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Antimicrobial susceptibility and ribotypes of *Clostridium difficile* isolates from a Phase 2 clinical trial of ridinilazole (SMT19969) and vancomycin

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Objectives: We evaluated the antimicrobial susceptibility and ribotypes of *Clostridium difficile* isolates from participants in a Phase 2 study of ridinilazole, a novel targeted-spectrum agent for treatment of *C. difficile* infection.

Methods: Participants received ridinilazole (200 mg twice daily) or vancomycin (125 mg four times daily) for 10 days (ClinicalTrials.gov: NCT02092935). The MICs of ridinilazole and comparators for *C. difficile* isolates from stool samples were determined by agar dilution. Toxin gene profiling was performed by multiplex PCR and ribotype identification by capillary electrophoresis.

Results: Eighty-nine isolates were recovered from 88/100 participants (one participant had two strains at baseline). The median colony count (cfu/g stool) was 1.9×10^4 (range: 2.5×10^2–7.0×10^6). Twelve participants (three received ridinilazole and nine received vancomycin) experienced recurrence, confirmed by immunoassays for free toxin in stool samples. The ribotype of eight out of nine isolates obtained at recurrence matched those of the initial isolates. All isolates, including those obtained at recurrence, were susceptible to ridinilazole within the expected range [median (range) MIC: 0.12 (0.06–0.5) mg/L]. The median (range) vancomycin MIC was 1 (0.5–4.0) mg/L. At baseline, 13.6% and 13.3% of samples in the ridinilazole and vancomycin groups were positive for VRE, increasing to 23.7% and 29.7% by day 40, respectively. Common ribotypes included 014-20 (14 isolates), 027 (13), 106 (7), 002 (7), 078-126 (4), 001 (4), 087 (3) and 198 (3). Toxin gene profiling of nearly all baseline isolates (98.9%) revealed a binary toxin gene (*cdtA/cdtB*) prevalence of 35%.

Conclusions: Ridinilazole potently inhibited recovered *C. difficile* isolates. Recurrence was not associated with altered susceptibility.

Introduction

Clostridium difficile infection (CDI) is a leading cause of morbidity and mortality and has seen a significant increase in global incidence, driven partly by the emergence of fluoroquinolone-resistant NAP1 (ribotype 027) strains.[1,2] Although the current mainstay antibiotics, vancomycin and metronidazole, are generally effective at treating initial infection, both agents are associated with unacceptably high rates of recurrent disease,[3] with each recurrent episode being associated with increased morbidity and mortality as well as a heightened economic burden.[4,5] Approximately 30% of patients experience a repeat infection following an initial episode, and the risk of recurrence doubles following a third episode.[6]

Ridinilazole (formerly SMT19969) is a novel targeted-spectrum oral antimicrobial under development for the treatment of CDI and for reducing the recurrence of CDI.[7] Through fluorescent labelling, confocal microscopy and scanning electron microscopy studies, ridinilazole has been shown to impact cell division and septum formation.[7,8] Preclinical efficacy studies have demonstrated its narrow spectrum of activity and potent bactericidal effect against a variety of clinical isolates of *C. difficile*, including less frequently isolated strains and isolates with varying antimicrobial resistance phenotypes.[9–13] Ridinilazole has been shown in a hamster model to be effective both at treating primary infections and preventing recurrent infections.[14] A Phase 1 study in healthy volunteers demonstrated single or multiple doses of ridinilazole to be safe and

well tolerated, with negligible plasma concentrations and minimal impact on normal gut microbiota.[15] A recent Phase 2 proof-of-concept clinical study compared the efficacy and safety of ridinilazole with that of vancomycin and demonstrated it to be non-inferior (15% margin) with regard to the primary efficacy measure, sustained clinical response (SCR), defined as clinical cure (≤3 unformed bowel movements within a 24 h period) at the test of cure (TOC) visit and lack of recurrence within 30 days of the end of treatment.[16] Statistical superiority at the pre-specified 10% level was also demonstrated with ridinilazole. Improved SCR rates persisted across patient subgroups based on age, baseline severity, history of recurrence and use of concomitant antibiotics at baseline. Moreover, ridinilazole was well tolerated and had an adverse event profile similar to that of vancomycin. We now report the microbiological findings of this Phase 2 trial, focusing on the antimicrobial susceptibility and ribotyping of isolates from participants with primary and recurrent infections.

Patients and methods

Study design

This Phase 2, double-blind, randomized, active-controlled, parallel group design study (ClinicalTrials.gov: NCT02092935) was conducted at 33 centres in the USA and Canada, with study sites primarily consisting of hospitals and outpatient clinics.[16]

Ethics

Institutional review boards at each centre provided ethics approval. Ethical principles as set forth in the Declaration of Helsinki and all principles of good clinical practice were complied with. Written informed consent was obtained from all participants.

Study procedures

One hundred participants randomized in a 1:1 ratio received either ridinilazole (200 mg orally twice daily) or vancomycin (125 mg orally four times daily) for 10 days. A complete description of all study procedures has been published previously.[16]

Sample collection

Faecal samples collected at baseline, on days 5, 10, 25 and 40, and at recurrence were used for the culture and isolation of *C. difficile* vegetative cells and spores. Quantitative counts of spores and vegetative cells were conducted on all vegetative isolates and germinated spores as detailed below. Isolates underwent susceptibility testing against ridinilazole, vancomycin, fidaxomicin, metronidazole and other comparators. Isolates at baseline and at recurrence were ribotyped by capillary electrophoresis and were subjected to toxin gene profiling by multiplex PCR to detect the presence or absence of *tcdA*, *tcdB* and *cdtA/B* as well as *tcdC* deletions (see methods below).

C. difficile isolation

Stool samples were diluted and plated on pre-reduced selective medium cycloserine–cefoxitin–fructose agar.[17,18] Plates were incubated for 48 h in an anaerobic chamber (5% CO_2/10% H_2/85% N_2) at 35±2°C and observed for characteristic growth and colonial morphology. Colonies that were ∼4 mm in diameter, yellow and had a ground-glass appearance were enumerated, with colony counts being reported as cfu/g stool. To determine the spore count, the stool sample was ethanol shocked prior to dilution and plated on cycloserine–cefoxitin–fructose agar with lysozyme.[19] After enumeration, representative colonies were subcultured onto anaerobic blood agar (CDC) for further identification and preparation of frozen stock culture. A proline disc test (Remel Products, Lenexa, KS, USA) and Gram stain were performed.[20] Isolates that were proline-positive, Gram-positive bacilli were further identified using rapid methodology with the API20A system (bioMérieux Inc., Durham, NC, USA). Identified isolates were kept frozen in skim milk at −80°C for susceptibility testing and/or reference.

Susceptibility testing

Susceptibilities of the isolates were assessed against a panel of antimicrobial agents that included ridinilazole, fidaxomicin, vancomycin, metronidazole, moxifloxacin, clindamycin, tigecycline, rifaximin, rifampicin, linezolid, imipenem and chloramphenicol. Susceptibility was determined by agar dilution methodology as described in CLSI M11-A8.[21,22] Inocula were prepared using direct colony suspension to achieve a turbidity equivalent to that of a 0.5 McFarland standard (∼10^7 cfu/mL for *C. difficile*). The antibiotic-containing plates were prepared on the day of the test. A Steer replicator was used to inoculate the agar plates, resulting in a deposit of ∼10^4 cfu on the surface of the agar. The plates were incubated at 35±2°C in an anaerobic chamber (5% CO_2/10% H_2/85% N_2) for 48 h.[21] The following reference organisms were included with each susceptibility testing run: *C. difficile* ATCC 700057, *C. difficile* ATCC 43255, *Bacteroides thetaiotaomicron* ATCC 29741 and *Staphylococcus aureus* ATCC 29213. Tests were repeated when the MICs of the control organisms were outside of the CLSI acceptable range.

PCR- and capillary electrophoresis-based ribotyping

Templates for amplicon generation were obtained by growing *C. difficile* on anaerobic blood agar medium in an atmosphere of 5% CO_2/10% H_2/85% N_2 at 37°C for 48 h and then transferring 3–5 colonies to 200 μL of 10% Chelex 100 in 10 mM Tris–HCl/1 mM EDTA (pH 8) buffer. Samples were boiled for 15 min and centrifuged briefly (5 min at 3400 rpm). Template DNA was then diluted 1:10 in 10 mM Tris–HCl/1 mM EDTA (pH 8) buffer and stored at −20°C until ready for use. Ribotyping was then performed as previously described.[23] Amplicons were stored at −20°C until ready to ship for fragment analysis. Plates were sent to the University of Michigan sequencing core facility for capillary electrophoresis (http://seqcore.brcf.med.umich.edu). Ribotypes were assigned as previously described,[23] using an online analysis tool at the Walk Laboratory at Montana State University (http://the walklab.com/tools).[24] This database can identify 116 distinct *C. difficile* ribotypes and is cognate with the ribotyping database in the UK (Leeds Reference Laboratory) for 35 of the most clinically relevant ribotypes based on prevalence in the USA.

Toxin gene profiling

Using template DNA as isolated above for ribotyping, toxin gene profiles for *C. difficile* were determined using PCR methodology as described by Persson *et al.*[25,26] Isolates were tested in duplicate and non-concordant tests were repeated. The following controls were included with each test: (i) a non-toxigenic strain of *C. difficile* VPI 11186 (ATCC 700057), with a PCR profile of *tcdA*−, *tcdB*−, *cdtA*−/*B*−, *tcdC*−, in which the entire PaLoc is absent and no functional *cdt* locus is present;[27] (ii) *C. difficile* VPI 10463, with a PCR profile of *tcdA*+, *tcdB*+, *cdtA*−/*cdtB*−, with no detected deletion in the *tcdC* region;[28,29] and (iii) *C. difficile* R20291, with a PCR profile of *tcdA*+, *tcdB*+, *cdtA*+/*B*+, with an 18 bp deletion in the *tcdC* hypervariable region.[30] Loss of PaLoc was confirmed in non-toxigenic strains by PCR performed using Lok1/Lok3 primers as previously described.[31]

Table 1. Isolation of *C. difficile* vegetative cultures and spores over time comparing ridinilazole and vancomycin treatment groups

	Vegetative cultures					Spores				
				$\log_{10}$ cfu/g stool					$\log_{10}$ cfu/g stool	
timepoint	number of samples	number positive	% *C. difficile* isolated	range	median	number of samples	number positive	% *C. difficile* isolated	range	median
Ridinilazole treatment group										
day −1	44	43	97.7	≤2.4–6.45	4.28	44	19	43.2	2.40–6.86	4.66
day 5	41	5	12.2	2.70–5.36	3.85	41	7	17.1	2.88–5.51	4.11
day 10	41	3	7.3	2.52–5.99	3.77	41	5	12.2	2.90–6.38	4.32
day 25	41	3	7.3	3.23–5.18	3.70	41	12	29.3	2.66–5.72	3.55
day 40	38	3	7.9	3.96–4.48	4.38	38	7	18.4	2.49–5.26	3.92
recurrence	3	3	100.0	3.56–4.20	3.72	3	0	0	all ≤2.4	NA
Vancomycin treatment group										
day −1	45	45	100.0	≤2.4–6.85	4.26	44	16	36.4	2.43–6.98	4.38
day 5	42	7	16.7	3.32–5.28	4.60	42	5	11.9	3.45–6.04	4.91
day 10	42	4	9.5	2.56–4.18	3.64	41	4	9.8	3.23–6.23	3.61
day 25	38	5	13.2	≤2.4–4.59	3.08	38	15	39.5	≤2.4–6.36	3.96
day 40	37	4	10.8	3.08–5.85	4.57	36	11	30.6	≤2.4–5.32	3.96
recurrence	6[a]	5	83.3	3.63–5.85	4.20	5[b]	4	80	2.72–5.67	4.98

NA, not applicable.
[a]There were nine recurrences, but only six recurrence samples were available for testing of vegetative cultures.
[b]There were nine recurrences, but only five recurrence samples were available for spore testing.

Identification and characterization of VRE

Samples were plated on selective-medium bile esculin azide agar with vancomycin (6 mg/L) for the isolation of VRE.[32,33] Cultures were incubated at 35°C for up to 72 h in an atmosphere of 7% CO_2 and then examined for a dark brown to black colour, indicating a positive esculin reaction. All esculin-positive, Gram-positive cocci were subcultured onto tryptic soy agar (Remel, Lenexa, KS, USA) for identification and preparation of frozen stock culture. A catalase test was then performed on all α- or non-haemolytic isolates. A test for confirmation of pyroglutamyl (PYR) aminopeptide activity and a test for confirmation of esculin hydrolysis (Visi-spot discs, Thermo Scientific Inc., Waltham, MA, USA) were performed on catalase-negative colonies. The PYR-positive isolates were presumptively identified as enterococci. Isolates were stored at −80°C for reference. Susceptibility of the enterococcal isolates to vancomycin was determined by agar dilution methodology as described in CLSI document M07-A10.[34]

Results

Vegetative isolates

Table 1 shows the rate of isolation and colony counts by treatment group of *C. difficile* isolated from stool at study entry (day −1), days 5, 10, 25 and 40, and at recurrence. Of the 100 randomized participants, 89 provided a baseline sample and *C. difficile* was successfully isolated from 43 participants at baseline in the ridinilazole group (2 strains from 1 participant) and 45 participants in the vancomycin group, for a total of 89 strains. Median counts and ranges at baseline were virtually identical in each arm. During the course of study drug dosing, there was a rapid decline in the proportion of participants from whom *C. difficile* could be isolated. By day 5, 12.2% of samples from the ridinilazole-treated participants had *C. difficile* recovered, compared with 16.7% of samples from vancomycin-treated participants. Day 5 median colony counts from the ridinilazole-treated participants from whom *C. difficile* was recovered were 3.85 $\log_{10}$ cfu/mL, compared with 4.60 $\log_{10}$ cfu/mL in the vancomycin arm. At day 40, 7.9% of the ridinilazole-treated participants had *C. difficile* isolated, compared with 10.8% of the vancomycin-treated participants.

Spore isolation

Data on isolation of *C. difficile* spores are also shown in Table 1. At baseline, 43.2% of samples from ridinilazole-treated participants had spores isolated, compared with 36.4% of samples from vancomycin-treated participants. The median counts were similar, although participants in the ridinilazole arm had marginally higher spore counts at baseline (4.66 versus 4.38 $\log_{10}$ cfu/mL for ridinilazole and vancomycin, respectively). The proportion of samples with detectable spore counts declined dramatically over the time of treatment to 12.2% and 9.8% on day 10 for the ridinilazole and vancomycin arms, respectively. By day 25 the proportion of spores increased in both arms (29.3% and 39.5% for ridinilazole and vancomycin, respectively). At day 40 the proportion of participants in the ridinilazole arm who had spores detected was 18.4%, compared with 30.6% in the vancomycin arm ($P = 0.10$).

Antimicrobial susceptibility

Table 2 presents the MICs of both study drugs and selected comparators for the *C. difficile* isolates recovered at baseline and at recurrence by treatment group for four selected agents. The distribution of MICs for ridinilazole was narrow, with an MIC_{90} of 0.25 mg/L and a range of 0.06–0.5 mg/L, consistent with the previously reported susceptibility of *C. difficile* to ridinilazole.[10,12,13] The MIC_{90} for vancomycin was 2 mg/L, with a range of 0.5–4 mg/L. In Table S1 (available as

Table 2. Susceptibility of *C. difficile* isolates at baseline and at recurrence by treatment arm

	n	MIC (mg/L)	Ridinilazole	Vancomycin	Metronidazole	Fidaxomicin
Ridinilazole group vegetative isolates						
day −1	44	MIC_{50}	0.12	1	0.5	0.12
		MIC_{90}	0.25	2	2	0.5
		range	0.06–0.5	1–4	0.12–4	0.06–1
recurrence	3	range	0.12–0.25	2–4	0.25–2	0.12–0.5
Vancomycin group vegetative isolates						
day −1	45	MIC_{50}	0.12	1	0.25	0.25
		MIC_{90}	0.5	2	1	0.5
		range	0.06–0.5	0.5–2	0.12–2	0.06–1
recurrence	5	MIC_{50}	0.12	1	0.25	0.25
		range	0.12–0.5	1–2	0.12–0.5	0.12–0.5

Table 3. Comparison of ribotype profiles of baseline isolates in the ridinilazole and vancomycin arms

Ribotype	Ridinilazole (*N* = 44), *n* (%)	Vancomycin (*N* = 45), *n* (%)	Total (*N* = 89), *n* (%)
014-020	8 (18.2)	6 (13.3)	14 (15.7)
027	8 (18.2)	5 (11.1)	13 (14.6)
106	2 (4.5)	5 (11.1)	7 (7.9)
002	4 (9.1)	3 (6.7)	7 (7.9)
001	0 (0.0)	4 (8.9)	4 (4.5)
078-126	0 (0.0)	4 (8.9)	4 (4.5)
Novel	2 (4.5)	2 (4.4)	4 (4.5)
087	3 (6.8)	0 (0.0)	3 (3.4)
198	2 (4.5)	1 (2.2)	3 (3.4)
Singletons (observed once)	15 (34.1)	15 (33.3)	30 (33.7)

Supplementary data at *JAC* Online) we list the susceptibility of all isolates against all agents tested.

Ribotyping of baseline isolates

The distribution of ribotypes of isolates obtained at baseline is shown in Table 3, which presents the data by treatment arm. Ribotype 014-020 was most commonly seen, with eight isolates in the ridinilazole arm and six in the vancomycin arm belonging to this ribotype. Ribotype 027 was seen in 13 of the enrolled participants. There was a slightly higher proportion of 027 in the ridinilazole arm [8/44 (18%)] compared with the vancomycin arm [5/45 (11%)]. There was differential distribution of certain ribotypes between the vancomycin and ridinilazole arms: ribotype 001 (four in the vancomycin arm versus zero in the ridinilazole arm), 078-126 (four versus zero) and 106 (five versus two). Four patients (two in each arm) had ribotypes novel to our database (see the Patients and methods section) and there were 19 different ribotypes that were isolated only once, for a total of 37 different ribotypes.

Toxin gene profiles

The toxin gene profiles by proportion at the time of diagnosis are shown in Figure 1. The most common toxin profiles observed were *tcdA tcdB* without (0 bp) any deletion in *tcdC* and *tcdA tcdB cdtA/B* with an 18 bp deletion in *tcdC*. The proportions were similar between the ridinilazole and vancomycin arms. There were two non-toxigenic strains, both of which were seen in the vancomycin arm. This may be due to infection with multiple strains, which has been shown to occur in 16% of CDI cases.[35] Notable toxin profiles were a strain with no *tcdA* signal and six strains with ∼39 bp deletions in *tcdC*, four of which belonged to ribotype 078-126. There were no strains with only binary toxin, *tcdB* deletions or *tcdC* with ∼54 bp deletions.

Identification and characterization of VRE

The rates of isolation of VRE at baseline were comparable in each treatment arm, with 13.6% and 13.3% of samples from ridinilazole- and vancomycin-treated participants being positive for VRE. Over time, the proportion of patients in whom VRE could be recovered increased in both arms, with 23.7% and 29.7% recovered at day 40 in the ridinilazole and vancomycin arms, respectively.

Recurrence isolates

A total of 12 participants (3 received ridinilazole and 9 received vancomycin) had a recurrence of CDI as diagnosed by a positive free-toxin enzyme immunoassay, with 9 of these participants providing a stool sample (3 received ridinilazole and 6 received vancomycin). Vegetative *C. difficile* was successfully isolated from samples from 8 of the 9 participants with recurrence from whom samples were available (3 received ridinilazole and 5 received vancomycin) (Table 1). Colony counts at the time of recurrence were similar to the baseline counts. All vegetative isolates recovered at recurrence maintained susceptibility to ridinilazole comparable with that of the baseline isolates. Of the 8 participants with strains isolated at baseline and at recurrence, ribotypes and toxin gene profiles were the same except for one ridinilazole-treated participant, in whom a recurrence isolate belonged to a novel ribotype (the baseline ribotype was 014-020).

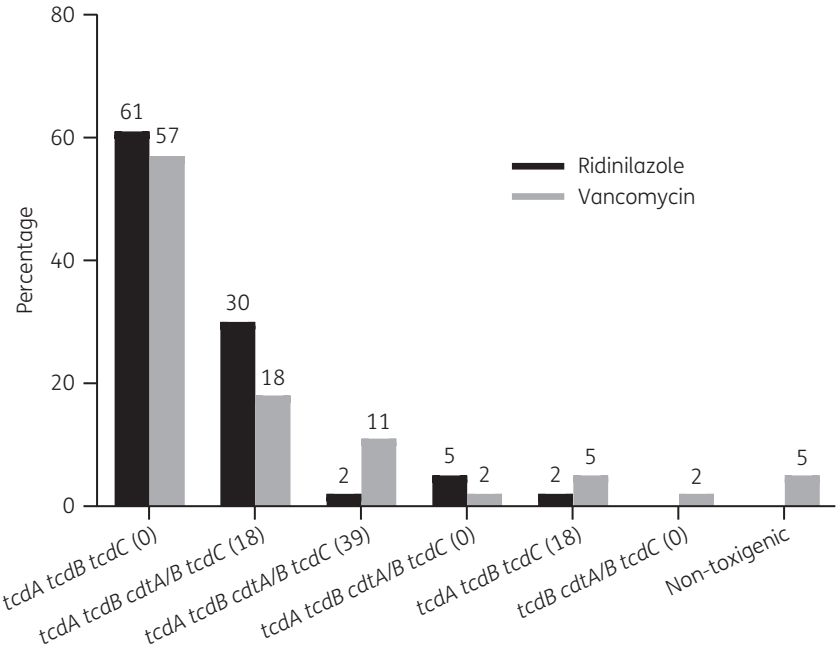

Figure 1. Percentage of toxin gene profiles at study entry by treatment group. Values in parentheses refer to the length of nucleotide base pairs missing/deleted in *tcdC*.

No spores could be isolated from the ridinilazole-treated participants at the time of recurrence, whereas four of five participants in the vancomycin arm had spores detected ($P = 0.07$; Fisher's exact test). One participant had insufficient sample to detect spores.

Discussion

In this small Phase 2 study, in which the two treatment groups were evenly matched at baseline from a microbiological perspective, *C. difficile* isolates obtained at baseline and at multiple timepoints during follow-up remained susceptible to both ridinilazole and vancomycin. There was no evidence for development of resistance to ridinilazole during the course of therapy and there was an absence of cross-resistance to ridinilazole and the other antimicrobial agents tested.

Ribotypes were evenly distributed between the two treatment groups. The overall percentage of patients with 027 (14.6%) was lower than that seen in most centres in a recent multicentre study in North America,[36] and may reflect a declining incidence, as was reported in England and the USA.[37,38] The exact reasons for this decline in incidence of 027 remain unclear. There was a wide array of ribotypes seen among participants enrolled in the trial, including a number of novel ribotypes. Where known, the majority of participants with recurrence were seen to have the same ribotype at recurrence as they did at baseline, suggesting that most recurrences were relapses of the initial infection rather than reinfection.

There were no significant differences between ridinilazole- and vancomycin-treated participants with respect to reductions in vegetative and spore counts during the course of therapy. At day 40 and at recurrence we noted only a small number of patients in the ridinilazole arm from whom spores could be isolated. This was unexpected, but with the small number of subjects studied, firm conclusions regarding this finding cannot be drawn. In addition, there were no significant differences in the presence or absence of VRE during therapy, although there was a trend towards an increasing presence of VRE in the post-dosing period in vancomycin-treated participants compared with ridinilazole-treated participants.

In summary, this study demonstrates the potent *in vitro* activity of the novel antimicrobial agent ridinilazole against *C. difficile*, with no emergence of resistance during treatment. Further clinical development of ridinilazole for the treatment of CDI is warranted.

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Transparency declarations

D. R. S. has received grants from Summit Therapeutics Plc, Actelion Pharmaceuticals and Merck, and is a consultant for Merck, Takeda and Sequiris. C. M. T. is a consultant for Summit Therapeutics Plc. R. J. V. is an employee of, and holds share options with, Summit Therapeutics Plc. All other authors: none to declare.

Medical writing assistance was provided by Prasad Kulkarni and Alexandra Rayser of the Healthcare Alliance Group, Voorhees, NJ, USA.

Author contributions

D. R. S. was involved in conceptualization of the study, funding acquisition, data analysis, project administration and writing of the manuscript. L. A. M. was involved in conceptualization of the study, data analysis and writing of the manuscript. C. M. T. was involved in conceptualization of the study, funding acquisition, data analysis, project administration and writing of the manuscript. J. C. and J. W. were involved in the investigation, methodology and writing of the manuscript. S. T. W. was involved in conceptualization of the study, data analysis and writing of the manuscript. R. J. V. was involved in conceptualization of the study, funding acquisition, data analysis and writing of the manuscript. All authors reviewed the manuscript and approved its submission for publication.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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