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ORIGINAL ARTICLE

Inhibition of activation of dsRNA-dependent protein kinase and tumour growth inhibition

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Abstract Inhibition of dsRNA-activated protein kinase (PKR), not only attenuates muscle atrophy in a murine model of cancer cachexia (MAC16), but it also inhibits tumour growth. In vitro the PKR inhibitor maximally inhibited growth of MAC16 tumour cells at a concentration of 200 nM, which was also maximally effective in attenuating phosphorylation of PKR and of eukaryotic initiation factor (eIF)2 on the α-subunit. There was no effect on the growth of the MAC13 tumour, which does not induce cachexia, even at concentrations up to 1,000 nM. There was constitutive phosphorylation of PKR and eIF2α in the MAC16, but not in the MAC13 tumour, while levels of total PKR and eIF2α were similar. There was constitutive upregulation of nuclear factor-κB (NF-κB) in the MAC16 tumour only, and this was attenuated by the PKR inhibitor, suggesting that it arose from activation of PKR. In MAC16 alone the PKR inhibitor also attenuated expression of the 20S proteasome. The PKR inhibitor potentiated the cytotoxicity of both 5-fluorouracil and gemcitabine to MAC16 cells in vitro. These results suggest that inhibitors of PKR may be useful therapeutic agents against tumours showing increased expression of PKR and constitutive activation of NF-κB, and may also prove useful in sensitising tumours to standard chemotherapeutic agents.

Keywords 20S proteasome · dsRNA-dependent protein kinase (PKR) · Eukaryotic initiation factor 2α · Nuclear factor-κB · Antitumour action

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Introduction

In cancer patients weight loss is not only an independent predictor of a shorter survival time, but it also decreases response to treatment, as well as predicting toxicity from treatment [34]. Weight loss is due to progressive atrophy of skeletal muscle and adipose tissue induced by cytokines and tumour factors, such as proteolysis-inducing factor (PIF) and lipid mobilising factor (LMF) [39]. Such factors may influence metabolism, not only in the host tissues, but also the primary tumour and metastases. Thus LMF induces expression of uncoupling protein (UCP)2 in tumours, which is thought to be involved in the detoxification of free radicals, and this protects tumour cells from cytotoxic drugs generating free radical damage [36]. Expression of the PIF core peptide, dermicidin, in breast cancer cells promotes cell growth and survival and reduces serum dependency [32]. PIF has been shown to promote muscle atrophy through activation of the transcription factor nuclear factor-κB (NF-κB) by a mechanism involving activation, by autophosphorylation, of the dsRNA-dependent protein kinase PKR [17]. A recent study [16] using a low molecular weight inhibitor of PKR in mice bearing the cachexia-inducing MAC16 tumour showed that it not only attenuated muscle atrophy, but also inhibited tumour growth. This was surprising, since like human tumours which induce cachexia, the MAC16 tumour is highly chemoresistant [15]. This is the first report indicating that inhibition of PKR induced tumour growth inhibition, although studies in mice bearing the B16-F10 melanoma have shown that inhibition of PKR also reduced the number of pulmonary metastatic nodules [1]. Thus studies into the mechanism of this effect may provide an insight into the treatment of chemoresistant tumours.

One possible link between PKR and tumour growth involves activation of NF-κB. Activation of NF-κB has been connected with tumour cell survival and proliferation [23], as well as invasion and angiogenesis, critical events for tumour metastasis [24]. Inhibition of the pulmonary metastatic potential of B16-F10 melanoma cells has been shown to be mediated by increases in the inhibitor protein I-κBβ, which would reduce translocation of NF-κB to the nucleus [1]. NF-κB has been reported to be constitutively activated in a number of tumour types including colorectal carcinoma [27], pancreatic adenocarcinoma [41] and hepatocellular carcinoma [38]. The factors responsible for constitutive activation of NF-κB include tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), pH and hypoxia [4]. It is possible that production of PIF by cachexia-inducing tumours may also lead to constitutive activation of NF-κB, as it does in the skeletal muscle of cachectic animals [43]. Inhibition of NF-κB activation in skeletal muscle by resveratrol also inhibited tumour growth in mice bearing the MAC16 tumour, although the mechanism was not investigated. NF-κB can activate the transcription of genes which suppress apoptosis, through the regulation of caspase activity [24]. Inhibition of apoptosis by NF-κB renders tumours resistant to chemotherapy and radiation [6], and could explain why cachexigenic tumours are so resistant to therapy.

This study compares the effectiveness of a PKR inhibitor on growth of the MAC16 tumour, with that on the MAC13 tumour, which is histologically similar to the MAC16 tumour, but does not induce cachexia, [5], and investigates the mechanism of tumour growth inhibition. The inhibitor is an oxindole/imidazole compound which prevents the autophosphorylation of PKR and rescues the translation blockade induced by PKR [21]. It is specific towards PKR and has no effect on the phosphorylated and total forms of PERK, another eIF2α kinase [19]. It is more potent and specific than 2-aminopurine which has received widespread use as an inhibitor of PKR kinase activity, but can inhibit other kinases at the high concentrations that have to be employed.

Materials and methods

Materials

Foetal calf serum (FCS) and RPMI 1640 tissue culture medium were purchased from Invitrogen (Paisley, Scotland). L-[2,6-^{3}H] Phenylalanine (sp.act 2.00TBqmmol^{-1}), hybond A nitrocellulose membranes and enhanced chemiluminescene (ECL) development kits were from Amersham Biosciences (Bucks, UK). Rabbit monoclonal antibodies to phospho (Thr^{451}) and total PKR were purchased from Calbiochem through New England Biolabs (Herts, UK). Rabbit polyclonal antisera to phospho eIF2α (Ser^{51}) was from Abcam (Cambridge, UK) and to total eIF2α from Santa Cruz Biotechnology (CA, USA) and mouse monoclonal antibody to 20S proteasome α-subunits was purchased from Affiniti Research Products (Exeter, UK). Peroxidase-conjugated goat anti-rabbit antibody was purchased from Dako Ltd. (Cambridge, UK). The PKR Inhibitor (8-[-(1H-imidazol-4-yl)meth-(*Z*)-ylidene]-6,8-dihydro-thiazol [5,4-e]indol-7-one) and PhosphoSafe™ Extraction Reagent were from Merck Eurolab Ltd. (Leics, UK), and electrophoretic mobility shift assay (EMSA) gel shift assay kits were from Panomics (CA, USA). Gemcitabine (Gemzar®) was a gift from Eli Lilly and Co (Basingstoke, UK) and 5-flurouracil was purchased from Sigma Aldridge (Dorset, UK).

Maintenance of tumours

The MAC16 and MAC13 tumours were propagated in vitro in RPMI 1640 medium containing 10% FCS at 37°C, under an atmosphere of 5% CO_2 in air. For cell growth assays, cells were seeded at either 0.5 (MAC13) or 1×10^5 cells per well (MAC16) in 24 well multi-well dishes and allowed to accumulate for 24 h prior to drug addition. The PKR inhibitor was dissolved in DMSO at such a concentration that the final concentration of DMSO in the culture medium was less than 1%. Cell number was determined three days later, whilst the cells were in exponential growth.

Both the MAC16 and MAC13 tumour were passaged in vivo in NMRI mice by transplanting fragments s.c. into the flank as described [7]. To maintain cachexia the MAC16 tumour for passage was selected from donor animals with established weight loss, and treatment was initiated when the average weight loss was 5%. Animals were randomised into groups of 6 to receive solvent (DMSO: PBS; 1:20) or the PKR inhibitor at 1 and 5 mg/kg administered daily by s.c. injection for 4 days as described [16]. Tumour volume was monitored daily. There was no toxicity associated with this dose and route of administration, although a single i.p. injection of 335 μg/kg has been found to be lethal in rats [19]. 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 meet the standards required by the UKCCR guidelines [42].

Measurement of protein synthesis

Protein synthesis in MAC16 and MAC13 cells was determined by the incorporation of L-[2,6-^{3}H] phenylalanine into protein over a 4 h period, as described [17]. The

reaction was terminated by removal of tissue culture medium, and washing three times with ice-cold sterile PBS. The PBS was removed and ice-cold 0.2 M perchloric acid was added, followed by incubation for 20 min at 4°C. Following removal of perchloric acid, 0.3 M NaOH was added, and incubation was continued for 30 min at 4°C, followed by a further incubation for 20 min at 37°C, and then 0.2 M perchloric acid was added and the mixture was left on ice for 20 min. Following centrifugation at 700 g for 5 min at 4°C, the protein-containing pellet was dissolved in 0.3 M NaOH, and the radioactivity determined, while the protein content was determined using a standard colorimetric protein assay (Sigma).

Western blot analysis

Samples (approximately 10 mg) of tumour were homogenised in 500 µl of PhosphoSafe™ Extraction Reagent and centrifuged at 15,000*g* for 15 min at 4°C. Portions of cytosolic protein (10 µg) were resolved on 10% sodium dodecylsulphate polyacrylamide gels (SDS/PAGE) (6% for eIF2α), and were then transferred onto 0.45 µm nitrocellulose membranes, which were blocked with 5% Marvel in Phosphate buffered saline, pH 7.5, for 1 h at room temperature. Membranes were then washed for 15 min in 0.5% Tween buffered saline, prior to adding the primary antibodies. The primary antibodies were used at a dilution of 1:1,000, except for phospho eIF2α (1:500) and incubated with membrane at 4°C overnight. The primary antibodies were washed off the membranes for 15 min, changing the wash every 5 min, using PBS-Tween (0.1%). The secondary antibodies were used at a dilution of 1:1,000, and were washed off after 2 h for a total time of 45 min, changing the wash every 15 min. Development was by ECL, and films were developed for 3–6 min. Blots were scanned by a densitometer to quantify differences.

Electrophoretic mobility shift assay

DNA-binding proteins were isolated from tumour samples by hypotonic lysis followed by high salt extraction of nuclei according to the method of Andrews and Faller [2]. The electrophoretic mobility shift assay (EMSA) was carried out using a Panomics EMSA 'gel shift' kit according to the manufacturer's instructions.

Statistical analysis

Results are presented as mean ± SEM for at least three replicate experiments. Differences in means between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. *P* values less than 0.05 were considered significant.

Results

Previous studies [16] showed a low molecular weight PKR inhibitor, (8-[1-(1H-imidazol-4-yl)meth-(*Z*)-ylidene]-6,8-dihydro-thiazol[5,4-e]indol-7-one), to attenuate the growth of the cachexia-inducing MAC16 tumour in mice. The results presented in Fig. 1 show that it also inhibited the growth of the MAC16 tumour in vitro, with a maximum effect at 200 nM, while it had no effect on the growth of the MAC13 tumour, even at a concentration up to 1,000 nM. Both tumours are adenocarcinomas of the large bowel in mice, induced by prolonged administration of 1,2-dimethylhydrazine [12], but the MAC16 induces cachexia [7], while the MAC13 does not. The results in Fig. 2 show high levels of expression of both phospho PKR (Fig. 2a) and phospho eIF2α (Fig. 2b) in the MAC16 tumour, but not in the MAC13 tumour. However, the total levels of both PKR and eIF2α were similar in the two tumour types. Treatment of mice bearing the MAC16 tumour with the PKR inhibitor caused complete attenuation of the increased phosphorylation of both PKR (Fig. 3a), and eIF2α (Fig. 3b), without an effect on the total levels of PKR and eIF2α. Treatment of MAC16 cells with the PKR inhibitor produced maximum inhibition of cell growth at a concentration of 200 nM with higher concentrations producing less effective inhibition (Fig. 1). To see if this effect correlated with inhibition of autophosphorylation of PKR the effect of the inhibitor on the phospho and total PKR was determined in both MAC16 and MAC13 cells (Fig. 4). As with the solid tumours in mice MAC16 cells showed high levels of phospho PKR, while MAC13 showed very low levels. The PKR inhibitor inhibited autophosphorylation of PKR in MAC16 cells with a maximum effect between 200 and 300 nM, whilst at higher concentrations it was less effective (Fig. 4a). There was no effect of the PKR inhibitor on the low levels of autophosphorylation of PKR in MAC13 cells (Fig. 4b). In neither cell line was there an effect of the inhibitor on the total PKR in the cell. Since PKR activation has been shown to induce expression of the 20S proteasome in skeletal muscle [17] the effect of the inhibitor was determined. Both MAC16 (Fig. 4c) and MAC13 (Fig. 4d) cells expressed the 20S proteasome α-subunits, but the expression was higher in MAC16 than MAC13 cells. Furthermore, the PKR inhibitor attenuated expression of the 20S proteasome α-subunits in MAC16 (Fig. 4c), but not MAC13 cells (Fig. 4d). Moreover there was a linear correlation (correlation coefficient 0.957) between expression of the 20S proteasome α-subunits (Fig. 4c) and expression of PKR (Fig. 4a), with the different concentrations of the PKR inhibitor (Fig. 4e), suggesting that expression of the 20S proteasome may also be controlled by expression of PKR in MAC16 cells.

Protein synthesis in the MAC16 tumour was significantly suppressed compared with the MAC13 tumour

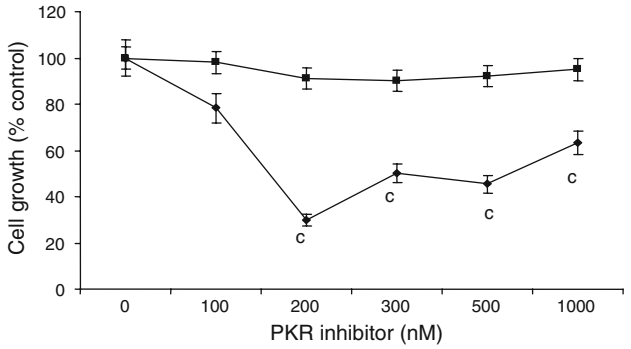

Fig. 1 Effect of increasing concentrations of the PKR inhibitor on growth of the MAC16 (*filled diamond*) and MAC13 (*filled square*) tumours in vitro. The experiment was repeated three times. Differences from control are indicated as *c*, $P < 0.001$

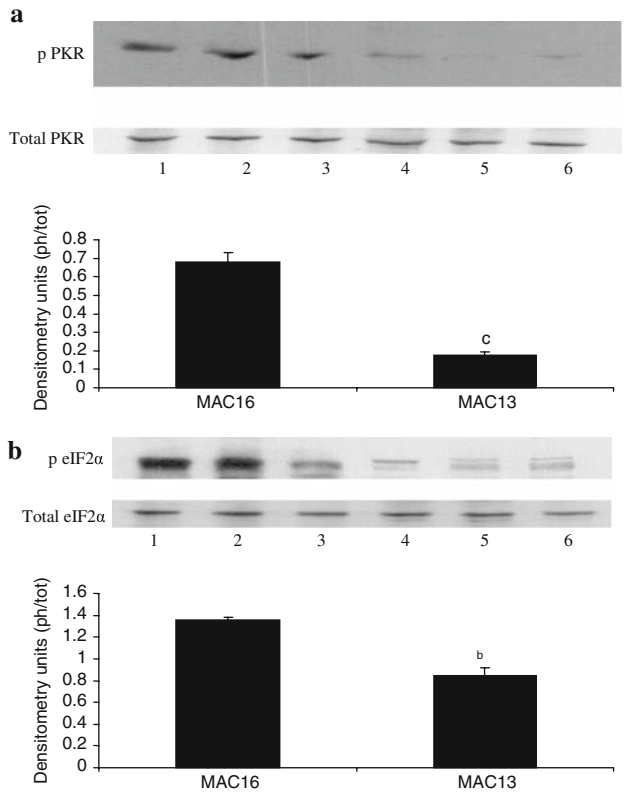

Fig. 2 Western blotting showing expression of phospho and total forms of PKR (**a**) and eIF2α (**b**) in MAC16 (*lanes 1–3*) and MAC13 tumours (*lanes 4–6*). The densitometric analysis shows the ratio of the phosphorylated (*p*) to total forms, and represents the average of three separate Western blots. Differences from the MAC16 tumour are shown as *b*, $P < 0.01$ or *c*, $P < 0.001$

(Fig. 5), possibly due to the increased phosphorylation of eIF2α. This suggests that phosphorylation of PKR may be important for the survival of the MAC16 tumour. One of the functions of PKR is that it is capable of activation of NF-κB [47]. The data in Fig. 6a show high levels of constitutive activation of NF-κB in the MAC16 tumour, but not

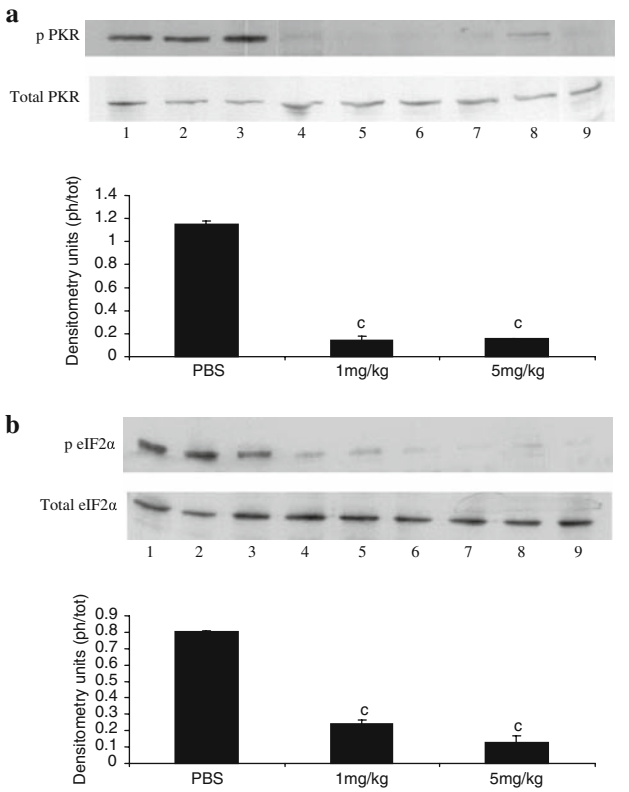

Fig. 3 Effect of treatment of mice bearing the MAC16 tumour with either solvent (DMSO:PBS, 1:20) control (*lanes 1–3*) or the PKR inhibitor at concentrations of 1 (*lanes 4–6*) or 5 (*lanes 7–9*) mg kg^{-1}, administered daily by s.c. injection (16) on phosphorylation of PKR (**a**) and eIF2α (**b**). The number of mice in each group $n = 6$. The densitometric analysis shows the ratio of phosphorylated (*ph*) to total forms, and represents the average of three separate Western blots. Differences from control are shown as *c*, $P < 0.001$

in the MAC13 tumour. Treatment of mice bearing the MAC16 tumour with the PKR inhibitor attenuated constitutive activation of NF-κB in the tumour, suggesting that it arose from activation of PKR.

Activation of NF-κB has been shown to play an important role in the chemoresistance of pancreatic cancer to gemcitabine [3] and stomach cancer to 5-flurouracil (5-FU) [40]. To determine whether downregulation of NF-κB by the PKR inhibitor would increase the sensitivity of MAC16 cells to gemcitabine and 5FU the effect of the agents alone, or in combination with the PKR inhibitor (at 100 or 200 nM) on cell growth was determined (Fig. 7). The 5FU alone produced significant inhibition of growth of MAC16 cells at concentrations between 1 and 10 μM, and this effect was significantly potentiated by the PKR inhibitor at both concentrations. Likewise gemcitabine induced inhibition of growth of MAC16 cells was also potentiated by the PKR inhibitors at both concentrations. These results suggest that PKR inhibitors may prove useful in the chemosensitisation of human tumours to cytotoxic agents.

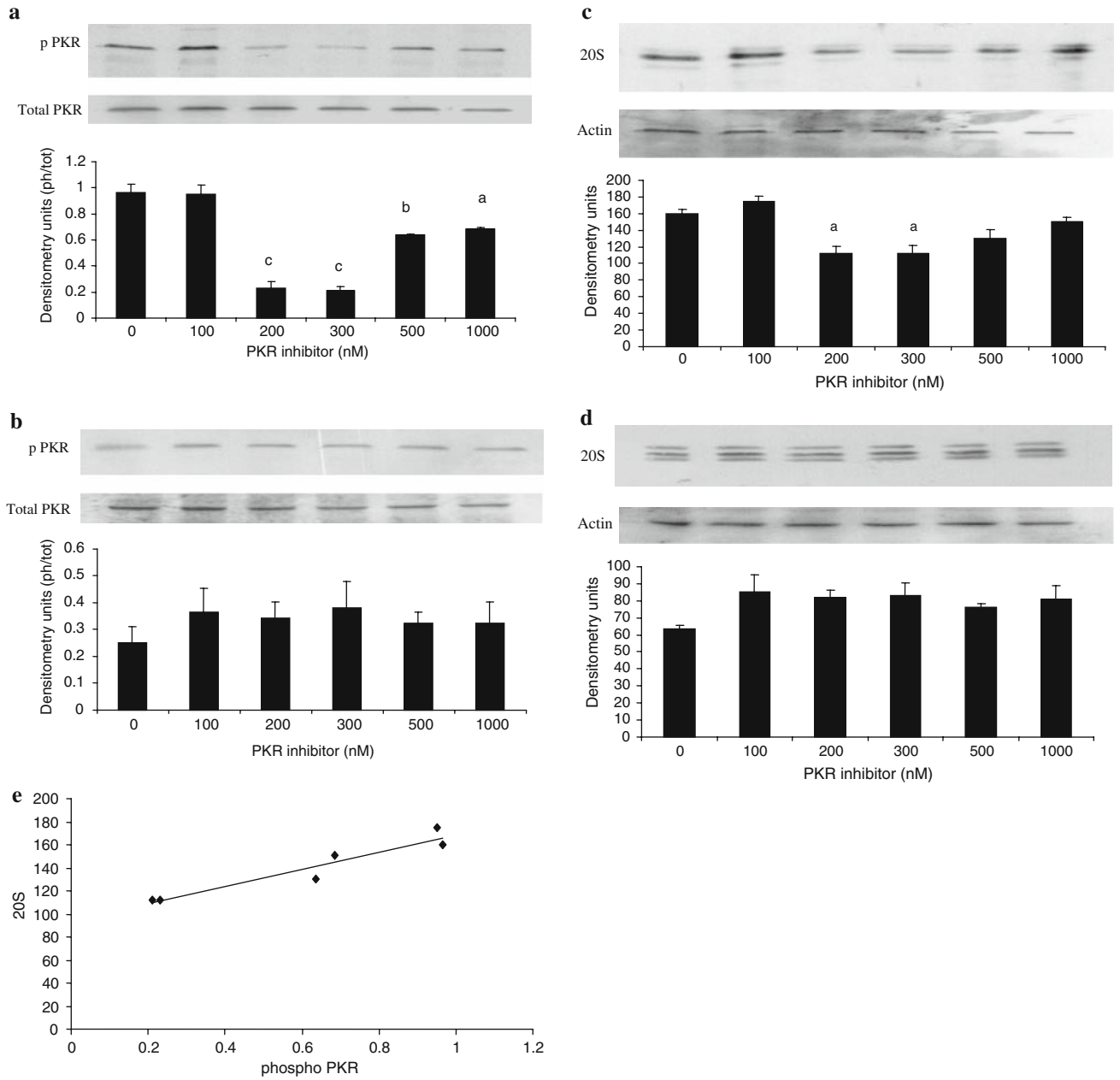

Fig. 4 Effect of concentration of the PKR inhibitor on autophosphorylation of PKR (**a**, **b**) and expression of the 20S proteasome α-subunits (**c**, **d**) in MAC16 (**a**, **c**) and MAC13 (**b**, **d**) cells. The antibody detects three bands at approximate molecular weights of 29, 32 and 35 kDa. The densitometric analysis shows the ratio of phosphorylated (*ph*) to total forms, and represents the average of three separate Western blots. Differences from control are shown as **a**, $P < 0.05$, **b**, $P < 0.01$ or **c**, $P < 0.001$. **e** Relationship between expression of 20S proteasome α-subunits measured densitometrically in MAC16 cells treated with the concentrations of the PKR inhibitor shown in (**c**) and the levels of phosphorylated PKR shown in (**a**). The correlation coefficient is 0.957

Discussion

Early studies suggested that PKR acted as a tumour suppressor, since transfection of 3T3 cells with a catalytically inactive mutant form of PKR led to cellular transformation [28], while upregulation of wild-type PKR activity in M1 myeloid leukaemia cells resulted in reversal of the transformed phenotype or apoptosis [33]. However, other studies [24, 45] cast doubt on this hypothesis. Thus PKR deficient transgenic mice are normal and do not show an increased tumour-incidence [45]. In addition autophosphorylation of PKR and phosphorylation of eIF2α is between 7 and 40-fold higher in lysates from breast carcinoma cell lines than in those from nontransformed epithelial cell lines [25], and is also higher in melanoma cells compared with nontransfected melanocytes in culture [26]. In addition

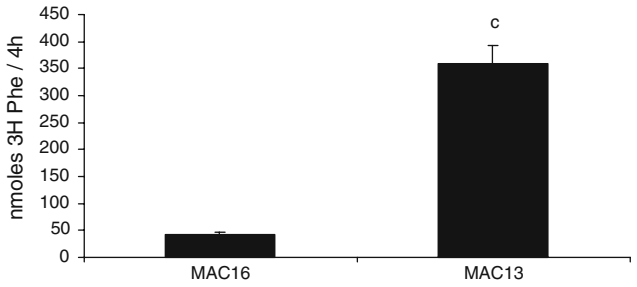

Fig. 5 Protein synthesis in MAC13 and MAC16 cells in vitro over a 4 h period as described in Materials and methods section. Difference from the MAC16 tumour is shown as *c*, $P < 0.001$

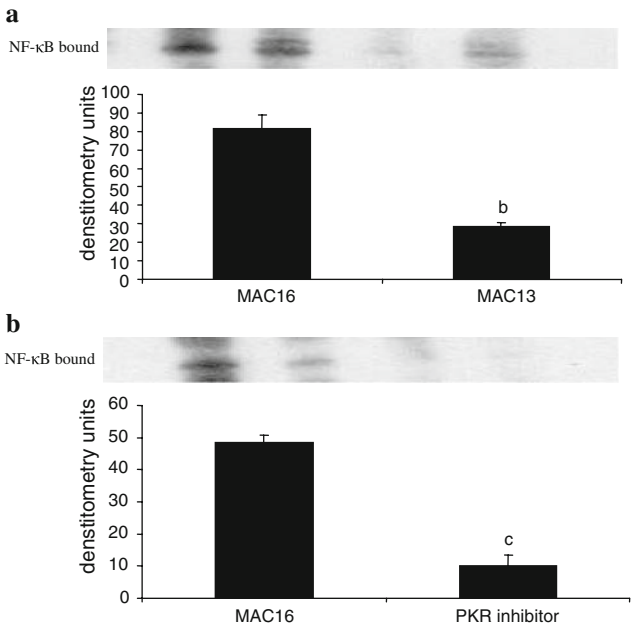

Fig. 6 Nuclear accumulation of NF-κB in MAC16 (*first two lanes*) and MAC13 (*second two lanes*) tumours (**a**) and (**b**) in the MAC16 tumour from mice treated with the PKR inhibitor at 5 mg kg^{-1} for 4 days or solvent control, as determined by EMSA. The densitometric analysis represents the average of three separate blots. Differences from the MAC16 tumour in (**a**) is shown as *b*, $P < 0.01$, while differences from the solvent control in (**b**) is shown as *c*, $P < 0.001$

transformation from normal mucosa to adenomas and carcinomas of the colon was coincident with an increase in PKR expression [26]. The lower PKR activity in nontransformed cell lines was partially due to lower PKR protein levels, and partially due to the presence of P58, a known cellular inhibitor of PKR [25].

The current study shows upregulated expression of autophosphorylated PKR in tumours from mice with cachexia. Activated PKR was associated with an increased nuclear binding of NF-κB, which was attenuated by inhibition of PKR activation. Activation of NF-κB in such tumours would correlate with the clinical data showing that cachexia is a pro-inflammatory state [31]. In the murine tumour pair (MAC16/MAC13), treatment with a low molecular weight PKR inhibitor inhibited the proliferation rate of MAC16, which showed upregulation of phosphorylated PKR, but had no effect on the MAC13 tumour, which did not show activation of PKR. This result suggests that cachexia-inducing tumours, showing activated PKR, may be more susceptible to the antitumour effect of PKR inhibitors. A surprising observation was the PKR inhibitor was maximally effective in inhibiting PKR at a concentration of 200 nM, with increasing concentrations having a reduced inhibitory effect. A similar observation was made in murine myotubes in the presence of PIF [17]. The PKR inhibitor is directed to the ATP-binding site in PKR, and a similar observation has been made with another ATP-binding site directed inhibitor, 2-aminopurine, in a cell-free translational assay [21]. This effect was attributed to non-specific inhibition of other components of the translational machinery. However, it is possible that higher concentrations of the inhibitor bind to PKR initiating a conformational change, which induces autophosphorylation, as it would with ATP [30].

Previous studies [47] have shown that PKR can directly activate NF-κB. PKR physically interacts, through its catalytic domain, with the upstream kinase IKK, which phosphorylates critical serine residues in IκB, leading to its degradation, releasing free NF-κB, which is then able to migrate to its specific binding sites on DNA in the nucleus [11]. Activation of IKK by PKR appears to occur through protein-protein interactions, which stimulate the autophosphorylation of IKKβ, and not by direct phosphorylation [10]. However, phosphorylation of eIF2 on the α-subunit has also been shown to activate NF-κB through a mechanism involving the release, but not the degradation of IκB [22]. This suggests another mechanism by which inhibition of PKR could serve to downregulate activation of NF-κB.

Inhibition of constitutive activation of NF-κB by the PKR inhibitor is likely to be at least partly responsible for the inhibition of tumour growth rate. PKR mediates apoptosis induced by many different stimuli through phosphorylation of eIF2α and activation of NF-κB [18]. PKR also participates in cell cycle arrest at GO/G1 [10] and G2/M phases of the cell cycle [13]. In addition to its ability to modulate the cell cycle the main function of PKR is the induction of stress-activated pathways that in turn mobilize somatic cell death programmes [20]. However, PKR also activates a survival pathway, also mediated by NF-κB, which delays apoptosis [14]. Thus like NF-κB, PKR may promote tumour cell survival or death. In addition to promoting growth NF-κB enhances the angiogenic potential of tumours by increasing the expression of proangiogenic factors, such as vascular endothelial growth factor [44], and NF-κB-regulated gene products promote migration and invasion of cancer cells [46]. Although NF-κB is involved

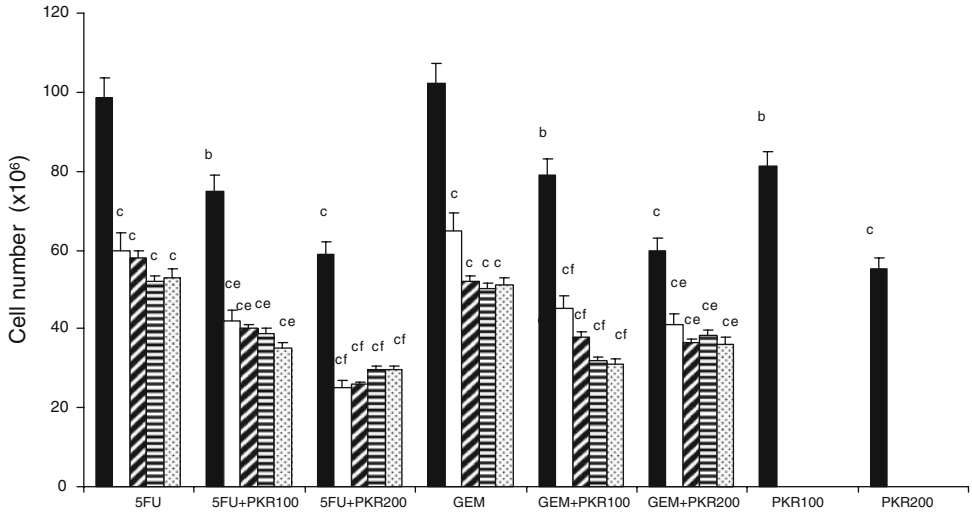

Fig. 7 Effect of 5FU alone at 0 (*filled square*), 1 (*open square*), 2.5 (*diagonal lines in square*), 5 (*horizonal lines in square*) and 10 μM (*dotted square*), or in combination with the PKR inhibitor (PKR) at 100 and 200 nM on growth of MAC16 cells in vitro, and effect of gemcitabine at 0 (*filled square*), 3.8 (*open square*), 9.5 (*diagonal lines in square*), 19 (*horizonal lines in square*) and 38 μM (*dotted square*) alone or in combination with the PKR inhibitor on growth of MAC16 cells. The effect of the PKR inhibitor alone at 100 and 200 nM is also shown. Differences from control are shown as *b*, $P < 0.01$ or *c*, $P < 0.001$, while differences in the presence of the PKR inhibitor are shown as *e*, $P < 0.01$ or *f*, $P < 0.001$

in the control of over 150 target genes, inhibition of its activation through inhibition of the autophosphorylation of PKR did not produce toxicity in mice, suggesting a new therapeutic regime for the treatment of cancer. A recent study [29] has shown that curcumin, an inhibitor of NF-κB activation, inhibits the growth of human pancreatic cancer cell lines in vitro, and potentiates the antitumour activity of gemcitabine in vivo. NF-κB has been shown to play a pivotal role in promoting gemcitabine resistance in pancreatic cancer [3], and in the chemoresistance to 5-FU and gemcitabine in human stomach cancer cell lines [40]. This suggests that inhibitors of PKR may be useful in sensitizing chemoresistant tumours to chemotherapeutic agents. In the current study the PKR inhibitor has been shown to sensitize MAC16 cells to the cytotoxic effect of both 5-FU and gemcitabine, suggesting another potential therapeutic role for such agents.

Activation of PKR may explain the low rate of proliferation of some tumours, which renders them insensitive to chemotherapy and radiation. In addition to activation of NF-κB, PKR also induces phosphorylation of eIF2α, which inhibits translation initiation by competitive inhibition of the guanine nucleotide exchange factor, eIF2B, which converts eIF2.GDP into eIF2.GTP [35]. However, in human breast cancer cells protein synthesis is not inhibited by the high eIF2α phosphorylation, possibly because they contain higher levels of eIF2B [25].

The results of this study show a direct relationship between the levels of phosphorylation of PKR and expression of the 20S proteasome α-subunits in the presence of the PKR inhibitor. This may provide another mechanism for tumour growth inhibition. The 26S proteasome, which is formed by combination of two 19S regulatory subunits with the 20S α-subunits, degrades proteins involved in cell cycle control such as p27 and p21 [8]. Targeted inhibition of the 26S proteasome with the dipeptide boronic acid analogue PS-341 (Velcade) has been shown to block proliferation and induce apoptosis in human pancreatic cancer cells and xenografts [37]. PS-341 has also been shown to sensitise human pancreatic cancer cells to gemcitabine [9]. Thus inhibition of proteasome expression in tumours by inhibitors of PKR autophosphorylation may be responsible for the attenuation of tumour growth and increasing sensitivity to standard chemotherapeutic agents.

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