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Megestrol acetate improves cardiac function in a model of cancer cachexia-induced cardiomyopathy by autophagic modulation

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Abstract

Background Cachexia is a complex metabolic syndrome associated with cancer. One of the features of cachexia is the loss of muscle mass, characterized by an imbalance between protein synthesis and protein degradation. Muscle atrophy is caused by the hyperactivation of some of the main cellular catabolic pathways, including autophagy. Cachexia also affects the cardiac muscle. As a consequence of the atrophy of the heart, cardiac function is impaired and mortality is increased. Anti-cachectic therapy in patients with cancer cachexia is so far limited to nutritional support and anabolic steroids. The use of the appetite stimulant megestrol acetate (MA) has been discussed as a treatment for cachexia.

Methods In this study the effects of MA were tested in cachectic tumour-bearing rats (Yoshida AH-130 ascites hepatoma). Rats were treated daily with 100 mg/kg of MA or placebo starting one day after tumour inoculation, and for a period of 16 days. Body weight and body composition were assessed at baseline and at the end of the study. Cardiac function was analysed by echocardiography at baseline and at day 11. Locomotor activity and food intake were assessed before tumour inoculation and at day 11. Autophagic markers were assessed in gastrocnemius muscle and heart by western blot analysis.

Results Treatment with 100 mg/kg/day MA significantly attenuated the loss of body weight ($-9 \pm 12\%$, $P < 0.05$) and the wasting of lean and fat mass ($-7.0 \pm 6\%$ and -22.4 ± 3 %, $P < 0.001$ and $P < 0.05$, respectively). Administration of 100 mg/kg/day MA significantly protected the heart from general atrophy (633.8 ± 30 mg vs. placebo 474 ± 13 mg, $P < 0.001$). Tumour-bearing rats displayed cardiac dysfunction, as indicated by the significant impairment of the left ventricular ejection fraction, the left ventricular fractional shortening, the stroke volume, the end dyastolic volume, and the end systolic volume. In contrast, MA significantly improved left ventricular ejection fraction, left ventricular fractional shortening, and left ventricular end systolic volume. Western blotting analysis showed an upregulation of the autophagic pathway in the gastrocnemius and hearts of the placebo-treated tumour-bearing rats. Treatment with MA, however, was able to modulate the autophagic markers (e.g. Beclin-1, p62, TRAF6, and LC3) in the gastrocnemius and in the hearts of tumour-bearing rats. Most importantly, 100 mg/kg/day MA reduced mortality [hazard ratio (HR): 0.44; 95%CI: 0.20–1.00; $P = 0.0486$].

Conclusions Megestrol acetate improved survival and reduced wasting through a marked downregulation of autophagy, occurring in both skeletal and heart muscle, the latter effect leading to a significant improvement of cardiac function. Our data suggest that MA might represent a valuable strategy to counteract the development of cancer cachexia-induced cardiomyopathy.

Keywords Cancer cachexia; Heart failure; Cardiac wasting; Autophagy; Body composition; Megestrol acetate

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Introduction

Cachexia is a complex metabolic disorder which has been shown to occur in late stages of chronic disease including cancer, characterized by involuntary weight loss caused by an ongoing wasting of skeletal muscle with or without loss of adipose tissue.[1] In particular, cancer cachexia is a predictor of poor quality of life, poor treatment response, increased chemotherapy toxicity, and higher mortality.[2] Cachexia affects 50–80% of patients with cancer and is responsible for 30% of cancer deaths.[3]

Generally, loss of muscle mass can be because of a decreased rate of protein synthesis, an increased rate of protein degradation or both. However, there is a general consensus that cancer cachexia is essentially because of a sustained proteolysis.[4]

Weight loss not only involves skeletal muscle and fat tissue but multiple organs, including the heart. Although heart atrophy and functional cardiac abnormalities have been described in patients with cancer in the late sixties[5,6] and even though the heart, like the skeletal muscle, is a striated muscle, the effects of cancer cachexia on cardiac atrophy and function have been undervalued for a long time. The postulate that cancer cachexia results in cardiac atrophy and cardiac dysfunction, which leads to congestive heart failure, is nowadays well supported by several preclinical studies.[7–11] However, the process underlying cardiac atrophy in patients with cancer cachexia is still a matter of debate. The compromised heart function observed in cancer cachexia experimental models seems to be related to cardiac alterations including marked fibrosis and loss of contractile protein such as troponin I and myosin heavy chain.[8,11] The skeletal and heart muscle atrophies seem to be linked to an hyperactivation of the ubiquitin-proteasome system (UPS) that provides a mechanism for selective protein degradation in many atrophy conditions, including cancer cachexia.[4,11–14] However, UPS is not the only proteolitic pathway that is activated during cachexia. The activation of autophagy-lysosomal pathway has been proposed as well.[15] Interestingly, the UPS and autophagy may be coordinated to augment protein degradation.[16]

Tumour necrosis factor receptor-associated factor 6 (TRAF6) is an adapter protein for toll like receptor-mediated nuclear factor-κB signalling pathway activation that induces the production of pro-inflammatory cytokines.[17] It is formerly known as a E3 ubiquitine ligase, but it has been reported to play an important role in coordinating the activation of autophagy and UPS in atrophying skeletal muscles.[18,19]

Autophagy is a highly conserved lysosome-driven degradation pathway of cellular constituents, normally activated at basal level to maintain cell homeostasis. Emerging data clearly show that induction of autophagy occurs in the skeletal muscle and in the heart in different experimental models of cancer cachexia and that it strongly contributes to the pathogenesis of muscle wasting.[15,20,21] Whether autophagy is also modulated in cachectic patient is subject to debate. Some reports showed that autophagy is induced in muscle biopsies of patients with lung and oesophageal cancer,[22,23] but an earlier report, which investigated some biological markers in patients with cachexia associated with either chronic obstructive pulmonary disease or lung cancer, showed that autophagy is significantly increased only in the cachectic skeletal muscle of patients with chronic obstructive pulmonary disease[24] .

Loss of appetite (i.e. anorexia) is frequent in patients with cachexia and is associated with poor prognosis and reduced quality of life. Moreover, loss of appetite is a multifactorial event that includes the increased expression of proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumour necrosis factor or interferon-γ,[25] which have been shown to have effects on peripheral metabolic pathways as lipolysis, proteolysis as well as on hypothalamic appetite regulation.[26,27] However, none of the nutritional strategies so far proposed for the treatment of cancer cachexia has been sufficient enough to reverse the syndrome.

Megestrol acetate (MA) is a synthetic, orally active derivative of the hormone progesterone, originally synthesized in 1963 as a contraceptive drug.[28] It was used in the treatment of breast cancer and later in the treatment of endometrial cancer. It was approved in the USA and in several European countries for the treatment of the anorexia–cachexia syndrome.[29] In humans, MA treatment results in an increased sense of appetite and improved body weight, as shown in several clinical trials.[30–34] Although many reports suggest that the weight gain is only because of an increase in fat mass,[35,36] a recent randomized phase III clinical trial showed that MA treatment positively affects muscle mass and performance,[37] probably because of reduced muscle wasting. Indeed, it has been demonstrated that MA is able to reduce the rate of protein degradation in incubated isolated skeletal muscle through a mechanism based on the inhibition of the UPS.[38] Moreover, MA treatment seems to lower humoral factors implicated in cachectic response, such as cytokines.[39] Nevertheless, the potential for MA in the treatment of cachexia-induced cardiomiopathy remains to be better clarified.

The aim of the present investigation was to assess the effects of MA on body weight, body composition, cardiac function, and quality of life as well as survival in a rat Yoshida AH-130 hepatoma cancer cachexia model. Further, we also extensively investigated its efficacy in modulating the autophagic catabolic pathway in the gastrocnemius and in the hearts of tumour-bearing rats.

Material and methods

Animals

Male Wistar Han rats (Harlan Laboratories, Rossdorf, Germany) of 8 weeks of age and weight of 199.8 ± 2.8 g were kept under standard laboratory conditions in a specific-

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pathogen-free animal facility and maintained at 22 ± 2°C with alternating 12 h light–dark cycle and free access to food and water. All the experimental procedures were performed in accordance with the European Commission guidelines for the animals used for scientific purposes.

Study design

Rats were randomized into two groups to be injected with either Yoshida 10^8 AH-130 hepatoma cells ($n = 21$) or saline ($n = 11$, sham) into the peritoneum. Tumour-bearing rats were further divided to be treated with placebo ($n = 11$) or with 100 mg/kg/day MA ($n = 10$). All treatments were given via gavage once daily over a period of maximum 16 days. Treatment with MA or placebo started one day after tumour inoculation. All operators involved in the study were blinded to treatment allocation. One day before tumour inoculation, baseline body weight, body composition, and echocardiographic analysis were assessed. Cardiac function was analysed again on day 11. Body composition and body weight were recorded on day 16 or the day of the euthanasia if the animals had to be sacrificed for ethical reasons. At the end of the study and for each tumour-bearing animals, the tumour was harvested from the peritoneum and its volume evaluated. Tumour cell number was determined using a Neubauer chamber. Organs and tissues were rapidly removed, weighed, and immediately frozen in liquid nitrogen.

Body composition analysis

Total body fat, lean mass, and body fluids were measured using the nuclear magnetic resonance spectroscopy device EchoMRI-700™ (Echo Medical System, Houston, TX, USA). Each rat was allocated in a tube for the measurement, which takes 90 s. The analysis of the body structures is based on nuclear magnetic resonance, which measures the resonance of magnetic active nuclei in the tissues.[40]

Spontaneous activity and food intake

Animals were housed individually, and spontaneous movement was recorded by an infrared monitoring system (Supermax, Muromachi, Tokyo, Japan) over a 24 h period. Food intake was also recorded during this period.[41]

Echocardiographic analysis

Echocardiographic analyses were performed using the high-resolution Vevo 770 system (VisualSonics Inc, Toronto, Canada), which was described previously.[42] Rats were anaesthetized with 1.5% isoflurane and laid in supine position on a heated surface to maintain body temperature and with all legs taped to ECG electrodes. All hair was removed from the chest using an electrical clipper prior to shave the animals with a chemical depilatory agent. Recordings were made in B-mode and M-mode to assess functional parameters, cardiac function, and dimensions.

Protein extraction and sodium dodecyl sulfate polyacrylamide gel electrophoresis western blot

Approximately 50 mg of heart and gastrocnemius muscle were separately homogenized in 500 μL ice-cold lysis buffer (20 mM Tris–HCl pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2,5 mM $Na_4P_2O_7$; 20 mM NaF; 1 mM dithiothreitol; 1 mM Na_3VO4; 1 mM β-glycerophosphate; and 10 μL/mL freshly added protease and phosphatase inhibitor cocktails), centrifuged at 14 000 × g for 20 min at 4°C and supernatant was collected. A total of 20 μL of the supernatant was used to determine the total protein concentration by Bradford assay (Biorad, Hercukes, California, USA) using bovine serum albumin as a standard. Proteins were heat denatured for 5 min at 95°C in sample-loading buffer (500 mM Tris/HCl, pH 6.8; 30% Glycerol; 10% sodium dodecyl sulfate; 5% β-mercaptoethanol; and 0024% bromophenol blu), and 30 μg of protein lysate was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked with Tris/HCl (pH 7.6) containing 0.1% Tween 20 and 5% BSA for 2 h and incubated overnight at 4°C with shaking with the following primary antibodies: Beclin-1 (3738, Cell Signaling Technology, Boston, USA), ATG12 (4180, Cell Signaling Technology, Boston, USA), LC3B (NB100-2220, Novus Biologicals, Littleton, USA), SQSTM1/p62 (5114, Cell Signaling Technology, Boston, USA), TRAF6 (ab33915, Abcam, Cambridge, UK), and GAPDH (G9545, Sigma-Aldrich, St. Louis, Missouri, USA). Membranes were then washed in TBS (pH 7.6) with 0.1% Tween-20 and incubated with a fluorescent-conjugated IgG secondary antibody (IRDye 680RD, Fisher Scientific International Inc., Hampton, New Hampshire, USA) for 1 h at RT with shaking. Immunoblot scanning and analyses were performed using an imaging system (Odyssey Classic, LI-COR Biosciences, Lincoln, NE, USA). Quantification of the bands was performed using the ImageJ software (NIH, Bethesda, Maryland, USA).

Statistical analysis

Data were analysed with GraphPad PRISM 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results are shown as mean ± SEM. Normality was tested using D'Agostino Pearson's test. Normally distributed data were analysed by

one way ANOVA followed by Tukey's test, while data without normal distribution were analysed using Kruskal–Wallis analysis of variance and subsequent Dunn's tests. Survival was tested by Cox-proportional hazard analysis, hazard ratio (HR), and 95% confidence interval (CI). A *P*-value of <0.05 was considered significant.

Results

Megestrol acetate improves survival in cancer cachexia

To assess whether the treatment with MA could have a positive effect on the lifetime in case of cancer cachexia development, we analysed the survival of the animals. Despite of the lack of statistically significant effect of MA treatment on tumour growth ($2996 \pm 222 \times 10^6$ and $2970 \pm 538 \times 10^6$ in placebo and MA-treated animals, respectively), tumour-bearing rats treated with 100 mg/kg/day MA showed a better survival compared with placebo animals (HR: 0.44; 95%CI: 0.20–1.00; $P = 0.0486$; *Figure* 1)

Megestrol acetate reduces wasting in cancer cachexia

Baseline weight and body composition, i.e. lean and fat mass, were similar in all the randomized groups before tumour inoculation ($P > 0.2$; *Figure* 2(A–C)). However, while tumour-bearing rats treated with placebo lost $25 \pm 6\%$ of their initial body weight, non-tumour-bearing, or sham, animals gained $30.4 \pm 2\%$ ($P < 0.001$; *Figure* 2D). Fat mass was reduced by $64.6 \pm 1\%$ in tumour-bearing rats treated with placebo compared with a gain of $55.4 \pm 1\%$ in sham animals ($P < 0.001$; *Figure* 2E). Lean body mass was reduced by $26.1 \pm 5\%$ in the tumour-bearing rats treated with placebo, while a muscle mass gain of $27.5 \pm 2\%$ was observed in sham rats ($P < 0.001$; *Figure* 2F). Treatment with 100 mg/kg/day MA significantly attenuated the loss of body weight ($-9 \pm 12\%$, $P < 0.05$; *Figure* 2D) and the wasting of fat mass and lean mass (-22.4 ± 3 and $-7.0 \pm 6\%$ %, $P < 0.05$ and $P < 0.001$, respectively; *Figures* 2E and 2F) in the tumour-bearing rats.

As expected, the tumour burden had a strong effect on the individual muscles and tissues. The weights of the gastrocnemius muscle, soleus muscle, extensor digitalis longus muscle, and tibialis muscle were significantly lower in placebo-treated tumour-bearing rats compared with sham animal ($P < 0.001$; *Table* 1). Treatment with 100 mg/kg/day MA markedly increased the weights of mixed fibre type gastrocnemius muscle and tibialis, fast-twitch extensor digitalis longus muscle, and slow-twitch soleus muscle in the tumour-bearing animals (all $P < 0.001$ vs. placebo; *Table* 1). Moreover, white adipose tissue and brown adipose tissue were also afftected by tumour cachexia (both $P < 0.001$ vs. sham; *Table* 1). The treatment with 100 mg/kg/day MA increased the weight of epididymal white and brown intrascapular fat in tumour-bearing rats ($P < 0.01$ vs. placebo, respectively; *Table* 1).

Figure 1 Kaplan–Meier survival curve and statistical analysis of survival of animals treated with 100 mg/kg/day megestrol acetate or placebo. A high mortality was observed in the placebo group (85%), which was significantly reduced by 100 mg/kg/day megestrol acetate.

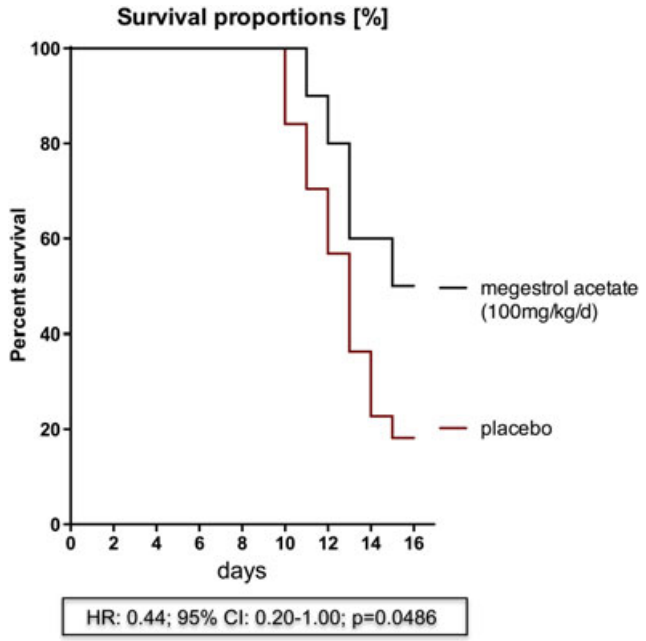

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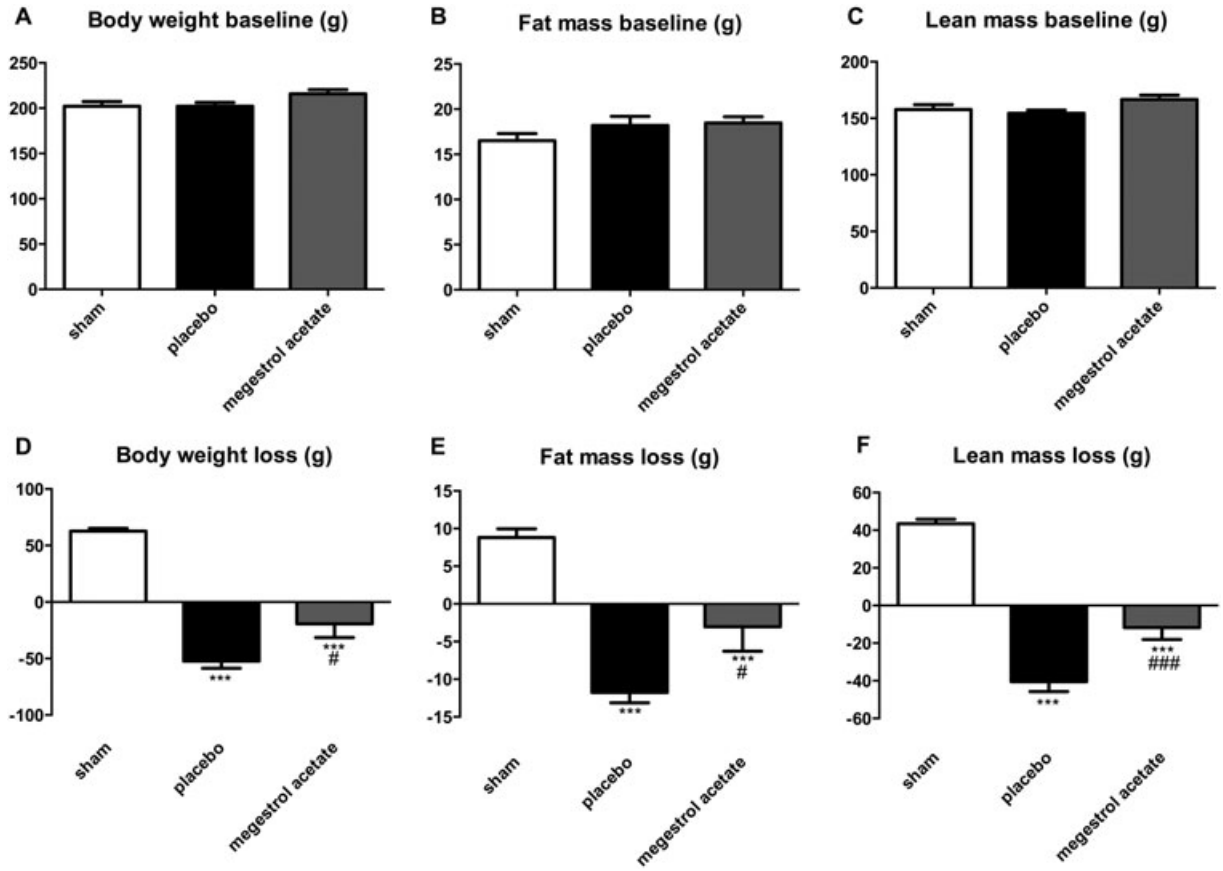
Figure 2 Effect of megestrol acetate treatment on body weight and body composition of the tumour-bearing rats. The lost of body weight (D), fat mass (E), and lean mass (F) are presented as the absolute difference between baseline (A, B, and C) and after removal the tumour at the end of the study. *White bars*: sham; *black bars*: placebo; *gray bars*: 100 mg/kg/day megestrol acetate. The data are presented as mean ± SEM. ***: $P < 0.001$ vs. sham, #:$P < 0.05$, ###:$P < 0.001$ vs. placebo. Sham $n - 11$, placebo $n - 11$, 100 mg/kg/day megestrol acetate $n - 10$.



Indices of morbidity

Baseline 24 h spontaneous activity and food intake were similar in all groups (all $P > 0.2$; Fig. S1). Cachectic rats treated with placebo showed a decreased food intake and activity on day 11 ($P < 0.001$ vs. sham; *Figure* 3A). Treatment with 100 mg/kg/day MA resulted in higher food intake compared with placebo ($P < 0.05$; *Figure* 3A) whilst no effect on activity was observed (*Figure* 3B).

Megestrol acetate improves tumour-induced cardiac dysfunction and heart wasting

Baseline echocardiography was similar in all groups before tumour inoculation ($P > 0.1$; Table S1). On day 11, tumour-bearing rats, treated with placebo, displayed an overall deterioration of cardiac function compared with baseline sham animals. However, a number of parameters of cardiac function were improved by treatment with MA. The left ventricular

Table 1. Tissues and muscles weight at the end of the study

	Gastrocnemius (mg)	Soleus (mg)	EDL (mg)	BAT (mg)	WAT (mg)
Sham	1235 ± 37.9	96.9 ± 2.7	105.2 ± 3.1	250.2 ± 13.9	1281 ± 66.7
Placebo	736.7 ± 34.1***	70.1 ± 1.6***	62.9 ± 2.6***	69.9 ± 3.4***	191.5 ± 9.1***
100 mg/kg/day MA	978.9 ± 48 ###	90.1 ± 4.7 ###	88.6 ± 4.8###	165.6 ± 33.5##	611.7 ± 148.7##

EDL, extensor digitalis longus; WAT, white adipose tissue; BAT, brown adipose tissue.
***$P < 0.001$ vs. sham.
##$P < 0.01$.
###$P < 0.001$ vs. placebo.

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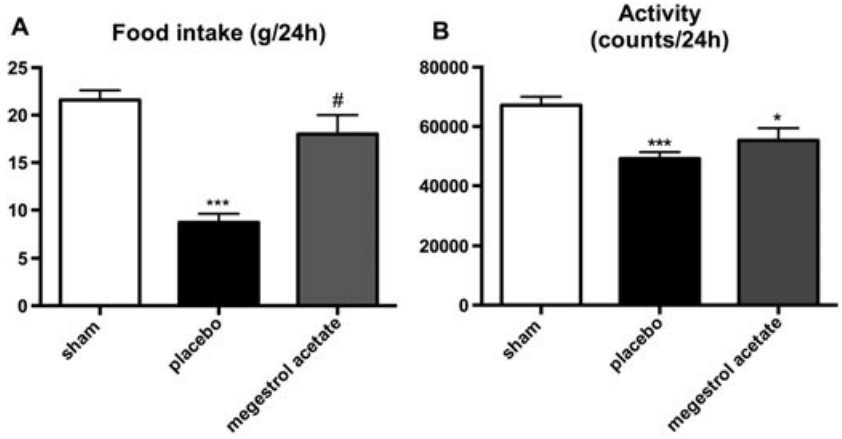
Figure 3 Effect of megestrol acetate treatment on (A) food intake and (B) spontaneous activity of tumour- bearing rats. Activity was not affected by the treatment, while food intake was increased by 100 mg/kg/day megestrol acetate. *White bars:* sham; *black bars:* placebo; *gray bars:* 100 mg/kg/day megestrol acetate. The data are presented as mean ± SEM. ***:$P < 0.001$, *$P < 0.05$ vs. sham; #:$P < 0.05$ vs. placebo. Sham $n - 11$, placebo $n - 11$, 100 mg/kg/day megestrol acetate $n - 10$.



ejection fraction (LVEF) was reduced in tumour-bearing rats treated with placebo compared with sham animals (58.7 ± 2% vs. sham 75.4 ± 2%, $P < 0.01$; *Figure* 4B). Treatment with 100 mg/kg/day MA significantly improved LVEF (73.5 ± 5%, $P < 0.05$ vs. placebo, *Figure* 4B). The left ventricular fractional shortening (LVFS) was also reduced in tumour-bearing rats treated with placebo in comparison to control animals (30.25 ± 1% vs. sham 51.6 ± 1%, $P < 0.001$; *Figure* 4C). The treatment with 100 mg/kg/day MA improved significantly LVFS (49 ± 4.6%; $P < 0.001$ vs. placebo; *Figure* 4C). The left ventricular stroke volume (LVSV), the end systolic volume (LVESV) and the end diastolic volume (LVEDV) were significantly impaired in tumour-bearing rats treated with placebo compared with sham animals ($P < 0.001$ for LVSV, $P < 0.05$ for LVESV and LVEDV vs. sham; *Figure* 4D, 4E, and 4F). Whereas the LVSV and the LVEDV were not improved in the MA-treated group, administration of 100 mg/kg/day MA was able to restore significantly the end systolic volume (66.5 ± 8.4 μL vs. placebo 98.4 ± 7.6 μL; $P < 0.05$; *Figure* 4E). At the end of the study, the average weight of the hearts of the tumour group treated with placebo was smaller compared with control rats (474 ± 13 mg vs. sham 776 ± 10 mg, $P < 0.001$; *Figure* 4A). Moreover, cancer cachexia had profound effects on cardiac diameters. For instance, a larger left ventricular internal diameter at end-systole (LVID sys) and a reduction of the left ventricular internal diameter at end-diastole (LVID dia) were observed in tumour-bearing rats treated with placebo compared with sham ($P < 0.01$ and $P < 0.001$ for LVID sys and LVID dia vs. sham, respectively; *Figures* 4G and 4H). Administration of 100 mg/kg/day MA significantly protected the heart from general atrophy (633.8 ± 30 mg vs. placebo 474 ± 13 mg, $P < 0.001$; *Figure* 4A) and from the loss of LV diameter in systole (2.9 ± 0.2 mm vs. placebo 3.8 ± 0.1 mm, $P < 0.01$; *Figure* 4G) and in diastole (5.8 ± 0.1 mm vs. placebo 4.5 mm, $P < 0.001$; *Figure* 4H). Finally, a striking reduction of left ventricular mass and posterior wall thickness (LVPWT sys), in systole, was observed in the tumour group treated with placebo compared with sham rats (both $P < 0.01$ vs. sham; *Figure* 4I and 4L). Treatment with 100 mg/kg/day MA significantly improved the value of the posterior wall thickness, in systole (2.75 ± 0.1 mm vs. placebo 2.3 mm, $P < 0.05$; *Figure* 4L).

Megestrol acetate downregulates autophagic catabolic pathway in the gastrocnemius muscle and in the heart of tumour-bearing rats

To assess the protective role exerted by MA in the muscle mass in the context of modulation of autophagic degradation, autophagic markers, in the gastrocnemius and in the heart of the tumour-bearing rats and in the control animals, were analysed. Although ATG12-ATG5 and Beclin-1, which play an important role in the initial steps and in the formation of the autophagosome, were not upregulated in the gastrocnemius muscle of tumour-bearing rats treated with placebo compared with control animals (*Figures* 5A and 5B), a significant increase of microtubule-associated protein 1 light chain 3B isoform I (LC3B-I) and its lipidated form LC3B-II was detected (both $P < 0.001$ vs. sham; *Figures* 5E and 5F). In addition, the ratio of LC3B-II to LC3B-I was measured as a marker of LC3 cleavage and therefore activation of the autophagic pathway. In the gastrocnemius, the LC3 ratio was increased in the tumour group treated with placebo compared with the control group ($P < 0.05$ vs. sham, *Figure* 5G). Moreover, p62, as a marker of substrate sequestration into autophagosome, was assayed. Levels of p62 were significantly higher ($P < 0.01$; *Figure* 5C) in the skeletal muscle of tumour-bearing rats treated with placebo than in sham rats. In addition, levels of the protein TRAF6 were greater in the gastrocnemius muscle of

Figure 4 The effect of megestrol acetate on cardiac function and heart wasting of cachectic rats. (A) At the end of the study, the weight of the heart in the tumour group treated with placebo was smaller compared with control rats. Treatment with 100 mg/kg/day MA significantly protected the hearts from atrophy. (B) Left ventricular ejection fraction and (C) left ventricular fractional shortening were reduced in tumour-bearing rats treated with placebo compared with sham animals. Treatment with 100 mg/kg/day MA significantly improved left ventricular ejection fraction and left ventricular fractional shortening. (D) The left ventricular stroke volume, (E) the left ventricular end systolic volume, and (F) the left ventricular end diastolic volume were significantly impaired in tumour-bearing rats treated with placebo compared with sham animals. Administration of 100 mg/kg/day MA was able to improve significantly the left ventricular end systolic volume (E), whereas the left ventricular stroke volume and the left ventricular end diastolic volume were not improved (D and F). (G) A larger left ventricular internal systolic diameter and (H) a reduction of the left ventricular internal diastolic diameter were observed in tumour-bearing rats treated with placebo compared with sham rats. Treatment with 100 mg/kg/day MA significantly protected from the loss of left ventricular diameter in systole (G) and in diastole (H). (I) A striking reduction of left-ventricular mass and left ventricular posterior wall thickness, in systole, was observed in the tumour group treated with placebo compared with sham rats. Treatment with 100 mg/kg/day MA significantly improved the value of the posterior wall thickness, in systole. The data are presented as mean ± SEM. *:$P < 0.05$, **:$P < 0.01$, ***:$P < 0.001$ vs. sham; $^{\#}$:$P < 0.05$, $^{\#\#}$:$P < 0.01$, $^{\#\#\#}$:$P < 0.001$ vs. placebo. Sham $n - 11$, placebo $n - 11$, 100 mg/kg/day megestrol acetate $n - 10$.

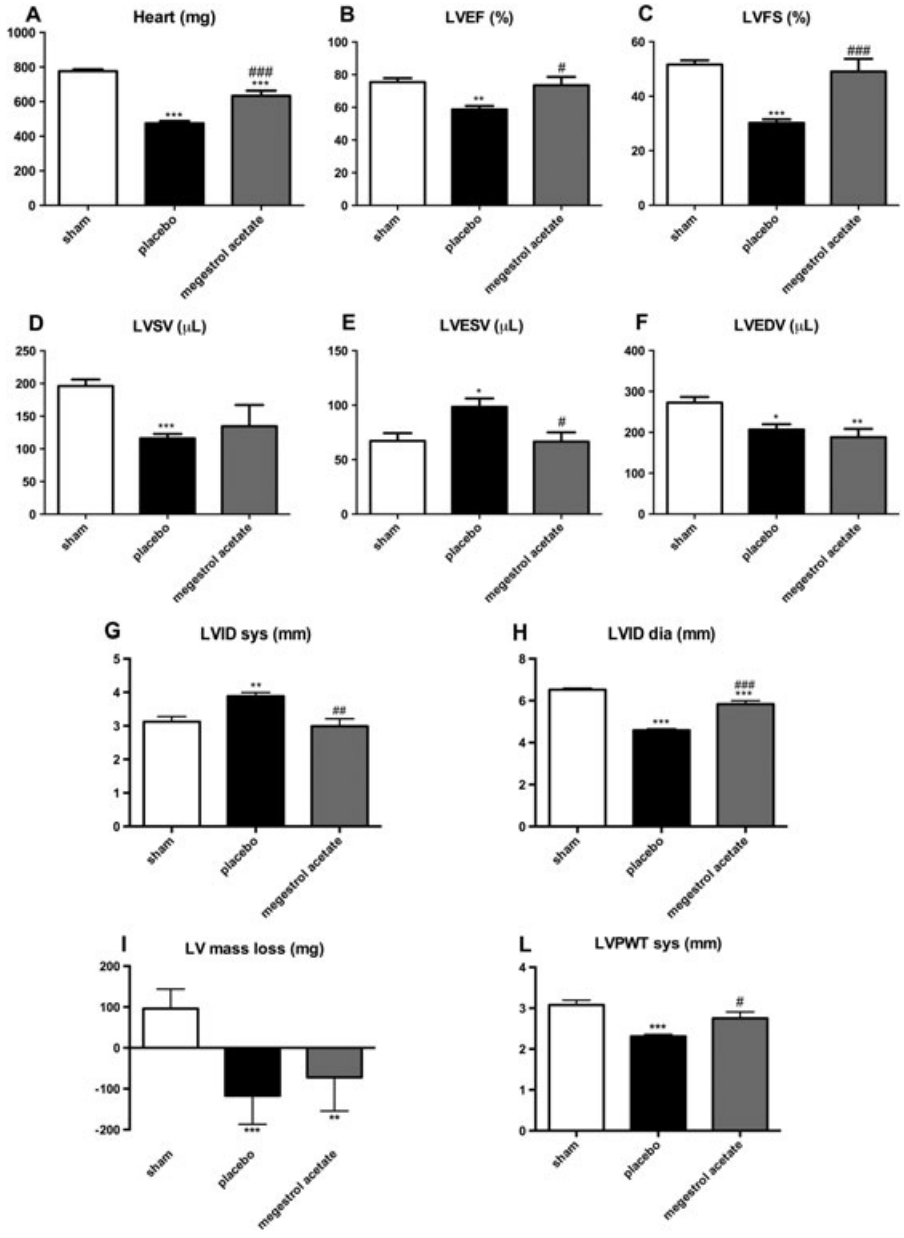


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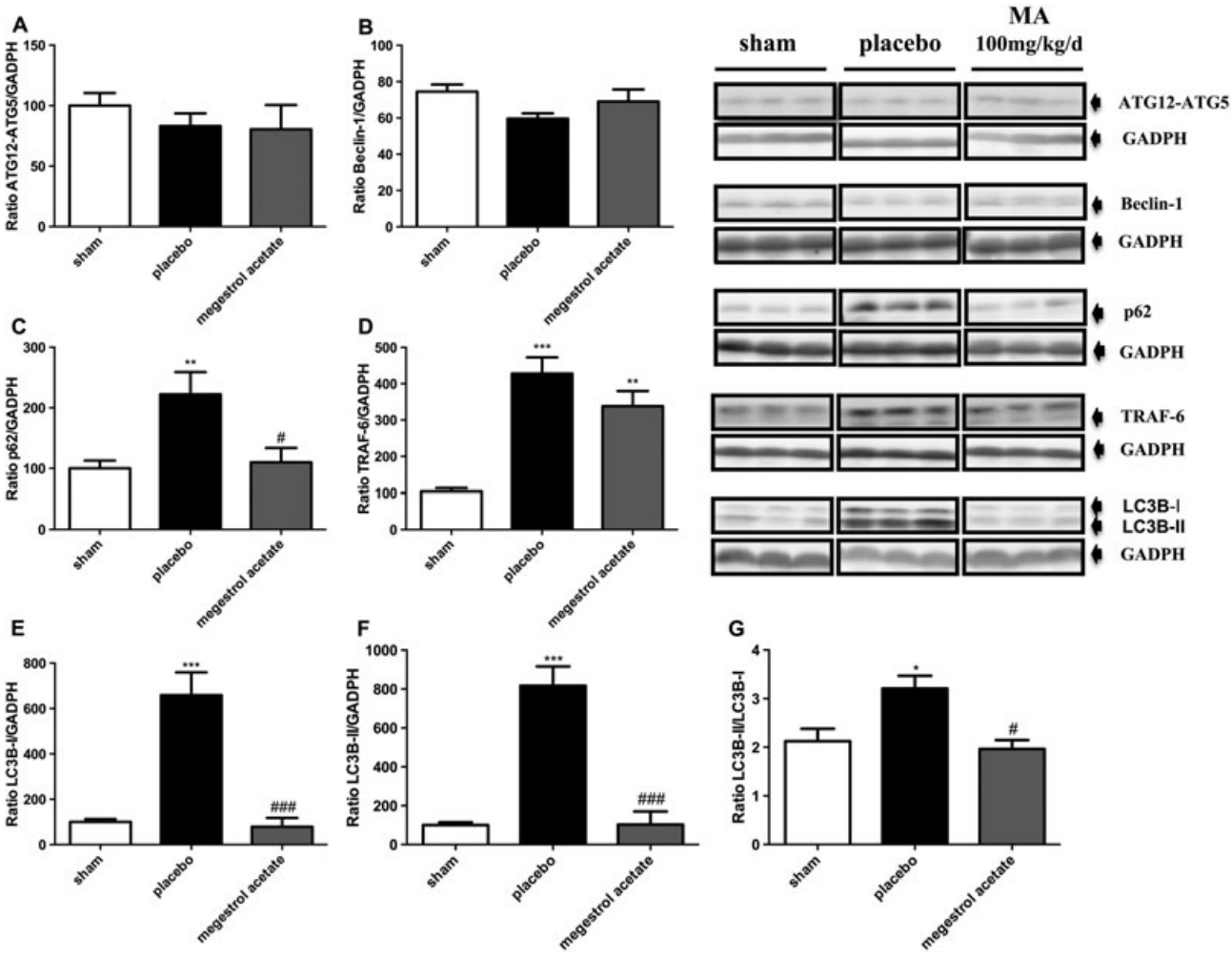
Figure 5 Megestrol acetate downregulates autophagy in the gastrocnemius muscle of tumour-bearing rats. (A) ATG12-ATG5 and (B) Beclin were not upregulated in the gastrocnemius muscle of tumour-bearing rats treated with placebo compared with control animals. (C) Levels of p62 were significantly higher in tumour-bearing rats treated with placebo than in sham rats. Treatment with 100 mg/kg/day MA significantly downregulated p62 levels. (D) TRAF6 levels were greater in placebo-treated tumour-bearing rats than in sham rats. Protein levels were not downregulated as a result of megesterol acetate treatment. (E) LC3B-I, (F) its lipidated form LC3B-II, and (G) the LC3 ratio were upregulated in placebo-treated tumour-bearing rats than in sham rats. Treatment with 100 mg/kg/day MA significantly downregulated LC3 in both forms (LC3-II and LC3-II), as well as LC3B-II/LC3B-I ratio was downregulated. The data are presented as mean ± SEM. *:$P < 0.05$, **:$P < 0.01$, ***:$P < 0.001$ vs. sham; $^{\#}$:$P < 0.05$, $^{\#\#\#}$:$P < 0.001$ vs. placebo. Sham $n - 11$, placebo $n - 11$, 100 mg/kg/day megestrol acetate $n - 10$.



placebo-treated tumour-bearing rats than in sham rats ($P < 0.001$; *Figure* 5D). Treatment with 100 mg/kg/day MA significantly reduced the autophagic markers in the gastrocnemius of the tumour-bearing rats. Indeed, LC3 was downregulated in both forms as well as LC3B-II/LC3B-I ratio ($P < 0.001$ for LC3B-I and LC3B-II, $P < 0.05$ for LC3B-II/LC3B-I vs. placebo, respectively; *Figure* 5E, 5F, and 5G). Similarly, p62 levels were downregulated as a result of megesterol acetate treatment ($P < 0.05$ vs. placebo; *Figure* 5C). Finally, TRAF6 protein levels demonstrated no significant difference between placebo and MA-treated tumour rats (*Figure* 5D).

Heart ATG12-ATG5 complex levels were not significantly different between any groups. However, heart Beclin-1 protein levels were significantly higher ($P < 0.05$; *Figure* 6B) in the tumour group treated with placebo compared with the sham group. Treatment with 100 mg/kg/day MA significantly reduced Beclin-1 levels in the tumour-bearing rats ($P < 0.01$ vs. placebo; *Figure* 6B). LC3B-I expression did not change in the hearts of the tumour-bearing rats treated with placebo (*Figure* 6E), whereas LC3B-II levels were significantly elevated compared with sham animals ($P < 0.01$; Figure 6F). The LC3 ratio was increased ($P < 0.001$; *Figure* 6G) in the tumour group treated with placebo compared with the control rats. Administration of 100 mg/kg/day MA significantly reduced LC3B-I ($P < 0.01$ vs. placebo; *Figure* 6E), LC3B-II ($P < 0.001$ vs. placebo; *Figure* 6F), and the

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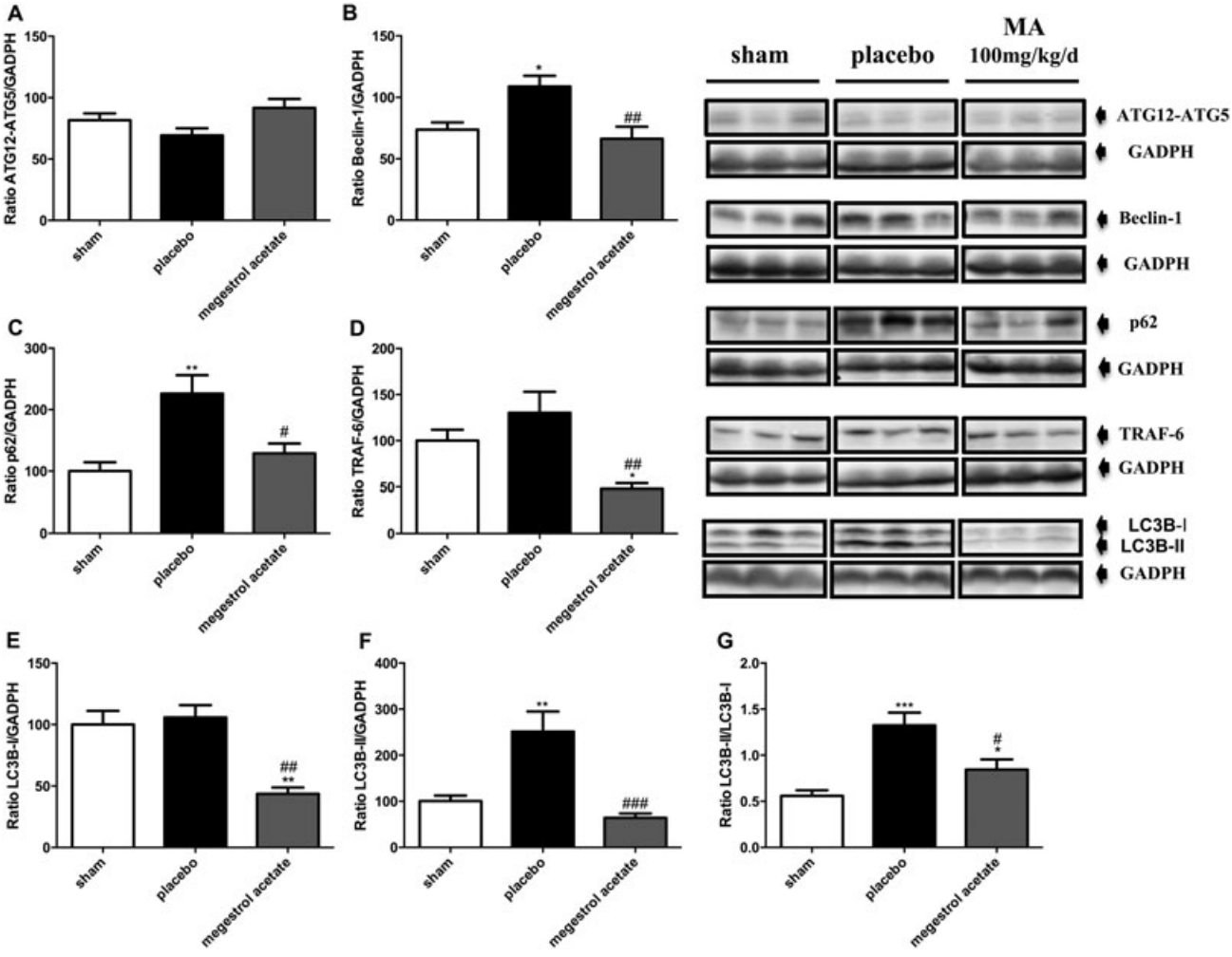
Figure 6 Megestrol acetate downregulates autophagy in the heart of tumour-bearing rats. (A) ATG12-ATG5 complex levels were not different between any groups. (B) Beclin-1 was significantly higher in the cachectic rats treated with placebo compared with the sham group. Treatment with 100 mg/kg/day MA significantly reduced Beclin-1 levels in the tumour-bearing rats. (C) The p62 expression was increased in the placebo-treated tumour group compared with sham animals, whereas 100 mg/kg/day MA significantly reduced p62 accumulation. (D) TRAF6 expression had an increasing trend, in the cachectic animals treated with placebo compared with sham rats. Treatment with 100 mg/kg/day MA significantly downregulated the expression of TRAF6 in the tumour group. (E) LC3B-I expression did not change in the hearts of the tumour-bearing rats treated with placebo, whereas (F) LC3B-II levels were significantly elevated compared with sham rats. (G) The LC3 ratio was increased in the tumour group treated with placebo compared with the control rats. Treatment with 100 mg/kg/day MA significantly reduced LC3B-I, LC3B-II levels and the LC3B-II/LC3B-I ratio of the cachectic animals. The data are presented as mean ± SEM. *:$P < 0.05$, **:$P < 0.01$, ***:$P < 0.001$ vs. sham; $^{\#}$:$P < 0.05$, $^{\#\#}$:$P < 0.01$, $^{\#\#\#}$:$P < 0.001$ vs. placebo. Sham $n - 11$, placebo $n - 11$, 100 mg/kg/day megestrol acetate $n - 10$.



LC3B-II/LC3B-I ratio in the hearts of the tumour group ($P < 0.05$ vs. placebo; *Figure* 6G). In addition, p62 heart expression was increased in the placebo-treated tumour group compared with sham animals ($P < 0.01$; *Figure* 6C), whereas 100 mg/kg/day MA significantly reduced p62 accumulation ($P < 0.05$ vs. placebo; *Figure* 6C). Although, heart TRAF6 expression had an increasing trend, which did not reach any statistical significance, in tumour-bearing rats of the placebo group compared with sham rats (*Figure* 6D), administration of 100 mg/kg/day MA significantly downregulated the expression of TRAF6 in the tumour group ($P < 0.01$ vs. placebo; *Figure* 6D).

Discussion

Tumour burden induces a severe wasting of skeletal muscle and fat tissue. In the present work we have demonstrated that Yoshida hepatoma is also associated with a form of cachexia, which induces cardiomyopathy in rats. In cancer cachexia, skeletal muscle and heart wasting are associated with an hyperactivation of autophagy.[15,20,21] We supported these data and, in addition, we demonstrated, for the first time, that MA downregulates autophagy in skeletal and heart muscle with a significant improvement of cardiac function.

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Here, we show that MA attenuates the loss of body weight in the Yoshida AH-130 hepatoma cancer cachexia model without effecting tumour growth. The AH-130 hepatoma rat model also develops a marked anorexia,[43] a condition often associated with cachexia in humans.[1] Previous reports have shown that intake of the drug resulted in an increased sense of appetite in rodents[38,44] and in humans.[35,36] The precise mechanism of action of MA is unknown, but its effect may be partially mediated by the neuropeptide Y, a potent centrally acting appetite stimulant.[45] Food intake and spontaneous activity are recognized as good indicators of the quality of life in animals.[46] In our study, treatment with MA had a positive effect on food intake, but it did not enhance physical performance, further supporting the previously described failure of MA to totally improve quality of life.[47] Although, several evidences[30–34] indicate the occurrence of a benefit in body weight gain subsequently to MA treatment, this seems to correlate with an increase only in fat mass,[35,36] with a direct action on adipocyte differentiation.[48]

However, other findings showed an increased bio-availability of IGF-I during MA treatment, which may contribute to the anabolic action of the drug on skeletal muscle in patients with advanced cancer[49] and a reduction of the muscle wasting process through a mechanism based on the modulation of the UPS.[38] Here, we show for the first time that MA may inhibit, to some extend, muscle atrophy by decreasing the catabolic pathway of autophagy.

Autophagy has been implicated in hearts and skeletal muscles wasting in several models of cancer cachexia.[50] We also demonstrated the hyperactivation of autophagy in rats bearing Yoshida AH-130 hepatoma by changes occurring in the well-recognized biomarkers of this catabolic pathway. In particular, we found MA-associated positive changes in Beclin-1, an inducer of the autophagosome formation, in LC3B-I to LC3B-II conversion, which was measured as a marker of autophagosome abundance, in p62, a marker of substrate sequestration and finally in TRAF6, an E3 ubiquitin ligase, involved in activation of autophagy and UPS in atrophying skeletal muscle.[19]

In particular, we found that in the skeletal muscle, autophagy was associated with increased LC3B lipidation and p62 accumulation, whereas no changes in ATG12-ATG5 or Beclin-1 levels were observed. Levels of p62 usually inversely correlate with autophagic degradation,[51] although our reports, and other studies clearly showed that this marker could also be increased in many atrophic conditions upon activation of autophagy.[15] The scaffold protein p62 assumes a main role in recognition of ubiquitinylated proteins or depolarized mitochondria during selective autophagy; interestingly, it has been described that p62 can also deliver ubiquitinylated cargos to the proteasome.[52] Looking for proteins that play a regulatory role in activation of signalling cascades related to muscle atrophy, TRAF6, might potentially be an upstream regulator for the activation of pathways involved in loss of muscle proteins in conditions of wasting.[19] TRAF6 expression is increased in several models of muscle atrophy, including fasting and cancer, leading to downstream activation of major catabolic pathways in skeletal muscle, including autophagy.[18,53] In accordance with these reports, in the gastrocnemius of the Yoshida AH-130 hepatoma model we found high levels of TRAF6. Our evidence supports the hypothesis that TRAF6 could modulate the autophagy also in this model. Although MA reduced skeletal muscle atrophy, modulating some steps of the complex autophagic pathway, the drug did not have a direct effect on TRAF6, at least in the gastrocnemius.

Cardiac atrophy has been described in several models of cancer cachexia.[11,20,21] Our results support and extend these data. We indeed observed a reduction of the posterior wall thickness, LV mass, and total heart weight in cachectic rats, which are features of cardiac atrophy. MA administration attenuated this wasting. Although the exact pathways leading to cardiac wasting are not fully elucidated, our results support the idea that the catabolic signalling pathways activated in the skeletal muscle are responsible for the atrophy of the heart.[54] In this scenario, autophagy has been proposed as the main catabolic pathway regulated in the heart, whereas UPS has a high basal activity.[55] However, it is likely that the activation of the main proteolitic pathways may differ between cancer models since using the Yoshida AH-130 hepatoma model, increased UPS activity was previously described.[11]

On the basis of the observation in the skeletal muscle and consistent with our findings of decreased cardiac mass, we observed that the heart atrophy in Yoshida AH-130 was associated with elevated level of Beclin-1, p62, and LC3, suggesting that changes in cardiac mass is clearly associated with autophagy. The results presented in our study clearly demonstrated that MA may inhibit atrophic mechanism, modulating autophagy also in the heart. MA might reduced the expression of humoral factors involved in chronic inflammation and oxidative stress, and contributing to the activation of protein degradation in muscle.[27,50] Although new studies are needed to better clarify these findings, we can further postulate that the decreased expression of TRAF6 in the hearts of cachectic rats treated with MA might be because of a reduction expression of pro-inflammatory cytokines, because TRAF6 suppression resulted in reduced IL-1β and IL-6 expression.[56] Overall, cardiac atrophy is associated with a clear heart dysfunction because of tumour growth and cachexia in the Yoshida AH-130 model. The cardiac parameters that we analysed in this study are signs for myocardial dysfunction, and they are similar to previous results.[57] Although the cardioprotective effect of MA has not been described so far, treatment with the drug could significantly improve LVEF, and LVFS of the cachectic rats compared with the placebo group, and therefore, it may prevent the development of cancer cachexia-induced cardiomyopathy.[10,57,58] Cardiac remodelling is a clear event in tumour-bearing rats and mice.[7,8,50] Even though our study is lacking some histological evidences, adverse cardiac remodelling in tumour-bearing rats, i.e. the reduction of

LVID dia and LVID sys, was significantly attenuated by MA treatment. Protecting heart and its function seems to be crucial in cachexia.[11] Cardiac function was dramatically improved by treatment with MA, and this is likely contributed to the survival benefits observed in our study.

In conclusion, our data show the action of MA, an appetite stimulant, on body weight and cardiac function in the well-known Yoshida AH-130 hepatoma rat model of cancer cachexia. In particular, MA improved survival and reduced wasting through a marked downregulation of autophagy, occurring in both skeletal and heart muscles, the latter effect leading to a significant improvement of cardiac function. Thus, our data suggest that MA might represent a valuable strategy to counteract the development of cancer cachexia-induced cardiomyopathy.

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The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia, and Muscle.[59]

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Conflict of interest

None declared.

Online supplementary material

Supporting information may be found in the online version of this article.

Figure S1. (a) Food intake and (b) spontaneous activity over 24 h were similar in all groups before tumour inoculation.
Table S1. Cardiac dimensions and function assessed before tumour inoculation

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