Melanocortin-4 receptor antagonist TCMCB07 ameliorates cancer- and chronic kidney disease-associated cachexia

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Conflicts of interest

The authors declare first-tier potential conflicts of interest. Drs. Callahan, Gruber and Marks have equity in Tensive Controls Inc. Drs. Gruber and Callahan were salaried Officers of Tensive Controls, Inc. Dr. Marks was the recipient of a Subaward from Dr. Gruber’s NIH SBIR grant 2R44CA150703.
Abstract

Cachexia, a devastating wasting syndrome characterized by severe weight loss with specific losses of muscle and adipose tissue, is driven by reduced food intake, increased energy expenditure, excess catabolism, and inflammation. Cachexia is associated with poor prognosis and high mortality, and frequently occurs in patients with cancer, chronic kidney disease, infection, and many other illnesses. There is no effective treatment for this condition. Hypothalamic melanocortins have a potent and long-lasting inhibitory effect on feeding and anabolism, and pathophysiological processes increase melanocortin signaling tone leading to anorexia, metabolic changes, and eventual cachexia. We utilized three rat models of anorexia and cachexia (LPS, methylcholanthrene sarcoma, and 5/6 subtotal nephrectomy) to evaluate efficacy of TCMCB07, a synthetic antagonist of the melanocortin-4 receptor. Our data show that peripheral treatment of TCMCB07 with intraperitoneal, subcutaneous, and oral administration increased food intake and body weight, and preserved fat mass and lean mass during cachexia and LPS-induced anorexia. Furthermore, administration of TCMCB07 diminished hypothalamic inflammatory gene expression in cancer cachexia. These results suggest that peripheral TCMCB07 treatment effectively inhibits central melanocortin signaling and therefore stimulates appetite and enhances anabolism, indicating that TCMCB07 is a promising drug candidate to treat cachexia.
Introduction

Cachexia is a devastating and multifactorial wasting syndrome, consisting of anorexia, loss of adipose tissue and lean body mass, and a paradoxical increase in energy expenditure and catabolism that accompanies a variety of illness conditions, such as cancer, chronic kidney disease (CKD), sepsis, chronic obstructive pulmonary disease, congestive heart failure, and HIV infection (1, 2). The severity of cachexia in these illnesses is often the primary determining factor for both quality of life and eventual mortality (2, 3). Cancer cachexia affects 50-80% of cancer patients and it causes 20-40% of all cancer deaths and aggravates toxicity and complications of cancer therapy (4-6). Advanced CKD particularly with uremia condition is frequently associated with cachexia. Survival with end-stage renal disease is even worse than with most cancers, and the mortality rate of maintenance dialysis patients is above 20% per year (7). At the patient level, longevity has consistently been observed in patients with CKD who have more muscle and /or fat, who report better appetite and who eat more (1).

It has been known for 30 years and well established that the hypothalamic melanocortin system plays a central role in regulation of appetite, body mass, and energy homeostasis (8, 9). Pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) expressing neurons located in arcuate nucleus of the hypothalamus are the primary regulators of melanocortin signaling in the brain. This system is unique not only in having the capability of sensing signals from a wide array of hormones, nutrients and afferent neural inputs, but also in having the ability of transducing both anorexigenic agonists (e.g. α-melanocyte-stimulating hormone, α-MSH) and orexigenic antagonists/inverse agonists (e.g. AgRP) of melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R). While MC3R neurons likely contribute to behavioral adaptation to fasting and nutrient partitioning, MC4R neurons are involved in feeding behavior, adaptive thermogenesis and glucose homeostasis (10). Therefore, this system provides a logical target for developing drugs to treat cachexia, obesity, and diabetes (8, 11-14). The pathophysiological processes of many illnesses increase the melanocortin tone that suppresses appetite and anabolism leading to anorexia and
body weight loss, with inflammation as an essential driver (15). Inflammatory signals produced from acute illness responses and chronic conditions exert great influence on the hypothalamus perturbing the homeostatic system (16-18). Direct experimental evidence demonstrates that stimulating the hypothalamus with inflammatory cytokines, such as IL-1, IL-6, TNF-α, and leukemia inhibitory factor, leads to anorexia via altering the activity of POMC and AgRP neurons (19-22). Furthermore, increasing evidence supports that pathogenesis of cachexia caused by cancer, CKD, and many other chronic illnesses is tightly linked to inflammation (1, 6, 23-29).

Due to the complexity of pathogenesis and multifactorial pathophysiology of cachexia, and despite increased understanding of the mechanisms and many years of drug development effort, currently no effective medical intervention completely reverses cachexia and there are no approved drug therapies (5). A few potential treatments have been reported, including ghrelin receptor agonists and leptin antagonists (30-36), but these have not yet gained approval for treatment of patients with cachexia. The recent consensus and strategy in cachexia therapy is that adequate nutritional support remains a mainstay, whereas it is important to develop drugs that target overactivation of inflammation, catabolic processes and cell injury (5, 6). Melanocortin antagonists, as powerful orexigenic agents in simulation of appetite, have been investigated for more than a decade (37-39). The efficacy of several compounds was evaluated in animal models including our previous preclinical trials (40-42). However, to date, there are no drugs in this class that are approved for clinical treatment, highlighting the need to develop novel drugs with maximum safety, high efficacy, and treatment therapeutic feasibility (e.g. oral administration, blood brain barrier penetration). In particular, despite robust effects when centrally administering of this type drugs, it is crucial to overcome the huge barrier associated with drug penetration cross the blood-brain barrier that extremely limits the clinical applications.

In the present study, we evaluated eleven TCMC compounds (TCMCs), a series of synthetic MC4R antagonists, using three rat models: 1) LPS-induced acute anorexia, 2) cancer cachexia induced by methylcholanthrene (MCA) sarcoma, and 3) CKD-associated cachexia induced by 5/6
subtotal nephrectomy. Particularly, with a number of pilot studies, we selected TCMCB07 from eleven TCMCs and tested its efficacy via a series of comprehensive approaches. Our results demonstrate that both central and peripheral treatment of TCMCB07 via four administration routes: a) intracerebroventricular (ICV) injection, b) intraperitoneal (IP) injection, c) subcutaneous (SC) injection, and d) oral (intragastric) gavage, increases food intake, attenuates body weight loss, and preserves fat mass and lean mass. In addition, peripheral TCMCB07 treatment diminishes hypothalamic inflammation in cancer cachexia. This preclinical trial suggests that TCMCB07 is a promising drug candidate with a high efficacy to ameliorate cachexia, indicating this is a promising target for treatment of patients with cancer, CKD, and infectious disease.
Results

Compound and dose selection. In order to select the most effective drug candidate and determine a safe and minimal effective dose for subsequently comprehensive evaluation, we initially performed dose-response experiments with a series of eleven compounds (TCMCB01-10 and a deamidated version of one compound TCMCB7A) using acute LPS model (Supplementary Table S1). LPS doses (100-250 µg/kg/day) were determined based on the results of a dose-response study (Figure S1) and other’s reports (43). Due to prior research showing that synthetic MC4R antagonists are most effective when given directly into the central nervous system (CNS), central administration via ICV injection was chosen for test initiation of all eleven TCMC compounds. The dose of 2 µg/rat/day (1.5 nmol/rat/day) was selected for ICV injection. Consequently, 1.5-3 mg/kg/day was selected for IP or SC injection, and 6-12 mg/kg/day for oral gavage. Results from initial tests validated that TCMCB07 has the most robust positive effects on both stimulation of appetite and attenuation of body weight loss in LPS-treated rats. Furthermore, TCMCB07 has the best solubility among eleven TCMC compounds. Therefore, TCMCB07 was selected for further evaluation. Specificity of TCMCB07 to the MCR3R and MC4R was characterized by EuroscreenFast (Gosselies, Belgium). For the MC3R, the IC\textsubscript{50} was 11.1 nmol and Hill coefficient was 0.93. For the MC4R, the IC\textsubscript{50} was 31.5 nmol and the Hill coefficient was 1.22.

Central and peripheral administration of TCMCB07 attenuates anorexia and body weight loss induced by LPS. We first examined the effects of central administration of TCMCB07 on food intake and body weight gain in rats with LPS (Figure 1A). LPS-treated rats receiving ICV injection of TCMCB07 at 2 µg/rat/day (1.5 nmol/rat/day) significantly increased 24 h food intake compared with LPS-treated rats receiving saline injection (Figure 1B). Consequently, 24 h body weight loss was attenuated in LPS/TCMCB07-ICV group compared with LPS/saline-ICV group (Figure 1C). Similar results were observed from a subset control experiment with LPS-treated rats receiving ICV injection of the MCR4 antagonist SHU9119 (1.5 nmol/rat/day) or TCMCB02 (1.5 nmol/rat/day, Figure S2A, S2B). To evaluate effects of peripheral administration of TCMCB07 in rats with LPS-
induced acute illness, we performed IP injection and intragastric gavage with TCMCB07 in LPS-treated rats through separate experiments, and then measured food intake at multiple time points within 24 h or 48 h (Figure 1A). There was no difference in nocturnal food intake at the first two time points (2 h and 4 h) post-IP injection between LPS/TCMCB07-IP (3 mg/kg/day) and LPS/saline-IP groups (Figure 1D), but cumulative food intake at 16 h and 24 h time points was significantly different between LPS/saline-IP and LPS/TCMCB07-IP groups (Figure 1D). 24 h body weight loss in LPS/TCMCB07 group was attenuated compared with the saline-treated group (Figure 1E). To compare with a previously reported derivative–of SHU9119 (PG932) (42), we performed a subset IP experiment with PG932 and TCMCB03 and found that both PG932 and TCMCB03 had no positive effects on LPS-treated rats (Figure S2C, S2D). Based on the positive effects of TCMCB07 through ICV and IP administration, we next investigated whether oral administration of TCMCB07 had similar effects. Intragastric gavage twice daily with water or TCMCB07 (10 mg/kg/day) was performed in LPS-treated rats (Figure 1A). Food intake post-treatment at 24 h (day 0-1) but not 4 h was significantly increased in LPS/TCMCB07-gavage group compared with LPS/water-gavage group (Figure 1F, 1G), and there was no difference at either baseline (previous day of treatment) or day1-2 (the second day post-treatment, Figure 1G). There was a difference in body weight gain between two groups at 24 h time point post-treatment but not baseline or day1-2 post-treatment (Figure 1H).

Central administration of TCMCB07 attenuates cancer cachexia. Previous studies demonstrated that cancer cachexia can be attenuated by genetic deletion of MC4R or pharmacologic blockade of MC4R signaling using peptide antagonists, such as AgRP and SHU9119, administered intracerebroventricularly (18, 44). Consistent with this, our data showed that cachexia associated with MCA sarcoma was significantly attenuated by ICV administration of AgRP. Our previous studies and others’ reports validated that MCA sarcoma produces reliable, reproducible and consistent cancer cachexia recapitulating key characteristics of the clinical condition (22, 34, 45). Using this model, we tested the efficacy of central administration of TCMCB07 in reversing cancer cachexia (Figure 2A). 6 days after tumor implantation, tumors became palpable and tumor-bearing
rats started decreasing food intake due to rapid tumor growth and cachexia development, and then experienced sustained decline in food intake for the rest of experimental period (Figure 2B). Starting at day 8, all tumor-bearing rats received ICV injection with either saline or TCMCB07 (1.5 nmol/rat/day) at 4 pm once daily for total 4 consecutive days. As a result of ICV injection of TCMCB07, food intake was increased in the tumor/TCMCB07 group following two doses of TCMCB07 treatment, and the increase was sustained for next two days while animals received TCMCB07 injections (Figure 2B). In contrast, a sustained decline in food intake was exhibited in tumor/saline group (Figure 2B). In comparison with TCMCB07 treatment, ICV administration of AgRP showed similar effects in a parallel study (Figure 2C). Consequently, there was a significant difference in cumulative food intake between the two treatment groups (saline versus TCMCB07 or saline versus AgRP, Figure 2D, 2E). Body weights (before and after tumors removal) in tumor/TCMCB07 group and tumor/AgRP group were greater than that in tumor/saline group (Figure S3 and Figure 2F, 2G), but no difference was found in tumor mass (Figure 2H, 2I). Tumor-bearing rats treated with saline lost substantial fat mass compared to the initial baseline, whereas tumor-bearing rats treated with TCMCB07 or AgRP preserved a significant amount of fat mass (Figure 2J, 2K). There was a trend toward greater preservation of lean mass in tumor/TCMCB07 or tumor/AgRP group, but it did not reach statistical significance (Figure 2L, 2M).

*Intraperitoneal administration of TCMCB07 ameliorates cancer associated anorexia.* We next evaluated the consequences of peripheral administration of TCMCB07 on cancer cachexia through IP injection. Starting at day 7, tumor-bearing rats received IP injection once daily with either saline or TCMCB07 (3 mg/kg/day) for total 6 consecutive days (Figure 3A). After two doses of treatment, tumor-bearing rats receiving TCMCB07 exhibited greater food intake compared with the tumor/saline group (Figure 3B). Accordingly, 6-day cumulative food intake post-treatment in the tumor/TCMCB07 group was significantly greater than that in tumor/saline group (Figure 3C). These data suggest that IP administration of TCMCB07 effectively stimulates appetite during cancer cachexia. However, we did not find a difference in body weights between two treatment groups (Figure S4 and Figure 3D). Tumor weights between two treatment groups were identical.
There was a significant change in food intake and body weight gain post-implantation in tumor-bearing rats versus sham-operation rats (Figure 3B-3D), representing key features of this cachexia model.

*Subcutaneous administration of TCMCB07 ameliorates cancer cachexia.* Because neither central nor peritoneal drug delivery is convenient or feasible for clinical application, we further sought an alternative peripheral route to deliver TCMCB07. Subcutaneous administration is a feasible clinical option, and might permit a lower dose of TCMCB07 (1.5 mg/kg/day) treatment. We split one dose into two injections that were administered in early morning and later evening to maintain a sustained effective drug concentration. Similar to that observed with IP injection, after 2-day SC treatment, tumor-bearing rats receiving TCMCB07 with either high dose (3 mg/kg/day) or low dose (1.5 mg/kg/day) significantly increased food intake compared with saline-treated tumor rats, and the increase remained for the rest of experimental period while animals continually received TCMCB07 injection (Figure 4A). 6-day cumulative food intake in tumor rats treated with TCMCB07 (low dose or high dose) was remarkably greater than that in tumor rats treated with saline. There were no differences between groups before treatment (Figure 4B). Furthermore, a trend toward dose-dependent increase in food intake was observed (Figure 4A, 4B). There was no difference in 6-day cumulative food intake post-treatment between tumor/TCMCB07H and sham/saline groups (Figure 4B). Accompanying this positive effect on food intake, tumor-bearing rats receiving TCMCB07 SC injections, particularly with high dose, maintained body weight relative to saline-treated tumor rats (Figure S5 and Figure 4C). There was a significant body weight loss among all tumor-bearing animals compared with sham animals, but the degree was different between TCMCB07 treatment groups and saline group (Figure 4C). No difference was found in tumor mass between three tumor groups (Figure 4D). In the end of the experiment, blood was collected from all animals via cardiopuncture, and plasma was assayed for TCMCB07 concentration by investigators who were blinded to group information. All animals receiving TCMCB07 but not saline had detectable plasma TCMCB07 levels that were tightly correlated with administered dose (Table 1). Plasma TCMCB07 concentration in tumor/TCMCB07H group was nearly 2-fold higher.
than that in tumor/TCMCB07L group, which replicated the dose ratio (high dose versus low dose: 3 versus 1.5 mg/kg/day) (Figure 4E).

Subcutaneous administration of TCMCB07 reverses cachexia associated with CKD. To validate whether TCMCB07 has a universal efficacy in reversing cachexia associated with various conditions, we utilized a common non-malignancy cachexia model, CKD-related renal failure induced by surgical 5/6 subtotal nephrectomy (Figure 5A). After a week (day 0-7) of recovery from stage-I nephrectomy (unilateral partial nephrectomy, Neph-I) or sham-operation (sham), all surgical rats gained a similar amount of body weight (Figure 5B). After a week (day 7-14) of recovery from stage-II nephrectomy (contralateral total nephrectomy, Neph-II) or sham-operation, all neph rats lost a notable amount of body weight compared with sham group (Figure 5B), but there was no difference between two neph groups (Figure 5C). With SC administration of TCMCB07 twice daily (3 mg/kg/day), neph rats persistently gained body weight over the 14-day treatment period and finally caught up to sham/saline group (Figure 5B). However, neph rats receiving saline treatment gained weight more slowly (Figure 5B). Total body weight gain post-14-day treatment was significantly less in the neph/saline group than in the neph/TCMCB07 group (Figure 5C). As expected, daily food intake in neph rats receiving TCMCB07 was higher than that in the neph/saline group, and was similar to that in the sham/saline group (Figure 5D). Cumulative food intake was significantly different between neph/saline and neph/TCMCB07 groups for the entire 14-day treatment (Figure 5E). Remarkably, 14-day SC administration of TCMCB07 reversed both fat mass and lean mass loss in neph rats (Figure 5F, 5G).

To confirm the renal failure and drug distribution, plasma was assayed at the end of the study for concentration of blood urea nitrogen (BUN), creatinine (Cr), and TCMCB07. Both BUN and Cr were increased in all neph rats relative to sham rats consistent with chronic renal failure, and there was no difference between neph/saline and neph/TCMCB07 groups (Figure 6A, 6B). TCMCB07 concentration in plasma was detectable among all rats receiving TCMCB07 SC injection (Table 1), and undetectable in all rats receiving saline (Figure 6C). Correlation analysis suggested that 14-
day food intake was not associated with plasma BUN levels (Figure 6D), but it negatively correlated with plasma Cr levels (Figure 6E). Both 14-day food intake and body weight gain were positively correlated with plasma TCMCB07 levels (Figure 6F and 6G).

Subcutaneous administration of TCMCB07 diminishes hypothalamic inflammation in cancer cachexia. Because excessive inflammation is a key driver for cachexia, we examined whether subcutaneous administration of TCMCB07 attenuates hypothalamic inflammation associated with cachectic conditions. As we previously observed in this model as well as other cancer cachexia models (28, 34), there was a significant upregulation in inflammatory gene expression of Il1b, Il1r1 and Il6 but not Tnf in tumor-bearing rats relative to sham rats (Figure 7A). Indeed, SC administration of TCMCB07 suppressed the expression of Il1b, Il1r1 and Il6 in tumor-bearing rats compared with saline treatment (Figure 7A). Selp, a gene encoding P-selectin, has been linked to development of cancer cachexia (46). We observed highly upregulated Selp in tumor-bearing rats compared with sham rats, which is also found in other cancer cachexia models and cancer patients (28, 46). Interestingly, TCMCB07 treatment dramatically suppressed Selp gene expression in tumor-bearing rats compared with saline treatment (Figure 7A). These data suggest that SC administration of TCMCB07 suppresses hypothalamic inflammation, and this may contribute to its beneficial effects during cachexia. In the CKD model, there was a trend toward increases in gene expression of Il1b, Il1r1, Il6, Tnf and Selp in neph rats compared with sham rats, but none reached statistical significance (Figure 7B). Furthermore, there was no difference in Pomc gene expression between tumor or neph and sham rats, although there was a trend toward decrease of the expression in tumor/saline rats. Compared with sham rats, Agrp gene expression was upregulated in cachexia rats particularly in those with cancer (Figure 7A, 7B). Remarkably, TCMCB07 treatment suppressed Agrp upregulation in both tumor and CKD animals, which resulted in a similar transcriptional level to that found in sham animals (Figure 7A, 7B).
Discussion

Over last two decades, several research groups contributed to the development of orexigenic agents to treat cachexia, including MC4R antagonists and ghrelin analogs. Our lab evaluated a number of drug candidates and demonstrated that some of them had promising effects in amelioration of cachexia associated with cancer, CKD and heart failure, and LPS-induced acute anorexia, etc. (18, 33, 34, 40, 41, 47). Some of these agents have found their way into clinical trial in patients with cachexia (32, 35, 36). In the present study, we evaluated efficacy of eleven TCMC MC4R antagonists, then specifically focused on TCMCB07 in three rat models of LPS and cancer- and CKD-associated cachexia. We sought to validate that peripheral administration of TCMCB07 was feasible for effectively inhibiting central melanocortin signaling. Our results demonstrate that peripheral treatment of TCMCB07 has remarkably positive effects in stimulation of appetite, retention of body weight and preservation of fat mass and lean mass under cachectic conditions. Furthermore, our data indicate that peripheral TCMCB07 treatment attenuates hypothalamic inflammation associated with cancer cachexia. It is possible that this is an independent effect of this compound, as melanocortin signaling is known to impact inflammation (48, 49). However, this effect is generally associated with melanocortin agonists, and it is therefore possible that this effect is secondary to improved appetite and reduced catabolism secondary to TCMCB07 treatment.

LPS is a bacterial endotoxin and is extensively used to mimic acute infection and inflammation condition commonly seen in patients. Based on the results of the LPS dose-response study (Figure S1), we chose a moderate dose (100-250 µg/kg/day) for IP injection to elicit reproducible sickness behaviors without extremely severe morbidity and mortality. Moreover, because we used a moderate LPS dose, we were able to observe possible side effects derived from the compounds. Other than the expected increases in food consumption, TCMCB07 administration did not produce notable behavioral alterations in the experimental rats except when given the high dose (20 µg/rat/day) of central administration (ICV injection, Supplementary Table S1). Our data showed
that both central and peripheral TCMCB07 treatment, including oral administration, increased food intake and attenuated body weight loss in LPS-treated rats. We noted that the benefits of TCMCB07 in the acute LPS model were consistent, but delayed and not observed in the first hours after compound administration. In addition, the effective dose with IP injection or intragastric gavage was much higher than that with ICV injection. We also tested TCMCB07 at a very low dose (0.3-0.6 mg/kg/day) through IP and oral routes but did not find significant positive effects in LPS-treated rats (data not shown). Because we previously observed that repeated LPS injections to rodents can cause either desensitization or mortality, we were not able to test TCMCB07 in a setting of LPS-induced chronic condition. Collectively, through a series of acute studies in the acute LPS model, we found that TCMCB07 was the best drug candidate among the eleven TCMC compounds and established effective doses for both central and peripheral treatment.

Cancer cachexia is a wasting syndrome characterized by a significant reduction of body weight resulting predominantly from losses of adipose tissue and skeletal muscle (4, 6, 50). Anorexia is often a major contributor to the weight loss and muscle wasting, and even with administration of drugs that target overactivation of catabolic processes and inflammation, adequate nutritional support still remains a mainstay of cachexia therapy (5). Appetite improvement and increased food intake can provide more nutritional support to reverse negative energy balance and promote anabolism, maintenance of body weight and physical activity associated with quality of life and eventual survival (51). Furthermore, normalized nutritional intake can increase treatment tolerance to cancer therapy (51). Our data demonstrated that both central and peripheral administration of TCMCB07 effectively stimulated appetite leading to a remarkable increase in food intake during the aggressive tumor growth and subsequently rapid cachexia development. It is important to note that weight gain is brought about by increased fat mass and lean mass not water retention. As was seen with the progestational agent megestrol acetate, which increased water weight but did not increase lean mass, weight gain without lean mass gain may not improve disease outcome (52, 53). Our data from body composition measurement validated there was no water retention after TCMCB07 measurement. Furthermore, a recent retrospective study in patients with head...
and neck squamous cell carcinoma (HNSCC) demonstrated that increased BMI was associated with significantly improved survival, and decreased overall survival was predicted by skeletal muscle depletion, suggesting that decreased skeletal muscle mass or BMI can predict oncologic outcomes for patients with HNSCC (54). In the cancer cachexia study with ICV administration, 4 doses of TCMCB07 (or AgRP) treatment significantly increased body weight and fat mass, and produced a positive trend toward increased lean mass. A few possibilities could explain the non-significant lean mass gain. First, fat mass loss or gain is more rapid than lean mass in cancer cachexia (55), and a marked increase of fat mass was observed after a short period (4 days) of treatment. It is possible that with further treatment lean mass would have continued to accrue. Second, because of ethical considerations and the increasingly morbid nature of tumor-bearing animals, the animals had to be euthanized on day 12-14 post-tumor implantation. A less aggressive cancer type might facilitate experiments that would show long-term effects with a bigger difference on lean mass between two treatment groups. Another point to note is the variation caused by the complexity of cancer cachexia model. Although in the beginning of each experiment, a same amount of fresh tumor tissue was implanted into similar locations in animals with same sex and similar age and body size, it was difficult to control the later progression of tumor growth and subsequent cachexia. In the cancer cachexia study with IP administration, we extended treatment period up to 6 doses in 6 days, but did not find a difference in body weight gain between saline and TCMCB07 treatment groups. In order to maintain a sustained drug concentration in the body, in the study with SC administration, we split one dose of TCMCB07 into two separate injections performed in early mornings and evenings. It is likely that an optimized dosing route, starting time point, frequency and duration would significantly enhance the drug efficacy and treatment outcomes, and would facilitate lower effective doses (3 or 1.5 mg/kg/day). It is also likely that early treatment improves the attenuation of cachexia (4). We found no association between tumor mass and TCMCB07 treatment within all cancer cachexia studies, demonstrating that increased energy intake does not lead to increased tumor growth.
Using the CKD model employed here, we previously demonstrated that treatment with ghrelin and its analogs increased food intake and lean mass and decreased circulating inflammatory cytokines in CDK-associated cachexia (33, 56). In the present study, renal failure rats receiving SC injection of TCMCB07 twice daily consistently increased food intake and body weight, and after 14 days of treatment, body weight reached the levels found in sham rats, suggesting that subcutaneous treatment of TCMCB07 effectively reverses anorexia and growth failure associated with CKD. Furthermore, this relatively long-term TCMCB07 treatment completely prevented the muscle loss normally observed with this model. These treatment outcomes are likely attributed to increased nutrient intake and improved daily physical activity.

Importantly, TCMCB07 was detectable in the circulation and the concentrations corresponded to administered drug doses. 14-day food intake and body weight gain were positively correlated with plasma TCMCB07 concentration, which supports the notion that SC administration of TCMCB07 stimulates appetite in a dose-dependent fashion. We did not find a significant difference in plasma BUN and Cr among all nephrectomy rats regardless of treatment, indicating that improvement in food intake, body weight and lean mass was related to factors other than a change in renal function. Furthermore, 14-day food intake was not correlated with plasma BUN but was negatively correlated with Cr. Plasma Cr is more reliable and accurate for reflecting renal function than BUN, because plasma BUN is highly affected by extrarenal factors, such as heart failure, dehydration, liver function or dietary protein (57, 58).

Route of delivery is a crucial factor that often determines an agent’s efficacy, and feasibility in a clinical setting. We initially utilized a central approach (ICV administration) and tested whether TCMC compounds have effective melanocortin antagonist properties. With one dose of ICV injection, all eleven TCMC compounds showed a robust effect in stimulation of appetite. However, direct central delivery of this type drugs is a barrier that would prevent clinical application. The capability of melanocortin antagonists crossing through blood brain barrier is a substantial challenge for development of this class of drug. For example, AgRP and SHU9119 (melanocortin
antagonists) or melanotan-II (melanocortin agonist) have no effects with peripheral administration, although they are capable of inducing robust responses when given centrally (44, 59, 60). Our data showed that TCMCB07 efficiently penetrated the BBB and effectively inhibited central melanocortin signaling. Furthermore, we observed that with the same dose of TCMCB07, initiation of early and frequent dosing via the SC route was the most effective to treat cachexia. Oral administration is the most convenient and feasible route for clinical application. With this in mind, we performed intragastric gavage of TCMCB07 to the animals of the acute LPS model in several experiments and found positive effects, suggesting its oral availability. However, we were not able to conduct the oral administration in the cancer and CKD cachexia models for several reasons. First, the gavage method is extremely difficult for sick and fragile animals particularly at the last stage of cachexia. Second, unlike dosing in the acute model, chronic models require serial handling and restraint for the gavage, which represents a chronic additional stress for the cachectic animals. The nonspecific stress would dramatically disrupt the rats’ feeding behavior and increase their morbidity and mortality. Third, alternative methods of oral delivery (e.g. through drinking water or mixed with diet, etc.) can be undertaken, but it is difficult to ensure effective and consistent therapeutic dosing with these methods.

Because hypothalamic inflammation is an essential driver for both acute illness responses and cachexia (61, 62), we specifically analyzed inflammatory gene expression in hypothalamus to examine whether TCMCB07 SC treatment diminishes hypothalamic inflammation associated with cancer and CKD cachexia. The expression of Il1b, Il1r1 and Il6 was significantly suppressed by TCMCB07 treatment in cachexia associated with cancer. Whether this represents an intrinsic property of this compound, or is simply reflective of the amelioration of end organ dysfunction (e.g. gut leak) secondary to catabolism will require further investigation. We note that our CKD model does not produce significant CNS inflammation, but this is correlated with the relatively mild (albeit prolonged) cachexia typical of this model. Furthermore, it is possible that the cachexia in this model is at least partially driven by relative hyperleptinemia, and this would also be expected to respond to melanocortin antagonism (30, 63). Selp gene encoding P-selectin is associated with
the development of cachexia in tumor-bearing rats, LPS-treated mice, and patients with cancer
(46, 64). Consistent with these, we found a marked increase of Selp gene expression in
hypothalamus among all tumor-bearing rats relative to sham rats. Interestingly, SC TCMCB07
treatment dramatically suppressed Selp gene expression in tumor-bearing rats compared with
saline treatment, suggesting that Selp gene expression is a sensitive predictor for cancer
cachexia and TCMCB07 treatment effectively inhibits inflammation during cancer progression and
cachexia development. Furthermore, previous studies demonstrated that both acute and chronic
inflammation decreases hypothalamic Pomc transcription and AgRP secretion, while
simultaneously increasing Agrp transcription and α-MSH secretion (20, 22). The alteration in
hypothalamic Pomc and Agrp gene expression was also observed in a unique setting of severe
muscle catabolism associated with essential amino acid deficiency (65). Consistent with these
findings, we observed a significant upregulation of hypothalamic Agrp transcription in both
cancer- and CKD-associated cachexia, and a trend toward reduced Pomc transcription in cancer
but not CKD cachexia. Notably, TCMCB07 SC treatment remarkably suppressed Agrp
transcription in both cancer- and CKD-associated cachexia, suggesting that endogenous Agrp
transcription remained sensitive to overall body weight and food intake status. Obviously, there
are many other factors regulating food intake that were not explored in this study and therefore
that deserve further study in the future, including the expression of peripheral factors (e.g. ghrelin)
and various ligands and receptors in the CNS (e.g. growth hormone secretagogue receptor 1,
neuropeptide Y, etc.).

Despite of a tremendous progress in understanding the mechanisms of cachexia, therapeutic
interventions for this common condition associated with many advanced illnesses are lacking.
Because cachexia is driven by a variable combination of reduced food intake, increased energy
expenditure, excess catabolism and inflammation (5), a single drug therapy is unlikely to be
sufficient to treat this condition. Instead, it will likely require optimized therapeutic combinations
with effective orexigenic, anti-inflammatory and anti-catabolic agents (66, 67). TCMCB07, a
synthetic orexigenic agent, was developed through a classical approach against the central
melanocortin signaling and it showed high efficacy in attenuation of anorexia, body weight loss, fat mass loss and muscle wasting associated with cachexia. This preclinical trial demonstrates that TCMCB07 is a promising drug candidate for cachexia therapy. We anticipate that combination therapy with TMCMB07 and additional drugs that target overactivation of catabolic processes and inflammation will greatly benefit patients with cachexia.
Methods

Animals. 225-275 g of Sprague Dawley (SD) and F344/CDF (F344) male rats (Charles River) were housed at two per cage, fed rat chow (Diet 5001; Purina Mills, Inc., St. Louis, MO), and acclimated for at least 7 d before use. SD strain rats were used for LPS and CKD models, and F344 for tumor model. One day before each experiment commenced, animals were weighed and divided into treatment groups such that the mean body weights of each group were similar. During experiments, food intake and body weight were measured at same time of each day, unless otherwise noted.

Compounds. Eleven TCMC compounds (TCMCB01-10 plus TCMCB07A) were synthetic MC4R antagonists that were designed and provided by Tensive Controls Inc (Columbia, MO, USA). Each compound was dissolved in distilled water by vortex or sonication for each experiment, and fresh working solution was prepared before administration. Test of each TCMC compound began with pilot experiments for selecting doses via four administration routes (ICV, IP, SC, oral). Among eleven TCMC compounds, TCMCB07 was finally chosen for comprehensive evaluation based on the results from a series of pilot studies. Melanocortin antagonists AgRP and SHU9119 (Phoenix Pharmaceuticals, Burlingame, CA, USA) were used as positive control reagents but only affected with central administration. PG932 (provided by Tensive Controls), a synthetic derivative of SHU9119, was previously reported as an effective reagent via IP injection in an LPS mouse model (42) that was also used as a potential positive control for peripheral treatment.

Compound administration. a) Central route and dose. We first investigated the effects on stimulation of appetite and attenuation of body weight loss after central administration of TCMC compounds. To establish a route for central drug administration, unilateral cannulation of the lateral ventricle was performed. Under isoflurane anesthesia, 22-gauge lateral ventricle cannulas (Plastics One, Roanoke, VA, USA) were placed in rats using a stereotactic instrument (Kopf,
Tujunga, CA, USA) at the following coordinates relative to bregma: 1.5 mm (X), -1.0 mm (Y) and -4.0 mm (Z). Rats were then individually housed and allowed to recover from surgery for at least 7 days. Compounds at 2 \( \mu g/\text{rat/day} \) (1.5 nmol/\( \text{rat/day} \)) or saline and positive control agents were administered in a total volume of 5 \( \mu l \) via ICV injection. b) Peripheral routes and doses. Three routes were applied for peripheral administration: IP, SC injection and oral intragastric gavage. The dose range of TCMC compounds was 0.6-12 mg/kg/day. Dosing frequency was between once and twice daily.

*Acute study in LPS-induced anorexia and body weight loss.* The effects of each TCMC compound in the attenuation of anorexia and body weight loss were first examined in LPS-treated rats. LPS doses were selected through a preliminary dose-response experiment. LPS (Sigma, St. Louis, MO, USA) was dissolved in vehicle (0.5% BSA in 0.9% saline) and injected into SD rats via IP injection at doses of 0 (vehicle), 10, 50, 100 and 250 \( \mu g/kg/day \), respectively. Under fasting condition, body weight change at 24 h post-LPS injection was measured and muscle catabolism was analyzed by qRT-PCR. LPS dose of 100-250 \( \mu g/kg/day \) was chosen for compound test. In order to accurately measure spontaneous food intake during acute response to LPS and compounds, we coordinated with rat nocturnal behavior and performed a series of night feeding studies in a very consistent way (29, 65). Briefly, rats were individually housed for at least 7 days for acclimation before starting experiments. One day prior to treatment, rats were weighed and placed in clean cages, and pre-weighted food pellets were placed into each cage at 2:30 pm for measuring baseline of 24 h food intake and body weight change. On the day of treatment at 2:30 pm, rats were weighed for initial body weight, and remaining food was weighed and removed from cages. LPS IP injection was performed at 3 pm, and at 4 pm saline or compound administration was performed through ICV injection (1.5 nmol/rat), IP injection (3 mg/kg/day), and intragastric gavage (10 mg/kg/day). Pre-weighted food pellets were placed into each cage at 5:30 pm, and then food weights were measured at 2 h, 4 h (under red light illumination during night phase), 16 h, 24 h and 48 h time...
points after food returned. Body weights were measured at 16 h, 24 h and 48 h. Care was taken to minimize non-specific stress to the animals during nighttime food measurements.

Cancer cachexia model. The cancer cachexia model was generated in F344 rats. Our previous studies and others' demonstrate that rat MCA sarcoma model produces reliable and reproducible cancer cachexia, and the MCA tumor is neither rejected by F344 rat strain and nor does it metastasize (34, 68). Based on our experience with this model for these experiments, we modified it by performing tumor implantation 6-10 days before treatment to allow for adequate tumor growth. Briefly, under isoflurane anesthesia, frozen tumor tissue was implanted subcutaneously into the flanks of donors for generating fresh tumor tissue. Approximate 16 days later, fresh tumor tissue (1.0-1.2 g) from a euthanized donor was implanted subcutaneously into the flanks of a rat under isoflurane anesthesia. Sham-operated rats received the procedure without tumor tissue and served as experimental controls. Rats were administered postoperatively with analgesic (buprenorphine 0.05 mg/kg, SC) and then individually housed. Daily body weight and food intake were measured. Tumors became palpable 6–7 d post-implantation and tumor size was measured daily thereafter. Tumor volume was calculated from the formula for a prolate sphere (\(V = \frac{1}{2}ab^2\)), where “a” is the longer and “b” the shorter dimension (69). On day 12-14, animals were euthanized, when tumor growth and overall condition of tumor-bearing animals had fallen within predetermined endpoints of the study, with particular attention paid to the volume of tumor and overall health.

CKD-associated cachexia model. Two-stage 5/6-nephrectomy surgery was performed in SD rats for a CKD-associated cachexia model, and sham controls experienced the same procedures without excision of kidney tissue, as described previously (33). Briefly, for the stage-I surgery (unilateral partial nephrectomy, Neph-I), the animals were anesthetized with isoflurane and placed prone in a clean environment. A 1-cm posterior incision was made on the left flank through which the left kidney was located. For animals undergoing nephrectomy, the renal capsule was removed.
and upper and lower one third of kidney was transected and resultant wound cauterized, leaving the middle one third of kidney with renal artery and vein intact. For control animals receiving a sham operation, the renal capsule was opened up to simulate the manipulations performed in the nephrectomy. The surgical wounds were then closed via suture at muscle and skin layers respectively. Surgical animals were allowed to recover and individually housed. Seven days after stage-I surgery, animals were again anesthetized and placed prone in the surgical area for the stage-II surgery (contralateral total nephrectomy, Neph-II). This time a right 1-cm incision was performed and the right kidney was isolated. For animals undergoing nephrectomy, the renal capsule was removed and vasculature was tied off with suture. The vascular bundle was then transected distal to the suture and entire kidney was removed. For animals in sham group, the renal capsule was removed. The surgical wounds were closed with suture at muscle and skin layers respectively. A dose of analgesic was administered after each stage surgery (buprenorphine 0.05 mg/kg, SC).

*Long-term studies with cachexia models of cancer and CKD.* To determine effects of TCMCB07 treatment in cachexia associated with cancer and CKD, we designed different experimental time frames for performing surgeries, measurements, treatments, and tissue collections (Figure 2A, 3A and 5A). Tumor implantation and two-stage 5/6-nephrectomy or sham operation were performed in the beginning of each experiment. Food intake and body weight were measured at a similar time point of each day, and body composition was measured before and after compound administration. In the study with cancer cachexia, when symptoms, such as anorexia and lethargy appeared in tumor-bearing animals, saline or compound was administered through ICV or IP injection once a day and SC injection twice a day for total 4-6 days. In the study with CKD cachexia, starting at day 14 (14 days after Neph-I), saline or compound was administered via SC injection twice a day for total 14 days.
Body composition. Body fat mass and lean mass were determined before and after administration of compounds using a magnetic resonance relaxometry (EchoMRI 4-in-1 Live Animal Composition Analyzer; Echo Medical System, Houston, TX, USA).

Blood and tissue collection. In the end of experiments with cancer and CKD cachexia, rats were euthanized. Blood was collected at approximately 2 hours after the last injection of TCMCB07 (estimated $C_{max}$) through cardiac puncture and plasma was isolated and stored in -80 °C until analysis. The brains were dissected, snap frozen and then stored in -80 °C until analysis. Tumors from tumor-bearing animals were dissected away from surrounding tissue and weighed. The residual kidneys in nephrectomized animals were examined for final confirmation of the procedure and residual renal survival.

Plasma TCMCB07, BUN and Creatinine assay and brain tissue qRT-PCR analysis. We developed an assay for TCMCB07 in body fluids using reverse-phase high performance liquid chromatograph (HPLC) with aromatic amino acid fluorescence detection. This method used a Hypersil GOLD C-18 column (4.6 mm ID/25 cm length, 5 µm particle size, 17.5 nm pore size), with a 15-50% acetonitrile-0.01% hydrochloric acid gradient. We used the unique fluorescence spectrum of these peptide's naphthylalanine (Nal) residue (229 excitation and 337 emission, nanometer) for post column detection of eluting Nal-containing peptides with a Fluoat-01 Panorama Spectrofluorometer (Arlington, VA). Plasma extraction was with acetonitrile w/0.01% hydrochloric acid. This one-step approach precipitated plasma proteins, while essentially extracting 100% of the drug. Standard curves were generated by spiking blank plasma samples with known peptide amounts. Plasma samples were assayed for concentration of BUN and creatinine (Cr) with a biochemistry analyzer (Siemens Dimension Vista 1500 Chemistry Analyzer). Total RNA was extracted from hypothalamic blocks using a QIAGEN RNA mini kit, and gene expression was analyzed by qRT-PCR as described previously (64).
Statistics. All data are expressed as mean ± standard error (SEM) for each group. Statistical analyses were performed using the unpaired Student’s t-Test, and one-way ANOVA or two-way ANOVA analysis followed by Bonferroni posttests using GraphPad Prism 8 (La Jolla, CA). The correlation was analyzed using Pearson’s correlation coefficient and linear regression. P < 0.05 was considered statistically significant.

Study approval. Studies were approved by the Institutional Animal Care and Use Committee of the Oregon Health & Science University and conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Author contributions

XZ and DLM designed the research studies. XZ, MFC and MS conducted experiments. KAG designed all the TCMC compounds. XZ and DLM analyzed data. XZ wrote the manuscript. DLM and MFC reviewed and edited the manuscript. All authors approved the final version of manuscript.
Acknowledgments

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References


Figures and figure legends

**Figure 1**
Central and peripheral administration of TCMCB07 attenuates anorexia and body weight loss in rats treated with LPS. (A) Schematic of LPS injection, TCMCB07 administration, and time points of measurements. (B) 24 h food intake and (C) 24 h body weight gain (%) in LPS-treated rats post-ICV injection of saline ($n = 3$) or TCMCB07 (1.5 nmol/rat/day, $n = 3$). (D) Cumulative food intake at 2 h, 4 h, 16 h and 24 h, and (E) 24 h body weight gain (%) in LPS-treated rats post-IP injection of saline ($n = 6$) or TCMCB07 (3 mg/kg/day, $n = 6$). (F) Cumulative food intake at 4 h and 24 h post-oral gavage, (G) 24 h cumulative food intake pre- and post-oral gavage, and (H) 24 h body weight gain (%) post-oral gavage of water ($n = 9$) or TCMCB07 (10 mg/kg/day, $n = 10$) in LPS-treated rats. All data in (B-H, except D) are expressed with each dot representing one sample, and data in (D) are expressed as mean ± SEM for each group. Unpaired Student’s t-Test (B, C, E) and Two-way ANOVA (D, F, G, H). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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**A**
TCMCB07 administration

**B**
LPS+saline-ICV
LPS+TCMCB07-ICV

**C**
LPS+saline-ICV
LPS+TCMCB07-ICV

**D**
LPS/saline-IP
LPS/TCMCB07-IP

**E**
LPS/saline-IP
LPS/TCMCB07-IP

**F**
LPS/water-gavage
LPS/TCMCB07-gavage

**G**
LPS/water-gavage
LPS/TCMCB07-gavage

**H**
LPS/water-gavage
LPS/TCMCB07-gavage
**Figure 2**

Central administration of TCMCB07 attenuates anorexia and body weight loss in rats with cancer cachexia that is similar to AgRP treatment. (A) Schematic of experimental design. Tumor donors were generated approximate 16 days prior to tumor implantation. Brain unilateral ventricle cannulation was performed and animals were allowed for recovery for a minimum of 7 days prior to tumor implantation. Fresh tumor tissue from donors was implanted into F344 rats. Daily food intake and body weights were measured post-tumor implantation. ICV injections of saline or TCMCB07 were performed between day 8-11 post-tumor implantation. Body composition (initial and terminal) was measured by MR. (B) Daily food intake in tumor-bearing rats received ICV injection once daily with saline (n = 6) or TCMCB07 (1.5 nmol/rat/day, n = 11). (C) In a separate experiment, tumor-bearing rats received ICV injection once daily with saline (n = 8) or AgRP (1 nmol/rat/day, n = 8) between day 10-13 post-tumor implantation. (D and E) Cumulative food intake pre- and post-compound treatment.
Central administration of TCMCB07 attenuates anorexia and body weight loss in rats with cancer cachexia that is similar to AgRP treatment. (F and G) Body weight gain (% tumor-free net gain normalized to baseline) pre- and post-treatment. (H and I) Tumors were dissected and weighed after animals were euthanized on day 12 or day 14. (J and K) Fat mass and (L and M) lean mass were determined by MR on day 0 and day 12 or day 14, and gain was calculated (% net gain normalized to baseline). All data in (B and C) are expressed as mean ± SEM for each group. All data in (D-M) are expressed with each dot representing one sample. Two-way ANOVA (B-G), and Unpaired Student's t-Test (H-M). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Daily food intake & body weight measurement

Tumor/sham implantation

Day -16

TCMCB07 or saline IP injection daily

Tissue collection

0

Daily food intake & body weight measurement

0

Days post-implantation

B

Daily food intake (g)

Tumor/saline

Tumor/TCMCB07

Sham/saline

Tumor/sham implantation

IP injection

0

1

2

3

4

5

6

7

8

9

10

11

12

13

14

Cumulative food intake (g, d 7-13)

Figure 3
Peritoneal administration of TCMCB07 diminishes anorexia in rats with cancer cachexia. (A) Schematic of experimental design. (B) Daily food intake in tumor/saline group \( (n = 9) \), tumor/TCMCB07 group \( (n = 8) \) and sham/saline group \( (n = 6) \) post-tumor implantation or sham operation. Rats received IP injection once daily with saline or TCMCB07 (3 mg/kg/day) between day 7 and 12 post-implantation. (C) Cumulative food intake post-saline or TCMCB07 treatment. (D) Body weight gain (% tumor-free net gain normalized to baseline of day 7) post-saline or TCMCB07 treatment. (E) Tumors were dissected and weighed after animals were euthanized on day 14. All data in (B) are expressed as mean ± SEM for each group, and all data in (C-E) are expressed with each dot representing one sample. Two-way ANOVA in (B) and sham/saline group was excluded for Two-way ANOVA analysis in order to clearly show a treatment comparison between two tumor groups (tumor/TCMCB07 versus tumor/saline). One-way ANOVA (C and D), and unpaired Student's t-Test (E). ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \). Red stars in (B): tumor/TCMCB07 group versus tumor/saline group.
A daily food intake in tumor/saline group (n = 8) versus tumor/TCMCB07L (low dose) group (n = 8), tumor/TCMCB07H (high dose) group (n = 8) and sham/saline group (n = 9) post-tumor implantation or sham operation. Rats received SC injection once (1x) or twice (2x) daily with saline or TCMCB07L (1.5 mg/kg/day) or TCMCB07H (3 mg/kg/day) between day 6 and 12 post-implantation. Cumulative food intake pre- and post-treatment.

Body weight gain post-treatment (%, tumor-free net gain normalized to baseline of day 6). Tumors were dissected and weighed after animals were euthanized on day 12. Terminal plasma TCMCB07 concentrations were measured by reverse-phase HPLC. All data in (A) are expressed as mean ± SEM for each group, and all data in (B-E) are expressed with each dot representing one sample. Two-way ANOVA in (A) and sham/saline group was excluded for Two-way ANOVA analysis in order to clearly show a treatment comparison between three tumor groups (tumor/saline, tumor/TCMCB07L, tumor/TCMCB07H) (A). One-way ANOVA (B-D). Unpaired Student’s t-Test (E). * and # P < 0.05, ** and ## P < 0.01, ### and ### # P < 0.001, #### P < 0.0001. Yellow stars in (A): Tumor/TCMCB07L group versus Tumor/saline group, and red pounds in (A): Tumor/TCMCB07H group versus Tumor/saline group.
Figure 5
Subcutaneous administration of TCMCB07 reverses cachexia associated with CKD in rats. (A) Schematic of experimental design. Two-stage 5/6 nephrectomy or sham operation was performed in SD rats. Stage-I of 5/6 nephrectomy (Neph-I) was unilateral partial nephrectomy and stage-II (Neph-II) was contralateral total nephrectomy. All animals experienced Neph-I and Neph-II or sham operation on day 0 and day 7, and received SC injection twice (2x) daily with saline or TCMCB07 (3 mg/kg/day) between day 14 and 28 post-nephrectomy. Neph/saline group (n = 11), Neph/TCMCB07 group (n = 11), and sham/saline group (n = 6). (B) Body weight change during entire experimental period (day 0-28, post-nephrectomy). (C) Body weight gain (% net gain normalized to baseline) post-nephrectomy and post-treatment. (D) Daily food intake in neph/saline, neph/TCMCB07 and sham/saline groups post-treatment. (E) Cumulative food intake post-treatment. (F) Fat mass and (G) lean mass were determined by MR pre- (day 14) and post- (day 28) treatment, and the gain (%) was calculated (net gain normalized to baseline). All data in (B and D) are expressed as mean ± SEM for each group, and all data in (C, E-G) are expressed with each dot representing one sample. Two-way ANOVA in (B-E), Unpaired Student's t-Test in (F-G), * and # P < 0.05, ** P < 0.01, *** and ### P < 0.001, **** P < 0.0001. Blue stars in (B & D): Neph/saline group versus sham/saline group, and red pounds in (B & D): Neph/TCMCB07 group versus Neph/saline group.
Figure 6
Plasma BUN, creatinine and TCMCB07 concentration, and the association with food intake and body weight gain in rats with CKD cachexia. Terminal plasma concentration of BUN (A), creatinine (B) and TCMCB07 (C) were measured after animals were euthanized. Correlation between plasma concentrations of BUN, creatinine, TCMCB07 and cumulative food intake and body weight gain post-treatment (D-G). All data from three groups (Neph/saline, n = 8, Neph/TCMCB07, n = 8, sham/saline, n = 6) are included in the statistical analysis, with each dot representing one sample (A-G). One-way ANOVA in (A and B). *** P < 0.001. The correlation was analyzed using Pearson’s correlation coefficient, and a linear regression-fitting curve is shown as a black line in (D-G).
Figure 7
Treatment of TCMCB07 diminishes inflammatory and P-selectin gene expression in hypothalamus in rats with cancer cachexia. The hypothalamic tissues were analyzed with qRT-PCR. Expression of inflammatory genes and anorexigenic (POMC) and orexigenic genes (AgRP) was profiled in rats with cancer cachexia (A) and CKD-associated cachexia (B) post-subcutaneous administration of TCMCB07. Tumor/saline group (n = 8), tumor/TCMCB07L group (n = 8), tumor/TCMCB07H group (n = 8) and sham/saline group (n = 6). Neph/saline group (n = 11), Neph/TCMCB07 group (n = 11) and sham/saline group (n = 6). All data in (A and B) are expressed with each dot representing one sample. One-way ANOVA in (A and B): * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, versus tumor/saline group (A) or Neph/saline group (B).
Table 1. TCMCB07 doses and plasma concentrations

<table>
<thead>
<tr>
<th>Cachexia-associated disease</th>
<th>TCMCB07 doses (mg/kg/day, SC injection)</th>
<th>TCMCB07 plasma concentrations (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>1.5</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>Cancer</td>
<td>3.0</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>CKD</td>
<td>3.0</td>
<td>1.37 ± 0.12</td>
</tr>
</tbody>
</table>

Table 1
TCMCB07 doses and plasma concentrations. At the end of experiments, plasma was collected after rats were euthanized. Plasma TCMCB07 concentrations were assayed using reverse-phase HPLC. Rats with cancer- and CKD-associated cachexia were treated with TCMCB07 at low dose (L, 1.5 mg/kg/day) or high dose (H, 3.0 mg/kg/day) via SC injection. Rats with cancer cachexia received 12 injections (both L and H groups, n = 8) in last 7 consecutive days. Rats with CKD-associated cachexia (n = 11) received 28 injections (H dose) in the last 14 consecutive days. Data for the plasma concentrations are expressed as Mean ± SEM for each group.