Microbiome Effect of Omadacycline on Healthy Volunteers

Jinhee Jo, Anne J Gonzales-Luna, Khurshida Begum, Chris Lancaster, M Jahangir Alam, and Kevin W. Garey

University of Houston College of Pharmacy

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Background and summary: Omadacycline is a novel aminomethylcycline antibiotic approved for the treatment of community-acquired bacterial pneumonia and acute skin and skin structure infections. In clinical trials, a remarkably low number of cases of *Clostridiodies difficile* infection (CDI) cases were observed in patients given omadacycline vs. comparators. In collaboration with the University of Houston College of Pharmacy, Dr. Garey and his research team have shown excellent *in vitro* susceptibility to a large number of *C. difficile* isolates. The clinical question that arises from this work is whether omadacycline should be considered the drug of choice in patients at risk for CDI? An effective antibiotic that does not increase the likelihood of CDI should have potent in vitro activity to *C. difficile* and also have a favorable microbiome disruption profile that minimizes dysbiosis. The purpose of this study is to define the microbiome effect of omadacycline. We will do this by conducting a phase I, healthy volunteer study. With a unique spectrum of activity that should limit destruction to the host gut microbiome, omadacycline is poised to become the drug of choice for patients at risk for CDI.

Specific aim 1. To identify microbiome changes associated with omadacycline vs. moxifloxacin.

Sub-aim 1.1. Quantitate changes over time on host fecal microbiota biomass of specific microbial families (Eubacteria and 5 different microbiota groups; Bacteroides, *C. coccoides*, *C. leptum*, Enterobacteriaceae, and Prevotella) using qPCR in healthy subjects given a 10-day course of omadacycline or comparators (moxifloxacin and oral vancomycin)

Sub-aim 1.2. Assess relative abundances of resident taxa, microbiota diversity, and taxonomic composition using high-throughput sequencing in health subjects given a 10-day course of omadacycline or comparators (moxifloxacin and oral vancomycin)

Significance: Using advanced microbiologic genomics analysis, this will be the defining study to assess the microbiome effects of omadacycline on the human gut microbiome in direct comparison to moxifloxacin and vancomycin.

Methods:

General overview: The goal of this study is to assess microbiome changes associated with omadacycline vs. comparators (moxifloxacin or oral vancomycin). Using a phase I study design, healthy volunteers aged 18-40 years will be given a 10-day course of either omadacycline 450 mg days 1 and 2 followed by 300 mg PO once daily, moxifloxacin 400 mg once daily, or oral vancomycin 125 mg given four times daily. Stool will be collected at baseline, daily during therapy, and at two follow-up time periods (days 13-14 and days 30-32). DNA will be extracted from stool and used for qPCR biomass and microbial metagenomic experiments. Results from the study will provide definitive data on the microbiome effects of omadacycline versus comparator antibiotics.

Overview of clinical trial, inclusion and exclusion, and sample collection: A 10-day, oral course of omadacycline, moxifloxacin, or vancomycin will be randomly assigned to healthy volunteers aged 18-40. All volunteers will be considered healthy based on medical history, none will have a history of cardiovascular, gastrointestinal, hepatic, or renal disease. Subjects will not have taken an antibiotic for at least three months prior to enrollment. Patients will not have taken a probiotic for at least a month prior to enrollment and during the entire study period. After screening for inclusion and exclusion and signing an informed consent, fecal samples will be collected at baseline prior to dosing, and on days 1 to 10. Follow-up stool samples will be collected between days 13-14 and days 30-32. Instructions on collection will be provided to all volunteers.

Safety assessment: See appendix 1

Sample size: Eight subjects, each will be randomized to receive either omadacycline, moxifloxacin, or vancomycin (total subjects = 24).

Drug supply: Omadacycline: Subjects will receive 3 tablets on day 1 and day 2 (450 mg) followed by 2 tablets (300 mg) for days 3 through 10. Thus, each subject will require 22 tablets. Total numbers of tablets required is 176 tablets (22 tablets per subjects X 8 subjects = 176).

Comparator antibiotics: Comparator antibiotics will be purchased from a local distributor. Moxifloxacin 400 mg tablets will be taken once per day for a total of 10 days. Thus, 10 tablets per subject will be required for a total of 80 tablets. Vancomycin 125 mg capsules will be taken four times daily for a total of 10 days. Thus, 40 capsules per subjects will be required for a total of 320 capsules.

Missed dose: If a dose of medication is missed, it will be given as soon as possible anytime up to 8 hours prior to the next scheduled dose. If less than 8 hours remains before the next dose, then dosing will occur at the next scheduled dose. A double dose will not be given to compensate for a missed dose. Subjects given vancomycin will follow the same protocol except a cut off time of three hours will be chosen. If the patient vomits following oral dosing and any tablet is visible, then another dose will be taken immediately. If the tablet is not visible, then the next scheduled dose will to be taken as planned.

Clinical trial supplies and sample collection: Volunteers will be instructed to collect the first stool sample passed each day into appropriate collection containers. The Collection accessory for use with OMNIgene GUT (DNA Genotek, Ottawa, CA) will be used for all DNA studies while the

Fisher Scientific commode collection container will be used for microbiologic studies. Samples will be brought to the research lab within 12 hour of collection.

Laboratory procedures: For each sample, weight and consistency (Bristol Stool chart) will be recorded. Approximately 500 mg of sample will be aliquoted into the OMNIgene GUT OMR-200 tube (DNA Genotek, Ottawa, CA) and stored via the manufacturer's directions. The remaining fecal samples will be aliquoted into appropriate storage containers and stored at -80C until analysis.

Stool DNA extraction: Prior to DNA extraction, the OMNIgene stool tube will be vortexed vigorously for at least 10 seconds. DNA will be extracted from the OMNIgene stool tube by aliquoting 300 µL of the tube sample using the AnaPrep system DNA extractor (AnaPrep 2, BioChain Instruments, Newark CA) and the following protocol. Approximately 300 mg stool will be dissolved in 250 µL of 1X PBS, homogenized for 60 seconds in a bead homogenizer (Bead Blaster24, Benchmark) for cell lysis, and added to 250 uL of lysis buffer BL2B with RNase A (Bacterial DNA Extraction Kit, BioChain Inc.). Approximately 400 uL of lysed bacteria will be mixed and loaded into the AnaPrep system and gDNA will be extracted using 400 microliter input volume and 100 uL elution volume. DNA will be eluted in 100µL of buffer and concentration measured using a Nanodrop 2000 (Fisher Scientific, Waltham, MA). If the DNA concentration is less than 10 µg/mL, the extraction will be repeated. A 50 µL portion of the extracted DNA will be used for qPCR analysis and the remainder used for 16S rDNA amplicon generation.

Methods for specific aim 1

*Quantitative PCR (qPCR) analysis***:** Ethanol precipitation of the extracted DNA will be done to eliminate inhibitors. The reconstituted DNA will be checked for purity and concentration using a Nanodrop 2000 (Fisher Scientific, Waltham, MA). Sample DNA will then be diluted with PCR grade water to 5 ng/µL. The DNA levels of bacterial groups will be assessed using the PCR primers/conditions used in the ridinilazole phase II study (Table 1) and published references [1-4]. Using the Viia 7 (Fisher Scientific, Waltham, MA), qPCR will be performed on each sample in triplicate in a final volume of 20 µL containing 25 ng DNA template, primers at 0.3 µM, and SYBR Green 2x Master Mix (QIAGEN), with a FAM-tagged probe at 0.25 µM for Eubacteria. Threshold cycle values will be converted to copies per ng of DNA using a standard curve. Standards will be prepared by performing PCR using species specific primers on appropriate bacterial strains or DNA from normal stool. The PCR products will be cloned using Invitrogen TOPO PCR Cloning Kit (Fisher Scientific, Waltham, MA), and verified by sequencing at the University of Houston Core Facility. A Basic Local Alignment Search Tool (BLAST) search will be performed to identify the closest matching database sequence (Table 2). A range of 10-fold serially diluted plasmid standard DNA (5 X 10^8 to 500 copies) will be run on each qPCR plate in triplicate. Standard curve R^2 values will be calculated for standards. Copies per gram of stool will be calculated, accounting for initial sample DNA concentrations and stool weights. The change in bacterial levels (Δlog10 copies/ gram stool) from entry level to each available successive time-point will be determined for each participant and median changes calculated

High-throughput metagenomics sequencing: Microbiome characterization will be performed by sequencing the V4 region of the 16S rRNA gene followed by extensive bioinformatics analysis related to microbial composition, diversity, and community structure. Microbial DNA extraction, 16S rRNA gene-based next-generation sequencing (using the Illumina MiSeq), quality filtering,

and microbiome-based analysis will be performed [5]. Each sample will be amplified using a barcoded primer, which yields a unique sequence identifier tagged onto each individual sample library. Illumina-based sequencing will yield > 15,000 reads per sample. Operational taxonomic units (OTUs) will be defined as ≥99% identity. To analyze differences between treatment groups and across timepoints, the OTU table will be normalized to the lowest number of sequences per sample, then consolidated by summing to species level and to each successively higher taxonomic level. The alpha diversity parameters Chao and Shannon (based on number of OTUs) and Phylogentic Diversity (PD) (based on phylogenetic relationships), will be generated in QIIME or R; alpha diversity is widely regarded as an indication of microbiota health. For beta diversity, which describes the relationships between samples, weighted Unifrac distance matrices will be generated in QIIME.

Statistical analyses. In general, the healthy volunteer analyses will assess changes in total and species-specific microbiota between groups and over time as well as changes in microbial diversity. For both qPCR and high-throughput sequencing data, significance differences between treatment groups at each time point will be calculated using Mann-Whitney U test while within each treatment arm, significance of differences between time-points will be assessed by the Wilcoxon Signed Rank test. Analyses will be performed using SAS (SAS Institute, Cary NC) or R on log-transformed data. For high-throughput sequencing data, the LEfSe (Linear Effect Size) algorithm [6] will be used to identify significant differences in microbiota composition between baseline and each study time point, followed by Wilcoxon Signed Rank tests on identified taxa. The Benjamin-Hochberg procedure will be used to control the false discovery rate at 0.10 [7]. The MaAsLin algorithm will be used to find associations of taxa with treatment. For beta diversity, principal co-ordinates analyses on weighted Unifrac distance matrices generated in QIIME will be performed in the vegan package in the statistical program R. Finally, using the methods described by Burdet at al, Shannon diversity (a measure of alpha diversity) will be compared at each time point for subjects that receive moxifloxacin or comparators.[8] For qPCR data, to analyze whether trajectories across time differed between treatment groups, repeated measures models with time and group as categorical fixed factors [9] will be used on log-transformed data, with diagnostics to assess the impact of potential influential points.

Deliverables: Results from this study will be presented at a major scientific meeting (ID-week, ECCMID, for example and submitted to a top tier medical journal (*Clinical Infectious Diseases / Journal of Antimicrobial Chemotherapy*, for example).

Budget (total costs): $204,725 (detailed budget attached)

Timeline: 1-year

Budget justification:

Dr. Gonzales-Luna will be the lead for all day to day activities on the grant. She will also be responsible for the healthy volunteer study with Dr. Lancaster. Drs. Begum and Alam will be responsible for all qPCR and metagenomics work in the study. Dr. Gonzales-Luna will also be responsible for analyses, generation of study reports, and first draft of the study manuscript. Dr. Garey will have oversight of the entire project as Principle Investigator. $77,781 in salary support is requested.

Supply costs are calculated $125 qPCR costs per sample and 16S rRNA (microbiome) costs of $125/sample. qPCR includes six relevant microbiota species. $28,800 and $38,400 are

requested for sequencing costs for the healthy volunteer. $10,000 is requested for clinical supplies and recruitment requirements for the phase I study along with study drug (omadacycline). Travel budget of $2500 is requested to present the results of the study at a major scientific meeting. Finally, $47,244 is requested for university indirect costs (mandatory per university policy).

Detailed budget:

Personnel		**Costs**
Kevin Garey, PI		$11,290
M Jahangir Alam, Co-I		$16,318
Khurshida Begum, Co-I		$16,318
Anne J. Gonzales-Luna, Co-I		$33,855
	Total Salary	**$77,781**
Material and Supplies		
Experiments		
Phase I clinical study supplies		$10,000
Healthy volunteer microbiome studies		
	qPCR	$28,800
	Metagenomics	$38,400
Travel (scientific meeting)		$2,500
	Total Materials & Supplies	**$79,700**
Total Direct Costs		**$157,481**
IDC Calculation		
IDC Base	Direct costs	$157,481
Total IDC	30%	$47,244
Total Costs		**$204,725**

References:

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6. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol **2011**; 12(6): R60.
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Table 1. DNA primers used for qPCR to quantify changes in major components of microbiota

Group	Primer name	Sequence (5'-3')	Reference
Bacteroides	Bac303F	GAA GGT CCC CCA CAT TG	Bernhard et al, 2000 (10)
	Bac708R	CAA TCG GAG TTC TTC GTG	
Clostridium coccoides	Erec482R	GCT TCT TAG TCA RGT ACC G	Louie et al, 2012 (11)
	Eub338F	ACT CCT ACG GGA GGC AGC	
Clostridium leptum	Sg-clept-F	GCA CAA GCA GTG GAG T	Matsuki et al, 2002 (12)
	Sg-clept-R	CTT CCT CCG TTT GTC AA	
Enterobacteriaceae	Eco1457F	CAT TGA CGT TAC CCG CAG AAG AAG C	Bartosch et al, 2004 (13)
	Eco1652	CTC TAC GAG ACT CAA GCT TGC	
Eubacteria	8F	AGT TTG ATC CTG GCT CAG	Jiang et al, 2009 (14)
	515R	GWA TTA CCG CGG CKG CTG	
	338P	FAM GCT GCC TCC CGT AGG AGT BHQ1	
Prevotella	CFB286F	GTA GGG GTT CTG AGA GGA	Louie et al, 2012 (11)
	CFB719R	AGC TGC CTT CGC AAT CGG	

Table 2. Sequences of plasmid standards for qPCR

Primer Set	Referenced Organism	Length	Match	Sequence
Bacteroides	Bacteroides sp. HGA0257 16S ribosomal RNA gene, partial sequence	419	99%	GAAGGTCCCCCACATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAAT ATTGGTCAATGGGCGAGAGCCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTG TAAACTTCTTTTATAAAGGAATAAAGTCGGGTATGGATACCCGTTTGCATGTACTTTATGGATAAG GATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCGAGCGTTATCCGGATTTAT TGGGTTTAAAGGGAGCGTAGATGGATGTTTAAGTCAGTTGTGAAAGTTTGCGACTCAACCGTAAA ATTGCAGTTGATACTGGATATCTTGAGTGCAGTTGAGGCAGGCGGAATTCGTGGTGTAGCGGTG AAATGCTTAGATATCACGAAGAACTCCGATTG
Clostridium coccoides	*Blautia coccoides* strain DSM 29138 16S ribosomal RNA gene,	139	95%	ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGC CGCGTGAGTGAAGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGAAAGAAAATGACGGTACCT GACTAAGAAGC
Clostridium leptum	*Clostridium leptum* 16S rRNA gene, strain DSM 753T Clostridium leptum ATCC 29065	241	90%	GCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT CGAGTGACGAACATAGAGATATGTTCTTCCTTCGGGACACGAAGACAGGTGGTGCATGGTTGTC GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGC TACGCAAGAGCACTCTAATGAGACTGCCGTTGACAAAACGGAGGAAG
Entero-bacteriaceae	*Escherichia coli* JJ1887, complete genome	190	100%	CATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATG TGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAG
Eubacteria	*Escherichia coli* str. K-12 substr. MG1655 strain K-12 16S ribosomal RNA, complete sequence (EUBACTERIA)	517	100%	AGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAAC AGGAAGAAGCTTGCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGG GGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACG GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACA CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGA GTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG CAGCCGCGGTAATAC
Prevotella	Prevotella denticola gene for 16S ribosomal RNA, partial sequence, strain: JCM 8528	446	86%	GTAGGGGTTCTGAGAGGAAGGTCCCCCACATTGGAACTGAGACACGGTCCAAACTCCTACGGGA GGCAGCAGTGAGGAATATTGGTCAATGGGCGAGAGCCTGAACCAGCCAAGTAGCGTGAAGGAT GACTGCCCTATGGGTTGTAAACTTCTTTTATAAAGGAATAAAGTCGGGTATGGATACCCGTTTGCA TGTACTTTATGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCGA GCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGATGGATGTTTAAGTCAGTTGTGAAAGTTT GCGGCTCAACCGTAAAATTGCAGTTGATACTGGATATCTTGAGTGCAGTTGAGGCAGGCGGAATT CGTGGTGTAGCGGTGAAATGCTTAGATATCACGAAGAACTCCGATTGCGAAGGCAGCT

Appendix 1 Safety assessments.

Adverse Events (AE), Serious Adverse Events (SAE), and Serious Adverse Drug Events (SADE):

Definitions and Reporting Procedures:

Adverse Events (AE)

Definition: an adverse event is defined as any event that was not present at baseline.

Criteria for Assessing Severity

The Investigator will evaluate the comments of the subject and the response to antibiotic treatment in order to judge the nature and severity of the adverse event. Severity refers to the intensity of discomfort/impairment of health and will be assessed according to the following criteria:

Mild: Awareness of sign, symptom, or event, but easily tolerated

Moderate: Discomfort enough to interfere with usual activity and may warrant intervention

Severe: Incapacitating with inability to do usual activities or significantly affects clinical status and warrants intervention

To clarify the difference between the terms "serious" and "severe", which are not synonymous, the following note is provided:

The term "severe" is often used to describe the intensity (severity) of a specific event (as in mild, moderate, or severe myocardial infarction); the event itself, however, may be of relatively minor medical significance (such as severe headache). This is *not* the same as "serious", which is based on patient/event *outcome or action* criteria usually associated with events that pose a threat to a patient's life or functioning. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations.

Criteria for Assessing Causality

The question of the relationship of an adverse event to study drug will be determined by the Investigator after thorough consideration of all facts that are available. Assessment of causality is based on considering associative connections (time or place), pharmacological explanations, previous knowledge of the drug, presence of characteristic clinical or pathological phenomena, exclusion of other causes, and/or absence of alternative explanations. The causal relationship of an adverse event to study drug will be assessed according to the following criteria:

The Investigator will be responsible for determining the relationship between the administration of study drug and the occurrence of an AE as not suspected or suspected.

Not suspected: The temporal relationship of the adverse event to study drug administration makes a causal relationship unlikely, or other drugs, therapeutic interventions, or underlying conditions provides a sufficient explanation for the observed event.

Not related: Temporal relationship to study drug administration is missing or implausible, or there is evidence of another cause.

Unlikely related: Temporal relationship to study drug administration makes a causal relationship improbable; and other drugs, chemicals, or underlying disease provide plausible explanations.

Suspected: The temporal relationship of the adverse event to study drug administration makes a causal relationship possible, and other drugs, therapeutic interventions, or underlying conditions do not provide a sufficient explanation for the observed event.

Possibly related: Reasonable time sequence to administration of study drug, but the event could also be explained by concurrent disease of other drugs or chemicals. Information on drug withdrawal may be lacking or unclear.

Definitely related: Plausible time relationship to study drug administration; event cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the drug (dechallenge) should be clinically plausible. The event must be definitive pharmacologically or phenomenologically, using a satisfactory re-challenge procedure if necessary.

Reporting of Adverse Events

All adverse events from the time of randomization will be recorded in the Adverse Events section of the case report form. All adverse events will be described in terms of duration, severity, possible relation to study medications and treatment. Previously documented side effects of each medication will be listed in each medication's respective section.

All adverse events will be followed until resolution; or for 30-days following the last dose of study drug or up until the last protocol-specified study visit, whichever occurs later.

If the adverse event meets any of the criteria for Serious Adverse Events, the event will also be documented on a separate SAE Form.

Serious Adverse Event (SAE)

Definition: serious adverse events are defined as the following

- Death
- Life Threatening
- Requiring prolonged hospitalization (greater than one week)
- Permanently disabling
- Causing cancer
- Congenital anomaly/ birth defect
- Any other clinically significant event

Reporting of SAE

In the event of a Serious Adverse Event, information will be collected and recorded on the Serious Adverse Event section of the case report form.

Serious Adverse Events will be reported from the time informed consent is obtained through 30-days following the last dose of study drug or to the last protocol-specified study visit; whichever occurs later. In addition, any SAE occurring after the observation period will be promptly reported if causal relationship to study drug is suspected. Serious adverse events will be followed until the event resolves or the event or its sequelae stabilize.

Serious Adverse Event (SAE) Reporting to IRB: The Principal Investigator will be responsible for reporting any SAE thought to be directly attributable to the study medication to the IRB.