Complete reversal of muscle wasting in experimental cancer cachexia: Additive effects of activin type II receptor inhibition and β-2 agonist

COMPLETE REVERSAL OF MUSCLE WASTING IN EXPERIMENTAL CANCER CACHEXIA: ADDITIVE EFFECTS OF ACTIVIN TYPE II RECEPTOR INHIBITION AND BETA-2 AGONIST

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Novelty: A new combined treatment based on the soluble receptor antagonist of myostatin and the β2-agonist formoterol resulted to be a very promising treatment for experimental cancer cachexia (cachectic Lewis lung carcinoma model).

Running title: Combined therapy on cachectic mice

Keywords: cancer cachexia, skeletal muscle, formoterol, anti-myostatin, multitherapy, ActRIIB

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ABSTRACT

Formoterol is a highly potent β_2-adrenoceptor-selective agonist, which is a muscle growth promoter in many animal species. Myostatin/Activin inhibition reverses skeletal muscle loss and prolongs survival of tumor-bearing animals. The aim of the present investigation was to evaluate the effects of a combination of the soluble myostatin receptor ActRIIB (sActRIIB) and the β_2-agonist formoterol in the cachectic Lewis lung carcinoma model (LLC). The combination of formoterol and sActRIIB was extremely effective in reversing muscle wasting associated with experimental cancer cachexia in mice. Muscle weights from tumour-bearing animals were completely recovered following treatment and this was also reflected in the measured grip strength. This combination increased food intake both in control and tumour-bearing animals. The double treatment also prolonged survival significantly without affecting the weight and growth of the primary tumour. In addition, it significantly reduced the number of metastasis. Concerning the mechanisms for the preservation of muscle mass during cachexia, the effects of formoterol and sActRIIB seemed to be additive, since formoterol reduced the rate of protein degradation (as measured *in vitro* as tyrosine release, using incubated isolated individual muscles) while sActRIIB only affected protein synthesis (as measured *in vivo* using tritiated phenylalanine). Formoterol also increased the rate of protein synthesis and this seemed to be favoured by the presence of sActRIIB. Combining formoterol and sActRIIB seemed to be a very promising treatment for experimental cancer cachexia. Further studies in human patients are necessary and may lead to a highly effective treatment option for muscle wasting associated with cancer.

INTRODUCTION

Cachexia is a multi-factorial syndrome found in 50 to 80% of cancer patients [1], particularly at advanced stages of disease, leading to a decrease in quality of life and physical performance. Cancer cachexia is clinically relevant since is considered a predictor of reduced survival, accounting for more than 20% of cancer deaths [2,3]. The pathogenesis of cancer cachexia is characterized by the loss of skeletal muscle mass and fat, often associated with anorexia. However, muscle wasting should be regarded as the most important trait, since it is responsible for most of the cancer cachexia - associated symptoms and decreased survival? [4].

The competition for nutrients between the tumour and the host and the inflammatory status promote profound metabolic disturbances that include a massive nitrogen flow from the skeletal muscle to the liver [5]. Consequently, muscle plays a central role in whole-body protein metabolism by serving as the principal reservoir for amino acids to maintain protein synthesis in peripheral tissues in the absence of amino acid absorption from the gut and by providing hepatic gluconeogenic precursors [5,6]. The negative protein balance results from altered rates on both sides of muscle protein turnover, reduced synthesis and increased degradation, as revealed by several preclinical studies of cancer cachexia and even cancer patients [7–9]. Multiple studies reported that muscle protein degradation in wasting conditions is primarily mediated by the ubiquitin-proteasome pathway, and two muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF-1, which play a crucial role in activating the degradation machinery [10–12]. Beyond the proteasome, there are other protein degradation systems such as autophagy-lysosomal pathway [13], calpains [14–16] or even apoptosis [17] that might play a role in muscle wasting associated with cancer cachexia.

However, there is still a missing link between the tumour-induced adaptation (i.e. both tumour [18] and host-derived [19] humoral factors) and protein degradation.

During the past, the Akt/FOXO signalling pathway has been causally connected with the induction of Atrogin-1 and MuRF-1 [12,20], albeit its relevance to cancer cachexia has been questioned [21]. Myostatin, also known as GDF-8 (growth and differentiation factor-8), is a member of the TGF-β superfamily of secreted growth factors and is a negative regulator of skeletal muscle growth [22,23]. The administration of a myostatin anti-sense oligonucleotide resulted in increased muscle mass in both healthy and cachectic mice [24]. Although the use of the deacetylase inhibitors to increase the levels of follistatin (the endogenous myostatin inhibitor) failed to prevent experimental cancer cachexia [25,26], the use of an activin receptor extracellular domain/Fc fusion protein (ACVR2B-Fc) was effective in similar experimental models [27]. Finally, Zhou et al. showed that the administration of a high-affinity activin type II receptor leads to prolonged survival in Colon26-bearing mice [26] and our group reported the improvement of muscle weight and function in the Lewis lung carcinoma model [28].

So far, the growing data regarding the cancer cachexia pathogenesis has still not turned into an effective therapeutic intervention available to the healthcare system. Studies in our laboratory have shown a very positive action of β-adrenergic agents upon experimental muscle wasting [29]. One of this compounds, formoterol, combines the clinical advantages of rapid onset of action with duration of action and it is currently in use in humans for the treatment of bronchospasm associated with asthma [30] . The suggested anti-wasting action is based on its ability to prevent muscle proteolysis. The anti-wasting effects of the drug were also observed in terms of total physical activity and grip force, surrogate for the estimation of muscle function and quality of life in rodents [31]. A clinical study involving formoterol treatment in cancer patients is also available; a cohort with advanced cancer cachexia, an 8-week course of megestrol and formoterol in combination was reported to be safe and well tolerated [32].

Taking into consideration the therapeutic potential of myostatin/activin blockers and β_2-adrenergic agonists for the treatment of cancer cachexia, the aim of the present investigation was to examine the effectiveness of the combined use of formoterol and

soluble receptor antagonist of myostatin/activin in a mouse model of cancer cachexia, in order to obtain a broad spectrum effect that targets both anabolism and catabolism, potentially being suitable for the majority of cancer patients.

METHODS

Animals, tumor inoculation and treatment

Male C57BL6 mice (Harlan, Barcelona, Spain) weighing about 20 g were used. The animals were maintained on a regular light-dark cycle (light on from 08:00 a.m. to 08:00 p.m.) and had free access to food and water. The diet (Panlab, Barcelona, Spain) consists of 54% carbohydrate, 17% protein and 5% fat (the residue was non digestible material). Mice received an intramuscular (hind leg) inoculum of $5x10^5$ Lewis lung carcinoma cells obtained from exponential tumours. The Lewis lung carcinoma is a highly cachectic rapidly growing mouse tumour containing poorly differentiated cells, with a relatively short doubling time. The animals were divided in two groups namely controls (C, 34 animals) and tumour-bearing mice (TB, 64 animals). Both groups were further divided into four subgroups: untreated (vehicle administered), treated with Formoterol (1mg/kg body weight, subcutaneous (s.c.), daily), treated with the soluble receptor antagonist of myostatin/activin, sActRIIB (10 mg/kg body weight, s.c., twice a week) and treated with both drugs. sActRIIB sequesters activin A and myostatin *in vivo* [27]. At day 14 after tumour transplantation the animals were weighed and anesthetized with a ketamine/xylacine mixture. The tumour was harvested from the hind leg, its volume and mass evaluated. For the visualization of metastases, the lungs were removed after blood removal and euthanasia of the mouse. Metastatic nodules were counted using an anatomical microscope and the metastases weights were evaluated according the methodology used by Donati et al [33]. Several tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

Total physical activity

Total physical activity (IR ACTIMETER System and ACTITRAK software from PANLAB, Barcelona) was determined during 20h on day 14 (the day before sacrifice) in the following subgroups: C: control mice; T: tumour-bearing mice; T+A: mice treated

with sActRIIB; T+F: mice treated with formoterol; T+A+F: mice treated with sActRIIB and with formoterol. Total physical activity was determined using activity sensors that translate individual changes in the infrared pattern caused by movements of the animals into arbitrary activity counts [34]. For the measure, animals remained in their home cage –a frame containing an infrared beam system was placed on the outside of the cage-. This minimizes stress to the animals.

Grip force assessment

Skeletal muscular strength in mice was quantified by the grip-strength test [34]. The grip-strength device (Panlab-Harvard Apparatus) comprised a bar connected to an isometric force transducer (dynamometer). In brief, the grip strength meter was positioned horizontally, and the mice were held by the tail and lowered towards the device. The animals were allowed to grasp the pull bar and were then pulled backwards in a horizontal plane. The force applied to the bar just before the grip was lost was recorded as the peak tension. At least three measurements were taken per animal at baseline and on test days, and the results were averaged for analysis. This force was measured in Newtons/100g initial body weight.

***In vivo* rate of protein synthesis**

Protein synthesis was quantified by determining the amount of Phe-^{3}H incorporated into the tissues, as previously reported [35]. Briefly, three hours prior to sacrifice, the animals received an IP dose of tritiated phenylalanine (500μCi/Kg). The muscles were then homogenized and the protein precipitated with trichloroacetic acid (12%). Finally, the samples were resuspended in sodium deoxycholate (0.4%), NaOH (4%) and boiled for 3 hours. Total radioactivity was measured with a β liquid scintillation counter at the Radioisotope Service of the Department of Biology (University of Barcelona).

***In vitro* rate of protein degradation**

Isolated EDL muscles were fixed to a stainless-steel clip in order to maintain the muscle under slight tension (making it comparable to resting length) during the incubation. Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 3 h incubation period [36]. The muscles were incubated in a shaking-thermostatized water bath at 35 °C for 3 h in 2 ml of Krebs-Henseleit physiological saline pH 7.4, containing 5 mM glucose and 20 mM HEPES. After the addition of the muscles to the vials the incubation started at a shaking rate of 45 cycles/min. Vials were gassed with O2/CO2 19:1 during the whole incubation period. The muscles were preincubated for 30 minutes in Krebs-Henseleit buffer and then incubated for 120 minutes in fresh supplemented medium.Total protein degradation was calculated as the rate of tyrosine released in the last two hours of incubation into the medium in the presence of 0.5 mM cycloheximide in order to block the reincorporation of tyrosine into tissue protein. Tyrosine was measured fluorimetrically as previously described [37].

RNA isolation and RT-PCR

Total RNA from tibialis muscle was extracted by TriPureTM kit (Roche, Barcelona, Spain). Reverse transcription (RT) reactions were prepared using by First Strand cDNA Synthesis Kit for RT-PCR (Roche, Barcelona, Spain) following the manufacturer's instructions. Analysis of mRNA levels for the genes from the different proteolytic systems was performed with primers designed to detect the following gene products: ubiquitin (FORWARD 5' GAT CCA GGA CAA GGA GGG C 3', REVERSE 5' CAT CTT CCA GCT GCT TGC CT3'); E2 (FORWARD: 5' AGG CGA AGA TGG CGG T 3'; REVERSE 5' TCA TGC CTG TCC ACC TTG TA 3'); C8 proteasome subunit (FORWARD 5' AGA CCC CAA CAT GAA ACT GC 3'; REVERSE 5' AGG TTT GTT GGC AGA TGC TC 3'); MuRF-1 (FORWARD 5' TGT CTG GAG GTC GTT TCC G 3'; REVERSE 5' ATG CCG GTC CAT GAT CAC TT 3'); Atrogin-1(FORWARD 5' CCA TCA

GGA GAA GTG GAT CTA TGT T 3'; REVERSE 5' GCT TCC CCC AAA GTG CAG TA 3'); m-calpain (FORWARD 5' TTG AGC TGC AGA CCA TC 3'; REVERSE 5' GCA GCT TGA AAC CTG CTT CT 3'), cathepsin B (FORWARD 5' CTG CTG AGG ACC TGC TTA C 3'; REVERSE 5' CAC AGG GAG GGA TGG TGT A3') and p0 (FORWARD 5' GAG GTC CTC CTT GGT GAA CA 3'; REVERSE 5' CCT CAT TGT GGG AGC AGA CA 3'). To avoid the detection of possible contamination by genomic DNA, primers were designed in different exons. The real-time PCR was performed using a commercial kit (LightCycler ^{TM} 480 SYBR Green I Master, Roche, Barcelona, Spain). The relative amount of all mRNA was calculated using comparative C_T method. Acidic Ribosomal Phosphoprotein P0 mRNA was used as the invariant control for all studies.

ELISA

Serum TNF-α, IL-1-β and IL-6 levels were detected by using Milliplex MAP mouse cytokine assay kit, used according to the manufacturer instructions (Millipore). Serum from each animal was assayed in duplicate.

Statistical analysis

Statistical analysis of the data was performed for each studied variable and the means and SE were calculated. Intergroup differences were evaluated statistically using multiple-way analysis of variance (ANOVA). *Post hoc* pairwise comparisons (Duncan test) were performed when was appropriated. Survival curves were computed with the Kaplan-Meier method, and differences in survival were validated with Log-rank test (Mantel-Cox). All statistical tests were performed using SPSS version 21.

RESULTS AND DISCUSSION

The results presented in Table 1 clearly show that treatment with either formoterol (F) or the myostatin soluble receptor antagonist sActRIIB (A) resulted in a significant increase in food intake in the control group. In tumour-bearing animals, only the combination of both treatments (A+F) resulted in a significant appetite improvement (Table 1). Both F and A determined a relevant increase in body weight in all the experimental groups studied and the combined treatment exerted a cumulative effect. The same effects were observed when the carcass weight – instead of total weight – was considered. Formoterol promoted a significant increase in skeletal muscle weights and heart, both in control and tumour hosts. These results agree with previous observations in our laboratory [38]. Interestingly, sActRIIB treatment also resulted in significant increments in gastrocnemius (21%), tibialis (52%) and diaphragm (48%) in control animals and also in tumour-bearing animals (29% and 31% for gastrocnemius and tibialis respectively). The combination treatment A+F resulted in larger increases in muscle weights than the individual treatments. It is noteworthy that the combination treatment significantly increased heart weight both in control and LLC-bearing animals. This is interesting because important abnormalities associated with heart function have been described during cancer cachexia [39,40].

All the above-reported observations did not associate with changes of primary tumour weight (Table 1). However, sActRIIB treatment resulted in a significant reduction of metastases content (92%). Such reduction was not modified by the combination with F.

As a consequence of the observed bigger muscles following the distinct treatments, a clear gain of muscle strength was recorded (Table 2). Both formoterol and sActRIIB caused an increase of this parameter in tumour-bearing mice. Interestingly the combined treatment induced a larger increase in muscle force as compared with sActRIIB or formoterol alone, suggesting additive effects.

The beneficial effects of gaining muscle mass were also seen when animal behaviour was monitored. As shown in Table 2, formoterol treatment was associated with a tendency to improve total physical activity while sActRIIB did not modulate this parameter. The recovery promoted by the β_2-agonist was mainly associated with locomotor movements as previously reported by our laboratory [41]. Interestingly the double treatment (F+A) resulted in a significant improvement of mean velocity and travelled distance (Table 2).

Since the improvement in terms of skeletal muscle mass and performance might impact on animal survival, an experiment was repeated in order to assay such parameter. The results presented in Figure 1A clearly show that while formoterol alone had no influence on survival, treatment with myostatin soluble receptor antagonist or the combination clearly resulted in enhanced survival. Thus while untreated tumour-bearing mice lived for 27 days, the double treatment was able to enlarge survival up to 36 days. This is likely due to the effect of sActRIIB on the number of metastases; Zhou et al. reported that treatment with sActRIIB during cancer cachexia caused myostatin and activin to be sequestered by this soluble receptor, leading to reduced availability of these ligands to activate the ActRIIB receptor, which leads to decreased activin signalling, thus preventing muscle atrophy [27]. Therefore, high levels of activin had been implicated in the pathogenesis of metastasis in different tumours [42–44]. Thereby, taking into consideration that the cause of death of this tumour model is associated with loss of respiratory capacity due to invasion of the lungs, it may be related with activin blockade promoted by ActRIIB treatment.

The results presented in Figure 1B demonstrated that tumour growth was associated with an important inflammatory response. Indeed, we observed a dramatic increase in the circulating levels of several cytokines: TNFα, IL-1β and IL-6 in agreement with previous observations [19,45,46]. The combined treatment with formoterol and sActRIIB elicited a significant decrease in TNFα and IL-6. Since the latter cytokine seems to have a very important role in different models of cancer cachexia [47], it

becomes clear that the observed effects upon muscle weights, force and activity can partly be explained by preventing high levels of the cytokines. The different treatments were also associated with an improvement in haemoglobin, haematocrit and platelet count (Figure 1B).

Our next step was to try and assess the mechanism by which the different treatments influenced muscle weight. We therefore estimated both protein synthesis and degradation in the skeletal muscle. The results presented in Figure 2A show the rate of protein synthesis as estimated by the incorporation of tritiated phenylalanine into protein. It can be seen that for all the skeletal muscle studied (gastrocnemius, tibialis and vastus lateralis) tumour burden resulted in very important decrease in protein synthesis rate. As can be observed treatment with formoterol resulted in an increment in protein synthesis only in the vastus lateralis muscle, while sActRIIB promoted increases in the rate of protein synthesis in both tibialis and vastus lateralis. Very interestingly, the combined treatment resulted in improvement of this parameter in all the skeletal muscle studied. Thus can be concluded that both formoterol and sActRIIB were able to improve somehow, depending the type of muscle, the rate of protein synthesis.

Concerning protein degradation, this parameter was measured in isolated EDL muscles from both control and tumour hosts. The results presented in Figure 2B clearly show that formoterol was able to decrease protein degradation in both control and tumour-bearing mice. Conversely sActRIIB did not affect the *in vitro* rate of protein degradation. Interestingly, the effects of formoterol on protein degradation were indirectly confirmed by analysing the transcript levels of genes belonging to different proteolytic systems (Table 3). Indeed, formoterol reduced the expression of several components of the ubiquitin-dependent proteolytic system, including atrogin-1 and MuRF-1 and of other proteolytic systems, such as m-calpain and cathepsin B, suggesting that the effects of β_2-agonist is upstream all the proteolytic systems.

Summarizing, the improved status triggered by the multifactorial treatment seems to be related with an improving in muscle mass and force where F decreases protein degradation and increases protein synthesis and sActRIIB acts mainly in favouring protein synthesis and reducing the number of metastasis, which is reflected by an increased survival.

In conclusion, the combined treatment presented here acts at two different levels (Figure 3). The first one influenced muscle mass by both increasing protein synthesis and reducing protein degradation. This is associated with recovery of physical activity and muscle strength, resulting in an improvement of quality of life. Second, the treatment diminished the number of metastases, this clearly being associated with extended survival. The results obtained in this preclinical setting originate solid fundamentals for the design of an effective therapy for cancer cachexia in humans. The combined approach will allow to face the anti-tumour treatments in improved health conditions, thus improving their overall effectiveness. On the other side, the anti-metastatic action reported here will strengthen the effect of anti-tumour drugs.

The results presented here clearly show that a multimodal approach including both anti-myostatin and β_2-adrenergic agonists seems to be the most suitable option for the treatment of cancer cachexia, particularly considering the high variability of pathogenic events typical of such syndrome due to the heterogeneity of neoplasms that causes cachexia. Not to mention that the combination of the two drugs will potentially allow a reduction of their effective dose, blunting their possible side effects.

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Table 1. Effects of the combination of formoterol and sActRIIB treatment on food intake, body weight and muscle weight in mice bearing the Lewis lung carcinoma.

Parameter	Experimental group							
	C (13)	**C+F** (7)	**C+A** (7)	**C+A+F** (7)	**T** (16)	**T+F** (16)	**T+A** (16)	**T+A+F** (16)
Food intake	226 ± 7 ^{ab}	258 ± 4 ^{c}	260 ± 8 ^{c}	256 ± 13 ^{c}	216 ± 7 ^{a}	221 ± 3 ^{a}	215 ± 3 ^{a}	240 ± 4 ^{b}
Tumour weight (g)					5,20 ± 0,16	5,03 ± 0,18	4,77 ± 0,24	4,65 ± 0,16
% lung metastases					19,2 ± 6,4 ^{b}	8,2 ± 3,2 ^{b}	1,6 ± 1,10^{a}	2,0 ± 1,48 ^{a}
Body Weight								
IBW	20,6 ± 0,4	20,9 ± 0,5	21,0 ± 0,8	21,0 ± 0,7	20,3 ± 0,3	20,4 ± 0,4	20,3 ± 0,4	20,5 ± 0,3
FBW	22,1 ± 0,6 ^{b}	24,5 ± 0,7 ^{c}	27,2 ± 0,9 ^{e}	27,3 ± 0,7 ^{e}	19,8 ± 0,5 ^{a}	21,6 ± 0,4 ^{b}	24,0 ± 0,6 ^{c}	24,9 ± 0,6 ^{d}
Weight change	7%	17%	29%	30%	-6%	6%	18%	21%
Carcass	15,1 ± 0,4 ^{b}	16,6 ± 0,5 ^{c}	18,8 ± 0,7 ^{e}	18,8 ± 0,5 ^{e}	13,1 ± 0,4 ^{a}	14,6 ± 0,2 ^{b}	16,4 ± 0,3 ^{c}	17,4 ± 0,5 ^{d}
Muscle Weight								
GSN	571 ± 13 ^{b}	690 ± 8 ^{c}	793 ± 14 ^{d}	837 ± 17 ^{e}	469 ± 13 ^{a}	579 ± 11 ^{b}	606 ± 17 ^{b}	727 ± 23 ^{c}
Tibialis	182 ± 4 ^{b}	213 ± 4 ^{cd}	277 ± 7 ^{e}	280 ± 10 ^{e}	146 ± 4 ^{a}	180 ± 7 ^{b}	191 ± 5 ^{bc}	227 ± 8 ^{d}
Diaphragm	288 ± 20 ^{a}	355 ± 20 ^{b}	426 ± 32 ^{c}	472 ± 10 ^{c}	247 ± 12 ^{a}	259 ± 17 ^{a}	296 ± 13 ^{a}	351 ± 16 ^{b}
Heart	559 ± 12 ^{a}	638 ± 12 ^{bc}	585 ± 22 ^{ab}	647 ± 34 ^{c}	553 ± 13 ^{a}	549 ± 11 ^{a}	565 ± 16 ^{a}	581 ± 10 ^{ab}

Results are mean ± S.E.M. for the number of animals indicated in parentheses. Food intake is expressed as g/100g of initial body weight (IBW) and refers to the cumulative intake (14 days). IBW: initial body weight is expressed as g. FBW: final body weight (without tumour) is expressed as g**.** Carcass is expressed as g. Tissue weights are expressed as mg/100 g IBW. GSN: gastrocnemius muscle. C: mice without tumour; T: tumour-bearing mice; C+F and T+F: treated with formoterol; C+A and T+A: treated with sActRIIB; C+A+F and T+A+F: treated with both sActRIIB and formoterol. Statistical significance of the results by full factorial three-way ANOVA (fixed factors: tumour, formoterol treatment and sActRIIB treatment). Statistical significance of tumour weight and metastases by two-way ANOVA (fixed factors: formoterol treatment and sActRIIB treatment). P-values of all the parameters detailed in Supplemental data. *Post hoc* Duncan tests were performed; different superscripts indicate significant differences between groups.

Table 2. Effects of the combination of formoterol and sActRIIB treatment on total physical activity and grip strength in mice bearing the Lewis lung carcinoma.

	C (10)	**T** (10)	**T + F** (10)	**T + A** (10)	**T + A + F** (7)	ANOVA
Physical activity						p-value
Total activity	65321 ± 2643 ^{b}	15451 ± 1152 ^{a}	19627 ± 1085 ^{a}	17041 ± 1383 ^{a}	19900 ± 631 ^{a}	0,000
Stereotyped movement	5312 ± 300 ^{b}	1893 ± 327 ^{a}	1545 ± 118 ^{a}	1769 ± 157 ^{a}	1888 ± 154 ^{a}	0,000
Locomotor movements	60010 ± 2636 ^{b}	13558 ± 1060 ^{a}	18082 ± 1019 ^{a}	15272 ± 1326 ^{a}	18012 ± 689 ^{a}	0,000
Velocity and distance						
Mean Velocity (cm/s)	0,64 ± 0,03 ^{c}	0,10 ± 0,03 ^{a}	0,14 ± 0,01 ^{ab}	0,13 ± 0,01 ^{ab}	0,17 ± 0,01 ^{b}	0,000
Travelled distance (cm)	46443 ± 990 ^{c}	7190 ± 737 ^{a}	10623 ± 1062 ^{ab}	9324 ± 784 ^{ab}	12172 ± 768 ^{b}	0,000
Time (%)						
Resting	73,3 ± 1,4 ^{a}	93,4 ± 0,6 ^{c}	89,7 ± 1,5 ^{bc}	90,3 ± 1,1 ^{bc}	87,3 ± 1,9 ^{b}	0,000
Slow movements	17,9 ± 1,2 ^{c}	6,2 ± 0,5 ^{a}	9,4 ± 1,3 ^{ab}	9,0 ± 1,0 ^{ab}	11,7 ± 1,7 ^{b}	0,000
Fast movements	8,8 ± 0,03 ^{b}	0,4 ± 0,08 ^{a}	0,9 ± 0,20 ^{a}	0,6 ± 0,11 ^{a}	1,0 ± 0,16 ^{a}	0,000
Grip strength						
Day 0	5,86 ± 0,26	6,04 ± 0,45	6,20 ± 0,27	5,73 ± 0,38	5,96 ± 0,32	ns
Day 14	6,78 ± 0,49 ^{b}	5,11 ± 0,47 ^{a}	6,80 ± 0,25 ^{b}	6,80 ± 0,26 ^{b}	8,24 ± 0,25 ^{c}	0,000

Results are mean ± S.E.M. for the number of animals indicated in parentheses. C: mice without tumour; T: tumour-bearing mice; T+F: treated with formoterol; T+A: treated with sActRIIB; T+A+F: treated with both sActRIIB and formoterol. Physical activity is expressed in activity units. Stereotyped movements include movements without displacement (eating and cleaning movements); conversely, locomotor movements include movements with displacement. Mean velocity is expressed in cm/s. Travelled distance is expressed in cm. Time is expressed as percentage of total time (24 hours). The thresholds of time are the following: time involving resting (sleeping, cleaning and eating time): [0-2] cm/s, time involving slow movements: [2-5] cm/s and time involving fast movements: [>5] cm/s. Muscle strength is expressed as Newtons/100g initial body

weight. Statistical significance of the results by one-way ANOVA following a *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

Table 3. Effects of the combination of formoterol and sActRIIB treatment on tibialis gene expression in mice bearing the Lewis lung carcinoma.

	C (6)	**T** (7)	**T + F** (7)	**T + A** (6)	**T + A + F** (7)	ANOVA
Proteolytic system						p-value
Ubiquitin-dependent						
Atrogin-1	100 ± 23 ^{a}	264 ± 20 ^{c}	196 ± 21 ^{bc}	250 ± 34 ^{bc}	173 ± 33 ^{ab}	0,000
MuRF-1	100 ± 22 ^{a}	206 ± 14 ^{b}	167 ± 29 ^{ab}	206 ± 45 ^{b}	178 ± 34 ^{ab}	0,000
Ubiquitin	100 ± 6 ^{a}	158 ± 8 ^{b}	118 ± 10 ^{a}	153 ± 9 ^{b}	109 ± 15 ^{a}	0,000
E2	100 ± 5 ^{ab}	118 ± 6 ^{c}	103 ± 6 ^{ab}	108 ± 7 ^{bc}	89 ± 4 ^{a}	0,000
C8 proteasome subunit	100 ± 6 ^{a}	160 ± 8 ^{c}	135 ± 7 ^{b}	145 ± 8 ^{bc}	120 ± 10 ^{ab}	0,000
Calcium-dependent						
m-Calpain	100 ± 8 ^{ab}	147 ± 7 ^{c}	116 ± 6 ^{b}	107 ± 6 ^{b}	86 ± 8 ^{a}	0,000
Lysosomal						
Cathepsin B	100 ± 6 ^{b}	126 ± 8 ^{b}	35 ± 20 ^{a}	92 ± 8 ^{b}	7 ± 2 ^{a}	0,000

Results are mean ± S.E.M. for the number of animals indicated in parentheses. C: mice without tumour; T: tumour-bearing mice; T+F: treated with formoterol; T+A: treated with sActRIIB; T+A+F: treated with both sActRIIB and formoterol. Statistical significance of the results by one-way ANOVA following a *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

FIGURE LEGEND

Figure 1. Survival (A) and Circulating cytokine levels (TNFα, IL-1β and IL-6), haemoglobin, haematocrit and platelet count in tumour-bearing mice (B).

Results are mean ± S.E.M. for the 8 animals per group. C: mice without tumour; T: tumour-bearing mice; T+F: treated with formoterol; T+A: treated with sActRIIB; T+A+F: treated with both sActRIIB and formoterol. **1A.** Kaplan-Meier survival analysis. Comparison of survival curves were analyzed by Log-rank test (Mantel-Cox). The global comparison for the treatments has a P value=0.000. Different subscripts means significant differences detected by pairwise comparisons (Bonferroni correction). P values < 0.05 were considered significant. **1B.** Statistical significance of the results by one-way ANOVA following a *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

Figure 2. Protein synthesis *in vivo* (A) and skeletal muscle proteolytic rate (B) in isolated EDL muscles *in vitro.*

Results are mean ± S.E.M. for 7 animals in control groups and 8 animals in tumour groups. C: control mice; T: tumour-bearing mice; C+A and T+A: mice treated with the soluble receptor antagonist of myostatin (sActRIIB); C+F and T+F: mice treated with formoterol; C+A+F and T+A+F: mice treated with sActRIIB and with formoterol. **2A. Protein synthesis** is expressed as dpm/g tissue. **2B. Skeletal muscle proteolytic rate in isolated EDL muscles *in vitro*** were measured in the presence of cycloheximide (0.5 mmol/L) and are expressed as nanomoles tyrosine per gram and 2 hours of incubation. Statistical significance of the results by full factorial three-way ANOVA (fixed factors: tumour, formoterol treatment and sActRIIB treatment). P-values of all the parameters detailed in Supplemental data. *Post hoc* Duncan tests were performed; different superscripts indicate significant differences between groups.

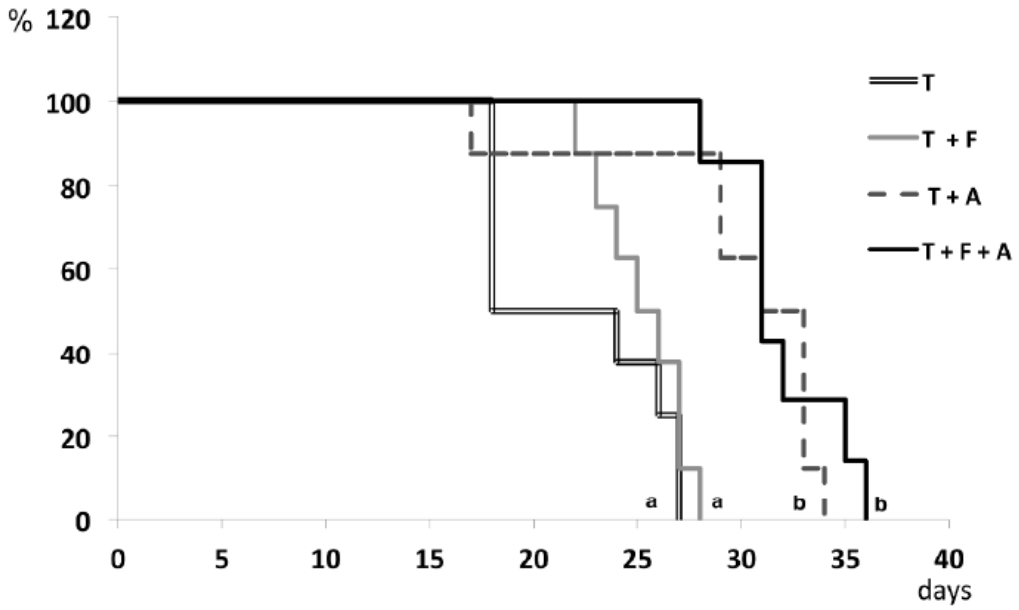
Figure 3

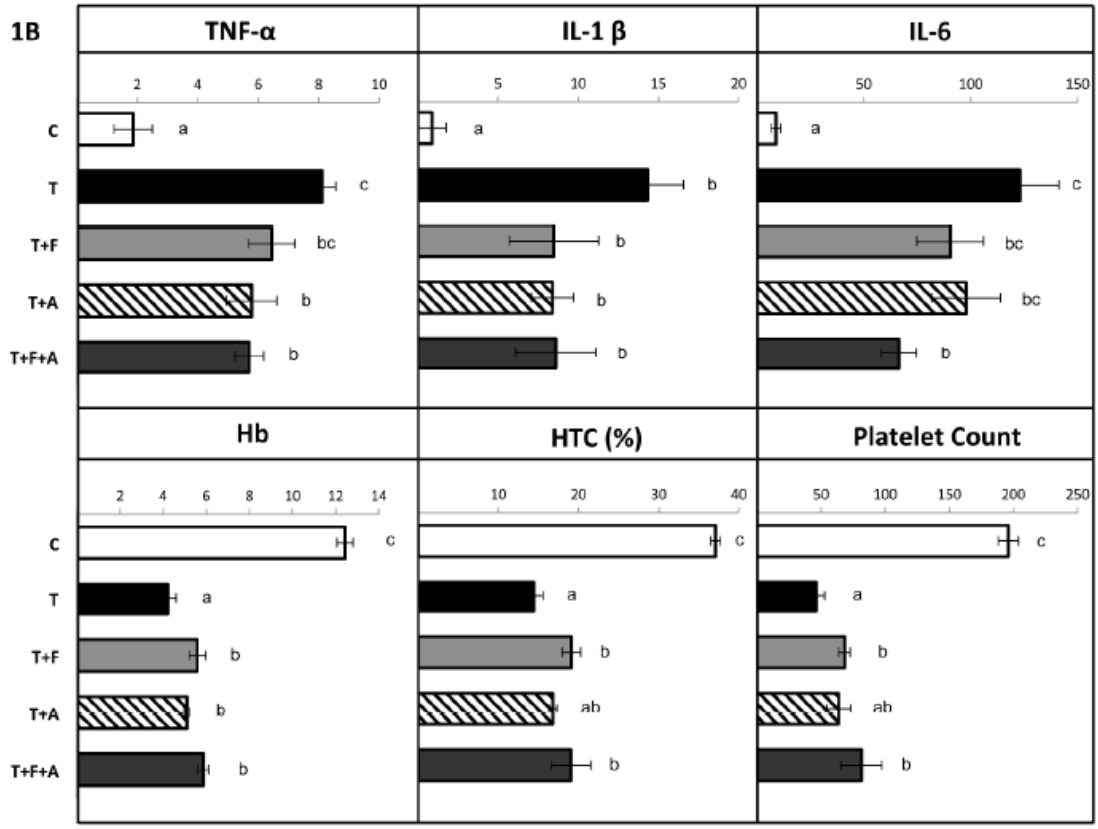
Proposed model of action of the multifactorial treatment.

Supplemental data

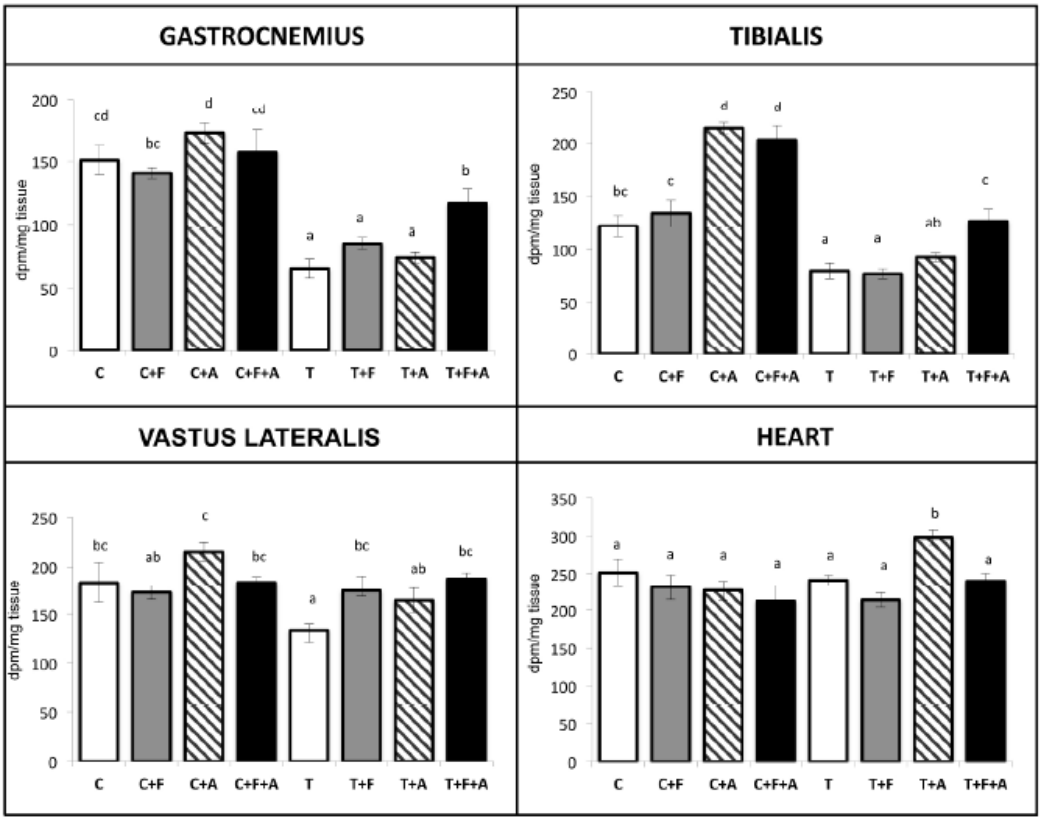
ANOVA P-values of all the parameters from Table 1, Figure 2A and Figure 2B.

1A




1B
TNF-α
IL-1 β
IL-6
Hb
HTC (%)
Platelet Count
C
T
T+F
T+A
T+F+A

2A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VASTUS LATERALIS
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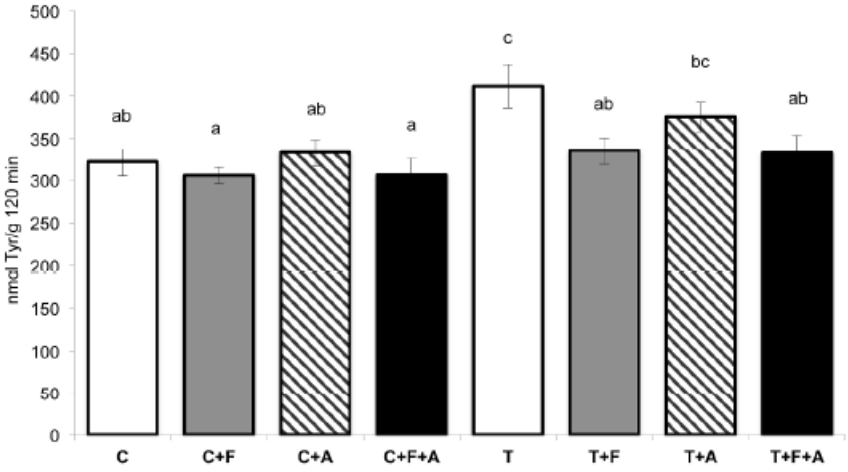
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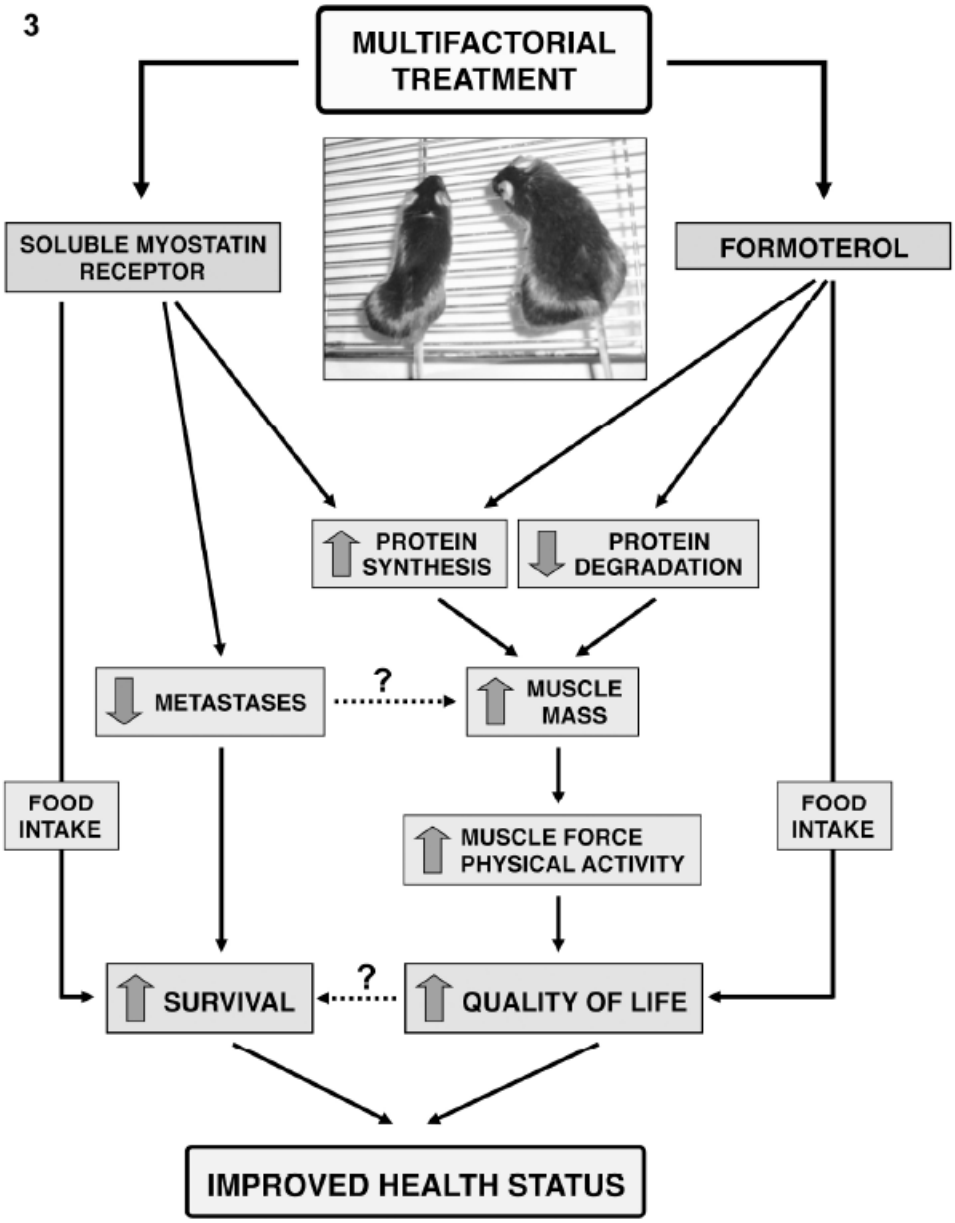
3
MULTIFACTORIAL TREATMENT
SOLUBLE MYOSTATIN RECEPTOR
FORMOTEROL
PROTEIN SYNTHESIS
PROTEIN DEGRADATION
METASTASES
?
MUSCLE MASS
FOOD INTAKE
MUSCLE FORCE PHYSICAL ACTIVITY
FOOD INTAKE
SURVIVAL
?
QUALITY OF LIFE
IMPROVED HEALTH STATUS