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Development of a TCM-Based Agent for the Treatment of Cancer Cachexia

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A concise and informative title: Development of a natural agent for cancer cachexia

Abstract

Background: Despite recent advances in understanding the pathophysiology of cancer cachexia, prevention/treatment of this debilitating disease remains an unmet medical need.

Methods: We developed an integrated, multi-tiered strategy involving both *in vitro* and *in vivo* muscle-atrophy platforms to identify Traditional Chinese Medicine (TCM)-based anticachectic agents. In the initial screening, we used inflammatory cytokine-induced atrophy of C2C12 myotubes as a phenotypic screening platform to assess the protective effects of TCMs. The selected TCMs were then evaluated for their abilities to protect *C. elegans* from age-related reduction of mobility and contractility, followed by the C-26 colon adenocarcinoma mouse model of cachexia to confirm the anti-muscle atrophy effects (body/skeletal muscle weights, fiber size distribution, grip strengths, and serum IL-6). Transcriptome analysis, quantitative real-time polymerase chain reaction (qPCR), and immunoblotting were performed to gain understanding of the potential mechanism(s) by which effective TCM protected against C26 tumor-induced muscle atrophy.

Results: Of twenty-nine widely used TCMs, Dioscorea radix (DR) and Mu Dan Pi (MDP) showed a complete protection (all *P* values, 0.0002) vis-à-vis C26 conditioned medium control in the myotube atrophy platform. MDP exhibited a unique ability to ameliorate age-associated decreases in worm mobility, accompanied by improved total body contractions, relative to control (P<0.0001 and <0.01, respectively), **which, however, was not noted with** DR. This differential *in vivo* protective effect between MDP and DR was also confirmed in the C-26 mouse model. MDP at 1,000 mg/kg (MDP-H) was effective in protecting body weight loss (*P*<0.05) in C-26 tumor-bearing mice without changing food or water intake, accompanied by the restoration of the fiber size distribution of hindleg skeletal muscles **(*P*<0.0001)** and the forelimb grip strength (*P*<0.05). MDP-treated C-26-tumor-bearing mice were alert, showed normal posture and better body conditions, and exhibited lower serum IL-6 levels (*P*=0.06) relative to vehicle control. This decreased serum IL-6 was associated with the *in vitro* suppressive effect of MDP (25 and 50 μg/mL) on IL-6 secretion into culture medium by C26 cells. RNA-seq analysis, followed by qPCR and/or immunoblotting, shows that MDP's anti-cachectic effect was attributable to its ability to reverse the C-26 tumor-induced reprogramming of muscle homeostasis-associated gene expression, including that of two cachexia drivers (MuRF1 and Atrogin-1), in skeletal muscles.

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Conclusion: All these findings suggest the translational potential of MDP to foster new strategies for the prevention and/or treatment of cachexia. The protective effect of MDP on other types of muscle atrophy such as sarcopenia might warrant investigations.

Keywords Cachexia; traditional Chinese medicine; muscle atrophy; C2C12 cell model; C-26 tumor-bearing model; *C. elegans* model

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Introduction

A major challenge in the treatment of certain types of cancers, e.g., pancreas, stomach, colon, is the accompanying cancer-induced cachexia, characterized by anorexia and loss of adipose tissues and skeletal muscle masses.[1] Although substantial advances in understanding the multifactorial pathophysiology of cancer cachexia were available, prevention and/or treatment of this debilitating disease remains an unmet medical need. Currently, no approved targeted therapy is available for cachexia, and nutritional support. The semi-synthetic progestational steroid, megestrol, is the only one agent used to ameliorate cachexia-associated symptoms. A number of Kampo medicines (i.e., multi-component herbal extracts) have been commonly prescribed in Japan to alleviate fatigue and chronic weakness in cachectic patients.[2] Recently, the hunger hormone ghrelin and ghrelin mimetics have received much attention in light of their potential to enhance apatite and quality of life but lacked clinical evidence to support cachexia treatment.[3]

The advantage of Traditional Chinese Medicines (TCMs) over small-molecule targeted agents for the treatment of chronic illnesses is multifold. First, the therapeutic utility of the polypharmacology is manifested by their long-standing history in the treatment of various complex diseases.[4] Second, many TCMs are consumed as dietary supplements on a daily basis. Third, TCMs are generally perceived in oriental societies as having fewer side effects, which might lead to better compliance in patients with chronic diseases.[5] We performed integrated, multi-tiered strategies to develop TCM-based anti-cachetic agents. We used inflammatory cytokine-induced atrophy of C2C12 myotubes (the C2C12 model) [6] as a phenotypic screening platform to assess the protective effects of a panel of TCMs. The selected TCMs were then evaluated for their abilities to protect *C. elegans* from age-related reduction of mobility **and contractility**, based on which the anti-muscle atrophy effect of the candidate TCM was confirmed in the C-26 colon adenocarcinoma mouse model of cachexia (C-26 model).[7] We found one of the common used TCM, Mu Dan Pi **(**or Moutan Radicis Cortex; MDP), which exhibited *in vivo* efficacy in protecting C-26 tumor-bearing mice from body weight losses. Mechanistically, RNA-seq and quantitative real-time polymerase chain reaction (qPCR) analysis reveals that MDP's anti-cachectic effect was associated with its ability to reverse the C-26 tumor-induced reprogramming of gene expression associated with muscle functions to that of non-cachectic muscles, therefore rescuing skeletal muscles (i.e., two cachexia drivers MuRF1 and Atrogin-1) from wasting. Together, these findings suggest the translational potential of MDP to foster new strategies for the prevention and/or treatment of cachexia in at-risk cancer patients.

Material and Methods

Source of the crude extracts of Chinese herbal medicine

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Dried root barks of *Paeonia suffruticosa* Andrews (MDP) were purchased from a local herbal medicine store and authenticated by KCW. Crushed MDP was ground into coarse powders (*ca.* 20-mesh), followed by extraction with 70% methanol (ACS grade; 1 L, three times) at room temperature. The combined supernatants were filtered, and concentrated under freeze-drying to generate MDP extracts for further use. We conducted high performance liquid chromatography (HPLC) fingerprint analysis after each batch of MDP extracts was prepared to ensure batch consistency. All other tested TCM extracts were prepared either by KCW or JKT in a similar manner.

Cell culture

C2C12 myoblasts[6] and mouse C26 colorectal cancer cells were purchased from the ATCC, and maintained in the respective recommended growth media supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37℃ in a humidified incubator containing 5% CO_2.

C26 conditioned medium preparation

Preparation of C26 conditioned medium (C26CM) was conducted according to a reported procedure.[7,8] Mouse C26 colorectal cancer cells were cultured in 10-cm dishes until reaching 95% confluence, washed twice with PBS, followed by one wash with serum-free DMEM, and grown in serum-free DMEM for 24 h. Collected medium was centrifuging at 1000 *g* for 5 min, filtered through 0.2 μm syringe filters and stored at −80℃.

Measurement of myotube width

C2C12 cells were cultured in 6-cm dishes containing 10% PBS in DMEM. When they reached over 95% confluence, the medium was changed to differentiation medium containing 2% horse serum (HS) in DMEM and further cultured for 4 days. The differentiated C2C12 cells were than incubated for additional 4 days in differentiation medium containing C26CM cells (1:1 ratio). The images of differentiated myotubes were obtained by phase-contrast microscopy, and the myotube width was analyzed by CellSens Software (Olympus, USA) and represented as percentage of control (%).[9]

Cell viability assays

C26 cells were seeded into 96-well plates at a density of 8×10^3 cells per well in the presence of 10% FBS. After overnight incubation, cells were exposed to MDP at indicated concentrations versus vehicle for 24 h. After treatment, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biomatik, Wilmington, DE) for one hour. The medium was then removed from each well

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and replaced with DMSO to dissolve reduced MTT dye for subsequent colorimetric measurement of absorbance at 565 nm.

***C. elegans* mobility test**

The age-dependent decline in mobility in *C. elegans* is a well-established model for age-related muscle atrophy,[10] characterized by declines in muscle quantity and quality. The nematode muscle does not contain stem cells, thus providing a suitable model to investigate how the assembled muscle contractile apparatus is maintained during aging in the absence of regeneration. *C. elegans* undergoes an age-dependent deterioration in the muscle myofilament lattice, which contributes to declines in mobility.[11] The microarray analysis has revealed that worm muscle sarcomere proteins undergo an age-dependent decrease starting as early as day 1 of adulthood.[12] Accordingly, the selected TCM samples were dissolved in either water or 1% DMSO at 10 mg/ml and stored at 4℃ (100x stock solutions). *E. Coli* OP50 bacteria was seeded on fresh NGM plates and left to grow for 2 days. One-hundred μL of TCM stock solutions were then added onto NGM plates with OP50 (total volume 10 mL; final TCM concentration, 100 ug/mL). After the TCM solution was completed absorbed into agar, the OP50 plates were then exposed to UV for 40 min to kill the bacteria. At least 50 synchronized eggs of CF512 worms were seeded on these TCM-containing plates and cultured at 25℃, which was repeated at least three times. Worm videos were recorded on day 1, 3, 5, and 7 of adulthood. To film the thrashing video of the worms, 2 mL of M9 buffer was added onto the plate. Plates with worms swimming in M9 buffer was then placed under a microscope. The video recording began exactly 2 min after the M9 buffer was added, which last for 1 min. The video was then analyzed using imageJ and wrMTrck (wrMTrck multiple object tracker - phage.dk).[13]

***C. elegans* body wall muscle contractility assay**

To quantify *C. elegans* body wall muscle contraction induced by levamisole, more than 30 worms at different ages (day 1, 5, 9,13 adults) were first incubated in drug-free solution (140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM dextrose, and 5 mM HEPES; 330 mOsm; pH adjusted to 7.2 with NaOH) and then in levamisole-containing solution (abovementioned solution with 100 mM levamisole) for 10 minutes. Images of animals before and after shifting to levamisole-containing solution were taken using regular dissecting scopes with digital imaging system. These images were then used to quantify the length of the worm body using ImageJ. The relative total body contraction was calculated as follow: [length $_{\text{(before Levamisole)}}$ – length $_{\text{(after levamisole)}}$] / length $_{\text{(before Levamisole)}}$.[14]

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Experimental animals

Experiments were performed on male CD2F1 mice (approximately 6 weeks of age) obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). These mice were kept in the animal center of China Medical University (CMU) with a controlled temperature of 22 ± 1℃ and a relative humidity of 55 ± 5% under a 12: 12 h light-dark cycle with a rodent diet and clean water *ad libitum*. The experimental protocol was approved by the institutional Animal Care and Use Committee (IACUC) (Protocol No.: CMUIACUC-2018-295).

Colon-26 carcinoma tumor-bearing mouse of cachexia

Previous studies have reported the use of various herbal extracts at 1,000 mg/kg,[15] 2,000 mg/kg[16] or so in mice for cancer researches. The dose of 1,980 mg/kg was used to study MDP protective effect on cardiac ischemia/reperfusion in rats.[17] Accordingly, we chose three doses, i.e., 100mg/kg (MDP-L), 500mg/kg (MDP-M), and 1,000mg/kg(MDP-H), for studying the anti-cachectic effect of MDP in C-26 tumor bearing mice. In addition, H3-14, a small-molecule histone deacetylase (HDAC)3 inhibitor[18] (aka, compound 28 in ref. 18; structure, Supplementary Fig s1.), was used as a positive control based on our previous screening findings. H3-14 was synthesized according to the published procedure, [18] of which the purity (≥98%) was verified by nuclear magnetic resonance (NMR) spectrometry. After an adaptation period of seven days, CD2F1 mice were randomly divided into the following six groups (*n*=6–10) for the first set of MDP experiments: (1) normal control group (tumor-free; NC), (2) C26/vehicle group (Veh), (3) C26/H3-14 (100 mg/kg) group, (4) C26/MDP-L group, (5) C26/MDP-M group, and (6) C26/MDP-H–group. To investigate the effect of prevention, the treatment groups were respectively pretreated by oral administration of different doses of MDP or H3-14 (all in 1% carboxymethylcellulose), starting seven days before C26 cell injection. Mice in the NC and vehicle groups received 1% carboxymethylcellulose only. Tumors were established by the subcutaneous injection of C-26 cells (0.5 x 10^6 cells in 0.1 mL) into the right flank of mice on day 7. The tumor volume was measured and calculated using the standard formula (length × width^2 × π/6). [19] On the endpoint of this experiment (day 17), blood and skeletal muscles of quadriceps (Quad), gastrocnemius (GC), and tibialis anterior (TA) samples were collected for analysis.[20] In the second set of MDP experiment, tumor-free and C26 tumor-bearing CD2F1 mice were treated with 1% carboxymethylcellulose (NC and Veh) or-MDP-H (all n = 5) by following the above protocol. Body weights, tumor sizes, and food and water consumptions of individual mice were monitored every other day. At sacrifice, hindleg skeletal muscles were dissected and stored at -80 ℃ for further analysis after body weight measurements. Furthermore, the C26

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tumor- bearing experiment of Dioscoreae rhizome (DR) was conducted by another batch of CD2F1 mice and divided into the NC (n = 3), Vehicle (n = 6), and DR (n = 6) groups.

IL-6 cytokines in serum

At sacrifice, blood samples were collected via cardiac puncture. After centrifugation at 3500 rpm for 10 min, mouse sera were collected and stored at -80℃ until analysis. Serum IL-6 and secreted IL-6 in C26 cell culture medium were measured by using the ELISA MAX^{TM} Deluxe Set Mouse IL-6 kit (BioLegend, Inc., San Diego, CA, USA).

Grip strength measurement

We measured the grip strength of the forelimbs of mice by using a grip strength meter (Bioseb, Vitrolles, France) as it has been reported that the muscle strength of forelimbs was comparable to that of hindlimbs in mice in the context of absolute twitch force and maximal isometric tetanic force.[21.] To ensure consistency, the grip strength measurements were carried out by the same person at same day of three different time points. Individual mice were allowed to grip with forelimbs, and pulled gently by tails with a consistent force. The maximal strength was recorded when the forelimbs were let go from the grid, and the grip strength was recorded as the average of three measurements.[22] However, hindlimbs were used in the following post-morten analyses due to their larger size relative to forlimbs.

Analysis of fiber cross sectional area (CSA)

GC muscles were embedded in paraffin and sectioned at a thickness of **3** μm. **Slides were subjected to Immunohistochemical staining with antidystrophin antibody (ab275391, abcam) to perform qualitative fiber size measurements.** Sections were examined and images were captured in a blinded manner with a BX-43 microscope (Olympus America, Melville, NY) outfitted with a SAGE Vision SGHD-3.6C high-resolution digital camera (SAGE VISION CO., LTD, Taipei, Taiwan). ImageJ software (National Institutes of Health, Bethesda, MD) was used to perform quantitative measurements. All of individual muscle fibers were manually traced, and fiber areas of 100–180 muscle fibers were recorded in each slide.

RNA isolation

Total RNA was extracted using a Rneasy kit according to the instruction manual. Purified RNA was quantified at OD260nm using a ND-1000 spectrophotometer (Nanodrop Technology, USA) and qualified by using a Bioanalyzer 2100 (Agilent Technology, USA) with RNA 6000 LabChip kit (Agilent Technology, USA).

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Library preparation, sequencing, alignment, and differential expression

Sequencing of mRNAs was performed by a commercial vendor (Welgene Biotech Co., Ltd, Taipei, Taiwan). All RNA sample preparation procedures were carried out according to the Illumina's official protocol (Illumina, USA). Agilent's SureSelect Strand-Specific RNA Library Preparation Kit was used for library construction followed by AMPure XP beads (Beckman Coulter, USA) size selection. The sequence was determined using Illumina's sequencing-by-synthesis (SBS) technology. Sequencing data (FASTQ reads) were generated using Welgene Biotech's pipeline based on Illumina's basecalling program bcl2fastq v2.20. Both adaptor clipping and sequence quality trimming were performed using Trimmomatic (v0.36). HISAT2 program was used for mRNA alignment. Differential expression analysis was performed using StringTie (v2.1.3) and Deseq (v1.39.0). Functional enrichment assay in differentially expressed genes of each experiment design was performed using clusterProfiler v3.6. Genes with p value ≤ 0.05 and ≥ 2-fold changes were considered significantly differentially expressed. The sample reports of RNA QC analysis are attached in Supplementary Fig s2.

qPCR

Total RNA was reverse transcribed to cDNA by using TOOLS Easy Fast RT kit (Biotools, Taipei, Taiwan) according to manufacturer's instructions. qPCR was performed using the CFX Connect Real-time qPCR Detection System (Bio-Rad) with Applied Biosystems™ Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific). The primer sequences used for quantitative qPCR analysis is attached in Supplementary Table s1.

Immunoblotting

Effect of MDP-H versus vehicle on the protein expression levels of two key muscle cachexia drivers, MuRF1 and Atrogin-1, in skeletal muscles of tumor-bearing mice was examined by immunoblotting. Skeletal muscle tissues were collected and homogenized in lysis buffer containing protease and phosphatase inhibitors. Protein concentrations of muscle lysates were determined by BCA protein assay (Thermo Fisher Scientific, Chicago, IL, USA). Equal amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto nitrocellulose membranes. After 1 h blocking with TBST (TBS containing 0.1% Tween 20) containing 5% nonfat milk, membranes were incubated with corresponding primary antibodies at 4 ℃ overnights, washed with TBST buffer three times and further incubated with secondary antibodies. Signals were visualized with enhanced chemiluminescence substrate (PerkinElmer, Boston, MA, USA).

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Statistical analysis

All data were expressed as means with standard deviation or standard error for continuous variables, and frequency or percentages for nominal variables. For experiments with repeated measurements, we applied generalized linear mixed-effects models with random intercept for individual subject and fixed effects for treatment group and days after tumor cells inoculation, accounting for the association of the same measure at different time points from the same subjects. To distinguish differences at each time point among treatment groups, Tukey-Kramer correction for multiple comparisons (e.g., =0.05/17=0.003 for the multiple comparisons with control or vehicle group) were used. For the experiment without repeated measures design, we examined the difference between two groups by using Wilcoxon rank-sum test, and the difference among 3 or more groups by using Kruskal Wallis test with Dunn's multiple-comparison test using Bonferroni adjustment. For the experiments of *C. elegans* mobility, we applied student's *t* test due to a large number of sample sizes. Analysis was conducted by using SAS 9.4 software (SAS, Inc; Cary, NC).

Results

Effects of TCMs on inflammatory cytokine-induced atrophy of C2C12 myotubes.

C2C12 murine muscle myoblasts have been proposed as a suitable *in vitro* model for developing therapeutic strategies for the treatment of disease- or age-associated muscle wasting.[23] Thus, we used this C2C12 model as the first-tiered screening platform to assess the protective effect of a panel of twenty-nine TCM extracts on inflammatory cytokine-induced muscle atrophy, in which C26CM-served as a source of inflammatory cytokines.[24] The experimental design is depicted in Fig. 1A, in which H3-14, a small-molecule HDAC3 inhibitor (aka, compound 28),[18] was used as a positive control based on our previous screening findings of a panel of known pan-, class I, and isoform-specific HDAC inhibitors obtained from commercial sources or through in-house synthesis. As shown, C26CM caused significant narrowing in C2C12 myotubes, and H3-14 at 1 μM was effective in counteracting this effect (Fig. 1B & C).

The selected TCMs (Supplementary Table s2) are traditionally classified as "heat-clearing, tonifying and replenishing" medicines in light of their diverse health-preserving effects,[25] including hyperglycemia, antioxidation, anti-inflammation, and immunomodulation. Among these TCM extracts, we found two widely used TCMs, DR and MDP, sharing the ability of H3-14 to fully protect C2C12 myotubes from C26CM-induced atrophy (all *P*s =0.0002 vis-à-vis C26CM control in the myotube atrophy platform, Fig. 1C), but other examined TCMs were either cytotoxic or lacked significant protective effects (for full data of those not shown here; Supplementary Fig. s3).

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Effects of DR and MDP on *C. elegans* mobility.

This phenotypic assay of nematode *C. elegans* demonstrated that MDP, but not DR, could ameliorate age-associated decreases in worm mobility relative to control (Fig. 2A & B). **In a separate experiment, the time-dependent effects of MDP on worm mobility versus total body contractions were analyzed to discern whether the ability of MDP to protect the worm mobility was muscle function-related. As shown, MDP showed parallel protective effects in both analyses (Fig. 2C & D).** Together, these findings suggest the unique ability of MDP to delay the rate of age-dependent decline in muscle functions, of which the underlying mechanism might warrant further investigations.

***In vivo* efficacy of MDP in protecting mice from C26 tumor-induced muscle wasting**.

In our first set of C-26 model experiments, which was reported to be associated with excessive IL-6 secretion by the tumor,[26] C-26 xenograft tumors were fast-growing, approaching the maximally allowable size of 1500 mm^3 in our approved IACUC protocol by day 17 after tumor cell implantation (Fig. 3A), due to the aggressive nature of C-26 cancer cells. Therefore, the experiment was terminated at day 17, at which time all mice were sacrificed. As shown, MDP-H was effective in ameliorating body weight losses in C-26 tumor-bearing mice, which was not noted with the two lower doses (Fig. 3A and B, with and without tumor mass, respectively; $*P < 0.05$). It is interesting to note that H3-14 did not show any protective effect despite its anti-atrophy effect in the C2C12 myotube model, suggesting complexity in the mechanism that drives muscle wasting *in vivo*. The anti-cachectic activity of MDP-H was not attributable to its ability to reduce tumor burden as MDP-H showed only a very modest tumor-suppressive effect on tumor growth (Fig. 3C). Moreover, MDP-H or any other MDP treatment had no significant effect on food or water intake throughout the course of this study (Supplementary Fig. s4), excluding the role of any dietary effect.

Moreover, this protective effect on body weight was paralleled by a similar effect on skeletal muscle weights. As shown in Fig. 3D, MDP-H was effective in protecting hindlimb muscles C-26 tumor-bearing mice treated with MDP-M and MDP-H exhibited an alert and active phenotype, lacking signs of cachexia observed in vehicle- or H3-14-treated counterparts (hunched posture and rough haircoat) (Fig. 3E), despite the finding that MDP-H exhibited no significant suppressive effect on tumor growth. This active phenotype albeit large tumor sizes again underscores the unique anti-cachectic effect of MDP. In contrast, DR at 100 mg/kg exacerbated body weight loss and caused deterioration of physical appearances relative to vehicle control (Fig. 4A & B), while no appreciative effect on tumor growth was noted (Fig. 4C).

In the second set of experiments, HPLC analysis showed an identical pattern of chromatographic fingerprints between two independent preparations of MDP used for the first and second sets of

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experiments, indicative of consistency in chemical compositions (Supplementary Fig. s5). Consistent with results from the first experiment, MDP-H showed *in vivo* efficacy in protecting mice from C-26 tumor-induced body weight loss with no significant suppressive effect on tumor burden at day 17 (Fig. 5A-C), which was evident by its ability to diminish cachexia-associated decreases in skeletal muscle weights (Fig. 5D). Further, MDP-H was able to rescue the fiber size distribution from shifting to smaller cross-sectional areas in cachectic muscles **through immunostaining with anti-dystrophin of GC myofibers followed by quantification of myofiber diameter (*P* < 0.0001) (Fig. 5E; H&E and anti-dystrophin staining images are shown in Supplementary Fig. s6.)**, accompanied by the restoration of forelimb grip strength at day 17 in response to MDP-H treatment (*P* < 0.001) (Fig. 5F).

MDP exerts the *in vivo* anti-cachectic effect by reversing tumor-induced reprogramming of muscle homeostasis-associated gene expression in skeletal muscles.

The mean serum IL-6 (a major driver in the C-26 tumor model of cachexia [26]) levels in MDP-H-treated C-26 tumor-bearing mice was lower but no statistically significant different from that of vehicle control (Fig. 6A, *P*= 0.06). This diminished serum IL-6 level was associated with the unique ability of MDP at 25 and 50 μg/mL to suppress IL-6 secretion into culture medium by C26 cells (*P* < 0.001) (Fig. 6B). As MDP at these two concentrations was non-cytotoxic to C26 cells (Fig. 6C), this MDP-mediated suppression of IL-6 secretion was not attributed to reduced viability of C26 cells (Fig. 6C).

The principal component analysis (PCA) plot shows that the two-dimensional projection of the variation in the T/MDP group was much closer to that of the TF/Veh group than to that of the T/Veh group (Fig. 7A). This clustering of expression profiles suggests that MDP was able to shift the gene expression profile of cachectic skeletal muscles (T/Veh) to a state similar to that of non-cachectic muscles (TF/Veh).

Venn diagram analysis reveals a total of 1849 differentially expressed genes shared by the two pairwise comparisons (center portion) that showed changed expression in the same direction (Fig. 7B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of these 1849 genes revealed that these genes are associated with signaling pathways related to proteasome, autophagy, and protein degradation (Fig. 7C, left). Moreover, analysis using the Gene Ontology (GO) Knowledgebase shows that these genes are linked to the biological processes of muscle cell differentiation, muscle system process, and muscle tissue development, and to the cellular component of proteasomes and lysosomes (Fig. 7C, center and right). These genetic analyses clearly demonstrate the ability of MDP to extensively reprogram the expression of genes associated with muscle homeostasis in cachectic skeletal muscles, which are reflected by the top twenty most up- versus down-regulated genes in response to MDP treatment (Supplementary Table s3).

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To validate these RNA-seq results, we selected 5 upregulated and 5 down-regulated genes to conduct qPCR analysis of skeletal muscles from vehicle- versus MDP-H treated mice, which included the upregulated genes *Lep*, *Kera*, *Chad*, *Ky*, and *Mettl21e* and the downregulated genes *Lcn1*, *Mt1*, *Ampd3*, *Fbxo32*, and *Trim63*. Especially noteworthy are *Fbxo32* and *Trim 63*, which encode the two muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF1, respectively. As shown, changes in the expression levels of these genes from qPCR analysis paralleled that of RNA-seq analysis (Fig. 8A). Consistent with the q-PCR results, MDP-H was effective in suppressing the protein expression of Atrogin-1 and MuRF1 in C26 tumor-bearing mice to the basal levels noted in the TF/Veh group upon Western blotting analysis (Fig. 8B).

Discussion

At the cellular level, the C2C12 myotube model provided an expedient platform for the initial screening of a panel of TCMs, which allowed us to advance MDP and DR for further assessments in two distinct *in vivo* models. From a drug discovery perspective, *C. elegans* proves to be a useful tool for the phenotypic screening of small-molecule agents/TCMs for aging,[12, 27] sarcopenia,[10] and a variety of diseases,[28,29] including cancer,[30] infectious diseases,[31] and neurodegenerative diseases.[32,33] Although MDP and DR were both active in the C2C12 myotube model, this *C. elegans* platform was able to discern the differential *in vivo* efficacy of MDP versus DR against muscle atrophy, consistent with results from the C26 mouse model studies.

The extract of DR and its bioactive compound allantoin were reported to regulate the myoblast differentiation and mitochondrial biogenesis of C2C12 myotubes,[34] and more recently, to improve skeletal muscle dysfunctions in diabetic mice.[35] In this study, although DR was shown to be effective in protecting C2C12 myotubes from C26CM-indued atrophy, this TCM was ineffective in protecting *C. elegans* and C26 tumor-bearing mice from age-related loss of mobility and tumor-induced muscle wasting, respectively. We rationalize that this discrepancy between the *in vitro* and *in vivo* efficacies of DR might be associated with the presence of steroidogenic and estrogen-stimulating compounds in this TCM.[36] Pharmacologically, these compounds might stimulate C2C12 myoblast differentiation and/or myotube proliferation, but are ineffective in protecting against loss of muscle functions or muscle wasting in whole animal models.

RAN-seq analysis in conjunction with qPCR and Western blot analyses suggests that MDP's anti-cachectic effect could be attributable to its ability to reverse tumor-induced reprogramming of genes governing muscle homeostasis, shifting the expression pattern similar to that of healthy muscles. This extensive genetic reprogramming is reminiscent to that reported with the pan-histone deacetylase (HDAC) inhibitor AR-42 in C26 tumor-bearing mice.[17] Nevertheless, the involvement of HDAC inhibition

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in MDP-mediated anti-cachectic effect was refuted by the inability of MDP to induce histone H3 hyperacetylation in cells (Supplementary Fig. s7). We hypothesize that MDP might act by altering the activation status/expression of relevant transcription factors to induce such an extensive genetic reprogramming.

Bioinformatic analyses of the 1,849 differentially expressed genes shared by the two pairwise comparisons (Fig. 7B) revealed that most of these genes were linked to pathways/biological processes associated with muscle homeostasis. For example, the products of the top two most upregulated genes are two chemokines Ccl21 and Ccl12 (Supplementary Table s3), which act as chemo-attractants through the respective receptors Ccr7 [37] and Ccr2 [38] in immune cells. Evidence suggests that these two chemokine receptors might be involved in regulating energy expenditure and muscle regeneration, respectively. Conversely, the products of several top 20 most downregulated genes in MDP-treated mice have been reported as pathological mediators or key players in cancer-induced cachexia (Supplementary Table s3). Specifically, the expression levels of MuRF1 and Atrogin-1, the selective muscle cachexia driver genes from skeletal muscle samples, were effectively reverted in MDP-H-treated tumor-bearing mice, as compared with that in vehicle-treated tumor bearing mice. The ability of MDP to downregulate the expression of these two cachexia derivers might represent the direct cause for its anti-cachectic effect in light of the finding in a recent study.[39] It is worth mentioning that the mode of action of MDP in targeting the expression of muscle homeostasis-associated genes appears to be different from the adaptogenic effects of Kampo medicines,[2] which warrants further investigation to explore the possibility of therapeutic combinations. **It is worth mentioning that the daily dose of MDP at 1,000mg/kg (MDP-H) in mice is attainable for human consumption. According to the U.S. FDA guideline,[40] a factor of 0.08 is used when translating a dose used in mice to the human equivalent dose in a clinical trial setting. Consequently, a daily dose of 6.4 g of MDP will be needed for a person with an average weight of 80 kg (=1g(1000mg)/kg×0.08×80kg), which is within the daily range of 6-12 g prescribed as a TCM depicted in the Taiwan Herbal Pharmacopeia.[25]**

This study is limited by lack of a clear understanding of the mechanism by which MDP regulate the expression of such a diverse array of muscle homeostasis-associated genes. Although the anti-muscle atrophy effect of MDP has been verified in the integrated, multi-tiered strategy, we would like to test the *in vivo* efficacy of MDP in another mouse model of cachexia (e.g., the newly developed mouse model of pancreatic adenocarcinoma-induced cachexia)[41] to-help shed light onto MDP's underlying mechanism in the future. In addition, the effect of MDP-H on tumor-specific pro-cachectic factors remains uncharacterized, which might also contribute to the reversal of cachectic phenotype or genotype in C-26 tumor-bearing mice (i.e., protective effect on the driver genes of MuRF1 and Atrogin-1 against cancer cachexia). For example, MDP-H could reduce the level of circulating IL-6, though

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not statistically significant, in C26 tumor-bearing mice. We obtained evidence that MDP was effective in blocking IL-6 secretion in the culture medium of C26 cells. Together with the findings that MDP-H could protect C2C12 myoblasts from C26CM-induced atrophy, we rationalize that the mechanism by which MDP exerts its anti-cachectic effect might be twofold by acting on tumors and skeletal muscles in a concerted manner, which warrants further investigations.

Another limitation is that it is unclear whether a single constituent or multiple compounds mediate MDP's anti-cachectic effect. Accordingly, further purification of MDP extracts *via* solvent fractionation in tandem with different types of column chromatography is currently underway. We expect that such information will help foster new strategies of combining MDP with chemotherapeutic regimens for the treatment of cancer patients at risk of cachexia or further to explore the effects of MDP on aging-related sarcopenia.

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Online supplementary material

The following are available online in the Supporting Information section:

Supplementary Table s1. The Primer sequences used for quantitative RT-PCR analysis.

Supplementary Table s2. List of twenty-nine TCMs used in the C2C12 myotube atrophy model screening and their reported pharmacological properties.

Supplementary Fig. s1. Chemical structure of H3-14

Supplementary Fig. s2. Sample RNA QC analysis reports

Supplementary Fig. s3. Lack of protective effect of 24 TCM extracts on C26CM-induced atrophy of C2C12 myotubes.

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Supplementary Fig. s4. Daily diet and water consumptions in different treatment groups. NC, tumor-free mice with vehicle treatment.

Supplementary Fig. s5. Superimposition of HPLC fingerprint chromatograms of two independent batches of MDP preparations used in the first (top) and second (bottom) sets of experiments.

Supplementary Fig. s6. Effects of MDP treatment on muscle fiber of gastrocnemius muscle in C-26 tumor-bearing mice through immunostaining with anti-dystrophin followed by quantification of myofiber diameter

Supplementary Fig. s7. Effects of MDP at indicated concentration versus AR-42, a pan-HDAC inhibitor, at 0.25 μM on histone H3 acetylation in MDA-MB-231 breast cancer cell lines.

Supplementary Table s3. Top 20 most upregulated and downregulated genes in skeletal muscles of MDP- versus vehicle-treated C-26 tumor-bearing mice

Author contributions

KCW, PCC, YJC, HWL, and CHL designed the study. KCW, PCC, YJC, JKT, HYW, SHW, ALH, and YCL performed the experiments. KCW, PCC, CIL, CIL, HHK, HWL, and CHL wrote the manuscript. KCW, PCC, CIL, CIL, HHK, HWL, and CHL revised the manuscript. All authors read and approved the final manuscript.

The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle. [42]

Conflicts of interest

The authors declare no conflict of interest.

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Reference

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1. Baracos VE, Martin L, Korc M, Guttridge DC, Fearon KCH. Cancer-associated cachexia. *Nat Rev Dis Primers.* 2018;**4**:17105.
2. Kuchta K, Cameron S. Phytotherapy for Cachexia: Where Do We Stand? *Front Pharmacol.* 2020;**11**:917.
3. Mansson JV, Alves FD, Biolo A, Souza GC. Use of ghrelin in cachexia syndrome: a systematic review of clinical trials. *Nutr Rev.* 2016;**74**(11):659-669.
4. Zhang R, Zhu X, Bai H, Ning K. Network Pharmacology Databases for Traditional Chinese Medicine: Review and Assessment. *Front Pharmacol.* 2019;**10**:123.
5. Lam TP. Strengths and weaknesses of traditional Chinese medicine and Western medicine in the eyes of some Hong Kong Chinese. *J Epidemiol Community Health.* 2001;**55**(10):762-765.
6. Sharples AP, Al-Shanti N, Stewart CE. C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: relevance to disease and ageing? *J Cell Physiol.* 2010;**225**(1):240-250.
7. Aulino P, Berardi E, Cardillo VM, Rizzuto E, Perniconi B, Ramina C, *et al*. Molecular, cellular and physiological characterization of the cancer cachexia-inducing C26 colon carcinoma in mouse. *BMC Cancer.* 2010;**10**:363.
8. Seto DN, Kandarian SC, Jackman RW. A key role for leukemia inhibitory factor in C26 cancer cachexia. *The Journal of biological chemistry.* 2015;**290**:19976-19986.
9. Li W, Moylan JS, Chambers MA, Smith J, Reid MB. Interleukin-1 stimulates catabolism in C2C12 myotubes. *Am J Physiol Cell Physiol.* 2009;**297**:C706–C714.
10. Christian CJ, Benian GM, Animal models of sarcopenia. *Aging Cell* 2020; **19**(10): e13223.
11. Glenn CF, Chow DK, David L, Cooke CA, Gami MS, Iser WB, *et al.*, Behavioral deficits during early stages of aging in Caenorhabditis elegans result from locomotory deficits possibly linked to muscle frailty. *J Gerontol A Biol Sci Med Sci* 2004; **59**:1251-1260.
12. A. Mergoud Dit Lamarche, Molin L, Pierson L, Mario MC, Bessereau JL, Gieseler K, *et al.*, UNC-120/SRF independently controls muscle aging and lifespan in Caenorhabditis elegans. *Aging Cell* 2018; **17**(2): e12713.
13. Gaffney CJ, Bass JJ, Barratt TF, Szewczyk NJ. Methods to assess subcellular compartments of muscle in C. elegans. *J Vis Exp.* 2014; **93**:e52043.
14. **Liu J, Zhang B, Lei H, Feng Z, Liu J, Hsu AL, et al., Functional aging in the nervous system contributes to age-dependent motor activity decline in *C. elegans*. *Cell Metabolism* 2013; 18(3): 392-402.**
15. Al-Afifi N, Alabsi A, Kaid F, Bakri M, Ramanathan A: Prevention of oral carcinogenesis in rats by Dracaena cinnabari resin extracts. *Clin Oral Investig* 2019; **23**(5):2287-2301.

This article is protected by copyright. All rights reserved.

16. Fang T, Fang Y, Xu X, He M, Zhao Z, Huang P, *et al*. Actinidia chinensis Planch root extract attenuates proliferation and metastasis of hepatocellular carcinoma by inhibiting epithelial-mesenchymal transition. *J Ethnopharmacol* 2019; **231**:474-485.
17. Dan H, Zhang L, Qin X, Peng X, Wong M, Tan X, Yu S, Fang N: Moutan cortex extract exerts protective effects in a rat model of cardiac ischemia/reperfusion. *Can J Physiol Pharmacol* 2016; **94**(3):245-250.
18. Hsieh H-Y, Chuang H-C, Shen F-H, Detroja K, Hsin L-W, Chen C-S. Targeting breast cancer stem cells by novel HDAC3-selective inhibitors. *Eur J Med Chem.* 2017;**140**:42-51.
19. Gumucio JP, Mendias CL. Atrogin-1, MuRF-1, and sarcopenia. *Endocrine.* 2013;**43**(1):12-21.
20. Tseng Y-C, Kulp SK, Lai I-L, Hsu E-C, He W-A, Frankhouser DE, *et al*. Preclinical Investigation of the Novel Histone Deacetylase Inhibitor AR-42 in the Treatment of Cancer-Induced Cachexia. *J Natl Cancer Inst.* 2015;**107**(12):djv274.
21. Takeshita H, Yamamoto K, Nozato S, Inagaki T, Tsuchimochi H, Shirai M, *et al.* Modified forelimb grip strength test detectsaging-associated physiological decline in skeletal muscle function in male mice. *Sci Rep* 2017; **7:** 42323.
22. Kim H, Cho SC, Jeong H-J, Jeong H-J, Lee H-Y, Jeong MH, *et al*. Indoprofen prevents muscle wasting in aged mice through activation of PDK1/AKT pathway. *J Cachexia Sarcopenia Muscle.* 2020;**11**(4):1070-1088.
23. Sharples AP, Al-Shanti N, Stewart CE. C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: relevance to disease and ageing? *J Cell Physiol.* 2010;**225**(1):240-250.
24. Seto DN, Kandarian SC, Jackman RW. A Key Role for Leukemia Inhibitory Factor in C26 Cancer Cachexia. *J Biol Chem.* 2015;**290**(32):19976-19986.
25. Ministry of Health and Welfare, Taiwan Herbal Pharmacopeia, 3rd Edition English Version. Taiwan, Taipei: Ministry of Health and Welfare Taiwan, Republic of China; December, 2019.
26. Carson JA, Baltgalvis KA. Interleukin 6 as a key regulator of muscle mass during cachexia. *Exerc Sport Sci Rev.* 2010;**38**(4):168-176.
27. Liu H, Guo M, Xue T, Guan J, Luo L, Zhuang Z. Screening lifespan-extending drugs in Caenorhabditis elegans via label propagation on drug-protein networks. *BMC Syst Biol.* 2016;**10**(Suppl 4):131.
28. O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC. C. elegans in high-throughput drug discovery. *Adv Drug Deliv Rev.* 2014;**69-70**:247-253.
29. Carretero M, Solis GM, Petrascheck M. C. elegans as Model for Drug Discovery. *Curr Top Med Chem.* 2017;**17**(18):2067-2076.

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30. Bae Y-K, Sung J-Y, Kim Y-N, Kim S, Hong K-M, Kim H-T, *et al*. An in vivo C. elegans model system for screening EGFR-inhibiting anti-cancer drugs. *PLoS One.* 2012;**7**(9):e42441.
31. Kim W, Hendricks GL, Lee K, Mylonakis E. An update on the use of C. elegans for preclinical drug discovery: screening and identifying anti-infective drugs. *Expert Opin Drug Discov.* 2017;**12**(6):625-633.
32. Ikenaka K, Tsukada Y, Giles AC, Arai T, Nakadera Y, Nakano S, *et al*. A behavior-based drug screening system using a Caenorhabditis elegans model of motor neuron disease. *Sci Rep.* 2019;**9**(1):10104.
33. Chen X, Barclay JW, Burgoyne RD, Morgan A. Using C. elegans to discover therapeutic compounds for ageing-associated neurodegenerative diseases. *Chem Cent J.* 2015;**9**:65.
34. Ma J, Kang SY, Meng X, Kang AN, Partk JH, Park Y-K, *et al*. Effects of Rhizome Extract of Dioscorea batatas and Its Active Compound, Allantoin, on the Regulation of Myoblast Differentiation and Mitochondrial Biogenesis in C2C12 Myotubes. *Molecules.* 2018;**23**(8):2023
35. Ma J, Meng X, Liu Y, Yin C, Zhang T, Wang P, *et al*. Effects of a rhizome aqueous extract of Dioscorea batatas and its bioactive compound, allantoin in high fat diet and streptozotocin-induced diabetic mice and the regulation of liver, pancreas and skeletal muscle dysfunction. *J Ethnopharmacol.* 2020;**259**:112926.
36. Lu J, Wong RN, Zhang L, Wang RYL, Ng TB, Lee KF, *et al.* Comparative Analysis of Proteins with Stimulating Activity on Ovarian Estradiol Biosynthesis from Four Different Dioscorea Species in vitro Using Both Phenotypic and Target-based Approaches: Implication for Treating Menopause. *Appl Biochem Biotechnol.* 2016;**180**(1):79-93.
37. Yoshida R, Nagira M, Kitaura M, Imagawa N, Imai T, Yoshie O. Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. *J Biol Chem.* 1998;**273**(12):7118-7122.
38. Sarafi MN, Garcia-Zepeda EA, MacLean JA, Charo IF, Luster AD. Murine monocyte chemoattractant protein (MCP)-5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. *J Exp Med.* 1997;**185**(1):99-109.
39. Yuan L, Han J, Meng Q, Xi Q, Zhuang Q, Jiang Y, *et al.* Muscle-specific E3 ubiquitin ligases are involved in muscle atrophy of cancer cachexia: An in vitro and in vivo study. Oncol Rep 2015; **33**(5): 2261-2268.
40. **U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers In.; 2005: 7.**

This article is protected by copyright. All rights reserved.

41. Erin E. Talbert, Maria C. Cuitiño, Katherine J. Ladner, Modeling Human Cancer-induced Cachexia, Cell Reports,2019; **28**(6):1612-1622.

42. Von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2019. *J Cachexia Sarcopenia Muscle.* 2019;**10**(5):1143-1145.

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