

A multifactorial anti-cachectic approach for cancer cachexia in a rat model undergoing chemotherapy

Míriam Toledo¹, Fabio Penna¹, Francesc Oliva³, Melania Luque¹, Angelica Betancourt¹, Enrica Marmonti¹, Francisco J. López-Soriano^{1,2}, Josep M. Argilés^{1,2} & Sílvia Busquets^{1,2*}

¹Cancer Research Group, Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; ²Institut de Biomedicina de la Universitat de Barcelona, Barcelona, Spain; ³Departament d'Estadística, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

Abstract

Background The effectiveness of drugs aimed at counteracting cancer cachexia is generally tested in pre-clinical rodent models, where only the tumour-induced alterations are taken into account, excluding the co-presence of anti-tumour molecules that could worsen the scenario and/or interfere with the treatment.

Methods The aim of the present investigation has been to assess the efficacy of a multifactorial treatment, including formoterol and megestrol acetate, in cachectic tumour-bearing rats (Yoshida AH-130, a highly cachectic tumour) undergoing chemotherapy (sorafenib).

Results Treatment of cachectic tumour-bearing rats with sorafenib (90 mg/kg) causes an important decrease in tumour cell content due to both reduced cell proliferation and increased apoptosis. As a consequence, animal survival significantly improves, while cachexia occurrence persists. Multi-factorial treatment using both formoterol and megestrol acetate is highly effective in preventing muscle wasting and has more powerful effects than the single formoterol administration. In addition, both physical activity and grip strength are significantly improved as compared with the untreated tumour-bearing animals. The effects of the multi-factorial treatment include increased food intake (likely due to megestrol acetate) and decreased protein degradation, as shown by the reduced expression of genes associated with both proteasome and calpain proteolytic systems.

Conclusions The combination of the two drugs proved to be a promising strategy for treating cancer cachexia in a pre-clinical setting that better resembles the human condition, thus providing a strong rationale for the use of such combination in clinical trials involving cachectic cancer patients.

Keywords Cancer cachexia; Skeletal muscle; Sorafenib; Tumour and chemotherapy

Received: 4 September 2014; Revised: 29 January 2015; Accepted: 16 March 2015

*Correspondence to: Sílvia Busquets, Cancer Research Group, Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal 643, 08028-Barcelona, Spain; Tel: 34–934034609, Fax: 34–934021559, Email: silviabusquets@ub.edu

Introduction

The percentage of cachexia in cancer patients is quite high: 50–80%, and is a useful tool for survival prediction, being held responsible for more than 20% of the deaths of cancer patients.¹ It is directly responsible for a reduction in physical activity² and quality of life, and decreases the efficacy and outcome of anti-cancer therapy.^{3,4} In an international consensus,⁵ cachexia was defined as a 'complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The

prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption, and hyperthyroidism, and is associated with increased morbidity'.⁵

The loss of weight is due to a reduction of adipose tissue and muscle, but muscle wasting is the key factor in the cancer cachexia outcome. Therefore, it determines the survival, and

muscle strength and function, the pillar of the recovery process.⁶

There have been many approaches and strategies to treat the cachexia syndrome, but none of them totally reverses the weight loss. Those strategies have basically two targets: counteracting anorexia and neutralizing metabolic disturbances.^{7,8} Many drugs are being developed and tested in clinical trials, but none of these treatments are efficient enough to be applied in clinical practice.⁹

The study of cancer cachexia is mainly based on experimental models (tumour-injected animals) not undergoing the same anti-tumoural treatments as in humans; therefore, it is difficult to translate the experimental results to humans: on the one hand, anti-cancer treatments have side effects that can worsen cachexia, while on the other hand, the reduction of tumour mass can mask or delay the appearance of cachexia. Indeed, in experimental conditions, cachexia occurs within days or a few weeks after tumour injection, while cancer cachexia in humans is often a chronic process.

Regarding anti-tumoural therapy, many significant advances have been made in cancer management with the development and introduction of new targeted agents, replacing non-targeted highly toxic chemotherapies. Such therapies make tumour cells more susceptible, without substantially increasing toxicity. However, several studies in humans demonstrated that weight loss associated with cancer was related to toxicity from treatment, anaemia, and shorter survival.^{10,11} Prado *et al.*¹⁰ focused on body composition as a potential determinant of toxicity in response to commonly used anti-neoplastic agents. They emphasized the importance of establishing an adjustment of the anti-tumoural treatment for lean body mass, due to the relationship between severe skeletal muscle depletion and excess toxicity during chemotherapy. Indeed, Antoun *et al.*¹² reported that muscle loss was specifically exacerbated by sorafenib (Sor) treatment and described it as an adverse effect of the drug related to asthenia, fatigue, and physical disability. Sorafenib is a multikinase inhibitor that showed efficacy against a wide variety of tumours in pre-clinical models¹³; it inhibits cell proliferation by targeting the Raf/MEK/ERK signalling pathways and exerts an anti-angiogenic effect by inhibition of tumour angiogenesis through vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR).^{14,15} It is already approved in humans for the treatment of advanced hepatocellular carcinoma¹⁶ and advanced renal cell carcinoma.¹⁷ Common side effects of Sor are cutaneous like hand-foot syndrome or rash and gastrointestinal like diarrhoea or nausea, as well as alopecia and fatigue. These side effects limit the patient's ability to receive full-dose Sor treatment.^{18,19}

Patient's ability to tolerate anti-cancer therapy will, in turn, be affected by their nutritional status preceding treatment, and it will determine the success of the therapy. So, nutritional support has to be considered rather as part of the oncological

treatment than as a separated action.²⁰ In spite of this, several studies demonstrated that nutritional strategies are not sufficient to reverse the cachectic syndrome. The use of total parenteral nutrition does not abolish weight loss. This points out the need that any therapeutic approach based on increasing food intake has to be combined with a pharmacological strategy to counteract metabolic changes.

One of the drugs most commonly used in cachexia is megestrol acetate (MA) due to its high efficacy and safety profile, as confirmed in multiple clinical trials.^{21,22} Megestrol acetate is a synthetic derivative of progesterone and has been described as a potent appetite stimulant and promotes weight gain. However, this gain had been mainly attributed to an increased fat mass, not to muscle mass and had no evidences of benefit in terms of quality of life or survival.^{23,24} The mechanisms of MA responsible for contributing to increased body weight in cachectic patients are not completely clear, but some studies showed that, in addition to stimulating appetite, it also had an effect on both metabolic and inflammatory mechanisms.^{25,26} Concerning skeletal muscle, previous studies performed in our laboratory in experimental animals showed a clear anabolic effect of MA in skeletal muscle. Thereby, Busquets *et al.*²⁷ demonstrated that the administration of MA to tumour-bearing (TB) rats resulted in an important reversal of the muscle-wasting process, as reflected by individual muscle weights. The mechanism for this effect seems to be, at least, partially explained by the ability of the drug to block the enhanced proteolysis associated with muscle wasting during cancer cachexia. In fact, the drug also acted to be improving appetite, and enhancing physical performance and muscle strength. In another study, Toledo *et al.*²⁸ observed that administration of MA to cachectic TB rats caused increased incorporation of orally administered labelled ¹⁴C-leucine into muscle protein, without altering the *in vivo* rate of the amino acid oxidation, suggesting that MA treatment is able to increase protein synthesis during cancer cachexia.

With regard to finding a pharmacological strategy to counteract metabolic changes, our laboratory introduced the use of formoterol (F)—a highly potent β_2 -adrenoceptor-selective agonist—as a possible drug for the treatment of cachexia.²⁹ The administration of beta-adrenergic agonists had been related with hypertrophy of skeletal muscles.^{30–33} Formoterol combines the clinical advantages of the rapid onset with the duration of the action, and it is currently in use in humans for the treatment of bronchospasm associated with asthma.³⁴ In animal models, data from our laboratory demonstrated that F had important anti-cachectic effects.²⁹ The mode of action of this drug is based on its ability to prevent muscle wasting by inhibiting proteolysis and apoptosis in skeletal muscle. Thereby, F decreased the activation of the ubiquitin-dependent proteolytic system, the main mechanism activated in muscle-wasting conditions, and decreased muscle apoptosis in TB animals.^{29,35} The anti-wasting effects

of the drug were also observed in terms of total physical activity and grip force, thus resulting in an improvement in physical performance in cachectic TB rats.³⁶

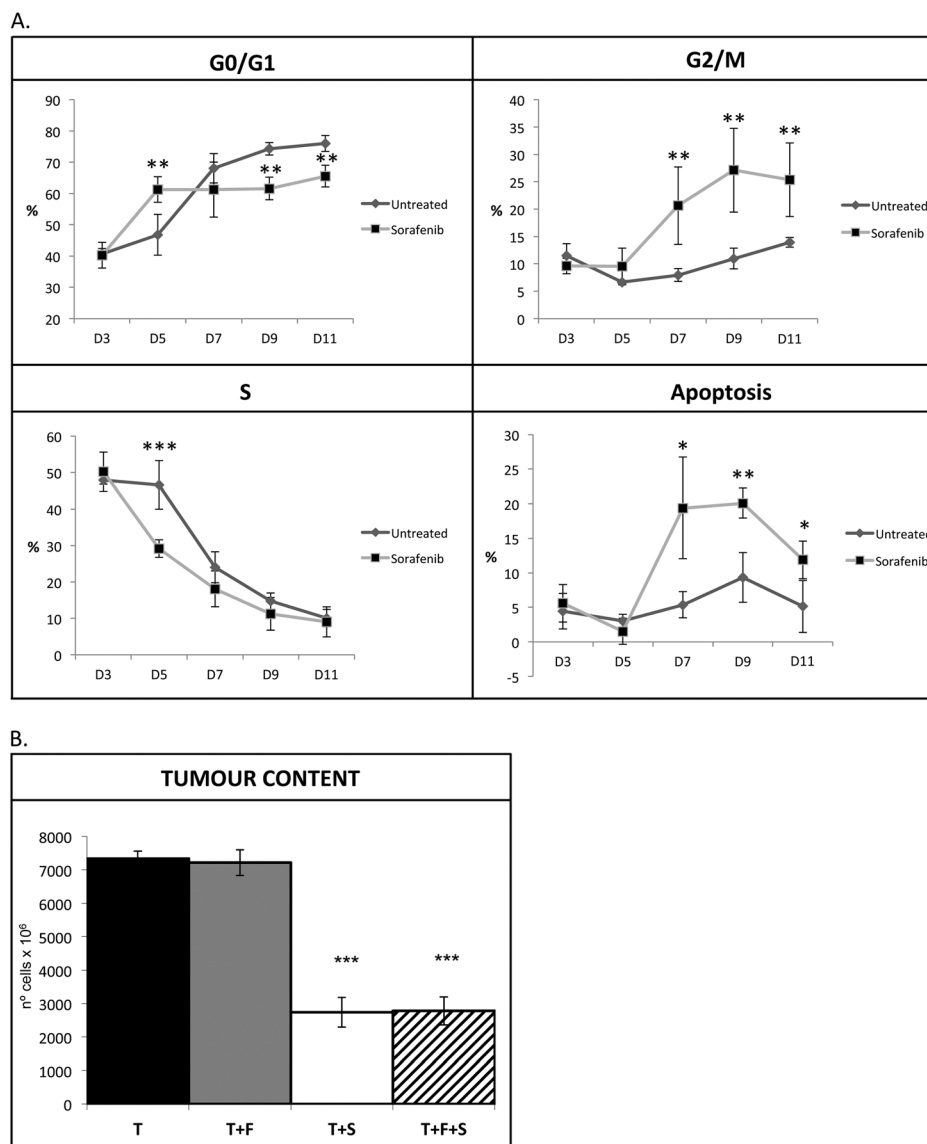
Bearing all this in mind, the aim of the present investigation was to assess the efficacy of a multi-factorial treatment, including F and MA, in cachectic TB rats undergoing chemotherapy.

Materials and methods

Animals

Five-week-old male Wistar rats (Harlan, Barcelona, Spain) were housed in individual cages and maintained at a constant temperature of $22 \pm 2^\circ\text{C}$ with a regular light–dark cycle (light

Figure 1 Effects of sorafenib on tumour cell cycle status and content in rats bearing the Yoshida AH-130 ascites hepatoma. (A) Cytometric analysis of the cell cycle distribution of AH-130 cells harvested every other day in both untreated and sorafenib-treated TB rats. A mixed model with repeated measures (factor days) was used to test the differences between sorafenib-treated and untreated rats over time. A first-order autoregressive covariance structure AR(1) was chosen, according to AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion). Significant differences detected between treatments along the days in all phases (G_0/G_1 , S, G_2/M , and apoptosis results in Table S2). The average values of population percentages at G_0/G_1 , S, G_2/M , and apoptosis phases are shown as mean \pm standard error from five animals per group. Pairwise comparisons for each day, significant differences noted as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Tumour cell content was assessed on day 10 after tumour inoculation. Results are mean \pm standard error for eight animals per group. T, tumour-bearing rats; T+S, treated with sorafenib; T+F, treated with formoterol; T+F+S, treated with both formoterol and sorafenib. Statistical significance of the results by two-way analysis of variance: sorafenib treatment ($P < 0.001$), formoterol treatment ($P = 0.86$), and non-significant interaction between factors ($P = 0.64$).



from 8 00 a.m. to 8 00 p.m.) and free access to food and water. Experimental cachexia was obtained through i.p. injection of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours as described previously.³⁷ The food intake was measured daily. The Bioethical Committee of the University of Barcelona approved the experimental protocol. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals.³⁸

Experimental design

Four distinct experiments were performed in order to test the following: (i) the action of anti-tumour drug (Sor) on Yoshida AH-130 ascites hepatoma cells; (ii) the effectiveness of the anti-cachectic agent F in TB rats either receiving chemotherapy (Sor) or not; (iii) the comparison of the survival of the untreated with Sor-treated TB rats; and (iv) the effectiveness of combined chronic (20 days) administration of F and MA against cachexia in Sor-treated TB rats.

Experiment I: TB animals were divided into two groups, untreated and treated daily with Sor (90 mg/kg body weight, intragastrically (i.g.), from day two after tumour injection). On days 3, 5, 7, 9, and 11, 100 μ L of ascites was extracted from each animal, and cells were analysed by flow cytometry (see below).

Experiment II: the animals were divided into two groups, namely controls (C) and TB. Both groups were further divided into four subgroups: untreated (vehicle administered), treated with F (0.3 mg/kg body weight, subcutaneous (s.c.),

daily), treated with Sor (90 mg/kg body weight, intragastrically (i.g.), daily from day two after tumour injection), and treated with both drugs. Ten days after tumour transplantation, the animals were weighted and anaesthetized with an i.p. injection of ketamine/xylazine mixture (3:1) (Imalgene® and Rompun®, respectively). Tumour volume and total cell number were assessed at the day of sacrifice. Tissues were rapidly excised, weighted, and frozen in liquid nitrogen.

Experiment III: TB animals were divided into two groups, untreated and treated with Sor (see above). The untreated group was administered with the corresponding volume of solvent. The treatment was stopped at day 20 after tumour inoculation in order to observe tumour relapse.

Experiment IV: the animals were divided into four groups, one C group (untreated and non-TB) and three TB groups. The latter three groups were treated from day two after tumour inoculation with Sor (see above). As for the anti-cachectic treatments, one TB group was administered the vehicle, one treated with F (see above), and the last TB group was treated with F and MA (100 mg/kg body weight, i.g., daily). The animals were sacrificed 20 days after tumour injection as previously stated.

Flow cytometry

The DNA distribution/cell cycle analysis was performed in AH-130 cells obtained from alternate day paracentesis in TB rats. Briefly, cells were washed in phosphate buffer solution (PBS), fixed in ice-cold 70% ethanol for at least 30 min, incubated at room temperature in PBS containing DNase-free RNase and propidium iodide at the final concentrations of 0.4 mg/mL

Table 1 Effects of formoterol treatment on food intake, body weight, and muscle and adipose weight in sorafenib-treated tumour-bearing rats at day 10 after tumour inoculation

Parameters	Experimental groups							
	C(6)	C + S(7)	C + F(7)	C + F + S(7)	T(8)	T + S(8)	T + F(8)	T + F + S(8)
Food intake	128 \pm 2 ^c	119 \pm 3 ^b	141 \pm 3 ^d	122 \pm 2 ^b	88 \pm 3 ^a	87 \pm 3 ^a	95 \pm 4 ^a	91 \pm 3 ^a
FBW	236 \pm 3 ^d	224 \pm 4 ^c	247 \pm 4 ^e	219 \pm 2 ^c	159 \pm 3 ^a	178 \pm 6 ^b	163 \pm 3 ^a	182 \pm 3 ^b
Muscle weight								
GSN	739 \pm 12 ^{de}	696 \pm 11 ^c	841 \pm 10 ^f	758 \pm 14 ^e	519 \pm 18 ^a	624 \pm 9 ^b	604 \pm 19 ^b	701 \pm 13 ^{cd}
Soleus	53 \pm 2 ^c	49 \pm 1 ^c	59 \pm 3 ^d	50 \pm 2 ^c	39 \pm 1 ^a	44 \pm 2 ^b	44 \pm 1 ^b	50 \pm 1 ^c
Tibialis	236 \pm 3 ^c	228 \pm 5 ^c	279 \pm 7 ^d	266 \pm 9 ^d	174 \pm 7 ^a	200 \pm 3 ^b	202 \pm 5 ^b	228 \pm 3 ^c
EDL	56 \pm 1 ^c	56 \pm 1 ^c	67 \pm 1 ^d	65 \pm 2 ^d	41 \pm 2 ^a	50 \pm 1 ^b	49 \pm 1 ^b	58 \pm 1 ^c
Heart	376 \pm 7 ^d	348 \pm 8 ^c	429 \pm 11 ^e	413 \pm 12 ^e	263 \pm 3 ^a	292 \pm 6 ^b	284 \pm 5 ^{ab}	330 \pm 8 ^c
Adipose weight								
dWAT	1390 \pm 91 ^d	1292 \pm 84 ^{cd}	1277 \pm 99 ^{cd}	1135 \pm 113 ^c	320 \pm 72 ^{ab}	535 \pm 65 ^b	211 \pm 51 ^a	373 \pm 53 ^{ab}
eWAT	1332 \pm 82 ^c	1208 \pm 107 ^c	1115 \pm 58 ^d	935 \pm 71 ^{bc}	542 \pm 106 ^a	926 \pm 49 ^{bc}	401 \pm 89 ^a	822 \pm 59 ^b
BAT	266 \pm 16 ^{bc}	241 \pm 22 ^b	283 \pm 14 ^c	238 \pm 17 ^b	110 \pm 7 ^a	125 \pm 4 ^a	117 \pm 6 ^a	118 \pm 8 ^a

Results are mean \pm standard error for the number of animals indicated in parentheses. Food intake is expressed as g/100 g initial body weight and refers to the cumulative intake (10 days). FBW, final body weight (without tumour) is expressed as g. Tissue weight is expressed as mg/100 g of initial body weight. GSN, gastrocnemius muscle; EDL, extensor digitorum longus; dWAT, dorsal white adipose tissue; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue; C, rats without tumour; T, tumour-bearing rats; C + S and T + S, treated with sorafenib; C + F and T + F, treated with formoterol; C + F + S and T + F + S, treated with both formoterol and sorafenib. Statistical significance of the results by full factorial three-way analysis of variance (fixed factors: tumour, sorafenib treatment, and formoterol treatment). *P*-values of all the parameters detailed in Table S1. Statistically significant differences by *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

and 10 µg/mL, respectively. Cells were then analysed using a Beckman Coulter Epics XL flow cytometer. Data were then analysed with the WinCycle software (Phoenix Flow Systems, San Diego, CA, USA). The percentage of apoptotic cells was assessed by evaluating the accumulation of cells having a $2n$ DNA content.

Blood haematochemical assays

Plasma albumin, triacylglycerides, glucose, and lactate were analysed by METROLAB 2300 (RAL S.A., Barcelona, Spain), a chemistry analyser that is based on the analysis of colorimetric reactions.

Biochemicals

Sorafenib was obtained from GENTAUR (Kampenhout, Belgium), F was kindly provided by Industriale Chimica s.r.l. (Saronno, Italy), and MA was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

RNA isolation and reverse transcription-PCR

Total RNA from the gastrocnemius muscle was extracted by TriPure™ kit (Roche, Barcelona, Spain), a commercial modification of the acid guanidinium isothiocyanate/phenol/chloroform method.³⁹ 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'); C2 proteasome subunit (FORWARD: 5' GTTCCATTGGGATTGTTGG 3'; REVERSE: 5' TGTTCCATTGGTTCATCAGC 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 A 3'); and hydroxymethylbilane synthase (FORWARD 5' TGC CAG AGA AAA GTG CCG TGG G 3'; REVERSE 5' TGC AGC TCA TCC AGC TTC CGT 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™ 480 SYBR Green I Master, Roche, Barcelona, Spain). The relative amount

of all mRNA was calculated using comparative C_T method. Hydroxymethylbilane synthase mRNA was used as the invariant control for all studies.

Total physical activity

Total physical activity (IR ACTIMETER System and ACTITRAK software from Panlab, Barcelona) was assessed during the last 24 h prior to the sacrifice of the animals in different experimental groups, using activity sensors that translate individual changes in the infrared pattern caused by movements of the animals into arbitrary activity counts.⁴⁰ For the measurements,

Figure 2 Effects of formoterol on total physical activity and grip force in sorafenib-treated tumour-bearing rats. Results are mean ± standard error for seven animals per group in control groups (without tumour) and eight animals per group in tumour-hosts groups. C, rats without tumour; T, tumour-bearing rats; C+S and T+S, treated with sorafenib; C+F and T+F, treated with formoterol; C+F+S and T+F+S, treated with both formoterol and sorafenib. Statistical significance of the results by full factorial three-way analysis of variance (fixed factors: tumour, sorafenib treatment, and formoterol treatment). *P*-values of all the parameters are detailed in Table S3. Statistically significant differences between all groups were assessed by pairwise comparisons *post hoc* Duncan test; different superscripts indicate significant differences between groups.

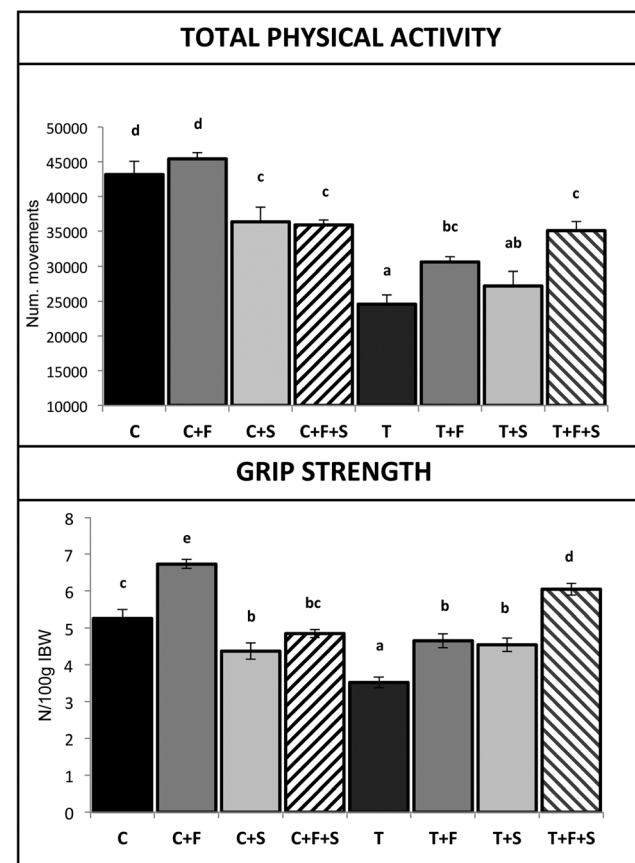
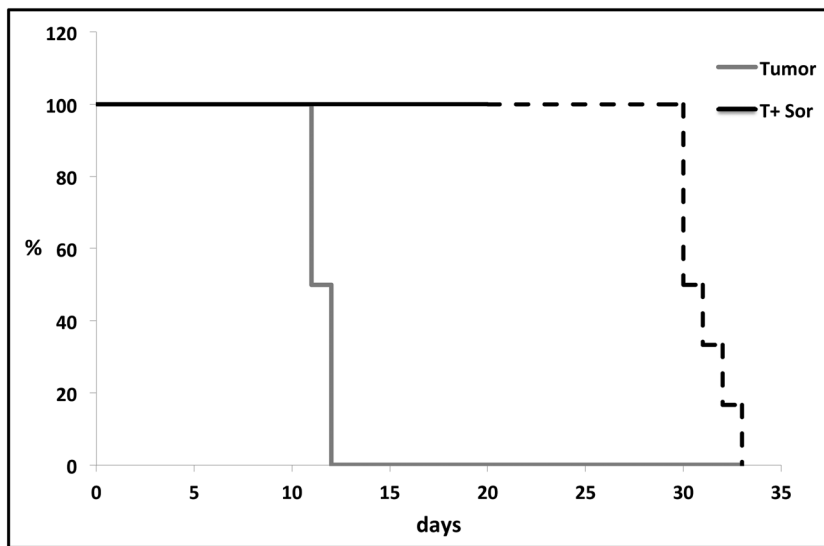


Figure 3 Survival. Kaplan–Meier survival analysis between untreated tumour-bearing rats and sorafenib-treated tumour-bearing rats (T + Sor). Sorafenib administration was stopped at day 20 after tumour inoculation (dotted line) in order to observe tumour relapse. Comparison of survival curves were analysed by log-rank test (Mantel–Cox). The global comparison for the treatments has a *P*-value < 0.001.



animals remained in their home cage. A frame containing an infrared beam system was placed on the outside of the cage; this minimized stress to the animals.

Grip-force assessment

Skeletal muscular strength in rats was quantified by the grip-strength test.⁴⁰ The grip-strength device (Panlab-Harvard

Figure 4 Effects of formoterol and formoterol+megestrol acetate on tumour cell content in sorafenib-treated tumour-bearing animals. Tumour cell content was assessed on day 20 after tumour inoculation. Results are mean ± standard error for eight animals per group. T + S, tumour-bearing rats treated with sorafenib; T + S + F, tumour-bearing rats treated with sorafenib and formoterol; T + S + F + MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. Statistical analysis of the results by one-way analysis of variance showed non-significant differences (*P* = 0.31).

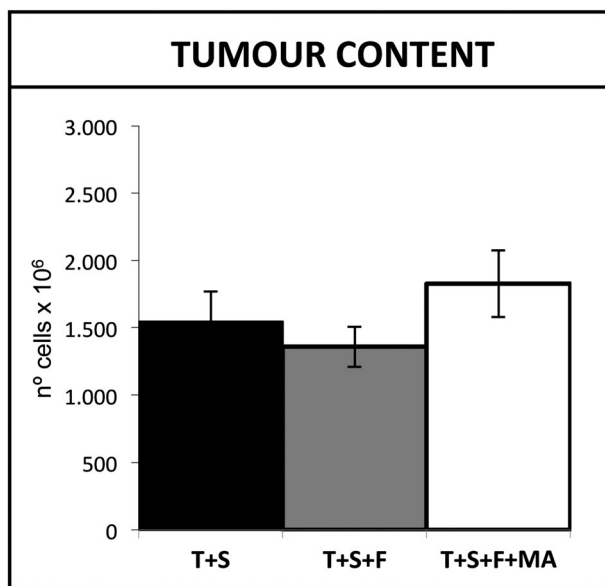
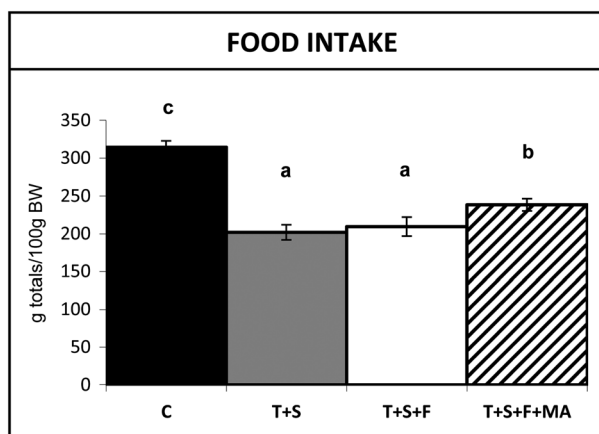


Figure 5 Effects of formoterol and formoterol+megestrol acetate on food intake in sorafenib-treated tumour-bearing animals. Food intake is expressed in g/100 g initial body weight and refers to the ingestion during the period of the experiment prior to sacrifice, which took place 20 days after tumour inoculation. Results are mean ± standard error for seven to nine animals per group. C, animals without tumour; T + S, tumour-bearing rats treated with sorafenib; T + S + F, tumour-bearing rats treated with sorafenib and formoterol; T + S + F + MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. Statistical significance differences between groups were detected by one-way analysis of variance (*P* < 0.001). Pairwise comparisons were performed by *post hoc* Duncan test; different superscripts indicate significant differences between groups.



Apparatus, Spain) comprised a pull bar connected to an isometric force transducer (dynamometer). Basically, the grip-strength meter was positioned horizontally, and the rats are held by the tail and lowered towards the device. The animals are allowed to grasp the bar and were then pulled backwards in the horizontal plane. The force applied to the bar just before it lost grip was recorded as the peak tension. At least three measurements were taken per rat, and the results were averaged for analysis. The data are presented as g/g initial body weight.

Statistical analysis

Average (arithmetic mean) and standard error were calculated for each studied variable. In Experiment I, a mixed model with repeated measures (longitudinal design) was performed. In Experiments II and IV, intergroup differences were evaluated statistically using analysis of variance (ANOVA); Experiment II: three-way ANOVA (fixed factors: tumour, Sor treatment, and F treatment); and Experiment IV: one-way ANOVA with four levels. *Post hoc* pairwise comparisons (Duncan test) were performed when appropriated. In Experiment III, 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 majority of studies involving anti-cachectic therapies are performed in TB animals not subjected to any anti-tumoural treatment. This fact may interfere with the translation of the results to human subjects, since in the clinical practice, anti-tumoural treatment is given as soon as cancer is diagnosed. For this reason in the present study, we adopted a well-established cachexia model (rats bearing the Yoshida

AH-130 ascites hepatoma) and added the administration of a new generation anti-tumour drug, Sor.¹⁷

The flow cytometry analysis of cell cycle phases in alternate-day tumour samples (*Figure 1*) showed that Sor has a bimodal action; a rapid (3 days after the first administration) exit from the cell cycle (reduced S (synthesis phase) and increased G0 (resting phase)/G1 (Growth 1/Gap 1 phase) was followed by a strong increase of G2 (pre-mitotic phase)/M (mitosis phase) arrest and apoptosis, suggesting that Sor is both an inhibitor of cell proliferation and an inducer of cell death.

Ten days after tumour inoculation, the cellularity in Sor-treated animals was reduced by 63% (*Figure 1B*). Formoterol treatment did not result in any changes of tumour cell content, as previously described.²⁹ Bearing in mind the strong anti-tumoural action of Sor, one would expect a consequent and consistent reduction of cachexia appearance. This was not the case, since Sor treatment was not free from side effects, such as hypophagia, even detectable in control (non-TB) animals (*Table 1*). However, Sor administration did not further reduce the food intake observed in TB animals, likely resulting from the combination of an attenuated tumour action and the drug-induced hypophagia. In the control group, the decreased food intake was associated with a reduction of body weight (*Table 1*). Again, similarly to the food intake, Sor treatment in TB animals did not worsen the body weight, possibly because of the decrease of tumour cell content induced by Sor. The effects of F on body weight were also distinct in control and TB animals: while F treatment increased the body weight significantly in the former ones, it did not produce any changes in the latter ones. Finally, the combination of F with Sor resulted in a decreased body weight in the control group, while an increase in the TB animals was observed. Again, this distinct behaviour may rely on the decreased cell content promoted by Sor, which possibly compensates the anorexigenic effects of Sor.

In non-TB rats, Sor induced a significant decrease in gastrocnemius (6%) and heart (7%) mass (*Table 1*). Actually, Antoun *et al.*¹² described skeletal muscle wasting as an

Table 2 Effects of the combination of formoterol and megestrol acetate treatment in sorafenib-treated tumour-bearing rats on muscle weight at day 20 after tumour inoculation

Parameters	Experimental groups				ANOVA P-value
	C (7)	T + S (8)	T + S + F (8)	T + S + F + MA (9)	
Muscle weight					
GSN	1005 ± 24 ^c	630 ± 19 ^a	723 ± 33 ^b	762 ± 28 ^b	< 0.001
Tibialis	314 ± 6 ^c	208 ± 5 ^a	247 ± 12 ^b	251 ± 8 ^b	< 0.001
Soleus	73 ± 1 ^d	50 ± 1 ^a	55 ± 2 ^b	62 ± 2 ^c	< 0.001
EDL	76 ± 2 ^c	53 ± 1 ^a	60 ± 3 ^b	62 ± 1 ^b	< 0.001
Diaphragm	474 ± 16 ^b	371 ± 12 ^a	342 ± 18 ^a	513 ± 23 ^b	< 0.001
Heart	549 ± 23 ^c	376 ± 16 ^a	401 ± 14 ^{ab}	429 ± 14 ^b	< 0.001

Results are mean ± standard error for the number of animals indicated in parentheses. Muscle weight is expressed as mg/100 g of initial body weight. GSN, gastrocnemius muscle; EDL, *extensor digitorum longus*; C, animals without tumour; T + S, tumour-bearing rats treated with sorafenib; T + S + F, tumour-bearing rats treated with sorafenib and formoterol; T + S + F + MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. Statistical significance of the results by one-way analysis of variance (ANOVA). Statistically significant differences by *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

adverse effect of Sor treatment. However, it is interesting to remark that Sor treatment did not significantly affect white adipose tissue mass. Therefore, it may be suggested that Sor side effects are more severe on skeletal muscle rather than on adipose tissue. As can be observed in *Table 1*, F treatment completely reversed the effects of Sor on skeletal muscle, without affecting adipose tissue. Sorafenib treatment in TB rats attenuated skeletal muscle and heart weight loss (*Table 1*), probably as a consequence of the reduction in tumour cell content induced by Sor. The other way round, bearing in mind the two-third reduction of tumour content, the animals were frankly cachectic, probably due to the combination of both residual tumour and Sor action. Consistently, F administration was able to ameliorate muscle mass in TB animals, either untreated (as previously reported³⁶) or Sor-treated.

The different effects on food intake, and body and muscle weights were compared with physiological parameters such as physical activity and muscle strength. In *Figure 2*, TB animals clearly showed a decrease in both parameters: total physical activity and grip strength. Treatment with Sor in healthy rats caused a reduction in physical activity (16%) and grip strength (17%). These changes agree with the previous effects of Sor on muscle weight (*Table 1*). Notably, Sor treatment, while prominently reducing the tumour burden, did not affect total physical activity and actually increased grip strength in TB animals (*Figure 2*). On the other hand, F increased both parameters in the control and TB animals. Remarkably, F administration to Sor-treated animals induced an even higher increase in physical activity (29%) and grip strength (33%) as compared with the TB animals only receiving the anti-tumoural treatment.

In summary, the use of F in Sor-treated animals seems to be highly effective in preventing muscle wasting and, thus, facilitating physical activity and, therefore, quality of life even in the presence of a chemotherapy regimen.

Taking into consideration that Sor treatment was able to strongly decrease tumour growth, a survival curve was performed (*Figure 3*). It can be seen that Sor was able to considerably increase survival (log-rank test, $P < 0.001$). After 20 days (approximately the double survival time of untreated TB rats), we stopped Sor treatment and observed tumour relapse, eventually leading to animal death around day 30.

Bearing this in mind, we decided to perform another series of experiments lasting 20 days following tumour inoculation in order to establish a potential chronic anti-cachectic protocol able to extend healthy lifespan. This experimental setting better resembles the human condition, where, after tumour diagnosis, an anti-cancer treatment is given and, despite the arrest of tumour growth, cachexia appears. At this time point, Sor-treated TB animals have only a very low residual tumour cell count, which cannot be assessed easily. We also incorporated a multi-factorial anti-cachectic treatment by combining F with MA. The rationale behind this was to provide a drug to compensate the hypophagia induced by Sor. Indeed, MA has been described as an

orexigenic drug, and its use in cancer patients is widely used.²³ In addition, our laboratory described the anabolic effects of the drug in skeletal muscle.²⁷

As shown in *Figure 4*, no effects were observed of either F or MA treatment on tumour cell content. The orexigenic action of MA can be observed in *Figure 5*; indeed, while no effects of F were seen, the combination increased food intake by 18%, despite being far from a complete recovery. In Sor-treated TB rats, F administration caused once again a positive effect on the majority of skeletal muscles studied

Figure 6 Effects of formoterol and formoterol+megestrol acetate on total physical activity and grip force in sorafenib-treated tumour-bearing animals. Total physical activity was assessed during the last 24 h prior to sacrifice of the animals on day 20 after tumour inoculation and was expressed as number of movements and grip force was assessed before the sacrifice. Results are mean \pm standard error for seven to nine animals per group. C, animals without tumour; T+S, tumour-bearing rats treated with sorafenib; T+S+F, tumour-bearing rats treated with sorafenib and formoterol; T+S+F+MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. Statistical significance between groups detected by one-way analysis of variance (Total physical activity; $P < 0.001$; grip strength, $P < 0.001$). *Post hoc* Duncan tests were performed; different superscripts indicate significant differences between groups.

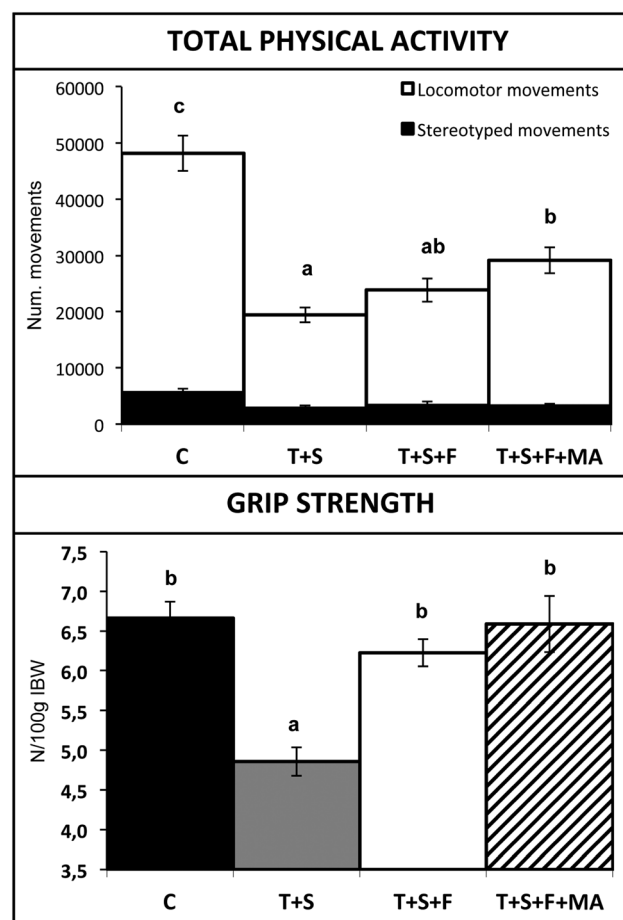
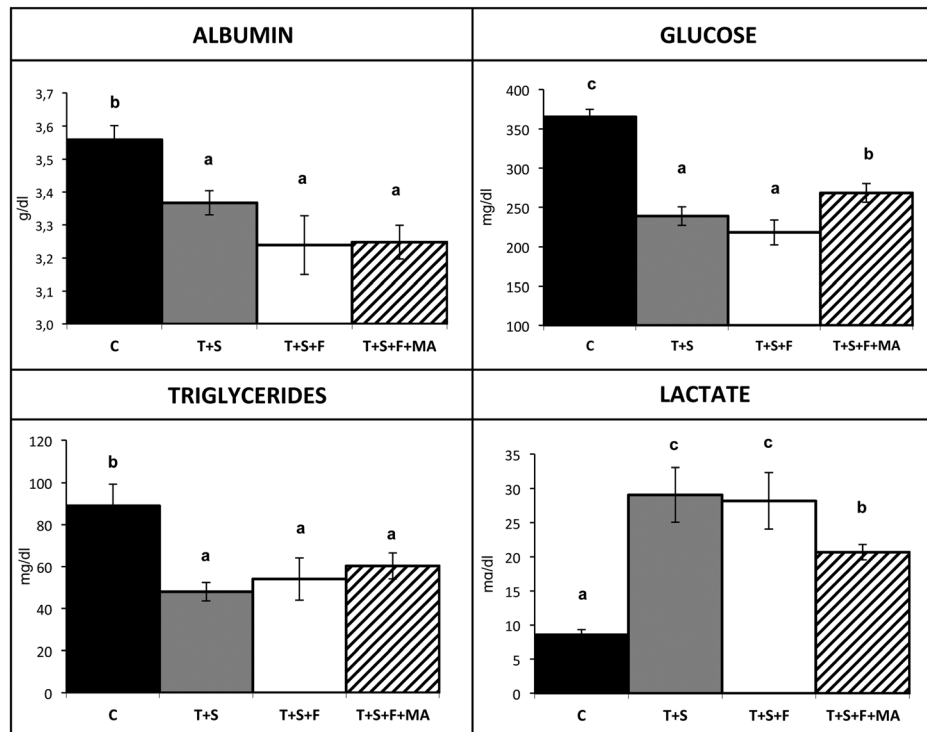


Figure 7 Effects of formoterol and formoterol + megestrol acetate on albumin and circulating metabolites in sorafenib-treated tumour-bearing animals on day 20 after tumour inoculation. Results are mean \pm standard error for seven to nine animals per group. C, animals without tumour; T + S, tumour-bearing rats treated with sorafenib; T + S + F, tumour-bearing rats treated with sorafenib and formoterol; T + S + F + MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. One-way analysis of variance denoted statistically significant differences between treatments (albumin, $P=0.003$; glucose, $P=0.008$; triglycerides, $P<0.001$; lactate, $P<0.001$). *Post hoc* pairwise comparisons (Duncan test) were performed; different superscripts indicate significant differences between groups.



(Table 2). Interestingly, the combination of F and MA was even more effective in the mainly oxidative muscles soleus and diaphragm, promoting an additional significant increase (13% for soleus and 50% for diaphragm) as compared with the animals that were treated only with F.

In relation to physical activity and muscle force, 20 days of tumour growth (18 of Sor administration) induced a significant decrease in both parameters (Figure 6). The combination of F and MA treatments was successful in significantly increasing both physical activity and grip strength, especially

Table 3 Effects of the combination of formoterol and megestrol acetate treatment on gastrocnemius gene expression in sorafenib-treated tumour-bearing rats at day 20 after tumour inoculation

Proteolytic system	Experimental groups				ANOVA <i>P</i> -value
	C (6)	T + S (7)	T + S + F (7)	T + S + MA + F (8)	
Ubiquitin-dependent					
Ubiquitin	100 \pm 22	117 \pm 21	102 \pm 10	106 \pm 28	0.95
E2	100 \pm 23 ^a	269 \pm 53 ^b	190 \pm 42 ^{ab}	194 \pm 49 ^{ab}	0.11
C8 proteasome subunit	100 \pm 23 ^a	224 \pm 20 ^b	280 \pm 58 ^b	235 \pm 49 ^b	0.035
C2 proteasome subunit	100 \pm 11 ^a	220 \pm 44 ^b	160 \pm 26 ^b	220 \pm 32 ^b	0.036
Murf-1	100 \pm 15 ^a	410 \pm 77 ^b	342 \pm 58 ^b	144 \pm 34 ^a	0.001
Atrogin-1	100 \pm 18 ^a	323 \pm 69 ^b	263 \pm 65 ^b	127 \pm 30 ^a	0.007
Lysosomal					
Cathepsin B	100 \pm 12	129 \pm 61	71 \pm 15	72 \pm 18	0.54
Calcium-dependent					
m-calpain	100 \pm 12 ^a	167 \pm 22 ^b	110 \pm 12 ^a	90 \pm 24 ^a	0.052

Results are mean \pm standard error for the number of animals indicated in parentheses. C, animals without tumour; T + S, tumour-bearing rats treated with sorafenib; T + S + F, tumour-bearing rats treated with sorafenib and formoterol; T + S + F + MA, tumour-bearing rats treated with sorafenib, formoterol, and megestrol acetate. Statistical significance of the results by one-way analysis of variance (ANOVA), following a *post hoc* Duncan test. Different superscripts indicate significant differences between groups.

for the latter, consistently with the rescue of muscle mass. Formoterol treatment alone was only able to induce an improvement in grip strength.

The presence of a tumour induces important metabolic changes which affect carbohydrate, lipid and protein metabolism.⁴¹ As readout of such alterations, tumour burden is associated with an increase in circulating lactate and a decreased glycaemia. The combined treatment was able to partially improve glycaemia and hyperlactemia in Sor-treated TB animals while had no effects on either circulating albumin or triglycerides (Figure 7).

Since one of the most deleterious events taking place in skeletal muscle during tumour growth is the increased protein breakdown, we also examined the effects of either F or F with MA treatments on gene expression of muscle proteolytic systems. As shown in Table 3, the combined treatment F+MA significantly reduced both Murf-1 and Atrogin-1 gene expression in gastrocnemius muscle, two Ub-ligases that represent the limiting step of proteasome-dependent degradation.⁴² Interestingly, the double treatment also decreased m-calpain gene expression, known to play a role in muscle proteolysis in cancer cachexia.^{43,44} Indeed, it was suggested that calcium-dependent proteases participate in the release of myofilaments from the sarcomere, and these myofilaments would be later degraded by the ubiquitin-dependent proteolytic system.⁴⁵ Sandri *et al.* reported the role for lysosome activity in muscle degradation during cancer cachexia. Actually, activation of FoxO3 stimulates lysosomal proteolysis in muscle by inducing the expression of autophagy-related genes.⁴⁶ It has been recently shown that autophagy plays a relevant role even in cancer cachexia.⁴⁷ Consistently, both F alone or in combination with MA effectively prevented the increase of Cathepsin B transcript observed in TB rats.

In summary, it is important to point out that this is one of the few studies that incorporates anti-tumour together with anti-cachectic treatments, since the majority of the data available from pre-clinical studies usually take into consideration only the cachectic effect induced in a short interval by tumour growth or by single anti-neoplastic drugs, not the combination. Such experimental settings, however, are far from the clinical practice, where an anti-tumour intervention is adopted as soon as the cancer is diagnosed and chronic cachexia occurs. The other way round, the study of drug toxicity and pharmacokinetics in the absence of the tumour might hide important aspects.⁴⁸ As a consequence, the use of inappropriate pre-clinical models complicates and limits the transfer of basic discoveries from the bench to the bedside. On the contrary, in the

present study, Sor administration effectively reduced tumour burden and prolonged survival at the cost of a dramatic cachectic state, reflecting the clinical conditions of cancer patients. Testing the effectiveness of candidate drugs in these experimental conditions offers a valid and reliable approach for translational research in the cancer cachexia field. Very recent data obtained in cancer patients using a similar combination strengthen this hypothesis.²² In conclusion, the present results reinforce the idea that a successful cachexia treatment has to be multifactorial. From this point of view, the combination of an orexigenic drug (MA) with an anti-catabolic one (F) resulted in clearly beneficial effects in TB animals.

Acknowledgements

F.P. was an AIRC/Marie Curie fellow when the study was performed. The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle (von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle. *J Cachexia Sarcopenia Muscle* 2010;1:7–8.).

Funding

‘Ministerio de Ciencia y Tecnología’ (SAF-26091-2011).

Supporting information

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

Table S1. ANOVA.

Table S2. Mixed-model with repeated measures (longitudinal design).

Table S3. ANOVA.

Conflict of Interest

None declared.

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