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ORIGINAL ARTICLE

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

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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_d. Low-dose TCMCB07 had a C_{max} of 2.1 ug ml^{-1} at day 28, compared to high-dose TCMCB07 which had a C_{max} 3.6 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.

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 hypothalamus.[1-4] The result is an increased metabolic rate, decreased appetite, and loss of lean body mass.[1,4,5] Loss-of-function mutations in the melanocortin-4 receptor (MC4R) gene is associated with an increased appetite and lean body mass in people, making MC4R antagonism a promising target for cachexia therapy.[3,6] 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 haploinsufficiency of MC4R have a reduction in autonomic tone, bradycardia, and increased incidence of obesity-associated hypertension.[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 features 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.[10,11] Peripheral treatment—subcutaneous, intraperitoneal, 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.[12]

Given the promising results of TCMCB07 in rodent models of cachexia, the objectives of this study were to examine pharmacokinetics and the safety profile of TCMCB07 administered subcutaneously to normal dogs. The hypothesis was that TCMCB07 would be well tolerated with no evidence of cardiotoxicity as measured by 24-hour ambulatory electrocardiography (ECG) and serial blood pressure, or systemic toxicity as measured by serial complete blood counts, and chemistry panels. In addition, histamine was measured throughout the study in dogs receiving high-dose treatment, as αMC is also known to promote secretion of histamine in vitro and in vivo, and antagonists of MC3 and MC4 can act as agonists for the MC1 and MC5 receptors.[13-15]

2 | MATERIALS AND METHODS

2.1 | Drug production

TCMCB07 was manufactured by CPC Scientific Inc. under cGMP conditions. Active pharmaceutical ingredient was dissolved in milliQ water at 10 mg ml^{-1}, sterile filtered with 0.22 μm Millex®GP PES Membrane (Merck Millipore Ltd, Tullagreen IRL) and frozen at −20°C until the day of use.

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 monitoring.[16] Holter monitoring occurred on an acclimation day when placebo was administered and on day 1, 5, and 28 during drug administration 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 orthogonal plane lead X. The ECG electrodes and leads were covered 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 abnormal pauses (pauses >3 secs), bradyarrhythmias, 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[17] 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 proteins 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 $p \leq .05$ 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 ($p \leq .054$), there was a significant overall effect of time on drug ($p \leq .001$) and a significant interaction between dose and time ($p \leq .001$) 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

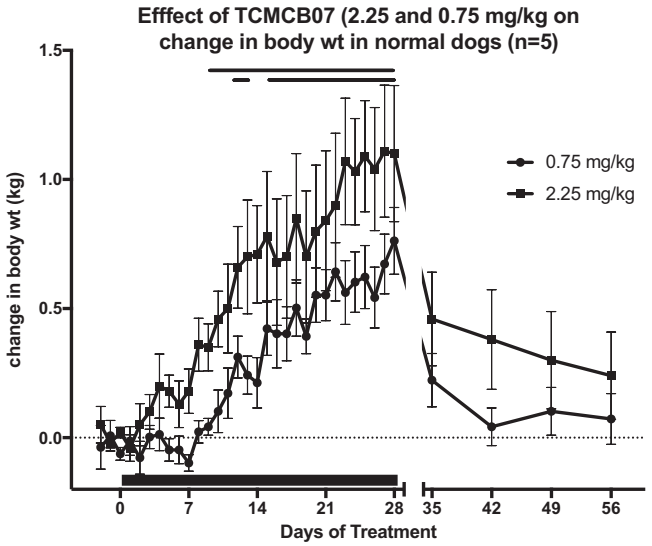

FIGURE 1 Bodyweight in dogs treated with TCMCB07; the mean and standard error of the mean are represented. Circle data points represent low dose (0.75 mg kg^{-1} per day; n = 5) and square high dose (2.25 mg kg^{-1} per day; n = 5). Bars above data indicate a significant difference between baseline average 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

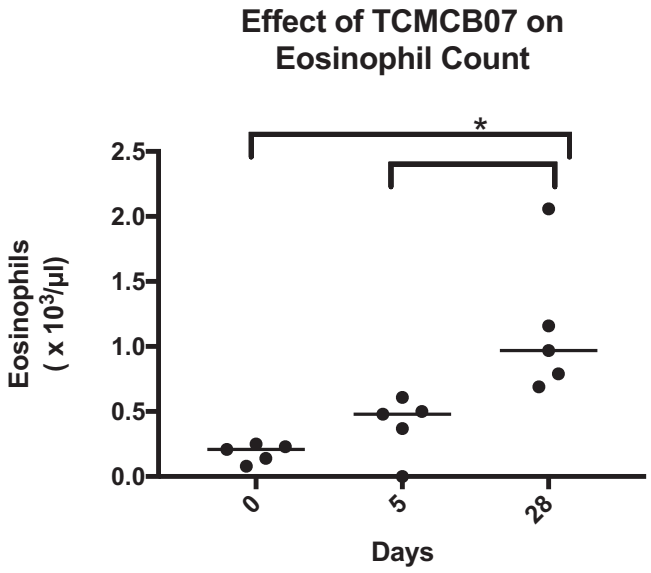

FIGURE 2 Eosinophil counts in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg^{-1} per day for 28 days. Eosinophil counts remained within the normal range, but were increased (RM ANOVA F=14.7, $p \leq .01$) at day 5 (0.48, 0–0.61, median and range) and 28 (0.97, 0.69–2.06),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

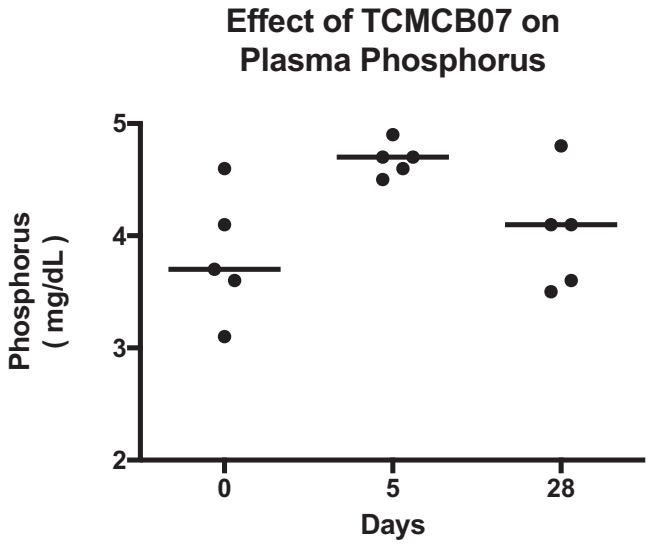

FIGURE 3 Serum phosphorous concentrations in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg^{-1} per day for 28 days. Overall RM ANOVA indicated a change in serum phosphorus (F = 6.5, $p \leq .03$), but there were no statistically significant changes at any timepoint and values remained within the normal range

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 × 10^3/µl; range 0.08–0.25 × 10^3/µl) 5 (median 0.48 ×10^3/µl, range 0–0.61 × 10^3/µl), and 28 (median 0.97 × 10^3/µl, range 0.69–2.06 × 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

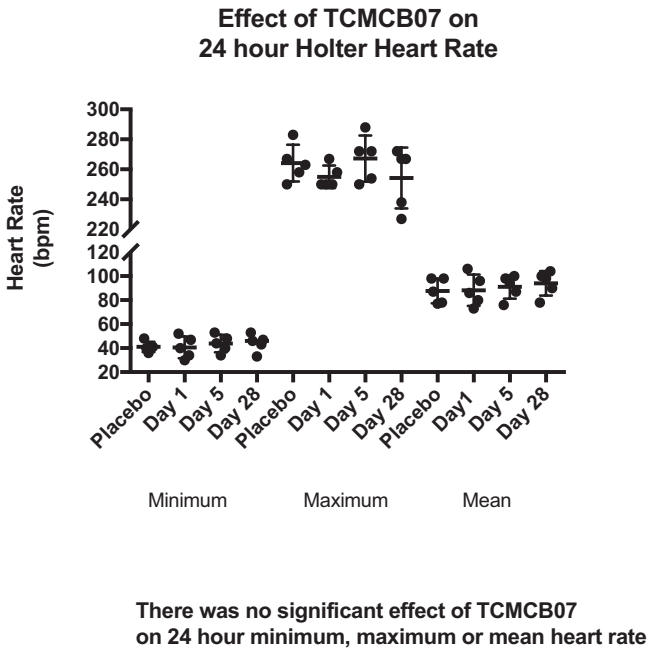

FIGURE 4 Heart rate measured by 24-hour Holter monitor in dogs treated with TCMCB07. Five dogs were acclimated to the Holter device and treated with a placebo and then treated at a dosage of 2.25 mg kg^{-1} per day for 28 days with additional Holter measurements on day 1, 5, and 28. The median and range of the minimum, maximum, and average daily heart rates are represented. There was no statistically significant difference in dogs on acclimation/placebo or day 1, day 5, day 28 of drug treatment in daily minimum (F=0.9, $p \leq .45$), maximum (F = 1.5, $p \leq .29$), or average (F = 1.8, $p \leq .23$) daily heart rate

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_{max} of 2.6 ug ml^{-1} at day 1 and 5 and 2.1 ug ml^{-1} at day 28, compared to high-dose TCMCB07 which had a C_{max} of 4.9 ug ml^{-1} at day 1, 5.0 ug ml^{-1} at day 5 and 3.6 ug ml^{-1} at day 28.

3.2 | Histamine Analysis

There was no elevation of plasma histamine within 24 hours of injection ($p \leq .63$). However, there was a significant elevation of plasma histamine across days ($p \leq .02$). 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 ($p \leq .04$) (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] 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] 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

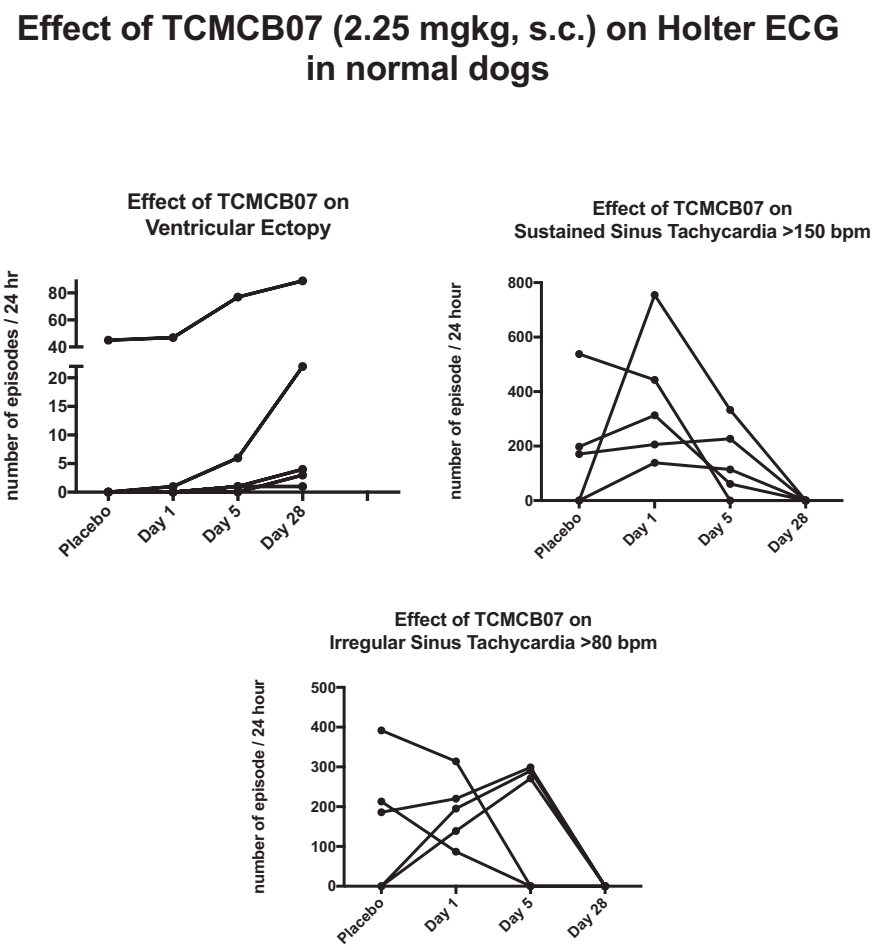

FIGURE 5 Arrhythmic events in the five dogs (female $n = 3$ and male $n = 2$) treated with TCMCB07 at a dosage of 2.25 mg kg^{-1} per day for 28 days. A: Ventricular ectopy as measured by 24-hour Holter monitor in five dogs during the study period. An increase in ventricular ectopy over the treatment 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

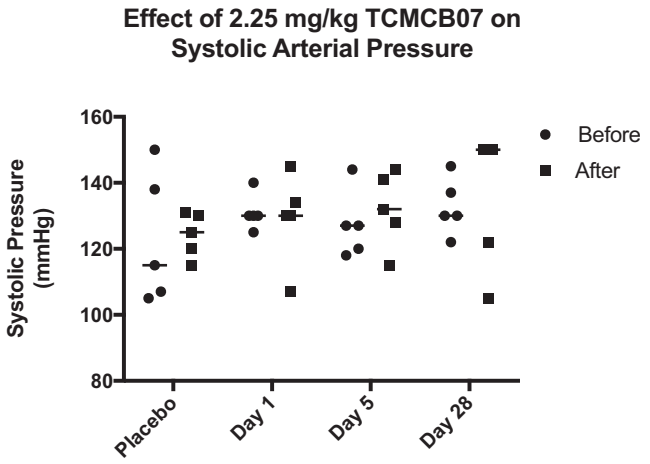

FIGURE 6 Systolic arterial pressure in dogs treated with TCMCB07. Five dogs were treated at a dosage of 2.25 mg kg^{-1} per day for 28 days. Circles represent systolic arterial pressure just prior to TCMCB07 on that particular treatment day, and squares represent systolic arterial blood pressure 4 hours following TCMCB07. No difference was found in dogs pre- and post-treatment or between treatment days

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 melanotropic 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.

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}	$T_{½}$ (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

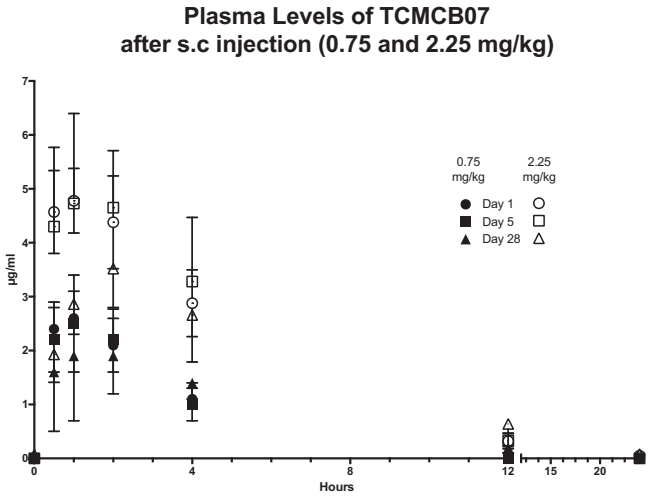

FIGURE 7 Plasma concentrations of TCMCB07 in dogs treated with TCMCB07; mean +/− standard deviation; solid circle data points represent low dose (0.75 mg kg^{-1} per day; n = 5) day 1 solid square low-dose day 5 and solid triangle low-dose day 28. Open circle represents high dose (2.25 mg kg^{-1} per 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.[3,13] In another study, a selective MC4R antagonist blocked the anti-inflammatory effects of α-MC.[7] 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,[3] 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.[7-9] TCMCB07 is a cyclic substituted melanocortin antagonist with the structure Ac-NIe-cyclo[Asp-DPro-DNal(2')-Arg-Trp-Lys]-DVal-DPro-NH_2

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

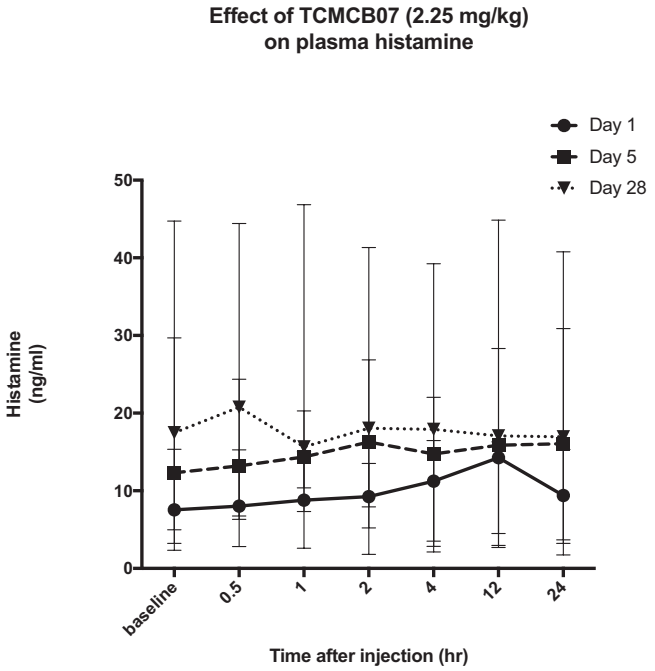

FIGURE 8 Plasma histamine in dogs tested with a high dose of TCMCB07 (2.25 mg kg^{-1} per day; $n = 5$). RM ANOVA showed that there was no effect ($F = 0.74$, $p \leq .63$) of time after injection up to 24 hours on plasma histamine levels. However, there was a significant effect of days ($F = 7.3$, $p \leq .02$) indicating that over the course of the study plasma histamine levels rose. There was a significant interaction between time after injection and days ($F = 2$, $p \leq .04$). Post hoc analysis indicated that at each time point after injection, values for day 5 and 28 differed from day 1 and each other, with the exception that at 24 hours after injection, day 5 and 28 were not significantly different

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 tachycardiac 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[23,24] and there can be a substantial variation of up to 80% from day-to-day.[25] 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.[26]

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.[27] αMC is also known to promote secretion of histamine in vitro and in vivo[13-15] 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.[28] 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.

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