Development of Therapeutic Agents that Protect the Colonic Microflora from Beta-Lactam Antibiotics for the Prevention of *Clostridium difficile* Infection

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sconnelly@syntheticbiologics.com

S. Connelly[1], T. Parsley[2], P. Koski[1], M. Kaleko[1]; [1]Synthetic Biologics, Inc., [2]SynPhaGen, LLC, Rockville MD

Abstract

Beta-lactam antibiotics are excreted in the bile, which can damage the colonic microflora and lead to serious illnesses such as *Clostridium difficile* infection. SYN-004 is a clinical stage oral beta-lactamase enzyme therapy for use with IV beta-lactam antibiotics to preserve the gut microbiome by degrading residual antibiotics in the intestine. SYN-004 degrades penicillins and cephalosporins, but not carbapenems. To expand this prophylactic approach to most beta-lactam antibiotics, we are evaluating the potential to develop product candidates from three broad spectrum carbapenemases, P2A, NDM-1, and KPC-1. Here we determined how effectively each could be manufactured and then assessed their relative potencies *in vitro* for 18 antibiotics.

Over 100 different *E. coli* production strains for P2A, NDM, and KPC were evaluated for expression (SDS/PAGE) and activity (CENTA chromogenic assay). For the metallo-enzymes, P2A and NDM, the addition of zinc was found to shift expression from inclusion bodies to the soluble fractions. The highest expressing strains for each enzyme were chosen for 5L bioreactor fermentation and chromatographic purification (95%). Final yields for each were ~600 mg/L. A microtiter assay using *E. coli* growth as the read-out for antibiotic degradation was used to assess the potencies of each beta-lactamase with 18 antibiotics. A total of 10 to 1000 μg/ml of each antibiotic was mixed with 10 or 100 ng/ml of each beta-lactamase. *E. coli* was added and growth quantified. Compared to SYN-004, P2A, NDM, and KPC all displayed broader antibiotic degradation profiles that included carbapenems. NDM was the most potent beta-lactamase and efficiently degraded all tested cephalosporins and carbapenems. NDM and P2A were resistant to the inhibitors sulbactam, tazobactam, and avibactam. KPC was the only beta-lactamase with activity against the monobactam, aztreonam. P2A retained biological activity in human chyme for at least 6 hrs.

These data indicate that all three beta-lactamase enzymes can be manufactured and have sufficient potency to be developed into oral therapeutics. Each has the potential to protect the gut microbiome from most beta-lactam antibiotics and provide prophylaxis for *Clostridium difficile* infection and antibiotic-associated diarrhea.

Background

IV beta-lactam antibiotics, including cephalosporins, are excreted via the bile duct into the intestine where they can disrupt the intestinal microflora and potentially lead to the outgrowth of pathogens like *Clostridium difficile*. SYN-004 is a clinical stage, oral beta-lactamase enzyme therapy for use with IV beta-lactam antibiotics to preserve the gut microflora by degrading residual antibiotics in the intestine. The intended indications are prevention of *Clostridium difficile* infection and antibiotic-associated diarrhea. Phase I clinical studies demonstrated SYN-004 safety and tolerability with a single dose of up to 750 mg and multiple doses of 300 mg q.i.d. for 7 days. SYN-004 was neither systemically bioavailable nor immunogenic in humans. A Phase 2a clinical study of SYN-004 was initiated in Q1 2015 and a Phase 2b clinical study is expected to begin in 2H 2015.

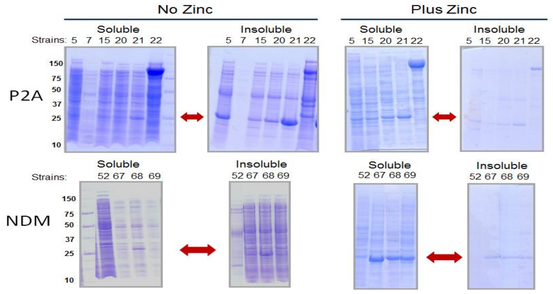
SYN-004 preclinical and clinical data are being presented in Poster 953.

While SYN-004 degrades penicillins and cephalosporins, it does not inactivate carbapenems. To expand this prophylactic approach to all beta-lactam antibiotic classes, we are evaluating the potential to develop product candidates from three broad spectrum carbapenemases, P2A, NDM-1, and KPC-1. P2A was derived from *Bacillus cereus*.

Results

Protein expression in *E. coli*

Over 100 different *E. coli* production strains for P2A, NDM, and KPC were evaluated for expression via SDS/PAGE and activity using the CENTA chromogenic assay. P2A and NDM are class B metallo-enzymes that require zinc for antibiotic hydrolysis activity. KPC is a class A serine enzyme that does not require zinc for activity. P2A and NDM were expressed without and with zinc in the bacterial growth media.

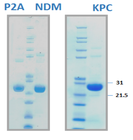


For the metallo-enzymes, P2A and NDM, the addition of zinc to the bacterial growth media was found to shift expression from inclusion bodies to the soluble fractions. The highest expressing strains that displayed biological activity, P2A-21, NDM-68, and KPC-101, were chosen for bioreactor fermentation.

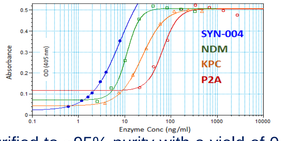
Protein Purification

E. coli carbapenemase-expressing strains, P2A-21, NDM-68, and KPC-101, were subjected to 5 liter bioreactor fermentation followed by a 2-step chromatographic purification. $ZnSO_4$ (100 μM) was added to bacterial growth media and all buffers used for enzyme purification.

SDS/PAGE



CENTA Standard Curve



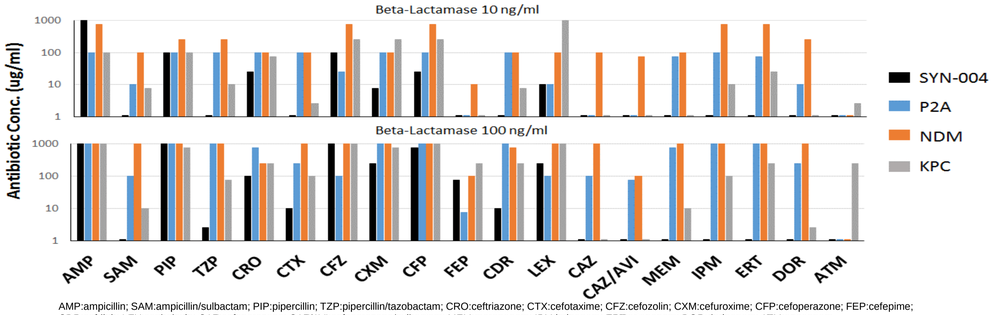
Relative Activities

Enzyme	Relative Activity with CENTA Substrate
SYN-004	1.00
P2A	0.12
NDM	0.71
KPC	0.33

All enzymes were purified to ~95% purity with a yield of 0.6 g/L. Biological activity was confirmed with the CENTA assay. Notably, the enzymes displayed different hydrolysis efficiencies of the CENTA reagent. These data demonstrate that the carbapenemase enzymes, P2A, NDM, and KPC, can be produced and purified from *E. coli* while retaining their biological activity.

Antibiotic Degradation Kinetics

The purified beta-lactamase enzymes were assessed for antibiotic hydrolysis potency with a microtiter plate activity assay using *E. coli* growth as the read-out for antibiotic inactivation. A total of 10 to 1000 μg/ml of each antibiotic was mixed with 10 or 100 ng/ml of each enzyme. *E. coli* was added and growth quantified. The graph displays the highest antibiotic concentration that allowed bacterial growth, indicating antibiotic inactivation.

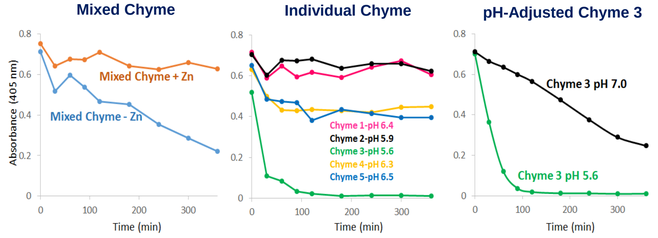


AMP:ampicillin; SAM:ampicillin/sulbactam; PIP:pipercillin; TZP:pipercillin/tazobactam; CRO:ceftriazone; CTX:cefotaxime; CFZ:cefozolin; CXM:cefuroxime; CFP:cefoperazone; FEP:cefepime; CDR:cefdinir; LEX:cephalexin; CAZ:ceftazidime; CAZ/AVI:ceftazidime/avibactam; MEM:meropenem; IPM:imipenem; ERT:ertapenem; DOR:doripenem; ATM:aztreonam

Compared to SYN-004, P2A, NDM, and KPC all displayed broader antibiotic degradation profiles that included carbapenems (MEM, IPM, ERT, DOR). NDM was the most potent enzyme and efficiently degraded all tested cephalosporins and carbapenems. NDM and P2A were resistant to the inhibitors sulbactam, tazobactam, and avibactam. KPC was the only enzyme with activity against the monobactam, ATM.

P2A Stability Was Evaluated in Human Chyme

Purified P2A was incubated in mixed human chyme or each of five individual samples from 30 to 360 minutes and enzyme activity was determined using the CENTA assay.



P2A displayed stable biological activity in human mixed chyme and 4 of 5 individual chyme samples in the presence of 100 μM $ZnSO_4$. Notably, P2A was rapidly inactivated in Chyme 3. As Chyme 3 had the lowest pH, P2A was evaluated in pH-adjusted Chyme 3. Increasing the pH to 7.0 improved P2A stability. These data demonstrate that P2A retains biological activity in human chyme for at least 6 hours.

Conclusions

- Three broad-spectrum beta-lactamases, P2A, NDM, and KPC, were manufactured and purified with retention of biological activity
- P2A, NDM, and KPC displayed antibiotic hydrolysis activity on a wide range of beta-lactam antibiotics, including penicillins, cephalosporins, and carbapenemens
- The metallo-enzymes, P2A and NDM, were resistant to the beta-lactam inhibitors sulbactam, tazobactam, and avibactam
- P2A displayed stability in human chyme
- All three carbapenemases have the potential to be developed into oral therapeutics to protect the gut microbiome from most beta-lactam antibiotics and to prevent *Clostridium difficile* infection and antibiotic-associated diarrhea
- SYN-004 preclinical and clinical data are being presented in Poster 953