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ORIGINAL ARTICLE

Attenuation of skeletal muscle atrophy in cancer cachexia by D-myo-inositol 1,2,6-triphosphate

S. T. Russell · P. M. A. Siren · M. J. Siren · M. J. Tisdale

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Abstract
Purpose To determine the effectiveness of the polyanionic, metal binding agent D-myo-inositol-1,2,6-triphosphate (alpha trinositol, AT), and its hexanoyl ester (HAT), in tissue wasting in cancer cachexia.
Methods The anti-cachexic effect was evaluated in the MAC16 tumour model.
Results Both AT and HAT attenuated the loss of body weight through an increase in the nonfat carcass mass due to an increase in protein synthesis and a decrease in protein degradation in skeletal muscle. The decrease in protein degradation was associated with a decrease in activity of the ubiquitin-proteasome proteolytic pathway and caspase-3 and -8. Protein synthesis was increased due to attenuation of the elevated autophosphorylation of double-stranded RNA-dependent protein kinase, and of eukaryotic initiation factor 2α together with hyperphosphorylation of eIF4E-binding protein 1 and decreased phosphorylation of eukaryotic elongation factor 2. In vitro, AT completely attenuated the protein degradation in murine myotubes induced by both proteolysis-inducing factor and angiotensin II.
Conclusion These results show that AT is a novel therapeutic agent with the potential to alleviate muscle wasting in cancer patients.

Keywords Muscle atrophy · Alpha trinositol · Protein kinase R (PKR) · Eukaryotic initiation factors · Eukaryotic elongation factors

S. T. Russell · M. J. Tisdale (✉)
Nutritional Biomedicine,
School of Life and Health Sciences,
Aston University, Birmingham B4 7ET, UK
e-mail: m.j.tisdale@aston.ac.uk

P. M. A. Siren · M. J. Siren
Bioneris Ab, IAM,
Adolf Fredriks Kyrkogata 13,
111 37 Stockholm, Sweden

M. J. Siren
JGK Memorial Research Library and Laboratory,
Töölön k 19 00260, Helsinki, Finland

Introduction

Patients with cancer cachexia show progressive atrophy of skeletal muscle, while visceral protein reserves are preserved [17]. Muscle loss can reach 75% prior to death, resulting in weakness (asthenia), immobility and respiratory failure. Although as many as half of all cancer patients suffer from this condition, therapy is limited and there are no therapies routinely used to counteract muscle atrophy [35]. There is thus a pressing need to develop new therapies for the treatment of this condition.

Atrophy of skeletal muscle can occur through a depression of protein synthesis [16], an increase in protein degradation [26], or a combination of both. The increased protein degradation is due to an increased expression of major components of the ubiquitin-proteasome pathway, such as the 20S proteasome subunits [21]. Activation of caspase-3 is also required to degrade actomyosin complexes before they can be further cleaved by the ubiquitin-proteasome system [11]. Recent results [15] show that activation of the dsRNA-dependent protein kinase (PKR) in skeletal muscle, leads to a depression of protein synthesis and to an increase in protein degradation through the ubiquitin-proteasome pathway. Thus in response to catabolic stimuli such as proteolysis-inducing factor (PIF) and angiotensin II (Ang II), PKR undergoes activation by

autophosphorylation, leading to increased phosphorylation of the translation factor, eukaryotic initiation factor 2 (eIF2), on the α-subunit. eIF2 mediates binding of Met tRNA to the 40S ribosomal subunit in a GTP-dependent manner, and is released in its GDP-bound state, which is then exchanged for GTP by the guanine nucleotide exchange factor eIF-2B. The phospho eIF2α acts as a competitive inhibitor of this process preventing translation initiation [28]. In addition PKR is known to activate the transcription factor, nuclear factor-κB (NF-κB), through phosphorylation of the inhibitor IκB [23], and this leads to induction of the ubiquitin-proteasome pathway [15]. This pathway is also activated in skeletal muscle of mice [15] and humans [14] with cancer cachexia, as shown by increased levels of phospho PKR and eIF2α, without changes in the total amount of these factors. In addition, an inhibitor of PKR autophosphorylation has been shown to attenuate muscle atrophy in the MAC16 murine cachexia model, through an increase in protein synthesis and a decrease in protein degradation [12]. This suggests that inhibitors of PKR activation may be useful in the treatment of cancer cachexia.

Inositols are naturally occurring derivatives of phytic acid, which are especially abundant in cereals. D-Myo-inositol-1,2,6-trisphosphate (alpha trinositol, AT) is a naturally occurring trisphosphorylated derivative of inositol. Alpha trinositol has previously been shown to have both analgesic and anti-inflammatory effects [10]. Alpha trinositol and its derivatives may interfere at some point in the phosphatidylinositol signalling pathway [3]. Being a polyanionic compound, AT readily interacts with inorganic cations such as Ca^{2+} and Zn^{2+}, which bind preferentially to phosphates in positions P1 and P6 in the inositol ring structure [18]. However, the exact mode of action of AT remains to be determined. There have been few studies on the role of metal ions in cachexia. However, Larsson et al. [24] found a gradually increasing zinc concentration in both skeletal muscle and tumour tissue during tumour progression in a rat cachexia model. Calcium concentrations also increased in skeletal muscle, which could be related to activation of PKR. Activation of PKR by aggregated β-amyloid peptide (Aβ) involves calcium release from the endoplasmic reticulum and activation of caspase 8, as upstream signals modulating the caspase-3 mediated activation of PKR [33]. The cell permeable calcium chelator BAPTA-AM has been shown to significantly reduce PKR phosphorylation in neurons [9], suggesting that calcium chelators may be effective in the treatment of muscle atrophy if activation of PKR is involved. This study investigates the effect of AT on the development of cachexia in a murine tumour model (MAC16) and investigates potential mechanisms involved.

Materials and methods

Materials

Foetal calf serum, horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies (Paisley, Scotland). L-[2,6-^{3}H] Phenylalanine (sp.act.1.96TBq/mmol^{-1}), Hybond A nitrocellulose membranes, m^{7}GTP (7-methyl-GTP) Sepharose 4B, and ECL development kits were from Amersham Biosciences Ltd (Bucks, UK). Mouse monoclonal antibodies to 20S proteasome α-subunits and p42 were from Affiniti Research Products (Exeter, UK). Rabbit monoclonal antibodies to phospho-4EBP1 (Thr$^{37/46}$), phospho mTOR (Ser^{2448}) and to phospho and total PKR, as well as rabbit polyclonal antisera to 4E-BP1, eIF4E, eIF4G, and to phospho (Thr^{56}) and total elongation factor 2 (eEF2) were purchased from New England Biolabs (Herts, UK). Rabbit polyclonal antisera to phospho eIF2α (Ser^{51}) and to total eIF2α was from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal antisera to myosin heavy chain was from Novocastra (Newcastle, UK). Rabbit polyclonal antisera to mouse β-actin, angiotensin II (Ang II) and the chymotrypsin substrate succinyl LLVY-7-amino-4-methylcoumarin were purchased from Sigma Aldridge (Dorset, UK). Peroxidase-conjugated rabbit anti-mouse antibody and peroxidase-conjugated goat anti-rabbit antibody were purchased from Dako Ltd (Cambridge, UK). 1-D-Myo-inositol 1,2,6-triphosphate (AT) and 1D tri-*O*-hexanoyl-myo-inositol 1,2,6-triphosphate (HAT) were supplied by JGK Memorial Research Library and Laboratory (Helsinki, Finland)/Bioneris Ab (Stockholm, Sweden). Phosphosafe™ extraction reagent was from Merck Eurolab Ltd (Leicestershire, UK). The caspase-3 and -8 substrates and inhibitors were purchased from Biomol International (Devon, UK).

Animals

The MAC16 tumour was passaged in pure strain NMRI mice (average weight 25 g), obtained from our own inbred colony, and maintained on a rat and mouse breeding diet (Special Diet Services, Witham, UK) and water ad libitum. Male mice were transplanted with fragments of the MAC16 tumour, selecting from donor animals with maximal weight loss. The tumour was implanted s.c. into the flank by means of a trochar, as previously described [12]. Weight loss began 12–15 days after tumour transplantation and animals were entered into the study when they had lost about 5% of their starting body weight. Preliminary dose-range finding assays in non-tumour bearing mice had established the tolerability range for the agents. For the study tumour-bearing animals were randomised into groups of six to receive either solvent (PBS), or AT (at doses of 10, 20 and

40 mg kg^{-1}), administered s.c. 3 times per day. HAT was administered at 6 and 8 mg/kg, also by the s.c. route 3 times per day. Tumour volume, body weight and food and water intake were monitored daily. Animals were terminated by cervical dislocation when the body weight loss reached 20%, as specified by the British Home Office. The ethical guidelines that were followed met the standards required by the UKCCR guidelines. After termination the body composition of the animals was determined as previously described in detail [30]. The carcasses were heated at 80–90°C for 48 h, or until a constant weight was achieved. The water content was determined from the difference between the wet and dry weight. Lipids were extracted from the dried carcass with chloroform/methanol (1:1), ethanol/acetone (1:1) and diethyl ether. The solvent was allowed to evaporate, and the fat content was determined from the weighed residue. The non-fat carcass mass was calculated as the difference between the initial weight of the carcass and the weight of water and fat. PIF was purified from solid MAC16 tumours, excised from mice with a weight loss between 20 and 25% using affinity chromatography with anti-PIF monoclonal antibody coupled to a solid matrix, as previously described [36].

Protein synthesis and degradation in gastrocnemius muscle

This was determined in a separate experiment in which mice ($n = 6$) bearing the MAC16 tumour were treated with AT (40 mg/kg) s.c., 3 times per day, for 5 days, while a control group received PBS. Protein synthesis and degradation were determined by the incorporation and release of L-[2,6-^{3}H] phenylalanine, as described [32]. On the fourth day of treatment, half of the group were administered 0.4 mmol/l L-[2,6-^{3}H] phenylalanine in PBS (100 µl) by i.p. administration. After 24 h the animals were terminated and gastrocnemius muscles were removed, washed with PBS and RPMI 1640, and the release of radioactivity during incubation for 2 h in RPMI 1640 was determined. Protein-bound activity was determined by homogenising the muscles in 2% perchloric acid and determining the radioactivity in the precipitate. Protein degradation was calculated by dividing the amount of radioactivity released into the medium over a 2 h period by the specific activity of the protein-bound radioactivity. To determine protein synthesis gastrocnemius muscles were incubated for 2 h in RPMI 1640, without phenol red, in the presence of L-[2,6-^{3}H] phenylalanine (37 MBq), and under an atmosphere of O_2/CO_2 (19:1). Muscles were then rinsed in nonradioactive media, and homogenised in 2% perchloric acid. The rate of protein synthesis was calculated by dividing the protein-bound radioactivity by the acid-soluble material. Myotubes were formed by allowing C_2C_{12} myoblasts to differentiate in DMEM containing 2% HS. Protein degradation in murine myotubes was determined by measuring the release of L-[2,6-^{3}H] phenylalanine from pre-labelled cells in the presence of 2 mM unlabelled phenylalanine to prevent reincorporation of radioactivity, as previously described [15].

Proteasome activity

The activity of the 20S proteasome was determined as the 'chymotrypsin-like' enzyme activity, the predominant proteolytic activity of the $\beta 5$ subunits of the proteasome. Gastrocnemius muscles were rinsed with ice-cold PBS and homogenised in 20 mM Tris–HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$ and 1 mM DTT, followed by sonication. The sonicate was centrifuged for 10 min at 18,000g at 4°C, and enzyme activity in the supernatant was determined by the method of Orino et al. [27] by determining the release of amino methyl coumarin (AMC) from the flurogenic substrate LLVY-AMC. Activity was measured in the absence and presence of the specific proteasome inhibitor lactacystin (10 µM). Only lactacystin-suppressible activity was considered to be proteasome specific.

Caspase activity

The activity of caspase-3 was determined by the release of 7-amino-4-methylcoumarin (AMC) from the specific substrate AcDEVD–AMC in the presence or absence of the caspase-3 inhibitor AcDEVD–CHO. Muscle (10 mg) was homogenised in lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris–HCl, pH 7.4, 0.25% sodium deoxycholate, 2 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 mM NaF and 1% proteasome inhibitor mixture), left at 4°C and then room temperature for 10 min, followed by centrifugation at 15,000g for 15 min. The supernatant (50 µg protein) was incubated with the caspase-3 substrate (10 µM) for 1 h, and the increase in fluorescence due to AMC was determined at an excitation wavelength of 370 nm and an emission wavelength of 430 nm. The difference in values in the absence and presence of the caspase-3 inhibitor (100 µM) was a measure of activity. The method for caspase-8 was similar with the substrate being Z-IETD-AFC (10 µM) and the inhibitor IETD–CHO (100 µM). The increase in fluorescence due to the release of 7-amino-4-trifluoro-methylcoumarin (AFC) was measured with an excitation wavelength of 400 nm and an emission of 505 nm.

Western blot analysis

Gastrocnemius muscle (10 mg) was homogenised in Phosphosafe™ Extraction Reagent (500 µl) and centrifuged at 15,000g for 15 min. Samples of the cytosolic proteins (5 µg) were loaded on either a 10% (mTOR, myosin, eIF4E and eIF4G), 12% (PKR, eIF2α and actin) or 15%

(4E-BP1) sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) and electrophoresed at 180 V for approximately 1 h. The extent of phosphorylation of 4E-BP1, and the association of 4E-BP1 and eIF4G with eIF4E was determined by Western blotting when eIF4E was extracted from the muscle samples by m^7GTP-Sepharose 4B-affinity binding, as previously described [13], by loading 20 μg of protein. The protein on the gels was then transferred to 0.45 mm nitrocellulose membranes, which were then blocked with 5% Marvel in Tris–buffered saline, pH 7.5, at 4°C overnight. The primary antibodies were used at a dilution of 1:1,000, except for phospho and total eIF2α (1:500) and myosin (1:250). The secondary antibodies were used at a dilution of 1:1,000. Incubation was either for 1 h at room temperature, or overnight, and development was by ECL. Blots were scanned by a densitometer to quantitate differences.

Statistical analysis

All results are shown as mean ± SE for at least three replicate experiments. Differences in means between groups were determined by one-way analysis of variance followed by Tukey–Kramer multiple comparison test. *P* values less than 0.05 were considered 'significant'.

Results

The effect of different concentrations of AT, administered tri-daily to mice bearing the MAC16 tumour, on body weight loss and tumour volume is shown in Fig. 1. At doses of 10 and 40 mg kg^{-1}, but not at 20 mg kg^{-1}, AT was effective in suppressing loss of body weight (Fig. 1a), while at 40 mg kg^{-1} there was also significant suppression of tumour growth rate (Fig. 1b). There was no significant effect of these concentrations of AT on food and water intake, and no toxicity was seen. A hexanoyl ester of AT (HAT) has been produced as a slow release form of AT, after hydrolysis by esterase. HAT was also effective in attenuating weight loss in the MAC16 model (Fig. 1c), but at lower concentrations than AT, the optimum dose being 6 mg kg^{-1}. There was no significant effect of HAT on tumour growth rate (Fig. 1d), suggesting that attenuation of

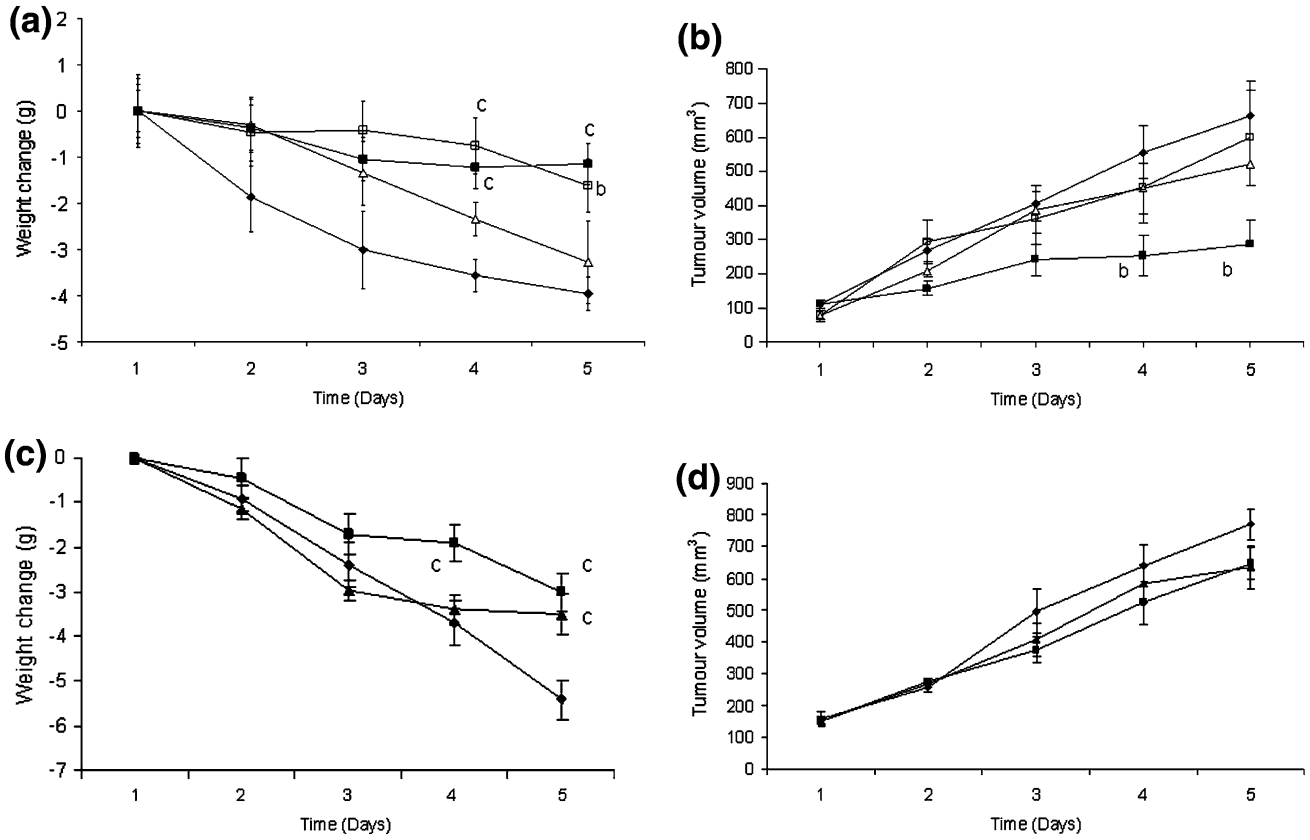

Fig. 1 Effect of AT administered s.c. three times per day at doses of 10 (*open square*), 20 (*open triangle*) or 40 mg kg^{-1} (*filled square*) in comparison with solvent (*PBS*) control (*filled diomand*) on body weight (**a**) and tumour volume (**b**). The effect of HAT also administered s.c. three-times per day at 6 (*filled square*) and 8 mg kg^{-1} (*filled triangle*) on body weight (**c**) and tumour volume (**d**) is shown in comparison with PBS control (*filled diomand*). The number of animals in each group $n = 6$. Differences from control are shown as **b**, $P < 0.01$ or **c** $P < 0.001$

body weight loss by AT was not related to its effect on tumour growth.

To determine the body compartment affected by AT, body composition analysis was performed on the animals at the end of the experiment shown in Fig. 1. The results presented in Table 1 show that AT produced a significant increase in the nonfat carcass mass, at doses of 20 and 40 mg kg^{-1}, with no effect on the body water content. At the optimum concentration (40 mg kg^{-1}) there was a 25% increase in the nonfat carcass mass, but, surprisingly, a large decrease in the fat mass, which reached 46% at a dose of AT of 20 mg kg^{-1} and did not further decrease at higher dose levels. Thus the change in body weight at various doses of AT is a balance between the loss of fat, and the increase in nonfat carcass mass. This would explain why AT, at a dose of 20 mg kg^{-1}, had no effect on total body weight, because although it increased lean body mass by 0.8 g from solvent controls, it decreased fat mass by 0.6 g, so the overall change in body weight did not differ from control. When corrected for hydration of lean body mass the difference in body weight of animals treated with AT at 40 mg kg^{-1} and PBS controls (Fig. 1a about 3 g) would be explained totally by the increase in lean body mass (Table 1). To determine whether changes in nonfat carcass mass arose due to attenuation of muscle atrophy, the effect of AT on protein synthesis and degradation in gastrocnemius muscle was determined after treatment with a dose of 40 mg kg^{-1} for 4 days (Fig. 2). The weight of the gastrocnemius muscles from mice treated with AT were significantly higher than from those treated with solvent alone (Fig. 2a), while there was a significant (almost threefold) increase in protein synthesis (Fig. 2b) and a 23% decrease in protein degradation (Fig. 2c). These results suggest that AT increases lean body mass through an increase in protein synthesis and a decrease in protein degradation in skeletal muscle.

To investigate further the mechanism by which AT altered the rates of protein synthesis and degradation, tumour-bearing animals were treated with either AT (40 mg kg^{-1}) or PBS daily for 4 days as described in the legend to Fig. 1, while a third group were not transplanted with the MAC16 tumour, nor received any therapy. In gastrocnemius muscle from mice bearing the MAC16 tumour there was a significant increase in the 20S proteasome functional activity, as measured by the 'chymotrypsin-like' enzyme activity (Fig. 3a) when compared with non-tumour bearing animals, as previously reported [37].

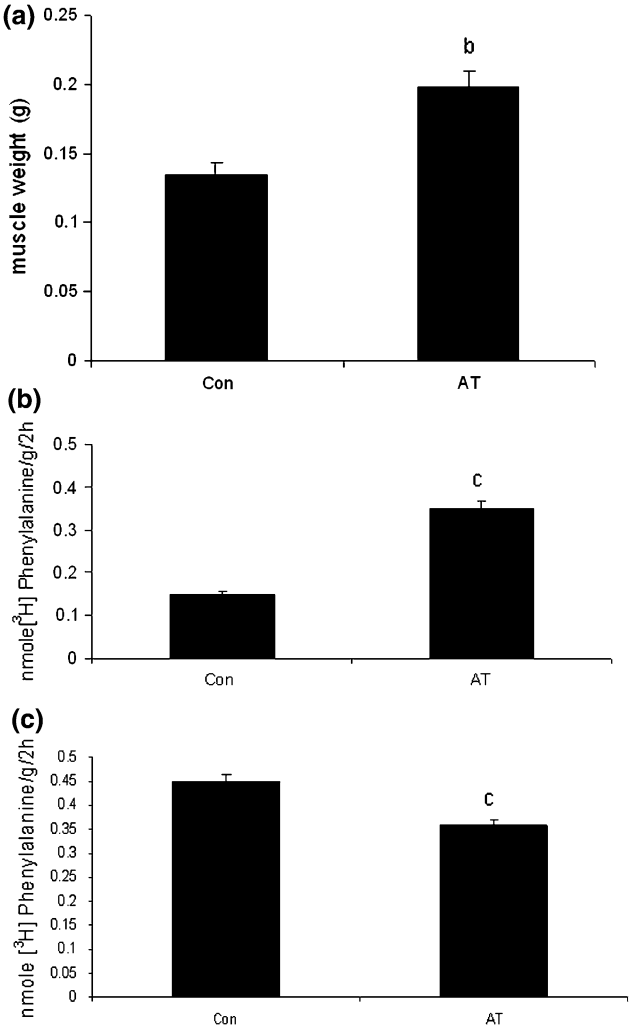

Fig. 2 Effect of AT (40 mg kg^{-1}) administered s.c. three times per day for 4 days in gastrocnemius muscle wet weight (**a**) and protein synthesis (**b**) and protein degradation (**c**) in gastrocnemius muscle. The number of animals in each group $n = 6$. Control animals received PBS at the same time as AT. Differences from control are shown as **c**, $P < 0.01$

Table 1 Body composition (%) of mice bearing the MAC16 tumour administered AT

AT (mg kg^{-1})	Lean mass		*P*	Fat		*P*	Water		*P*
	g	%		g	%		g	%	
0	5.7 ± 0.7	26.1 ± 1.6	–	1.4 ± 0.6	6.1 ± 2.1	–	14.7 ± 1.8	67.8 ± 1.2	–
10	6.3 ± 1.0	27.1 ± 3.0	NS	1.2 ± 0.6	4.9 ± 2.3	NS	15.8 ± 1.2	68.0 ± 2.0	NS
20	6.5 ± 0.3	28.6 ± 1.7	0.05	0.8 ± 0.1	3.3 ± 0.5	0.01	15.6 ± 0.7	68.1 ± 1.7	NS
40	7.1 ± 0.6	30.7 ± 2.5	0.01	0.9 ± 0.4	3.7 ± 1.9	0.05	15.2 ± 1.8	65.6 ± 2.2	NS

Values are mean ± SD. *P* values are from O AT measured after 4 days of treatment as shown in Fig. 1a

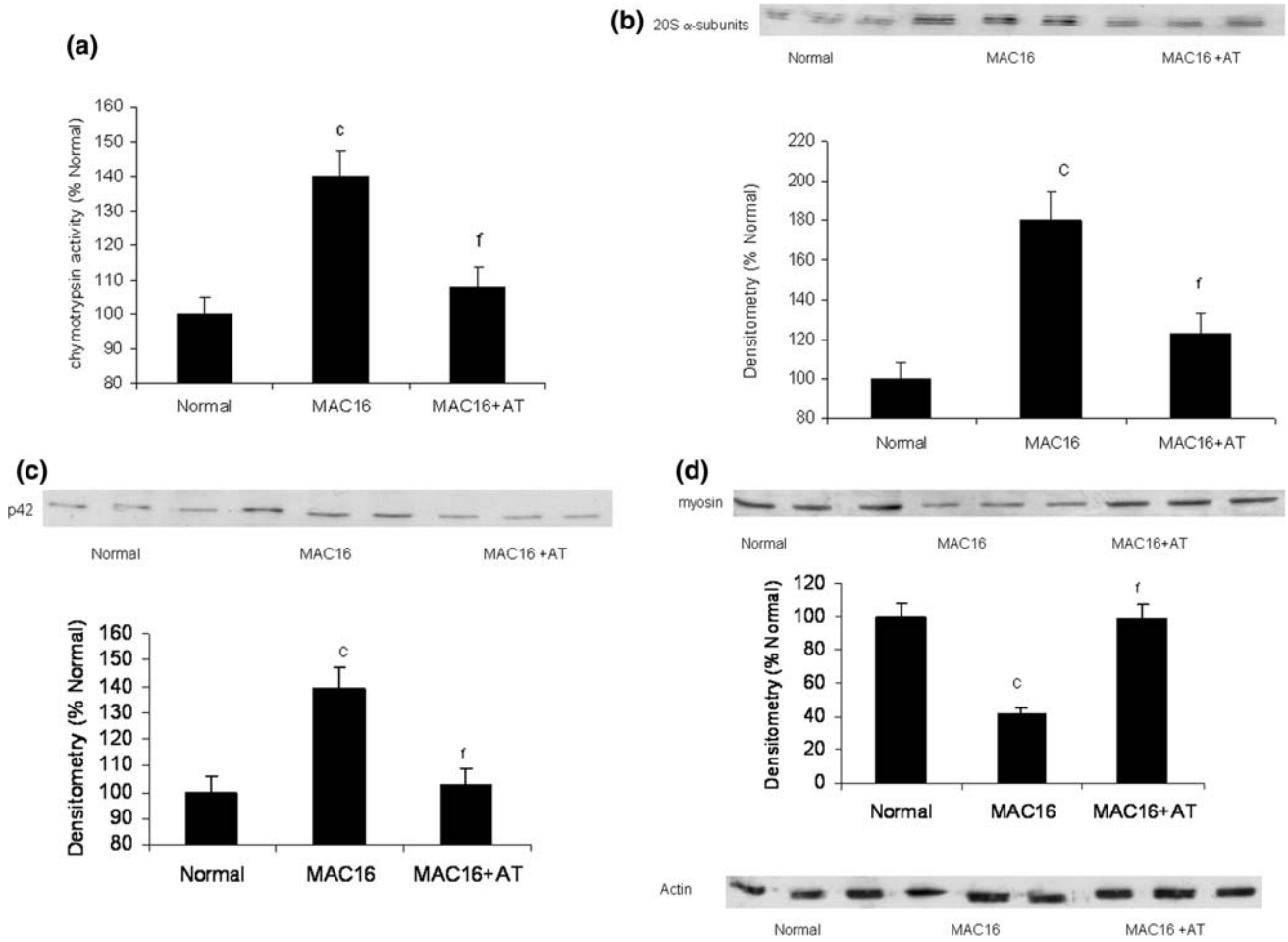

Fig. 3 Effect of AT (40 mg kg^{-1}) administered s.c. three-times per day for 4 days on the chymotrypsin-like enzyme activity (**a**), expression of the 20S proteasome α-subunits (**b**), p42 (**c**) and myosin levels (**d**) determined by Western blotting of protein from gastrocnemius muscle of mice bearing the MAC16 tumour (MAC16 + AT), in comparison with control tumour-bearing animals administered PBS (MAC16), and non tumour-bearing animals of the same age and starting weight (25 g) (Normal). An actin loading control is shown in **d**. Each of the experiments was repeated three times and there were 6 animals in each group. Representative Western blots are shown and the densitometric analysis is the average of three separate Western blots. Differences from normal are shown as **c**, $P < 0.001$, while differences from MAC16 are shown as **f**, $P < 0.001$

Treatment with AT for 4 days reduced the increased 'chymotrypsin-like' enzyme activity down to that found in non-tumour bearing animals (Fig. 3a). This suggests that AT attenuates protein degradation in skeletal muscle by down-regulating the increased activity of the ubiquitin-proteasome pathway. This was confirmed by measurement of expression of the 20S proteasome α-subunits (Fig. 3b), as well as expression of p42, an ATPase subunit of the 19S regulator, which promotes association with the 20S proteasome to form the 26S proteasome (Fig. 3c). In both cases expression was increased by 40–80% in gastrocnemius muscle of mice bearing the MAC16 tumour, but after 4 days of treatment with AT, expression levels were not significantly different from that found in non tumour-bearing animals. Expression levels of proteasome components correlated inversely with the level of myosin in gastrocnemius muscle, which were reduced by 60% in mice bearing the MAC16 tumour, and increased back to values found in non tumour-bearing animals after treatment with AT for 4 days (Fig. 3d). As reported for animals bearing the cachexia-inducing colon 26 adenocarcinoma, despite extensive loss of myosin there was no reduction in the expression of β-actin. The activity of caspase-3 (Fig. 4a) and caspase-8 (Fig. 4b) was elevated 2.5 to 3-fold in gastrocnemius muscle of mice bearing the MAC16 tumour, compared with non tumour-bearing animals, and this was significantly attenuated in animals treated with AT, although the levels were still significantly higher than those found in non-tumour-bearing animals.

As previously reported [15], expression of the phosphorylated forms of both PKR (Tyr^{451}) and eIF2α (Ser^{51}) were significantly increased (fivefold) in gastrocnemius muscle of mice bearing the MAC16 tumour compared with non tumour-bearing animals, while levels of total PKR and

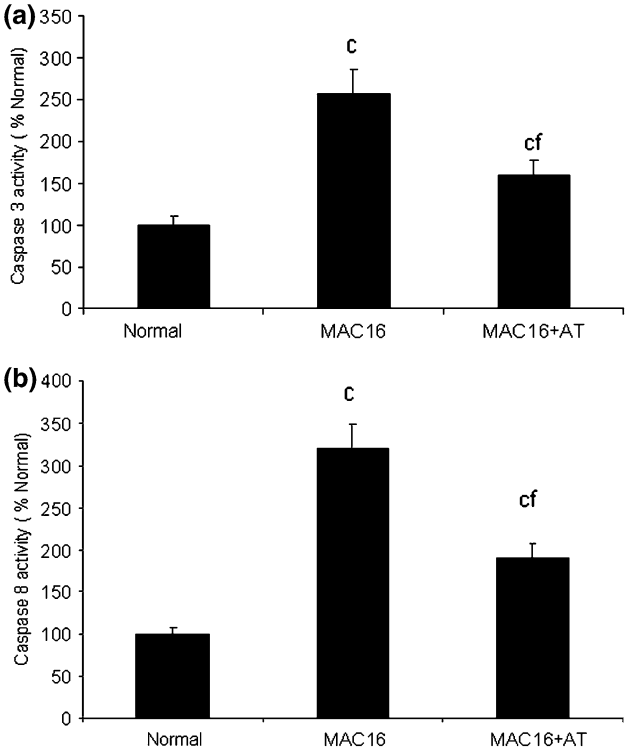

Fig. 4 Activity of caspase-3 (**a**) and caspase-8 (**b**) in gastrocnemius muscle of mice bearing the MAC16 tumour (MAC16), mice bearing the MAC16 tumour and treated with AT (40 mg kg^{-1}) for 4 days (MAC16 + AT) as described in the legend to Fig. 1, and in non tumour-bearing animals of the same age and starting weight as those bearing the MAC16 tumour (Normal). Each assay was repeated at least three times and there were six animals in each group. The values given are the average of three separate determinations. Differences from normal animals are shown as **c**, $P < 0.001$, while differences from MAC16 are shown as **f**, $P < 0.001$

eIF2α did not change (Fig. 5a, b). After 4 days of treatment with AT there was a significant reduction in the levels of both phospho PKR and eIF2α, which did not differ significantly from non tumour-bearing animals, and this correlated with the increase in protein synthesis (Fig. 2b). One reason for the decreased phosphorylation of PKR is an increased expression of protein phosphatase 1 (PP1) after treatment with AT (Fig. 5c), which is known to dephosphorylate PKR [34]. Levels of the phosphorylated form of eEF2 (Thr^{56}) were also increased in gastrocnemius muscle of mice bearing the MAC16 tumour (Fig. 5d), and this was reduced down to the levels found in non tumour-bearing animals after treatment with AT for 4 days. This suggests that AT could raise global protein synthesis in mice bearing the MAC16 tumour, up to the levels found in non-tumour-bearing animals, through attenuation of the decrease in translation elongation.

There was also a fivefold reduction in the level of the phosphorylated (Ser^{2448}) form of mTOR in gastrocnemius muscle of mice bearing the MAC16 tumour (Fig. 6a), and this was completely reversed up to the values found in non tumour-bearing animals after 4 days of treatment with AT. The effect of cachexia on the amount of eIF4E available for formation of the active eIF4G. eIF4E complex in gastrocnemius muscle and the effect of AT is shown in Fig. 6b–d. Animals bearing the MAC16 tumour showed a 60% reduction in the level of phosphorylation of 4E-BP1 ($Thr^{37/46}$) (Fig. 6b) in line with the decreased activation of mTOR (Fig. 6a), but there was no effect on phosphorylation of eIF4E (Ser^{209}) (Fig. 6c). Weight loss increased the amount of 4E-BP1 associated with eIF4E (Fig. 6c) and decreased formation of the active eIF4G.eIF4E complex (Fig. 6d). These effects were completely attenuated by AT, such that the levels of eIF4F were the same as in non-tumour bearing controls.

In order to determine whether AT had a direct effect on the cachexic process, or whether the observed anti-cachexic effect was due to AT's anti-tumour activity, protein degradation was measured in vitro in murine myotubes in the presence of either PIF (Fig. 7a) or Ang II (Fig. 7b) both of which have been shown to initiate protein degradation by a PKR-mediated process [15]. At a concentration of 100 μM AT completely attenuated the increase in protein degradation induced by both agents confirming a direct antagonistic effect. Further studies will evaluate the mechanism by which AT produces this effect.

Discussion

This study provides evidence for the effectiveness of AT and its hexanoyl ester to attenuate loss of body weight in mice bearing the MAC16 tumour, through preservation of lean body mass, despite the fact that AT accentuates loss of adipose tissue. Loss of adipose tissue would not pose a problem in the treatment of patients with cancer cachexia, since this can readily be replenished by treatment with an appetite stimulant such as megestrol acetate [25]. AT also attenuated tumour growth rate, but this was not essential for the anticachectic effect, since the hexanoyl derivative of AT attenuated loss of body weight without an effect on tumour growth. In addition, if the change in body weight induced by AT was solely due to the inhibition of tumour growth, then both fat and non-fat carcass mass should be increased, and only the latter was increased, while fat mass showed a progressive decrease with increasing concentrations of AT. Also there was no correlation between inhibition of tumour growth by AT and its ability to attenuate weight loss. We have also shown that AT can attenuate muscle protein degradation induced by PIF and Ang II in vitro confirming a direct effect of AT on the cachectic process, since both PIF [6] and Ang II [5] have been shown to induce a cachexic syndrome in mice with enhanced degradation of skeletal

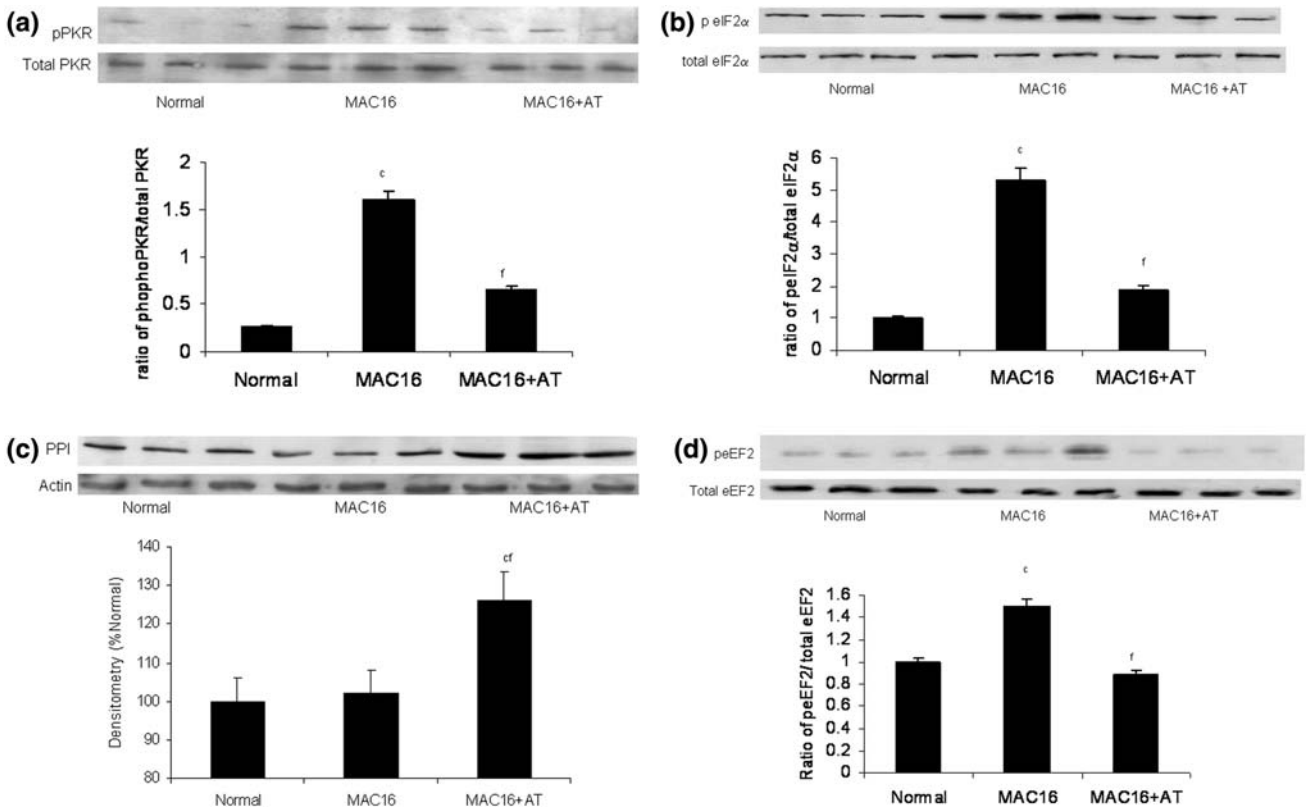

Fig. 5 Western blots showing expression of phosphorylated forms of PKR (pPKR) (**a**) and eIF2α (peIF2α) (**b**) in relation to total PKR and eIF2α, **c** PP1, with actin loading control and **d** phospho-eEF2 (peEF2) and total eEF2, in gastrocnemius muscle of mice bearing the MAC16 tumour, treated with PBS for 4 days (MAC16), or with AT (MAC16 + AT) (40 mg kg^{-1}), as described in the legend to Fig. 1, in comparison with non tumour-bearing controls (Normal). Representative Western blots are shown for three separate animals for each group, and the densitometric analysis represents the average of the determinations. Differences from normal animals are shown as **c**, $P < 0.001$, while differences from MAC16 are shown as **f**, $P < 0.001$

muscle. The change in body composition is unusual, since other agents which attenuate loss of body weight in mice bearing the MAC16 tumour, such as β-hydroxy-β-methylbutyrate (HMB) increase both the fat and non-fat body compartments [32]. Only one other agent, an oxindole/imidazole derivative, has been found to produce a similar effect, and that is an inhibitor of PKR autophosphorylation, and this was also found to attenuate tumour growth, although it was unrelated to the anticachectic effect [12].

As with the PKR inhibitor [12], preservation of lean body mass resulted from both the attenuation of the depression of protein synthesis and the increase in protein degradation in skeletal muscle. Very few agents are capable of influencing both processes. Thus eicosapentaenoic acid (EPA) preserves muscle mass solely from an effect on protein degradation, while having no effect on protein synthesis [31]. To affect both processes, EPA must be combined with amino acids, such as leucine, arginine and methionine [31].

The effect of AT on protein degradation is associated with down-regulation of the activity and expression of key components of the ubiquitin-proteasome pathway, resulting in retention of the myofibrillar protein myosin. AT also down-regulated the increased activity of caspases-3 and -8, which have previously been shown to be elevated in skeletal muscle of mice bearing the MAC16 tumour, without evidence for DNA fragmentation typical of apoptosis [2]. This effect may arise from the ability of AT to attenuate the elevated levels of autophosphorylated PKR seen in the skeletal muscle of mice bearing the MAC16 tumour, which has been shown to regulate this pathway through activation of NF-κB [15]. The effect on PKR autophosphorylation may be related to an increase in expression of PP1, which has been shown [8] to reduce the dsRNA-mediated autoactivation of PKR, while inhibiting PKR transphosphorylation activity. The mechanism by which AT increases the expression of PP1 is not known, but branched-chain amino acids (BCAA) such as leucine [13], as well as growth factors such as insulin [8], are known to stimulate expression of PP1. Plasma levels of both BCAA and insulin [1] have been shown to be low in weight-losing mice bearing the MAC16 tumour. Alternatively the attenuation of PKR autophosphorylation by AT could be mediated through inhibition of Ca^{2+} release from the endoplasmic reticulum, since

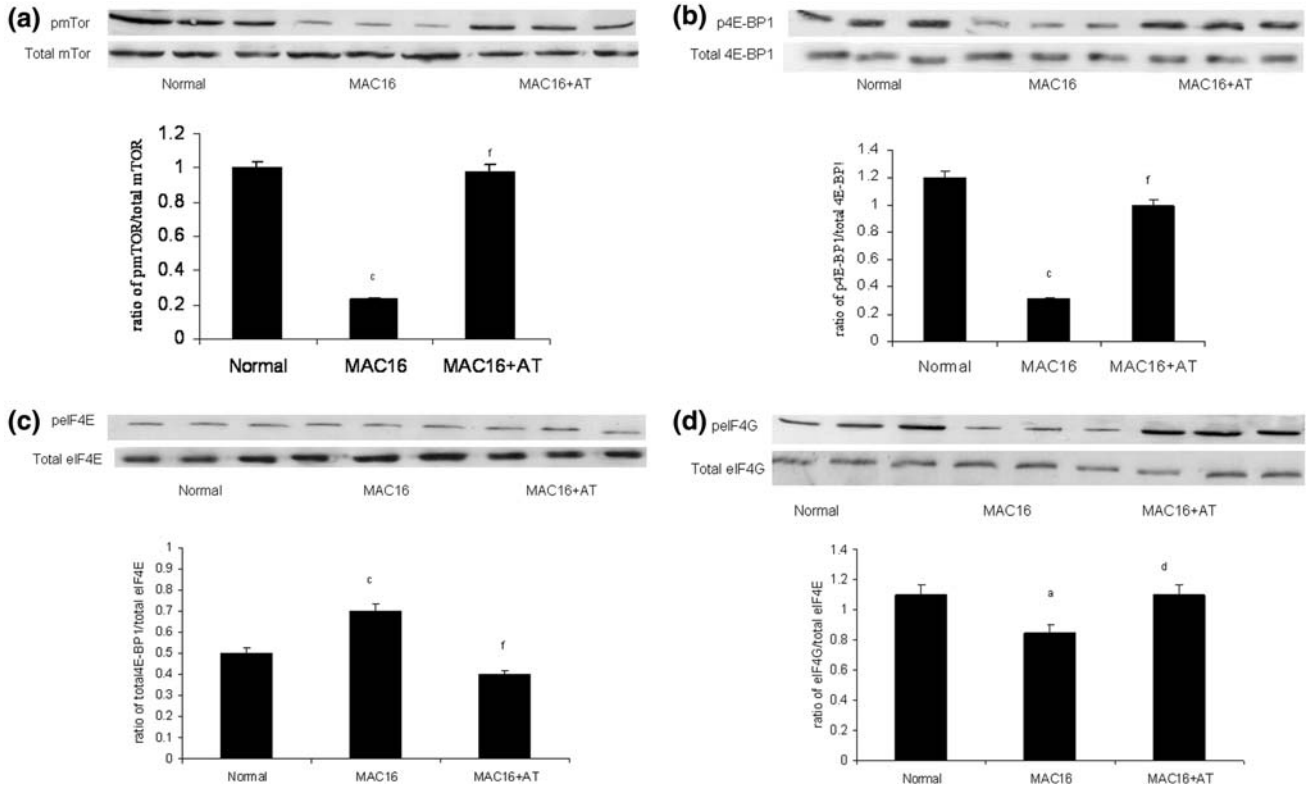

Fig. 6 Western blots showing expression of phosphorylated (pmTOR) and total mTOR (**a**), phospho (p4E-BP1) and total 4E-BP1 (**b**), phospho (peIF4E), total eIF4E and the ratio of 4E-BP1 to eIF4E (**c**), phospho (peIF4G) and total eIF4G and the ratio of eIF4G to eIF4E (**d**) in gastrocnemius muscle of mice bearing the MAC16 tumour, and treated with PBS (MAC16), or AT (40 mg kg^{-1}) (MAC16 + AT), in comparison with non tumour-bearing controls (Normal). Representative Western blots are shown for three separate animals for each group, and the densitometric analysis represents the average of the determinations. Differences from normal animals are shown as either **a**, $P < 0.05$ or **c**, $P < 0.001$, while differences from MAC16 are shown as either **d**, $P < 0.05$ or **f**, $P < 0.001$

AT also attenuated the elevated levels of caspases-3 and -8 (Fig. 4a, b), which have also been suggested to require Ca^{2+} for activation [33]. However, AT is highly charged, and is unlikely to enter muscle cells, so it would have to influence intracellular Ca^{2+} from the outside. One possible mechanism could involve chelation of extracellular Zn^{2+} [18], since extracellular Zn^{2+} has been shown to trigger Ca^{2+} release from thapsigargin-sensitive intracellular pools, through formation of inositol 1,4,5-triphosphate, by binding to a G-protein coupled receptor [19]. Further experiments are required to detail the pathway and test this hypothesis.

Restoration of protein synthesis in skeletal muscle by AT would result from its ability to attenuate the increased phosphorylation of eIF2 on the α-subunit, which inhibits the first step of translation initiation, by preventing binding of Met tRNA to the 40S ribosomal subunit [29]. In addition protein synthesis would be increased by AT through an increase in the eIF4F triad of translation initiation factors (eIF4A, eIF4G, eIF4E), which recruits the 40S ribosomal subunit to mRNA through the 5′cap recognition structure (m^7GpppX). eIF4E is present in low molar amounts in the cell, and its concentration is regulated by its association with its binding protein (4E-BP1). Hypophosphorylation of 4E-BP1, as found in the gastrocnemius muscle of weight-losing mice bearing the MAC16 tumour, would block formation of the eIF4F complex, because it competes with eIF4G for binding eIF4E [29]. Treatment with AT increased phosphorylation of 4E-BP1, releasing eIF4E to form the active eIF4F complex. mTOR regulates phosphorylation of 4E-BP1 at multiple sites [29], and is likely to be responsible for the changes in phosphorylation seen in gastrocnemius muscle of animals treated with AT, since the low levels of phosphorylation seen in mice bearing the MAC16 tumour were restored up to control values after treatment with AT. Amino acid starvation, especially of leucine, causes a rapid impairment of mTOR signaling [22], and this may be responsible for the low levels of mTOR phosphorylation seen in the cachectic state because of the low plasma levels of BCAA [1]. The tuberous sclerosis complex gene products TSC1 and TSC2 also act as negative regulators of mTOR, and this in turn is regulated by the energy status of the cell through AMP-activated protein kinase (AMPK) [29]. The Akt/mTOR pathway has been shown to be upregulated during muscle hypertrophy and

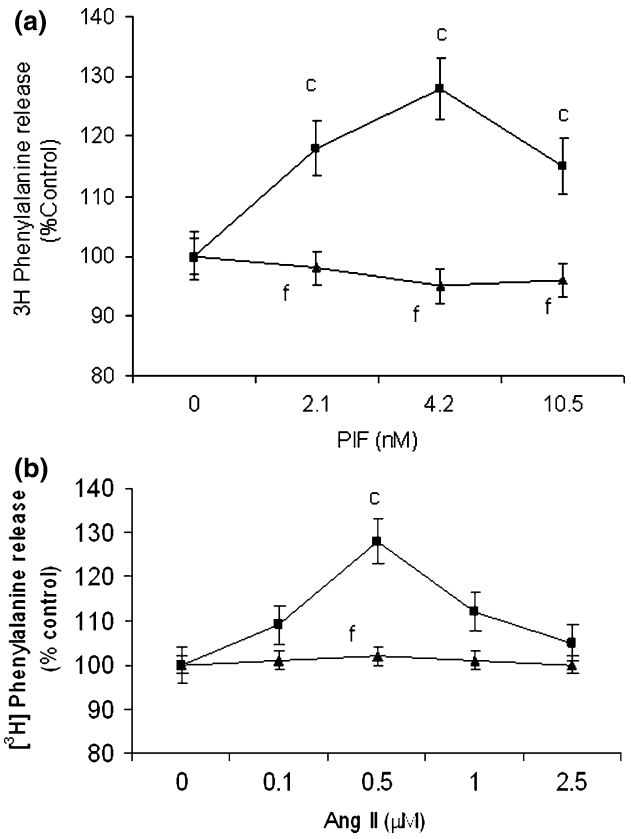

Fig. 7 Effect of PIF (**a**) and Ang II (**b**) on total protein degradation in murine myotubes over 24 h in the absence (*filled square*) or presence (*filled triangle*) of AT (100 μM). AT was dissolved in PBS and added to the myotubes 2 h prior to PIF or Ang II. Differences from control are shown as **c**, $P < 0.001$, while differences in the presence of AT are shown as **f**, $P < 0.001$

downregulated during atrophy, and together with downstream targets such as 4E-BP1 is involved in regulating the size of skeletal muscle [4]. Further studies are required on the mechanism of activation of mTOR in skeletal muscle by AT.

Skeletal muscle of mice bearing the MAC16 tumour also showed an increased phosphorylation of the elongation factor eEF2, which would decrease global protein synthesis by decreasing its affinity for the ribosome [7]. Treatment with AT restored phosphorylation levels of eEF2 up to that seen in non tumour-bearing animals, and this would also increase protein synthesis. eEF2 kinase is dependent on both Ca^{2+} and calmodulin for activity [20], and at resting levels of Ca^{2+} phosphorylation of eEF2 kinase at Ser^{500} leads to kinase activation leading to increased phosphorylation and inactivation of eEF2 [7]. If AT affects intracellular Ca^{2+} levels then it would be expected to lead to hypophosphorylation of eEF2.

The results of this study demonstrate the potential usefulness of AT in the treatment of muscle atrophy in cancer patients and suggest that it may also possess antitumour activity. Further studies are required to evaluate its exact mode of action and the role of divalent metal ions, in muscle atrophy.

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