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TITLE: Preclinical Advancement of Novel Mechanism-of-Action Therapeutics to Combat Type 2 Diabetes in US Veterans

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14. ABSTRACT

The purpose of this project is to complete rigorous nonclinical studies to support an IND application submission to the US FDA for our most promising Type 2 diabetes (T2D) drug candidate. These studies encompass advanced drug metabolism and pharmacokinetic (DMPK), synthesis/process development, engineered GMP-like batch API, and GLP safety pharmacology, genotoxicity, and toxicology studies in rodents and non-rodents, with the goal to fully identify potential liabilities that might prevent our current clinical candidate drugs from achieving successful clinical endpoints. Over the first year of this project (July, 2019-2020), we have successfully completed all milestones defined by our SOW including: i) process research and development, analytical method development, proof-of-concept synthesis/route optimization, and demonstration of 0.5 kg batch of our lead drug; ii) cross-species pharmacokinetic and drug metabolism characterization, dose-ranging efficacy study in obese mouse models of T2D, and dose-range finder and no observable adverse effect determination by oral dosing in rats. In addition, we have engaged with regulatory experts to set the stage for pre-IND meeting preparations. Meaningful and significant outcomes from all these activities have guided the continued advancement of our project milestones for years 2 and 3 to ultimately develop a paradigm-shifting class of drugs to improve the lives of millions of US Veterans and civilians battling T2D.

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1. INTRODUCTION:

T2D is one of the leading medical issues concerning the Department of Defense and the Veterans Health Administration with an alarming 2-fold higher incidence of T2D among Veterans compared to the general US adult population (Liu 2017). Obesity is a major predictor for T2D, which generally progresses to numerous chronic health comorbidities including cardiovascular disease, stroke, blindness, kidney disease, limb loss, and early death. Over 40% of US Veterans are obese or overweight and either suffer from T2D or battle prediabetes (Administration 2017; Liu 2017; Das 2005; Moin 2016). Even modest (e.g., 5-7%) weight loss improves glycemic control and prevents or delays the progression of T2D/prediabetes in obese individuals (Goldberg 2017). Unfortunately, first-line interventions such as diet and exercise that are adopted to promote body weight loss and provide glucose control rarely provide effective and sustained disease management, with nearly 85% of adults unable to sustain a 10% weight loss over a 2-year period (Fildes 2015; Center, 2016). Consequential adoption of pharmacological treatments to control elevated glucose levels by obese patients with T2D or prediabetes associates with lifelong administration, significant adverse effects, high rates of non-adherence, extensive healthcare costs, and overall limited success altering disease progression (Barja-Fernandez 2014; Kalkan 2017). Our innovative drug development project targets this critical unmet need by centering on novel mechanism-of-action drugs that can accelerate and sustain weight loss, restore glucose homeostasis, and reverse T2D/prediabetes that is driven by obesity. This project's overarching purpose is to successfully advance our *first-in-class* drug candidates toward clinical trials and, ultimately, as front-line therapeutics to treat T2D. The scope of this project broadly covers manufacturing of the candidate drug substances under regulated pharmaceutical standards to support and complete the appropriate DMPK and advanced IND-enabling studies under GLP conditions necessary for IND filing with the US FDA. These studies are being performed in partnership with Cambrex and Covance Laboratories, two world-leading and well-established organizations known for advancing candidates through preclinical/clinical development and US market approval. At the conclusion of this 3-yr Expansion Award, our high-reward T2D drug candidate will seamlessly advance to current Good Manufacturing Practice (cGMP) synthesis and drug product development,
completion of GLP studies in non-rodent species, followed by IND application filing for Phase I human trials.

2. KEYWORDS:

API	active pharmaceutical ingredient
cGMP	current Good Manufacturing practice
CMC	chemistry, manufacturing, and controls
DMPK	drug metabolism and pharmacokinetics
FDA	USA Food and Drug Administration
GLP	good laboratory practice
IND	investigational new drug
LC-MS/MS	liquid chromatography tandem mass spectrometry
NNMT	nicotinamide N-methyltransferase
PK	pharmacokinetics
qNMR	quantitative nuclear magnetic resonance
T2D	Type 2 diabetes
XRPD	X-ray powder diffraction

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals as stated in the SOW are the Aims 1-3 of this project highlighted in blue font in the below table. All-important activities for this reporting period (07/2019 – 07/2020) under the three main goals are highlighted in green font. The corresponding target dates (months) and actual completion dates where applicable (month/year) are also included for each of the highlighted activities (green font) in the table. Percentage of milestone completed are noted in **bold** font under each Goal/Aim.

Goals / Activities		Target dates (mo)	Completion dates (mo/yr)
Project setup			
0.1	Complete Ridgeline team hires (initiated upon Notice of Award)	0	10-11/2019
0.2	Finalize project contracts and CRO personnel assignments	0-2	08-11/2019
0.3	Submit IACUC and USAMRMC ACURO documentation *ongoing (on a study-by-study basis)	0-3*	11/2019-02/2020*
Milestones			
	IACUC and ACURO approvals *ongoing (on a study-by-study basis)	5*	**100% of necessary approvals completed**
Aim 1	Develop and optimize GMP-like scale-up process parameters and analytical methods for drug candidate RLT-72484 (timelines revised to reflect contract, timeline, deliverables for drug candidate RLT-72484 as negotiated in signed contract with Cambrex)		
1.1	Project management and oversight of Cambrex studies *ongoing	0-32	10/2019-*
1.2	Receipt of raw materials	3	10/2019
1.3	Process development and optimization	3-5	12/2019
1.4	Analytical method development and validation *planned prelim validation activities completed, additional validation activities will occur as needed	3-5*	01/2020*
1.5	Synthesis and analysis of 500 g non-GLP demonstration batch	5-6	02/2020
1.6	Salt screening *initial salt form evaluated and deemed acceptable	10-12	02/2020*
1.7	Analytical method qualification *planned prelim qualification activities completed, additional qualification activities will occur as needed	5-8*	02/2020*
1.8	Stability studies (short and long term) *planned forced degradation, solid state, solubility, and short-term stability studies completed; long-term studies are continuing	6-30+	07/2020*

1.9	Synthesis and analysis of 2 kg scale-up GMP-like batch	20-22	
1.10	Release of 2 kg GMP-like batch	21-22	
1.11	Report completion *development report for activities highlighted above is ongoing	24*	08/2020*
Milestones			
	Delivery of 2 kg batch	24	**30% completed**
	Provision of CMC report *development report ongoing and will continue into CMC report	25*	**30% completed**
Aim 2	Complete non-GLP DMPK characterization and optimization of NNMT inhibitor drug candidate RLT-72484		
2.1	Project management and oversight of Covance DMPK studies	0-12	08-2020
2.2	Metabolic stability and metabolite ID profiling	3-6	07/2020
2.3	Bioanalytical method development	6-9	07/2020
2.4	DMPK/PD optimization of RLT-72484 *activities ongoing and on-track	6-15*	04/2020-*
2.4a	Non-GLP acute IV/oral PK (rodent, non-rodent)	6-9	06/2020
2.4b	Non-GLP mouse PK/PD (subcutaneous in DIO animals)	12-15	
2.5	*In vitro* and *in vivo* development of alternative RLT-72484 analogues *activities ongoing and on-track	0-21*	10/2019-*
2.5a	*In vitro* off-target safety pharmacology screen	0-15	
2.5b	Rat IV/oral PK studies *activities ongoing and on-track	0-18	03/2020-*
2.5c	*In vivo* non-GLP dose range finder studies	12-18	
2.5d	*In vivo* efficacy studies in DIO mice *activities ongoing and on-track	12-18	08/2019*
2.5e	Non-GLP 7-day repeat dosing toxicity/TK study in rats	15-21	
2.6	FDA pre-IND study consultation	21-24	
Milestone			
	Positive FDA review of CMC data and GLP study design *discussions with CMC and regulatory consultants ongoing	24	**20% completed***
Aim 3	Complete IND-directed nonclinical toxicology/safety studies of a GMP-like batch of RLT-72484 under GLP conditions		
3.1	Project management and oversight of Covance safety/toxicity studies *activities ongoing	6-36	01/2020-*
3.2	Regulated bioanalytical method validation (rodent, non-rodent plasma)	18-24	
3.3	Formulation verification and analysis	6-12	02/2020

3.4	GLP study to assess modulation of hERG ion channels	24-27	
3.5	Non-GLP toxicity; Phase 1 & 2 (rodent)	9-12	08/2020
3.6	Non-GLP toxicity; Phase 1 & 2 (non-rodent) *Phase 1 initiated	18-24*	08/2020*
3.7	Genotoxicity studies (GLP)	24-30	
3.8	GLP 28-day oral toxicity and TK study; Phase 3 (rodent)	25-34	
3.9	GLP cardiovascular safety study (non-rodent)	30-33	
3.10	GLP respiratory safety study (rodent)	28-32	
3.11	GLP CNS safety study (rodent)	30-33	
3.12	Report completion and provision of report	30-36	
3.13	Compilation of study results for FDA IND review	34-40	
3.14	Pre-IND submission and review meeting with FDA	42-48	
Milestones			
	Favorable therapeutic window and safety profile established (rodent) *optimization activities ongoing	34*	**30% completed***
	IND package submitted to FDA	48	

What was accomplished under these goals?

Reporting period: 07/2019 – 07/2020

Goal/Aim 1: Develop and optimize GMP-like scale-up process parameters and analytical methods for drug candidate RLT-72484 (Performance site: Cambrex)

Major Activities:

1. ***Process development and optimization.*** The main objective was to complete process research and development activities to enable manufacturing of 100 g of proof-of-concept batch, 500 g demonstration batch, and eventually a 2 kg GMP-like batch of RLT-72484 via an optimized, robust, scalable, and reproducible manufacturing scheme to support GLP safety/toxicity studies in rodent and non-rodent species.

   Process development summary.
   - Two-step synthetic scheme was evaluated followed by counter-ion exchange and recrystallization.
   - Two approaches (nitro reduction followed by methylation [path A] and vice-versa [path B]) were tested for the intermediate production from the starting material; initial efforts were dedicated to testing path B, as path A (i.e., nitro reduction followed by methylation) was predicted to have likely operational challenges and issues associated with selectivity in the subsequent methylation step.
   - In path B, the first step of methylation was reproducible, but the reduction of the quinolinium intermediate posed scalable issues due to limitations of solubility profiles of the compounds. Hence, development efforts were shifted toward testing path A.

- In path A synthetic scheme, eight variations of reagents for the nitro reduction and five variations of methylating agents were tested to evaluate efficient and scalable synthesis of the intermediate and final products, respectively. For the final anion exchange, two resins were tested.

Process optimization summary.

- Based on the results of testing completed in process research and development activities, path A was chosen for scalable synthesis of 500 g batch of RLT-72484 using sodium dithionite as the reducing agent (step 1), methyl tosylate as the methylating agent (step 2), followed by ion exchange using IPA/water and concentrated hydrochloric acid and re-slurry with IPA for making the final anhydrous product.
- The above process was established to be reproducible and scalable with 30% yield and acceptable purity profile.
- Further optimization efforts were focused on testing solvent swap in step 1 to increase purity and/or yield of the intermediate, exploring reslurry or recrystallization of the tosylate product with optimal solvents to improve overall purity of the RLT-72484 chloride form, testing lower volumes of IPA for ion exchange step without impact on product, and exploring alternative solvent for the final reslurry for better purity upgrade. Since two forms of RLT-72484 were identified in the process chemistry work, the anhydrous and a hydrate form, final reslurry further enables the conversion of the initial hydrate to the final anhydrous form.

Key outcomes.

- 100 g proof-of-concept batch of RLT-72484 was generated using a non-optimized synthetic route to provide to Covance for early research and development activities.
- 500 g demonstration batch of RLT-72484 was generated using a fully scalable and an optimized process, with 40% yield and 92.2% purity pre-ion exchange product. Final product after ion exchange and reslurry had 99.3% purity and 98.2% potency.

2. ***Analytical method development.*** The primary objective was to develop sound analytical methods (and phase appropriate qualification and validation activities) to support testing of starting materials, intermediates as in process research chemistry and development, and the manufacture of the target drug substance RLT-72484.

Analytical activities summary.

- Analytical methods were developed for verifying purity of the starting material and intermediate, identification of the final RLT-72484 product, as well as impurities in the final product by an ultra performance liquid chromatographic (UPLC) assay; protocol qualification for the latter two methods have been initiated.
- Analytical method was developed for detection of chloride content in the final RLT-72484 product by a high performance liquid chromatography (HPLC)-charged aerosol detector (CAD) system and protocol qualification has been initiated.
- Analytical method (Karl Fischer (KF) method) was developed for detection of water content in the final RLT-72484 product and method qualification to verify anhydrous form (to be performed under USP <921>) has been initiated.
- Analytical method development activity for the detection of all possible residual solvents by headspace gas chromatography (GC-HS) has been completed.
- All other analytical activities performed during this reporting period are summarized below.

Table 1. Analytical activities summary

Analytical methods:

Test	Method	Test Method
Appearance	Visual	RM0090
Identity by High Res MS	HPLC-MS	RM0781
Identity by ^{1}H NMR	NMR	Research
Chromatogsaphic Purity/Impurities	UPLC	RM1451
Chloride Content	HPLC-CAD	RM1455
Water Content	KF	RM1453
Residual Solvents	GC-FID	RM1454
Residue on Ignition	ROI	USP <281>
Differential Scanning Calorimetry	DSC	RM0806
Polymorphic Form	XRPD	RM0858

NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; XRPD, X-ray powder diffraction.

Key outcomes.

- Analytic methods for impurities and counter ion content were successfully applied to characterize the initial 100 g proof-of-concept batch of RLT-72484, which was defined as a monochloride salt (17.8% w/w) with qNMR potency of 79.81% w/w and 99.7% purity.
- Qualified-equivalent analytic methods were successfully applied to characterize the 500 g demonstration batch of RLT-72484. Demonstration batch of RLT-72484 appeared as red-orange solid, was identified to be consistent with its structure by 1H NMR, and defined by 80.9% potency (by compositional mass balance), 0.17% w/w water content, 99.3% purity with intermediate present at 0.45% and an unknown impurity at 0.26%, 18.3% w/w chloride content, 0.02% w/w residue on ignition, melting point onset at 252.3°C (heat flow -315.14 J/g, DSC method), and isopropanol detected residual solvent at 0.0305%.

3. ***Forced degradation, solid state characterization, and stability assessments.*** Forced degradation, solid state characterization, and stability assessment activities were performed for the demonstration batch of RLT-72484. The main objective of the forced degradation study was to assess the stability indicating nature of the impurity detection analytical method by UPLC. Solid state activities were performed to determine the relationship between the anhydrous and hydrate forms of RLT-72484 and a critical water activity for conversion from the anhydrous to the hydrate form. Stability studies utilizing various relative humidity and temperature conditions were conducted to determine the stability of both the hydrate and anhydrous forms of RLT-72484.

Forced degradation summary.

- Forced degradation test conditions included thermolytic, photolytic, oxidative, and hydrolytic stress conditions, with a target of 5-20% total degradants calculated for each condition; duration of study was 14 days.
- Data generated were used to assess the stability indicating nature of the purity analytical method developed for RLT-72484 by evaluating the purity of the photodiode array peak in terms of the spectral homogeneity using Empower software.
- Passing threshold for peak purity was set by a lower purity angle than that of the purity threshold.
- A ratio was calculated for the final degradation samples using either the solid or solution control for calculation of a crude potency/mass balance for the stressed samples. The RLT-72484 peak area of each sample was compared to the peak area of the appropriate control sample and expressed as a percentage.
- Experimental details of the stress conditions tested are summarized below.

Table 2. Forced degradation conditions

Stress Condition	Duration	Conditions	Control	Comments
Thermolytic	1, 4, 7 and 14 days	80 °C	Solid Control	Solid State
Hydrolytic (humidity)	1, 4, 7 and 14 days	60 °C/75% RH	Solid Control	Solid State
Photolytic	2X ICH 3X ICH	Photo chamber, ambient temperature	Dark Control in photo chamber	Solid State
Hydrolytic, acid	1, 4, 7 and 14 days	0.1N HCl, 50 °C	Solid Control	Solution
Hydrolytic, base	1 day	0.1N NaOH, 50 °C	Solid Control	Solution
Hydrolytic, base	2, 3, 4.5 and 5.5 hours	0.1N NaOH, ambient temperature	Solid Control	Solution
Oxidative	14 days	0.3% H2O2, 50 °C	Solid Control	Solution

Major findings.

- At 14 days, there was no significant degradation of RLT-72484 in the unstressed solid-state and aqueous solution (in water) control forms held at ambient conditions protected from light.
- At 14 days, there was no significant degradation (less than 1%) of RLT-72484 at the thermolytic, hydrolytic (humidity), photolytic, and hydrolytic acid conditions.
- Under oxidative condition (noted in **Table 2**), a small level of 0.32% degradation was observed.
- Under hydrolytic base condition (noted in **Table 2**), degradation that was beyond the target 20% was observed within one day; the heated basic stress condition was aborted. When held at ambient temperature, 15% degradation was observed by 5.5 h, suggesting significant degradation under strongly basic conditions.
- Peak purity passed under all conditions at or below target degradation range, suggesting spectral homogeneity of the main band. These findings support the purity analytical method as suitable for evaluation of RLT-72484 drug substance.

Solid state, solubility, and stability studies.

- Since two forms of RLT-72484 were identified in process chemistry and development (hydrate and anhydrous forms), full solid state characterization was performed on each of the forms, including XRPD, polarized light microscopy (PLM), and dynamic vapor sorption/desorption (DVS).
- The water activity of each form was studied to determine the critical water activity required for conversion of RLT-72484 from the anhydrous to the hydrate form.
- The equilibrium solubility of anhydrous and hydrate forms was determined in various buffers across a pH range of 1.6 to 7.5, water, and simulated gastric fluids.
- Initial stability studies were performed using both forms of RLT-72484 under an accelerated study design. Vials containing solids of either the anhydrous or hydrate form were placed uncapped in either 25 °C/60% relative humidity (RH) or 40 °C/75% RH conditions. Visual observations and XRPD were performed at 1, 2, and 4 weeks.
- To further study the nature of RLT-72484 hydrate and anhydrous under various storage and shipping conditions, an extended stability study was conducted with various temperature, relative humidity and desiccant conditions. Chemical stability, visual observations, and XRPD were collected after 12 weeks

of storage in -20°C with desiccant, 48 h and 2 weeks at ambient temperature, and 48 h at 20% and 95% relative humidity at ambient temperature (only anhydrous form tested).

Major findings.

- RLT-72484 anhydrous consists of small blades/rods around 20 µm as identified by PLM. The DVS profile showed a weight gain and loss of 19% with significant hysteresis upon desorption. XRPD of the post-DVS solids showed no form change, with sharp peaks. The water activity was measured to be 0.125.
- RLT-72484 hydrate consists of larger blades around 75 µm by PLM. The DVS profile shows a weight gain and loss of 18% with significant hysteresis upon desorption. XRPD of the post-DVS solids shows conversion to the anhydrous form. The water activity was measured to be 0.062.
- Conversion of the anhydrous to hydrate form was observed at water activity between 0 and 0.26.
- RLT-72484 hydrate has solubility >100 mg/mL in solvents tested, while RLT-72484 anhydrous displayed slightly greater solubility (>150 mg/mL) in all solvents. No pH changes were noted for both forms in all buffered fluids, but for in water where a pH drop was noted by 24 h.
- XRPD results at each time point tested in the accelerated stability conditions indicated that all samples had converted to RLT-72484 hydrate plus peaks, regardless of starting material. Thermogravimetric analysis conducted at 2 weeks showed a range of weight losses between samples suggesting that RLT-72484 hydrate plus peaks may be a channel hydrate. A single crystal analysis will be performed in the future for confirming the crystal structure of the hydrate forms.
- In all stability conditions tested (48 h and 2 weeks at ambient temperature and 12 weeks at -20°C with desiccant), additional peaks were observed in XRPD patterns of RLT-72484 hydrate, suggesting an additional hydrated form or a channel hydrate exists.
- RLT-72484 anhydrous began to covert to the hydrate form after 48 hours at ambient temperature and 20% RH, while fully converting to hydrate plus peaks at 48 hours at 95% RH and after 2 weeks at ambient temperature.
- RLT-72484 anhydrous remained stable when stored with desiccant at -20°C suggesting it as a stable and a recommended form for development.

Conclusions. Seventy percent of the activities under goal 1 have been successfully completed, including full process chemistry and research activities, analytical development and qualification, and demonstration batch production and characterization of RLT-72484. A development report is in progress that will account for 30 percent of milestone completion and will directly support the final CMC report completion at the end of this project.

Goal/Aim 2: Complete non-GLP DMPK characterization and optimization of NNMT inhibitor drug candidate RLT-72484 (Performance sites: Covance/Wuxi AppTec)

Major Activities:

1. ***Bioanalytical method development.*** The purpose of this activity was to develop a robust and sensitive bioanalytical method by a liquid chromatography tandem mass spectrometry (LC-MS/MS) for the detection and quantitation of RLT-72484 in biofluids (including rat and dog plasma) and application in non-GLP rodent and non-rodent pharmacokinetic (PK) studies.

Method development summary.

- An initial method was developed by our academic collaborator research group (Texas Southern University), chromatographic separation was achieved using an ACE® Excel™ C18 column (2 µm, 50×2.1 mm) and analysis was performed using an API 4000 QTRAP hybrid triple quadruple mass

spectrometer and multiple reaction monitoring (MRM) in positive mode at m/z transitions of 159.100 → 90.00 and 162.200 → 117.200 for RLT-72484 and the internal standard (IS), respectively.

- The above method was validated according to the 2018 United States Food and Drug Administration "Guidance for Industry: Bioanalytical Method Validation" (USFDA, Guidance for Industry: Bioanalytical Method Validation, 2018).
- The above developed and validated bioanalytical method was adapted and further modified by Covance for application in non-GLP rodent (rat) and non-rodent (dog) oral dosing PK studies. Chromatographic separation was achieved using Waters Atlantis Hilic column (5 μm, 50×2.1 mm). Analysis was performed using a Sciex API-5000 LC-MS/MS instrumentation that was used in the positive ion mode. Calibration curve ranged from 10-10,000 ng/mL and quality control (QC) samples were tested at 30, 400, 4000, and 8000 ng/mL. m/z transitions for RLT-72484 and the internal standard (IS) were at 159.1 → 116.1 and 162.1 → 116.1, respectively.

Key outcomes.

- The standard curves of RLT-72484 plasma samples were linear in the concentration range of 10 – 2500 ng/mL, as determined by the ACE® Excel™ C18 column and API 4000 QTRAP LC-MS/MS instrumentation. The intra-day and inter-day precisions and accuracies at four concentration levels in rat plasma and the accuracy and precision of RLT-72484 quantification in samples diluted up to 20-fold using blank plasma were found to be within the 15% US FDA acceptance range. The extraction recoveries and matrix effects at three concentration levels of rat plasma samples ranged from 99.5% - 110.6% and -6.1% - 14.1%, respectively. 5-AMQ was stable in rat plasma samples subjected to standard storage, preparation, and handling conditions, with less than 15% variation noted at two concentration levels. The validated, sensitive, and reproducible LC-MS/MS method for RLT-72484 in rat plasma was effectively applied to a pharmacokinetic study in rats with IV and oral administration of RLT-72484 (see Appendix for full manuscript draft).
- The method developed by Covance was qualified and met the following acceptance criteria: specificity and carryover, < lower limit of quantitation (LLOQ); concentration range, 10-10,000 ng/mL; calibrated precision and accuracy in biofluids, ≥75% of STDS within ±25% (±30% LLOQ); QC sample precision and accuracy, ≥67% of QCs within ±25%. This method was successfully applied to compete acute oral and intravenous RLT-72484 dosing pharmacokinetic studies in Wistar Han rats and Beagle dogs.

2. ***Non-GLP acute IV/oral PK (rodent, non-rodent).*** The purpose of this study was to determine the pharmacokinetics (PK) of RLT-72484 after intravenous (IV) and oral dose to rats and dogs and establish dose proportionality via a range of oral dose testing in both species.

PK summary.

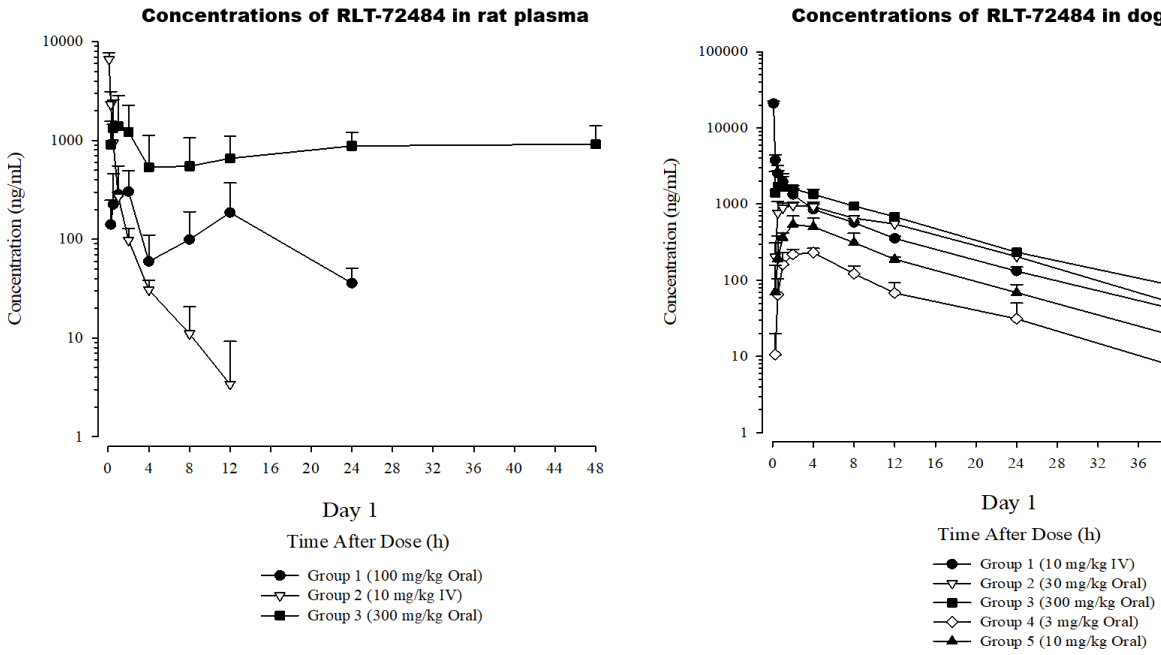
- Male, Wistar Han rats received a single IV (10 mg/kg) or oral (100 or 300 mg/kg) administration of RLT-72484.
- Male, Beagle dogs received a single IV (10 mg/kg) or oral (3, 10, 30, or 300 mg/kg) administration of RLT-72484.
- Blood samples were collected pre-dose and at approximately 0.083 (IV only), 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 hours post-dose in the oral and IV groups, and the processed plasma samples were evaluated using the bioanalytical LC-MS/MS method described above.
- Noncompartmental analysis using Phoenix WinNonlin (Certara USA; version maintained in the data) was applied to the individual plasma RLT-72484 concentration data for male rats and dogs to establish a range of PK parameters including oral bioavailability.

Results.

- RLT-72484 demonstrated substantial systemic exposures in rats and dogs when administered both via IV and oral routes (**Figure. 1**).

- After IV administration, RLT-72484 concentrations declined generally in a bi-phasic (rat) and mono-exponential (dog) manner, with a mean $t_{1/2}$ value of 2.78 (rat) and 9.03 (dog) hours. Clearance (CL) values in rats ranged from 3210 to 5280 mL/hr/kg that was consistent with liver blood flow in a 0.25 kg rat (3312 mL/hr/kg) suggesting substantial hepatic clearance. CL in dogs ranged from 439 to 524 mL/hr/kg and were generally less than liver blood flow in a 10 kg dog (1854 mL/hr/kg), indicating that RLT-72484 is not highly extracted by the liver after IV administration. V_{SS} values ranged from 2400 to 4000 mL/kg (rat) and 3350 to 4110 mL/kg, exceeding the total body water of a 0.25 kg rat (668 ml/kg) and 10 kg dog (603.6 mL/kg), suggesting high distribution to the tissues.
- After oral dosing of rats, systemic exposures as assessed by RLT-72484 mean C_{max} and AUC_{0-48} values, increased with the increase in RLT-72484 oral gavage dose level from 100 to 300 mg/kg. The increases in mean Cmax values were generally dose proportional and the increases in mean AUC0-48 values were greater than dose proportional. Oral bioavailability was estimated to be 13.4% and 49.9% at 100 and 300 mg/kg, respectively.
- After oral dosing of dogs, systemic exposures as assessed by RLT-72484 mean C_{max} and AUC_{0-48} values, generally increased with the increase in RLT-72484 dose level from 3 to 300 mg/kg. The increases in RLT-72484 mean Cmax and AUC_{0-48} values were dose proportional between 3 and 10 mg/kg. Oral bioavailability was estimated to be 46.2% and 33.7% at 3 and 10 mg/kg, respectively. Dose proportionality and oral bioavailability assessments at 30 and 300 mg/kg were unreliable due the observed instances of vomiting.

Figure 1. Plasma concentration-time profiles of RLT-72484 in rodent and non-rodent species



3. ***Metabolic stability and metabolite ID profiling.*** The objective of this study was to determine metabolic stability and identify metabolite profiles of RLT-72484 *in vitro* using co-cultured plates comprised of primary cryopreserved hepatocytes from rat, dog, and human cultured with non-parenchymal stromal cells (HμREL® System).

Study summary.

- The HμREL hepatic co-culture model that is comprised of primary cryopreserved hepatocytes from rat (a pool of male Wistar Han rats), dog (a Beagle dog), and human (mixed-sex pool) cultured together

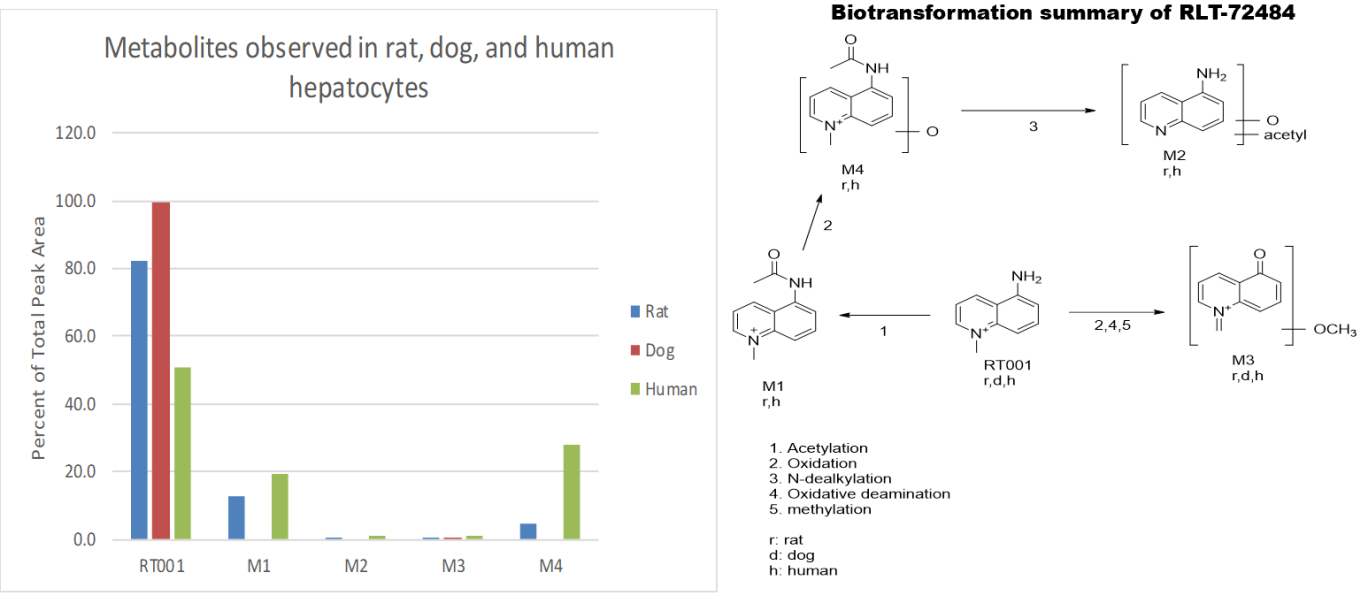
with non-parenchymal stromal cells, and has demonstrated cellular competence to persist for weeks in culture was employed in the metabolic stability and metabolite ID studies for RLT-72484.

- *In vitro* metabolism of RLT-72484 was evaluated by incubating 1 and 10 µM concentrations of RLT-72484 in the HµREL hepatic co-culture system; 0, 3, 6, 12, 24, 48, and 72 hours of incubation samples were terminated by the addition of 200 µL ACN containing IS and causing cell lysis. Lysed cell samples were centrifuged at 2200x *g* for 10 minutes. Supernatants were analyzed LC-MS/MS.
- Based on the estimated half-life of intrinsic clearance for RLT-72484 in the hepatic co-culture systems from the three species studied, metabolites of RLT-72484 were identified and characterized at 72 hours for rat and dog and 48 hours for human by LC-MS/MS.

Results.

- Results indicated time-dependent, concentration-dependent, and species-dependent rates of metabolism for RLT-72484. Time-dependent disappearance of RLT-72484 was observed in rat and human hepatocyte incubations, but little to no metabolism was observed in dog hepatocyte incubations. The mean percent remaining concentrations (percent of 0-hour time point) from 1 µM and 10 µM incubations were determined to be 49.3, 82.6, and 4.08%, and 65.2, 87.6, and 8.06%, respectively, after 72 hours of incubation in rat, dog, and human HµREL hepatocytes, respectively.
- The half-lives ($t_{1/2}$) from 1 and 10 µM RLT-72484 incubations were 75.1 and 124 hours for rat, and 15.7 and 20.3 hours for human, respectively. The rat half-lives were extrapolated to >72 hours (the longest time point tested) in rats, while in the dog incubations predicted half-life of >400 hours; these values are less accurate and reliable for predictions of half-life or other clearance parameters and indicate high stability of RLT-72484 in rat and dog hepatocytes.
- The intrinsic clearance was calculated as 2.61 and 1.58 mL/min/kg for rat, and as 5.14 and 3.96 for human from 1 and 10 µM RLT-72484 incubations, respectively, using known physiological parameters for each species.
- All identified metabolites of RLT-72484 were detected in rat and human hepatocyte incubation with no unique metabolites in any one species. M3 (ox-deamino-dehydro-methyl-RLT-72484) was the only metabolite observed in the dog hepatocytes; data are summarized in **Figure 2**.

Figure 2. Biotransformation summary of RLT-72484.



4. ***In vivo efficacy study in DIO mice.*** The objective of this study was to fully validate dose-ranging effects of RLT-72484 against diabetic markers/endpoints, including insulin sensitivity, hyperlipidemia, and liver pathologies in industry-standard, translationally-relevant, diet-induced obese (DIO) mice.

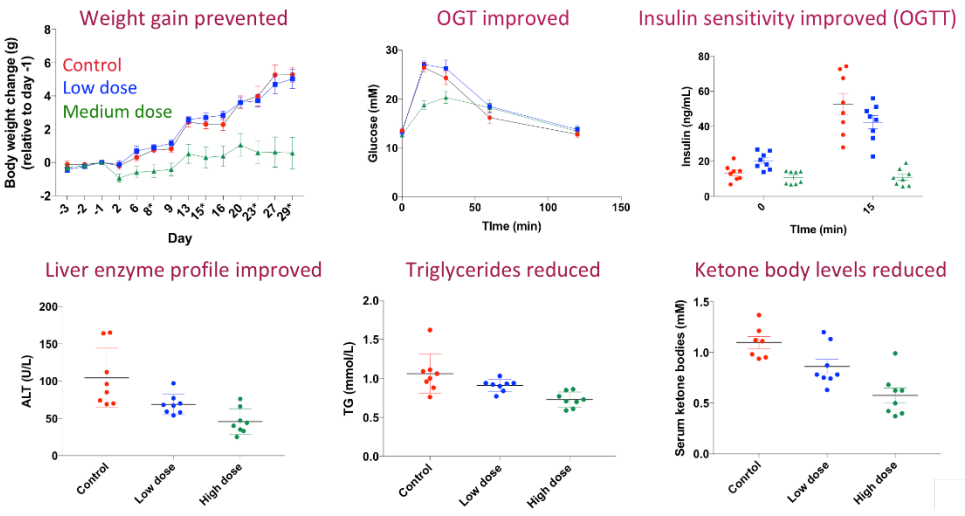
Study summary.

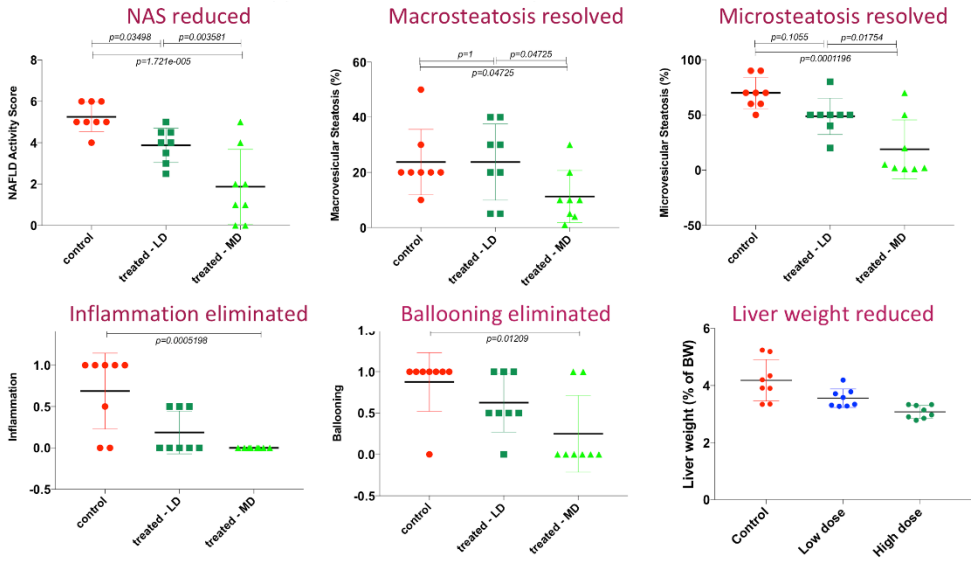
- Mice were treated once-daily subcutaneously with RLT-72484 (low dose: 7.5 mg/kg; mid dose: 25 mg/kg) for 4 weeks; body weight and food intake were recorded twice weekly, body composition (including adiposity and lean mass measures by Echo MRI scan) and non-fasted blood glucose and insulin levels were measured weekly, oral glucose tolerance test (glucose and insulin measures) was completed on day 21. Study was terminated on day 28 with serum, liver, and adipose tissues collected and processed further for evaluating serum markers (liver and cardiac enzymes, lipid profile, ketone bodies), and liver and EWAT histology.

Results.

- RLT-72484 dose-dependently and robustly limited body weight gain over 28 days with a 10% overall reduction in body weight compared to controls. Over 70% of the body weight loss was accounted for by whole body fat loss (i.e., limited fat mass gain on high-fat diet). Overall, liver and epididymal fat pad weights had reduced with treatment compared to control DIO mice.
- RLT-72484 treatment blocked the development of hyperinsulinemia, which was otherwise observed in the DIO control mice between days 21 and 28, indicating prevention of prediabetes/insulin insensitivity development. Consistently, treatment improved oral glucose tolerance and overall insulin sensitivity (**Figure 3**).
- Consistent with improved insulin sensitivity, serum ketone bodies, TG levels, and levels of the liver enzymes (AST and ALT) dose-dependently reduced in RLT-72484 treated mice compared to control mice. Reductions in the whole liver weights and serum TG levels also reflected in reduced liver TG levels, and correspondingly reduced hepatic steatosis, inflammation, and ballooning (**Figure 3**) in RLT-72484 treated DIO mice. The overall NAFLD activity score (NAS) indicated a dose-dependent reduction in the RLT-72484 treatment group, relative to controls; data summarized in **Figure 3**.
- Taken together, the NNMT inhibitor RLT-72484 convincingly demonstrated significant pharmacological efficacy in reducing body weight and adiposity and improving diabetic and NAFLD/NASH-related liver pathologies in DIO mice.

Figure 3. Effects of RLT-72484 in DIO mice.





Conclusions. Forty percent of the activities under goal 2 have been successfully completed, including advanced dose-ranging efficacy measures for RLT-72484 in a DIO model, in vitro metabolic stability and metabolite identification in safety-relevant species, and dose linearity and cross-species exposure assessments for RLT-72484 in rats and dogs via oral dosing. In preparation for the main milestone listed under this goal, i.e., an official FDA review of the CMC and GLP program for RLT-72484 clinical candidate, we have initiated discussions with our CMC and regulatory expert consultants. The data generated are currently being reviewed and strategically planned for compilation for review by FDA in the upcoming project period.

Goal/Aim 3: Complete IND-directed nonclinical toxicology/safety studies of a GMP-like batch of RLT-72484 under GLP conditions (Performance sites: Covance)

Major Activities:

1. ***Formulation verification and analysis.*** The primary objective of this activity was to perform dose formulation analysis using a qualified analytical method to verify concentration and test for homogeneity in the oral dosing formulation of RLT-72484.

   Formulation verification summary.
   - Formulation verification was performed to test for homogeneity of the RLT-72484 dosing formulations prepared for dosing rats in the Covance study 8416067 – "*A 2-Phase Oral Gavage Single Dose Range-Finding (Phase I) and 7-Day Repeat Dose (Phase II) Toxicity and Toxicokinetic Study in Rats*"; formulations prepared on day 1 of Phases I and II were tested for homogeneity.
   - Four samples (1 mL each) were drawn from 1, 10, 50, and 100 mg/mL concentrations of RLT-72484 from the top, middle, and bottom strata, respectively. Duplicate samples from each stratum were analyzed for homogeneity/test article content and the overall test article concentration of the dose preparations were verified.

   Results.
   - The concentration and homogeneity of dosing solutions of RLT-72484 made up as clear solutions in reverse osmosis water were verified and confirmed to range from 93.1% to 106.4% of target concentrations with a less than 1% RSD across samples.

2. ***Non-GLP toxicity; Phase 1 & 2 (rodent).*** The purpose of this study is to evaluate acute toxicity and establish the maximum tolerated dose (MTD) of RLT-72484 administered once (Phase I) and evaluate

toxicity and toxicokinetics, and establish the no observable adverse effect limit (NOAEL) after dosing for 7 consecutive days (Phase II) via oral gavage to rats.

Non-GLP rodent toxicity study summary.

- In Phase I, individual groups of male and female Wistar Han rats were administered 10, 100, 500, and 1000 mg/kg of RLT-72484 once on day via oral gavage. Cage side observations were performed during dosing, at 1, 4, and 8 h post-dose, and on day 3 postdosing (included detailed observations on day 3). Body weights on the day prior to dosing and on day 3 and food consumption from day 1 to 3 were recorded. All surviving animals were necropsied on day 4 after an overnight fast, and the external features of the carcass; external body orifices; abdominal, thoracic, and cranial cavities; organs; and tissues were examined.
- In Phase II, individual groups of male and female Wistar Han rats were administered vehicle (reverse osmosis water), 100, or 300 mg/kg of RLT-72484 once daily for seven consecutive days via oral gavage. Separate sub-groups of rats were administered the same doses of RLT-72484 for toxicokinetic assessments. General daily observations were performed twice daily, body weights and cage side observations were recorded daily during dosing phase. Detailed observations were collected on days 1, 3, and 7. Food consumptions were recorded on days 1 to 3 and 3 to 7. In the toxicity groups, blood was collected on day 8 prior to sacrifice for hematology and serum chemistry panel assessments. All surviving animals were necropsied on day 8 after blood collection, and the external features of the carcass; external body orifices; abdominal, thoracic, and cranial cavities; organs; and tissues were examined; lesioned tissues were further processed and macroscopically evaluated. In the toxicokinetic group, blood was collected from rats at predose (day 1 only), 0.5, 1, 4, 8, 12, and 24 h postdose on days 1 and 7, and RLT-72484 concentrations were evaluated via LC-MS/MS.

Results.

- In the single-dose phase (Phase I), administration of 1000 mg/kg/dose of RLT-72484 resulted in mortality of two females. Compared with animals administered the low dose of RLT-72484 (10 mg/kg/dose), decreased body weight gains were noted in males administered 100 mg/kg/dose and females administered ≥100 mg/kg/dose. These decreases generally correlated with decreased food consumption. Taken together, dose levels ≥500 mg/kg/dose were considered not suitable for longer term dosing and doses of 100 and the estimated maximum tolerated dose of 300 mg/kg/dose were selected for the 7-day dosing during Phase II.
- In Phase II, 7 day repeat oral dosing of RLT-72484 at 100 mg/kg/day limited body weight gain by 50% in males, relative to a much smaller (~10%) decrease in food intake noted in both males and females compared to controls. This is aligned with the pharmacological effects of RLT-72484.
- No RLT-72484-related macroscopic observations were noted during necropsy in the animals administered 100 mg/kg/day over 7 days. GI tissues (esophagus, GALT, stomach, duodenum, jejunum, ileum, colon, rectum, and cecum) were processed microscopically and reported to have no remarkable changes in all groups. Females administered 100 mg/kg/day were noted with increased heart and liver weights and decreased splenic weights, compared to controls, however, the organ weights differences were considered of uncertain relationship to RLT-72484.
- Minor clinical pathology effects were noted in males and females administered 100 mg/kg/day RLT-72484; lower absolute reticulocyte count (average -20% across sexes) that was consistent with decreased red blood cell production; overall higher leukocyte counts and lower plasma albumin in females suggesting an inflammatory response. Despite some clinical pathology parameter changes, this dose level was clinically well tolerated by both sexes and considered the no observable adverse effect limit (NOAEL) of RLT-72484 in rats.
- In Phase II, 7 day repeat oral dosing of RLT-72484 at 300 mg/kg/day was not well tolerated with adverse effects noted in both sexes. Since ~20% mortality was observed by days 3-4 in both sexes, all animals were humanely euthanized by days 4-5 of the study. Body weights had significantly reduced by 7-11% compared to controls with >50% reduction in food consumption in both sexes. Marked clinical

pathology changes were noted, which were aligned with changes noted with the 100 mg/kg/day dose, but the magnitude of effects being dose-dependent and higher. In males, higher red cell mass was suggestive of dehydration. In females, higher leukocyte counts were suggestive of an inflammatory response, and lower absolute reticulocyte count was consistent with decreased red blood cell production in both sexes.

- Exposure, as assessed by C_{max} and AUC_{0-24}, generally increased with the increases in RLT-72484 dose level from 100 to 300 mg/kg/day on Day 1 in a dose-proportional manner. Accumulation of RLT-72484 was noted by day 7 after multiple oral dosing of the drug at 100 mg/kg/day.
- Taken together, these findings provided insight into the NOAEL dose of RLT-72484 in rodents and the tolerable exposures for estimating the therapeutic index of this drug lead.

Conclusions. Sixty percent of the activities listed under goal 3 for this project period have been successfully completed, including verification and analysis of homogeneity for RLT-72484 oral dosing formulations and non-GLP rodent toxicity and toxicokinetic assessments for RLT-72484 in rats. These studies have provided insights into the overall safety margin and therapeutic window estimates for RLT-72484 that support further optimization of the clinical candidate for selection as drug lead to advance to clinical trials as an anti-T2D therapeutic.

What opportunities for training and professional development has the project provided?

Training/professional development.

Dr. Harshini Neelakantan (Executive Director of Research and Development, Ridgeline Therapeutics)

- ***Periodic discussions and interactions with CMC, drug development, and regulatory consultants.*** During this project period, Dr. Neelakantan periodically discussed strategic directions and outcomes from the major activities with established drug development leaders (Drs. Paul Tarantino and Radford Decker), CMC (Dr. Joseph Chen) and regulatory (Ms. Lisa Hulle) subject matter experts (SME), Ridgeline's Executive Chair (Neil Warma), Ridgeline's key opinion leaders in obesity and liver diseases (Drs. Stephen Harrison, Michael Charlton, Quentin Anstee), as well as SMEs from our CRO partners' teams (Covance and Cambrex). These periodic discussions focused on drug development guidance and regulatory procedures, alongside self-learnings through US FDA regulatory materials provide continuous training and professional development opportunities for Dr. Neelakantan to hone skills in nonclinical through clinical drug development areas.

- ***esqLABS GmbH hosted ASCPT2020 2-day Webinar (2 day).*** Basics in physiologically based pharmacokinetic modeling (PBPK) and physiologically based quantitative systems pharmacology (PB-QSP) with PK-sim® and MoBi® (March 16-17, 2020)
  Key Speakers: Stephan Schaller, PhD: CEO and Founder, esqLABS
  Summary. This two-day workshop covered the general concepts of PBPK modeling and its applications in modeling drug-drug interactions and clinical PK predictions in diseased and special clinical populations such as pediatric and elderly (Day 1) and using two open systems pharmacology suites, PK-sim® and MoBi®, and PBPK/PD and mechanistic modeling (e.g., for biologics) using MoBi® platform (Day 2). Training in these cutting-edge approaches provided the fundamental knowledge to apply these modeling systems to the data generated from the project. Dr. Neelakantan will be using these platforms to model the PK data generated from this project to predict clinical outcomes, including systemic exposures, safety profile, and safe starting doses of RLT-72484 in T2D clinical populations.

- ***BIO Convention, June 8-11, 2020.*** Ridgeline team (Drs. Neelakantan and Watowich) participated in one of the largest biotechnology conferences held digitally for the first time. During this meeting, Ridgeline team

members had the opportunity to attend several presentations related to drug development and the biotechnology industry at-large, engaged in meaningful discussions with leaders in the field of obesity and lover diseases, as well as learnt novel techniques and scientific approaches relevant to this project by networking and connecting with scientists affiliated with contract research organizations.

Dr. JoAnne Babula (Research Scientist, Ridgeline Therapeutics)

- ***On the job training.*** Dr. Babula regularly attends team meetings with our CRO partners (Cambrex and Covance) to review outcomes of the major CMC and nonclinical drug development activities pertaining to this project and drug development consultants to engage in strategic and scientific discussions. These opportunities continually provide avenues for Dr. Babula to expand her knowledge-base in early-phase drug discovery through advanced nonclinical development fields.

- ***Drug Discovery workshop hosted by Gulf Coast Consortia (GCC), Houston.*** Computational Drug Discovery and Lead Optimization (Dec 5, 2019)
  Key Speakers: Jason Cross, PhD: Structural Chemistry group leader for applied cancer science and MD Anderson Matt Repasky, PhD: VP of Life Sciences Products at Schrodinger
  Summary. This seminar included a comparison of strategies used by academic and industry groups for computation-based drug discovery and design followed by a networking reception and dinner. The seminar was very insightful and introduced several programs and techniques utilized by the two groups to improve various aspects of drug design and outcomes predictions. It introduced Dr. Babula to the available programs for affinity predictions, and the strategy of using the results of several programs to compile higher accuracy predictions tables. New software using machine learning algorithms to predict binding affinities was also introduced by Schrodinger. Since Dr. Babula extensively uses the Schrodinger software suite in-house to continue lead optimization and second-generation compound development as part of this project, this training allowed her to get adept with the upcoming modules and developments in the software. It additionally provided her a networking opportunity to connect with leaders in the field.

- ***esqLABS GmbH hosted ASCPT2020 2-day Webinar (2 day).*** Basics in physiologically based pharmacokinetic modeling (PBPK) and physiologically based quantitative systems pharmacology (PB-QSP) with PK-sim® and MoBi® (March 16-17, 2020)
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How were the results disseminated to communities of interest?

- *Nothing to report in this project period.*

What do you plan to do during the next reporting period to accomplish the goals?

- As listed in the SOW table (pg. 5), several major activities have been initiated and are ongoing for the next reporting period to accomplish the goals and milestones under the 3 Aims. These primarily include:

- Goal 1/Aim 1:
  - Analytical method qualification and validation activities to support CMC activities.
  - Continued longer-term stability studies for RLT-72484.
  - Launch and completion of the production of 2 kg batch for GLP studies.
  - While salt screening is currently not considered necessary, the hydrous/anhydrous forms of RLT-72484 in the chloride salt form will be further evaluated to confirm the stable salt form of the API.

- Goal 2/Aim 2:
  - Complete DMPK/PD studies for RLT-72484 optimization to support launch of toxicity assessments in non-rodent species and launch GLP studies.
  - Evaluate optimized RLT-72484 analogues through *in vitro* safety and *in vivo* efficacy, PK/PD studies
  - Compile documentation and questions to discuss with the FDA with guidance from our CMC and regulatory consultants and launch FDA pre-IND consultation.

- Goal 3/Aim 3:
  - Validate regulated bioanalytical method for RLT-72484 in rat and dog plasma.
  - Complete non-GLP non-rodent toxicity/toxicokinetic assessments in Beagle dogs.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

- Demonstration of the efficacy of our *first-in-class* small molecule NNMT inhibitor clinical candidate in translationally-relevant DIO mouse models of T2D validated NNMT as a novel target to regulate insulin resistance and associated hyperlipidemia and liver pathologies by modulating whole-body adiposity, an underlying driver of obesity-linked T2D and metabolic dysfunctions.
- Our innovative research, in an extended way, currently focuses on deciphering the molecular mechanisms-of-action of NNMT inhibitors, including the downstream pathways regulated by these inhibitors to cause metabolic improvements in the adipose, liver, and muscle tissues. Particularly, we have observed that treatment of DIO mice with our lead drug candidate RLT-72484 combined with a reduced calorie diet displays unique metabolic signature and mitigates dysregulated lipid, amino acid, and energy metabolism in the adipose tissue compared to the metabolome profile associated with obesogenic diets. These findings translate to clinically meaningful indications for the use of NNMT inhibitors as adjunct therapeutics to enhance and sustain the effects of lifestyle interventions such as dietary alterations among target obese populations.
- Furthermore, our data directly support application of NNMT inhibitors as therapeutics to treat non-alcoholic fatty liver disease/steatohepatitis (NAFLD/NASH) that is strongly linked with obesity. To this end, we have demonstrated in DIO mouse models that RLT-72484 significantly improves hepatic adiposity, triglyceride content, steatosis, inflammation, ballooning, and the overall NAFLD activity score, which are all hallmark features of NAFLD/NASH. There are currently no FDA approved drugs to resolve NAFLD/NASH and demonstration of safety and successful clinical development of our clinical lead as a T2D drug can support its application as a dual therapeutic to address T2D and/or NAFLD/NASH in obese individual with comorbid conditions.

What was the impact on other disciplines?

- The process chemistry research activities for RLT-72484 completed in this reporting period validated an FDA-acceptable synthesis procedure with the ability to support scalability in kilogram amounts for IND-enabling studies and into clinical development. These findings provide novel insights for adaptability and

applications to the synthesis of similar chemical molecules, thereby directly contributing to the field of medicinal and synthetic chemistry.
- Overall, all major CMC and drug development research activities and studies completed for RLT-72484 during this reporting period provide guidance for continued optimization and development of optimized RLT-72484 analogues.

What was the impact on technology transfer?

- *Nothing to Report during this project period.*

What was the impact on society beyond science and technology?

- *Nothing to Report during this project period.*

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The following changes were incurred during the reporting period of this project that has been reviewed and approved by the awarding agency grants official:

- SOW Revision 1, Oct. 08, 2019
  - Ridgeline in-house laboratory site information added;
  - Contracted API manufacturing operations switched from ARCINOVA to Cambrex. This change occurred because Cambrex provided (i) more competitive pricing for the desired deliverables, (ii) more extensive and robust capabilities, (iii) synthesis and manufacturing processes more aligned with US FDA requirements, (iv) an accelerated timeline to provide deliverables, and (v) a wholly domestic operation for the manufacturing.

- SOW Revision 2, March 10, 2020
  - PI changed to Dr. Neelakantan; Dr. Watowich continues to serve as the Sponsored Program and Grant Signing Official.

- SOW Revision 3, June 16, 2020
  - Adjustments in the timeline for major manufacturing activity in Aim 1 and GLP activities in Aim 3. These changes have been necessitated as guided by the findings from this reporting period; early therapeutic index estimates for RLT-72484 warrants further optimization of the DMPK/PD profile of the lead candidate to provide better safety margins for continued development and clinical testing. These have been noted as expanded activities under Aim 2 (see activities 2.5a-e, pg. 6).

Actual or anticipated problems or delays and actions or plans to resolve them

- No delays were incurred, and ALL major activities proposed in this reporting period were successfully completed as reported here.
- As noted above, findings from studies in this reporting period suggest a few optimization activities for RLT-72484 that have been initiated and will be completed in the next project period.

Changes that had a significant impact on expenditures

- Nothing to report in this project period.
- Expanded activities under Aim 2 (see activities 2.5a-e, pg. 6) per SOW Revision 3 that have been initiated and will be completed in the next reporting period do not add any impact on expenditures for this program and are largely supporting through other funding support to Ridgeline.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

- *Not applicable.*

Significant changes in use or care of vertebrate animals

The following amendments were executed to study protocols as guided by outcomes and/or the need to expand studies, as needed. These amendments were promptly notified to and approved by Covance (CRO site for study execution) Institutional Animal Care and Use Committee and DoD ACURO teams, respectively.

- Covance Study 8416177: Determination of the Pharmacokinetics of RT001 (a.k.a RLT-72484) after a Single Intravenous or Oral Dose to Rats; study was expanded to add 300 mg/kg oral dose of RLT-82484 testing in rats to assess dose proportionality; protocol was amended to test 300 mg/kg in separate cohorts of rats.
  Covance IACUC approval date: 01/28/2020

- Covance Study 8416178: Determination of the Pharmacokinetics of RT001 (a.k.a RLT-72484) after a Single Intravenous or Oral Dose to Dogs; due to adverse event outcomes at the 30 and 300 mg/kg oral doses of RLT-82484 in dogs (reported in detail to AUCRO), protocol was amended to test lower oral doses of 3 and 10 mg/kg in separate cohorts of Beagles.
  Covance IACUC approval date: 01/22/2020

- Covance Study 8416071: RT001: Oral Gavage Dose Toxicity Study in Dogs; based on Covance study 8416178 observations, doses in the protocol were revised from 3, 10, 30, and 300 mg/kg/day (in Amendment #1) to 5, 15, 25, and TBD mg/kg/day and TBD mg/mL (in Amendment #2).
  Covance IACUC approval date:

Significant changes in use of biohazards and/or select agents

- *Nothing to Report during this project period.*

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Journal publications.

1. ***Authors.*** Ololade Awosemo, Harshini Neelakantan, Stanley Watowich, Jing Ma, Lei Wu, Diana SL, Chow, Liang Dong.
   Title. Development & Validation of LC-MS/MS Assay for 5-Amino-1-Methyl Quinolinium in Rat Plasma: Application to Pharmacokinetic and Oral Bioavailability Studies
   Journal. Journal of Pharmaceutical and Biomedical Analysis
   Status. Under review; *Acknowledgement of federal support – No.*

2. ***Authors.*** Catherine M. Sampson, Andrea L. Dimet, Harshini Neelakantan, Kehinde O. Ogunseye, Heather L. Stevenson, Jonathan D. Hommel, and Stanley J. Watowich
   Title. Nicotinamide N-Methyltransferase Inhibition Enhances the Metabolic and Physiological Benefits of Calorie Restriction in Obese Mice
   Journal. Nature Metabolism
   Status. In preparation; *Acknowledgement of federal support – Yes.*

   Books or other non-periodical, one-time publications.

   - *Nothing to Report during this project period.*

   Other publications, conference papers and presentations.

   - *Nothing to Report during this project period.*

- **Website(s) or other Internet site(s)**

  - *Nothing to Report during this project period.*

- **Technologies or techniques**

  - *Nothing to Report during this project period.*

- **Inventions, patent applications, and/or licenses**

  - *Nothing to Report during this project period.*

- **Other Products**

  - *Nothing to Report during this project period.*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	*Dr. Harshini Neelakantan*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-2913-1824
Nearest person month worked:	*9.6*
Contribution to Project:	Dr. Neelakantan is an assigned key personnel on this grant. During this project period, she transitioned full time to Ridgeline as the Executive Director of Research and Development and took over to

serve as the PI/PD for this DoD program. As the PI, she has been fully overseeing and handling the scientific and management aspects of this grant. During this reporting period, she reviewed and managed CRO proposal/contracts, study protocols and reports from Cambrex and Covance, remotely and closely oversaw and monitored the activities at the performance sites (Cambrex and Covance), led all communications pertaining to the grant activities with the internal Ridgeline team, external consultants, KOLs, and CRO research teams. She has served as the primary contact at Ridgeline for communication with the DoD team, including periodic meetings the Scientific Officer, ad-hoc email communications with the Contract Specialist, and required communications with the ACURO management teams.

Funding Support: *1.8 Cal months (NIAMS STTR Grant 1R41AR076871-01)*

Name: *Dr. Stanley Watowich*
Project Role: *Grant & Contract Signing Official*
Researcher Identifier (e.g. ORCID ID): *N/A*
Nearest person month worked: *N/A*
Contribution to Project: Dr. Watowich is an assigned key personnel on this grant. During this project period, he relinquished his PI role to Dr. Neelakantan and continues to serve as the Grant & Contract Signing Official for this DoD program. He primarily manages and oversees financials at Ridgeline and serves as the main contact for communications with contract specialists. During this reporting period, Dr. Watowich oversaw contracts and proposals officially signed with our CMO and CRO partners, and activities led by Dr. Neelakantan. He actively engaged in all discussions with our CRO research teams, external consultants, and KOLs, to ensure synergy among the major activities performed during this reporting period and the overarching goals for our program and Ridgeline Company as a whole.

Funding Support: *N/A*

Name: *Dr. JoAnne Babula*
Project Role: *Research Scientist*
Researcher Identifier (e.g. ORCID ID): 0000-0002-7953-179X
Nearest person month worked: *10.8*
Contribution to Project: As a trained Computational Scientist, Dr. Babula was hired as a Research Scientist to lead the design and early *in vitro* ADMET drug development activities for second-generation lead candidate NNMT inhibitors as proposed in this project. During this project period, Dr. Babula has been engaging in meetings with out CRO partners to closely follow RLT-72484 activities and transfer that knowledge to second-generation analogues development activities. She has been applying advanced computational and modeling/simulations techniques to design novel molecules and rank-order compounds based on favorable drug-like properties. She oversees and manages the synthesis of new generation compounds by our medicinal chemistry academic or CRO collaborators as well as early screening activities for these

analogues by CROs. Drs. Neelakantan and Watowich supervise these activities.

Funding Support: *N/A*

Name: *Dr. Joseph Chen*
Project Role: *CMC Consultant*
Nearest person month worked: *1.5*
Contribution to Project: Dr. Chen is a leader in pharmaceutical sciences with over 20 years working experience in development of pharmaceutical drug products (biologicals and small molecules); extensive expertise in both technical leadership and management oversight in drug product development and manufacturing in-house and/or at CRO/CMO sites. He has also prepared several relevant CMC sections for regulatory submissions, including IND's, IND amendments and NDA's or BLA's, and engaged directly with regulatory agencies. During this reporting period, Dr. Chen engaged in proposal and contract discussions with our CMO, reviewed and approved CMC activities, aligned the milestones for our CMC program, attended TC meetings with CMO teams and led discussions, reviewed incoming data for accuracy and approved studies.

Funding Support: *N/A*

Name: *Ms. Lisa Hulle*
Project Role: *Regulatory Consultant*
Nearest person month worked: *2.4*
Contribution to Project: Ms. Lisa Hulle is an executive regulatory affairs leader with over 25 years of experience advising small pharmaceutical companies on strategic regulatory development. She has extensive experience leading, preparing, reviewing, and maintaining regulatory filings with several agencies, including the U.S. FDA. Her role with Ridgeline is to fully lead the regulatory activities defined in this project and serve as the prime contact for U.S. FDA at Ridgeline. During this reporting period, Ms. Hulle reviewed all nonclinical study protocols and reports for studies performed by Covance CRO to ensure they were acceptable according to US FDA guidance. She ensured our CRO partner provided the needed supporting documentations (e.g., SEND files) and checked their accuracies to support the development of the documentations for IND filing. She attended TC meetings with our CMO and CRO teams and led regulatory-relevant discussions.

Funding Support: *N/A*

Name: *Dr. Paul Tarantino*
Project Role: *Safety/toxicology and nonclinical drug development Consultant*
Nearest person month worked: *0.35*
Contribution to Project: Dr. Tarantino has extensive experience in nonclinical support of small molecule therapeutics from drug discovery through clinical development and regulatory approval. In his past years, he has designed and managed the execution of several nonclinical

	safety/toxicology programs by developing protocols with CROs, monitoring studies, reviewing, and reporting results. Further, he has authored regulatory documentations (including INDs, meeting requests, briefing materials, etc.) and interfaced with US FDA and international regulatory authorities. During this project period, Dr. Tarantino reviewed the protocols and results of the PK/TK and safety studies performed by Covance, provided his advice on study design, and discussed the study outcomes in relation to the overall drug development program of RLT-72484 with the Ridgeline team.
Funding Support:	*N/A*
Name:	*Dr. Rad Decker*
Project Role:	*DMPK Consultant*
Nearest person month worked:	*0.35*
Contribution to Project:	Dr. Decker has 25+ years of in-depth experience in DMPK and nonclinical drug development for small molecule therapeutics in several therapeutic areas. He has managed several drug development programs from early discovery through clinical development and regulatory approval, and designed/developed the reporting of drug metabolism and pharmacokinetics studies for review by the US FDA. During this project period, Dr. Decker reviewed the protocols and results of the DMPK studies performed by Covance and Wuxi, provided his advice on the study designs, and discussed the study outcomes in relation to the overall drug development program of RLT-72484 with the Ridgeline team.
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- Effective March 10, 2020, Dr. Harshini Neelakantan began officially serving as the PI for this DoD Grant (PR180216/ Contract no. W81XWH-19-1-0290).
- During this reporting period, a funding support from the NIH NIAMS Agency that was previously pending was approved and awarded to Ridgeline (PI: Neelakantan). This does not change Dr. Neelakantan's percent effort towards this project, which remains at 80% effort over the project period.

What other organizations were involved as partners?

Organization Name: **Cambrex** (CMO)
Location of Organization: Longmont, CO
Partner's contribution to the project (identify one or more)

- Facilities (facilities for completion of project activities on a fee-for-service basis);
- Project management and subject matter experts' support on site (Project Manager and Scientific experts from Organization providing oversight leadership and coordination of deliverables and activities).

Organization Name: **Covance** (CRO)
Location of Organization: Madison, WI
Partner's contribution to the project (identify one or more)

- Facilities (facilities for completion of project activities on a fee-for-service basis);

- Project management and Drug Development Leader support on site (Project Manager, Drug Development Leader, and Individual Study Directors providing oversight leadership and coordination of deliverables and activities).

Organization Name: **Xenobiotics Laboratories** (a Wuxi ApptTec subsidiary; CRO)
Location of Organization: Plainsboro, NJ
Partner's contribution to the project (identify one or more)

- Facilities (facilities for completion of early-phase development project activities on a fee-for-service basis);
- Study Director/Coordinator on site (Study Coordinator/Director coordinating deliverables and activities).

Organization Name: **Eurofins Cerep** (CRO)
Location of Organization: France
Partner's contribution to the project (identify one or more)

- Facilities (facilities for completion of early-phase *in vitro* safety/pharmacology panel screening activities on a fee-for-service basis);
- Study Director/Coordinator on site (Study Coordinator/Director coordinating deliverables and activities).

Organization Name: **Center for Innovative Drug Discovery** (UTSA, Academic collaborator)
Location of Organization: San Antonio, TX
Partner's contribution to the project (identify one or more)

- Facilities (facilities for medicinal chemistry/synthesis of second-general analogues activities on a fee-for-service basis);
- Director and Lead Scientists (Center Director and Lead Scientist execute synthesis proposals and deliverables).

Organization Name: **Texas Southern University** (Analytical Core, Academic collaborator)
Location of Organization: Houston, TX
Partner's contribution to the project (identify one or more)

- Facilities (facilities for initial analytical activities for RLT-72484 as a collaborative project);
- PI and Graduate Student (PI and Graduate Student in the lab developed and validated and analytical method for RLT-72484 that is being published and adapted by Covance for this project support).

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

- *Not applicable.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

- *Not applicable.*

9. APPENDICES:

Major activity: Analytical method development for RLT-72484 (a.k.a 5-amino-1-methylquinilinium) – original journal article submitted to the Journal of Pharmaceutical and Biomedical Analysis (under review)

Development & Validation of LC-MS/MS Assay for 5-Amino-1-Methyl Quinolinium in Rat Plasma: Application to Pharmacokinetic and Oral Bioavailability Studies

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Abbreviations: 5-AMQ, 5-amino-1-methyl quinolinium; NNMT, nicotinamide N-methyl transferase; MRM, multiple reaction monitoring; IS, internal standard; AUC, area under the curve; Cmax, maximum drug concentration in plasma; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantitation; SD, Sprague Dawley; QC, quality control; t1/2, half-life

ABSTRACT
5-amino-1-methyl quinolinium (5-AMQ) is a potent Nicotinamide N-methyl transferase (NNMT) inhibitor. NNMT is an enzyme that catalyzes the N-methylation of the endogenous substrate nicotinamide, as well as exogenous xenobiotics. NNMT is fundamental to cellular metabolism; NNMT is overexpressed in select tissues (e.g., adipose tissue, skeletal muscle, etc.) in pathophysiological conditions, making it a clinically relevant target for drug development in
several chronic diseases including obesity and diabetes. The objective of this study was to develop and validate a simple, sensitive, and reproducible liquid chromatography-tandem mass
spectrometry (LC-MS/MS) method for the quantification of 5-AMQ in rat plasma and urine
samples. 5-AMQ was extracted from plasma and urine by protein precipitation. Chromatographic separation was achieved using an ACE® Excel™ C18 column (2 µm, 50×2.1 mm) with a binary gradient solvent system comprising of water (A) and acetonitrile (B) containing 0.1% formic acid as the mobile phase. Analysis was performed using an API 4000 QTRAP hybrid triple quadruple mass spectrometer and multiple reaction monitoring (MRM) in positive mode at *m/z* transitions of 159.100 → 90.00 and 162.200 → 117.200 for 5-AMQ and the internal standard, respectively. The standard curves of 5-AMQ in rat urine and plasma samples were linear in the concentration range of 10 – 2500 ng/mL. The intra-day and inter-day precisions and accuracies for 5-AMQ at four concentration levels in rat plasma and urine samples were found to be within the 15% FDA acceptance range. Similarly, the accuracy and precision of 5-AMQ quantification in samples diluted up to 20-fold using blank plasma were within the 15% acceptable range. Furthermore, the extraction recoveries and matrix effects at three concentration levels of rat plasma samples ranged from 99.5% - 110.6% and -6.1% - 14.1%, respectively. 5-AMQ was stable in rat plasma samples subjected to standard storage, preparation, and handling conditions, with less than 15% variation noted at two concentration levels. The validated, sensitive, and reproducible LC-MS/MS method for 5-AMQ in rat plasma and urine samples was effectively applied to a pharmacokinetic study in rats with IV and oral administration of 5-AMQ. 5-AMQ displayed substantial plasma exposures via IV and oral route, with modest half-life and good oral bioavailability (F% = 38.4).

Keywords:
LC-MS/MS
5-amino-1-methyl quinolinium
Obesity
Pharmacokinetics
Bioavailability

Highlights

· A simple, specific and reproducible LC-MS/MS assay for 5-AMQ quantification in rat plasma and urine samples was developed and validated
· The assay was used to characterize pharmacokinetic properties and oral bioavailability of 5-AMQ in rats
· Results demonstrate that 5-AMQ was well absorbed with an average oral bioavailability of 38.4%