TITLE PAGE

Study Title:	A Phase 1 Placebo-controlled Study of the Safety and Tolerability of Rectally Administered Single Ascending Doses of IW-3300 in Healthy Volunteers
Protocol Number:	C3300-101
Version:	1.0
Date of Plan:	06 Apr 2022
Study Drug:	IW-3300
Study Phase:	1
Sponsor:	Ironwood Pharmaceuticals, Inc. 100 Summer Street, Suite 2300 Boston, MA 02110 USA (617)-621-7722

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. ABBREVIATIONS

Abbreviation	Term
ADaM	Analysis Data Model (a CDISC standard)
AE	adverse event
BBMD	bladder and bowel movement diary
BM	bowel movement
BMI	body mass index
BSFS	Bristol Stool Form Scale
CDISC	Clinical Data Interchange Standards Consortium
cGMP	cyclic guanosine monophosphate
CI	confidence interval
CRO	clinical research organization
CRP	C-reactive protein
CRU	clinical research unit
CTCAE	Common Terminology Criteria for Adverse Events
DMC	data monitoring committee
ECG	electrocardiogram
eCRF	electronic case report form
ESR	erythrocyte sedimentation rate
GC-C	guanylate cyclase C
IC/BPS	interstitial cystitis/bladder pain syndrome
ICF	informed consent form
IPD	important protocol deviations
ITT	intention-to-treat
LLN	lower limit of normal
MedDRA	Medical Dictionary for Regulatory Activities
MRP	multidrug resistance protein
PCS	potentially clinically significant
PD	pharmacodynamic
PK	pharmacokinetic
PT	preferred term
QTcF	QT Interval corrected for heart rate using Fridericia's formula
SAE	serious adverse event

Abbreviation	Term
SAP	statistical analysis plan
SD	standard deviation
SE	standard error
SI	Système International
SOC	system organ class
SDTM	Study Data Tabulation Model (a CDISC standard)
TEAE	treatment-emergent adverse event
TESAE	treatment-emergent serious adverse event
ULN	upper limit of normal

2. INTRODUCTION

Study C3300-101 is a Phase 1, placebo-controlled study of the safety and tolerability of rectally-administered single-ascending doses of IW-3300 in healthy volunteers.

IW-3300 is being developed for the treatment of bladder pain associated with interstitial cystitis/bladder pain syndrome (IC/BPS). IW-3300 is a novel, 13-amino-acid, guanylate cyclase C (GC-C) agonist peptide. GC-C, the target of IW 3300, is predominantly expressed on the luminal surface of the small and large intestines. When GC-C receptors are stimulated, intracellular cyclic guanosine monophosphate (cGMP) is secreted across the basolateral membrane of colonic epithelial cells in the submucosa by multidrug resistance proteins (MRP)4 and MRP5, decreasing the activity of afferent nerve fibers located in the colonic wall, resulting in reduced visceral pain, which ultimately produces an analgesic effect in other organs of the abdominopelvic region via action mediated through the common afferent pathways.

This statistical analysis plan (SAP) was developed in accordance with ICH E9 guideline. All decisions regarding final analysis, as defined in this SAP document, will be made prior to database lock and unblinding of the study data. Further information can be found in the protocol.

This SAP describes the statistical analysis methods for the analysis of safety and pharmacodynamic (PD) data. The statistical analysis methods for the pharmacokinetics (PK) assessments will be described in a PK analysis plan. The data are collected as described in the following documents:

- Study protocol version 4/amendment 3 dated 10 January 2022
- electronic case report form (eCRF) version 1.02 dated 19 Jan 2022.

The final SAP must be finalized, approved by the sponsor, Ironwood Pharmaceuticals, Inc. and formally archived before database is locked. Deviations from the final approved plan will be noted in the clinical study report.

3. OBJECTIVES, ESTIMAND(S), AND ENDPOINTS

3.1. Objectives

3.1.1. Primary Objective

To assess the safety and tolerability of single ascending doses of IW-3300 administered rectally via enema in healthy volunteers.

3.1.2. Exploratory Objectives

- To summarize the single-dose pharmacokinetics of IW-3300 administered rectally via enema in healthy volunteers.
- To summarize the single-dose pharmacodynamics of IW-3300 administered rectally via enema in healthy volunteers based on bladder and bowel assessments.

3.2. Endpoints

3.2.1. Safety Endpoints

- Incidence of treatment-emergent adverse events (TEAEs)
- Incidence of treatment-emergent serious adverse events (TESAEs)

3.2.2. Pharmacokinetic Endpoints

- Area under the plasma concentration time curve from time zero to the time at which the last measurable concentration is observed (AUC_{0-t})
- Area under the plasma concentration time curve from time zero to time infinity ($AUC_{0-\infty}$)
- Maximum plasma concentration (C_{max})
- Time to maximum plasma concentration (T_{max})
- Terminal elimination half-life ($t_{1/2}$)
- Total amount of IW-3300 in stool (concentration and percent recovery)

3.2.3. Pharmacodynamics Endpoints

- Change from baseline in bowel movement (BM) frequency during the Clinic Period
- Change from baseline in stool consistency (Bristol Stool Form Scale [BSFS] score) during the Clinic Period
- Change from baseline in urinary frequency during the Clinic Period for a 24-hour duration
- Change from baseline in urinary urgency during the Clinic Period for a 24-hour duration
- Change from baseline in nocturia during the Clinic Period for a 24-hour duration

- Change from baseline in pain or burning in bladder or pelvic area during the Clinic Period for a 24-hour duration

4. STUDY DESIGN

4.1. GENERAL DESCRIPTION

This is a Phase 1, single-center, randomized, double-blind, placebo-controlled, single-ascending dose study assessing the safety, tolerability, and PK of IW-3300 administered rectally as a low volume enema in healthy adult volunteers. Each cohort will progress through 3 study periods: (1) Screening Period, (2) Clinic Period, and (3) Follow-up Period; these periods and the progression between cohorts are illustrated in the study schematic (Figure 1).

This first-in-human study in healthy adult volunteers will assess all subjects for safety, PK, and effect on stool frequency/form and urination.

A Dose Escalation Committee will conduct blinded reviews of all the safety/tolerability parameters of the dosing cohort through Discharge (Day 2) in order to make decisions regarding dose escalation. Following blinded review, safety data for individual subjects may be unblinded as described below. The Dose Escalation Committee will include sponsor and contract research organization (CRO) representatives.

4.1.1. Number of Subjects

A maximum of 40 subjects will be randomized in the study (up to 5 cohorts of 8 subjects each); within each cohort, 6 subjects will be randomized to IW-3300 and 2 subjects will be randomized to placebo.

4.1.2. Intervention Groups and Duration

The study will evaluate single ascending doses of IW-3300 in a double-blind manner. The 8 subjects within each cohort will be randomized to receive a single dose of IW-3300 (6 subjects) or placebo (2 subjects), administered rectally (as a low-volume [20 mL] enema) following a fast of at least 6 hours (refer to the Schedule of Activities [SoA], Section 12.3 for additional details regarding dosing instructions). The planned cohorts are:

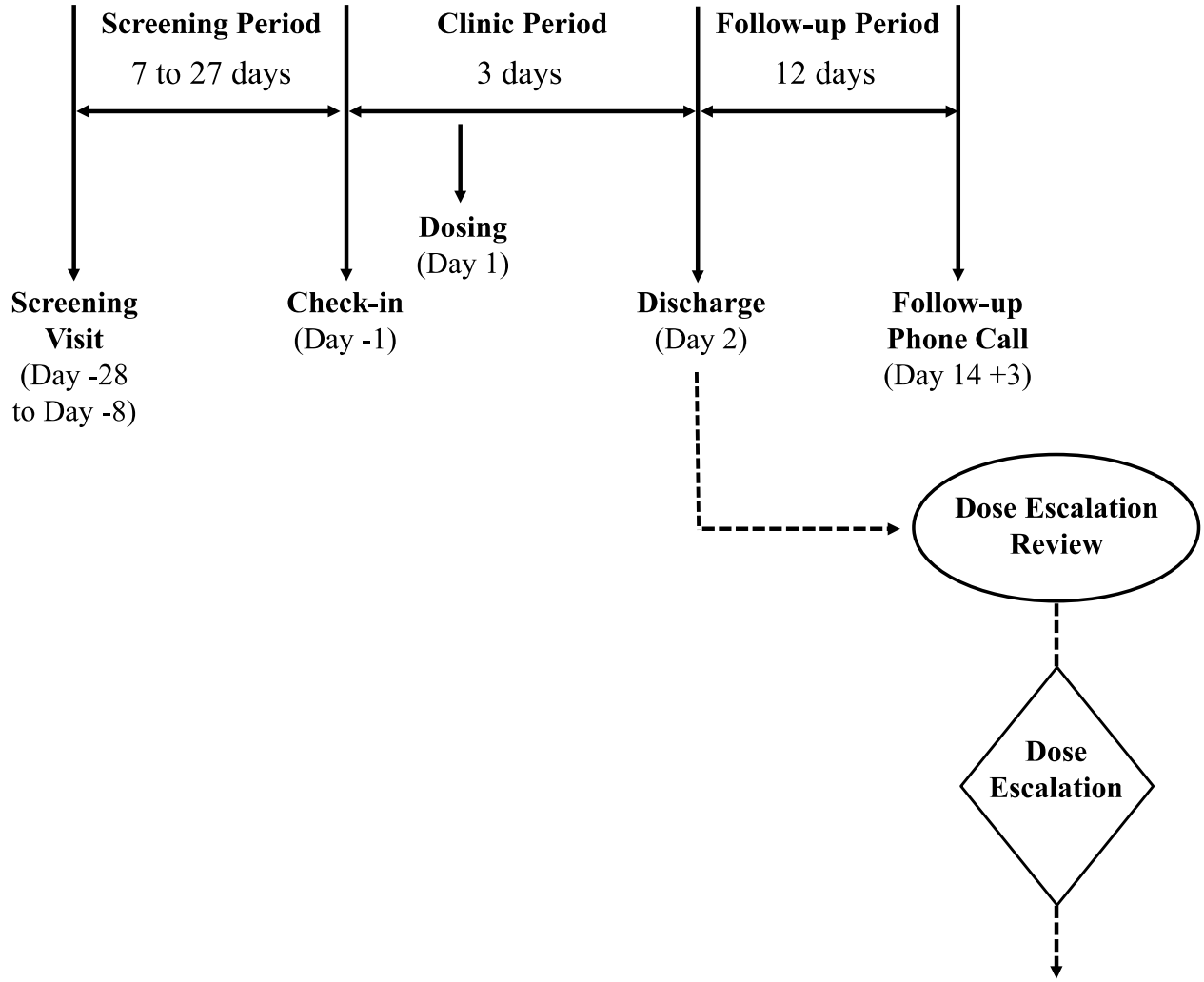
- Cohort 1: 100 µg IW 3300 or matching placebo single rectal dose
- Cohort 2: 300 µg IW 3300 or matching placebo single rectal dose
- Cohort 3: 900 µg IW 3300 or matching placebo single rectal dose
- Cohort 4: 2500 µg IW 3300 or matching placebo single rectal dose
- Cohort 5 (optional): ≤ 2500 µg IW 3300 or matching placebo single rectal dose (specific dose level to be determined after safety reviews of previous cohorts; doses higher than 2500 µg will not be tested)

In Cohort 1, sentinel dosing will be implemented. The first 2 subjects will be randomized in a 1:1 ratio to IW-3300 or placebo and dosed at least 4 hours before the remaining subjects in the cohort. If no safety signals are noted following an observation period of at least 4 hours postdose, the remaining 6 subjects in Cohort 1 will be dosed. Dosing in Cohorts 2 through 5 will only proceed following a review of the prior dosed cohort(s), including adverse events (AEs), clinical laboratory test results, vital signs, and 12-lead ECGs. The determination of dose escalation will be made at a meeting of the Dose Escalation Committee.

Treatment duration will be 1 day; subjects will be followed in the Phase 1 clinical research unit (CRU) for approximately 24 hours after dosing and contacted by phone for follow-up approximately 2 weeks after dosing. Total subject participation will be 22 to 45 days, including the Screening, Clinic, and Follow-up Periods.

The schematic of the study design is presented below (Figure 1).

Figure 1: Schematic of Study Design



4.2. Discussion of Study Design, Including Choice of Control Group

A randomized, double-blind, placebo-controlled study design was chosen to investigate the safety, PK, and PD of IW-3300, and to determine the tolerability of a range of doses of IW-3300 administered rectally as a low-volume enema. Subjects will be randomized within each cohort to ensure that the treatment groups are comparable and to minimize the potential for selection bias. The study will be double-blind to ensure that the subjects and CRU staff are unaware of the treatment assignment and to minimize the potential for bias in study assessments or AE reporting. Placebo was chosen as the control so that the rate of spontaneously occurring AEs can be determined and to reduce the potential for bias in the reporting of AEs.

Subjects will complete the BM portion of the BBMD during the last 7 days of the Screening Period to establish a baseline without study drug. Subjects will be confined to the CRU for the duration of the Clinic Period, from Check-in (Day -1) through 1 day of dosing until Discharge (Day 2), which will occur after completion of the assessments (at least 24 hours after administration of study drug) and at the investigator's discretion. CRU staff will contact subjects for the Follow-up Phone Call 12 (+3) days after Discharge for safety follow-up; at the discretion of the investigator, subjects may be requested to return to the CRU for their follow-up contact.

Because this is the first-in-human study with IW-3300, Cohort 1 will feature sentinel dosing whereby the first 2 subjects will be randomized in a 1:1 ratio to receive IW-3300 or placebo, and they will be dosed at least 4 hours before the remaining subjects in the cohort. Subsequently, the cohorts will be enrolled sequentially, following a safety review of prior dosed cohorts. In addition, stopping criteria have been established to ensure that dosing at the same or higher dose levels (a lower dose could be used) will stop should a safety signal be detected.

4.3. Method of Assigning Subjects to Treatment Groups

Randomization numbers encoding the subjects' treatment assignments will be based on a randomization schedule that is computer-generated; the randomization schedule will be generated prior to the study, by an independent statistician from a CRO who is not otherwise associated with the study.

4.4. Blinding

The investigator and all other CRU staff, sponsor study personnel, and the subject will remain blinded to individual subject treatment assignments throughout the study, except as noted below.

The Dose Escalation Committee, which will include sponsor and CRO representatives, will conduct blinded reviews of all the safety/tolerability parameters of the dosing cohort through Discharge (Day 2) in order to make decisions regarding dose escalation.

Specific designated personnel in the Ironwood may be unblinded to the treatment assignment of individual subjects for regulatory reporting purposes. All other sponsor study personnel, except as described, will remain blinded until the study is complete and the database is locked, unless warranted by emerging safety or tolerability issues.

The designated independent study statistician, who will not be involved in study data analysis and interpretation, will have access to the randomization schedule including treatment assignments.

Specifically designated unblinded CRU pharmacy staff will be responsible for preparing dosing for each cohort and providing doses to the study coordinator to administer to subjects. The investigator (except as detailed here) and the remaining CRU staff will be blinded as to treatment. In the event of a Quality Assurance audit, the auditor(s) will be allowed access to unblinded study intervention records at the CRU to verify that randomization/dispensing has been done accurately.

Site unblinding of a subject's treatment assignment is restricted to emergency situations that necessitate identifying the study drug for the welfare of the subject. Subject safety must always be the first consideration in making such a determination. If the investigator decides that unblinding is warranted, the investigator, or person designated by the investigator, should contact the sponsor's medical monitor directly to discuss the need for emergency unblinding. Individual sealed unblinding envelopes, which can be opened to identify the treatment assignment for an individual subject in an emergency, will be provided to and retained by the CRU pharmacist. The reason for breaking the blind will be recorded.

4.5. Sample Size and Power Considerations

No statistical sample size determination process was performed. The sample size chosen for each cohort (8 subjects) is considered sufficient for evaluation of safety, tolerability, PK, and PD.

5. STATISTICAL ANALYSIS SETS

5.1. Screened Set

The Screened Set consists of all subjects who signed the informed consent.

5.2. ITT Set

The ITT Set consists of all subjects in the Screened Set who have been randomized to a treatment regimen. Analysis will be performed according to the allocated treatment regimen regardless of the treatment regimen actually received.

5.3. Safety Analysis Set

The Safety Analysis Set consists of all subjects who received any amount of study drug. Analysis will be performed according to the treatment actually received regardless of the allocated treatment.

5.4. PK Analysis Set

The PK Analysis Set consists of all subjects in the Safety Analysis Set who have at least 1 postdose PK assessment. Analysis will be performed according to the treatment actually received regardless of the allocated treatment.

5.5. PD Analysis Set

The PD Analysis Set consists of all subjects in the Safety Analysis Set who have at least 1 postdose BBMD entry in the Clinic Period. Analysis will be performed according to the treatment actually received regardless of the allocated treatment.

All subjects on placebo arm will be pooled from available cohorts, whenever appropriate, to conduct analyses. All data will be summarized by cohort or treatment group, whenever it is appropriate.

6. STUDY SUBJECTS

6.1. Disposition of Subjects

The number and percentage of subjects who were included in each defined analysis set (ie, Screened, ITT, Safety, PK, and PD) will be summarized by treatment group and overall, except for the Screened Set, which will only be summarized overall.

In rare instances, a subject may withdraw from the study prior to completion of the Clinic Period. The number and percentage of subjects who completed or who prematurely discontinued will be presented for each treatment group and overall for the Safety Analysis Set. Reasons for premature discontinuation as recorded on the termination page of the eCRF will be summarized (number and percentage) by treatment group and overall.

6.2. Demographic and Other Baseline Characteristics

Descriptive summaries of demographic and baseline characteristics will be presented by treatment group and overall, for the Safety Analysis Set.

The following demographic characteristics will be summarized in the tables: age (years and by category), sex, ethnicity, race, weight, height, and BMI.

6.3. Medical and Surgical History

Medical and surgical history will be collected at the Screening Visit and will be coded using MedDRA Version 24.0. A data listing will be provided for the Safety Analysis Set.

The medical history will be summarized by system organ class (SOC) and preferred term (PT) for each treatment group and overall, for the Safety Analysis Set.

6.4. Prior Medications, Therapies, Procedures

Prior medications will be coded using the WHO Drug Dictionary dated 03 March 2020. Prior therapies and procedures will be coded using MedDRA Version 24.0.

Prior medications, therapies, and procedures are defined as that with a start date prior to the date of the first dose of study drug.

The prior medication, therapy, and procedure usage will be summarized by the number and percentage of subjects in each treatment group and overall, within each preferred term for the Safety Analysis Set. Multiple uses of the same medication, therapy, or procedure by a subject in the same category will be counted only once.

All prior medications, therapies, and procedures will be listed for the Safety Analysis Set.

6.5. Concomitant Medications, Therapies, and Procedures

Concomitant medications will be coded using the WHO Drug Dictionary dated 03 March 2020. Concomitant therapies and procedures will be coded using MedDRA Version 24.0.

Concomitant medications, therapies, and procedures are defined as those with a start date prior to the date of the first dose of study drug and continuing on or after the first dose of study drug, or

with a start date between the dates of the first and last doses of study drug, inclusive. Any medication, therapy, or procedure with a start date after the date of the last dose of study drug will not be considered a concomitant medication, therapy, or procedure.

The concomitant medication, therapy, and procedure and usage will be summarized by the number and percentage of subjects in each treatment group and overall, within each preferred term for the Safety Analysis Set. Multiple uses of the same medication, therapy, or procedure by a subject in the same category will be counted only once.

All concomitant medications, therapies, and procedures will be listed for the Safety Analysis Set.

6.6. Exposure to Study Drug

A data listing will be created giving the date and time of dose administration for each subject in the Safety Analysis Set.

6.7. Protocol Deviations

Protocol deviations will be identified and recorded by the site and tracked separately from the clinical database. Clinical team members on the Ironwood Study Execution Team (SET) will determine which protocol deviations are important protocol deviations (IPDs) and will categorize IPDs based on a blinded review of all protocol deviations prior to database lock and unblinding. The SET members reviewing the protocol deviations will include the study clinical operations lead, the medical director, the study biostatistician, and other personnel as appropriate.

The number and percentage of subjects with IPDs will be presented by treatment group and IPD category for the Safety Analysis Set. Protocol deviations and IPDs will be provided by subject in a data listing for the Safety Analysis Set.

A separate listing of all inclusion/exclusion criteria deviations will be provided for all randomized subjects (ie, the ITT Set).

7. SAFETY ANALYSIS

All safety parameters will be summarized using descriptive statistics. Safety analyses will be performed on the Safety Analysis Set. The safety parameters will include AEs, clinical laboratory evaluations, vital signs, and ECGs. For each laboratory parameters and ECG site interpretation, the last nonmissing assessment before the first dose of study drug will be used as the baseline for all analyses of that laboratory parameter or ECG interpretation. For numeric ECG measurements, the average of the last triplicate ECG before dosing will be used as baseline. For vital signs, the last supine measurement before dosing will be used as baseline.

All safety analyses will be performed according to the treatment actually received regardless of the allocated treatment.

7.1. Adverse Events

AEs will be coded by SOC and PT using MedDRA Version 24.0. The study investigator will assess the severity of the AE and assign a grade using the Common Terminology Criteria for Adverse Events (CTCAE). An AE is considered a treatment-emergent AE (TEAE), if the AE started after initial study drug administration and within 1 day of the last dose of study drug. In addition, an AE that started before initial study drug but worsened after the first dosing of the study drug is also considered a TEAE.

The number and percentage of subjects reporting TEAEs in each treatment group will be tabulated:

- by SOC and PT
- by SOC, PT, and CTCAE grade
- by SOC, PT, and relationship to study drug.

If a subject has more than 1 TEAE coded to the same PT, the subject will be counted only once for that PT by identifying the TEAE with the highest CTCAE grade and the closest relationship to study drug.

The incidence of the following TEAEs will be summarized by PT:

- Treatment-emergent Serious Adverse Events (TESAEs)

All AE summary tables will be sorted alphabetically by SOC and each PT within the SOC will be sorted by decreasing frequency for the IW-3300 group.

A TEAE summary table with all the non-SAEs by SOC and PT will be generated to support the clinicaltrials.gov reporting requirements.

AE listings will be presented for all subjects with AEs, all screened subjects with SAEs, all SAEs (regardless of treatment-emergent status), and subjects who died (if any), respectively.

7.2. Clinical Laboratory Data

Descriptive statistics for clinical laboratory values (in SI units) and changes from baseline at each postbaseline time point will be presented by treatment group for each clinical laboratory parameter. All laboratory data will be listed for the Safety Analysis Set.

Table 1: Laboratory Assessments

<table>
<tr><th>Laboratory Assessments</th><th colspan="4">Parameters</th></tr>
<tr><td>Hematology</td><td>Platelet count
RBC count
Hemoglobin
Hematocrit</td><td colspan="2">RBC Indices:
MCV
MCH
MCHC
% Reticulocytes</td><td>WBC Count with Differential:
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils</td></tr>
<tr><td rowspan="5">Clinical Chemistry</td><td>Blood urea nitrogen (BUN)</td><td>Potassium</td><td>AST</td><td>Total and direct bilirubin</td></tr>
<tr><td>Creatinine</td><td>Sodium</td><td>ALT</td><td>Total protein</td></tr>
<tr><td>Glucose</td><td>Calcium</td><td>Alkaline phosphatase</td><td>Magnesium</td></tr>
<tr><td>Chloride</td><td>Albumin</td><td>Bicarbonate</td><td>Phosphate</td></tr>
<tr><td>Cholesterol</td><td>Uric acid</td><td></td><td></td></tr>
<tr><td>Routine Urinalysis</td><td colspan="4">• Specific gravity
• pH, glucose, protein, blood, and ketones, by dipstick
• Microscopic examination (if blood or protein is abnormal)
• Urine culture (if urinary tract infection is suspected)</td></tr>
<tr><td>Inflammatory markers</td><td colspan="4">• ESR (analyzed at an outside laboratory)
• CRP</td></tr>
<tr><td>Hemoccult testing</td><td colspan="4">• Point-of-care hemoccult test</td></tr>
<tr><td>Other Screening Tests</td><td colspan="4">• Alcohol and drug screen (to include at minimum: amphetamines, barbiturates, cocaine, opiates, cannabinoids, and benzodiazepines).
• Urine cotinine test
• SARS-CoV-2 testing (Screening and Check-in)
• HIV antibody and hepatitis panel (HBsAg, and HCV antibody).</td></tr>
</table>

For each clinical laboratory parameter, the number and percentage of subjects with potentially clinically significant (PCS) postbaseline clinical laboratory values will be tabulated by treatment group. The criteria for PCS laboratory values are described in Table 2. The percentages will be calculated relative to the number of subjects with non-PCS baseline values and at least 1 postbaseline assessment. The numerator will be the total number of subjects who had non-PCS values at baseline and who had at least 1 postbaseline PCS value.

A supportive listing for subjects with PCS postbaseline values will be provided and will include the subject ID number, all lab values, both pre- and postbaseline, and a flag marking the PCS laboratory values.

Table 2: Criteria for Potentially Clinically Significant Laboratory Tests

Parameter	SI Unit	Lower Limit	Higher Limit
Chemistry			
Albumin	g/L	<0.9 × LLN	>1.1 × ULN
Alanine aminotransferase	U/L	—	≥3 × ULN
Alkaline phosphatase	U/L	—	≥3 × ULN
Aspartate aminotransferase	U/L	—	≥3 × ULN
Bicarbonate	mmol/L	<0.9 × LLN	>1.1 × ULN
Bilirubin, total	µmol/L	—	>1.5 × ULN
Calcium	mmol/L	<0.9 × LLN	>1.1 × ULN
Chloride	mmol/L	<0.9 × LLN	>1.1 × ULN
Cholesterol, total	mmol/L	—	>1.6 × ULN
Creatinine	µmol/L	—	>1.3 × ULN
Glucose	mmol/L	<0.8 × LLN	>1.4 × ULN
Magnesium	mmol/L	<0.9 × LLN	>1.1 × ULN
Phosphate	mmol/L	<0.9 × LLN	>1.1 × ULN
Potassium	mmol/L	<0.9 × LLN	>1.1 × ULN
Protein, total	g/L	<0.9 × LLN	>1.1 × ULN
Sodium	mmol/L	<0.9 × LLN	>1.1 × ULN
Urea nitrogen	mmol/L	—	>1.2 × ULN
Uric acid	µmol/L	<0.9 × LLN	>1.1 × ULN
Hematology			
Basophils, absolute cell count	10^9/L	—	>3 × ULN
Eosinophils, absolute cell count	10^9/L	—	>3 × ULN
Hematocrit	Ratio	<0.9 × LLN	>1.1 × ULN
Hemoglobin	g/L	<0.9 × LLN	>1.1 × ULN
Lymphocytes, absolute cell count	10^9/L	<0.8 × LLN	>1.5 × ULN
Mean corpuscular hemoglobin	Pg	—	>3 × ULN
Mean corpuscular hemoglobin concentration	g/L	—	>3 × ULN
Mean corpuscular volume	fL	<0.9 × LLN	>1.1 × ULN
Monocytes, absolute cell count	10^9/L	—	>3 × ULN
Neutrophils, absolute cell count	10^9/L	<0.8 × LLN	>1.5 × ULN
Platelet count	10^9/L	<0.5 × LLN	>1.5 × ULN

Parameter	SI Unit	Lower Limit	Higher Limit
Red blood cell count	10^{12}/L	<0.9 × LLN	>1.1 × ULN
White blood cell count	10^{9}/L	<0.7 × LLN	>1.5 × ULN

LLN: Lower limit of normal value provided by the laboratory
ULN: Upper limit of normal value provided by the laboratory

7.3. Vital Signs

Descriptive statistics for body weight and vital signs (ie, oral temperature, supine systolic and diastolic blood pressure, and pulse) and changes from baseline values at each time point will be presented by treatment group, and all the data will be listed. If there are multiple measurements for the same time point, the first one will be used for the by-time point analysis.

The number and percentage of subjects with PCS postbaseline vital signs will be tabulated by treatment group. A vital sign value will be considered PCS if it meets both the observed value criterion and the change from baseline criterion. The criteria for PCS vital sign values are detailed in Table 3. The PCS percentages will be calculated relative to the number of subjects with baseline values and at least 1 assessment in the corresponding postbaseline period. The numerator will be the total number of subjects with available baseline values and at least 1 postbaseline PCS value.

A supportive data listing for subjects with PCS postbaseline values will be provided, including the subject ID number, baseline and postbaseline values for each visit, and a flag indicating the PCS vital sign values.

Table 3: Criteria for Potentially Clinically Significant Vital Signs

Vital Sign Parameter	Flag	Criteria[a]	
		Observed Value	Change from Baseline
Supine systolic blood pressure (mmHg)	High	≥180	Increase of ≥20
	Low	≤90	Decrease of ≥20
Supine diastolic blood pressure (mmHg)	High	≥105	Increase of ≥15
	Low	≤50	Decrease of ≥15
Supine pulse (beats per minute)	High	≥120	Increase of ≥15
	Low	≤50	Decrease of ≥15
Weight (kg)	High	-	Increase of ≥7%
	Low	-	Decrease of ≥7%

[a] A postbaseline value is considered as a PCS value if it meets the criteria for both observed value and change from baseline.

In addition to supine measurements, at the predose measurement and at postdose time points 1, 6, and 24 hours, standing blood pressure and pulse will be measured 3 minutes after the supine measurements are taken. The standing blood pressure will be used to assess indicators of orthostatic hypotension. The criteria for testing positive for the orthostatic hypotension indicators are presented in Table 4.

Table 4: Criteria for Orthostatic Hypotension Indicators

Blood Pressure Parameter	Change from Baseline
Supine systolic blood pressure (mmHg)	Increase of ≥20 mmHg
Supine diastolic blood pressure (mmHg)	Increase of ≥10 mmHg

Subjects meeting either one of these two criteria are considered to be positive for indicators of orthostatic hypotension. The number and percent of subjects who are positive for these indicators will be tabulated by treatment group and by period (baseline versus postbaseline).

7.4. Electrocardiogram

Triplicate 12-lead ECGs will be obtained as outlined in the Schedule of Activities. An average of the 3 consecutive ECG values for each parameter (heart rate, PR interval, QRS interval, QT interval, and QTc interval), or 1 or 2 values if all 3 are not available, will be used for generating the summary statistics. Descriptive statistics for ECG parameters and their changes from baseline at each assessment time point will be presented by treatment group. QTc interval will be calculated using Fridericia correction ($QTcF=QT/(RR)^{1/3}$); if RR is not available, it will be replaced with 60/hr in the correction formula. ECG interpretation (designated as normal, abnormal not clinically significant, abnormal clinically significant) will be summarized by time point. A shift table from baseline to each visit for qualitative ECG results will be presented and all the data will be listed.

In addition, the number and percentage of subjects with PCS postbaseline ECG results will be tabulated by treatment group. An ECG value will be considered PCS if it meets the observed value criterion. The criteria for PCS ECG values are detailed in Table 5. The percentages will be calculated relative to the number of subjects with available non-PCS baseline values and at least 1 postbaseline assessment. The potential numerator will be the total number of subjects with available non-PCS baseline values and at least 1 postbaseline PCS value in the corresponding postbaseline period.

A supportive data listing for subjects with PCS postbaseline values will be provided, including the subject ID number, baseline and postbaseline values for each visit, and a flag indicating the PCS ECG values.

Table 5: Criteria for Potentially Clinically Significant ECG Values

ECG Parameter	Unit	Higher Limit
QRS Interval	msec	≥150
PR Interval	msec	≥250
QTc Interval	msec	>500

8. PHARMACOKINETIC ANALYSIS

Pharmacokinetic (PK) assessments and analyses will be described in a separate PK analysis plan.

9. PHARMACODYNAMIC ANALYSIS

The analysis of the bladder and bowel movement diary (BBMD) parameters will be based on the PD Analysis Set. All PD data will be captured on the BBMD. Subjects will enter BM-related information into a paper diary, the BBMD, on an event-driven basis (ie, following each BM) during the Screening Period (beginning at Day -8) and throughout the Clinic Period. The BM related information will include the day and time of BMs and a report of stool consistency for each BM using the BSFS (1=Separate hard lumps like nuts [difficult to pass], 2=sausage shaped but lumpy, 3=like a sausage but with cracks on surface, 4=like a sausage or snake, smooth and soft, 5=soft blobs with clear-cut edges (passed easily), 6=fluffy pieces with ragged edges, a mushy stool and 7=Watery, no solid pieces [entirely liquid]).

Subjects will complete the bladder potion of the BBMD in the morning of Dosing Day 1 (which represents the baseline responses) and in the morning of Discharge Day 2. The bladder diary will include the following questions:

In the past 24 hours…

- Did you have to urinate more frequently than normal? (yes/no)
- How many times did you urinate? (0 to 2; 3 to 6; 7 to 10; or more than 10)
- Did you feel the strong need to urinate with little or no warning? (yes/no)
- Did you have to get up to urinate during the night more frequently than usual? (yes/no)
- How many times did you have to get up at night to urinate? (0, 1, 2, 3+ times)
- Did you have pain or burning in your bladder or pelvic area? (yes/no)

9.1. Pharmacodynamic Analysis

Bowel movement data will be summarized by treatment for the baseline period and the 24-hour period after dosing using descriptive statistics. Change from baseline for the following BM parameters will be calculated:

- BM frequency
- Stool consistency (BSFS)

The baseline period for BM data is exactly 1 week and consists of the seven 24-hour periods that end at the time of first dose. Baseline values will be derived from the BBMD data collected during that period. The baseline BM daily frequency rate (BMs per day) will be calculated as the number of BMs the subject reports during this period divided by 7. Baseline stool consistency will be calculated as the average of the non-missing BSFS scores associated with the BMs reported by the subject during this period.

For BM data, the postdosing frequency and averages will be based on events and reports that occur during the 24-hour period that starts at the time of dosing and ends when subjects completes the BBMD 24 hours later on Day 2. Change from baseline will be defined as the postdosing values minus the baseline value. PD data will be provided in listings.

The PD data regarding bladder symptoms and urination is categorical. The baseline values are simply the responses to the 6 relevant questions asked, predose, in the morning of Dosing Day 1, and the postdosing values are the responses to the BBMD on Day 2.

For the bladder and urination data, shift tables by time point will be created for each of the 6 questions, showing the responses for the 24 hours before dosing (completed on the morning of dosing, Day 1) versus the responses for the 24 hours postdose (from Discharge, Day 2).

For the Day 1 BBMD diary, summary analyses will only include diaries that were completed before dosing. Any diaries that were completed after the first dose will not be used. Similarly, for the Day 2 BBMD diary, only diaries that were completed 20 hours or more after dosing will be used.

10. DATA MONITORING/REVIEW COMMITTEE

This study will not utilize a DMC. A Dose Escalation Committee will be utilized to review safety data for each cohort in order to make decisions about dose escalation.

11. REFERENCES

Not applicable

12. APPENDICES

12.1. Data Handling Conventions

12.1.1. General Data Reporting Conventions

Continuous variables will be summarized using the following descriptive statistics: n, mean, median, standard deviation (SD), minimum and maximum. Categorical variables will be summarized by the number of subjects (n) and the percent of subjects in each category.

12.1.2. Repeated or Unscheduled Assessments of Safety Parameters

If a subject has repeated assessments for a time point, any by-time point analyses will use the last result for that time point.

12.1.3. Definition of Visit Windows

Study day will be calculated in accordance with CDISC standards defined in the SDTM and ADaM implementation guides. The date of first dose is defined as study day 1, and the day before is study day -1. Here are the formulas appear below:

- If the assessment date is on or after the date of first dose of study drug:
  Study day = assessment date – date of first dose + 1
- If the assessment date is before the date of first dose of study drug:
  Study day = assessment date – date of first dose

12.1.4. Missing Date of Adverse Events

For AEs with partial start dates, nonmissing date elements, such as start year, will be used to determine if the AE is treatment-emergent or not. If a determination cannot be made using the nonmissing date elements as to when the AE occurred relative to study drug administration (eg, AE start day is missing and the year and month are the same as the year and month of the first dose of study drug), then the AE will be classified as treatment-emergent.

To facilitate categorization of AEs as treatment-emergent, the start date will be imputed. The stop date will be imputed if it is needed to determine whether an AE is treatment emergent.

12.1.5. Missing Date of Prior or Concomitant Medications, Therapies, and Procedures

For prior or concomitant medications, therapies, and procedures including rescue medications, incomplete (ie, partially missing) start date and stop dates will be imputed following the same rules that are used for adverse events, as detailed in the previous section.

12.1.6. Character Values of Clinical Laboratory Variables

It is possible that the laboratory will report a non-numeric value for a clinical lab result that is normally provided as a numeric value. This can happen if the test result is below the level of quantification. In this situation, analyses that require a numeric result will use an appropriately determined value. For example, if the reported result for ALT is “<5”, any relevant analyses will use a value of 0. However, the actual values as reported by the laboratory will be stored in the laboratory database will be presented in data listings.

Table 6: Coding of Special Character Values for Clinical Laboratory Results

Clinical Laboratory Test	Possible Results (in SI units)	Coded Value for Analysis
Chemistry: ALT	<5	0
Chemistry: AST	<5	0
Chemistry: Total Bilirubin	<2	0
Urinalysis: Glucose	≥55	Positive
	≤0	Negative
Urinalysis: pH	≥8.0	8.0

12.2. Analysis Software

Statistical analyses will be performed using of SAS® 9.4 on a validated, suitably qualified environment.

12.3. Schedule of Activities

Table 7: Schedule of Activities

Study Period →		**Screening Period**	**Clinic Period**			**Follow-up Period**
Study Procedure ↓	**Visit/Day →**	**Screening Visit Day -28 to Day -8**	**Check-in Day -1**	**Dosing Day 1**	**Discharge Day 2[a]**	**Follow-up Phone Call Day 14 (+3 days)**
Informed consent		X				
Eligibility criteria		X	X			
Medical history		X				
Demographics		X				
Body weight & height[b]		X	X		X	
Urine drug & alcohol screen		X	X			
Urine cotinine test		X				
Physical examination[c]		X	X		X	
Vital signs[d]		X		Pre: 0 (≤25m) Post: 0.5, 1, 2, 4, 6, and 8h (±10m)	24h post-Day 1 dose (±25m)	
Triplicate 12-lead ECG[e]		X	X	Pre 1h (±30m) Post: 2h (±30m)	X[f]	
Clinical chemistry, hematology, urinalysis		X	X	Pre: 0 (≤15m) Post: 6h (±30m)	24h post-Day 1 dose (±15m)	
ESR and CRP			X		24h post-Day 1 dose (±15m)	

Study Period →		**Screening Period**	**Clinic Period**			**Follow-up Period**
Study Procedure ↓	**Visit/Day →**	**Screening Visit Day -28 to Day -8**	**Check-in Day -1**	**Dosing Day 1**	**Discharge Day 2[a]**	**Follow-up Phone Call Day 14 (+3 days)**
SARS-CoV-2 testing		X	X			
HIV & hepatitis panel		X				
BBMD training		X				
BBMD dispensed		X	X			
Daily BBMD completion[g]		Day -8 through Day -2	X	X	X	
BBMD collection and review[h]			X		X	
Concomitant medications		X	X	X	X	X
AE monitoring		X	X	X	X	X
Subject confinement to clinic[i]			X	X	X	
Start of predose fast[j]				Pre: ≥6h		
Randomization				X		
Study drug administration[k]				X		
PK blood draws[l]				Pre: 0 (≤15m) Post: 0.5 (±2m), 1h (±10m), 2, 4, 6, and 8h (±5m)	24h post-Day 1 dose (±15m)	
Stool collection[m]				Post: 0h to Discharge		
Hemoccult testing			X	Post: 0h to Discharge		

Study Period →		Screening Period	Clinic Period			Follow-up Period
Study Procedure ↓	**Visit/Day →**	**Screening Visit Day -28 to Day -8**	**Check-in Day -1**	**Dosing Day 1**	**Discharge Day 2[a]**	**Follow-up Phone Call Day 14 (+3 days)**
Follow-up phone call[n]						X

AE=adverse event; BBMD=bladder and bowel movement diary; CRP=C-reactive protein; CRU=clinical research unit; ECG=electrocardiogram; ESR=erythrocyte sedimentation rate; HIV=human immunodeficiency virus; PK=pharmacokinetic; Post=postdose; Pre=predose; SARS-CoV-2=severe acute respiratory syndrome coronavirus 2

[a] Subjects will be discharged on Day 2 unless they have physical examination findings or laboratory abnormalities that are considered by the investigator to be clinically meaningful. In the event that a subject discontinues from the study, an early termination visit will be performed prior to discharge from the CRU. The early termination assessments will be the same as those conducted at Discharge (Day 2). Every effort should be made and documented to ensure that safety procedures scheduled for Discharge are performed at the early termination visit.

[b] Height will only be measured at the Screening Visit. At Check-in (Day -1) and Discharge (Day 2), subjects will be weighed in the morning upon awakening and before they ingest any water or food.

[c] Rectal examination should be performed at each physical examination; the rectal examination at all timepoints should include a digital rectal examination and inspection of the perianal area for redness or irritation.

[d] Vital signs will include oral temperature, blood pressure, and pulse rate. Side-lying blood pressure and pulse will be obtained at the 0.5 hour postdose timepoint on Day 1 (consistent with the position required following dosing). Blood pressure and pulse at the 2, 4, and 8 hour postdose timepoints should be preceded by at least 5 minutes of rest for the subject in a quiet setting without distractions (eg, television, cell phones). At all other timepoints (ie, predose and 1, 6, and 24 hours postdose), orthostatic blood pressure and pulse measurements will be obtained; subject must lie quietly for ≥5 minutes before supine/semisupine blood pressure and pulse measurements are taken, then assume standing position for 3 minutes before standing blood pressure and pulse measurements are taken. If a subject has orthostatic symptoms upon standing (eg, palpitation, dizziness), they will be assisted and asked to lie down without waiting the 3 minutes for vital sign assessment. Orthostatic measurements may be taken at other vital signs collection timepoints if clinically indicated, at the discretion of the investigator. When applicable, vital sign measurements will be obtained immediately before blood draws.

[e] ECGs should be obtained after the subject has been supine for at least 5 minutes. At each timepoint required, 3 individual ECG tracings should be obtained as closely as possible in succession, but no more than 2 minutes apart. The full set of triplicates should be completed in less than 4 minutes.

[f] Procedure will be done in the morning shortly after the subject awakens but before the subject eats breakfast.

[g] The BM portion of the BBMD will be completed by the subject on an event-driven basis. For subjects with Screening Visits earlier than Day -9, CRU staff will call subjects on Day -9 to remind them to begin recording their BMs in the BBMD the following day, answer any questions, and provide replacement BBMD pages via email, if needed. Bladder and urination information in the BBMD will be collected on Day 1 predose and Day 2, with a 24-hour recall period at each timepoint.

[h] CRU staff will review the subject's Screening Period BBMD at Check-in to confirm that subjects have at least 3 bowel movements during the 7 days prior to Check-in (Days -8 through -2) and no more than 3 bowel movements per day to be eligible for the study; CRU staff will collect the subject's Clinic Period BBMD at Discharge.

[i] Subjects will remain at the CRU from Check-in (Day -1) through Discharge (Day 2).

[j] Subjects will fast for at least 6 hours prior to dosing on Day 1 and for at least 1 hour following dosing.

[k] Study drug (IW-3300 or placebo low-volume enema) will be administered rectally after a fast of at least 6 hours. Subjects will be encouraged to empty their bowels in the morning prior to dosing, if possible. Subjects will be instructed to lie on their left side with their left leg extended and their right leg slightly bent. CRU staff will remove the cap from the applicator tip and gently insert the tip into the subject's rectum, then slowly squeeze the bottle to empty the contents into the rectum. After using the enema, subjects will lie on their left side for at least 30 minutes to allow the liquid to distribute throughout their intestines, followed by at least 30 additional minutes in the semisupine position. CRU staff will monitor subjects for leakage of the study drug from the rectum during the initial 30 minutes post administration. Subjects should avoid using the bathroom and hold in the enema for at least 1 hour.

[l] PK blood draws at 0.5 and 1 hour postdose should be collected while the subject maintains the position noted in the dosing instructions above.

[m] Beginning immediately after dosing on Day 1, all stool passed up to the time of Discharge on Day 2 will be collected and the wet weight will be measured, to calculate IW-3300 concentrations in stool.

[n] CRU staff will contact subjects by phone for safety follow-up (at the discretion of the investigator, subjects may be requested to return to the CRU for their follow-up contact).

12.4. Bowel Movement Log

Study Visit: ________________ **Date:** ____________ _____, _______
Month Day Year

Bowel Movements		
Did you have a Bowel Movement today? ☐ Yes ☐ No If yes, please complete the table below.		
Please complete one row below for ***each bowel movement*** (BM). If you have more than one BM in a day, please complete additional rows as needed. At the end of each day, please confirm that all BMs for that day have been entered and complete a row for each BM you may have forgotten to record.		
Time of Bowel Movement	**Describe the bowel movement form** (Please refer to the Bristol Stool Form Scale)	**Initials**
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	
_____ : _____ ☐ AM ☐ PM	☐ Type 1 ☐ Type 2 ☐ Type 3 ☐ Type 4 ☐ Type 5 ☐ Type 6 ☐ Type 7	

12.5. Bladder and Urination Log

Study Visit: _____ **Date:** _____ _____, _____
Day 1 or Day 2 Month Day Year

Bladder and Urination		
Time of Completion: _____ : _____ ☐ am ☐ pm Please answer the questions below upon awakening for the day, thinking back over the past 24 hours		
	In the past 24 hours…	
1	Did you have to urinate more frequently than normal?	☐ Yes ☐ No
2	How many times did you urinate?	☐ 0-2 ☐ 3-6 ☐ 7-10 ☐ more than 10
3	Did you feel the strong need to urinate with little or no warning?	☐ Yes ☐ No
4	Did you have to get up to urinate during the night more frequently than usual?	☐ Yes ☐ No
5	How many times did you have to get up at night to urinate?	☐ 0 ☐ 1 ☐ 2 ☐ 3 or more
6	Did you have pain or burning in your bladder or pelvic area?	☐ Yes ☐ No