NEW ANTIBACTERIAL DISCOVERY & DEVELOPMENT

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Indole-based inhibitors of MreB-mediated bacterial cytoskeleton polymerization discovered in whole-cell screens employing efflux-compromised *Pseudomonas aeruginosa* strains.

G.T. ROBERTSON, T.B. DOYLE, Q. DU, L. DUNCAN, K. MDLULI, K. COMBRINK, C. DING, and A.S. LYNCH.

Cumbre Pharmaceuticals Inc., Dallas, TX

Abstract

Efflux pumps contribute to drug resistance in *Pseudomonas aeruginosa* and are largely responsible for the failure of both current antibacterial therapies and attempts to discover new agents against this pathogen. Cumbre has employed a panel of *Pseudomonas aeruginosa* systematic efflux knock-out (SEKO) strains lacking 53 individual efflux pumps in whole-cell screening to discover compounds that inhibit the growth of efflux compromised, but not wild-type cells. One active compound from this screen, CBR-4830, was found to be subject to efflux by the MexAB-OprM system and as such, would not have been discovered using conventional whole-cell screens employing wild-type efflux competent bacteria. Using resistance genetics, we identified an essential protein, MreB, as the direct cellular target for CBR-4830 in Δ*mexAB Pseudomonas aeruginosa*. MreB is a bacterial homologue of actin that polymerizes into cytoskeleton-like filaments and is thought to interact with numerous other proteins to coordinate cell wall biosynthesis with chromosomal segregation during the cell division process (3, 5). Combined results from microscopic examination of CBR-4830-treated bacteria, the characterization of CBR-4830-resistant mutants, and biochemical studies of the effect of CBR-4830 on *in vitro* polymerization of MreB, substantiate this compound (and closely related analogs) as a specific inhibitor of this essential cell division protein. SAR development pertaining to potency, efflux avoidance and "on-target" versus "off-target" activities will be discussed.

Introduction

Despite the promise that bacterial genomics, combinatorial chemistry and/or target-based HTS efforts would deliver new classes of antibacterial agents, no new agents based on these efforts have been introduced into the clinic (2, 8). To inhibit bacterial growth, a compound that is active *in vitro* must also be able to penetrate the bacterial cell surface, avoid elimination by a range of efflux mechanisms and avoid inactivation through modification by bacterial enzymes.

Pseudomonas aeruginosa displays a high level of intrinsic resistance to a variety of structurally unrelated antimicrobial agents due to interplay of both the low-permeability outer membrane and broad-specificity drug efflux systems (7). Six RND efflux pumps have been extensively studied and are functionally involved in the efflux of a variety of antibacterial agents including β-lactams, fluoroquinolones, tetracyclines, chloramphenicol, and erythromycin (9). Although the MFS, MATE, SMR and ABC efflux systems have not been as well studied as the RND systems, members of these efflux families clearly play a role in antibiotic efflux in other organisms (6) and it seems quite likely that they play a similar role in *P. aeruginosa*.

Given that drug efflux systems have impeded target- and cell-based discovery efforts for new antibacterial agents, particularly in *P. aeruginosa*, we sought to identify unique compounds that specifically inhibit the growth of individual *P. aeruginosa* strains that are each deficient in a single efflux pump system. By definition, leads identified in this manner will already possess cell-based activity under specific efflux-deficient conditions and thus have a distinct advantage over compounds discovered in target-based biochemical assays. Furthermore, these compounds should be unique to Cumbre, since to our knowledge, no other company has conducted a screen in this manner. Once attractive compounds are identified, efforts are focused toward the identification the compounds' molecular target(s). Thereafter, this knowledge plus the efflux assets used to identify these leads are employed to help guide compound optimization aimed at discovering compound analogues that have activity against wild-type cells because they are of increased potency and/or otherwise able to evade the effects of the identified efflux system.

A portion of this work was presented at the 44th ICAAC meeting in Washington D.C.

Panel 1: SEKO HTS screening platform and optimization of leads.

Step	Activity	*Haemophilus*	*Streptococcus*	*Pseudomonas*
1	Efflux Pumps Targeted	6 + (*tolC*)	13	55 + (*tolC*)
2	Efflux Pumps Inactivated	6 + (*tolC*)	11	53 + (*tolC*)
3	Combine deletions to reduce panel size	6	8	20
4	HTS Validation via Pilot Screens	Completed	Completed	Completed
5	Compounds Tested in HTS	~220,000	~220,000	~150,000
6	Single Pump - "Serendipity" - Hits	635	51	47
7	Target ID through Resistance Genetics			
8	Target Validation			
9	Est. SAR Guiding Biochemical Assays			
10	Secondary Evaluation of Leads			CBR-4830 series
11	Chemical Optimization of Leads			

- Efflux-compromised and wild-type strains employed in whole cell screens to identify "Serendipity" Hits active against a single efflux compromised strain but not wild-type bacteria.
- Re-discovery of progenitors of multiple drug classes validates SEKO screening approach.
- One series CBR-4830 selected as exploratory 'proof of concept' for further optimization.

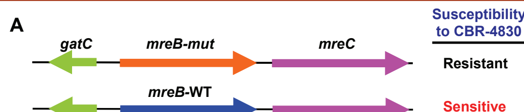
Panel 2: CBR-4830 is subject to MexAB-mediated efflux.

Compound	MIC in μM for:						
	CB046 WT PA01	CB391 Δ(*mexAB*)	CB392 Δ(*mexAB*) Δ(*mexCD*)	CB393 Δ(*mexAB*) Δ(*mexEF*)	CB394 Δ(*mexAB*) Δ(*mexXY*)	CB398 Δ(*mexAB*) Δ(*mexCD*) Δ(*mexXY*) Δ(*mexJK*) Δ(*opmH*)	CM336 Δ(*mexAB*) Δ(*mexCD*) Δ(*mexXY*) Δ(*mexJK*) Δ(*opmH*) pUC-*mexAB*-*oprM*$^{+}$
CBR-4830	128	2	1	4	4	1	32
Chloramphenicol	> 128	4	4	4	4	2	64
Triclosan	> 128	64	32	64	64	8	>128
Rifampicin	16	16	16	16	16	16	16

- Increased susceptibility to chloramphenicol or CBR-4830 is attributable to loss of MexAB-OprM.
- Sensitivity to triclosan does not deconvolute to any one single RND efflux system (see also ref. 1).
- Role of MexAB-OprM pump confirmed via plasmid-based complementation (CM336).

Panel 3: Identification of MreB as the direct target of CBR-4830.

A



B

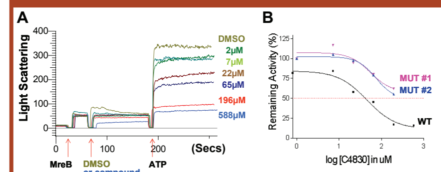
MreB Amino Acid Changes	Susceptibility to CBR-4830
WT	Sensitive
Pro$_{113}$ - Arg	Resistant
Pro$_{113}$ - Leu	Resistant
Ile$_{124}$ - Ser	Resistant
Glu$_{141}$ - Gly	Resistant

- **Altered amino acids (yellow) mapped onto crystal structure of MreB from *Thermotoga maritima***

Panel 3A:
- Resistance to CBR-4830 in Δ(*mexAB*) arises at a frequency of 10^{-9} (4-16×MIC).
- Genomic library from pooled CBR4830-resistant strains used to identify plasmid conferring heritable and stable CBR4830-resistance to naïve Δ(*mexAB*) strain.
- Mutant *mreB* allele(s) necessary and sufficient to confer CBR-4830 resistance.

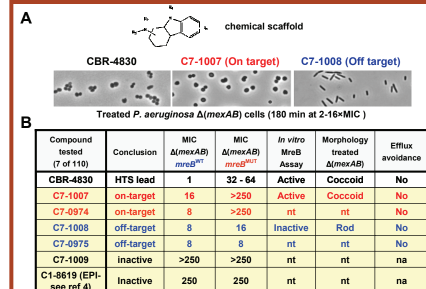
Panel 3B:
- CBR-4830 resistance localized to *mreB* in 14 of 14 independent spontaneous CBR-4830^{R} selectants.
- Mutations map near ATP-binding site based on *Thermotoga* MreB crystal structure.
- MreB essentiality in *Pseudomonas* confirmed at Cumbre by inability to knock out endogenous *mreB* gene unless balanced by second *mreB*+ copy (not shown).
- Mutant *mreB*, alone or in merodiploids, confers resistance to CBR-4830 (not shown).

Panel 4: CBR-4830 MOA validation through biochemistry.



- Purified recombinant *Pseudomonas* MreB employed in biochemical assays.
- Dose-dependent effects of CBR-4830 on MreB (WT) light scattering observed (panel A).
- Minimal effects of CBR-4830 on mutant MreB (Mut) light scattering observed (panel B).

Panel 5: Preliminary SAR for CBR-4830 analogs.



Treated *P. aeruginosa* Δ(*mexAB*) cells (180 min at 2-16×MIC)

B

Compound tested (7 of 110)	Conclusion	MIC Δ(*mexAB*) *mreB*^{WT}	MIC Δ(*mexAB*) *mreB*^{MUT}	*In vitro* MreB Assay	Morphology treated Δ(*mexAB*)	Efflux avoidance
CBR-4830	HTS lead	1	32 - 64	Active	Coccoid	No
C7-1007	on-target	16	>250	Active	Coccoid	No
C7-0974	on-target	8	>250	nt	nt	No
C7-1008	off-target	8	16	Inactive	Rod	No
C7-0975	off-target	8	8	nt	nt	No
C7-1009	inactive	>250	>250	nt	nt	na
C1-8619 (EPI- see ref 4)	Inactive	250	250	nt	nt	na

Methods to guide further compound selection:
- Analogs first sorted based on MIC potency and cross resistance to CBR-4830^{R} mutant alleles (*mreB*^{MUT}).
- "On-target" activity confirmed *in vitro* through biochemical inhibition of MreB polymerization and through cell morphological studies following treatment.
- Efflux avoidance tested through plasmid-based complementation with MexAB pump in a multiple pump knock-out strain.

Summary and Conclusions

Summary:
- Novel efflux sensitized strain panels were employed in HTS.
- The discovery of a novel compound (CBR-4830) that inhibits the growth of an efflux-deficient strain of *P. aeruginosa* and that exerts its effect by interfering directly with MreB function substantiates the utility of the SEKO (Systematic Efflux Knock-Out) screening approach.

Conclusions:
- Use of an efflux-sensitized *P. aeruginosa* strain panel has resulted in the identification of potential anti-*Pseudomonas* compounds.
- Preliminary SAR studies with one series (CBR-4830) identified features necessary for retaining "on-target" activity, but have thus far failed to identify critical features necessary for avoiding efflux.

References

1. **Chuanchuen, R., Beinlich, K., Hoang, T.T., Becher, A., Karkhoff-Schweizer, R.R., and H.P. Schweizer.** 2001. *Antimicrob Agents Chemother.* **45**:428-32.
2. **Coates, A., Hu, Y., Bax, R., and C. Page**. 2002. *Nat Rev Drug Discov.* **1**:895-910.
3. **Daniel, R.A., and J. Errington**. 2003. *Cell.* **113**:767-776.
4. **Kaczmarek, F., Sweeney, M.T., Dib-Hajj, F., *et al.*,** 2005. 45th ICAAC, Washington D.C.
5. **Kruse, T., Blagoev, B., Løbner-Olesen, *et al.*,** 2006. *Gene and Dev.* **20**:113-124.
6. **Paulsen, I.T., Brown, M.H., and R.A. Skurray.** 1996. *Microbiol Rev.* **60**:575-608.
7. **Poole, K.** 2002. *Curr Pharm Biotechnol.* **3**:77-98.
8. **Projan, S.J.** 2002. *Curr Opin Pharmacol.* **2**:513-522.
9. **Schweizer, H.P**. 2003. *Genet Mol Res.* **2**:48-62.