Exploring Cell Wall Targets to Overcome *Mycobacterium tuberculosis* (*Mtb*): Ceftriaxone (CRO) Inhibits Ldt_{Mt2}, a Major Peptidoglycan (PG) Synthase

David C. Nguyen MD[1], Sarah N. Redmond MD[2], Khalid M. Dousa MD[1,3], Christopher R. Bethel[4], Magdalena A. Taracila[4], Qing Li[3], Sebastian G. Kurz MD[5], Martin S. Pavelka, Jr. PhD[6], Krisztina Papp-Wallace PhD[3,4,7], Stephen M. Holland MD[8], Barry Kreiswirth PhD[9], W. Henry Boom MD[3,10,11], Robert A. Bonomo MD[1,3,7,11,12]

[1]Medical Service, Veterans Affairs Northeast Ohio Healthcare System (VANEOHS), Cleveland, OH; [2]University Hospitals (UH) Cleveland Medical Center and UH Rainbow Babies & Children's Hospital, Cleveland, OH; [3]Dept of Medicine, Case Western Reserve University (CWRU), Cleveland, OH; [4]Research Service, VANEOHS, Cleveland, OH; [5]Dept of Internal Medicine VIII, Medical Oncology and Pneumology, University of Tübingen, Germany; [6]Dept of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY; [7]Dept of Biochemistry, CWRU, Cleveland, OH; [8]National Institute of Allergy and Infectious Diseases, Bethesda, MD; [9]Center for Discovery and Innovation, Hackensack, NJ; [10]Division of Infectious Diseases & HIV Medicine, UH, Cleveland, OH; [11]Dept of Molecular Biology and Microbiology, CWRU, Cleveland, OH; [12]Depts of Pharmacology and Proteomics and Bioinformatics, CWRU and Cleveland Geriatrics Research Education and Clinical Center (GRECC), VANEOHS and CWRU-Cleveland VAMC Center for Antibiotic Resistance and Epidemiology (Case VA CARES), Cleveland, OH

Introduction

Infections caused by drug-resistant (DR) *Mycobacterium tuberculosis (Mtb)* continue *to* challenge our health care systems. 500,000 cases of DR TB occur annually resulting in 150,000 deaths. Novel therapies are urgently needed. Bedaquiline, delamanid, linezolid, and pretomanid possess serious limitations.

β-lactams such as amoxicillin/clavulanate, imipenem, and meropenem/clavulanate have not received significant attention as clinical regimens since these agents may add to the complexity of treatment

Mechanism of Action (MOA) - β-lactams

- **Targets of β-lactams are D,D-transpeptidases (Ddts) and L,D-transpeptidases (Ldts).[1]**
- **Inhibition of BlaC is a chromosomally encoded class A β-lactamase of *Mtb* limits β-lactam therapy. Clavulanate (CLA) inhibits BlaC.[2]**

Can Ceftriaxone be used to treat *Mtb?*

- **BlaC hydrolyzes CRO less efficiently compared to other cephems.[2] CRO inhibits Ldt_{Mt1}.[1,3]**
- **The activity of CRO on Ldt_{Mt2}, a major PG synthase of *Mtb*, is unknown.**

HYPOTHESIS

• Based upon work done with *M. abscessus* we hypothesized that CRO, as an expanded spectrum cephem, can inhibit Ldt_{Mt2}

•If so, the combinations of CRO with meropenem (MEM) or imipenem (IPM) will also lower MICs more than each alone supporting the thesis of target redundancy

Methods

- **Protein purification and electrospray ionization mass spectrometry (ESI-MS)**
- **Inhibition kinetics (K_{iapp}) with Ldt_{Mt2};**
- **AST and FIC index**

Results

Table 1. β-lactam and CLA MICs for *Mtb* H37Ra, H37Rv, and Clinical Isolates

Antibiotic(s)	MIC (μg/mL) by *Mtb* Isolate										
	H37Ra	H37Rv	#11.1	#15.1	#16.1	#17.2	#18.1	#19.2	#22.2	#23.1	#25.2
CRO	0.25	4	1	**4**	**16**	**4**	**8**	**4**	**2**	**8**	**0.5**
MEM	1	4	2	2	8	4	32	8	4	4	2
IPM	4		2	0.5	16	8	>64	>64	32	16	32
CLA	16	32	64	>64	16	32	>64	>64	32	16	16
MEM + CRO 1:1	0.25	1	2	0.5	4	0.5	1	4	0.25	0.25	0.125
IPM + CRO 1:1			2	2	4	1	2	4	0.25	0.25	0.125
CRO + CLA 2.5 μg/mL	0.125		2	**1**	**1**	**≤0.06**	**2**	**1**	**0.125**	**0.125**	**≤0.06**

designates specific isolate from our panel

← 6 *Mtb* isolates had FIC Index < 0.5 suggesting synergy with CRO and MEM/IPM. Fixed concentration CLA lowered CRO MIC by > 2-fold dilution for 8 of 10 isolates tested (MICs bolded).

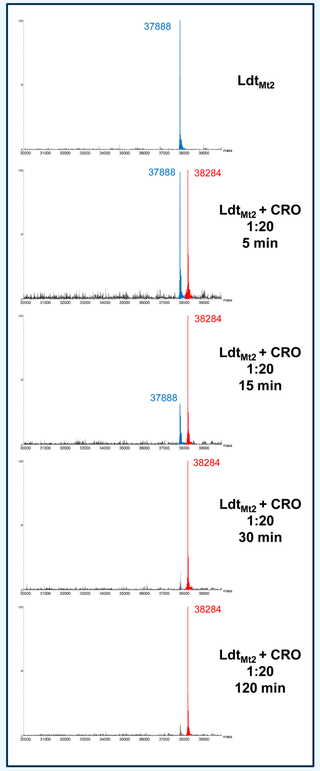
Synergy (FIC Index <0.5) Key:
CRO + MEM only
CRO + MEM and IPM
No isolate with FIC Index < 0.5 with CRO and IPM alone

Biochemical Analyses

- **Ldt_{Mt2} is a 408 amino acid protein.**
- **Δ (1-55) Ldt_{Mt2} was cloned with a His-tag at the N terminus, expressed in *E. coli* BL21(DE3) (IPTG induction) and purified using a nickel column**
- **A Synapt G2-Si high-resolution QTOF mass spectrometer was used to acquire mass spectra.**
- **ESI MS revealed the MW of Ldt_{Mt2} is 37888 ± 5 Da**
- **Timed ESI-MS Analysis showed:**
  - **CRO (MW= 554) forms an adduct with Ldt_{Mt2} (MW= 38284) that undergoes post-acylation modification (loss of R2 group) and is stable for 120 minutes.**
  - **MEM (MW=383) formed a 382 Da adduct; IMP (MW= 299) formed a 299 Da adduct; and CRO and MEM formed a 384 Da adduct. All species were stable for 120 minutes**

Table 2. $K_{i\ app}$ of Ldt_{Mt2} with β-lactams

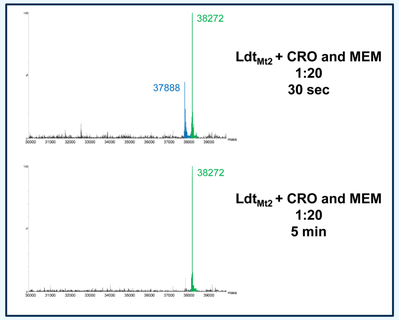
Compound	$K_{i\ app}$ (μM)
CRO	0.07 ± 0.007
MEM	0.09 ± 0.009
IPM	0.01 ± 0.002



← Figure 1. ESI-MS chromatograms with Ldt_{Mt2} alone and incubated with CRO 5 min, 15 min, 30 min, and 120 min. [Ldt_{Mt2}] = 13.2 μM and [CRO] = 264 μM (1:20 ratio). Mass peaks of Ldt_{Mt2} are in blue and mass peaks of Ldt_{Mt2}-CRO complexes are in red.

Observed changes in MW were +396 Da which is less than the nominal MW of CRO. This can be accounted for by post-acylation elimination of the R2 side group (see Fig. 3).

Figure 2. ESI-MS chromatograms with Ldt_{Mt2} incubated with CRO and MEM together. Only mass peaks corresponding to complexes with MEM were captured. [Ldt_{Mt2}] = 13.2 μM and both [CRO] and [MEM] = 264 μM (1:20 ratio). Mass peaks of Ldt_{Mt2} are in blue and mass peaks of Ldt_{Mt2}-MEM complexes are in green.



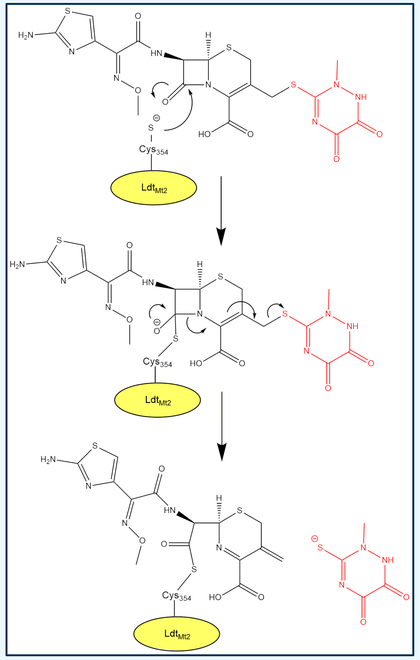


Figure 2. CRO-Ldt_{Mt2} adduct formation leading to elimination of the **R2 side group**.

Conclusion

- **Acknowledging that** MEM and IPM inhibit other PG synthases (PonA1, Ldt_{Mt1}, Ldt_{Mt3}), **these results lead us to hypothesize that the synergistic combination of CRO + MEM/IPM inhibit the growth of *Mtb* by the combined inactivation of multiple cell wall synthesizing enzymes.**
- Our observations support further exploration of the notion of "**target redundancy**" as an approach to treat multidrug-resistant mycobacteria with **β-lactams**.
- The clinical implications of using a "once a day" administered cephalosporin in treating Mtb infections remain to be tested in animal models.

References:

1. Lavollay M, Arthur M, Fourgeaud M, et al. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol.* 2008; 190: 4360-4366.
2. Hugonnet J-E, Blanchard JS. Irreversible inhibition of the *Mycobacterium tuberculosis* β-lactamase by clavulanate. *Biochemistry*. 2007; 46: 11998-12004.
3. Dubée V, Triboulet S, Mainardi J-L, et al. Inactivation of *Mycobacterium tuberculosis* L,D-transpeptidase Ldt-$_{Mt1}$ by carbapenems and cephalosporins. *Antimicrob Agents Chemother*. 2012; 56: 4189-4195.
4. Triboulet S, Dubée V, Lecoq L, et al. Kinetic features of L,D-transpeptidase inactivation critical for β-lactam antibacterial activity. *PLoS ONE.* 2013; 8: e67831.

Acknowledgements: DCN received support from Rainbow Babies & Children's Foundation and NIH T32 AI07024; contact: dcn21@case.edu