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P4A, a Novel Oral β-Lactamase for the Prevention of Cephalosporin-Induced C. *difficile* Infection

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Abstract

Background: The β-lactam antibiotics are excreted into the intestine where they can damage the microflora. Such antibiotic-mediated disruption of the intestinal flora is a known risk factor for C. *difficile* infection (CDI). P1A, a β-lactamase developed by Ipsat Therapies, Finland, is given orally with IV penicillins to degrade antibiotics in the gut and protect the microflora. In five clinical trials, P1A preserved the microbiome and prevented overgrowth of resistant coliforms without altering systemic antibiotic levels. However, P1A has limited utility as it does not efficiently degrade cephalosporins (CEFs), major offenders for CDI. Therefore, we engineered P4A from P1A as a next generation therapeutic for use with CEFs to prevent CDI.

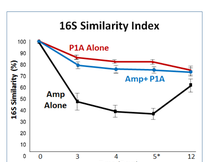
Methods: P1A mutants were screened for degradation of a panel of CEFs. P4A was compared to P1A in a microtiter plate activity assay that used E. *coli* growth as the read-out for antibiotic degradation. 10 to 1000 μg/ml of antibiotic were mixed with each β-lactamase; E. *coli* added; and growth quantified. The graphs display the highest concentration of each antibiotic with bacterial growth.

Results: Amino acid changes that improved CEF degradation were: A2323G, A237S, A238G, S240D, and D276N. P4A combined all five changes. P4A displayed 10-1000 fold more potency than P1A for every CEF tested, while retaining its AMP activity. While P1A showed low activity with ceftriaxone, cefotaxime, and ceftazidime, P4A displayed broad cephalosporinase activity. Importantly, 100 ng/ml of P4A enabled E. *coli* growth at 1000 μg/ml for every CEF.

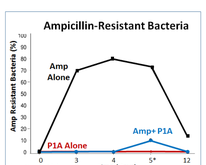
Conclusions: Modification of five amino acids in P1A boosted its cephalosporinase activity 10-1000-fold. Furthermore, P4A degraded ceftriaxone, cefotaxime, and ceftazidime, antibiotics not efficiently hydrolyzed by P1A. Oral administration of P4A is anticipated to protect the gut flora from commonly used CEFs and to extend the clinical utility of this prophylactic strategy to the prevention of CDI.

Background

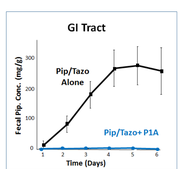
The β-lactam antibiotics are excreted via the bile duct into the intestine where they can disrupt the intestinal microflora. In clinical trials, P1A given orally with IV penicillins preserved the diversity of the intestinal microbiome, reduced the selection for antibiotic-resistant coliforms, efficiently degraded pipperacillin/tazobactam in the intestine, and did not alter plasma antibiotic levels. However, P1A has limited utility as it does not degrade cephalosporins, a major risk factor for C. *difficile* infection.



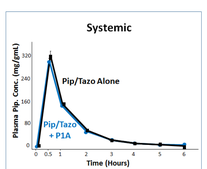
16S similarity index was significantly different between Amp Alone and Amp+P1A and P1A alone groups



Rates of ampicillin resistance remained below 10% for Amp+P1A



P1A degraded Piperacillin/Tazobactam in GI tract



P1A did not alter antibiotic plasma levels

Therefore, P1A was selectively modified following comparison to other cephalosporin-hydrolyzing β–lactamases, including the CTX-M enzymes. In addition to single aa differences, two major areas of sequence divergence were observed, Block 1 (3 aa) and Block 2 (4 aa). A panel of single and multiple P1A aa changes were generated and characterized in kinetic and antibiotic hydrolysis assays.

Results

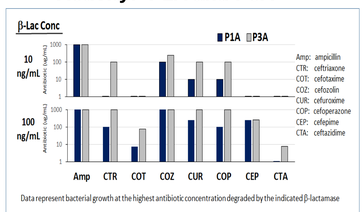
The first P1A mutant characterized contained a single aa change, D276N, and was designated P3A.

Kinetics: P1A and P3A

Antibiotic	P1A			P3A (D276N)		
	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1} \times s^{-1}$ (x10^{-3})	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1} \times s^{-1}$ (x10^{-3})
Piperacillin	49	939	**19,163**	53	816	**15,396**
Ampicillin	157	3,369	**21,458**	161	2,160	**13,416**
Amoxicillin	119	2,956	**24,840**	219	2,789	**12,735**
Ceftriaxone	400	0.05	**0.12**	38	83	**2,184**
Cefoperazone	7	10	1,429	2	17	8,500
Cefazolin	22	93	4,227	37	192	5,189
Cefotaxime	363	246	678	213	36	169
Cefuroxime	107	233	218	277	35	126
Cefepime	n/a*	n/a	n/a	1,357	133	98
Ceftadizime	n/a	n/a	n/a	1,505	274	182

n/a*-No activity

Activity: P1A and P3A



Data represent bacterial growth at the highest antibiotic concentration degraded by the indicated β-lactamase

Michaelis-Menten kinetics were determined using non-linear regression analyses. P3A displayed a dramatic improvement in ceftriaxone degradation while maintaining activity against the penicillins.

Relative antibiotic hydrolysis was evaluated using a microtiter plate activity assay that used E. *coli* growth as the read-out for antibiotic degradation that was designed to model the activity of β-lactamases in the gut in the presence of high antibiotic concentrations. P3A displayed improved activity against ceftriaxone, cefotaxime, cefuroxime, cefoperazone, and ceftazidime while retaining activity against ampicillin.

Screening of P1A Mutants

Mutant	AA Change	Kinetics					
		Ceftriaxone			Cefotaxime		
		K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1} \times s^{-1}$ (x10^{-3})	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1} \times s^{-1}$ (x10^{-3})
P1A (wt)	none	400	0.05	0.12	363	246	678
227	I72S, Q135M, T160F	n/a*	n/a	n/a	n/a	n/a	n/a
219	**A232G, A237S, A238G, S240D, D276N**	**47**	**171**	**3638**	**164**	**60**	**366**
221	**A232G, A237S, A238G, S240D, D276K**	**30**	**103**	**3433**	**66**	**43**	**652**
229	A232G, A237S, A238G, S240D, R244T	n/a	n/a	n/a	574	136	237
158	F33Y, D276N	33	45	1364	144	20	139
230	F33Y, S240P, **D276N**	18	32	1777	21	23	1095
232	F33Y, A238T, **D276N**	4	27	6750	14	23	1643
234	T243I, S266N, **D276N**	37	53	1432	33	30	909

n/a*-No activity

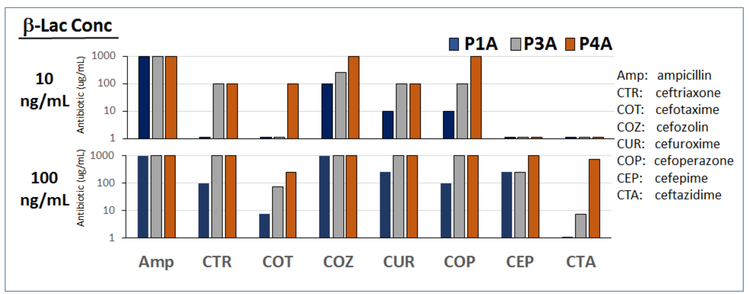
Additional P1A mutants were screened by kinetic analysis. Some mutants containing the Block 2 changes (219 and 221) displayed improved degradation of ceftriaxone. Some mutants containing the D276N change (230, 232, and 234), displayed improved degradation of both ceftriaxone and cefotaxime.

The Block 2 and D276N (P3A) mutations were combined to generate P4A.

Results

P4A antibiotic degradation activity was compared to P1A and P3A in the microtiter plate assay.

Activity: P1A, P3A, and P4A



Compared to P1A, P4A displayed increased activity against all tested cephalosporins. P1A, P3A, and P4A displayed full activity against penicillins.

Conclusions

- Modification of five amino acids in P1A boosted its cephalosporinase activity 10-1000-fold.
- P4A displayed improved degradation of all cephalosporins evaluated while retaining its activity against penicillins.
- Oral administration of P4A is anticipated to protect the gut flora from commonly used cephalosporins and to be used prophylactically to prevent C. *difficile* infection.

References

1. Harmoinen, J, Vaali, K, Koski, P, Syrjanen, K, Laitinen, O, Lindevall, K, Westemarck, E. (2003). Enzymatic degradation of a beta-lactam antibiotic, ampicillin, in the gut: a novel treatment modality. J. Antimicrob. Chemother. 51:361-365.
2. Pitout, JD. (2009). IPSAT P1A, a class A beta-lactamase therapy for the prevention of penicillin-induced disruption to the intestinal microflora. Curr. Opin. Investig. Drugs. 10:838-844.