

In Vitro and In Vivo Activities of the Novel Ketolide RBx 14255 against *Clostridium difficile*

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The MIC₉₀ of RBx 14255, a novel ketolide, against *Clostridium difficile* was 4 µg/ml (MIC range, 0.125 to 8 µg/ml), and this drug was found to be more potent than comparator drugs. An *in vitro* time-kill kinetics study of RBx 14255 showed time-dependent bacterial killing for *C. difficile*. Furthermore, in the hamster model of *C. difficile* infection, RBx 14255 demonstrated greater efficacy than metronidazole and vancomycin, making it a promising candidate for *C. difficile* treatment.

Clostridium difficile is a Gram-positive spore-forming anaerobic bacterium that causes a range of infections from mild and self-limiting diarrhea to life-threatening pseudomembranous colitis and death (6, 7). *C. difficile* spores excreted from infected patients are prevalent in hospitals and health care facilities and can persist in the lower intestinal tract of human beings (6, 16). The use of broad-spectrum antibiotics causes disruption of the normal gut flora and predisposes the gut to *C. difficile* intestinal colonization (11, 12). During the past decade, there has been a striking increase in the number of cases of *C. difficile* infection (CDI) due to the emergence of epidemic strains such as the *C. difficile* B1/NAP/027 strain (3, 5).

Metronidazole and vancomycin are the drugs of choice to treat CDI patients. Although both drugs are used for treating the infection, they have shortcomings, including unacceptably high rates of recurrence of infection (15 to 35%) (2, 18). The drawbacks of current CDI therapy have necessitated the search for new and improved therapies.

Ketolides belong to a novel class of macrolide antibiotics with improved antibacterial activity against macrolide-resistant organisms (17). In this study, we have investigated for the first time the activity of RBx 14255, a novel ketolide (Fig. 1), against *C. difficile* *in vitro* and in a hamster model of CDI and identified a prospective candidate for the treatment of *C. difficile* infection.

The bacterial strains included in this study were from the Ranbaxy Research Laboratories (Gurgaon, India) culture collection, the American Type Culture Collection (ATCC; Manassas, VA), the National Collection of Type Cultures (London, United Kingdom), and a tertiary care medical center (PGIMER, Chandigarh, India). The BI/NAP1/027-type strain 2009155 (epidemic strain) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). Reference anaerobe strains *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were also included when performing antibiotic susceptibility assays.

Erythromycin, clarithromycin, vancomycin, and metronidazole were purchased from Sigma (St. Louis, MO). Telithromycin and clindamycin phosphate were obtained from a commercial source. RBx 14255 (Fig. 1) was synthesized at Ranbaxy Research Laboratories, Gurgaon, India.

MICs of RBx 14255, telithromycin, erythromycin, clarithromycin, vancomycin, metronidazole, and clindamycin were determined in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines by the agar dilution method (13).

Further, the antimicrobial activities of RBx 14255 and comparators against the spores of *C. difficile* ATCC 43255 and *C. difficile* ATCC 9689 were determined by the broth microdilution method in 96-well plates. The spores of *C. difficile* ATCC 43255 and ATCC 9689 were prepared as described earlier (9).

Time-kill kinetics studies of RBx 14255, vancomycin, and telithromycin against *C. difficile* ATCC 43255 and *C. difficile* 6387 were performed at multiple concentrations ranging from 1× to 16× MIC as described earlier (4, 10).

The frequency of resistance development by *C. difficile* ATCC 43255 and *C. difficile* 6387 in the presence of 4× and 16× MICs of RBx 14255, vancomycin, metronidazole, and rifampin was determined by a previously described method (10). In order to determine the ability of *C. difficile* ATCC 43255 and *C. difficile* 6429 strains to develop resistance to RBx 14255, vancomycin, metronidazole, and telithromycin, a previously published serial passage method was used (10). All the above *in vitro* experiments were performed in an anaerobic chamber (Bactron IV; Sheldon Mfg., Cornelius, OR).

Golden Syrian hamsters weighing 80 to 100 g were used for the study. They were purchased from the National Institute of Nutrition (NIN, ICMR, Hyderabad, India). Hamsters were given a single subcutaneous dose of clindamycin phosphate (30 mg/kg of body weight), to render them susceptible to *C. difficile* infection (10, 14). On the following day, hamsters were infected by oral gavage with a 1-ml suspension (5 × 10⁷ CFU/ml) of *C. difficile* ATCC 43596 prepared from a culture grown overnight. Treatment was started 24 h postinfection with vehicle, RBx 14255 (50

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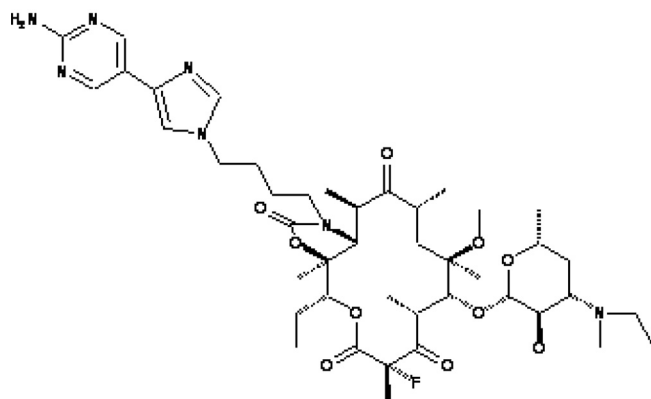


FIG 1 Structure of RBx 14255.

mg/kg), vancomycin (5 mg/kg), or metronidazole (150 mg/kg) and was given twice a day for 5 consecutive days. RBx 14255 and metronidazole were prepared in 0.25% methylcellulose, while vancomycin was prepared in sterile Milli-Q water. An infected and an uninfected control (administered clindamycin only) were also included in this study. Six hamsters per group were taken and monitored twice daily for morbidity and mortality. The statistical analysis was performed using GraphPad Prism-5.03 (GraphPad Software, San Diego, CA).

The MIC range, MIC₅₀s, and MIC₉₀s for the tested organisms are presented in Table 1. RBx 14255 showed 2-fold-higher MICs against all the clinical and reference strains of *C. difficile* than did metronidazole, 2-fold-lower MICs than did vancomycin, and more-than-2-fold-lower MICs than did other comparators. RBx 14255 showed MICs of 0.25 µg/ml against *C. difficile* BI/NAP1/027, whereas MICs of clindamycin, erythromycin, and clarithromycin were 16, 8, and 4 µg/ml, respectively. Moreover, against other *Clostridium* spp., including *C. perfringens*, the activities of RBx 14255 were higher than those of reference antibiotics, with MIC₉₀ being 0.125 µg/ml. RBx 14255 showed poor activity against Gram-negative anaerobes *B. fragilis* and *Fusobacterium nucleatum* (Table 1). RBx 14255 and comparator antibiotics showed comparable activities against spores of *C. difficile* ATCC 43255 and *C. difficile* ATCC 9689 (Table 2).

The time-kill studies demonstrated that RBx 14255 at 4× MIC and above showed bactericidal (99.9%) activity against both strains with no regrowth even at MIC level (Fig. 2). Vancomycin showed bactericidal activity against *C. difficile* ATCC 43255 and bacteriostatic activity against *C. difficile* 6387 at 2× MIC, whereas telithromycin at 2× MIC produced a bacteriostatic effect.

The frequency of resistance to RBx 14255 observed for *C. difficile* ATCC 43255 was 2×10^{-8} and $<7 \times 10^{-9}$ whereas that for *C. difficile* 6387 was 1.5×10^{-9} and 4.0×10^{-9} at 4× and 16× MIC, respectively. The frequency of resistance to vancomycin and metronidazole was also comparable to that to RBx 14255 for both the *C. difficile* strains (Table 3). Twenty-five serial passages of *C. difficile* ATCC 43255 and *C. difficile* 6429 resulted in no significant increase in RBx 14255 MICs. Similarly, *C. difficile* strains did not develop any kind of shift for vancomycin and metronidazole MICs (data not shown).

As expected from a ketolide, the sub-MIC of RBx 14255 in a mechanism of action (MOA) study resulted in 70% inhibition of protein synthesis at 6 h of incubation (data not shown). Moreover, RBx 14255 inhibited the [¹⁴C]erythromycin binding to 70S ribo-

some with a 50% inhibitory concentration (IC₅₀) of 0.23 µM; in comparison, telithromycin showed the same inhibition at 0.65 µM (8). RBx 14255 displayed greater potency for the 70S ribosome than did telithromycin. This finding supports the excellent *in vitro* activity and time-kill kinetics profile of RBx 14255.

The *in vivo* efficacy of RBx 14255 in the hamster model of CDI is presented in Fig. 3. All infected hamsters treated with placebo

TABLE 1 Antibacterial activity of RBx 14255 against *Clostridium* spp. and other anaerobic bacteria

Organism (n) and antimicrobial agent	MIC (µg/ml)		
	MIC ₅₀	MIC ₉₀	Range
<i>C. difficile</i> (28) ^a			
RBx 14255	0.5	4	0.125–8
Telithromycin	0.5	8	0.5–16
Erythromycin	2	16	2–>16
Clarithromycin	1	>16	1–>16
Vancomycin	1	4	0.5–8
Metronidazole	0.25	2	0.06–4
Clindamycin	8	>16	2–>16
<i>Clostridium</i> spp. (11) ^b			
RBx 14255	0.06	0.125	0.03–0.25
Telithromycin	0.125	2	0.5–4
Erythromycin	2	8	2–16
Clarithromycin	0.5	16	0.06–16
Vancomycin	0.5	1	0.5–4
Metronidazole	0.25	1	0.03–1
Clindamycin	0.5	1	0.125–8
<i>Propionibacterium acnes</i> (6) ^c			
RBx 14255	0.03	1	0.015–1
Telithromycin	0.125	4	0.06–>4
Erythromycin	0.125	0.25	0.125–0.25
Clarithromycin	0.125	0.25	0.03–0.5
Vancomycin	1	1	0.125–1
Metronidazole	16	16	16
Clindamycin	0.25	1	0.25–1
<i>B. fragilis</i> (17) ^d			
RBx 14255	2	8	1–16
Telithromycin	2	16	2–16
Erythromycin	8	>16	8–>16
Clarithromycin	8	>16	8–>16
Vancomycin	16	16	16–>16
Metronidazole	0.5	2	0.25–2
Clindamycin	1	16	1–>16
<i>F. nucleatum</i> (3) ^e			
RBx 14255	8	16	8–>16
Telithromycin	2	4	2–16
Erythromycin	>16	>16	>16
Clarithromycin	>16	>16	>16
Vancomycin	16	16	8–>16
Metronidazole	0.25	0.5	0.06–0.5
Clindamycin	0.06	0.125	0.03–0.125

^a *Clostridium difficile* ATCC 43255, *C. difficile* ATCC 43596, *C. difficile* ATCC 9689, *C. difficile* NCTC 11223, *C. difficile* BI/NAP1/027-2001155, and *C. difficile* clinical isolates (23).

^b *Clostridium perfringens* ATCC 13124, *C. perfringens* clinical isolates (4), *Clostridium cadaveris* 6400, *Clostridium bifermentans* (3), and *Clostridium subterminale* (2).

^c *Propionibacterium acnes* ATCC 6919, *P. acnes* clinical isolates (5).

^d *Bacteroides fragilis* ATCC 25285, *B. fragilis* (16).

^e *Fusobacterium nucleatum* 5356, *F. nucleatum* (2).

TABLE 2 Antibacterial activity of RBx 14255 against *C. difficile* vegetative cells and spores

Antimicrobial agent	MIC against <i>C. difficile</i> ($\mu\text{g/ml}$)			
	ATCC 43255		ATCC 9689	
	Vegetative cells	Spores	Vegetative cells	Spores
RBx 14255	0.5	2	0.25	2
Telithromycin	0.25	2	0.25	2
Erythromycin	1	2	1	2
Clarithromycin	0.5	2	32	>32
Vancomycin	1	4	2	4
Metronidazole	0.125	0.25	0.25	2
Clindamycin	8	>16	8	>32

TABLE 3 *In vitro* frequency of resistance by *C. difficile* against RBx 14255 and other standard drugs

<i>C. difficile</i> strain/drug	Frequency of spontaneous resistance	
	4 \times MIC (mg/liter)	16 \times MIC (mg/liter)
ATCC 43255		
RBx 14255	2×10^{-8}	$<7.0 \times 10^{-9}$
Vancomycin	3.5×10^{-9}	$<7.0 \times 10^{-9}$
Metronidazole	2.0×10^{-9}	6.0×10^{-9}
Rifampin	1.0×10^{-7}	3.5×10^{-7}
6387		
RBx 14255	1.5×10^{-9}	4.0×10^{-9}
Vancomycin	4.5×10^{-9}	$<6.5 \times 10^{-9}$
Metronidazole	1.0×10^{-8}	$<6.5 \times 10^{-9}$
Rifampin	1.5×10^{-7}	2.0×10^{-7}

developed clinical symptoms of infection, including a wet tail, within 4 days of infection and died by day 5. The feces and cecal contents collected from dead animals were found to be positive for *C. difficile* vegetative cells and *C. difficile* toxins (A/B). The first mortality in the RBx 14255-treated group occurred at day 13 with no sign of diarrhea; however, the cecal content of the dead animal was found to be positive for *C. difficile* and its toxin. The remaining hamsters in the RBx 14255-treated group showed no mortality

until day 18. In the metronidazole-treated group, no mortality occurred during treatment; however, within 4 days of stoppage of treatment, hamsters started showing signs of diarrhea, followed by 100% mortality by day 13. While no mortality occurred in the vancomycin-treated group until 7 days after treatment, all animals subsequently succumbed to diarrhea within the next 3 days. The dose of vancomycin in our study was low (5 mg/kg); however,

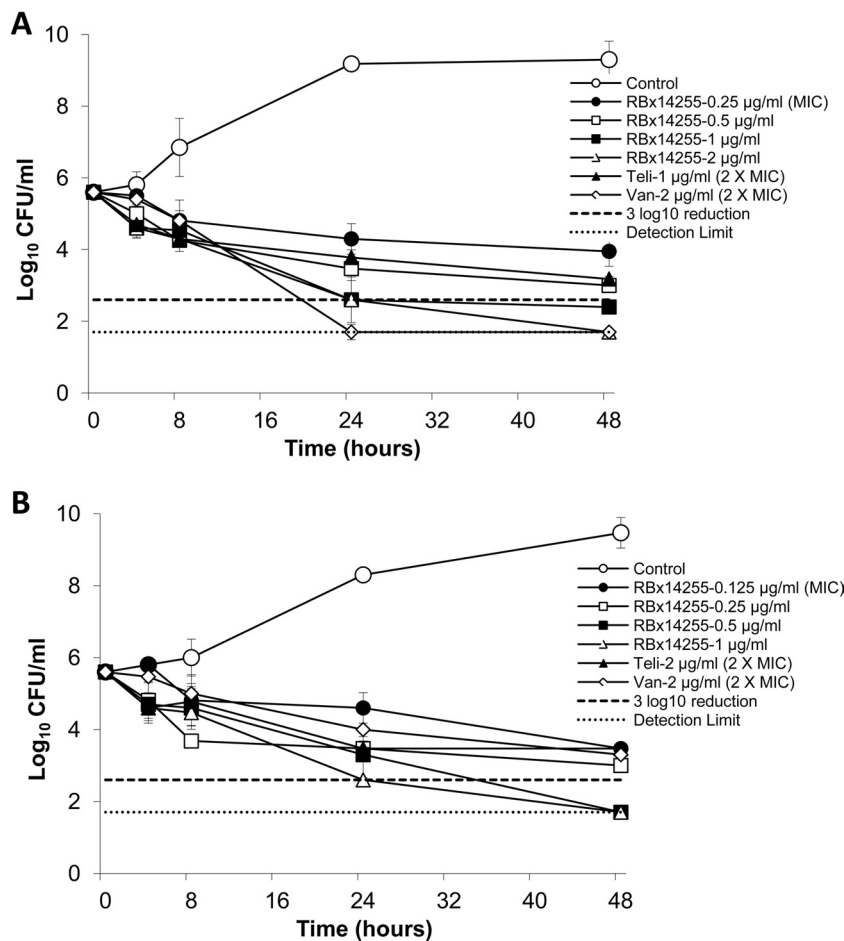


FIG 2 *In vitro* time-kill kinetics of RBx 14255 against *C. difficile* ATCC 43255 (A) and *C. difficile* 6387 (B). Viability counts are presented at various time periods between 0 and 48 h.

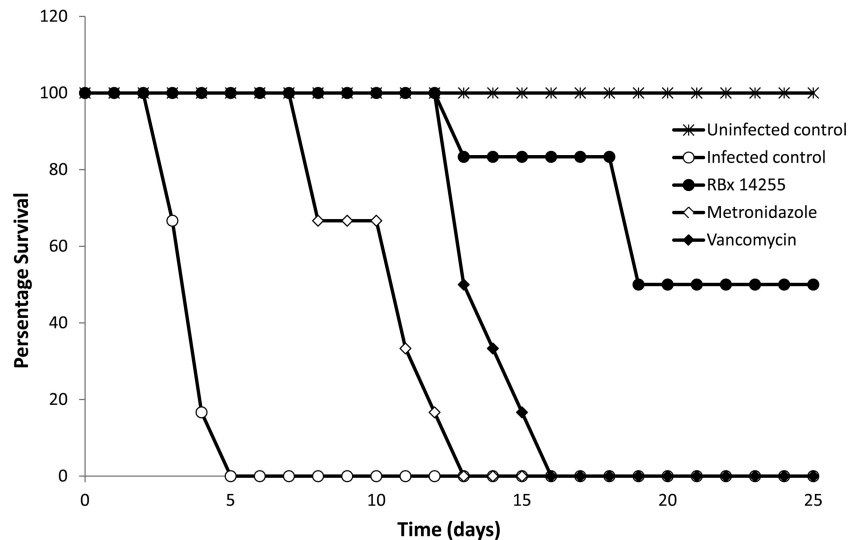


FIG 3 *In vivo* efficacy of RBx 14255 in a hamster model of *C. difficile* infection. Hamsters were pretreated with 30 mg/kg of clindamycin subcutaneously on day – 1 and infected orally with *C. difficile* on day 0. Hamsters were treated with RBx 14255 (50 mg/kg), metronidazole (150 mg/kg), and vancomycin (5 mg/kg) orally twice a day on day 1 until day 5 and observed for clinical signs of disease and mortality. All RBx 14255-treated animals showed extended survival compared to metronidazole-treated animals ($P < 0.0001$) and vancomycin-treated animals ($P < 0.002$).

another study reported that a higher dose of vancomycin (50 mg/kg), which is equal to the dose of RBx 14255 administered in this study, resulted in comparable efficacy (1).

In summary, we have investigated and reported the activity of RBx 14255, a novel ketolide, against anaerobic bacteria for the first time. It was found that RBx 14255 is active against erythromycin- and clarithromycin-resistant *C. difficile* strains, including the epidemic BI/NAP1/027 strain, and several bacterial species with different resistance patterns; these findings could indicate a special advantage of RBx 14255 over existing macrolides and ketolides (15, 17). RBx 14255 is a novel antibacterial agent that prevents the growth of *C. difficile* by inhibiting protein synthesis and could be investigated for *C. difficile* treatment. Based on the above promising data, RBx 14255, a novel investigational antibacterial agent, may be explored further for treatment of CDI in humans.

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There are no competing interests to declare.

The institutional animal ethics committee (IAEC) of Ranbaxy Research Laboratories, Gurgaon, India, approved all experimental protocols (no. 159/07 E2/10) for the use of animals.

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