

ATTENUATION OF MUSCLE ATROPHY BY AN N-TERMINAL PEPTIDE OF THE
RECEPTOR FOR PROTEOLYSIS-INDUCING FACTOR (PIF)

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Abstract

Atrophy of skeletal muscle in cancer cachexia has been attributed to a tumour-produced highly glycosylated peptide called proteolysis-inducing factor (PIF). The action of PIF is mediated through a high affinity membrane receptor in muscle. This study investigates the ability of peptides derived from the 20 N-terminal amino acids of the receptor to neutralise PIF action both *in vitro* and *in vivo*. PIF was purified from the MAC16 tumour using an initial pronase digestion, followed by binding onto DEAE cellulose, and the pronase was inactivated by heating to 80°C, before purification of the PIF using affinity chromatography. This process resulted in almost a 23,000-fold purification, but with a recovery of only 0.004%. Both the D- and L-forms of the 20mer peptide attenuated PIF-induced protein degradation in murine myotubes through the ubiquitin-proteasome proteolytic pathway and increased expression of myosin. *In vivo* studies in mice bearing the MAC16 tumour showed that neither the D- or L-peptides significantly attenuated weight loss, although the D-peptide did show a tendency to increase lean body mass. These results suggest that the peptides may be too hydrophilic to be used as therapeutic agents, but confirm the importance of the receptor in the action of the PIF.

Keywords: Proteolysis-inducing factor (PIF); PIF-receptor; muscle protein degradation; cancer cachexia.

Introduction

Half of all cancer patients experience a progressive loss of body mass due to depletion of adipose tissue and skeletal muscle. This process, called cachexia, is estimated to be responsible for at least 20% of cancer deaths through cardiac/respiratory failure (Inagaki et al, 1974) and the patients quality of life is also reduced through immobility. Tissue wasting is progressive and extensive, with loss of 85% of adipose tissue and 75% of skeletal muscle mass, when the weight loss reaches 30%, which is close to death (Fearon, 1992).

In order to provide appropriate therapy it is necessary to understand the mechanism of tissue atrophy in cachexia. A number of factors, including cytokines such as tumour necrosis factor- α (TNF- α) (Oliff et al, 1987) and interleukin-6 (IL-6) (Strassman et al, 1992) have been implicated as playing a role in the development of cachexia. In addition our own studies have shown that those tumours that initiate muscle atrophy produce a novel sulphated glycoprotein of Mr 24,000 called proteolysis-inducing factor (PIF) (Todorov et al, 1996a), which induces muscle wasting, both by depressing protein synthesis and increasing protein degradation (Lorite et al, 1997). Protein degradation induced by PIF is mediated through an upregulation of the expression and activity of the ubiquitin-proteasome proteolytic pathway, which is considered to play the major role in the degradation of skeletal muscle in cachectic cancer patients (Lorite et al, 1998). The central mediator for the depression of protein synthesis and induction of protein degradation induced by PIF is phosphorylation of the RNA-dependent protein kinase (PKR) (Eley and Tisdale, 2007). Autophosphorylation of PKR causes activation, resulting in phosphorylation of its substrate eukaryotic initiation factor 2 (eIF2) on the α -subunit, leading to inhibition of translation

initiation by preventing binding of the initiator methionyl-tRNA to the 40S ribosomal subunit. Activation of PKR also initiates protein degradation through the ubiquitin-proteasome pathway by increasing nuclear binding of the transcription factor nuclear factor- κ B (NF- κ B) (Eley and Tisdale, 2007). The action of PIF is mediated through a Mr 40,000 membrane receptor with high affinity (K_d 2.1 to 21nmol L⁻¹) present on muscle and liver, but not on kidney or adipose tissue (Todorov et al, 2007). Antisera reactive to a 19mer corresponding to the N-terminal amino acids of the PIF receptor was effective in attenuating weight loss in mice bearing the cachexia-inducing MAC16 tumour, through an increase in lean body mass, attributed to an increase in protein synthesis and decrease in degradation in skeletal muscle.

This study investigates the possibility of using the PIF receptor N-terminal 20mer to neutralise PIF action *in vitro*, as well as its ability to neutralise muscle atrophy in mice bearing the MAC16 tumour. Both the D- and L-versions of the peptide were investigated, since the D-form would be resistant to proteolytic cleavage and more suitable for *in vivo* studies. This study also reports a new method for the preparation of PIF from the MAC16 tumour, because of the reported difficulties in the isolation and identification of PIF (Wieland et al, 2007).

Materials and Methods

Materials

Foetal calf serum (FCS), horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Paisley, UK). L-[2,6-³H] Phenylalanine (sp.act.37 Bqmmol⁻¹) was from American Radiolabelled Chemicals (Cardiff, UK). Mouse monoclonal antibodies to 20S proteasome α -subunits and p42 were from Affiniti Research Products (Exeter, UK) and mouse monoclonal antibody to myosin heavy-chain type II was from Novocastra (via Leica Biosystems, Newcastle, UK). Polyclonal rabbit antibody to mouse β -actin was purchased from Sigma Aldrich (Dorset, UK). Peroxidase-conjugated sheep antirabbit and antimouse antibodies were purchased from GE Healthcare (Bucks, UK), as were Hybond A nitrocellulose membranes. Enhanced chemiluminescence (ECL) development kits were from Pierce through Thermo Fischer Scientific (Northumberland, UK). The D- and L- amino acid versions of the N-terminal 20 amino acids of the PIF receptor Asp-Ile-Asn-Gly-Gly-Gly-Ala-Thr-Leu-Pro-Gln-Lys-Leu-Tyr-Leu-Ile-Pro-Asn-Val-Leu were synthesised by Severn Biotech Ltd (Worcs, UK). For the D-amino acid version the amino acids were in the reverse order, so that the Leu became the N-terminal amino acid and the Asp the C-terminal.

Animals

Fragments (1x2mm) of the MAC16 tumour, obtained from donor animals with maximum weight loss, were implanted s.c. into the flank of pure strain male NMRI mice, obtained from our own inbred colony, by means of a trochar, as previously described (bibby et al, 1987), Animals were fed a rat and mouse breeding diet (Special Diet Services, Witham, UK) and water ad libitum, and weight loss was evident 12 to 15 days after transplantation when the

tumour became palpable. When the mice had lost approximately 5% of their starting body weight they were randomized into groups of 6 to receive either D- or L-peptide or solvent (PBS) administered daily by i.v. injection. Both tumour volume and body weight were monitored daily and animals were terminated by cervical dislocation when the body weight loss reached 20%. All animal experiments followed a strict protocol approved by the British Home Office, and the ethical guidelines that were followed met the standards required by the UKCCR guidelines (Workman et al, 1998). After termination by cervical dislocation the body composition was determined as previously described in detail (Russell et al, 2004). Carcasses were heated at 80-90°C for 48h, or until a constant weight was achieved and the water content was determined as the difference between the wet and dry weights. Lipids were extracted with chloroform/methanol (1:1), ethanol/acetone (1:1) and diethyl ether and after evaporation of the solvent 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.

Cell culture

C₂C₁₂ murine myoblasts were maintained in vitro in DMEM supplemented with 10% FCS and glutamine under an atmosphere of 10% CO₂ in air at 37°C. When the myoblasts reached about 80% confluency they were differentiated into myotubes in DMEM containing 2% HS, with medium changes every 2 days. Differentiation was complete in 3-5 days, and the myotubes remained viable for a further 4-5 days.

Measurement of total protein degradation

This was performed as previously described (Whitehouse and Tisdale, 2003). Briefly myotubes were labelled for 24h with L-[2,6-³H] phenylalanine and then washed extensively in PBS followed by a further incubation for 2h to eliminate degradation of short-lived proteins. Protein degradation was determined over a 24h period in the presence of 2mM non-labelled phenylalanine to prevent reincorporation of radioactivity, as a fraction of the total radioactivity incorporated into the myotubes.

Measurement of proteasome activity

The functional activity of the 20S proteasome was determined as the “chymotrypsin-like” enzyme activity, as previously described in detail (Whitehouse and Tisdale, 2003). Activity was determined by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC in the absence and presence of the specific proteasome inhibitor lactacystin (10 μ M). Only lactacystin-suppressible activity was considered to be proteasome-specific. The activity was normalised to the protein content which was determined using a standard colourimetric protein assay (Sigma).

Purification of PIF

Solid MAC16 tumours (5g) were homogenised in 25ml 10mM Tris-HCl, pH 8.0, on ice, and incubated with pronase (1mgml⁻¹) overnight at 37°C. The soup was centrifuged at 4,000g for 15min to remove the pellet, and the supernatant was concentrated to a volume of 1ml against PBS using an Amicon Ultra-15 centrifugal filter with a molecular weight cut-off of 1000. DEAE cellulose (2g), previously washed with 100mM Tris-HCl, pH 8.8 for 30min, and 10mM Tris-HCl, pH 8.8 for another 30min, was resuspended in 20ml 10mM Tris-HCl, pH8.8,

and was incubated with the protein concentrate for 2h at room temperature. The PIF bound to the DEAE cellulose, which was sedimented by centrifugation at 4,000g for 15min, and the PIF was eluted with 0.3M NaCl in 20ml 10mM Tris pH8.8, by incubation for 30min. The DEAE cellulose was removed by centrifugation at 4,000g for 15min, and the supernatant was concentrated to 1ml against PBS using an Amicon Ultra-15 centrifugal filter with a molecular weight cut-off of 10,000. Proteolysis assays suggested that the concentrate still contained pronase. To inactivate this the sample was heated to 80°C for 15min. Two methods were investigated for the isolation of PIF: 1) Wheat germ agglutinin agarose, based on the observation (Todorov et al, 1996b) that this lectin will bind the oligosaccharide chains of PIF. The sample was loaded onto the column and washed with 10mM Tris-HCl, pH 7.4 and eluted with 0.1mM N-acetylglucosamine. 2) Affinity chromatography using anti-PIF monoclonal antibody coupled to protein A on an Affi-Gel column. The retained proteins were eluted with 100mM glycine-HCl, pH 2.5, as described (Todorov et al, 1996b).

Western blotting

Samples of cytosolic protein (5-15µg), formed by centrifugation of cellular homogenates from the 'chymotrypsin-like' enzyme activity at 18,000g for 10min at 4°C, or from steps in the purification of PIF were resolved on 12% SDS-PAGE at 180V for approximately 1h, followed by transference to 0.45µm nitrocellulose membranes. These were blocked with 5% Marvel in Tris-buffered saline, pH7.5, for 1-2h at room temperature (20S, p42) or at 4°C overnight. Both primary and secondary antibodies were used at a dilution of 1:1000 except for myosin (1:40). Incubation was for 2h at room temperature and development was by ECL. Blots were scanned by a densitometer to quantify differences.

Statistical analysis

Data are presented as means \pm SE for at least 3 replicate experiments. Differences in means between groups were determined by one-way ANOVA followed by Tukey-Kramer multiple comparison test. p values <0.05 were considered significant.

Results

A new purification scheme has been devised for PIF, the steps and yields of which are given in Table 1. Initially the MAC16 tumour homogenate was subjected to pronase digestion, because PIF, unlike the majority of tumour proteins, is not affected by pronase, being predominantly carbohydrate in composition (Todorov et al, 1996a). PIF can be recovered from the digest by binding to DEAE cellulose, (Fig. 1A) since the molecule has a high overall negative charge, but enzyme assays showed co-purification with the pronase, which would preclude further purification by affinity chromatography. To overcome this problem the pronase was inactivated by heating at 80°C for 15min, after which time Western blotting revealed that the PIF was not destroyed by this treatment (Fig. 1A). This allowed a final purification of PIF by affinity chromatography using an anti-PIF monoclonal antibody (Fig. 1B) giving an overall purification of 22,936 -fold (Table 1). Alternatively the PIF can be purified by lectin affinity chromatography using wheat germ agglutinin agarose and elution with N-acetylglucosamine (Fig. 1). However, the capacity of this type of affinity chromatography was less than with antibody and some of the PIF came through with the column washings (Fig. 1)

To determine the ability of the N-terminal 20mer of the PIF receptor to attenuate protein degradation induced by PIF, experiments were conducted in murine myotubes as a surrogate model of skeletal muscle. A dose-response curve for total protein degradation induced by PIF in murine myotubes in the absence and presence of the L-peptide is shown in Fig. 2A. As in previous studies (Whitehouse and Tisdale, 2003) optimal protein degradation by PIF was achieved at a concentration of 4.2nM, and this concentration was used for all further in vitro studies. Protein degradation by PIF was completely attenuated by the L-

peptide at all concentrations of PIF (Fig. 2A). The D-peptide also attenuated total protein degradation in myotubes, as did the anti-PIF antibody (Fig. 2B). To determine if the effect on total protein degradation was due to attenuation of the ubiquitin-proteasome pathway, functional activity was measured as the 'chymotrypsin-like' enzyme activity. As with total protein degradation PIF increased the 'chymotrypsin-like enzyme activity (Fig. 2C), and this was attenuated by both the D- and L-forms of the receptor N-terminal 20mer. Further support for the involvement of the ubiquitin-proteasome pathway was provided by the attenuation, by both the D- and L-forms of the receptor peptide, of the PIF-induced increase in the expression of the 20S proteasome α -subunits (Fig. 3A), p42, an ATPase subunit of the 19S regulator (Fig. 3B), and the muscle atrophy promoting E3 ligases MuRF1 (Fig. 3C) and MAFbx/atrogen-1 (Fig. 3D). Myotubes treated with either the D- or L-forms of the peptide also showed an increase in expression of the myofibrillar protein myosin (Fig. 3E). These results suggest that the PIF receptor N-terminal peptides may be effective in the attenuation of muscle wasting in cachexia.

To determine if this was correct mice bearing the MAC16 tumour were treated daily with either the L- or D- peptide at dose levels up to 100 μ g per mouse by iv injection. The L-peptide was completely ineffective in the attenuation of the loss of body weight, while the D-peptide treated animals showed a reduced rate of weight loss (Fig. 4), but this did not reach statistical significance. There was also a tendency to increase lean body mass (D-peptide 22.1 ± 1.5 % versus PBS 17.4 ± 1.9 %; $p=0.09$), but again this did not reach statistical significance. These results suggest that the peptides may be too hydrophilic to be used as therapeutic agents.

Discussion

PIF is a sulphated glycoprotein with a short central polypeptide chain, which is derived from dermicidin (Schitteck et al, 2001), to which are attached sulphated oligosaccharide residues through both N- and O-glycosidic linkages (Todorov et al, 1997). PIF is strongly catabolic to skeletal muscle by depressing protein synthesis and increasing protein degradation through the ubiquitin-proteasome proteolytic pathway (Eley and Tisdale, 2007). Its presence in urine (Cariuk et al, 1997; Williams et al, 2004) and tumours (Cabal-Manzano et al, 2001) is strongly correlated with the development of cachexia in cancer patients. However, a study in patients with metastatic gastric/oesophageal cancer based on Western blotting of urine extracts (Wieland et al, 2007) has questioned the role of PIF in weight loss. It is questionable whether these investigators were actually measuring PIF, since the material was relatively abundant, and previous studies (Cariuk et al, 1997) had shown PIF to represent just 5×10^{-4} % of the total urinary proteins. The material was later identified as immunoglobulin light chain, which cross-reacted with the anti-PIF antibody. This antibody has been shown to be reactive to the carbohydrate chains of PIF (Todorov et al, 1997), and may cross-react with other oligosaccharides having a similar antigenic determinant. However, a more recent study (Wang et al, 2010) in patients with non-small-cell lung cancer (NSCLC) using immunohistochemical staining with the same antibody identified PIF in cancer, but not normal tissue. The positive rate of PIF expression (90%) was significantly higher in patients with weight loss, than those without, and PIF expression was negatively related to survival in medium to advanced stages of disease (II-IV)

To overcome problems with western blotting of PIF this study reports a new method for the purification of PIF from the MAC16 tumour, which could also be adopted to Western

blotting of urine extracts. This method is simple and overcomes the problems associated with the purification of a molecule which is only present in tumours at 1 part per 10^7 of the total tumour protein (Todorov et al, 1996a). It utilises the unique characteristics of PIF as a sulphated glycoprotein, which is resistant to pronase and heat denaturation (Todorov et al, 1996a). This enables the major tumour proteins to be digested giving a 22,936 -fold purification after extraction of PIF by DEAE cellulose (Table 1). It is likely that the oligosaccharide chains in PIF bind around the core peptide protecting it from both heat and proteolytic degradation. A final purification can be achieved by affinity chromatography using either *Triticum vulgare* (wheat germ agglutinin), which has been shown by lectin blotting studies to show a strong reaction with PIF (Todorov et al, 1996b), or anti-PIF monoclonal antibody, previously employed in purification studies (Todorov et al, 1996b). The PIF produced by this scheme was biologically active and induced protein degradation in murine myotubes at the same concentration as that previously employed (Whitehouse and Tisdale, 2003).

Previous studies (Todorov et al, 2007) have shown that antisera raised against the N-terminal portion of the PIF receptor effectively attenuate protein degradation induced by PIF in vitro and protected atrophy of skeletal muscle in mice bearing the cachexia-inducing MAC16 tumour. This occurred through binding of antibody to the receptor, which would sterically exclude PIF from binding. This study reports a similar phenomenon with both the D- and L-forms of the N-terminal 20mer of the PIF receptor, which therefore must be the site to which PIF binds. However, studies in mice bearing the MAC16 tumour show that the peptides were less effective at blocking weight loss than the antibody, possibility due to

pharmacokinetic differences. Despite this the ability of the peptides to block PIF action in vitro confirms the importance of the receptor in the biological effects of PIF.

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References

Bibby MC, Double JA, Ali SA, Fearon KCH, Brennan RA, Tisdale MJ (1987) Characterization of a transplantable adenocarcinoma of the mouse colon producing cachexia in recipient animals. *J Natl Cancer Inst* **78**: 539-546

Cabal-Manzano R, Bhargava P, Torres-Duarte A, Marshall J, Bhargava P, Wainer IW (2001) Proteolysis-inducing factor is expressed in tumours of patients with gastrointestinal cancers and correlates with weight loss. *Br J Cancer* **84**: 1599-1601

Cariuk P, Lorite MJ, Todorov PT, Field WN, Wigmore SJ, Tisdale MJ (1997) Induction of cachexia in mice by a product isolated from the urine of cachectic cancer patients. *Br J Cancer* **76**: 606-613

Eley HL, Tisdale MJ (2007) Skeletal muscle atrophy, a link between depression of protein synthesis and increase in degradation. *J Biol Chem* **282**: 7087-7097

Fearon KCH (1992) The mechanisms and treatment of weight loss in cancer. *Proc Nutr Sci* **51**: 251-265

Inagaki J, Rodriguez V, Bodey GP (1974) Causes of death in cancer patients. *Cancer* **33**: 568-571

Lorite MJ, Thompson MG, Drake JL, Carling G, Tisdale MJ (1998) Mechanism of muscle protein degradation induced by a cancer cachectic factor. *Br J Cancer* **78**: 850-856

Lorite MJ, Cariuk P, Tisdale MJ (1997) Induction of muscle protein degradation by a tumour factor. *Br J Cancer* **76**: 1035-1040

Oliff A, Defco-Jones D, Boyer M, Martinez D, Kiefer D, Vuocolo G, Wolfe A, Socher SH (1987) Tumors secreting human TNF/cachectin induce cachexia in mice. *Cell* **50**: 555-563

Russell ST, Zimmerman TP, Domin BA, Tisdale MJ (2004) Induction of lipolysis in vitro and loss of body fat in vivo by zinc- α_2 -glycoprotein. *Biochem Biophys Acta* **1636**: 59-68

Schittek B, Hipfel R, Sauer B, Bauer J, Kalbacher H, Stevanovic S, Schirle M, Schroeder K, Blin M, Meier F, Rassner G, Garbe C (2001) Dermicidin: a novel human antibiotic peptide secreted by sweat glands. *Immunol Nat* **2**: 1133-1137

Strassman G, Fong M, Kenney JS, Jacob C (1992) Evidence for the involvement of interleukin 6 in experimental cancer cachexia. *J Clin Invest* **89**: 1681-1684

Todorov PT, Wyke SM, Tisdale MJ (2007) Identification and characterization of a membrane receptor for proteolysis-inducing factor on skeletal muscle. *Cancer Res.* **67**: 11419-11427

Todorov PT, Deacon M, Tisdale MJ (1997) Structural analysis of a tumor-produced sulphated glycoprotein capable of initiating muscle protein degradation. *J Biol Chem* **272**: 12279-12288

Todorov PT, Cariuk P, McDevitt T, Coles B, Fearon K, Tisdale M (1996a) Characterisation of a cancer cachectic factor. *Nature* **379**: 739-742

Todorov PT, McDevitt TM, Cariuk P, Coles B, Deacon M, Tisdale MJ (1996b) Induction of muscle protein degradation and weight loss by a tumour product. *Cancer Res* **56**: 1256-1261

Wang Q, Hu J-B, Wu B, Hao L-Y (2010) Expression and clinicopathologic significance of proteolysis-inducing factor in non-small-cell lung cancer: an immunohistochemical analysis.

Clin Lung Cancer **11**: 346-351

Whitehouse AS, Tisdale MJ (2003) Increased expression of the ubiquitin-proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF- κ B. Br J Cancer **89**: 1116-1122

Wieland BM, Stewart GD, Skipworth RJE, Sangster K, Fearon KCH, Ross JA, Reiman TJ, Easaw J, Mourtzakis M, Kumar V, Pak BJ, Calder K, Filippatos G, Kremastinos DT, Palcic M, Baracos VE (2007) Is there a human homologue to the murine proteolysis-inducing factor? Clin Cancer Res **13**: 4984-4992

Williams ML, Torres-Duarte A, Brant LJ, Bhargava P, Marshall J, Wainer IW (2004) The relationship between a urinary cachectic factor and weight loss in advanced cancer patients. Cancer Investigation **22**: 866-870

Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embelton J, Newell D, Raymond R, Stables J, Stephens T, Wallace J (1998) United Kingdom Co-ordinating Committee on Cancer Research (UKCCR). Guidelines for the welfare of animals with experimental neoplasia (second edition). Br J Cancer **77**: 1-10

Table 1 Purification of PIF from solid MAC16 tumours

Stage	Protein (mg)	Recovery (%)	Purification (fold)
Tumour homogenate	2,500	-	-
Pronase digest	564	22.56	4.4
DEAE cellulose	2.1	0.08	1,190
Heat to 80°C	2.0	0.08	1,190
Affinity chromatography (antibody)	0.109	0.004	22,936

Figure 1. Purification of PIF from the MAC16 tumour (A) The tumour homogenate (lane 1) was incubated with pronase (1 mg ml^{-1}) overnight at 37°C , and the PIF was concentrated by binding to DEAE cellulose (lane 2). Pronase was inactivated by heating to 80°C for 15 min (lane 3). The PIF was purified by affinity chromatography using either anti-PIF antibody (B) or WGA (C). In (C) lane 1 shows the tumour extract after heating to 80°C , lane 2 shows material not adherent to the WGA column while lane 3 shows retained material eluted with 10 mM tris-HCl , $\text{pH } 7.4$ containing $0.1 \text{ M N-acetylglucosamine}$ as for purification of the PIF receptor (Todorov et al, 2007). The figures are western blots using the anti-PIF monoclonal antibody (Todorov et al. 1996, b)

Figure 2. Effect of receptor peptides on protein degradation by PIF in C_2C_{12} myotubes (A) Total protein degradation after 24h in response to PIF alone (■) or in the presence of the L-receptor peptide (□) ($10\mu\text{M}$) added 2h prior to the addition of PIF. (B) Comparison of D- and L- peptide (both $10 \mu\text{M}$) and anti-PIF antibody ($10 \mu\text{g ml}^{-1}$) on protein degradation induced by PIF (4.2 nM) over a 24h period. (C) Effect of the D- and L-peptide (both $10\mu\text{M}$) on the proteasome 'chymotrypsin like' enzyme activity in response to PIF (4.2 nM) after 24h incubation. The experiment was repeated three times. Differences from control are indicated as either b, $p < 0.01$ or c, $p < 0.001$, while differences between groups are indicated as d, $p < 0.05$, e, $p < 0.01$ or f, $p < 0.001$.

Figure 3. Effect of PIF-receptor peptides on expression of components of the ubiquitin-proteasome pathway in the presence of PIF (4.2 nM). Western blotting showing expression of the 20S proteasome α -subunits (A), p42, a component of the 19S regulator (B) and the E3 ligases MURF1 (C) and MAFbx (D) in murine myotubes 24h after addition of PIF in the absence or presence of the L- and D- peptides (both $10 \mu\text{M}$). The expression of the

myofibrillar protein myosin is shown in (E). Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with L-receptor peptide (10 μ M) 2h prior to the addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 η M PIF (lanes 5 and 10). The densitometric analysis is based on three separate blots, and values in the presence of PIF are indicated as (■), whilst for myotubes co-incubated in the presence of the receptor peptide they are shown as (□). Differences from controls are shown as c, $p < 0.001$, while differences between groups are shown as f, $p < 0.001$.

Figure 4. Rate of weight loss in NMRI mice transplanted with the MAC16 tumour and administered either PBS (100 μ l) (□) or D-peptide (100 μ g in 100 μ l PBS) (■) daily by i.v. injection. There was no difference in food and water intake between the two groups, and no statistically significant change in the rate of weight loss.