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The Discovery of the Novel Diazabicyclooctane (DBO) ETX0462: A Single Antibacterial Agent to Treat Gram-negative Infections, Including MDR *Pseudomonas aeruginosa*

Thomas Durand-Reville, Mark Sylvester, Janelle Comita, Frank Wu, Xiaoyun Wu, Jing Zhang, Jan Romero, Satenig Guler, Camilo Velez-Vega, Adam Shapiro, Nicole Carter, April Chen, Ramkumar Iyer, Samir Moussa, Sarah McLeod, Alita Miller, John O'Donnell and Ruben Tommasi

Entasis Therapeutics, Waltham, MA

Entasis Therapeutics
t.durand-reville@entasistx.com

Abstract

Background: The DBO class of compounds includes effective serine β-lactamase inhibitors such as avibactam, relebactam and the Entasis phase 3 clinical candidate, durlobactam. Their ability to inhibit Penicillin Binding Protein 2 (PBP2) can result in enhanced antibacterial activity when partnered with a β-lactam. Potent stand-alone PBP2-selective inhibitors have been reported, but their high spontaneous frequency of resistance (FOR) *in vitro* and lack of efficacy *in vivo* challenges progression of these compounds. We hypothesized that conversion of a PBP2-selective DBO into a potent PBP1 and PBP3 inhibitor would be an effective solution to this problem. Herein we report the discovery of ETX0462, a novel, single agent DBO, with potent PBP1a and PBP3 inhibition, excellent antibacterial activity versus contemporary clinical isolates of *Pseudomonas aeruginosa* (*P.a.*), as well as other Gram-negative bacteria.

Methods: Synthesis was conducted at Entasis Therapeutics. Protein-ligand co-crystal structures were obtained by X-ray diffraction at Advanced Photon Source. Iterative design, synthesis, and test cycles were progressed using results from *Pa* PBP fluorescence anisotropy assays, Pa broth microdilution (MIC) assays, and titratable outer membrane permeability whole cell assays. The final hypothesis was evaluated with FOR measurements and *in vivo* efficacy.

Results: We discovered four key features needed for a DBO to covalently link to the *P.a.* PBP3 active site serine (Ser294) and to exhibit potent *P.a.* PAO1 minimal inhibitory concentration (MIC) values. These are: (1) a hydrogen bond acceptor to bind with Asn401, (2) to fit within the pocket defined by Tyr407 and Tyr409, (3) a small lipophilic group in a specific conformation to displace an ordered water, and (4) a hydrogen bond donor for translocation through porins while minimizing efflux by efflux pumps. Measured *kinact/Ki* values for ETX0462 were 1.1 (± 0.2) x 10^3 $M^{-1}s^{-1}$ for *P.a.* PBP1a, <0.6 $M^{-1}s^{-1}$ for *P.a.* PBP2, and 2.6 (± 0.4) x 10^5 $M^{-1}s^{-1}$ for *Pa* PBP3. The ETX0462 MIC for *P.a.* PAO1 was 0.5 mg/L with permeation through multiple porins. The FOR of *P.a.* PAO1 at 4x MIC was <1.7 x 10^{-10}.

Conclusions: ETX0462 demonstrated potent *P.a.* PBP1a and PBP3 inhibition, excellent wild-type *P.a.* MIC values, was found to translocate *via* multiple porins and had a very low FOR. ETX0462 also showed excellent *in vivo* activity in murine thigh and lung models. These results support further development of ETX0462 as a first-in-class single agent DBO to treat multidrug-resistant Gram-negative infections.

Introduction and Methods

Multidrug-resistant (MDR) Gram-negative infections represent an urgent threat worldwide. A recent and important advance against this formidable challenge was the discovery of the diazabicyclooctane (DBO) scaffold. Its first example, Avibactam, is a reversible, covalent inhibitor of Ambler Class A, C and a few Class D β-lactamases, and is used in combination with ceftazidime to treat many Gram-negative infections [1]. In addition to their β-lactamase inhibition, certain DBOs also selectively inhibit penicillin-binding protein 2 (PBP2), leading to *in vitro* antibacterial activity [2,3]. However, these compounds cannot be developed as monotherapies as they present multiple liabilities, including high frequency of resistance and poor *in vivo* efficacy. We hypothesized that the selective inhibition of PBP2, which is non-essential for bacterial growth in vivo upon compensatory activation of stress response pathways [4] (or stringent response) could be responsible for these liabilities.

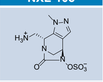
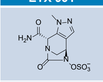
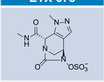
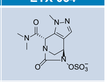
The goal of this program was therefore to expand the spectrum of inhibition to PBP3 and PBP1a and discover a novel single IV DBO to treat Gram-negative infections, including MDR *Pseudomonas aeruginosa* (*P.a.*).

Synthesis of analogs was conducted at Entasis Therapeutics. Protein-ligand co-crystal structures were obtained by X-ray diffraction at the Advanced Photon Source. Iterative design, synthesis, and test cycles were progressed using results from *P.a.* PBP fluorescence anisotropy assays [5], *P.a.* broth microdilution (MIC) assays according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M07-A10, and titratable outer membrane permeability whole cell assays [6]. The *in vivo* murine neutropenic infection models were conducted as previously described [7].

Re-engineering DBOs for *In Vivo* Efficacy

Modeling studies to identify the features behind the *P.a.* PBP2 selectivity of NXL-105 [8] suggested the presence of a salt bridge between the carboxylate of ASP409 and the positively charged aminomethyl group (R1) of the DBO inhibitor. The sequence alignment of *P.a.* PBP3 and PBP1a showed an asparagine in place of the PBP2 ASP409.

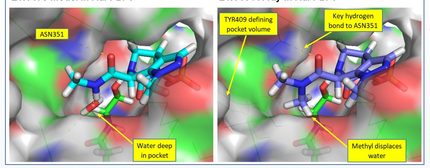
To form a hydrogen bond with the PBP3 ASN351 side chain and therefore design out the PBP2 selectivity, we decided to prepare a series of amide analogs.

	NXL-105	ETX'991	ETX'678	ETX'054
Structure				
P.a. PBP2 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	5,200	131	110	<8
P.a. PBP3 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	11	230	610	582,000
P.a. PAO1 MIC (mg/L)	0.125	>64	>64	4
FOR *P.a.* PAO1 (4x MIC)	2.10^{-5}	Not tested	Not tested	< 1.10^{-9}
P.a. MIC_{90} (mg/L)	0.5 (N=600)	Not tested	Not tested	32 (N=200)
Murine thigh infection model	**Not active *in vivo***	Not tested	Not tested	**Active *in vivo***

This resulted in reduced PBP2 acylation and a modest gain in PBP3 acylation for **ETX'991** and **ETX'678**. A concomitant loss of antibacterial activity vs. wild-type *P.a.* (PAO1) was also observed, which could be attributed to relatively weak PBP inhibition. In contrast, the R1 dimethyl amide analog **ETX'054** showed greatly increased inhibition of PBP3 and PBP1a, no detectable inhibition of PBP2, and an MIC of 4 mg/L against wild-type *P.a.* PAO1. In addition, **ETX'054** had a low frequency of resistance and was active *in vivo*, confirming our hypothesis of the liabilities associated with selective PBP2 inhibition.

The docking of **ETX'054** in our *P.a.* PBP3 model showed that one of the methyl groups of the dimethyl amide displaced a high-occupancy water identified in the **ETX'678** model. Displacement of this ordered water could favor proper placement of the ligand for the covalent reaction, and result in an entropic gain upon the return of that water to bulk solvent.

ETX'678 model in *P.a.* PBP3 **ETX'054 X-ray in *P.a.* PBP3**



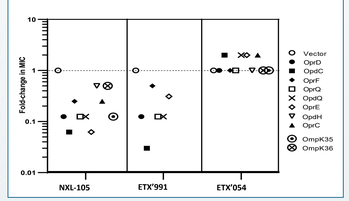
From the SAR exploration, we identified the following key features for *P.a.* PBP3 inhibition:

1. Engineer a hydrogen-bond with ASN351
2. Displace the high-occupancy water deep in binding pocket
3. Fit within the limited volume of the pocket defined by TYR409

Despite excellent biochemical potency, **ETX'054** exhibited limited antibacterial activity against a panel of *P.a.* contemporary clinical isolates (MIC_{90} = 32 mg/L). Biological studies identified poor outer membrane permeation as the main reason for the **ETX'054** decreased activity against certain *P.a.* isolates.

To investigate the limited permeation of **ETX'054** into bacteria, we employed the previously described TOMAS assay [6] to establish structure-porin permeation relationships (SPPR). This optimized cell-based porin assay confirmed that, while **NXL-105** and **ETX'991** permeate through multiple *P.a.* and *K. pneumoniae* porins, **ETX'054** does not. It became clear from this SPPR that a hydrogen bond donor at the R1 position of the DBO was required for effective uptake. Multi-porin uptake mediated by the primary amide of **ETX'991** indicated that a positive charge is not the only means to afford bacterial permeation.

Effect on MIC when overexpressing porins in TOMAS assay



Improving Permeation by Rational Design

Based on these preliminary findings, the medchem plan incorporated designs to fulfill the PBP3/1a inhibition (hydrogen bond with ASN, water molecule displaced and small volume) and multi-porin permeation (hydrogen bond donor). Initial attempts to modify the pyrazole ring or its substituents failed to significantly improve the MIC data compared to **ETX'054**.The focus of the medchem plan turned to novel R1 groups. To expedite the SAR exploration, we decided to prepare the analogs as a trans racemic mixture with the prior knowledge that only one of the enantiomers was active against the PBP enzymes.

The oxime analog of **ETX'054** (**ETX'312**) introduced a hydrogen bond donor which helped permeation but had a negative effect on PBP3 inhibition. A series of heterocycles were prepared but failed to improve the *P.a.* PAO1 MIC, either due to a lack of biochemical potency (**ETX'307**, **ETX'434**, **ETX'337**) or limited porin permeation (ETX'372). An sp2 carbon at R1 seemed required for PBP inhibition, either for optimal binding conformation or reactivity of the cyclic urea.

	ETX'054 (chiral)	ETX'312 (racemic)	ETX'307 (racemic)	ETX'434 (racemic)	ETX'337 (racemic)	ETX'372 (racemic)
	R1 =	R1 =	R1 = 	R1 = 	R1 = 	R1 = 
P.a. PBP2 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	<8	<6	<6	<6	32	7
P.a. PBP3 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	582,000	13,900	8	3,730	2,870	121,000
P.a. PBP1a acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	2,300	572	8	273	115	360
P.a. PAO1 MIC (mg/L)	4	32	>64	64	16	16
Porin permeation (TOMAS assay)	None	+	Not tested	Not tested	++	+

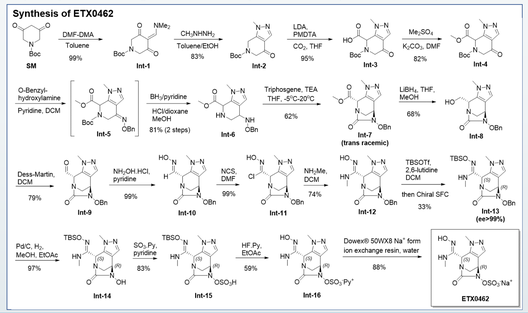
Several additional hydroxyamidines were synthesized to further explore the R1 pocket and led to the discovery of **ETX0462**. The unique *cis* conformation of this hydroxyamidine forces the methyl group away from the oxime while presenting 2 potential hydrogen bonds to enable multi-porin permeation.

	ETX'359 (racemic)	ETX'353 (racemic)	ETX'368 (racemic)	ETX0462 (chiral)
	R1 =	R1 = 	R1 =	R1 =
P.a. PBP2 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	7	7	6	<0.6
P.a. PBP3 acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	1,610	85,100	410	400,000
P.a. PBP1a acylation rate $k_{(on)}$ ($M^{-1}.s^{-1}$)	53	344	136	1,000
P.a. PAO1 MIC (mg/L)	16	64	>64	0.5
Porin permeation (TOMAS assay)	+++	+	None	+++

As observed during this lead optimization program, very subtle structural modifications (a simple methyl group!) can have dramatic effects on the PBP binding and porin permeation.

While the medchem synthetic route is long and linear, each transformation is high yielding. **Int-7** represented a versatile intermediate for analog synthesis and was prepared on hundreds of grams. Access to optically active analogs was obtained by chiral SFC separation (**Int. 13**).

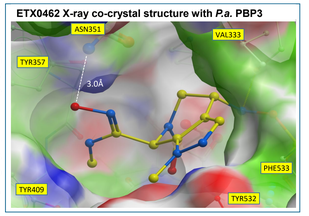
Synthesis of ETX0462



ETX0462: a Novel Class of Gram-negative Antibiotics

Our design hypothesis was confirmed by an X-ray co-crystal structure (PDB:7JWL). The R1 hydroxyamidine group of ETX0462 adopted a unique conformation and was able to make a hydrogen bond with ASN351, displace the high occupancy water at the bottom of the active site and fit in the small pocket defined by TYR409 and TYR357.

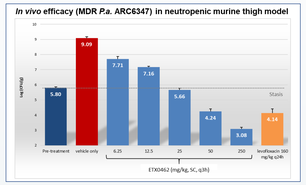
ETX0462 X-ray co-crystal structure with *P.a.* PBP3



ETX0462 is active against a broad range of Gram-negative pathogens. Excellent potency was observed against the 'KAPE' pathogens and also against *S. maltophilia* and biothreat pathogens, including *B. pseudomallei* and *B. mallei*.

Bacterial Species	N	ETX0462 (mg/L)		Comparator Agent (mg/L)	
		MIC Range	$MIC_{90/90}$	MIC Range	$MIC_{90/90}$
2017-2018 global clinical isolates				Imipenem	
P. aeruginosa	205	≤0.06 - 4	0.5/1	0.06 - >16	1/16
A. baumannii	200	0.25 - >16	2/4	0.06 - >16	8/>16
S. maltophilia	101	0.25 - 2	2/4	>16	>16/>16
E. coli	204	0.12 - 16	0.25/1	0.03 - 0.25	0.12/0.12
K. pneumoniae	201	0.25 - >16	0.5/4	0.06 - >16	0.12/0.5
Biothreat pathogens				Doxycycline	
F. tularensis	5	0.25 - 0.5	NA	0.5	NA
Y. pestis	5	0.25	NA	1 - 2	NA
B. anthracis	16	0.5 - 1	NA	≤0.01 - 0.06	NA
				Ceftazidime	
B. pseudomallei	13	0.25 - 2	NA	1 - 8	NA
B. mallei	6	0.25 - 0.5	NA	1 - 4	NA

***In vivo* efficacy (MDR *P.a.* ARC6347) in neutropenic murine thigh model**



ETX0462 is active *in vivo* as illustrated in the murine neutropenic thigh infection model. A strong dose response was observed against this MDR *P.a.* clinical isolate (ARC6347: OXA-486, PDC-24; MIC (imipenem) > 4 mg/L) with greater than 2.5-log kill achieved at the high dose. The MIC of ETX0462 for this strain is 0.25 mg/L.

Conclusions

- A unique rational design approach was used by incorporating Structure-Porin Permeation Relationships into the medchem plan.
- The critical biochemical spectrum (*P.a.* PBP3 and PBP1a) was engineered in the DBO scaffold using Structure-based Drug Design.
- Excellent broad-spectrum activity was observed against Gram-negative 'KAPE' and biothreat pathogens as a single agent (*in vitro* and *in vivo*). ETX0462 maintained activity in the presence of all 4 classes of β-lactamases tested.
- ETX0462 was well tolerated in 14-day rat GLP toxicology study to a limit dose of 2000 mg/kg.
- If successfully developed, ETX0462 would represent the first new antibiotic class in over 25 years to treat MDR Gram-negative and biothreat infections.

References

[1] Ehmann, D. E., *et al.* (2012) Proc. Natl. Acad. Sci. USA 109, 11663-11668, doi:10.1073/pnas.1205073109; 10.1073/pnas.1205073109; [2] Livermore, D. M., *et al.* (2017) J. Antimicrob. Chemother. 72, 1373-1385, doi: 10.1093/jac/dkw593; [3] Durand-Reville, T. F., *et al.* (2017) Nature Microbiol. 2, 17104, doi:10.1038/nmicrobiol .2017.104; [4] Doumith, M., *et al.* (2016) J. Antimicrob. Chemother. 71, 2810-2814, doi:10.1093/jac/dkw230; [5] Shapiro, A. B., *et al.* (2013) Anal. Biochem. 439, 37-43, doi:10.1016/j.ab.2013.04.009; [6] Iyer, R., *et al.* (2017) ACS Infect. Dis. 3, 310-319, doi:10.1021/acsinfecdis.6b00197; [7] Gerber, A. U., *et al.* (1983) Journal Infect. Dis. 147, 910-917, doi:10.1093/infdis/147.5.910; [8] Levasseur, P., *et al.* (2010). WO/2010/041112.

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