SATURDAY - 277

# Reversibility of β-lactamase inhibition by the broad-spectrum diazabicyclooctenone serine β-lactamase inhibitor ETX2514

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## Abstract

### Introduction

ETX2514 is a new broad-spectrum non-β-lactam serine β-lactamase (BL) inhibitor (BLI) in clinical development that restores β-lactam activity against multidrug-resistant Gram-negative bacteria. ETX2514 is more potent and has a broader spectrum of inhibition than the related BLI avibactam. After acylating BLs, with opening of its cyclic urea ring, avibactam can recyclize and dissociate intact from some BLs. We investigated the reversibility of BL acylation by ETX2514.

### Methods

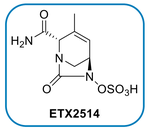
Dissociation rate constants ($k_{off}$) of acyl-enzyme complexes were measured by jump-dilution with 10 serine BLs representing classes A, C and D. The masses of the complexes were measured by protein mass spectrometry (MS). Recyclization of ETX2514 was tested by acylation exchange, the ability of ETX2514 to transfer from one BL to another. Partition ratios were measured by the residual activity after incubating a BL with various molar ratios of ETX2514. Degradation of ETX2514 was measured by compound MS. MICs were measured by broth microdilution.

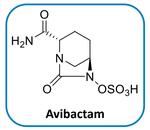
### Results

Values of $k_{off}$ ranged from 3.4 x $10^{-6}$ $s^{-1}$ for OXA-10 to 4 x $10^{-3}$ $s^{-1}$ for *Pseudomonas aeruginosa* AmpC. MS of the acyl-enzyme complexes showed that for *P. aeruginosa* AmpC, CTX-M-15, *Enterobacter cloacae* P99, SHV-5, TEM-1, OXA-23, OXA-24 and OXA-48 the mass increase of the ETX2514 adduct was the same as that of ETX2514, indicating that opening of the cyclic urea ring was the only change upon acylation. OXA-10, however, was acylated with a mixture of the full-mass adduct and one reduced in mass by 80 Da, consistent with desulfation. With KPC-2, only the adduct missing 80 Da was observed. Acylation exchange experiments showed that intact ETX2514 was released from AmpC, CTX-M-15, P99, SHV5 and TEM-1, demonstrating ETX2514 recyclization. There was no significant exchange from KPC-2, OXA-10, OXA-23, OXA-24, or OXA-48. Partition ratios were about 1.0 for all BLs except for KPC-2. The partition ratio for KPC-2 increased from 1.2 initially to 3.0 after 2 hours. ETX2514 (20 μM) was fully degraded by 6 μM KPC-2 within 2 hours at 37°C, whereas ETX2514 by itself was stable. Nevertheless, ETX2514 restored piperacillin and ceftazidime MICs in *P. aeruginosa* expressing KPC-2.

### Conclusions

Like avibactam, ETX2514 dissociated intact from most class A and C BLs by recyclization. Inhibition of class D enzymes was virtually irreversible, with partition ratios of 1. KPC-2 was the only BL tested that had measurable degradative activity, which was insufficient to counteract the ability of ETX2514 to protect β-lactams.



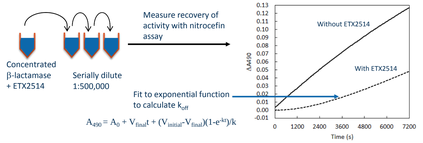




## Inhibition of β-lactamases

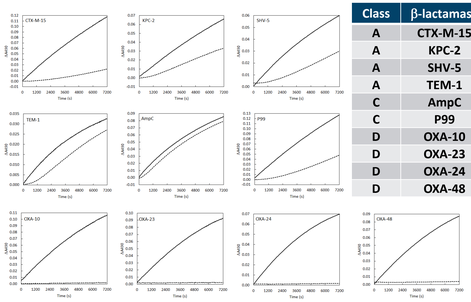
| BLI | $k_{inact}/K_i$ in $M^{-1}s^{-1}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Class A | | | Class C | | Class D | | | |
| | CTX-M-15 | TEM-1 | KPC-2 | AmpC | P99 | OXA-10 | OXA-23 | OXA-24 | OXA-48 |
| ETX2514 | 7,400,000 | 14,000,000 | 940,000 | 920,000 | 2,300,000 | 8,600 | 5,100 | 9,300 | 830,000 |
| Avibactam | 800,000 | 400,000 | 6,000 | 3,000 | 8,000 | 70 | nd | 80 | 5,000 |

ETX2514 inhibits a broad range of class D β-lactamases, as well as classes A and C.

## Off-rate constants measured by jump dilution



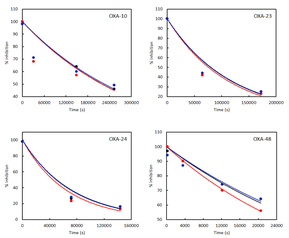

## Continuous jump-dilution




| Class | β-lactamase | $k_{off}$ ($s^{-1}$) |
|---|---|---|
| A | CTX-M-15 | 2.2 x $10^{-4}$ |
| A | KPC-2 | 1.0 x $10^{-3}$ |
| A | SHV-5 | 5.5 x $10^{-4}$ |
| A | TEM-1 | 1.4 x $10^{-3}$ |
| C | AmpC | 4 x $10^{-3}$ |
| C | P99 | 3.4 x $10^{-4}$ |
| D | OXA-10 | - |
| D | OXA-23 | - |
| D | OXA-24 | - |
| D | OXA-48 | - |

Dissociation from class D enzymes too slow to measure.

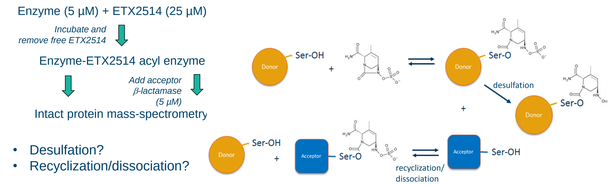
## Discontinuous jump-dilution



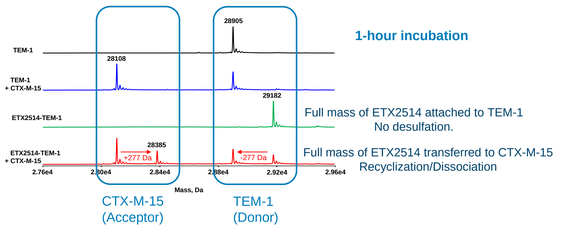

| β-lactamase | $k_{off}$ ($s^{-1}$) |
|---|---|
| OXA-10 | 3.4 x $10^{-6}$ |
| OXA-23 | 1.1 x $10^{-5}$ |
| OXA-24 | 1.7 x $10^{-5}$ |
| OXA-48 | 2.5 x $10^{-5}$ |

Very slow dissociation of ETX2514 from class D β-lactamases.

## Acylation products by intact protein mass spectrometry




- Desulfation?
- Recyclization/dissociation?



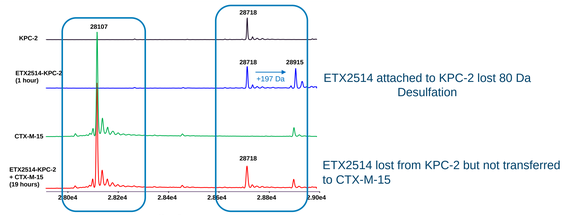

1-hour incubation

Full mass of ETX2514 attached to TEM-1
No desulfation.

Full mass of ETX2514 transferred to CTX-M-15
Recyclization/Dissociation

CTX-M-15 (Acceptor) TEM-1 (Donor)

Similar results with CTX-M-15, SHV-5, *P. aeruginosa* AmpC, and *E. cloacae* P99.

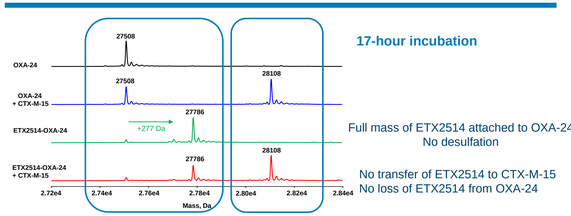



ETX2514 attached to KPC-2 lost 80 Da Desulfation

ETX2514 lost from KPC-2 but not transferred to CTX-M-15

CTX-M-15 (Acceptor) KPC-2 (Donor)

ETX2514 was not intact when released by KPC-2.




17-hour incubation

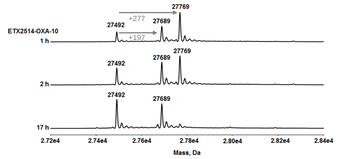
Full mass of ETX2514 attached to OXA-24
No desulfation

No transfer of ETX2514 to CTX-M-15
No loss of ETX2514 from OXA-24

OXA-24 (Donor) CTX-M-15 (Acceptor)

Similar result with OXA-23 and OXA-48

OXA-10 197 and 277 Da adducts from ETX2514 to OXA-10
Slow loss of 277 adduct from OXA-10
No acyl transfer from OXA-10 to CTX-M-15 (not shown)
Consistent with very slow degradation of ETX2514 by OXA-10

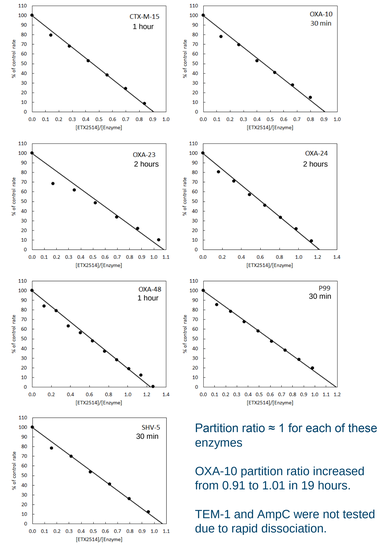



## Partition ratio – the average number of molecules of ETX2514 required to inhibit one molecule of β-lactamase

Enzyme (3 μM) + ETX2514 (0-6 μM)

preincubation

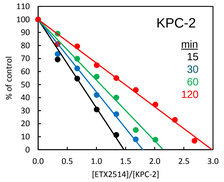
Dilute into β-lactamase assay with nitrocefin.
Measure initial rate of reaction.




Partition ratio ≈ 1 for each of these enzymes

OXA-10 partition ratio increased from 0.91 to 1.01 in 19 hours.

TEM-1 and AmpC were not tested due to rapid dissociation.




Slow degradation of ETX2514 by KPC-2 observed as a time-dependent increase in the partition ratio.

## Restoration of β-lactam potency by ETX2514 in vitro

| BLI at 4 mg/L | Piperacillin MIC (mg/L) in isogenic *P. aeruginosa* PAO1-derived strains expressing a single β-lactamase | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | None | Class A | | | Class C | | Class D | | | |
| | | CTX-M-15 | TEM-1 | KPC-2 | AmpC | P99 | OXA-10 | OXA-23 | OXA-24 | OXA-48 |
| None | 2 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| ETX2514 | 2 | 4 | 4 | 4 | 4 | 4 | 8 | 8 | 8 | 4 |
| Avibactam | 4 | 4 | 8 | 8 | 8 | 4 | >64 | >64 | >64 | 8 |

| BLI at 4 mg/L | Ceftazidime MIC (mg/L) in isogenic *P. aeruginosa* PAO1-derived strains expressing a single β-lactamase | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | None | Class A | | | Class C | | Class D | | | |
| | | CTX-M-15 | TEM-1 | KPC-2 | AmpC | P99 | OXA-10 | OXA-23 | OXA-24 | OXA-48 |
| None | 1 | 64 | 2 | 64 | 32 | >64 | 2 | 2 | 2 | 2 |
| ETX2514 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
| Avibactam | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

Despite the measurable rate of ETX2514 degradation by KPC-2, ETX2514 is capable of rescuing the potency of β-lactamase in isogenic *P. aeruginosa* strains expressing KPC-2

## Conclusions

- ETX2514 dissociated from each of 10 β-lactamases representing classes A, C, and D, with a wide range of $k_{off}$s.
- Dissociation from class D β-lactamases was much slower than from class A and C enzymes.
- In some cases, intact ETX2514 that dissociated from one enzyme reacted with a second enzyme, demonstrating that ETX2514 recyclized, similarly to avibactam[1].
- Loss of 80 Da from the ETX2514 adduct with some enzymes suggests sulfate hydrolysis, which was also observed with avibactam.
- Of the enzymes tested, only KPC-2 showed a significant, but low rate of ETX2514 degradation (~1/h), as seen with avibactam[2]. For the other enzymes, a single molecule of ETX2514 per enzyme molecule sufficed for complete inhibition.
- ETX2514 restored antibacterial potency of β-lactams in vitro due to potent inhibition of class A, C, and D β-lactamases, despite slow dissociation and/or hydrolysis.

References
1. Ehmann et al (2012) Avibactam is a covalent, reversible, non–β-lactam β-lactamase inhibitor. *Proc Natl Acad Sci USA* **109**, 11663-11668
2. Ehmann et al (2013) Kinetics of avibactam inhibition against class A, C, and D β-lactamases. *J Biol Chem* **288**, 27960-27971

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