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ETX0462 Inhibits Penicillin-binding Protein 3 of *Pseudomonas aeruginosa* and *Escherichia coli*

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Abstract

Background: ETX0462 is a novel, rationally designed, diazabicyclooctane (DBO) penicillin-binding protein (PBP) inhibitor with potent in vitro and in vivo activity against Gram-negative pathogens, including *P. aeruginosa* (*Pa*) and *E. coli* (*Ec*). Previously reported DBOs with antibacterial activity selectively inhibited PBP2. Selective inhibition of PBP2 is associated with poor *in vivo* efficacy. We characterized PBP inhibition by ETX0462.

Methods: *Pa* cells (PAO1 strain) were treated with ETX0462 for 3 hours and observed with phase contrast microscopy. Inhibition by ETX0462 of PBPs *in situ* in *Pa* membranes was determined by blocking BOCILLIN labeling, detected by SDS-PAGE. Second-order rate constants for inactivation (k_{inact}/K_I) were measured with purified, soluble PBP constructs using fluorescence anisotropy-based assays. The partition ratio was measured by inhibition of the initial rate of the PBP3 reaction with BOCILLIN after incubating *Pa* PBP3 with ETX0462 at various molar ratios. Intact protein mass spectrometry was used to measure the masses of covalent adducts of ETX0462 with proteins. Reversibility of PBP3 acylation by ETX0462 was detected by exchange of covalently bound ETX0462 from *Pa* PBP3 to an acceptor protein, P99 β-lactamase, which does not detectably hydrolyze ETX0462.

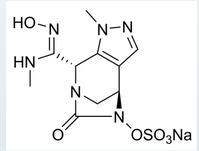
Results: ETX0462 caused *Pa* cell filamentation, consistent with PBP3 inhibition. ETX0462 targeted PBP1a, 1b, 3, 4 and 5 in *Pa* membranes. Values of k_{inact}/K_I were 1.1 (± 0.2) x 10^3 $M^{-1}s^{-1}$ for PBP1a, 2.6 (± 0.4) x 10^5 $M^{-1}s^{-1}$ for PBP3,but <0.6 $M^{-1}s^{-1}$ for PBP2 of *Pa*. The value of k_{off} with *Pa* PBP3 was 1.4 (± 0.2) x 10^{-4} s^{-1}. Values of k_{inact}/K_I were 3.2 (± 0.7) x 10^3 $M^{-1}s^{-1}$ for PBP3 and 3 (±3) x 10^2 $M^{-1}s^{-1}$ for PBP2 of *Ec*. The partition ratio for inhibition of *Pa* PBP3 was 1.0 and did not increase with time from 15 min to 4 h, showing that there was no catalytic turnover of ETX0462. ETX0462 formed stable, covalent adducts with *Pa* PBP3, consisting mostly of the full mass (346 Da), with some desulfation. The non-zero dissociation rate constant is explained by the ability of ETX0462 to recyclize and dissociate from *Pa* PBP3, as shown by exchange of the 346 Da adduct from PBP3 to P99.

Conclusions: Unlike previously reported DBOs with antibacterial activity, ETX0462 inhibits *Pa* PBP1a and PBP3 instead of PBP2. *Pa* PBP3 is incapable of catalytic hydrolysis of ETX0462, but ETX0462 can recyclize to its original state and slowly dissociate intact from PBP3.

Introduction

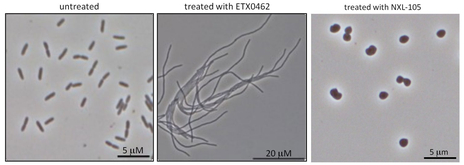
ETX0462 is a novel, rationally designed diazabicyclooctane (DBO) penicillin-binding protein (PBP) inhibitor with potent in vitro and in vivo activity against Gram-negative pathogens, including *P. aeruginosa* (*Pa*) and *E. coli* (*Ec*).Previously reported DBOs with antibacterial activity selectively inhibited PBP2, but selective inhibition of PBP2 is associated with poor in vivo efficacy. We characterized PBP inhibition by ETX0462.

ETX0462



Microscopy

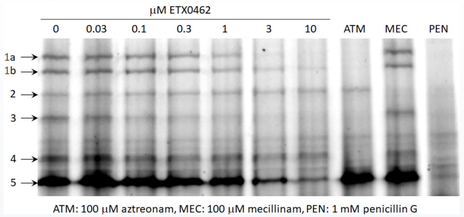
Pa cells (PAO1 strain) were treated with 0.25 µg/mL (1/2X MIC) ETX0462 for 3 hours and observed with phase contrast microscopy.



Cell filamentation is a characteristic response to PBP3 inhibition, whereas PBP2 inhibition results in the formation of spherical cells, as shown for cells treated with the PBP2-specific inhibitor NXL-105 at 0.06 µg/mL (0.5X MIC).

PBP inhibition *in situ*

Inhibition by ETX0462 of PBPs in situ in *Pa* membranes was determined by blocking BOCILLIN labeling, detected by SDS-PAGE and fluorescence imaging.



Aztreonam inhibits labeling of PBP1a, 1b, 3 and 4.Mecillinam inhibits labeling of PBP2.Penicillin inhibits labeling of all PBPs, by definition.

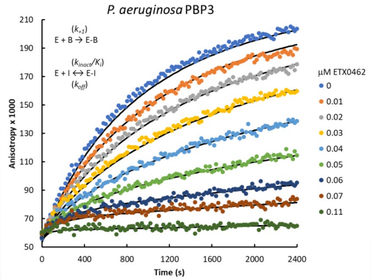
ETX0462 inhibits labeling of PBP1a, 1b, 3 and 5.

Purified PBP inhibition

Second-order rate constants for inactivation (k_{inact}/K_I) were measured with purified, soluble PBP constructs using fluorescence anisotropy-based assays.[1,2,3]

Top: an example of *Pa* PBP3 inhibition by ETX0462. ETX042 concentrations in µM are shown at right (some curves omitted for clarity). Dots are measurements, lines are global fits. The kinetic model is shown in the inset. E: PBP3 enzyme, B: BOCILLIN, I: ETX0462 inhibitor. The kinetics do not distinguish between reversal of inhibitor binding and hydrolysis of the inhibitor to form an inactive product.

Bottom: Values of k_{inact}/K_I and k_{off} measured for several PBPs.

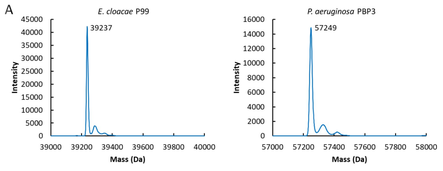


Enzyme	n	k_{inact}/K_I ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
P. aeruginosa PBP1a	7	1.0 (± 0.2) x 10^3	none detected
P. aeruginosa PBP2	1	<0.6	N/A
P. aeruginosa PBP3	15	4 (± 1) x 10^5	1.3 (± 0.8) x 10^{-4}
E. coli PBP2	2	1.4 (± 0.1) x 10^2	none detected
E. coli PBP3	6	3.2 (± 0.7) x 10^3	2 (± 2) x 10^{-4}
K. pneumoniae PBP3	1	4 x 10^3	4 x 10^{-4}

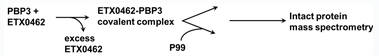
ETX0462 inhibits PBP1a and PBP3 but not PBP2 in *Pa*. In *Ec*, however, ETX0462 inhibits PBP2 and PBP3, but is more potent against PBP3. ETX0462 inhibits *Klebsiella pneumoniae* PBP3 with similar potency as *Ec* PBP3. ETX0462 slowly dissociates from PBP3.

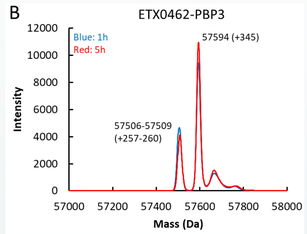
Acylation exchange

Intact protein mass spectrometry was used to measure the masses of covalent adducts of ETX0462 with proteins. *Panel A*: Masses of unliganded *Enterobacter cloacae* β-lactamase P99 acceptor (*left*) and *Pa* PBP3 donor (*right*). Masses were measured to a precision of about ±1 part in 10,000.



Panel B: Mass spectrum of *Pa* PBP3 after reaction with ETX0462, removal of excess, unbound ETX0462, and incubation for 1 or 5 hours. The larger peak consists of the mass of PBP3 with the full-mass adduct of ETX0462, which reacts with the active site serine nucleophile. The smaller peak is believed to be 2 unresolved peaks of similar intensities consisting of the adduct with mass losses of 80 Da (SO_3) and 96 Da (SO_4). The result is essentially the same after 1- and 5-hour incubations. No unliganded PBP3 is seen, consistent with a lack of hydrolysis of ETX0462 by *Pa* PBP3.

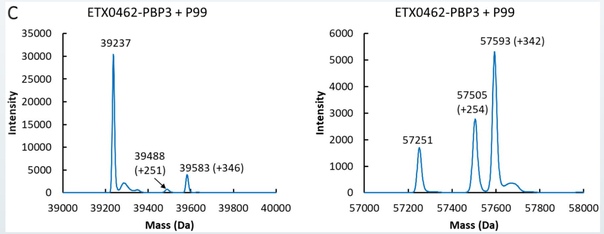




Panel C: Reversibility of PBP3 acylation by ETX0462 was detected by exchange of covalently bound ETX0462 from *Pa* PBP3 to P99, which does not detectably hydrolyze ETX0462 but is inhibited by it.

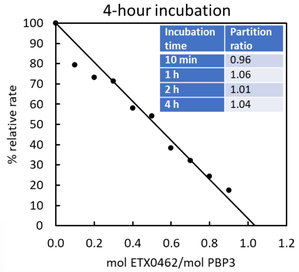
P99 was added to the ETX0462-PBP3 covalent complex with excess, unbound ETX0462 removed, and incubated for 1 hour.

The appearance of unliganded PBP3 shows that ETX0462 dissociated from PBP3 (*right panel*). The appearance of P99 with the full-mass adduct of ETX0462 (*left panel*) shows that ETX0462 dissociated from PBP3 in its original cyclic form and reacted with P99, since the cyclic urea ring-opened product of ETX0462 in the covalent complex with PBP3 is not reactive. Partial desulfation of the ETX0462-P99 covalent complex was also observed.



Partition ratio

The partition ratio (or turnover number) is the average number of molecules of a covalent enzyme inhibitor required to obtain complete inhibition given sufficient time. The partition ratio of ETX0462 with *Pa* PBP3 was measured with the fluorescence anisotropy assay by inhibition of the initial rate of the PBP3 reaction with BOCILLIN after incubating *Pa* PBP3 with ETX0462 at various molar ratios.



Incubation time	Partition ratio
10 min	0.96
1 h	1.06
2 h	1.01
4 h	1.04

The incubation time-independent partition ratio of 1 shows that one molecule of ETX0462 per molecule of *Pa* PBP3 is sufficient for complete inhibition, and it confirms that *Pa* PBP3 does not hydrolyze ETX0462.

Conclusions

- **ETX0462 is a new diazabicyclooctane (DBO) antibacterial compound that reacts covalently with the essential high-molecular-mass PBPs 1a, 1b and 3 of *Pa* and *Ec*.**
- **ETX0462 does not react with *Pa* PBP2, and it reacts relatively weakly with *Ec* PBP2.**
- ***Pa* PBP3 does not hydrolyze covalently bound ETX0462.**
- **ETX0462 can slowly dissociate from *Pa* PBP3 by reforming the cyclic urea ring, restoring the compound to its original, reactive form.**

Acknowledgments

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References

1. Shapiro et al (2013) *Anal. Biochem.* 439, 37-432.
2. Shapiro et al (2014) *Anal. Biochem.* 463, 15-223.
3. Shapiro et al (2019) *ACS Infect. Dis.* 5, 863-872