

F1-2101

47th ICAAC Meeting
Chicago, Illinois
Sept. 17 - 20, 2007

In Vitro Mode-of-Action Studies of CBR-2092: a Novel Rifamycin-Quinolone Hybrid Antibiotic.

A.S. LYNCH, E.J. BONVENTRE, T.B. DOYLE, Q. DU, L. DUNCAN, G.T. ROBERTSON, E.D. ROCHE
Cumbre Pharmaceuticals Inc., Dallas, TX.

Abstract

Background: CBR-2092 is a novel rifamycin-quinolone hybrid antibiotic in development for the treatment of serious bacterial infections. The multi-functional antibiotic nature of CBR-2092 is substantiated herein through studies that encompass biochemistry, microbiology, and resistance genetics.

Methods: CLSI guidelines were followed throughout with polysorbate-80 (0.002%) supplementation of broth medium as indicated. Additional studies employed standard methods; biochemical assays employed recombinant forms of *S. aureus* enzymes.

Results: Biochemical studies indicate that CBR-2092 retains rifampin-like potency as an inhibitor of RNAP (IC₅₀ 30 nM), exhibits balanced activity against DNA gyrase (CC₅₀ 1.5 μM) and DNA topoisomerase IV (CC₅₀ 1.7 μM), and retains activity against a common quinolone-resistant variant (ParC^{R380F}) of DNA topoisomerase IV (CC₅₀ 2.7 μM). MIC values determined for derivatives of *S. aureus* ATCC# 29213 bearing rifamycin and/or quinolone resistance alleles confirm that CBR-2092 retains rifampin-like potency (0.016 μg/mL) against quinolone-resistant strains and garofloxacin-like potency (0.12 μg/mL) against rifampin-resistant strains. A serial stepwise passage resistance study conducted with CBR-2092 over a period of 26 days yielded a terminal isolate with broth and agar MICs of >16 and 4 μg/mL, respectively, that possessed five target mutations (*rpoB*^{R484H}, *gyrA*^{R452R,S44L}, *parC*^{R236R,H103Y}) but was unaltered in efflux properties. Data from time-kill and metabolic labeling experiments also support the notion that the primary activity of CBR-2092 in *S. aureus* is driven by its rifamycin pharmacophore and that activity against rifampin-resistant strains is mediated by the quinolone pharmacophore.

Conclusions: Data from biochemistry, microbiology, and resistance studies indicate that CBR-2092 exhibits antimicrobial activity via combined effects on RNA polymerase, DNA gyrase and DNA topoisomerase IV.

Introduction

Antibiotics of the rifamycin class have proven efficacy in the treatment of persistent bacterial infections including tuberculosis and biofilm-associated infections of indwelling medical devices (4, 7, 8). However, the relative ease with which bacteria develop resistance to the rifamycins restricts their clinical use to antibiotic combination regimens (1). In a program directed toward the synthesis and evaluation of rifamycin-based multi-functional antibiotics, a series of compounds were prepared that covalently combine rifamycin and quinolone pharmacophores to form stable hybrid antibiotic agents.

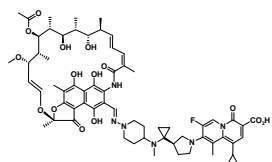
CBR-2092 combines the rifamycin SV and 4H-4-oxoquinolizone (5) pharmacophores via a chiral linking group. Herein we report the characterization of the mode-of-action of CBR-2092 as an anti-staphylococcal agent through biochemical, microbiological, genetic and cell biology studies. The combined data suggest that the antimicrobial properties of CBR-2092 reflect its activity as an inhibitor of three essential cellular targets: RNA polymerase, DNA gyrase, and DNA topoisomerase IV.

Methods and Materials

Inhibition of transcription by *S. aureus* σ⁴ RNA polymerase holoenzyme forms was measured by quantitative gel electrophoresis of a single RNA species to determine the minimal concentration necessary to inhibit 50% (IC₅₀) of the RNA product formed in the absence of test agents (6). Inhibition of the *in vitro* activity of Type II topoisomerases employed recombinant forms of the indicated enzymes in electrophoresis assays wherein the minimal concentration necessary to induce 50% cleavage of a ccDNA substrate (CC₅₀) was determined. Macromolecular biosynthesis assays were carried out in a time-course fashion in the presence of 0.03 μg/mL rifampin, 0.2 μg/mL CBR-2092 or 2 μg/mL ciprofloxacin and employed [Methyl-³H]-Thymidine (DNA), [5,6-³H]-Uridine (RNA), L-[3,4,5-³H]-Leucine (protein) or [2,3-³H]-D-Alanine (cell wall). Radiolabel incorporated at T₀ was set to 100% for estimation of the relative change in total incorporation in the presence of test agents.

Determination of broth and agar microdilution MIC endpoints (2) and assessment of bactericidal activity by the time-kill methodology was done in accordance with CLSI methodology in cation adjusted Mueller Hinton (MHII) supplemented with 0.002% (vol:vol) Polysorbate-80 (P-80). Mutant Prevention Concentrations (MPCs) were determined through plating 10⁹ viable test organisms (3). Resistance frequency was similarly determined using 10¹² viable organisms and agar plates with a fixed dose of 1 μg/mL test agent. Step-wise passage for multi-step resistance selection was undertaken in glass tubes with MHII supplemented with P-80 initially inoculated with 10⁸ CFU/mL at sub-MIC doses of the CBR-2092 test agent. Cultures were incubated with shaking at 37°C for 20-24 hours. Thereafter, the cell inoculum was prepared from the highest consecutive drug concentration which has supported growth to an absorbance equivalent ≥ 10⁸ CFU per mL. Daily passages were performed until compound solubility issues were limiting.

Panel 1: CBR-2092 Structure



- Chemical Name:** R-3-[[4-(1-[1-(3-Carboxy-1-cyclopropyl-7-fluoro-9-methyl-4-oxo-4H-quinolizine-8-yl)-pyrrolidin-3-yl-cyclopropyl]-methylamino)-piperidin-1-ylimino]-methyl-1-yl]-rifamycin SV
- Chemical Formula:** C₅₅ H₅₁ F N₉ O₁₅
- Molecular Weight:** 1205.38 Daltons

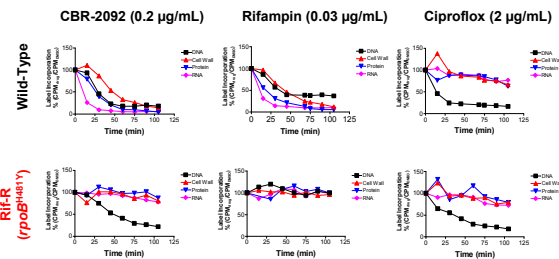
Panel 2: CBR-2092 MOA – Biochemical studies

Compound	IC ₅₀ (μM)		CC ₅₀ (μM)				
	RNAP-σ ⁴ (WT)	RNAP-σ ⁴ (RpoB ^{R481Y})	Topo IV (WT)	Gyrase (WT)	Topo IV (ParC ^{R380F})	Gyrase (GyrA ^{R544L})	hTOP2α
CBR-2092	0.034	> 25	1.7	1.5	2.7	> 150	> 150
Rifampin	0.015	> 25	—	—	—	—	—
Ciprofloxacin	—	—	0.4	5	31	> 150	> 150
Garofloxacin	—	—	0.2	0.7	13	18	> 150

CBR-2092 profiled against purified *S. aureus* enzymes and human TOP2α.

- Potent inhibitor of RNA polymerase
- Balanced (equipotent) inhibitor of DNA gyrase and topoisomerase IV
- Retains activity against key Fq-R mutant Topo IV enzyme (ParC^{R380F})
- No activity detected versus human DNA topoisomerase IIα

Panel 3: CBR-2092 MOA - Metabolic labeling studies



CBR-2092 profiled by metabolic labeling with wild-type or rifampin-resistant *S. aureus*.

- Primary activity on RNA synthesis in rifampin-sensitive strain
 - Anticipated secondary effects on other pathways
- Primary quinolone-like effects on DNA synthesis in Rif-resistant strain (*rpoB*^{R481Y})

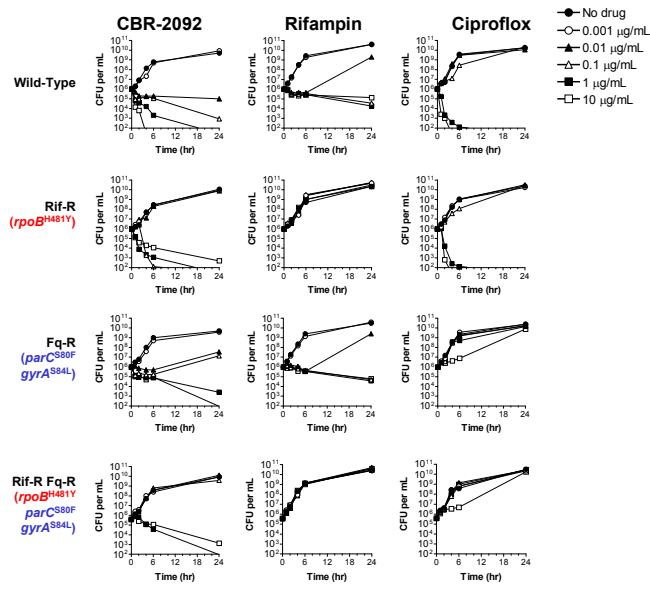
Panel 4: CBR-2092 MOA - MIC activity versus resistant strains

Compound	WT parent (29213)	MIC in μg/mL for:						
		<i>gyrA</i> ^{R544L}	<i>parC</i> ^{R380F}	<i>gyrA</i> ^{R544L} <i>parC</i> ^{R380F}	<i>rpoB</i> ^{R481Y}	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R544L}	<i>rpoB</i> ^{R481Y} <i>parC</i> ^{R380F}	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R544L} <i>parC</i> ^{R380F}
CBR-2092 broth	0.015	0.015	0.015	0.015	0.12	0.5	0.24	2
CBR-2092 agar	0.008	0.008	0.008	0.008	0.06	0.12	0.06	0.25
Rifampin	0.008	0.008	0.008	0.008	>250	>250	>250	>250
Ciprofloxacin	0.24	0.24	2	16	0.24	0.24	2	16
Levofloxacin	0.24	0.24	1	8	0.24	0.24	1	8
Garofloxacin	0.06	0.12	0.24	4	0.06	0.12	0.24	4

CBR-2092 activity assessed through MIC assays with isogenic *S. aureus* resistant panel.

- MIC potency shifts of CBR-2092 consistent with genuine triple targeting characteristic
- Potent antimicrobial activity observed versus rifampin-sensitive strains
 - Suggests primary activity driven by rifamycin pharmacophore
- Secondary activity retained in rifampin-resistant strains
 - Indicative of activity of fluoroquinolone pharmacophore
 - Biochemical and other data indicates activity vs. the triple mutant is Topo IV-directed

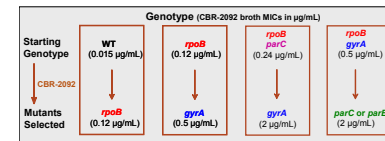
Panel 5: CBR-2092 MOA – Time-kill studies



CBR-2092 activity in time-kill assays with isogenic *S. aureus* resistance panel.

- Improved kill compared to rifampin against rifampin-sensitive strains
- Rapid quinolone-like kill against rifampin-resistant strains

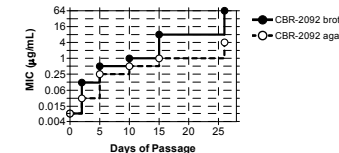
Panel 6: CBR-2092 MOA – single-step selections



CBR-2092 profiled in single-step resistance studies versus resistant *S. aureus*.

- Appearance of single-step mutations consistent with target preference: RNA polymerase → DNA gyrase → DNA topoisomerase IV

Panel 7: CBR-2092 MOA - Step-selection studies



Isolate tested	Relevant genotype	MIC in μg/mL for:					In vitro doubling time (min)
		CBR-2092 (Broth)	CBR-2092 (Agar)	Rif	Cipro	EtBr	
WT	<i>S. aureus</i> 29213 (Wild-type parent)	0.008	0.008	0.008	0.24	4	38
Day 2	<i>rpoB</i> ^{R484H}	0.12	0.03	> 250	0.24	4	43
Day 5	<i>rpoB</i> ^{R484H} <i>gyrA</i> ^{R452Q}	0.5	0.25	> 250	0.5	4	41
Day 10	<i>rpoB</i> ^{R484H} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236R}	1	0.5	> 250	1	4	45
Day 15	<i>rpoB</i> ^{R484H} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236R}	8	1	> 250	1	4	47
Day 26	<i>rpoB</i> ^{R484H} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236R,H103Y}	64	4	> 250	8	4	65

CBR-2092 profiled in step-wise resistance studies versus wild-type *S. aureus*.

- Order of mutations consistent with proposed target preference (*rpoB* > *gyrA* ≥ *parC*)
- Unique first step mutations in *gyrA*^{R452Q} and *parC*^{R236R}
- Thereafter, canonical Fq-R mutations in *gyrA* or *parC* QRDR's
- No apparent role of efflux in contributing to CBR-2092-resistance development
- In vitro* fitness cost apparent for terminal isolate

Panel 8: CBR-2092 MOA - step-selections with resistant mutants

Isolate tested	Relevant genotype	MIC in μg/mL for:				
		CBR-2092 (Agar)	CBR-2092 (Broth)	Rif	Cipro	EtBr
CB190, Parent	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q} <i>parC</i> ^{R236Y}	0.008	0.008	0.008	0.24	4
CB190+26 days	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236R,H103Y}	64	4	> 250	8	4
CB370, Parent	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q} <i>parC</i> ^{R236Y}	0.12	0.03	> 250	0.24	4
CB370+20 days	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y}	4	1	> 250	2	4
CB814, Parent	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y}	0.008	0.06	0.008	16	4
CB814+21 days	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y} <i>parE</i> ^{R202Y}	31	4	> 250	63	4
CB815, Parent	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y}	1	0.25	> 125	16	4
CB815+7 days; #1	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y} <i>parE</i> ^{R202Y}	16	2	> 125	63	4
CB815+7 days; #2	<i>rpoB</i> ^{R481Y} <i>gyrA</i> ^{R452Q,S44L} <i>parC</i> ^{R236Y} <i>parE</i> ^{R202Y}	16	2	> 125	63	4

CBR-2092 profiled in step-wise resistance studies versus resistant *S. aureus*.

- Resistance development impacted by the Rif-R and/or Fq-R genotype of test strain
- Unique mutations in *gyrA* and *parC* again apparent with CBR-2092
- No apparent role of efflux in contributing to CBR-2092-resistance development

Summary and Conclusion

Summary:

- CBR-2092 is a novel rifamycin-quinolone hybrid antibiotic in development for the treatment of serious bacterial infections.
- Biochemical studies with purified bacterial enzymes and metabolic labeling assays with *S. aureus* demonstrate dual-pharmacophore nature of CBR-2092.
- CBR-2092 exhibits potent antimicrobial activity against an otherwise isogenic resistance panel of *S. aureus* that is equivalent or improved over individual reference agents of the parental classes.
- Single-step and multi-step resistance selection studies support both the multi-targeting nature of CBR-2092 and its overall target preference.
- Unique mutations selected by CBR-2092 in DNA gyrase and DNA topoisomerase IV may be indicative of distinct target interactions.
- No apparent role of efflux in contributing to CBR-2092 resistance.

Conclusion:

- The studies described herein indicate that the covalently coupled hybrid antibiotic CBR-2092 exhibits antimicrobial activity via combined effects on three essential cellular targets: RNA polymerase, DNA gyrase and DNA topoisomerase IV.

References

- Chaisson RE. 2003. Treatment of Chronic Infections with Rifamycins: Is Resistance Likely To Follow? *Antimicrob. Agents Chemother.* 47: 3037-9
- Clinical and Laboratory Standards Institute. 2006. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approved Standard- Seventh Edition CLSI Document M7-A7, 26(2). CLSI, Wayne, Pa.
- Drica K. 2003. The mutant selection window and antimicrobial resistance. *J. Antimicrob. Chemother.* 52: 11-7
- Floss HG, Yu TW. 2005. Rifamycin: mode of action, resistance, and biosynthesis. *Chem Rev.* 105: 621-32.
- Li Q, Chu DT, Balcombe A, et al. 1996. Synthesis and structure-activity relationships of 2-pyridones: a novel series of potent DNA gyrase inhibitors as antibacterial agents. *J. Med. Chem.* 39: 3070-88.
- Lynch AS, Du Q. 2007. Methods to Identify and Characterize Inhibitors of Bacterial RNA Polymerase. Published in 'New Antibiotic Targets'; Molecular Medicine series published by Humana Press Inc., Totowa, NJ.
- Pace JL, Rupp ME, Finch RG. 2006. Biofilms, Infection, and Antimicrobial Therapy. CRC Press, Taylor & Francis Group, Boca Raton, FL.
- Waldvogel FA, Bisno AE. 2000. Infections associated with indwelling medical devices, 3rd Edition. ASM Press, Washington, D.C.

Acknowledgements

The authors wish to acknowledge past contributors to the rifamycin-quinolone program at Cumbre Pharmaceuticals including Doug Beeman, Keith Combrink, Jing Li, Zhenkun Ma, Timothy Morris and Dalai Yan and the contributions of current colleagues Donghui Bao, Charles Ding, Steve Madden, Paul Renick and William Weiss.