Pharmacokinetics and safety of TCMCB07, a melanocortin-4 antagonist peptide in dogs

Sandra M. Axiak-Bechtel¹ | Stacey B. Leach¹ | David G. Scholten² | Jessica R. Newton-Northup² | Brendan J. Johnson¹,² | H. E. Durham¹ | Kenneth A. Gruber²,³ | Michael F. Callahan²

¹Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO, USA
²TCI Peptide Therapeutics, Columbia, MO, USA
³Department of Medical Pharmacology & Physiology and the Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO, USA

Abstract
The melanocortin-4 receptor (MC4R) antagonistic peptide TCMCB07 was developed for the treatment of cachexia. The objectives of this study were to examine pharmacokinetics and safety of TCMCB07 administered subcutaneously to healthy dogs. Dogs were treated with high- (2.25 mg kg⁻¹) (n = 5) and low-dose TCMCB07 (0.75 mg kg⁻¹) (n = 5) once daily for 28 days with a 14-day washout period between groups. Histamine levels, complete blood count, chemistry panel, blood pressure, 24-hour Holter recording, and pharmacokinetic parameters were monitored in the high-dose group. Physical examination changes were limited to weight gain and darkening of the coat color. There was no elevation of plasma histamine within 24 hours of injection but there was a significant elevation of plasma histamine across time. An approximately doubled eosinophil count and an approximately 25% increase, and then 25% decrease back to pre-treatment plasma phosphorous were also found, although both remained within the reference interval. Serial blood pressure and 24-hour Holter monitors revealed no clinically relevant changes. A difference was found in the AUC between dosing groups and a significant effect of dose, time, and interaction was noted for $V_p$. Low-dose TCMCB07 had a $C_{max}$ of $2.1 \text{ug ml}^{-1}$ at day 28, compared to high-dose TCMCB07 which had a $C_{max}$ of $3.6 \text{ug ml}^{-1}$ at day 28. Once-daily subcutaneous administration of TCMCB07 was well-tolerated for up to 28 days in dogs when administered at doses one and three times (0.75 mg kg⁻¹ and 2.25 mg kg⁻¹) the predicted therapeutic dose and pharmacokinetic parameters are described.

Significance Statement: Melanocortin-4 receptor (MC4R) antagonistic peptide TCMCB07 is safe at both low and high doses in dogs. Therapy was tolerated well as determined by physical examination, clinical pathology, and cardiovascular parameters; darkening of the coat was noted with treatment and resolved with discontinuation. Pharmacokinetics are described and further study in the naturally occurring canine model is warranted.

Abbreviations: MC4R, Melanocortin-4 receptor; API, Assay of active pharmaceutical ingredient.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. Pharmacology Research & Perspectives published by British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics and John Wiley & Sons Ltd.

Pharmacol Res Perspect. 2021;9:e00777.
https://doi.org/10.1002/prp2.777
1 | INTRODUCTION

Cachexia is a devastating consequence of numerous acute and chronic
disease processes and is caused by proinflammatory cytokines acting
as hormonal messengers, stimulating melanocortin neurons in the hy-
pothalamus.\textsuperscript{1–4} The result is an increased metabolic rate, decreased
appetite, and loss of lean body mass.\textsuperscript{1,4,5} Loss-of-function mutations
in the melanocortin-4 receptor (MC4R) gene is associated with an in-
creased appetite and lean body mass in people, making MC4R antago-
nism a promising target for cachexia therapy.\textsuperscript{1,6} The MC4R antagonistic
peptide TCMCB07 was developed to improve the pharmacokinetics of
MC4R antagonists and avoid possible cardiovascular effects, as MC4R
plays a role in cardiovascular physiology. People with haploinsuffi-
ciency of MC4R have a reduction in autonomic tone, bradycardia, and
increased incidence of obesity-associated hypertension.\textsuperscript{3,4,6–9}

TCMCB07 is a cyclic substituted melanocortin antagonist with the
structure Ac-Nle-cyclo[Asp-Pro-DNal(2')-Arg-Trp-Lys]-DVal-
DPro-NH$_2$. The compound was designed through a series of iterative
amino acid substitutions designed to mimic common structural fea-
tures of peptides with desirable characteristics (blood–brain barrier
transport, oral activity, or hepatic active transport). These structural
features include a C-terminus with nonpolar amino acid residues or
chemical groups and cyclization or proline residue in or adjacent to
the peptide’s pharmacophore. Several peptides targeting MC4 were
produced and TCMCB07, as the most promising candidate, was
further studied.\textsuperscript{10,11} Peripheral treatment—subcutaneous, intraper-
itoneal, or oral—in rodent models of LPS, renal disease, and cancer-
induced cachexia resulted in retention of both lean and fat body
mass, appetite stimulation, and stable body weight. Furthermore,
TCMCB07 plasma concentrations were correlated with drug dose
and the 14-day food intake and body weight gain were correlated
with TCMCB07 plasma concentration.\textsuperscript{12}

Given the promising results of TCMCB07 in rodent models of
cachexia, the objectives of this study were to examine pharma-
okinetics and the safety profile of TCMCB07 administered subcuta-
naneously to normal dogs. The hypothesis was that TCMCB07 would
be well tolerated with no evidence of cardiotoxicity as measured by
24-hour ambulatory electrocardiography (Holter recording). Dogs
were treated with high-dose TCMCB07 (2.25 mg kg$^{-1}$) ($n = 5$) or
low-dose TCMCB07 (0.75 mg kg$^{-1}$) ($n = 5$) once daily for 28 days.
The female dogs ($n = 3$) were treated in both the high and low
dosages after a minimum 14-day washout period, and the male
dogs ($n = 4$) were treated with high-dose only ($n = 2$) or low-dose
only ($n = 2$) TCMCB07. Dosages of TCMCB07 were determined
based on allometric scaling of efficacious doses in rat models. In
the high-dose study ($n = 5$), dogs were monitored with a complete
blood count and chemistry panel, blood pressure, and 24-hour
Holter recordings on day 5 and 28. In the low-dose study ($n = 5$),
two parameters—daily bodyweight and examination weekly—were
used for monitoring. Pharmacokinetics were performed for both
the low- and high-dose groups.

2.2 | Study design

This study was a prospective, one-armed open-label trial in
healthy dogs. All work was approved by the University of Missouri
Animal Care and Use Committee, protocol approval number 7452,
prior to study initiation. Seven purpose-bred adult Beagles, three
intact females and four intact males, were obtained, housed, and
handled according to the approved protocol. Dogs were fed three
cups of a standard adult canine diet daily with water ad libitum.
Dogs were determined healthy prior to study initiation using
physical examination and results within the reference range for
complete blood count, chemistry panel, blood pressure, and 24-
hour ambulatory electrocardiography (Holter recording). Dogs
were treated with high-dose TCMCB07 (2.25 mg kg$^{-1}$) ($n = 5$) or
low-dose TCMCB07 (0.75 mg kg$^{-1}$) ($n = 5$) once daily for 28 days.
The female dogs ($n = 3$) were treated in both the high and low
dosages after a minimum 14-day washout period, and the male
dogs ($n = 4$) were treated with high-dose only ($n = 2$) or low-dose
only ($n = 2$) TCMCB07. Dosages of TCMCB07 were determined
based on allometric scaling of efficacious doses in rat models. In
the high-dose study ($n = 5$), dogs were monitored with a complete
blood count and chemistry panel, blood pressure, and 24-hour
Holter recordings on day 5 and 28. In the low-dose study ($n = 5$),
two parameters—daily bodyweight and examination weekly—were
used for monitoring. Pharmacokinetics were performed for both
the low- and high-dose groups.

2.3 | Holter monitoring

A 5-electrode, 2-channel ambulatory electrocardiographic system
(Decipher, Medicomp Inc.) was utilized for 24-hour Holter moni-
toring.\textsuperscript{16} Holter monitoring occurred on an acclimation day when
placebo was administered and on day 1, 5, and 28 during drug ad-
ministration at 2.25 mg kg$^{-1}$. Fur on the sternum and lateral chest
area was clipped and the skin cleaned with isopropyl alcohol wash.
Four adhesive electrodes were placed on the right and left lateral
thoracic and ventrolateral thoracic area (near the costochondral
junction) with a fifth ground electrode placed in the midsternal
region. This configuration most closely corresponded with the or-
thogonal plane lead X. The ECG electrodes and leads were cov-
ered with cotton and adhesive elastic bandages (Vetwrap, 3 M).
Monitoring equipment was housed in a jacket. Elizabethan collars
were used to prevent dog access to Holter equipment and animals
were individually housed during the recording period. The Holter
data were analyzed by proprietary software and was subjected to
manual visual inspection and correction by a trained technician.
Holter monitoring data were evaluated for the presence of abnor-
mal pauses (pauses >3 secs), bradycardrhythms, tachyarrhythmias,
sustained sinus tachycardia, and ventricular ectopy. Other parameters evaluated included the minimal, maximal, and average daily heart rate.

2.4 | Blood pressure

Arterial blood pressure (ABP) was estimated noninvasively using the Riva–Rocci principle of detecting arterial blood flow past a pressurized cuff on a distal limb as previously described. A pediatric cuff (Critikon, GE Medical Pittsburg PA) with a width corresponding to approximately 40% of the circumference of the limb was placed just proximal to the carpal joint and systolic pulses were identified using acoustic Doppler flow detection (Ultrasonic Doppler Flow Detector, Model 811-b, Parks Medical Electronics). The final systolic ABP was logged as the average of three measurements that were observed to be within 6 mmHg of each other to allow for the elimination of stress hypertension that may be observed with the first 1–2 inflations.

2.5 | Pharmacokinetics

Pharmacokinetic (PK) parameters were assessed on days 1, 5, and 28 in both the high and low-dose studies at the following time points: baseline (0), 30 minutes, and 1, 2, 4, 12, and 24 hours following TCMCB07 administration. Whole blood (1–3 mls) was collected using jugular or peripheral venipuncture into lithium heparin tubes and kept on ice until centrifugation. Blood was centrifuged at 13,000 rpm at 4°C for 2.5 minutes, and plasma was extracted and stored at −80°C for batch analysis. Noncompartmental pharmacokinetic parameters were determined using PK Solutions software (Summit Research Services, Montrose CO). Plasma concentrations were modeled on a two-phase elimination and distribution/absorption model.

2.6 | Assay of active pharmaceutical ingredient (API)

In order to quantify API within canine plasma, the majority of the plasma proteins were precipitated out of solution with a 1:4 ratio of plasma to acetonitrile followed immediately by vigorous vortexing for 30 seconds. These precipitates were then pelleted, the supernatant transferred to a new tube, and the acetonitrile evaporated. These samples were then diluted to between 400 and 500 μl using purified water and run on a reverse phase—high-pressure liquid chromatography (RP-HPLC) system (Gilson) with a Hypersil GOLD C18 column (ThermoScientific) for separation and analysis of API. API was detected via the innate fluorescence of the non-natural D-amino acid naphthylalanine (D-Nal) using a spectrofluorometer (Panorama Fluorat-02, Lumex Ltd) set at an excitation of 229 nm and detecting the emission at 337 nm. Quantification of API included using area under the curve (AUC) calculations performed by PanoramaPro, Version 2.2.0 (Lumex Ltd). These AUC values were then compared to AUC values of a standard curve and the concentration of API calculated.

2.7 | Histamine Analysis

Histamine levels within the plasma were probed using an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp.). Plasma treated with heparin was frozen at −80°C until the day of analysis. On the day of analysis, samples were brought to room temperature and diluted by a factor of two prior to use within the ELISA protocol. In short, this kit provided an ELISA plate precoated with anti-histamine antibody for use within a competitive inhibition procedure between histamine within the plasma (or standard) and biotin-labeled histamine (provided within the kit). The amount of bound biotinylated histamine was then quantified using avidin conjugated to horseradish peroxidase resulting in an inverse correlation between histamine concentrations within the sample to signal intensity.

2.8 | Statistical Analysis

Bodyweight, blood chemistry values, complete blood count values, blood pressure, heart rate, and pharmacokinetic parameters were analyzed by one or two way with repeated measures ANOVA (Prism 6, Graph Pad), with Bonferroni corrected post hoc tests in the event of significant main or interaction effects. Analysis of arrhythmia events per 24 hours was conducted with a Friedman nonparametric ANOVA. A p ≤ .05 was considered significant for all comparisons.

2.9 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,18 and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.19

3 | RESULTS

Dogs receiving 0.75 and 2.25 mg kg⁻¹ of TCMCB07 once daily showed progressive weight gain throughout the 28-day study period (Figure 1). While the effect of dose approached significance (p ≤ .054), there was a significant overall effect of time on drug (p ≤ .001) and a significant interaction between dose and time (p ≤ .001) indicating that the higher dose produced a greater response in body weight. Overall, dogs in the low-dose group had an increased body weight with a median increase of 0.45 kg (range 0.3–1.0 kg) and dogs in the high dose increased in body weight with a median increase of 0.9 kg (range 0.5–1.25 kg). Following discontinuation of
treatment, weight returned to baseline by day 60. Diffuse darkening of coat color was noted in all dogs in both the low-dose and high-dose groups beginning at day 14. Other physical examination parameters (heart rate, respiratory rate, auscultation, abdominal palpation, alertness, activity level) remained within normal limits throughout the study duration. No differences were detected in the following parameters throughout the 28-day study period: total white blood cell count, neutrophil count, lymphocyte count, basophil count, platelet count, blood urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, sodium, potassium, and chloride. An increase in eosinophil count was found between days 0 (median 0.21 $\times$ 10$^3$/μl; range 0.08–0.25 $\times$ 10$^3$/μl) and 5 (median 0.48 $\times$ 10$^3$/μl, range 0–0.61 $\times$ 10$^3$/μl), and 28 (median 0.97 $\times$ 10$^3$/μl, range 0.69–2.06 $\times$ 10$^3$/μl), although the eosinophil count was still considered within normal limits throughout the study period (Figure 2). Changes in plasma phosphorous were also noted, with the phosphorus remaining within the normal range at all-time points: day 0: median 3.7 (range 3.1–4.6 mg dl$^{-1}$); day 5: median 4.7 (range 4.5–4.9 mg dl$^{-1}$) and day 28: median 4.1 (range 3.5–4.8 mg dl$^{-1}$) (Figure 3).

Results of Holter heart rate and arrhythmia monitoring are illustrated in Figures 4 and 5. There were no differences in the minimum, maximum, or average daily heart rate measured on day 1 (minimum median 40, range 30–52; maximum median 250, range 250–267; average median 86, range 73–101), day 5 (minimum median 34, range 44–53; maximum median 272, range 250–288; average median 94, range 76–100), and day 28 (minimum median 46, range 33–53; maximum median 267, range 227–272; average median 98, range 78–104) of treatment compared to placebo treatment (minimum median 40, range 36–48; maximum median 263, range 250–283; average median 87, range 77–98) (Figure 4). Although there

**FIGURE 1** Bodyweight in dogs treated with TCMCB07; the mean and standard error of the mean are represented. Circle data points represent low dose (0.75 mg kg$^{-1}$ per day; n = 5) and square high dose (2.25 mg kg$^{-1}$ per day; n = 5). Bars above data indicate a significant difference between baseline average weight and treated average weight for 2.25 mg kg$^{-1}$ (upper bar, $p \leq .001$) group and between baseline average weight and average weight for dogs in the 0.75 mg kg$^{-1}$ treatment group (lower bar $p \leq .001$). Bodyweight was consistently increased by day 15 in the low-dose group and day 10 in the high-dose group. Following discontinuation of TCMCB07 at day 28, body weight returned to baseline. Results were analyzed by one or two way with repeated measures ANOVA, with Bonferroni corrected post hoc tests in the event of significant main or interaction effects.

**FIGURE 2** Eosinophil counts in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg$^{-1}$ per day for 28 days. Eosinophil counts remained within the normal range, but were increased (RM ANOVA F=14.7, $p \leq .01$) at day 5 (0.48, 0–0.61, median and range) and 28 (0.97, 0.69–2.06), compared to baseline (0.21, 0.08–0.25 $\times$ 10$^3$/μl; median, range).

**FIGURE 3** Serum phosphorous concentrations in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg$^{-1}$ per day for 28 days. Overall RM ANOVA indicated a change in serum phosphorus (F = 6.5, $p \leq .03$), but there were no statistically significant changes at any timepoint and values remained within the normal range.
were no clinically significant pathologic arrhythmias noted during the course of study, there was a significant increase in ventricular ectopy over time \((p < .001)\) (Figure 5). VE increased from a baseline median of 0 (range of 0 to 45) to a day 28 median of 4 (range from 1 to 89). In one dog, ventricular ectopic complexes doubled from a baseline of 45 complexes over 24 hours to 89 complexes over 24 hours on day 28. No episodes of complex ventricular ectopy (i.e., couplets, triplets, runs of ventricular tachycardia) were noted. There was no significant change in the occurrence of bradycardic episodes \((p < .47)\) or in the occurrence of bradycardic pauses \((p < .37)\) over the treatment period. Treatment with TCMCB07 was associated with a decrease in the occurrence of sustained sinus tachycardic episodes \((p < .023)\). No subjects showed sustained tachycardic events by day 28 (Figure 5). Furthermore, no difference in arterial blood pressure was found in dogs between pre- and 4 hours post-treatment, and between placebo treatment and days 1, 5, and 28 of TCMCB07 treatment (Figure 6).

### 3.1 Pharmacokinetics

Pharmacokinetics are illustrated in Table 1 and Figure 7. A difference was found in the area under the curve (AUC) between the low- and high-dose groups and a significant effect of dose, time, and interaction was noted for volume of distribution \((V_d)\). In addition, low-dose TCMCB07 had a \(C_{\text{max}}\) of 2.6 \(\mu\)g ml\(^{-1}\) at day 1 and 5 and 2.1 \(\mu\)g ml\(^{-1}\) at day 28, compared to high-dose TCMCB07 which had a \(C_{\text{max}}\) of 4.9 \(\mu\)g ml\(^{-1}\) at day 1, 5.0 \(\mu\)g ml\(^{-1}\) at day 5 and 3.6 \(\mu\)g ml\(^{-1}\) at day 28.

### 3.2 Histamine Analysis

There was no elevation of plasma histamine within 24 hours of injection \((p ≤ .63)\). However, there was a significant elevation of plasma histamine across days \((p ≤ .02)\). Within any day there was no significant difference between any time points after injection (Table 2). Post hoc analysis indicated that within any time point there was a significant difference in histamine across days with the exception of no difference between Day 5 and 28 at 24 hours after injection \((p ≤ .04)\) (Figure 8).

### 4 DISCUSSION

Once-daily subcutaneous administration of TCMCB07 was well-tolerated for 28 days in dogs when administered at dosages of 0.75 mg kg\(^{-1}\) and 2.25 mg kg\(^{-1}\). Physical examination changes were limited to weight gain and coat color. On serial complete blood counts, the only change was an increased overall eosinophil count over time, although the actual eosinophil count remained within the reference interval. On serial chemistry panels, plasma phosphorous changed over time, but also remained within the reference range. Holter analysis revealed a decrease in sustained tachycardia episodes over time, though the minimum, maximum, and average daily heart rates remained unchanged. Administration of high-dose TCMCB07 was associated with an increase in ventricular ectopy with an average number of ventricular ectopic events at baseline compared to day 28 of therapy. Serial blood pressure did not change throughout the study period.

The melanocortin system involves five G-protein-coupled melanocortin receptors in a signaling pathway that regulates a diverse number of physiologic functions, such as energy homeostasis, food intake, skin pigmentation, and exocrine gland secretion in addition to many more. MC4R is a G-protein-coupled receptor expressed primarily in the central nervous system and brain, including the cortex, thalamus, hypothalamus, brainstem, and spinal cord.\(^{3,6}\) In the hypothalamus, MC4R is expressed in the ventromedial, lateral, dorsomedial, and paraventricular nuclei.\(^{3,6}\) During fetal development of rats, MC4R is also expressed in the heart, lung, and kidney.\(^{3,6}\) MC4R binds both α- and β-melanocortin (MC) and has a low affinity for γ-MC. MC4R plays an important role in regulating food intake and energy homeostasis—when MC4R is activated, the result is a decrease in food intake coupled with an increase in energy expenditure.\(^{3,8}\)

The MC4R plays a role in the development of cachexia associated with many primary disease processes. Chronic diseases cause an increase in proinflammatory cytokines; pro-opiomelanocortin...
neurons in the arcuate nucleus express type I interleukin (IL)-1 receptor and respond to IL-1β (a pro-inflammatory cytokine) stimulation by increasing the release of α-MC, which then stimulates MC4R. MC4R stimulation results in decreased appetite and increased energy expenditure.1–3,8,9 Several studies have shown that antagonism of MC4R has therapeutic potential for cytokine-induced cachexia.4,5,7–9,20 The MC4R in dogs is fully functional and plays as important a role in physiology as in people, supporting dogs as a large animal model for translation to people.3,21 The MC4R is widely expressed in the brain and has a role in body weight regulation. Mice lacking MC4R lose weight and this implies an inhibitory role for MC4R in body energy balance and metabolism.3,4,9,21 Weight gain was anticipated in dogs treated with TCMCB07 and weight in all dogs returned to baseline within 60 days of treatment discontinuation.

Coat color changes were also noted in treated dogs and were similar to that described previously with administration of a melanotrophic peptide in a dog.22 In preliminary studies, TCMCB07 was found to be an agonist of melanocortin receptor (MCR)1 and 5 in addition to its MC4R antagonist actions (and poor antagonist of MCR2). MCR1, similar to MC4R, is a G-protein-coupled receptor that plays a primary role in skin pigmentation—when stimulated, melanin production is increased resulting in increased pigmentation and MCR1 controls coat color in mice.3 The darkening of coats in dogs in this study is most likely a result of the MCR1 stimulation by TCMCB07 and this underlines the complex physiology of MCR and their role in physiology.

**FIGURE 5** Arrhythmic events in the five dogs (female n = 3 and male n = 2) treated with TCMCB07 at a dosage of 2.25 mg kg⁻¹ per day for 28 days. A: Ventricular ectopy as measured by 24-hour Holter monitor in five dogs during the study period. An increase in ventricular ectopy over the treatment period was noted (p < .001) and in one dog ventricular ectopy increased from a baseline of 44 complexes in 24 hours to 89 complexes in 24 hours on day 28. B: Episodes of sustained sinus tachycardia as measured by 24-hour Holter monitor in five dogs during the study period. A decrease in the occurrence of sustained sinus tachycardia was noted over the study period (p < .023). Analysis of arrhythmia events per 24 hours was conducted with a Friedman nonparametric ANOVA

**FIGURE 6** Systolic arterial pressure in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg⁻¹ per day for 28 days. Circles represent systolic arterial pressure just prior to TCMCB07 on that particular treatment day, and squares represent systolic arterial blood pressure 4 hours following TCMCB07. No difference was found in dogs pre- and post-treatment or between treatment days.
An increased eosinophil count was noted over time in dogs on study; of note, the eosinophil count remained within the reference interval in all dogs and there were no examination or behavior changes. In humans, α-MC activation of MC4R results in reduced inflammation in the brain by causing a reduction in nitric oxide and inducible nitric oxide synthase. It is possible that blockade of some of the anti-inflammatory effects of MC4R stimulation may have led to the increased eosinophil count, however, the mechanism of this is not clear and the clinical relevance was unknown. Plasma phosphorous also changed over time, both increasing and decreasing during the study period and remaining within normal limits throughout the study. This had no clinically detectable effect on dogs based on examination and behavior, and these values remained within reference intervals. Since a trend in phosphorous was not detected and no research links phosphorus metabolism and excretion to the melanocortin system, we hypothesize these changes reflect normal variation over time.

MC4R is expressed in the nucleus of the solitary tract, which can regulate cardiovascular functions, therefore, monitoring for cardiovascular side effects was an important objective of this study. Activation of the MC4R can result in increased blood pressure and central antagonism of MC4R using different medications in other studies have resulted in decreased mean arterial pressure and bradycardia with increased food intake and weight gain. TCMCB07 is a cyclic substituted melanocortin antagonist with the structure Ac-Nle-cyclo[Asp-Pro-Dnal(2')-Arg-Trp-Lys]-DVal-DPro-NH₂

### TABLE 1  Pharmacokinetic parameters of TCMCB07 at both low (0.75 mg kg⁻¹ once daily) and high (2.25 mg kg⁻¹ once daily). Mean +SEM; n = 5

|          | T_max (hours) | AUC (area) µg hr ml⁻¹ | T₁/₂ (hours) | V_d (area) ml | C_max µg ml⁻¹ |
|----------|--------------|------------------------|--------------|--------------|---------------|
| **Low dose** |              |                        |              |              |               |
| Day 1    | 0.9 ± 0.3    | 11.7 ± 1.3             | 2.0 ± 0.1    | 1730 ± 141   | 2.6 ± 0.3     |
| Day 5    | 1.2 ± 0.2    | 11.6 ± 1.5             | 1.9 ± 0.2    | 1612 ± 225   | 2.6 ± 0.4     |
| Day 28   | 1.5 ± 0.4    | 14.1 ± 2.2             | 2.9 ± 0.2    | 223 ± 482    | 2.1 ± 0.5     |
| **High dose** |             |                        |              |              |               |
| Day 1    | 1.1 ± 0.3    | 30.5 ± 2.7             | 2.6 ± 0.2    | 2452 ± 249   | 4.9 ± 0.3     |
| Day 5    | 1.6 ± 0.3    | 32.3 ± 5.2             | 2.5 ± 0.1    | 2406 ± 327   | 5.0 ± 0.8     |
| Day 28   | 2.2 ± 0.5    | 29.1 ± 5.0             | 3.7 ± 0.5    | 4241 ± 415   | 3.6 ± 0.3     |

### FIGURE 7  Plasma concentrations of TCMCB07 in dogs treated with TCMCB07; mean +/- standard deviation; solid circle data points represent low dose (0.75 mg kg⁻¹ per day; n = 5) day 1 solid square low-dose day 5 and solid triangle low-dose day 28. Open circle represents high dose (2.25 mg kg⁻¹ per day; n = 5) day 1, open square high-dose day 5, and open triangle high-dose day 28.

An increased eosinophil count was noted over time in dogs on study; of note, the eosinophil count remained within the reference interval in all dogs and there were no examination or behavior changes. In humans, α-MC activation of MC4R results in reduced inflammation in the brain by causing a reduction in nitric oxide and inducible nitric oxide synthase. In another study, a selective MC4R antagonist blocked the anti-inflammatory effects of α-MC. It is possible that blockade of some of the anti-inflammatory effects of MC4R stimulation may have led to the increased eosinophil count, however, the mechanism of this is not clear and the clinical relevance was unknown. Plasma phosphorous also changed over time, both increasing and decreasing during the study period and remaining within normal limits throughout the study. This had no clinically detectable effect on dogs based on examination and behavior, and these values remained within reference intervals. Since a trend in phosphorous was not detected and no research links phosphorus metabolism and excretion to the melanocortin system, we hypothesize these changes reflect normal variation over time.

MC4R is expressed in the nucleus of the solitary tract, which can regulate cardiovascular functions, therefore, monitoring for cardiovascular side effects was an important objective of this study. Activation of the MC4R can result in increased blood pressure and central antagonism of MC4R using different medications in other studies have resulted in decreased mean arterial pressure and bradycardia with increased food intake and weight gain. TCMCB07 is a cyclic substituted melanocortin antagonist with the structure Ac-Nle-cyclo[Asp-Pro-Dnal(2')-Arg-Trp-Lys]-DVal-DPro-NH₂

### TABLE 2  Histamine median and range in ng ml⁻¹

|          | Baseline | 30 minutes | 1 hour | 2 hours | 4 hours | 12 hours | 24 hours |
|----------|----------|------------|--------|---------|---------|----------|----------|
| Day 1    | Median   | 7.55       | 8.02   | 8.8     | 9.25    | 11.2     | 14.3     | 9.4      |
|          | Range: Low | 2.33     | 2.81   | 2.6     | 1.82    | 2.13     | 2.7      | 1.73     |
|          | High     | 15.3      | 15.3   | 15      | 13.5    | 16.5     | 16.3     | 15.3     |
| Day 5    | Median   | 12.3      | 13.2   | 14.4    | 16.3    | 14.8     | 15.9     | 16.1     |
|          | Range: Low | 3.22    | 6.31   | 7.32    | 5.23    | 2.85     | 2.97     | 3.22     |
|          | High     | 29.7      | 24.4   | 20.3    | 26.9    | 22       | 28.3     | 30.9     |
| Day 28   | Median   | 17.5      | 20.8   | 15.7    | 18.1    | 17.9     | 17.1     | 17       |
|          | Range: Low | 4.97    | 6.75   | 10.4    | 7.94    | 3.51     | 4.5      | 3.68     |
|          | High     | 44.7      | 44.4   | 46.8    | 41.3    | 39.3     | 44.9     | 40.8     |
designed through a series of iterative amino acid substitutions to avoid potential cardiovascular side effects. Thus, while TCMCB07 is a cyclic melanocortin 3/4 receptor antagonist, it also includes degradation-resistant N- and C-terminal extensions designed to prevent exposure of free RFamide pharmacophore which is part of basic melanocortin peptides. These features were incorporated to reduce or eliminate cardiovascular side effects. In this group of dogs, no changes in blood pressure or heart rate were detected in the high-dose group over the 28-day study period. However, treatment with TCMCB07 was associated with a decrease in the occurrence of sustained sinus tachycardic episodes. Holter analysis revealed a decrease in sustained tachycardic episodes over time, whereas the minimum, maximum, and average daily heart rates remained unchanged. While this effect may be mediated by antagonism of MC4R, it is important to note that no episodes of bradycardia were noted in any dog on physical examination or on 24-hour Holter monitoring on days 5 and 28. It is more likely that the dogs became acclimated to laboratory personnel and to wearing the Holter over time, thus reducing surges in sympathetic tone and resultant sinus tachycardic episodes. Treatment with TCMCB07 was found to be associated with an increase in ventricular ectopy. It is possible that TCMCB07 is proarrhythmic; however, it has been previously shown that clinically healthy dogs can have ventricular ectopic events noted on Holter recordings and there can be a substantial variation of up to 80% from day-to-day. While heart rate variability was not directly measured in this study due to the lack of available software on the Holter systems used in this study, the minimum daily heart rate has been shown to correlate with several measures of heart rate variability, and thus serves as a simpler surrogate marker in this study.

Histamine levels rose over the 28 days of the clinical trial. A number of cationic peptides are known for their ability to degranulate mast cells or basophils including mast cell degranulating peptide, a cyclic 22 amino acid cationic peptide, and a component of bumble bee venom. αMC is also known to promote secretion of histamine in vitro and in vivo and it is well established that MC3/4 receptor antagonists can act as agonists on the MC1 and MC5 receptors, thus the ability to TCMCB07 to increase plasma histamine may be due to agonist actions at these receptors. Alternatively, a number of cationic peptide drugs have been shown to activate mast cell degranulation via activation of Mas-related G-protein-coupled receptors.

The physiological significance of the increased histamine following administration of TCMCB07 remains unknown due to the lack of a change in behavior, physical examination findings (including appetite and activity level), and arterial pressure in either the short term (on the day of injection) or over the course of the study.

Once-daily subcutaneous administration of TCMCB07 was safe and well-tolerated for up to 28 days in dogs when administered at 0.75 mg kg⁻¹ and 2.25 mg kg⁻¹. The only clinically relevant side effect noted in this study was weight gain. No changes in blood pressure or heart rate were detected in either group. Based on this data and the described pharmacokinetics, a clinical trial in companion dogs with spontaneously occurring cachexia is warranted to confirm safety and determine efficacy.

CONFLICT OF INTEREST

MC and KG are shareholders in Tensive Controls, Inc. SA-B, SL, DS, JN-N, BJ, and HD have no conflicts of interest to disclose.

AUTHORS CONTRIBUTION

Participated in research design: Axiak-Bechtel, Leach, Scholten, Newton-Northup, Johnson BJ, Durham, Gruber, Callahan. Conducted experiments: Axiak-Bechtel, Leach, Scholten, Newton-Northup, Johnson BJ, Durham, Gruber, Callahan. Performed data analysis: Axiak-Bechtel, Leach, Scholten, Newton-Northup, Gruber, Callahan. Wrote or contributed to the writing of the manuscript: Axiak-Bechtel, Leach, Scholten, Newton-Northup, Johnson BJ, Durham, Gruber, Callahan.

RECOMMENDED SECTION ASSIGNMENT

Drug Discovery and Translational Medicine.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.
REFERENCES

1. Evans WJ, Morley JE, Argilés J, et al. Cachexia: a new definition. Clin Nutr. 2008;27(6):793-799. https://doi.org/10.1016/j.clnu.2008.06.013
2. Argilés JM, Busquets S, Stemmler B, López-Soriano FJ. Cancer cachexia: understanding the molecular basis. Nat Rev Cancer. 2014;14(11):754-762. https://doi.org/10.1038/nrc3829
3. Tao Y-X. The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. Endocr Rev. 2010;31(4):506-543. https://doi.org/10.1210/er.2009-0037
4. Fearon K, Arends J, Baracos V. Understanding the mechanisms and treatment options in cancer cachexia. Nat Rev Clin Oncol. 2013;10(2):90-99. https://doi.org/10.1038/nrclinonc.2012.209
5. Argilés JM, López-Soriano FJ, Toledo M, Betancourt A, Serpe R, Busquets S. The cachexia score (CASCO): a new tool for staging cachectic cancer patients. J Cachexia Sarcopenia Muscle. 2011;2(2):87-93. https://doi.org/10.1007/s13539-011-0027-5
6. Humphreys MH, Ni X-P, Pearce D. Cardiovascular effects of melanocortins. Eur J Pharmacol. 2011;660(1):43-52. https://doi.org/10.1016/j.ejphar.2010.10.102
7. Dallmann R, Weyermann P, Anklin C, et al. The orally active melanocortin-4 receptor antagonist BL-6020/979: a promising candidate for the treatment of cancer cachexia. J Cachexia Sarcopenia Muscle. 2011;2(3):163-174. https://doi.org/10.1007/s13539-011-0039-1
8. DeBoer MD, Marks DL. Therapy insight: Use of melanocortin antagonists in the treatment of cachexia in chronic disease. Nat Clin Pract Endocrinol Metab. 2006;2(8):459-466. https://doi.org/10.1038/ncpendmet0221
9. Cheung WW, Mak RH. Melanocortin antagonism ameliorates muscle wasting and inflammation in chronic kidney disease. Am J Physiol Renal Physiol. 2012;303(9):F1315-1324. https://doi.org/10.1152/ajprenal.00341.2012
10. Hu Y, Gruber KA, Smith DE. Characterization of the cellular transport mechanisms for the anti-cachexia candidate compound TCMCB07. J Cachexia Sarcopenia Muscle. 2020;11(6):1677-1687. https://doi.org/10.1002/prp2.777
11. Gruber KA, Cowan JA, Cowan A, et al. Vector-mediated transport producing drug-like peptides. Pharmacol Toxicol. 2018. https://doi.org/10.1111/ptx.12538
12. Zhu X, Callahan MF, Gruber KA, Szumowski M, Marks DL. Melanocortin-4 receptor antagonist TCMCB07 ameliorates cancer- and chronic kidney disease-associated cachexia. J Clin Invest. 2020;130(9):4921-4934. https://doi.org/10.1172/JCI138392
13. Adachi S, Nakano T, Vilagostis H, Metcalfe DD. Receptor-mediated modulation of murine mast cell function by alpha-melanocyte stimulating hormone. J Immunol. 1999;163(6):3363-3368.
14. Shimizu K, Andoh T, Yoshihisa Y, Shimizu T. Histamine released from epidermal keratinocytes plays a role in u- melanocyte-stimulating hormone-induced itching in mice. Am J Pathol. 2015;185(11):3003-3010. https://doi.org/10.1016/j.ajpath.2015.07.015
15. Jain S, Panyutin A, Liu N, et al. Melanotan II causes hypothermia in mice by activation of mast cells and stimulation of histamine 1 receptors. Am J Physiol Endocrinol Metab. 2018;315(3):E357-E366. https://doi.org/10.1152/ajpendo.00024.2018
16. Ware W. Practical use of holter monitoring. Comp Cont Educ Pract Vet. 1998;20:167-177.
17. Schellenberg S, Glaus TM, Reusch CE. Effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious untrained beagles. Vet Rec. 2007;161(12):418-421. https://doi.org/10.1136/vr.161.12.418
18. Harding SD, Sharmans RJL, Faccenda E, et al. The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucleic Acids Res. 2018;46(D1):D1091-D1106. https://doi.org/10.1093/nar/gkx1121
19. Alexander SPH, Cidlowski JA, Kelly E, et al. THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Nuclear hormone receptors. Br J Pharmacol. 2019;176(51): https://doi.org/10.1111/bph.14750
20. Kanat O, Cubukcu E, Avci N, et al. Comparison of three different treatment modalities in the management of cancer cachexia. Tumori. 2013;(2013March-April). https://doi.org/10.1700/1283.14197
21. Yan J, Tao Y-X. Pharmacological characterization of canine melanocortin-4 receptor and its natural variant V213F. Domest Anim Endocrinol. 2011;41(2):91-97. https://doi.org/10.1016/j.domianend.2011.05.002.
22. Johnson PD, Dawson BV, Dorr RT, Hadley ME, Levine N, Hruby VJ. Coat color darkening in a dog in response to a potent melanotropic peptide. Am J Vet Res. 1994;55(11):1593-1596.
23. Meurs KM, Spier AW, Wright NA, Hamlin RL. Use of ambulatory electrocardiography for detection of ventricular premature complexes in healthy dogs. J Am Vet Med Assoc. 2001;218(8):1291-1292.
24. Noszczyk-Nowak A. ECG Parameters in 24-hour Holter monitoring in healthy dogs. Bull Vet Inst Pulawy. 2009;53:499-502.
25. Spier AW, Meurs KM. Evaluation of spontaneous variability in the frequency of ventricular arrhythmias in Boxers with arrhythmogenic right ventricular cardiomyopathy. J Am Vet Med Assoc. 2004;224(4):538-541.
26. Burr RL, Motzer SA, Chen W, Cowan MJ, Shulman RJ, Heitkemper MM. Heart rate variability and 24-hour minimum heart rate. Biol Res Nurs. 2006;7(4):256-267. https://doi.org/10.1177/1099800405285268
27. Lu L, Kulkia M, Unsworth LD. Peptide-mediated mast cell activation: ligand similarities for receptor recognition and protease-induced regulation. J Leukoc Biol. 2017;102(2):237-251. https://doi.org/10.1189/jlb.3RU1216-539R
28. McNeil BD, Pundir P, Meeker S, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. Nature. 2015;519(7542):237-241. https://doi.org/10.1038/nature14022

How to cite this article: Axiak-Bechtel SM, Leach SB, Scholten DG, et al. Pharmacokinetics and safety of TCMCB07, a melanocortin-4 antagonist peptide in dogs. Pharmacol Res Perspect. 2021;9:e00777. https://doi.org/10.1002/prp2.777