ASM Microbe 2018
Saturday-637

Molecular Characterization of Clinical Trial Isolates Exhibiting Increased MIC Results during Fosfomycin (ZTI-01) Treatment in a Phase 2/3 Clinical Trial for Complicated Urinary Tract Infections (ZEUS)

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Introduction

- Gram-negative organisms producing extended spectrum β-lactamases (ESBLs) that show resistance to many antibiotics have been steadily increasing in the hospital and community settings to alarming levels
- The spread of ESBL-producing organisms has led to increased use of carbapenem agents, which has promoted the selection of carbapenem-resistant *Enterobacteriaceae* (CRE)
  - CRE isolates are categorized as an urgent threat by the US Centers for Disease Control and Prevention and the World Health Organization
- Fosfomycin has been clinically available in many European countries, Japan, South Africa, and Brazil in both oral and parenteral formulations for up to 4 decades
  - Intravenous fosfomycin (known as ZTI-01) is under development in the United States for treatment of complicated urinary tract infections (cUTIs)
  - The broad spectrum of activity of fosfomycin and ability of the IV form to achieve significant plasma, urine, and tissue concentrations render it well-suited for treating multidrug-resistant (MDR) gram-negative bacterial infections
- A Phase 2/3 trial (ZEUS) was recently completed that compared the efficacy and safety of fosfomycin (ZTI-01) to piperacillin-tazobactam (TZP) for treating cUTI, including acute pyelonephritis
  - ZTI-01 was found to be noninferior to TZP for overall success at the test-of-cure (TOC) visit in the m-MITT population with an overall success of 64.7% in the ZTI-01 group compared to 54.5% in the TZP group
  - The treatment difference favored ZTI-01 (10.2%; 95% CI [-0.4, 20.8])
  - The primary endpoint was re-analyzed using results from a post-hoc blinded pulsed-field gel electrophoresis classification (PFGE) and resulted in higher overall success rates for both treatment groups, further favoring ZTI-01
  - Overall success occurred in 69.0% of ZTI-01 patients and 57.3% TZP patients, with a treatment difference of 11.7% (95% CI [1.3, 22.1])
- The number of patients who developed decreased susceptibility to either study drug received was defined by a >4-fold increase in ZTI-01 or TZP MIC relative to that of the baseline strain; this number was low in both treatment groups (10 ZTI-01 patients, 9 TZP patients)
  - This study evaluated the potential mechanisms of reduced susceptibility observed in isolates obtained in the Phase 2/3 study from the fosfomycin treatment arm

Materials and Methods

Bacterial isolates

- Isolates selected for this analysis were recovered during a Phase 2/3 trial (ZEUS) to evaluate the efficacy and safety of ZTI-01 to treat cUTIs
- The bacterial identification of all isolates collected from the clinical trial was confirmed by JMI Laboratories (North Liberty, Iowa, USA) using matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany)
  - JMI was blinded to randomization and treatment codes
- Isolates for this study were selected when pathogen(s) recovered during any of the follow up (FU) patient visits exhibited fosfomycin MIC value(s) >4-fold higher than that observed for the respective baseline isolate (day 1 [D1])
- A total of 10 patients had pathogens recovered during FU visits (day 5 [D5], TOC, end of treatment [EOT], and late follow-up [LFU]) that met the study inclusion criterion and previously reported study classifications for microbiologic evaluation and clinical outcome

Resistance mechanism characterization by next-generation sequencing

- Total genomic DNA was extracted using the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, Ohio, USA)
- DNA samples were quantified using the Qubit™ High Sensitivity DS-DNA assay (Invitrogen, ThermoFisher Inc.) and normalized to 0.2 ng/µL
  - A total of 1 ng high-quality genomic DNA was used as input material for library construction
- DNA libraries were prepared using the Nextera XT™ library construction protocol (Illumina, San Diego, California, USA) following the manufacturer's instructions and sequenced on a MiSeq Sequencer (Illumina) at JMI Laboratories
- Each raw sequencing data set was quality assured, error corrected, and assembled *de novo* using the SPAdes genome assembler
- Assembled genomes were subjected to a proprietary software (JMI Laboratories) to screen for fosfomycin resistance genes including *fomA, fomB, fosA, fosA2, fosA4, fosA5, fosB, fosB1, fosB2, fosC, fosD, fosE, fosF, fosG, fosK*, and *fosX*
- Alterations in intrinsic genes associated with the fosfomycin binding site (MurA) and transport system regulators (*glpT, uhpT, uhpABC, cyaA, crp*, and *ptsI*) were investigated

Epidemiology typing

- PFGE was performed on genomic DNA prepared in agarose blocks and digested with appropriate restriction endonuclease (New England Biolabs, Ipswich, Massachusetts, USA) for the bacterial species being treated
- Restriction fragments were resolved on the CHEF-DR II apparatus (BioRad, Richmond, California, USA) using standard conditions previously determined
  - When a coefficient of similarity of 100% was obtained, a follow-up isolate was considered identical to baseline (each baseline and follow-up isolate was designated as PFGE type A)
  - When a coefficient of similarity of ≥85%–<100.0% was obtained, a follow-up isolate was considered related to baseline (PFGE profile of follow-up isolate designated as type A, followed by a number [A1, A2, A3, etc.] to indicate a subtype)
  - When a coefficient of similarity of <85% was obtained, a follow-up isolate was considered unrelated to baseline (designated as PFGE type other than that of baseline [ie, B, C, D, etc.])
  - The percentage of similarity was determined based on band position tolerance and optimization. The parameters were set to have the interpretations obtained from percentage of similarities aligned with visual inspections (number of band differences) and the criteria established by Tenover et al. (1995)
- Multilocus sequence typing (MLST) was performed by extracting previously defined sets of 7 housekeeping gene fragments (~500 bp) for each species included in the study
  - Each fragment was compared to known allele variants for each locus (housekeeping gene) on the MLST website (http://www.mlst.net)

Results

- Table 1 presents genetically unrelated baseline and follow-up isolates, as determined by PFGE and MLST typing
  - Three patients (1, 2, and 3) had isolates in which fosfomycin MIC results obtained from FU isolates were at least 16-fold higher than the respective baseline strain
  - Gram-negative isolates that were unrelated to the respective baseline strain consisted of 1 FU *Escherichia coli* isolate that did not have any of the genes associated with fosfomycin resistance mechanisms that were investigated, whereas 2 *E. coli* isolates from patient 2 and 1 *Proteus mirabilis* isolate from patient 3 showed alterations in various proteins (Table 1)

Table 1 Fosfomycin resistance mechanisms detected among baseline and FU isolates that were genetically unrelated

Subject	Time point[a]	Date	Organism	JMI isolate no.	Fosfomycin MIC (µg/mL)	Epidemiology typing[b]		Resistance mechanisms[c]
						PFGE	MLST	
1	D1	12/7/2016	*E. coli*	945	0.5	A	1193	NA
1	LFU	1/3/2017		1167	16	B	131	NF
2	D1	9/20/2016	*E. coli*	324	1	A	648	NA
2	EOT	9/26/2016		586	>512	NT	162	CyaA (L1-V20Δ); GlpT (W161R, T383M)
2	TOC	10/10/2016		587	512	B	162	CyaA (L1-V20Δ); GlpT (W161R, T383M); UhpT (C143R)
3	D1	9/27/2016	*P. mirabilis*	589	64	A	MNA	Mur (V70I); GlpT (N437S, V425A); UhpC (S6L, S282P, I328M)
3	EOT	10/3/2016		590	>512	B	MNA	GlpT[d], UhpC[d] and CyaA[d]

[a] Time point when isolate culture was performed: D1, day 1 visit; EOT, end-of-treatment visit; TOC, test-of-cure visit; LFU, last follow-up visit.
[b] PFGE, pulsed-field gel electrophoresis. NT, not tested; MNA, MLST method not available for *P. mirabilis*.
[c] NF, no gene or alteration found; NA, not applicable, baseline isolate used as control.
[d] Amino acid alterations caused a frame-shift mutation.

Table 2 Fosfomycin resistance mechanisms detected among baseline and FU isolates that were genetically related

Subject	Time point[a]	Date	Organism	JMI isolate no.	Fosfomycin MIC (µg/mL)	Epidemiology typing[b]		Resistance mechanisms[c]
						PFGE	MLST	
4	D1	8/4/2016	*E. coli*	105	0.5	A	405	NA
4	LFU	8/30/2016		188	64	A	405	NF
5	D1	9/5/2016	*K. pneumoniae*	193	4	A	101	NA
5	D5	9/9/2016		253	32	NT	101	*fosA*
5	TOC	9/27/2016		255	4	A1	NT	NT
6	D1	10/4/2016	*K. pneumoniae*	370	16	A	340	*fosA*
6	EOT	10/10/2016		492	>512	NT	340	*fosA*; GlpT (G358D)
6	TOC	10/25/2016		626	>512	A	340	*fosA*; GlpT (W149R); UhpT (A431Δ); UhpC (E296G)
7	D1	10/21/2016	*E. cloacae*	417	64	A	93	*fosA*
7	D5	10/25/2016		418	512	NT	93	*fosA*
7	EOT	10/27/2016		508	>512	NT	93	*fosA*
7	TOC	11/8/2016		894	>512	A	93	*fosA*
7	LFU	11/15/2016		893	256	NT	93	*fosA*
8	D1	11/8/2016	*P. aeruginosa*	756	64	A	621	*fosA*
8	TOC	11/28/2016		833	64	A	NT	NT
8	LFU	12/2/2016		834	>512	NT	621	*fosA*; MurA (M102V); PtsI (P407A)
9	D1	8/17/2016	*P. aeruginosa*	101	64	A	234	*fosA*
9	EOT	8/23/2016		124	>512	NT	234	*fosA*; GlpT (T278-281Δ)[d]
9	TOC	9/4/2016		216	>512	A	234	*fosA*; GlpT (P277-278Δ)[d]
10	D1	11/24/2016	*P. aeruginosa*	1142	64	A	654	*fosA*
10	TOC	12/14/2016		1144	>512	A	654	*fosA*

[a] Time point when isolate culture was performed: D1, day 1 visit; D5, day 5 visit; EOT, end-of-treatment visit; TOC, test-of-cure visit; LFU, last follow-up visit.
[b] PFGE, pulsed-field gel electrophoresis. NT, not tested.
[c] NT, not tested; NF, no gene or alteration found; NA, not applicable, baseline isolate used as control.
[d] Amino acid alterations caused a frame-shift mutation.

- Table 2 presents genetically related baseline and FU isolates
  - *E. coli* recovered from patient 4 at LFU tested with a fosfomycin MIC value (64 µg/mL) 128-fold higher than the baseline isolate (0.5 µg/mL) (Table 2); none of the resistance mechanisms investigated was detected in this isolate
  - *Klebsiella pneumoniae* isolates recovered at baseline and D5 visits from patient 5 tested with fosfomycin MIC results of 4 and 32 µg/mL, respectively (Table 2); the FU isolate recovered at D5 carried the *fosA* gene
  - Patient 6 in whom *K. pneumoniae* isolates were recovered at D1, EOT, and TOC tested with fosfomycin MIC values of 16, >512, and >512 µg/mL, respectively (Table 2)
    - All 3 isolates harbored *fosA*
    - Alterations in GlpT and in GlpT, UhpT, and UhpC were observed in both isolates tested with fosfomycin MIC values of >512 µg/mL
  - Patient 7 had 5 isolates of *Enterobacter cloacae* belonging to ST93; these isolates displayed fosfomycin MIC results of 64 – >512 µg/mL and all carried *fosA*
    - Additional determinants or mutations in the investigated intrinsic genes were not detected in any isolates (Table 2)
  - Three patients (8, 9, and 10) had 2 to 3 *Pseudomonas aeruginosa* pathogens recovered during baseline and FU visits (Table 2)
    - All tested baseline and FU *P. aeruginosa* isolates carried *fosA*
  - The isolate from patient 8 recovered at LFU visit had alterations at MurA (M102V) and PtsI (P407A), whereas FU isolates from patient 9 had a 4 amino acid deletion at GlpT that caused a frame-shift alteration (Table 2)
  - The *P. aeruginosa* isolate recovered from patient 10 at TOC visit did not show any alterations of the genes investigated in this study
  - Table 3 presents clinical and microbiologic response at TOC and LFU visits from the Phase 2/3 Zeus trial
    - Most of the patients who were infected by a pathogen with decreased susceptibility had a combination of clinical cure and microbiologic persistence at the TOC visit
    - One patient failed therapy at TOC while 90% (9/10) of these patients were deemed clinical cures based upon resolved/improved signs/symptoms that warranted no further antibiotic treatment
  - At LFU visit, most patients had a combination of sustained clinical cure and continued persistence; 1 patient clinically relapsed and 8/9 (89%) were deemed sustained clinical cures

Table 3 Clinical and microbiologic response of patients at TOC and LFU with pathogens exhibiting decreased susceptibility to fosfomycin

Patient	Clinical response at TOC	Microbiologic response at TOC	Clinical response at LFU	Microbiologic response at LFU
1	Cure	Indeterminate	Sustained clinical cure	Indeterminate
2	Cure	Persistence	Relapse	Continued persistence
3	Failure	Persistence	Clinical failure[a]	Indeterminate
4	Cure	Persistence	Sustained clinical cure	Continued persistence
5	Cure	Persistence	Sustained clinical cure	Continued persistence
6	Cure	Persistence	Sustained clinical cure	Continued persistence
7	Cure	Persistence	Sustained clinical cure	Continued persistence
8	Cure	Persistence	Sustained clinical cure	Continued persistence
9	Cure	Persistence	Sustained clinical cure	Continued persistence
10	Cure	Persistence	Sustained clinical cure	Continued persistence

[a] At TOC visit, patient received additional antibiotics and was failing those medications at LFU visit.
TOC, test-of-cure visit; LFU, last follow-up visit.

Conclusions

- This data showed that increases in the fosfomycin MIC results could be attributed to mutations in the drug target binding site (MurA) and/or proteins associated with decreased drug permeability (ie, GlpT, UhpC, UhpT, CyaA, or PtsI)
- The lack of fosfomycin resistance mechanisms in 2 *E. coli* (JMI #1167 and 188), 4 *E. cloacae* (#418, 508, 894, and 893), and 1 *P. aeruginosa* (#1144) indicate that these isolates may have resistance mechanisms other than those investigated in this study and warrant further investigation
- Results indicate that the presence of *fosA* increased the fosfomycin MIC results 8-fold (*K. pneumoniae*; patient 5), whereas alterations in the binding site and/or other investigated genes increased the MIC results 16- to 64-fold
- Despite the identified mutations, a majority (90%) of patients were considered clinically cured and warranted no further antibiotic treatment
  - LFU visits of these patients continued to show a low rate of clinical relapse, with 8/9 patients continuing without other antibiotics and were considered sustained clinical cures
- The presence of resistance mechanisms altering the drug's MIC results may not always be immediately reflective of patients' clinical outcome, as shown within this data set; however, these data provide a potentially important context when interpreting molecular characterization of isolates with elevated MIC results to that of available clinical outcome data

Acknowledgements

This study was supported by Zavante Therapeutics Inc., San Diego, California. JMI Laboratories received compensation for services related to preparing this poster.

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