Attenuation of muscle atrophy by an N-terminal peptide of the receptor for proteolysis-inducing factor (PIF)

KA Mirza¹, SM Wyke¹ and MJ Tisdale*,¹
¹Nutritional Biomedicine, School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

BACKGROUND: 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.

METHODS: Proteolysis-inducing factor was purified from the MAC16 tumour using an initial pronase digestion, followed by binding on DEAE cellulose, and the pronase was inactivated by heating to 80°C, before purification of the PIF using affinity chromatography. In vitro studies were carried out using C2C12 murine myotubes, while in vivo studies employed mice bearing the cachexia-inducing MAC16 tumour.

RESULTS: The process resulted in almost a 23 000-fold purification of PIF, but with a recovery of only 0.004%. Both the D- and L-forms of the 20mer peptide attenuated PIF-induced protein degradation in vitro through the ubiquitin-proteolytic pathway and increased expression of myosin. In vivo studies showed that neither the D- nor the L-peptides significantly attenuated weight loss, although the D-peptide did show a tendency to increase lean body mass.

CONCLUSION: 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 on muscle protein degradation.

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

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).

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 (Ollif et al, 1987) and interleukin-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 by 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 have 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 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-κB (Eley and Tisdale, 2007). The action of PIF is mediated through an Mr 40 000 membrane receptor with high affinity (Kd 2.1–21 nmol l⁻¹) present on muscle and liver, but not on kidney or adipose tissue (Todorov et al, 2007). Antibodies 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, as 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

Fetal 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 Bq/nmol -1) was from American Radiolabelled Chemicals (Cardiff, UK). Mouse monoclonal antibodies to 20S proteasome z-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 antimuscle 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 (1 x 2 mm) 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 (Ribby 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 12–15 days after transplantation when the tumour became palpable. When the mice had lost ~5% of their starting body weight they were randomised into groups of six 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 48 h, 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 glucose 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 24 h with L-[2,6-3H] phenylalanine and then washed extensively in PBS followed by a further incubation for 2 h to eliminate degradation of short-lived proteins. Protein degradation was determined over a 24-h period in the presence of 2 mM 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 μM). 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, Dorset, UK).

Purification of PIF

Solid MAC16 tumours (5 g) were homogenised in 25 ml 10 mM Tris-HCl, pH 8.0, on ice, and incubated with pronase (1 mg ml⁻¹) overnight at 37°C. The soup was centrifuged at 4000 g for 15 min to remove the pellet, and the supernatant was concentrated to a volume of 1 ml against PBS using an Amicon Ultra-15 centrifugal filter (Millipore, Cork, Rep of Ireland) with a molecular weight cutoff of 1000. DEAE cellulose (2 g), previously washed with 100 mM Tris-HCl, pH 8.8 for 30 min and 10 mM Tris-HCl, pH 8.8 for another 30 min, was resuspended in 20 ml 10 mM Tris-HCl, pH 8.8, and was incubated with the protein concentrate for 2 h at room temperature. The PIF bound to the DEAE cellulose, which was sedimented by centrifugation at 4000 g for 15 min, and the PIF was eluted with 0.3 M NaCl in 20 ml 10 mM Tris pH 8.8, by incubation for 30 min. The DEAE cellulose was removed by centrifugation at 4000 g for 15 min, and the supernatant was concentrated to 1 ml against PBS using an Amicon Ultra-15 centrifugal filter with a molecular weight cutoff of 10 000. Proteolysis assays suggested that the concentrate still contained pronase. To inactivate this, the sample was heated to 80°C for 15 min. 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 10 mM Tris-HCl, pH 7.4 and eluted with 0.1 mM N-acetylglucosamine. (2) Affinity chromatography using anti-PIF monoclonal antibody coupled with protein A on an Affi-Gel column. The retained proteins were eluted with 100 mM glycine-HCl, pH 2.5, as described (Todorov et al., 1996b).

Western blotting

Samples of cytosolic protein (5–15 μg), formed by centrifugation of cellular homogenates from the ‘chymotrypsin-like’ enzyme activity at 18 000 g for 10 min at 4°C, or from steps in the purification of PIF, were resolved on 12% SDS–PAGE at 180 V for ~1 h, followed by transference onto 0.45 μm nitrocellulose membranes. These were blocked with 5% Marvel in Tris-buffered saline, pH 7.5, for 1–2 h at room temperature (208, 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 2 h at room temperature and development was by ECL. Blots were scanned by a densitometer to quantify differences.

Statistical analysis

Data are presented as mean values ± s.e. for at least three replicate experiments. Differences in mean values 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). Proteolysis-inducing factor can be recovered from the digest by binding to DEAE cellulose (Figure 1A), as 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 15 min, after which time western blotting revealed that the PIF was not destroyed by this treatment (Figure 1A). This allowed a final purification of PIF by affinity chromatography using an anti-PIF monoclonal antibody (Todorov et al., 1996b).

Table 1  Purification of PIF from solid MAC16 tumours

| Stage                      | Protein (mg) | Recovery (%) | Purification (fold) |
|----------------------------|--------------|--------------|---------------------|
| Tumour homogenate          | 2500         | —            | —                   |
| Pronase digest             | 564          | 22.56        | 4.4                 |
| DEAE cellulose             | 2.1          | 0.08         | 1190                |
| Heat to 80°C               | 2.0          | 0.08         | 1190                |
| Affinity chromatography (antibody) | 0.109      | 0.004        | 22936               |

Abbreviations: DEAE = diethylaminoethylcellulose; PIF = proteolysis-inducing factor.

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, pH 7.4 containing 0.1 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., 1996b).

Figure 2  Effect of receptor peptides on protein degradation by PIF in C2C12 myotubes. (A) Total protein degradation after 24 h in response to PIF alone (B) or in the presence of the L-receptor peptide (C) (10 μM) added 2 h before the addition of PIF. (B) Comparison of D- and L-peptides (both 10 μM) and anti-PIF antibody (10 μg ml⁻¹) on protein degradation induced by PIF (4.2 nM) over a 24-h period. (C) Effect of the D- and L-peptides (both 10 μM) on the proteasome ‘chymotrypsin-like’ enzyme activity in response to PIF (4.2 nM) after 24 h incubation. The experiment was repeated three times. Differences from control are indicated as a, P < 0.05; 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.
and presence of the L-peptide is shown in Figure 2A. There was not a significant difference in protein degradation at concentrations of PIF between 2.1 and 10.5 nM, and the bell-shaped dose–response was less pronounced than in previous studies (Whitehouse and Tisdale, 2003). However, PIF was used for all further in vitro studies at a concentration of 4.2 nM to maintain continuity with other investigations. Protein degradation by PIF was completely attenuated by the L-peptide at all concentrations of PIF (Figure 2A). The D-peptide also attenuated total protein degradation in myotubes, as did the anti-PIF antibody (Figure 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 (Figure 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 a-subunits (Figure 3A), p42, an ATPase.

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 a-subunits (A), p42, a component of the 19S regulator (B), the E3 ligases MuRF1 (C), MAFbx (D) in murine myotubes 24 h after addition of PIF in the absence or presence of the L- and D-peptides (both 10 μM). The expression of the myofibrillar protein myosin with actin as loading control is shown in (E). The densitometric analysis is based on three separate blots, and values in the presence of PIF are indicated as (■), while for myotubes co-incubated in the presence of the receptor peptide are shown as (□). Differences from controls are shown as c, P<0.001, while differences from PIF alone are shown as e, P<0.001 or f, P<0.001.
while the D-peptide-treated animals showed a reduced rate of tumour growth after 6 days but statistically significant reduction in tumour growth after 6 days. There was no difference in food and water intake between the two groups, and no statistically significant change in the rate of weight loss. Differences from control are shown as a, P<0.05.

subunit of the 19S regulator (Figure 3B), the muscle atrophy promoting E3 ligases MuRF1 (Figure 3C), and MAFbx/atrogen-1 (Figure 3D). Myotubes treated with either the D- or L-forms of the peptide also showed an increase in expression of the myofibrillar protein myosin (Figure 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 D- or L-peptide at dose levels up to 100 µg per mouse by i.v. 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 (Figure 4A), but this did not reach statistical significance. There was also a tendency to increase lean body mass (D-peptide 22.1 ± 1.5% vs PBS 17.4 ± 1.9%; P = 0.09), but again this did not reach statistical significance. There was a small but statistically significant reduction in tumour growth after 6 days treatment with the D-peptide (Figure 4B), but there was no effect with the L-peptide. These results suggest that the peptides may be too hydrophilic to be used as therapeutic agents.

Figure 4 Rate of weight loss (A) and tumour growth (B) in NMRI mice transplanted with the MAC16 tumour and administered either PBS (100 µl) (C) or D-peptide (100 µg in 100 µl 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. Differences from control are shown as a, P<0.05.

**DISCUSSION**

Proteolysis-inducing factor is a sulphated glycoprotein with a short central polypeptide chain, which is derived from dermicidin (Schittek et al, 2001), to which are attached sulphated oligosaccharide residues through both N- and O-glycosidic linkages (Todorov et al, 1997). It 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 × 10⁻⁵ percent 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 crossreact 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 using immunohistochemical staining with the same antibody identified PIF in cancer, but not in 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 adapted 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 10⁷ 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 vulgaris (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 antibodies possibility due to their hydrophilic nature, which would promote rapid excretion. Despite this the ability of the peptides to block PIF action in vitro confirms that the N-terminal region of the receptor is responsible for binding of PIF initiating the signalling pathway leading to protein degradation through the ubiquitin-proteasome proteolytic pathway.

**ACKNOWLEDGEMENTS**

We thank Mr W Fleary for the tumour transplantation and animal experiments. This work has been supported by a grant from Halsas Pharmaceuticals, TX, USA.
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 cachetic 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, Cariuk P, Tisdale MJ (1997) Induction of muscle protein degradation by a tumour factor. Br J Cancer 76: 1035 – 1040

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

Oliff A, Defo-Jones D, Boyer M, Martinez D, Kiefer D, Vucolo 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-α2-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) Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Immunol Lett 78: 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, Cariuk P, McDevitt T, Coles B, Fearon K, Tisdale M (1996a) Characterisation of a cancer cachectic factor. Nature 379: 739 – 742

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

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-kB. Br J Cancer 89: 1116 – 1122

Wieland BM, Stewart GD, Skipworth RJ, Sangster K, Fearon KCH, Ross JA, Reiman TJ, Easaw J, Mouritzakis 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 Invest 22: 866 – 870

Workman P, Twentman 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