambutol.

Time-kill curves of *M. kansasii* against SPR719 and ethambutol (EMB) show a stronger killing effect than these drugs alone (Fig. 2B). The kill rate is inversely concentration-dependent, as the lowest (0.5× MIC) combination concentration yields the highest kill rate, with bacterial loads below the detection limit at day 10.

SPR719 alone showed a slightly stronger growth-inhibiting effect against *M. abscessus* CIP104536 than clarithromycin alone (Fig. 2C). While not impacting the initial kill

FIG 1 (A to D) Dose-response time-kill kinetics of SPR719 against *M. avium* ATCC 700898 (MIC, 2 mg/liter) (A), *M. kansasii* ATCC 25221 (MIC, 0.06 mg/liter) (B), and *M. abscessus* CIP 104536 (MIC, 4 mg/liter) (C) with the percentage of the bacterial population resistant to 5× MIC SPR719 in the time-kill assay of *M. abscessus* CIP104536 (D). The x axis shows time; the y axis shows log₁₀ CFU/ml, mean and standard deviation, performed in duplicate.

FIG 2 (A) Time-kill kinetics of *M. avium* ATCC 700898 against SPR719 (MIC, 2 mg/liter) alone and in combination with ethambutol (MIC, 8 mg/liter). (B) Time-kill kinetics of *M. kansasii* ATCC 25221 against SPR719 (MIC, 0.06 mg/liter) alone and in combination with ethambutol (MIC, 8 mg/liter). (C) Time-kill kinetics of *M. abscessus* CIP104536 against SPR719 (MIC, 4 mg/liter) alone and in combination with clarithromycin (MIC, 0.5 mg/liter). The x axis shows time; the y axis shows log₁₀ CFU/ml, mean and standard deviation, performed in duplicate.

rate, the SPR719-clarithromycin combination showed more pronounced activity, especially beyond day 5.

Response surface analysis. The sigmoidal maximum effect ($E_{\max}$) curves for SPR719 on *M. avium*, *M. kansasii*, and *M. abscessus* are shown in Fig. S1A to C. For *M. avium*, the fitted $E_{\max}$ was 105.85 log₁₀ CFU/ml · day, the 50% effective concentration (EC_{50}) was 0.34 × MIC, and the estimated Hill slope was 1.11. For *M. kansasii*, the fitted $E_{\max}$ was 104.96 log₁₀ CFU/ml · day, the EC_{50} was 0.21 × MIC, and the estimated Hill slope was 4.08. For *M. abscessus*, the fitted $E_{\max}$ was 18.31 log₁₀ CFU/ml · day, the EC_{50} was 0.2 × MIC, and the estimated Hill slope was 1.96. The calculated difference between observed and expected effect (ΔE) percentages, as well as the observed effect ($E_{\text{comb,obs}}$), a combination under Bliss independence ($E_{\text{comb,BI}}$), and the effect sizes of the companion drugs in the combination time-kill (TK) assays are shown in Table 3.

Combinations of SPR719 with ethambutol against *M. avium* show no interaction, with ΔE clustering around 0%. The combination of SPR719 with ethambutol against *M. kansasii* was synergistic, which was most pronounced at lower concentrations. Though the maximum effect size of SPR719 against *M. abscessus* is low, combinations with clarithromycin proved synergistic with ΔE around 50% for all combinations, without a concentration-dependent trend.

DISCUSSION

SPR719 shows potent activity against nontuberculous mycobacteria and warrants further evaluation in pharmacodynamic models and clinical trials. As for most

TABLE 3 Response surface analysis results of time-kill data, as well as interaction effect size, by deviation from expected combination effect under Bliss independence in percent ΔE

Combination (concn)	Effect (log ₁₀ CFU/mL × day)				
	SPR719	Companion	Observed, combination	Expected, combination ^a	ΔE (%)
<i>M. avium</i>					
SPR719/ethambutol (2 × MIC)	88.1	165	116	116	0.49
SPR719/ethambutol (1 × MIC)	86.3	122	120	109	9.80
SPR719/ethambutol (0.5 × MIC)	84.0	98.9	100	104	−3.77
<i>M. kansasii</i>					
SPR719/ethambutol (2 × MIC)	101	184	128	108	19.0
SPR719/ethambutol (1 × MIC)	103	168	146	106	37.4
SPR719/ethambutol (0.5 × MIC)	109	94.3	184	105	74.8
<i>M. abscessus</i>					
SPR719/clarithromycin (2 × MIC)	23.1	8.44	34.7	20.9	65.9
SPR719/clarithromycin (1 × MIC)	22.6	5.42	31.5	21.4	47.6
SPR719/clarithromycin (0.5 × MIC)	14.0	4.63	23.7	15.1	56.6

^aUnder Bliss independence.

antimycobacterial drugs, its *in vitro* activity is strongest against *M. kansasii*, followed by MAC and then *M. abscessus* (7). This contrasts with the unmet clinical need, which is most grave for *M. abscessus* and MAC (1–4).

SPR719 shows promising *in vitro* activity against MAC isolates, and our MIC data confirm previously published MIC data (6). Its bacteriostatic activity is no real drawback, as the cornerstone of current treatment regimens is macrolides which are also bacteriostatic only (8). The checkerboard assay suggested synergy between SPR719 and ethambutol against *M. avium*. This could not be confirmed with time-kill kinetics (TKK) response surface analysis, in which this combination shows no interaction. However, the kill curves show that in the presence of ethambutol, killing by SPR719 is not necessarily enhanced, but lasts longer; ethambutol suppresses the emergence of SPR719-tolerant subpopulations. This interaction with ethambutol suggests that cell wall permeability is an important driver of SPR719 activity against *M. avium* (9) and is helpful for rational regimen design.

SPR719 proved bactericidal against *M. kansasii*, with very low MICs. In the time-kill experiments, the effect was dose-dependent, up to 1 $\times$ MIC, with a high maximum effect size, and there was evident synergy with ethambutol. *M. kansasii* disease is rare in most settings and readily cured by currently recommended treatment regimens (rifampicin and ethambutol with either isoniazid or a macrolide antibiotic) (10), but the promising *in vitro* activity of SPR719 suggests a possible role of SPR720 as a second-line agent for patients failing on or intolerant of currently recommended regimens.

SPR719 proved least active against *M. abscessus*, so rightfully dubbed an “antibiotic nightmare” for its intrinsic resistance to most classes of antibiotics (11). In the TK assay, SPR719 showed modest inhibition of growth only. This is partially explained by the observation that 30% of the unexposed bacterial population was already tolerant of SPR719 at a 5 $\times$ MIC concentration (Fig. 1D). SPR719 enhances the activity of the macrolides by preventing the emergence of tolerance. Still, SPR720 is unlikely to have a role in treatment of *M. abscessus* pulmonary disease, except perhaps in the oral continuation phase after rigorous preclinical and clinical evaluation (10, 12). Its modest *in vitro* activity and synergistic potential resemble those of clofazimine, which is now recommended as part of *M. abscessus* treatment regimens (1, 10, 12, 13).

This study included a large set of reference and clinical NTM isolates of most clinically relevant species and applied time-kill kinetics and multimodal assessments of

synergy for SPR719. Ongoing studies of intracellular activity in dynamic models such as the hollow fiber model and animal models of NTM disease provide additional support for the continued development of SPR720. Additionally, human pharmacokinetic and safety data have been gathered in a recently completed phase 1 trial (ClinicalTrials.gov; study ID NCT03796910), and a phase 2A clinical trial (ClinicalTrials.gov; study ID NCT04553406) on safety, tolerability, pharmacokinetics, and efficacy in MAC pulmonary disease has recently started recruiting.

In conclusion, SPR719 is active *in vitro* against nontuberculous mycobacteria; its activity is strongest against *M. kansasii*, followed by MAC and *M. abscessus*. SPR719 shows potential for combination therapy with ethambutol against MAC and *M. kansasii* and synergy with clarithromycin against *M. abscessus*; in *M. avium*, the combination of SPR719 and ethambutol suppresses the emergence of SPR719-tolerant subpopulations. SPR720 has the most potential in the treatment of *M. avium* complex pulmonary disease. Based on the current *in vitro* findings, replacing rifampicin in the current rifampicin-ethambutol-macrolide regimens (10, 12) with SPR720 is a logical strategy for a clinical trial.

MATERIALS AND METHODS

Strains and compounds. The reference strains *M. avium* ATCC 700898, *M. intracellulare* DSM43223, *M. chimaera* DSM44623, *M. kansasii* ATCC 25221, *M. xenopi* ATCC 19250, *M. simiae* ATCC 252275, and *M. abscessus* CIP 104536 were purchased from their respective strain collections and freshly cultured for all experiments. Clinical isolates were obtained from the department of Medical Microbiology, Radboud University Medical Center, the Netherlands.

SPR719 was kindly provided by Spero Therapeutics (Cambridge, MA, USA). Rifampicin, ethambutol, amikacin, clarithromycin, cefoxitin, and clofazimine were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands, and St. Louis, MO, USA). Components were reconstituted in water, except for clarithromycin and clofazimine, which were dissolved in acetone and DMSO, respectively. Tigecycline was obtained from Pfizer (Capelle aan den IJssel, the Netherlands), as Tygacil powder for infusion and dissolved in water.

Susceptibility and stability testing. MICs of SPR719 were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB; BD Bioscience, Drachten, the Netherlands) according to CLSI guidelines (14). We tested concentrations ranging from 0.03 to 32 mg/liter.

SPR719 MICs were determined for reference and clinical strains of *M. avium* (51 clinical isolates), *M. intracellulare* (9 clinical isolates), *M. chimaera* (10 clinical isolates), *M. kansasii* (20 clinical isolates), *M. abscessus* (31 clinical isolates; 15 *M. abscessus* subsp. *abscessus*, 3 *M. abscessus* subsp. *bolletii*, 13 *M. abscessus* subsp. *massiliense*), *M. malmoense* (3 clinical isolates), *M. simiae* (3 clinical isolates), and *M. xenopi* (4 clinical isolates).

MBCs were determined for all reference strains stated above by quantification of log₁₀ CFU/ml on Middlebrook 7H10 agar plates (Becton, Dickinson and Company, Drachten, the Netherlands) for SPR719 concentrations that showed no visible growth in microdilution; the MBC was defined as the lowest concentration that yielded no visible growth. We defined bactericidal activity as an MBC/MIC ratio of <8 and bacteriostatic activity as a ratio of ≥8 (15).

We tested SPR719 stability by preincubating broth microdilution assay plates for 7 and 14 days at 30°C before inoculating them with *M. abscessus* CIP104536.

Synergy testing. Synergy between SPR719 and key antimycobacterial drugs was assessed using checkerboard microdilution assays against clinical and reference strains of MAC (*n* = 10), *M. kansasii* (*n* = 6), and *M. abscessus* (*n* = 5). SPR719 was evaluated in combination with amikacin, clarithromycin, clofazimine, ethambutol, and rifampicin for MAC and *M. kansasii* and with amikacin, clarithromycin, clofazimine, cefoxitin, and tigecycline for *M. abscessus*. All synergy tests were performed in CAMHB; only clofazimine synergy tests were performed in Middlebrook 7H9, as clofazimine is insoluble in CAMHB (13).

Drug interactions were interpreted according to the fractional inhibitory concentration index (FICI) calculated with the formula $FICI = (MIC_A^{combi}/MIC_A^{alone}) + (MIC_B^{combi}/MIC_B^{alone})$. We defined synergy as a FICI of ≤0.5, no interaction as FICI between 0.5 and 4.0, and antagonism as FICI of >4 (16).

Time-kill kinetics. To determine the pharmacodynamics of SPR719 alone and in combination with the antibiotics that proved most synergistic in checkerboards assays, we performed time-kill kinetics (TKK) assays for SPR719 against reference strains of *M. avium*, *M. abscessus*, and *M. kansasii*, in duplicate, using previously published methods (13, 17). In brief, bacteria were cultured in bottles with 10 ml CAMHB (supplemented with 0.05% Tween 80 and, for *M. avium* and *M. kansasii*, 10% oleate-bovine albumin-dextrose-catalase [OADC]) with SPR719 in concentrations ranging from 0.125× to 16× the MIC for the dose-response experiment and 0.5× MIC to 2× MIC each of SPR719 and the companion antibiotic for the combination experiments. The bacterial population size, expressed in CFU/ml, was determined by culture on Middlebrook 7H10 agar after 0, 1, 2, 3, 4, 5, 7, 10, and 14 days of incubation for *M. abscessus* and after 21 and 28 days for *M. avium* and *M. kansasii* (17).

To assess the size of the SPR719-resistant *M. avium*, *M. kansasii* and *M. abscessus* population in the TKK assays, we plated samples on Middlebrook 7H10 (M7H11) agar plates containing 5× MIC of SPR719. M7H11 plates were prepared from powder (Sigma-Aldrich), with 10% OADC supplementation and SPR719 added after autoclaving. M7H11 plates were read after a maximum of 10 days incubation at 30°C for *M. abscessus* and 21 days at 37°C for *M. avium* and *M. kansasii*.

Response surface analysis. To assess the synergy according to Bliss independence from the time-kill curves, we performed response surface analysis as previously described (18). The area under the concentration-time curve (AUC) was calculated from the log CFU over time plots using the trapezoidal rule after averaging the result from the two replicates and normalizing to the baseline colony count. The effect was then calculated according to $\text{Effect}_X = \text{AUC}_{\text{Growth control}} - \text{AUC}_X$, where X is any given curve other than the growth control.

To assess potential interactions in the TK experiments, we calculated the expected effect for a combination under Bliss independence ($E_{\text{comb,Bl}}$) to be compared with the observed effect ($E_{\text{comb,obs}}$). Since Bliss independence builds on probability theory, the maximum effect to be evaluated is limited to 1, and therefore all effects were normalized to the E_{max} of SPR719. After simplification, this gives the following formula: $E_{\text{comb,Bl}} = E_A + E_B - (E_A \cdot E_B)/E_{\text{maxhigh}}$, where E_A and E_B are the effect sizes of drug A or B separately and E_{maxhigh} is the highest maximum effect. The E_{max} of SPR719 was determined by fitting a sigmoidal E_{max} model to the concentration-response data using ordinary least squares.

The difference between observed and expected effect (ΔE) was quantified as a percentage difference relative to the expected, $\Delta E = (E_{\text{comb,obs}} - E_{\text{comb,Bl}})/E_{\text{comb,Bl}}$. We defined a ΔE of $0\% \pm 10\%$ as no interaction; ΔE of less than -10% was defined as antagonistic, and ΔE of $>10\%$ was defined as synergistic (17).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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