Mas Receptor Activation Slows Tumor Growth and Attenuates Muscle Wasting in Cancer

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Abstract

Cancer cachexia is a multifactorial syndrome characterized by a progressive loss of skeletal muscle mass associated with significant functional impairment. Cachexia robs patients of their strength and capacity to perform daily tasks and live independently. Effective treatments are needed urgently. Here, we investigated the therapeutic potential of activating the "alternative" axis of the renin-angiotensin system, involving ACE2, angiotensin-(1-7), and the mitochondrial assembly receptor (MasR), for treating cancer cachexia. Plasmid overexpression of the MasR or pharmacologic angiotensin-(1-7)/MasR activation did not affect healthy muscle fiber size in vitro or in vivo but attenuated atrophy induced by coculture with cancer cells in vitro. In mice with cancer cachexia, the MasR agonist AVE 0991 slowed tumor development, reduced weight loss, improved locomotor activity, and attenuated muscle wasting, with the majority of these effects dependent on the orexigenic and not antitumor properties of AVE 0991. Proteomic profiling and IHC revealed that mechanisms underlying AVE 0991 effects on skeletal muscle involved miR-23a-regulated preservation of the fast, glycolytic fibers. MasR activation is a novel regulator of muscle phenotype, and AVE 0991 has orexigenic, anticachectic, and antitumorigenic effects, identifying it as a promising adjunct therapy for cancer and other serious muscle wasting conditions.

Significance: These findings demonstrate that MasR activation has multiple benefits of being orexigenic, anticachectic, and antitumorigenic, revealing it as a potential adjunct therapy for cancer.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/4/706/F1.large.jpg.

Introduction

Cancer cachexia is a complex, multifactorial syndrome characterized by a progressive loss of skeletal muscle mass that is associated with significant functional impairments (1). It affects 40% to 80% of all patients with advanced cancer with the highest prevalence in those with pancreatic, gastric, esophageal, colorectal, and lung cancer, and in patients with advanced prostate, head/neck, liver, osteosarcoma, cervical, ovarian, or breast cancer (2, 3). The devastating consequences include profound weakness, impaired mobility and fatigue, reduced functional independence, and in the worst cases, compromised survival and death from metabolic, respiratory (diaphragm), or cardiac muscle (heart) failure (4). Cachexia is estimated to account for 20% to 30% of...
all cancer-related deaths (5), and weight loss and body mass index are predictors of survival in patients with cancer (6, 7). Unfortunately, effective treatment options for cancer cachexia are lacking, and therefore identifying new therapeutic targets is critical.

A potential target that has received relatively little attention is the renin-angiotensin system (RAS). The "classical" RAS axis, which involves conversion of angiotensin I (Ang I) to angiotensin II (Ang II) by the angiotensin-converting enzyme (ACE) and signaling via the angiotensin type 1 (AT1) receptor, has been well-described as a negative regulator of skeletal muscle mass. Circulating Ang II levels are elevated in several muscle wasting conditions (8), and Ang II treatment induces muscle fiber atrophy in vitro and in vivo, effects associated with reduced Akt phosphorylation, increased myonuclear apoptosis, and enhanced expression of the muscle-specific E3 ligases MuRF-1 and atrogin-1 (MAFbx; refs. 9–12). In contrast, the "alternative" RAS axis counteracts signaling by the classical axis and so skeletal muscle size can be regulated by the balance between the two RAS axes. The alternative ACE2/Ang-(1-7)/MasR axis involves conversion of Ang I to angiotensin-(1-7) [Ang-(1-7)] via one of two pathways: (i) direct hydrolysis of Ang II to Ang-(1-7) via ACE2; or (ii) indirect hydrolysis of Ang I to angiotensin-(1-9) [Ang-(1-9)] via ACE2 and subsequent conversion of Ang-(1-9) to Ang-(1-7) via ACE. ACE2 mediates production of Ang-(1-7) by two distinct pathways, although the catalytic activity of ACE2 is approximately 400-fold higher with Ang II as a substrate than Ang I (13). Ang-(1-7) signals through the G-protein-coupled transmembrane mitochondrial assembly receptor (MasR; ref. 14). Ang-(1-7) treatment counteracts muscle atrophy induced by Ang II administration in mice via mechanisms involving reduced TGFβ signaling, MuRF-1 and atrogin-1 expression, and myonuclear apoptosis, and increased myosin heavy chain (MyHC) expression and Akt phosphorylation (9, 10, 15). Therefore, activation of the alternative RAS axis has therapeutic potential for muscle wasting conditions associated with increased Ang II/AT1 signaling. In this respect, infusion or administration of Ang-(1-7) in mice attenuated the muscle wasting and weakness associated with disuse (16) and endotoxemia (17), and reduced muscle fibrosis and enhanced strength in dystrophic mice (18, 19). Recent studies reported increased plasma Ang II mRNA in cachetic but not noncachetic cancer patients (20) and SNPs in the ACE gene, resulting in increased ACE activity, were associated with concurrent weight loss and low skeletal muscle index in patients with cancer (21). The therapeutic potential of activating the alternative RAS axis for cancer cachexia has not been investigated. We tested the hypothesis that activation of the alternative ACE2/Ang-(1-7)/MasR axis attenuates muscle wasting in cancer and report for the first time that activating the alternative ACE2/Ang-(1-7)/MasR axis modules muscle phenotype with the multiple benefits of having orexigenic, anticachetic, and antitumorigenic effects.

Materials and Methods

Animals

All animal experiments were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). Mice were obtained from the Animal Resources Centre and housed in the Biological Research Facility at The University of Melbourne under a 12:12-hour light–dark cycle. Water was available ad libitum, and both water and standard laboratory chow were provided, changed, and monitored daily. Methods for production and infection of recombinant adeno-associated virus (rAAV) vectors are described in the Supplementary Information, and the primers with the restriction sites are shown in Supplementary Table S1.

In vivo AVE 0991 study

The Colon-26 (C-26) mouse model of cancer cachexia is described in detail in the Supplementary Information. In the low-dose AVE 0991 study, CD2F1 mice (18–19-week-old) received an s.c. injection of C-26 cells (day 1), and 3 days later, began receiving the nonpeptide, orally-active MasR agonist, AVE 0991, via oral gavage at a dose of 1 mg/kg/day (n = 8) or an equivalent volume of vehicle (sterile 0.9% NaCl containing 11.8% DMSO, n = 8), given 10 times over a 13-day period (days 4–17). The volume given was 5 μl per gram body mass (i.e., 150 μl for a 30 g mouse). Seventeen days after C-26 injection (day 18), mice were anesthetized with sodium pentobarbitone (Nembutal, 60 mg/kg) via i.p. injection, and the tumor was excised, and tumor length, width, and depth were measured using digital calipers to facilitate determination of tumor volume. Mice were killed as a consequence of the cancer condition.

In the high-dose AVE 0991 study, CD2F1 mice (10.5-month-old) received an s.c. injection of C-26 cells (day 1), and 3 days later, began receiving AVE 0991 via oral gavage at a dose of 15 mg/kg/day (n = 16) or an equivalent volume of vehicle (sterile 0.9% NaCl containing 11.8% DMSO, n = 8), given 9 times over a 10-day period (days 4–14). The volume given was 6 μl per gram body mass (i.e., 180 μl for a 30 g mouse). Mice in the vehicle group and in one cohort of AVE 0991-treated mice (n = 8) were fed ad libitum, and the second cohort of AVE 0991-treated mice was pair-fed (PF) to the control group to account for effects on food intake (n = 8). Water was available ad libitum for all groups. Sixteen days after C-26 injection (day 17), whole body metabolism and locomotor activity was assessed using the Promethion Metabolic Analyzer (Sable Systems International). Mice were acclimated for 12 hours before data were collected every 5 minutes over a 12-hour light period and a 12-hour dark period. Oxygen consumption (VO2), energy expenditure, locomotor activity (Pedometers, meters moved), and the number of beam breaks (sum of breaks in x, y, and z beams) were recorded. During the entire data collection period, mice received drinking water (ad libitum). Mice also received food ad libitum, with the exception of the PF animals that were only provided with the average amount of food consumed by the vehicle-treated mice over the 24-hour period. Eighteen days after C-26 injection (day 19), mice were anesthetized with sodium pentobarbitone (Nembutal, 60 mg/kg) via i.p. injection, and dissections were performed as in the low-dose study.

Culture of C2C12 cells

Murine C2C12 myoblasts (ATCC) were plated in 6- or 12-well plates and cultured in DMEM (Life Technologies) supplemented with 10% (v/v) FCS (Life Technologies) and 1% l-glutamine.
Other cells were co-cultured with cancer cells and analyzed by SDS-PAGE and Western blot. For immunohistochemistry, the noncancer controls were undergoing surgery for bile duct complications (7 females/5 males) and 12 patients with pathologically diagnosed pancreatic ductal adenocarcinoma (PDAC) cancer (7 females/5 males) were classed as being cachectic (>5% weight loss in the previous 12 months, n = 12). At the beginning of surgery, a sample of approximately 100 mg of rectus abdominus muscle was excised under aseptic conditions, immediately frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

**Results**

Muscle-specific MasR overexpression does not alter muscle fiber size in healthy mice

Although activation of the alternative RAS axis can counteract Ang II–induced muscle wasting (9, 10, 15), its effects on skeletal muscle size in healthy basal (in vivo) conditions was unknown. We
therefore used rAAV vectors to directly increase MasR expression in skeletal muscle of healthy mice and examined its effect on muscle fiber size. This method achieves effective overexpression and is useful for such proof-of-principle studies. rAAV-mediated overexpression of the AT1 was used as a comparison as it would be anticipated to reduce skeletal muscle size. rAAV9 vectors expressing the MasR or AT1 were injected into the right tibialis anterior (TA) muscle, and rAAV9 expressing an empty control vector was injected into the contralateral left TA muscle of healthy CD2F1 mice and analyzed 21 days (d) later. Intramuscular injection of rAAV9:MasR increased MasR protein abundance by approximately 5.3-fold compared with muscles injected with empty vector (P < 0.001; Supplementary Fig. S1A), but had no effect on muscle fiber CSA (Supplementary Fig. S1B and S1C). In comparison, intramuscular injection of rAAV9:AT1 increased AT1 protein abundance by approximately 3.2-fold (P < 0.001; Supplementary Fig. S1D) and reduced average muscle fiber CSA by 11% (P < 0.05; Supplementary Fig. S1E), an effect attributed to an increased proportion of smaller fibers (P < 0.05; Supplementary Fig. S1F). Supporting these results, pharmacologic activation of the ACE/Ang II/AT1 axis in healthy C2C12 cells using Ang II or A779 induced myotube atrophy (P < 0.01; Supplementary Fig. S2A and S2B). These findings confirm that skeletal muscle fiber size in healthy basal conditions can be negatively regulated by increasing expression or activation of the AT1 but is not affected by direct MasR overexpression.

Genetic MasR overexpression and pharmacologic Ang-(1-7)/MasR agonist attenuate serum starvation–induced muscle atrophy in vitro

Because activation of the ACE2/Ang-(1-7)/MasR axis protects against muscle wasting in various conditions including diise (16) and endotoxin-induced sepsis (17), we investigated the downstream mediators of this response in a nontargeted and unbiased manner to direct the focus of our subsequent in vivo analyses. We also wanted to avoid the complications of inflammatory and cachetic factors in media from cancer cells to better understand the mechanisms within skeletal muscle cells. To this end, we used a serum starvation model of muscle atrophy in vitro. C2C12 myotubes that had been differentiated for 4 days were incubated for a further 48 hours in serum-free media (serum-starved), causing a 40% reduction in myotube diameter (P < 0.05; Supplementary Fig. S2C and S2D). The starvation-induced atrophy was associated with a decrease in protein synthesis (P < 0.05; Supplementary Fig. S2E and S2F). 37% increase in AT1 protein abundance (P < 0.05) but no change in MasR protein abundance (Supplementary Fig. S2G–S2I).

To induce MasR overexpression in myotubes, we transfected C2C12 myoblasts with a control plasmid (rAAV-CMV-GFP) or plasmid expressing the MasR (rAAV-CMV-MasR), and after 4-day differentiation, myotubes were incubated for 48 hours in HS or serum-starved. MasR overexpression had no effect on healthy myotube size but completed prevented starvation-induced atrophy (P < 0.0001; Fig. 1A and B). To confirm that similar findings were seen when MasR overexpression was induced only in myotubes and not expressed at the myoblast stage, we transfected C2C12 myoblasts with a Tet-regulated lentivirus without (empty) or containing the MasR gene, differentiated for 4 days and then incubated myotubes for 48 hours in HS or serum-starved in the presence of doxycycline. MasR overexpression was confirmed by Western blotting (Supplementary Fig. S3A). Inducing MasR overexpression in differentiated myotubes had no effect on healthy myotube size, but attenuated starvation-induced atrophy by 51% (P < 0.02; Supplementary Fig. S3B and S3C). The similar findings between experiments inducing MasR overexpression in myotubes or myoblasts strongly support the protective effects of MasR overexpression for starvation-induced atrophy.

For pharmacologic activation of the Ang-(1-7)/MasR axis, differentiated myotubes were incubated for 48 hours in HS or serum-starved without or with Ang-(1-7) or AVE 0991 (MasR agonist). DMSO-treated myotubes were used as a vehicle control for the AVE 0991 experiments. Neither Ang-(1-7) nor AVE 0991 altered the size of healthy myotubes, but both attenuated starvation-induced atrophy, by 64% and 41%, respectively (P < 0.001; Fig. 1C–F). Neither Ang-(1-7) nor AVE 0991 increased phosphorylation of Akt or mTOR (Fig. 1G–I) or enhanced protein synthesis (Fig. 1J; Supplementary Fig. S3D).

Next, we performed stable isotope dimethyl labeling and LC-MS/MS to identify, in a nontargeted and nonbiased manner, proteins and pathways altered by AVE 0991 compared with vehicle treatment of serum-starved C2C12 myotubes (Fig. 2A). AVE 0991 was used because unlike Ang-(1-7) (H-1715), it is orally active and resistant to proteolytic enzymes (26) and hence more clinically relevant. A total of 911 proteins were identified, and among them, 22 were increased (by a fold change > 1.3) and 18 were decreased (by a fold change < 0.85) in AVE 0991 compared with DMSO-treated serum-starved myotubes (Supplemental Tables S3 and S4). The top-ranked downregulated proteins and the biological processes and molecular functions representing these proteins are shown in Supplementary Fig. S3E and S3F.

The top-ranked upregulated protein was myosin-4 (fast myosin heavy chain, MyHC; Iib, Fig. 2B), and consistent with its upregulation, functional analyses using DAVID revealed the most prominent biological process, and molecular function of upregulated proteins in AVE 0991-treated serum-starved myotubes involved striated muscle contraction and motor activity, respectively (Fig. 2C). Western blotting confirmed upregulation of fast MyHC in AVE 0991-treated serum-starved myotubes (P < 0.05; Fig. 2D), indicating that the protective effect of AVE 0991 in atrophied myotubes was associated with a shift toward a greater abundance of larger, fast type II muscle fibers. Quantitative PCR analysis confirmed that serum starvation was associated with a 64% decrease in MHCIb mRNA expression (P < 0.05, Fig. 2E).

Plasmid overexpression and pharmacologic activation of the MasR attenuate cancer-induced muscle atrophy in vitro

We next examined whether the benefits of MasR activation extended to cancer-based muscle wasting. Because AT1 was elevated in serum-starved myotubes, we first examined whether AT1 expression was similarly increased in humans (Supplementary Table S2) and mice with cancer cachexia. AT1 mRNA was approximately 100% higher in rectus abdominus muscle from cachectic (5% body weight loss) patients with PDAC cancer compared with noncancer controls (P < 0.05, Fig. 3A). However, the cachetic PDAC group were significantly older than controls (P < 0.05, Supplementary Table S2), and so we examined the relationship between age and AT1 mRNA expression but found no significant correlation (r² = 0.058; P = 0.292, Fig. 3B). These findings indicate that the increase in AT1 mRNA in the cachetic PDAC group was not simply due to age. Similar to serum-starved myotubes, MasR mRNA expression was not different between controls and patients with PDAC (P = 0.16; Fig. 3C) and was not...
significantly correlated with age ($r^2 = 0.038; P = 0.399$, Fig. 3D).

There was no significant correlation between AT1 or MasR mRNA expression and the percentage weight loss in the previous 12 months in the patients with PDAC (AT1, $r^2 = 0.18, P = 0.18$; MasR, $r^2 = 0.26, P = 0.10; n = 12$).

In gastrocnemius muscles from the C-26 mouse model of cancer cachexia, which had a 23% smaller mass than controls (PBS, 4.43 ± 0.12; C-26, 3.42 ± 0.13 mg/g initial body mass, unpaired $t$ test; $P < 0.001; n = 7$), AT1 protein expression was 240% higher ($P < 0.01$; Fig. 3E), and MasR protein expression was 21% lower than controls ($P < 0.01$; Fig. 3F).

As depicted in Fig. 4A, C2C12 myoblasts were transfected with GFP control or MasR plasmid, and after 4-day differentiation, a transwell insert containing no cells (control) or C-26 cancer cells was added and cocultured for 48 hours. Coculture with C-26 cells induced a 45% reduction in myotube size ($P < 0.05$, compare Con+GFP and C-26+GFP in Fig. 4B and C). However, MasR overexpression attenuated the atrophy from C-26 coculture by 46% ($P < 0.05$; Fig. 4B and C).

To investigate the effect of pharmacologic MasR activation on C-26–induced muscle atrophy and to assess whether greater protection could be conferred by combined antagonism of the AT1, differentiated C2C12 myotubes were treated for 48 hours with vehicle (Veh, DMSO), the AT1 antagonist telmisartan (Tel), AVE 0991 (MasR agonist; E and F), and cell diameter was assessed (D and F). Two-way ANOVA; $*P < 0.05$ vs. HS + control/vehicle; $\dagger P < 0.05$ vs. HS + Ang-(1-7)/AVE 0991; $\ddagger P < 0.01$ vs. serum starved + control/vehicle; $n = 8$. G–J, Representative Western blots (G) and quantification (H) of phosphorylated and total Akt, phosphorylated and total mTOR (I), and puromycin stain (J) to assess protein synthesis in myotubes incubated for 48 hours in serum-free media and treated at the same time with or without Ang-(1-7), vehicle control, or AVE 0991. One-way ANOVA; $* P < 0.05$ vs. serum starved + Control; $n = 3$. 

Figure 1. Genetic MasR overexpression and pharmacologic Ang-(1-7)/MasR activation protects against serum starvation-induced muscle fiber atrophy in vitro. A and B, C2C12 myoblasts were transfected with GFP control or MasR plasmid and, after 4 days of differentiation into myotubes, were incubated in normal HS or serum-free media (SS, serum-starved; A) and cell diameter assessed after 48 hours (B). Two-way ANOVA; ‘$P < 0.001$ vs. HS + GFP; ‘$P < 0.001$ vs. HS + MasR; ‘$P < 0.001$ vs. serum starved + GFP; $n = 6$. Pharmacologic Ang-(1-7)/MasR activation was induced by incubating differentiated C2C12 myotubes for 48 hours in HS or serum starved and treating at the same time: with or without Ang-(1-7) (C and D); or with vehicle control (DMSO) or AVE 0991 (MasR agonist; E and F), and cell diameter was assessed (D and F). Two-way ANOVA; ‘$P < 0.05$ vs. HS + control/vehicle; ‘$P < 0.05$ vs. HS + Ang-(1-7)/AVE 0991; ‘$P < 0.01$ vs. serum starved + control/vehicle; $n = 8$. G–J, Representative Western blots (G) and quantification (H) of phosphorylated and total Akt, phosphorylated and total mTOR (I), and puromycin stain (J) to assess protein synthesis in myotubes incubated for 48 hours in serum-free media and treated at the same time with or without Ang-(1-7), vehicle control, or AVE 0991. One-way ANOVA; ‘$P < 0.05$ vs. serum starved + Control; $n = 3$. 

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63%, respectively, indicating no additive effect of the two treatments ($P < 0.05$, Fig. 4D and E). Treatment with telmisartan alone had no effect on myotube size (Fig. 4D and E).

The combination of plasmid-mediated MasR overexpression and AVE 0991 treatment was also investigated and compared with either intervention alone (MasR overexpression, +34%; AVE 0991, +25%). Combination treatment conferred the greatest increase in the size of myotubes cocultured with C-26 cells, with effects being additive (+66%, $P < 0.0001$, Fig. 4F and G).

These findings demonstrate that genetic overexpression or pharmacologic activation of the MasR can attenuate cancer-induced wasting when treatment is given prior to or at the same time as coculture is initiated. However, whether it could reverse atrophy was not known. We therefore cocultured C2C12 myotubes with C-26 cells for 24 hours, which induced a 37% decrease in myotube size ($P < 0.0001$, Fig. 4H and I), before adding vehicle or AVE 0991 and assessed myotube diameter after another 24 hours. AVE 0991 treatment initiated 24 hours after coculture
completely reversed the C-26–induced atrophy, with myotube size not significantly different from controls (Fig. 4J and K). Coculture with C-26 cells for 48 hours induced a 72% decrease in MHCIIb mRNA expression ($P < 0.02$, Fig. 5A). We used Ingenuity Pathway Analysis (IPA) to identify potential interacting pathways between MasR and Myh4 (MHCIIb). This identified miR-23a (miR-23a-3p) as a mediator of MasR regulation of Myh4, with MasR signaling inhibiting miR-23a expression, and Myh4 being a direct target of miR-23a-3p (Fig. 5B). The 3'UTR of Myh4 contains a binding site for the seed sequence of miR-23a-3p (Fig. 5B). We therefore investigated whether increasing miR-23a-3p expression attenuated the protective effects of AVE 0991 for cancer-induced myotube wasting. C2C12 myoblasts were transfected with a miR-23a-3p mimic or mimic negative control, and after 4 days of differentiation, myotubes were cocultured for 48 hours with C-26 cells and treated without (DMSO) or with AVE 0991. The miR-23a-3p mimic attenuated the AVE 0991-induced increase in myotube size by 19% ($P < 0.01$, Fig. 5C and D). By identifying relevant pathways, these findings provide further insight into the mechanism of fiber protection by MasR signaling.

Pharmacologic MasR activation slows tumor growth and attenuates muscle wasting in mice with cancer cachexia by inducing an oxidative-to-glycolytic muscle fiber transition

To test the clinical relevance of our in vitro findings, we next examined whether MasR activation could attenuate cancer-induced muscle wasting in vivo. To this end, we used oral gavage of AVE 0991 rather than rAAV-mediated MasR overexpression because oral administration is more likely to be implemented in
Figure 4.
Plasmid MasR overexpression and pharmacologic Ang-(1-7)/MasR activation attenuates and reverses C-26 cancer cell–induced muscle fiber atrophy in vitro. A, Schematic of the C2C12-C-26 coculture system, with C2C12 myotubes incubated for 48 hours with a transwell insert containing no cells (control) or C-26 cancer cells. B and C, C2C12 myoblasts were transfected with GFP control or MasR plasmid and, after 4 days of differentiation, were cocultured with or without C-26 cancer cells and assessed 48 hours later for myotube diameter (one-way ANOVA; *, P < 0.05 vs. control + GFP; †, P < 0.05 vs. C-26 + GFP; n = 7–8). D and E, C2C12 myotubes that had been differentiated for 4 days were treated with vehicle (DMSO), the AT1 antagonist telmisartan (Tel), the MasR agonist AVE 0991 (AVE), or both telmisartan and AVE 0991 (AVE + Tel) and at the same time were cocultured with or without C-26 cancer cells and assessed 48 hours later for myotube diameter (one-way ANOVA; *, P < 0.05 vs. control + vehicle; †, P < 0.05 vs. C-26 + vehicle; ‡, P < 0.05 vs. C-26 + telmisartan; n = 8). F and G, C2C12 myoblasts were transfected with GFP control or MasR plasmid and, after 4 days of differentiation, were treated with vehicle (DMSO) or AVE 0991 and at the same time were cocultured with or without C-26 cancer cells and assessed 48 hours later for myotube diameter (one-way ANOVA; *, P < 0.05 vs. control + vehicle; †, P < 0.05 vs. C-26 + vehicle; ‡, P < 0.001 vs. C-26 + GFP + AVE 0991; n = 6). Pharmacologic Ang-(1-7)/MasR activation reverses C-26 cancer cell–induced muscle fiber atrophy in vitro. H and I, C2C12 myotubes were cocultured with or without C-26 cancer cells and assessed 24 hours later for myotube diameter (unpaired t test; ***: P < 0.001; n = 6). J and K, C2C12 myotubes were cocultured with or without C-26 cancer cells and, after 24 hours, were treated with vehicle or AVE 0991 and assessed for myotube diameter at 48 hours after coculture was initiated (one-way ANOVA; ***, P < 0.01 vs. control + vehicle; †, P < 0.05 vs. C-26 + vehicle; n = 4).
the clinic and have greater patient compliance, and because gene therapy approaches still need to be refined for clinical use. Although whole-body effects of AVE 0991 may complicate findings within the muscle, additional systemic benefits would further strengthen its therapeutic potential and interest to patients and clinicians. C-26 tumor–bearing mice were treated with 1 mg/kg AVE 0991 via daily oral gavage from days 4 to 17 and on day 18, skeletal muscle mass and fiber size were assessed (Supplementary Fig. S4A). AVE 0991 induced small but significant improvements in cumulative food and water intake, which are indicators of quality of life (Supplementary Fig. S4B and S4C). It also attenuated loss of body mass without altering tumor size (Supplementary Fig. S4D and S4E). Despite low-dose AVE 0991 having no effect on the percentage change in tumor-free body mass, the mass of various hindlimb muscles or other tissues (Supplementary Fig. S4F–S4H), or on average muscle fiber CSA (Supplementary Fig. S5A and S5B), it did induce a shift in fiber proportions from oxidative type IIa fibers toward the higher force-producing fast, glycolytic type IIb fibers (Supplementary Fig. S5A and S5C). Furthermore, AVE 0991 decreased mRNA expression of the slow isoforms of both troponin I and troponin C and reduced Smad3 mRNA (~44%, P < 0.01; Supplementary Fig. S5D and S5E).

Because AVE 0991 had previously been administered to rodents over doses ranging from 1 to 20 mg/kg (27–30), we next investigated whether a higher dose of AVE 0991 (15 mg/kg) would confer greater benefits in mice with severe cancer cachexia (Fig. 6A). In addition, an AVE 0991-treated group that was PF to the vehicle control was included to examine the relationship between observed benefits and food intake (Fig. 6A). In mice fed ad libitum, high-dose AVE 0991 increased food intake from day 15 (Fig. 6B), water intake from day 13 (Supplementary Fig. S6A), and attenuated loss of body mass from day 16 (Fig. 6C). As expected, PF AVE 0991-treated mice had a similar food intake (Fig. 6B), water intake (Supplementary Fig. S6A), and loss of relative body mass to vehicle controls (Fig. 6C). In mice fed ad libitum but not in PF mice, high-dose AVE 0991 decreased tumor area from day 12 (Fig. 6D) and at the end of the experimental period, reduced tumor volume by 30% (C-26+Vehicle, 2,743 ± 269 mm³; C-26+AVE 0991, 1,917 ± 186 mm³; C-26+AVE 0991 PF, 2,331 ± 394 mm³; one-way ANOVA; P < 0.05 C-26+AVE 0991 vs. C-26+Vehicle; n = 8–16). Whole-body metabolism and locomotor activity were assessed 16 days after C-26 injection, and there was a main effect for AVE 0991-treated mice either fed ad libitum or PF to controls to have a lower oxygen consumption (VO₂) than vehicle controls (P < 0.05, Fig. 6E). Energy expenditure was not different between groups (Fig. 6F), but AVE 0991-treated mice fed ad libitum had greater motor activity (Pedometers, P < 0.05 treatment main effect, Fig. 6G) and movement as assessed by the number of beam breaks (P < 0.05 treatment main effect; Supplementary Fig. S6B) compared with both vehicle control.
Figure 6. High-dose AVE 0991 (15 mg/kg) attenuates wasting and slows tumor growth in severely cachectic C-26 tumor-bearing mice. A, Experimental timeline involving treatment of C-26 tumor-bearing mice with high-dose AVE 0991 (15 mg/kg) or vehicle control. B–D, Daily cumulative food intake (B), relative body mass (C), and tumor area (D) were improved in AVE 0991-treated mice fed ad libitum (two-way ANOVA; *, P < 0.05 C-26 + AVE 0991 vs. C-26 + vehicle; †, P < 0.05 C-26 + AVE 0991 PF vs. C-26 + vehicle; ‡, P < 0.05 C-26 + AVE 0991 PF vs. C-26 + AVE 0991; n = 4–16). On day 17, mice were placed in a Promethion metabolic analyzer for 24 hours, and average oxygen consumption (VO2; E), energy expenditure (F), locomotor activity (Pedmeters; G), and energy expenditure relative to locomotor activity (H) were assessed for each of the 12-hour light and dark cycles, and for the average of both cycles (24-hour period; two-way ANOVA; *, P < 0.05 treatment main effect C-26 + AVE 0991 vs. C-26 + vehicle; †, P < 0.05 treatment main effect C-26 + AVE 0991 PF vs. C-26 + vehicle; ‡, P < 0.05 treatment main effect C-26 + AVE 0991 PF vs. C-26 + AVE 0991; n = 5–6). At the end of treatment (day 19), the tumor was excised, weighed, and measured for size and mass (representative tumors shown in inset in D), and the percentage change (I) in tumor-free body mass from initial (day 1) was calculated (one-way ANOVA; *, P < 0.01 vs. C-26 + vehicle; †, P < 0.05 C-26 + AVE 0991 vs. C-26 + vehicle; ‡, P < 0.05 C-26 + AVE 0991 PF vs. C-26 + vehicle; ‡, P < 0.05 C-26 + AVE 0991 PF vs. C-26 + AVE 0991; n = 8–16).
and PF mice. Energy expenditure normalized to locomotor activity was lower in AVE 0991-treated mice fed ad libitum compared with vehicle control and PF mice (P < 0.05, Fig. 6H). Furthermore, AVE 0991-treated mice fed ad libitum but not PF had an attenuated loss of tumor-free body mass (~9%, P < 0.01), and greater plantaris (+22%, P < 0.05), TA (+9%), and quadriceps muscle mass (+14%, P < 0.05) compared with vehicle controls (Fig. 61 and J). In AVE 0991-treated mice, heart and liver mass were both reduced by 10% in PF mice compared with those fed ad libitum (P < 0.05, Fig. 6K).

AVE 0991 treatment of mice fed ad libitum but not PF to controls enhanced average TA muscle fiber CSA by 16% (P < 0.05) due to increased CSA of the type Ix fibers (+20%, P < 0.05) and type Iib fibers (+18%, P < 0.05, Fig. 7A and B). In mice fed ad libitum, AVE 0991 also induced a shift in fiber type proportions away from type Ix fibers toward glycolytic type Iib fibers (P < 0.05; Fig. 7A and C). Moreover, AVE 0991 reduced mRNA expression of the slow isoforms of troponin I and C (P < 0.05), with the decrease in slow troponin C being independent of food intake (Fig. 7D).

To assess whether the observed beneficial effects of AVE 0991 were simply due to inhibition of tumor growth, a subset of mice with similar tumor burden were analyzed. As shown in Supplementary Fig. S6C–S6F, comparisons were made between mice in each group that had similar tumor burden (tumor mass as a percentage of total end body mass): 2.05%, 2.28%, and 2.28% (low tumor burden) and 4.96%, 5.09%, and 5.06% (high tumor burden). Because only one mouse per group was assessed, statistical analyses were not performed. The attenuated loss of tumor-free body mass and increase in quadriceps muscle mass with AVE 0991 remained in mice fed ad libitum with both low and high tumor burden (Supplementary Fig. S6C and S6D). The increase in plantaris and TA muscle mass with AVE 0991 was only maintained in mice fed ad libitum with low tumor burden (Supplementary Fig. S6C and S6D). Also maintained in AVE 0991 treated C-26 tumor-bearing mice (one-way ANOVA; μ = 0.05 vs. C-26 + vehicle; μ = 0.05 vs. C-26 + AVE 0991; C-26 + vehicle, n = 16; C-26 + AVE 0991, n = 15; C-26 + AVE 0991 PF, n = 7). Gene expression of the fast and slow isoforms of troponin I and troponin C (D), the ubiquitin ligases MuRF-1 and atrogin-1, TGFβ1, Smad-3, and caspase-3 in TA muscles from vehicle- or AVE 0991-treated C-26 tumor-bearing mice (one-way ANOVA; μ = 0.05 vs. C-26 + vehicle; μ = 0.05 vs. C-26 + AVE 0991; C-26 + AVE 0991 PF, n = 7–8; E). Using a 20 Objective, total magnification for images in A is ×126. Scale bar, 50 μm.

Relevance to other cancers
We investigated the therapeutic potential of AVE 0991 for attenuating wasting induced by other cancer cells and found that 48-hour coculture with Lewis lung carcinoma cells induced a 23% (P < 0.05) decrease in C2C12 myotube size, which was attenuated by 18% with AVE 0991 treatment (Supplementary Fig. S7A and S7B).
Discussion

The two regulatory axes of the RAS exert opposing effects in several tissues including skeletal muscle (31, 32), with the “classical” ACE/Ang II/AT1 axis inducing muscle wasting, which can be counteracted by the “alternative” ACE2/Ang-(1-7)/MasR axis (9, 10, 15). Activation of the alternative RAS axis therefore has therapeutic potential for muscle wasting conditions associated with hyperactivity of the classical axis. Here, we show that AT1 expression is elevated in preclinical and clinical cancer cachexia and that pharmacologic activation of the alternative axis attenuates cancer cell–induced atrophy in vitro, and improves locomotor activity, enhances muscle mass and fiber size, reduces weight loss, and slows tumor growth in mice with cancer cachexia. These effects were dependent on the orexigenic properties of the MasR agonist AVE 0991. Thus, the multifactorial benefits of AVE 0991 having orexigenic, anticachectic, and antitumorigenic effects identify this strategy as a novel and promising adjunct therapy for cancer.

Systemic activation of the ACE2/Ang-(1-7)/MasR axis reduces many of the deleterious effects of Ang II infusion in mouse skeletal muscle (9, 10, 15), and infusion of Ang-(1-7) in mice attenuates muscle wasting in several conditions, including the muscular dystrophies (18, 33), hindlimb immobilization (16), and endotoxin-induced sepsis (17). However, its role in the regulation of skeletal muscle size in healthy, basal conditions had not previously been examined. We demonstrate for the first time that increasing ACE2/Ang-(1-7)/MasR signaling has no direct effect on the regulation of healthy skeletal muscle size in vivo, with muscle-specific MasR overexpression not altering muscle fiber size in healthy mice. The lack of effect was not due to an absence of endogenous ligand as serum Ang-(1-7) levels are higher than Ang II in the healthy condition (34). Furthermore, genetic MasR overexpression or pharmacologic Ang-(1-7)/MasR activation had no effect on the size of healthy myotubes in vitro. Conversely, we demonstrated that muscle-specific AT1 overexpression or pharmacologic AT1 activation induced fiber atrophy in healthy mice. These experiments were done in conjunction with those involving intramuscular injection of aAV9.MasR and together confirm that the size of healthy skeletal muscle can be regulated by overexpression of the AT1, but not the MasR. In contrast, MasR overexpression or pharmacologic activation of the ACE2/Ang-(1-7)/MasR axis attenuated myotube atrophy induced with serum starvation or coculture with cancer in vitro. The latter findings were not specific to C-26-induced wasting, with AVE 0991 also attenuating wasting induced by coculture with Lewis lung carcinoma cells, indicating efficacy across multiple cancer types. Because the combination of increasing MasR levels and enhancing receptor activity conferred greater protection against cancer-induced atrophy in vitro, with effects being additive of the individual interventions, it is unlikely that the addition of ligands will induce further improvements in AVE 0991 activation. The findings also support the notion that the eventual best treatment may come from combining AVE 0991 with receptor overexpression when gene therapy approaches become suitable for clinical use. Because cancer cachexia is one of the most prevalent muscle wasting diseases affecting 40% to 80% of all patients with advanced cancer (2, 3, 35) and estimated to account for up to 30% of all cancer-related deaths (5), we investigated whether ACE2/Ang-(1-7)/MasR axis activation conferred protective effects against cancer-based wasting in vivo. This was demonstrated through low-dose (1 mg/kg) AVE 0991 treatment improving food and water intake and weight loss, with even greater benefits with higher dose (15 mg/kg) AVE 0991 administration, which also improved locomotor activity, enhanced muscle mass and fiber size, attenuated the loss of tumor-free body mass, and reduced tumor development in the C-26 mouse model of cancer cachexia. Although anticancer properties of Ang-(1-7) have been reported (36), this is one of the first reports of the clinically applicable nonpeptide MasR agonist AVE 0991 having antitumor effects. Most of the benefits of AVE 0991 remained when mice of similar tumor burden were compared but were diminished or lost in PF mice, indicating that the anticachectic effects were at least in part mediated by the orexigenic properties of AVE 0991 and were not simply due to slowed tumor growth. However, our findings in the cancer-free serum starvation cell culture model confirmed that AVE 0991 also has direct effects on skeletal muscle cells. In this regard, the identification of AVE 0991 as a regulator of skeletal muscle phenotype by preserving the large, fast type IIb fibers (myosin-4, fast MyHC) reveals a novel role for MasR signaling across multiple cancer types. Because cancer cachexia is one of the most prevalent muscle wasting diseases affecting 40% to 80% of all patients with advanced cancer (2, 3, 35) and estimated to account for up to 30% of all cancer-related deaths (5), we investigated whether ACE2/Ang-(1-7)/MasR axis activation conferred protective effects against cancer-based wasting in vivo. This was demonstrated

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No potential conflicts of interest were disclosed.

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References


# Mas Receptor Activation Slows Tumor Growth and Attenuates Muscle Wasting in Cancer

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