Development of SYN-004, an oral beta-lactamase treatment to protect the gut microbiome from antibiotic-mediated damage and prevent *Clostridium difficile* infection

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**A B S T R A C T**

The gut microbiome, composed of the microflora that inhabit the gastrointestinal tract and their genomes, make up a complex ecosystem that can be disrupted by antibiotic use. The ensuing dysbiosis is conducive to the emergence of opportunistic pathogens such as *Clostridium difficile*. A novel approach to protect the microbiome from antibiotic-mediated dysbiosis is the use of beta-lactamase enzymes to degrade residual antibiotics in the gastrointestinal tract before the microflora are harmed. Here we present the preclinical development and early clinical studies of the beta-lactamase enzymes, P3A, currently referred to as SYN-004, and its precursor, P1A. Both P1A and SYN-004 were designed as orally-delivered, non-systemically available therapeutics for use with intravenous beta-lactam antibiotics. SYN-004 was engineered from P1A, a beta-lactamase isolated from *Bacillus licheniformis*, to broaden its antibiotic degradation profile. SYN-004 efficiently hydrolyses penicillins and cephalosporins, the most widely used IV beta-lactam antibiotics. In animal studies, SYN-004 degraded ceftriaxone in the GI tract of dogs and protected the microbiome of pigs from ceftriaxone-induced changes. Phase I clinical studies demonstrated SYN-004 safety and tolerability. Phase 2 studies are in progress to assess the utility of SYN-004 for the prevention of antibiotic-associated diarrhea and *Clostridium difficile* disease.

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1. Introduction

The gut microflora and their collective genomes, referred to as the microbiome, play a key role in health and disease. The gut microbiome works symbiotically with the body to aid digestion, facilitate the absorption of nutrients, synthesize vitamins, block the invasion of pathogenic bacteria across the intestinal mucosal barrier, and provide protection against virulent organisms [1,2]. Recent evidence has demonstrated that the influence of the gut microbiome is far reaching, affecting immune, metabolic, cardiovascular, and neurological functions [3]. Alterations or imbalances in the ecology of the gut, termed dysbiosis, have been linked to a variety of disease states including obesity, allergies, asthma, autism, and a rapidly growing list of other conditions [3–5]. In fact, the gut microbiome functions like an organ [3], and our understanding of its role in human physiology is still evolving.

Anything that disrupts the normal ecology of the gut microbiome can have a negative impact on human health. While antibiotics are life-saving, their use is associated with dysbiosis in the GI tract, which can increase susceptibility to pathogens such as *Clostridium difficile* [4,6,7] and lead to overgrowth of resistant bacteria [8–10]. Beta-lactams are the most commonly used...
intravenous (IV) broad spectrum antibiotics, accounting for 72% of IV antibiotic use in the United States annually [11]. The most frequently prescribed beta-lactams are piperacillin, including piperacillin/tazobactam, and ceftriaxone, both of which are associated with an increased risk of *C. difficile* infection (CDI), particularly the latter [12].

Current treatments for CDI remain relatively limited, with metronidazole and vancomycin still the cornerstones of therapy. Antibiotic-mediated treatment of CDI is characterized by high rates of recurrence [13]. Recognition of the importance of the gut microbiome in prevention of CDI has prompted investigation into therapies aimed at restoring the normal microbial balance, such as the use of probiotics [3] and fecal microbiota transplantation (FMT) [14,15]. FMT reconstitutes the gut microflora with organisms native to the fecal donor and therefore does not necessarily re-establish the patient’s pre-disease microbiome. As it is becoming increasingly apparent that host genetics influence the structure of the microbiome, and that the gut microbiome affects many, if not all organ systems, the long-term outcomes of FMT must be carefully evaluated [14,15]. While each of these interventions provides treatment for CDI once the infection is established, prevention of antibiotic-associated dysbiosis may represent a complementary strategy to preclude the occurrence of CDI.

One novel strategy to protect the microbiome from antibiotic-mediated dysbiosis and to prevent CDI is the use of beta-lactamase enzymes to degrade the antibiotics in the GI tract.

1.1. Beta-lactamases: from enemies to therapies

Beta-lactamases are natural enzymes that degrade beta-lactam antibiotics, confer antibiotic resistance, and dramatically complicate the treatment of bacterial infections. Thus, they are rightfully considered the “enemy”, and substantial efforts have been directed towards their characterization and inhibition [16]. However, beta-lactamases can also be harnessed as therapeutics to protect the “good”, commensal gut microflora from unwanted damage incurred by antibiotic use. In 1999, Ipsat Therapies, OY (Helsinki, Finland) had the foresight to recognize that an orally administered beta-lactamase had the potential to degrade residual antibiotics in the proximal GI tract, before they could adversely affect the colonic microflora. The ensuing beta-lactamase treatment, termed P1A, was designed to be used in conjunction with parenterally administered beta-lactam antibiotics, which are secreted through the biliary system into the proximal small intestine.

P1A is a naturally-occurring penicillinase isolated from *Bacillus licheniformis* (the PenP protein) [17]. It was manufactured in *Bacillus subtilis* and formulated into pellets with a Eudragit®-coated sucrose pellets designed to release active enzyme at pH 5.5 or greater [26]. The pellets contained approximately 15% SYN-004 [26]. Gelatin capsules suitable for oral delivery were filled with the pellets for a total SYN-004 content of 75 mg/capsule.

2. Methods

2.1. Production of beta-lactamases

P1A was manufactured in *B. subtilis* as described [22]. SYN-004, a protein of 29 kDa, was manufactured in *Escherichia coli*. SYN-004 was formulated for oral delivery by incorporation into Eudragit®-coated sucrose pellets designed to release active enzyme in the proximal small intestine. SYN-004 was engineered from P1A with the intent of broadening its antibiotic degradation profile to include cephalosporins. SYN-004 is currently undergoing Phase 2 clinical analyses [25]. Here, we report the data supporting the clinical application of SYN-004.

2.2. In vitro antibiotic degradation analyses

The antibiotic degradation kinetics of P1A and SYN-004 enzymes were determined for ampicillin, amoxicillin, piperacillin, ceftriaxone, ceftazidime, cefotaxime, and cefuroxime. The steady-state *Km* and *kcat* values for each antibiotic were determined by measuring substrate hydrolysis under initial rate conditions with Hanes linearization [27] of the Michaelis-Menten equation. The reactions were performed in 20 mM phosphate buffer (pH 7.0) at 30 °C. Briefly, dilutions of each antibiotic were prepared in sodium phosphate buffer and distributed to individual wells of a 96-well plate. The plate was pre-incubated at 30 °C for 5 min, after which P1A (1.0 nM) or SYN-004 (1.0 nM) was added to the plate. The absorbance at 235 nm (ampicillin and piperacillin), 242 nm (amoxicillin), 257 nm (ceftriaxone), and 264 nm (ceftazidime, cefotaxime, cefepoxide, cefotaxime) of the individual wells was determined every 5–8 s over a 10–20 min period of incubation. The initial velocity was calculated as the change in absorbance per min (μmol/min) of the reaction as determined using SoftMax Pro 5.4 software from the slope of the curve within the linear range of the reaction. Following the kinetic analysis, an endpoint reading was taken to determine the pathlength (cm) of the specimen in each individual well. The velocity (μmol/min) values were normalized to a 1 cm pathlength by dividing the values by the measured pathlength. The normalized velocity values (μmol/min-cm) were then converted to velocity (nmol/sec-cm) using an experimentally determined extinction coefficient specific for each individual antibiotic at the given wavelength. The data were imported into Prism GraphPad 5 for determination of Michaelis-Menten kinetics by non-linear regression. The analysis was performed a minimum of 3 times for each antibiotic substrate. Kinetic data (*Km*, *kcat*, and *kcat/Km*) were calculated from the mean of the experiments. Data are presented as the mean ± standard deviation.

The antibiotic inactivation activities of P1A and SYN-004 were
evaluated using a bacterial growth microtiter plate assay that was
designed to mimic the activity of the beta-lactamases in the gut in
the presence of high antibiotic concentrations. The degradation of
the antibiotics Amp, ampicillin; CRO, ceftriaxone; CFZ, cefazolin;
CXM, cefuroxime; CFP, cepoferezone; FEP, cephempe; CTX, cefotax-
ime; and CAZ, cezafidime were evaluated. Purified P1A or SYN-004
(10 or 100 mg/mL) was added to wells of a microtiter plate con-
taining high antibiotic concentrations (10, 100, or 1000 μg/mL) after
which E. coli (ATCC strain 25922) were immediately added. The
beta-lactamase activity of each culture supernatant was deter-
mined as positive or negative based on the appearance of bacterial
growth in the individual wells. An OD625 of 0.8 or greater indicated
maximal bacterial growth, therefore complete antibiotic degrada-
tion and high beta-lactamase activity. An OD625 of less than 0.8
indicated low bacterial growth and, therefore, incomplete antibi-
otic degradation, hence low beta-lactamase activity. Wells with an
OD625 of ≥0.8, indicating full growth of the E. coli, were recorded.
The highest concentration of antibiotic that was inactivated by the
beta-lactamase present in each well is represented on the bar
graph. The assay, therefore, was read as a growth or no growth
endpoint. Analyses were performed in duplicate wells, and the
studies were repeated at least twice for each beta-lactamase and
antibiotic concentration. In the rare instance of a disparity in bac-
terial growth between duplicate wells, the analyses were repeated.

2.3. In vitro dissolution and human chyme studies

For the dissolution analyses, SYN-004 enteric-coated pellets
were held in a pH 1.0 solution for 2 h after which the pH was raised
to 5.5, 5.8, or 6.8. Samples collected at the indicated times over 4 h
were assessed for SYN-004 biological activity using the chromo-
genic cephalosporin, CENTA, as the substrate, as described [28].
Human chyme, collected from 5 different donors, was obtained
from the Oklahoma Foundation for Digestive Research (OFDR), an
Oklahoma not-for-profit corporation. Human chyme samples were
characterized by pH, liquid content, and protease activity. Protease
activity of the chyme specimens was determined using a com-
mercial Protease Activity Fluorometric Assay Kit (Biovision, Milpi-
tas, CA) following the manufacturer’s recommended procedures.
Chyme specimens were diluted 1:20 in protease assay buffer prior
to performing the protease assay. The pHs ranged from 5.58 to 6.56,
liquid content ranged from 55% to 78%, and protease activity ranged
from 5.57 to 8.96 mU/mL. SYN-004 enteric-coated pellets were
mixed with the five individual human chymes. Samples collected
over a 6 h period were assessed for SYN-004 biological activity
using the chromogenic cephalosporin, CENTA, as the substrate, as
described [28].

2.4. Animal studies

All animal studies were performed with adherence to animal
use guidelines and with the required institutional animal assur-
ances. Dog studies were performed at Ipsat, OY (Helsinki, Finland)
as described [22]. Pig studies were performed at Tufts University
(North Grafton, MA) as described [29]. Briefly, jejunal-fistulated
dogs (n = 6) received IV ceftriaxone (30 mg/kg) alone or ceftriaxone + oral SYN-004 enteric-coated pellets (0.44 mg/kg) in
two serial studies on separate days. The animals were fed shortly
before dosing and then again 6 h later; however, two of the dogs
did not eat prior to antibiotic dosing in the second study with
ceftiraxone + SYN-004. Ceftriaxone and SYN-004 were measured in
the chyme at the indicated times over 10 h. Animal dosing and
sample collections, and beta-lactamase and ceftriaxone assays
were performed as described [22–24,26].
Pigs (n = 7) were delivered via Caesarian section and housed
separately in microisolator cages to maintain them as gnotobiotic
animals [30]. Five days after birth (day 5), the GI tract was popu-
lated with human adult fecal microflora as described [29], and
on day 8, the appropriate animals (approximately 1 kg each), received
SYN-004 (75 mg capsule, PO, QID) for 7 consecutive days (days
8–14). On day 9, animals received ceftriaxone (50 mg/kg, IP, SID) for
4 consecutive days (days 9–12). The cohorts were untreated (n = 2,
but one animal was sickly and did not continue in the study), cef-
triaxone alone (n = 2), and ceftriaxone + SYN-004 (n = 3). Feces
were collected directly from the rectum using sterile swabs on days
14, 20, and 21. Dilutions of day 20 fecal samples were plated on
LB + ampicillin plates and incubated overnight. Distinct colonies on
plates from equal feces dilutions were counted.

For microbiome analyses, DNA was isolated from day 14
(designated T1) and day 21 (designated T2) fecal samples
and subjected to 16S and/or shotgun deep sequencing analyses. The 16S
sequencing was performed with the day 14 fecal DNA using
HiSeq2000 Illumina sequencer with an average of 4 million 250
nucleotide single reads with 3000 sequences randomly selected
from each sample as described [29]. Data analyses were performed
using open-source software Mothur version 1.25 as described [29].
Shotgun sequence analyses were performed on fecal DNA from day
14 and day 21 samples (designated gDNA, Fig. 5A) as well as
additional DNA isolated from frozen day 21 feces (designated Fecal,
Fig. 5A). Shotgun sequencing was performed using Illumina HiSeq
RAPID RUN, targeting 100 bp single reads to achieve 10 million
reads per sample. Sequenced datasets were taxonomically classi-
fied using the GENUIS® software package [31,32]. Heatmaps were
created based on the log of the relative abundance of bacteria
strains in each sample using the NMF R package [33]. Samples were
grouped using the maximum distance function and the Ward hi-
erarchical clustering algorithm to create a dendrogram grouping
samples with similar compositions into clades. The centroid clas-
sification method from the pamr R package [34] was used to
compare the average frequency (absolute abundance) of each
bacterial strain in the samples. The deviation of the centroid of
each bacterial strain from each study cohort from the overall average
frequency of all study groups was calculated.

3. Results

3.1. Beta-lactamase antibiotic degradation profile

P1A, a naturally occurring beta-lactamase enzyme was engi-
eered to broaden its antibiotic degradation profile to include
cephalosporins such as ceftriaxone. This modified beta-lactamase,
originally designated P3A, is currently referred to as SYN-004. To
generate SYN-004, the negatively charged aspartic acid at position
276 using the Ambler numbering system for class A beta-
lactamases [35] was replaced with a neutral amino acid, aspara-
gine. This one amino acid substitution was the only change made
to the molecule and was intended to change the structure of the beta-
lactamase enzyme. Specifically, the aspartic acid forms a salt bond
with an arginine at position 244, proximal to the active site of
the enzyme. The asparagine substitution at 276 was expected to alter
the interaction with the arginine to enlarge the enzymatic cavity
and to potentially accommodate a broader range of beta-lactam
substrates.

To determine if this one amino acid substitution changed the
antibiotic degradation profile of SYN-004 compared to P1A, two in
vitro analyses including kinetic studies and a microtiter plate
bacterial growth assay were performed. The kinetic parameters
of the P1A and SYN-004 enzymes were determined for hydrolysis of
a series of beta-lactam antibiotic substrates (Fig. 1A). The Km, kcat and
kcat/Km values calculated for SYN-004 were similar to P1A for
amoxicillin, amoxicillin, and piperacillin verifying that the single amino acid substitution did not adversely affect hydrolysis of the penicillins. Notably, SYN-004 displayed a marked improvement for the hydrolysis of ceftriaxone. Whereas the $k_{cat}/K_m$ was 157 for P1A, it was 50-fold higher at 8554 for SYN-004 (Fig. 1A). Therefore, the single amino acid substitution generated a beta-lactamase enzyme that efficiently degraded penicillins and exhibited improved kinetics for ceftriaxone.

While the kinetic analyses revealed a broader degradation profile for SYN-004, it was not readily apparent how to use these parameters to predict in vivo efficacy. Thus, a second in vitro assay was developed to more closely model the in vivo enzyme requirements. This microtiter plate assay mixed the beta-lactamases with various antibiotics and used bacterial growth as a readout for antibiotic degradation. Low concentrations of the beta-lactamase enzyme (10 and 100 ng/mL) were mixed with high concentrations of the antibiotics, (10, 100, or 1000 μg/mL) in microtiter wells. Bacteria were immediately added and bacterial growth was measured the next day. Bacterial growth indicates that the beta-lactamase inactivated the antibiotic. Thus, this assay was scored using a growth or no growth endpoint. The bars on the graph represent the highest concentration of each antibiotic that was inactivated by the beta-lactamase. Amp, ampicillin; CRO, ceftriaxone; CFZ, cefazolin; CXM, cefuroxime; CFP, cefoperazone; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime. Data presented in part at ICAAC 2014 [55].

3.2. SYN-004 stability in human chyme

In contrast to P1A, which was manufactured in B. subtilis [22], SYN-004 was produced in E. coli [26]. This change in manufacturing platform improved process yields from ~1 g/L to 14 g/L [36]. A purification strategy that includes one chromatographic step produced final yields of ~45% with >95% purity [36]. For protection from stomach acid, SYN-004 was incorporated into Eudragit®-coated sucrose pellets designed for release at pH 5.5 [26,36]. Compared to the original P1A pellets [18], the SYN-004 formulation was modified to increase drug loading and reduce the Eudragit® content [26]. The pellets were confirmed to be uniform spheres of ~1 mm diameter by electron microscopy and were used to fill capsules suitable for oral delivery [26,36]. To verify pH-based dissolution, SYN-004 pellets were held in a 0.1 N HCl solution for 2 h followed by incubation in buffers at varying pHs for 4 h (Fig. 2A) [36]. SYN-004 pellets were protected at low pH while dissolution occurred at pHs >5.5, with pHs of 5.8 and 6.8 showing more rapid dissolution than pH 5.5. Therefore, these data confirm that the enteric coating remained intact at low, stomach pH and active beta-lactamase enzyme was released at pH > 5.5, the pH levels found in the duodenum.

Resistance to degradation by intestinal proteases is a key attribute for beta-lactamases intended for GI-targeted therapy. To determine if SYN-004 retained biological activity in human intestinal fluid, SYN-004 enteric-coated pellets were incubated in human jejunal fluid, chyme, and then evaluated for biological activity (Fig. 2B). Human chyme was collected from five donors with ileostomies and was characterized based on pH, liquid content, and protease activities. SYN-004 pellets were incubated in each chyme sample, and aliquots taken over 6 h were assessed for biological activity. As expected, based on the initial pH dissolution studies (Fig. 2A), SYN-004 pellets dissolved rapidly, within 30–60 min, in human chyme. Notably, SYN-004 biological activity was maintained at high levels for at least 6 h in the five chyme samples (Fig. 2B). These data suggest that enteric-coated SYN-004 will retain activity in the GI tract for a sufficient period to degrade residual beta-lactam antibiotics and protect the downstream microbiota.

3.3. Efficacy of SYN-004 in a canine model

To assess the ability of SYN-004 to degrade antibiotics in the GI tract, six jejunal-fistulated dogs were subjected to two serial studies on separate days. In the first study, each dog received IV ceftriaxone alone. In the second study, the dogs received the IV...
ceftriaxone along with oral SYN-004 pellets [36] (Fig. 3). The animals were fed shortly before dosing and then again 6 h later. In the first study, with ceftriaxone alone, the antibiotic was excreted at high levels into the intestine with a first peak at 100 min and a second peak 6 h later, after the additional feeding. The mean \( C_{\text{max}} \) for the two peaks were 1500 \( \mu \text{g/g} \) of chyme (range of 1020–2760 \( \mu \text{g/g} \) of chyme) and 167 \( \mu \text{g/g} \) (range of 49–440 \( \mu \text{g/g} \) of chyme), respectively. As ceftriaxone is excreted into the duodenum through the bile, and bile is released after eating, the second peak was likely due to residual biliary ceftriaxone. In the second study, SYN-004 was rapidly detected in the chyme from 4 of the 6 dogs (peak from 12.6 to 30.9 \( \mu \text{g/g} \) of chyme) and the ceftriaxone concentrations remained low (<5 \( \mu \text{g/g} \) chyme) for at least 5 h (Fig. 3, left panels). Interestingly, even though SYN-004 became undetectable (<0.1 \( \mu \text{g/g} \) of chyme) by 6 h, the second ceftriaxone peak was also noticeably reduced. The other 2 dogs did not eat prior to antibiotic dosing. Only one of these animals produced enough chyme for both the ceftriaxone and beta-lactamase analyses. The analyzed dog exhibited slower accumulation of SYN-004 in the chyme, presumably due to delayed gastric emptying. Not unexpectedly, the first ceftriaxone peak was present in the chyme, but the second peak was substantially reduced (Fig. 3, right panels).

These data indicate that, whenever SYN-004 was present in the small intestine, it efficiently eliminated ceftriaxone from the chyme. Moreover, the SYN-004 remained functional for more than 8 h, the duration that antibiotic was released into the intestine. Finally, the timing with which SYN-004, and perhaps the antibiotic, enter the small intestine may be variable. Thus, SYN-004 should be dosed more frequently than once per day to ensure its continuous presence in the GI tract.

3.4. SYN-004 protects the intestinal microflora of humanized gnotobiotic pigs

SYN-004 was evaluated in “humanized” neonatal pigs [29] to assess its capacity to prevent antibiotic-induced microbiome changes. In this pilot study with limited cohort sizes, the GI tracts of 5 day old gnotobiotic pigs were populated with human adult fecal microflora and randomly assigned to experimental groups: no antibiotic treatment; ceftriaxone alone; and ceftriaxone + SYN-004 [37,38]. Ceftriaxone was administered by intraperitoneal injection once each day for four days, and SYN-004 was delivered orally four times a day for seven days beginning one day before the ceftriaxone. Protection of the fecal flora and microbiome from antibiotic-mediated dysbiosis was monitored in several ways.

First, levels of ampicillin-resistant facultative aerobes were quantified by counting colonies on LB + Amp plates (Fig. 4A). This specific subset of fecal bacteria, which includes the phylum Proteobacteria, was chosen based on its anticipated sensitivity to ceftriaxone. As expected, the colony counts from the animals that received ceftriaxone alone were two orders of magnitude lower than those from the no antibiotic control animal. Notably, when SYN-004 was administered with the ceftriaxone, the colony counts were as high as those of the control. Thus, SYN-004 protected the fecal bacteria from ceftriaxone.

Second, to obtain a phylum-level taxonomic classification of the GI microflora, fecal DNA samples collected 2 days after the antibiotic was stopped were subjected to 16S rRNA V6 region sequencing (Fig. 4B). The 16S sequence analyses revealed that the bacterial profile from the no antibiotic control animal comprised Bacteroidetes, Proteobacteria, and Firmicutes, while the profiles from the ceftriaxone alone cohort were distorted, with Bacteroidetes as the most predominant phylum (Fig. 4B). When SYN-004 was administered along with the ceftriaxone, the fecal bacteria were protected from the antibiotic and maintained 16S profiles similar to the control. As predicted from the colony count data (Fig. 4A), Proteobacteria were underrepresented in the ceftriaxone-alone animals but were preserved in the animals treated with SYN-004.

To further assess the microbiome of the neonatal pigs, whole genome shotgun sequencing was performed using the pig fecal DNA samples (Fig. 5). Heatmaps of the bacterial taxa were constructed based on the relative abundance of each bacterial strain and a dendrogram was generated with the Ward hierarchical clustering algorithm to group samples with similar compositions into clades. The data revealed that the no antibiotic control and ceftriaxone + SYN-004 samples were clustered together while samples from the animals treated with ceftriaxone alone formed a separate clade (Fig. 5A). Thus, the bacterial populations in the control and ceftriaxone + SYN-004 groups were more similar to each other than to those in the ceftriaxone alone group.

The sequencing data were also analyzed using the nearest shrunken centroid classification [34] to compare the average deviation of the frequency of each bacterial species among the samples within each cohort to the overall average frequency of all study

Fig. 2. SYN-004 Enteric-Coated Pellet Dissolution and Stability Profiles. A) SYN-004 enteric-coated pellets were held in a pH 1.0 solution for 2 h after which the pH was raised to 5.5, 5.8, or 6.8. Samples collected over 4 h were assessed for SYN-004 biological activity using the chromogenic cephalosporin, CENTA, as the substrate [28]. B) SYN-004 enteric-coated pellets were mixed with human chyme obtained from 5 different donors. Samples collected over 6 h were assessed for SYN-004 biological activity as described. The pH of each chyme sample is displayed. Data presented in part at IDWeek 2014 [36].
groups (Fig. 5B). The SYN-004 + ceftriaxone cohort displayed less severe distortion of species abundance compared to the ceftriaxone alone cohort. Likewise, the no antibiotic control and ceftriaxone + SYN-004 cohorts were more similar to each other than to the ceftriaxone alone cohort. Interestingly, in the ceftriaxone alone cohort, the methanogen, *Methanobrevibacter smithii*, was overrepresented while *Turicibacter* spp. were underrepresented compared to the control and ceftriaxone + SYN-004 cohorts. Overgrowth of *M. smithii* was reported to be associated with constipation and irritable bowel syndrome as well as obesity in humans [39], while reduction of *Turicibacter* spp. was associated with idiopathic inflammatory bowel disease and acute hemorrhagic diarrhea in dogs [40, 41].

Taken together, both the 16S and whole genome sequencing data demonstrate that SYN-004 protected the gut microbiota from the adverse effects of antibiotic use and maintained the microbiome diversity. Further studies are in progress using normal juvenile pigs to confirm the results of this pilot study.

4. Discussion

The intestinal microbiome plays a significant role in regulating
human physiology and preserving health. Whereas microbiome function was once relegated to aiding digestion and protecting from colonization by potential pathogens, it is now recognized to modulate host immune, metabolic, cardiovascular, and neurologic activities [3–5]. Thus, preserving a patient’s native microbiome can be considered a high medical priority. Unfortunately, when antibiotics are used to treat serious infections, they can enter the GI tract and damage the native microflora. Recovery from this collateral damage can require protracted times, and some bacterial species may be permanently lost [6,7].

SYN-004 has the potential to protect the GI microbiome from beta-lactam antibiotics. The most tangible application for this technology is the prevention of antibiotic-associated C. difficile infection. In this regard, SYN-004 has a major advantage over its predecessor, P1A, in that it extends utility to commonly used cephalosporins. Whereas both the penicillins and cephalosporins predispose to CDI, the risk is substantially greater with the cephalosporins [7,42–44]. Moreover, cephalosporins were reported to increase C. difficile acquisition during hospitalization, while penicillins were associated with a decrease in C. difficile carriage [45]. Beyond CDI, it is noteworthy that SYN-004 has the potential to protect patients from hospital acquired secondary infections with other drug-resistant pathogens including both gram negative co-/forms and gram positive cocci [20,21,46]. Antibiotic treatment can promote gastrointestinal overgrowth of these organisms, which can then lead to the seeding of other organs.

Based on its extended activity profile, SYN-004 is anticipated to have clinical utility for a large number of patients. In the U.S. and E.U. intravenous beta-lactams are administered to approximately 27 million patients annually for a total of 123 million patient days [11]. SYN-004 efficiently inactivates ceftriaxone and piperacillin. These two antibiotics, including piperacillin/tazobactam, account for approximately 50% of the beta lactam usage with 12.6 million patients treated annually and 66 million patient days of therapy per year.

**Figure 5.** SYN-004 Protects the Microbiome from Antibiotic-Mediated Changes in Pigs. Whole genome shotgun sequencing was performed with the pig fecal DNA samples. The fecal DNA samples included DNA from day 14 (T1), day 21 (T2), and DNA isolated from day 21 frozen feces (Fecal). A) Heat map of the log of the relative abundance of bacteria strains in each sample. Samples were grouped using the maximum distance function and the Ward hierarchical clustering algorithm. A dendrogram was constructed that displays samples with similar compositions organized into clades (left side of the figure). The yellow box illustrates that the no antibiotic control and the majority of the SYN-004 + ceftriaxone samples clustered together in one clade. B) Nearest shrunken centroid classification comparing the average deviation of the frequency of each bacterial species among the samples within each cohort to the overall average frequency of all study groups. The species displayed in red text, *Methanobrevibacter smithii*, was overrepresented, while *Turicibacter* spp. were underrepresented in the antibiotic-alone group. No Abx, no antibiotic; SYN-004 + CRO, SYN-004 + ceftriaxone; CRO alone, ceftriaxone alone. Data presented in part at DDW 2015 [37], and ICAAC 2015 [38].
We presented data from in vitro analyses, formulation studies, and two large animal models. The kinetic and bacterial growth microtiter assays suggest that SYN-004 has the potential to degrade multiple beta-lactam antibiotics, and the dissolution studies confirm that the formulated pellets will successfully traverse the stomach to release biologically active enzyme in the duodenum. Most importantly, the chyme stability studies demonstrate that SYN-004 has the remarkable capacity to avoid digestion in the GI tract, thereby circumventing a major limitation to the development of therapeutic proteins for oral use. The canine data verify that SYN-004 persists in the GI tract and can efficiently degrade ceftriaxone in the chyme. The humanized neonatal pig data support the potential to protect the microbiome from antibiotics. Importantly, the neonatal pig data also suggest that SYN-004 degrades ceftriaxone to levels that are sufficiently low, below the minimum inhibitory concentrations (MICs), to protect much of the antibiotic-sensitive colonic microflora.

The canine studies reveal the complex pharmacokinetic nuances related to dosing. Since gastric emptying, biliary secretion, and intestinal transit times will differ among patients, it may be difficult to coordinate the SYN-004 dosing with that of the antibiotic. This limitation is effectively addressed by dosing more than once per day to achieve continuous bioavailability in the GI tract. Moreover, SYN-004 has a broad therapeutic window to enable an upward dose adjustment if necessary. Specifically, the beta-lactamase is not toxic to the GI tract and does not alter systemic antibiotic levels [26, 47–49]. Thus, an administration frequency and dose can be established to mitigate patient to patient variability.

One interesting finding is that both P1A and SYN-004 are inhibited in vitro by commonly used beta-lactamase inhibitors including tazobactam and sulbactam [20, data not shown]. Yet P1A was efficacious in a clinical trial with piperacillin/tazobactam [19] and in canine studies with piperacillin/tazobactam, amoxicillin/clavulanate, and ampicillin/sulbactam [19]. While this paradoxical result remains unexplored, it is possible that the antibiotic and the inhibitor have different biliary pharmacokinetics such that the antibiotic enters the duodenum separately from the inhibitor. Alternatively, the P1A may simply have overwhelmed the inhibitor. Importantly, these results suggest that SYN-004 may have utility for the beta-lactam/inhibitor combinations in current use and in development.

SYN-004 was evaluated in two canine GLP toxicology studies in which it was well tolerated, was not systemically absorbed, and did not affect ceftriaxone blood levels [26, 47–49]. The currently anticipated therapeutic dose is 150 mg orally four times per day. Two Phase 1 studies were completed, which demonstrated safety and tolerability with a single dose of up to 750 mg and multiple doses of 300 mg four times per day for 7 days. SYN-004 was neither systemically bioavailable nor immunogenic in humans [47–49].

Two Phase 2a Proof-of-Mechanism studies are in progress in ileostomy patients to enable facile sampling of intestinal chyme. Patients received ceftriaxone along with SYN-004. Plasma ceftriaxone pharmacokinetics are being determined and chyme is being assayed for both ceftriaxone and SYN-004. A Phase 2b study, also in progress, is intended to evaluate the capacity of SYN-004 to reduce the risk of CDI and antibiotic-associated diarrhea in hospitalized patients treated with ceftriaxone for lower respiratory tract infections [25].

One aspect of the beta-lactamase strategy that is currently unexplored is the potential for CDI prophylaxis in patients who receive multiple antibiotics, either concurrently or before/after the course of a beta-lactam. In particular, fluoroquinolones and clindamycin carry a high risk for CDI [7]. In the U.S., the percentage of patients treated with ceftriaxone who concurrently receive a quinolone or clindamycin is 0.4% and 1.4%, respectively [11]. For piperacillin/tazobactam, the data are 6.6% and 1.4%, respectively [11]. Since the likelihood of acquiring CDI may be to roughly correlated with the total antibiotic exposure [50], it is reasonable to assume that removing the contribution from the beta-lactam will diminish the total risk. Quantitative support for this hypothesis will require future clinical evaluation.

A technology that may have potential to complement SYN-004 for patients concurrently treated with a beta-lactam and fluoroquinolone involves the use of activated charcoal as a binding agent. When orally administered to animals and humans, activated charcoal was able to bind fluoroquinolones in the intestines [51–53]. However, there are potential limitations to the clinical implementation of a charcoal-based product. First, efficacy may require a substantial amount of charcoal since the potency is limited by its binding capacity; and second, activated charcoal has the potential to nonspecifically bind other medications and nutrients in the GI tract.

To further broaden the utility of the beta-lactamase technology, two SYN-004 pipeline products are being developed. The first is designed to extend the utility of SYN-004 to use with oral antibiotics. This product will provide continuous microbiome protection for hospitalized patients treated with IV beta-lactams and then discharged on oral antibiotics. In the U.S. and E.U. 19% and 13% of patients, respectively [11], are transitioned to oral antibiotics. This first pipeline product is also being designed for general outpatient use in conjunction with commonly prescribed antibiotics including amoxicillin, amoxicillin/clavulanate, and cephalaxin. The second pipeline product will expand the antibiotic degradation profile to include the carbapenems [54]. Approximately 2.8 million courses of carbapenems are prescribed annually in the U.S. and E.U. [11]. This third class of beta-lactam antibiotic is gaining in importance due to changing patterns of bacterial resistance.

The ultimate goal of the antibiotic inactivation strategy described herein, and for SYN-004 in particular, is to enable a patient to leave the hospital with the same gut microbiome with which he or she entered. In the short-term, this should provide protection from acute illnesses such as CDI and secondary infections with drug resistant pathogens. In the long-term, the symbiotic relationship between the patient and his or her GI microflora will be preserved. Finally, by mitigating the GI collateral damage, SYN-004 has the potential to eliminate many of the risks associated with the use of beta-lactam antibiotics.

**Conflict of interest**

MK, JAB, SH, JK-K, JS, and SC are employees of Synthetic Biologics, Inc. and receive stock option compensation. AJ is a paid consultant for Synthetic Biologics, Inc. PK was an employee of Ipsat Therapies. GW and ST received financial support for the pig microbiome study under a Sponsored Research Agreement with Synthetic Biologics, Inc. SynPhaGen, LLC and Cosmos ID, Inc. are fee-for-service providers engaged by Synthetic Biologics, Inc.

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