

Original Article

## Pyrrolidine dithiocarbamate exerts anti-proliferative and pro-apoptotic effects in renal cell carcinoma cell lines

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### Abstract

**Background.** The activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) has been implicated in the development, progression and metastasis of renal cell carcinoma (RCC). This study investigates the effect of pyrrolidine dithiocarbamate (PDTC), a NF- $\kappa$ B inhibitor, on two metastatic human RCC cell lines, ACHN and SN12K1.

**Methods.** RCC cell lines and normal cells were exposed to 25 or 50  $\mu$ M of PDTC. Apoptosis was measured by flow cytometry and TdT-mediated nick end labelling methods. Cell viability and proliferation were measured by MTT and BrdU assays, respectively. Expression of NF- $\kappa$ B subunits, I $\kappa$ Bs, I $\kappa$ B Kinase (IKK) complex and apoptotic regulatory proteins were analysed by western blotting and/or immunofluorescence. DNA-binding activity of NF- $\kappa$ B subunits were measured by ELISA.

**Results.** RCC cell lines had a higher basal level expression of all the five subunits of NF- $\kappa$ B than normal primary cultures of human proximal tubular epithelial cells or HK-2 cells. PDTC decreased the viability and proliferation of RCC, but not normal cells. Of the two RCC cell lines, ACHN had a higher basal level expression of all the five NF- $\kappa$ B subunits than SN12K1 and was more resistant to PDTC. While PDTC induced an overall decrease in expression of all the five NF- $\kappa$ B subunits in both RCC cell lines, unexpectedly, it increased the nuclear expression of NF- $\kappa$ B in ACHN, but not in SN12K1. PDTC reduced the DNA-binding activity of all the NF- $\kappa$ B subunits and the expression of the IKK complex (IKK- $\alpha$ , IKK- $\beta$  and IKK- $\gamma$ ) and the inhibitory units I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$ . PDTC induced a significant increase in

apoptosis in both RCC cell lines. This was associated with a decrease in expression of the anti-apoptotic proteins, Bcl-2 and Bcl-X<sub>L</sub>, without marked changes in the pro-apoptotic protein Bax.

**Conclusion.** These data suggest that PDTC has the potential to be an anticancer agent in some forms of RCC.

**Keywords:** apoptosis; IKK complex; NF- $\kappa$ B; renal cell carcinoma

### Introduction

The treatment of renal cell carcinoma (RCC) has made little progress in the past 30 years and no chemotherapeutic agents currently available are effective against it [1,2]. The biological heterogeneity of RCC, its resistance to anti-cancer drugs and the side effects of chemotherapeutics are the major obstacles in the effective treatment of RCC. Radical nephrectomy of localized RCC is effective only in a few cases because the rate of systemic metastasis is high with nearly 50% of the patients developing metastasis after surgical resection [3,4]. Patients with metastatic RCC have a median survival rate of 10 months and <2% of patients survive beyond 5 years [3]. Therefore, the search for effective therapeutic agents for this malignancy is urgently needed.

Of the many candidate molecules that have been implicated in the development of RCC, the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) has received much attention in recent years. NF- $\kappa$ B is a collective term for transcription factors of the Rel family of DNA-binding proteins that recognize a common sequence motif (5'-GGG(A/G)NN(T/C)(T/C)CC-3', where N is any base) called the  $\kappa$ B site [5–8]. The five known members of the mammalian Rel family are RelA (p65), RelB, c-Rel, NF- $\kappa$ B1 (p105/p50) and

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NF- $\kappa$ B2 (p100/p52). In normal states, NF- $\kappa$ B is sequestered in the cytoplasm in an inactive form bound to one of many inhibitory molecules (I $\kappa$ Bs), such as I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\gamma$ , I $\kappa$ B- $\epsilon$ , p100, p102 and Bcl-3. The I $\kappa$ Bs contain two conserved serine residues in their N-terminal domain. Upon stimulation, phosphorylation of these serine residues by the I $\kappa$ B (IKK) kinase complex leads to polyubiquitination of the I $\kappa$ B proteins. The IKK complex consists of two catalytic units, IKK- $\alpha$  and IKK- $\beta$  and a regulatory unit, IKK- $\gamma$ . IKK- $\gamma$  is a bifunctional protein required for the assembly of the IKK complex and the activation of IKK- $\alpha$  and IKK- $\beta$  [6,8,9]. Phosphorylated and ubiquitinated I $\kappa$ B is degraded by the 26S proteasome, leading to the translocation of the active NF- $\kappa$ B to the nucleus where it binds to  $\kappa$ B elements and regulates transcription of genes mediating inflammation, carcinogenesis and pro- or anti-apoptotic functions [5–8].

The constitutive activation of NF- $\kappa$ B has been implicated in many human solid tumours and haematological malignancies [10]. There are emerging reports that suggest a role for NF- $\kappa$ B in the development of RCC as well [11–13]. Some RCC cell lines have enhanced constitutive activation of NF- $\kappa$ B [11]. In addition, the progression, invasion and metastases of RCC has been shown to correlate with the increased activation of NF- $\kappa$ B [12]. NF- $\kappa$ B up-regulates anti-apoptotic, angiogenic and multi-drug resistance genes, which play key roles in proliferation, invasion and metastasis of cancer [7,12]. The observations that NF- $\kappa$ B is constitutively activated in many cancers have elicited an interest in its inhibition as a potential treatment strategy [7]. To our knowledge, there are no reports on the role of specific NF- $\kappa$ B inhibitors on RCC. However, interferon- $\alpha$  (an immunomodulatory agent), TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), bortezomib (a proteasome inhibitor) and erythropoietin have been shown to induce apoptosis in RCC through the inhibition of NF- $\kappa$ B [11,13–15].

Dithiocarbamates (DTCs) represent a class of antioxidants that mediate a wide variety of effects in biological systems. The diethyl derivatives of DTC have been used in the treatment of metal poisoning, endotoxic shock, diabetic retinopathy and acquired immunodeficiency syndrome [16,17]. In addition to their metal chelating and antioxidant properties, DTCs are effective inhibitors of NF- $\kappa$ B [16]. Of these, the pyrrolidine derivative, pyrrolidine dithiocarbamate (PDTC) is the most potent NF- $\kappa$ B inhibitor [16]. The NF- $\kappa$ B inhibitory property of PDTC is independent of its anti-oxidant behaviour [18]. PDTC has been shown to enhance the cytotoxicity of the chemotherapeutic agent 5-fluorouracil in animal models of colorectal cancer [19,20], although the role of NF- $\kappa$ B was not investigated in these studies. PDTC has also been shown to induce pro-apoptotic and anti-proliferative effects in prostate cancer [21], T-cell leukaemia [22], lymphoma [23], myelogenous leukemia, [24] and gastric cancer cells [25]. PDTC has also been

shown to inhibit IKK- $\alpha$  [26], IKK- $\beta$  [27] and I $\kappa$ B- $\alpha$  [28], in some experimental models. To our knowledge, the effect of PDTC on RCC has not been reported. In the current study, we explore the effect of PDTC on NF- $\kappa$ B, I $\kappa$ B and the IKK complex and the subsequent changes in proliferation and apoptosis of two metastatic human RCC cell lines.

## Subjects and methods

### Antibodies

The following primary and secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA): NF- $\kappa$ B p50 (sc-1191), NF- $\kappa$ B p52 (sc-7386), NF- $\kappa$ B p65 (sc-372), RelB (sc-226), c-Rel (sc-70), I $\kappa$ B- $\alpha$  (sc-847), I $\kappa$ B- $\beta$  (sc-945), IKK- $\alpha$  (sc-7182), IKK- $\beta$  (sc-8014), IKK- $\gamma$  (sc-8032), Bcl-2 (sc-783), Bcl-x<sub>L</sub> (sc-7195), Bax (sc-493), goat anti-rabbit IgG (sc-2004), mouse anti-goat IgG (sc-2354) and bovine anti-mouse IgG (sc-2371). The following fluorescent secondary antibodies were purchased from Molecular Probes (Eugene, Oregon, USA): Alexa fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 rabbit anti-goat IgG and Texas Red-X goat anti-mouse IgG.

### Cell culture

Human metastatic RCC cell lines (ACHN and SN12K1) and HK-2 cells, an immortalized human proximal tubular cell line, were cultured in DMEM/F12 (Gibco, Invitrogen, CA, USA) containing 10% foetal bovine serum (Gibco, Invitrogen, CA, USA) supplemented with penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml) and amphotericin B (0.125  $\mu$ g/ml). Primary cultures of human proximal tubular epithelial cells (PTEC) from the normal pole of human nephrectomy samples were isolated and cultured in serum-free defined medium, which is DMEM/F12 supplemented with epidermal growth factor (10 ng/ml), insulin (10  $\mu$ g/ml), transferrin (5  $\mu$ g/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml) and amphotericin B (0.125  $\mu$ g/ml) as per published methods [29–31]. The collection and culture of PTEC from nephrectomy samples were approved by the ethics committees of the Royal Brisbane and Women's Hospital Foundation and the Queensland Institute of Medical Research, Brisbane, Australia. Informed consent was obtained from patients for the collection and use of cells from the nephrectomy samples.

### Pilot studies

RCC cells were exposed to 25, 50 or 100  $\mu$ M of PDTC (Sigma, Missouri, USA), (dissolved in culture medium) for up to 72 h. Cell viability and cell proliferation were measured (see below) at 24, 48 and 72 h. A dose- and time-dependent decrease in cell viability and proliferation were observed (data not shown). Western blotting of various proteins at 24 h showed that PDTC (100  $\mu$ M) completely inhibited the expression of Bcl-2 in SN12K1 cells (no bands were detected in treatment group; data not shown). Therefore, further studies were carried out using 25 and 50  $\mu$ M of PDTC at 24 h.

### Experimental protocol

RCC or HK-2 cells were seeded in 24-well plates ( $1 \times 10^5$  cells) in DMEM/F12 supplemented with 10% foetal bovine serum. For other culture plates, the cell numbers were adjusted accordingly. All the experiments for RCC and HK-2 cells were carried out in this medium. The cells were treated with 25 or 50  $\mu$ M of PDTC, dissolved in the culture medium after 24 h. PTEC ( $5 \times 10^4$  cells) were seeded in 96-well plates in serum-free defined medium and grown to approximately 80–90% confluence. The experiments for PTEC were carried out in serum-free medium. The cells were analysed for various parameters 24 h after exposure to PDTC.

### Cell viability assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Cell viability was measured using MTT (Sigma, Missouri, USA). In brief, cells were seeded in 96-well culture plates and treated with PDTC. The volume of culture medium was 100  $\mu$ l. Ninety minutes before the end of the experiment, 10  $\mu$ l of MTT solution [5 mg/ml in phosphate buffered saline (PBS)] was added to each well and incubated at 37°C for 90 min. The culture medium was removed and the purple crystals formed were dissolved in 150  $\mu$ l of 0.1 N hydrochloric acid in isopropanol. The absorbance was measured in a microplate reader at 570 nm with a reference wavelength of 690 nm.

### Cell proliferation assay using 5-bromo-2'-deoxyuridine (BrdU)

Cell proliferation was determined using a commercially available kit (Cell Proliferation ELISA, BrdU Calorimetric kit, Roche Diagnostics Corporation, IN, USA), following the instructions of the manufacturer. In brief, cells were seeded in 96-well culture plates and treated with PDTC. The volume of culture medium was 100  $\mu$ l. Ninety minutes before the end of the treatment periods, 10  $\mu$ l of BrdU labelling solution were added to each well and incubated at 37°C. After an incubation period of 90 min, the culture medium was removed and the cells were treated with 200  $\mu$ l of Fixdenat solution for 15 min. The Fixdenat solution was removed and the cells were incubated with 100  $\mu$ l of anti-POD labelling solution at room temperature for 60 min. The anti-POD solution was removed and the cells were washed three times with the BrdU wash solution. The substrate solution (100  $\mu$ l) was added to the cells, and the colour developed was measured in a microplate reader at a wavelength of 370 nm with a reference wavelength of 492 nm.

### Apoptosis measurement using flow cytometry

Apoptosis was measured using a commercially available annexin-V fluores staining kit (Roche Diagnostics, IN, USA). In brief, cells were grown in 24-well plates and exposed to 25 or 50  $\mu$ M of PDTC. After 24 h, non-adherent cells were pelleted and added to trypsinized and pelleted adherent cells. The cells were re-suspended in 100  $\mu$ l of binding buffer containing fluorescein isothiocyanate (FITC) conjugated annexin-V and propidium iodide and

incubated at room temperature for 15 min. After the incubation period, 300  $\mu$ l of binding buffer was added and the cells were analysed in a FACS Calibur (Beckton Dickinson, USA). Ten thousand events were recorded from each treatment group.

### Apoptosis assay using TdT-mediated nick end labelling (TUNEL)

Apoptosis was measured using a commercially available ApopTag Apoptosis Detection Kit (Serological Corporation, Norcross, GA, USA) as per the instructions of the manufacturer. In brief, cells grown on Thermanox cover slips (Nalge Nunc, Rochester, NY, USA) in 24-well plates were exposed to 50  $\mu$ M of PDTC. After 24 h, the cells were washed in PBS and fixed in 1% paraformaldehyde for 15 min at room temperature. After quenching the endogenous peroxidase with 3% hydrogen peroxide, the cells were labelled with the reaction buffer containing TdT-digoxigenin-nucleotide for 1 h at 37°C. The reaction was stopped by adding the stop solution. After washing the cells in PBS, the cells were incubated with anti-digoxigenin peroxidase conjugate at room temperature for 30 min. The cells were washed and incubated with peroxidase substrate and the colour development was monitored under a microscope. The cells were counterstained with light haematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in DePex mounting medium. The cells were viewed under a 40 $\times$  objective and the cells that fell within the 100 squares of an eye graticule, were counted. The number of apoptotic cells was expressed as a percentage of the total cells counted from five random fields for each cover slip.

### Western blotting

The cytoplasmic and nuclear proteins from cells were prepared using a commercially available nuclear extraction kit (Active Motif, Carlsbad, CA, USA). For whole cell lysates, cells were lysed in radio immuno precipitation assay (RIPA) buffer (1 $\times$  PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged for 15 min at Sigma 113 centrifuge, rotor number 12029 at 4°C. The supernatant was collected and the protein content measured using bicinchoninic (BCA) protein assay reagent (Pierce, Rockford, IL, USA). The lysates were aliquoted and stored at  $-80^\circ\text{C}$  until further use. The proteins were resolved in 12% Tris-HCl gel (Biorad, Hercules, CA, USA) and electro-transferred into Hybond-C nitrocellulose membrane (Amersham Biosciences, UK). Equal loading of proteins was further confirmed by staining the membranes with electrophoresis gel stain (Gradipore Ltd, Australia). Standard western blotting procedures were followed, and the proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The difference in intensity of the signals was analysed by Scion Image software (Scion Corporation, MD, USA).

### Immunofluorescent microscopy

The cells were grown in Thermanox cover slips and treated with PDTC. Twenty-four hours later, the cells were washed in PBS and fixed in 4% paraformaldehyde for 15 min at



room temperature. Non-specific binding was blocked by incubating the cells in serum-free protein block (DakoCytomation, CA, USA) at room temperature for 1 h. The antibodies were diluted in antibody diluent (DakoCytomation, CA, USA) and immunofluorescent staining was carried out as per standard procedures. The cells were mounted in vectashield mounting medium with DAPI (Vector laboratories Inc., CA, USA).

#### *DNA binding activity assay for NF- $\kappa$ B Sub units by ELISA*

Nuclear transcription activity of all the five subunits of NF- $\kappa$ B was analysed using a commercially available ELISA-based TransAM NF- $\kappa$ B family assay kit (Active Motif, Carlsbad, CA, USA). Each kit contains two 96-well plates, all reagents, antibodies and positive and negative controls required for the assay. The 96-well plates contain immobilized oligonucleotides of the NF- $\kappa$ B consensus site (5'-GGGACTTCC-3'). The preparation of reagents and the assay were carried out following the instructions of the supplier. In brief, 5  $\mu$ g of nuclear extracts were added to each well and incubated at room temperature for 1 h. Appropriate positive and negative controls were maintained. During this process, the active form of NF- $\kappa$ B contained in the nuclear extract specifically binds to the oligonucleotides immobilized in the 96-well plates. The wells were washed using the washing buffer, and incubated with respective primary antibodies that recognize an epitope on p50, p52, p65, c-Rel or RelB that is accessible only when NF- $\kappa$ B is activated and bound to its target DNA. After 1 h of incubation at room temperature, the wells were washed and incubated with HRP-conjugated secondary antibodies and incubated at room temperature for 1 h. After washing the cells, 100  $\mu$ l of developing solution was added and observed for the development of blue colour. The reaction was stopped by adding 100  $\mu$ l of stop solution and the absorbance was read using a microplate reader at 450 nm with a reference wavelength of 655 nm.

#### *Statistical analyses*

The results are expressed as mean  $\pm$  SE ( $n=6$  for each data point). Western blotting and immunofluorescence results are representative of two separate experiments. Comparisons between groups were analysed using analysis of variance (ANOVA) with Tukey's *post hoc* test or Student's *t*-test, where appropriate. Analyses were performed using Graphpad Instat software (San Diego, CA, USA).  $P < 0.05$  was considered significant.

## **Results**

### *RCC cell lines have a higher expression of NF- $\kappa$ B subunits than normal cells and PDTC decreases the viability of RCC but not normal cells*

Western blotting revealed that both the RCC cell lines showed a higher basal level expression of all the five subunits of NF- $\kappa$ B when compared with PTEC or

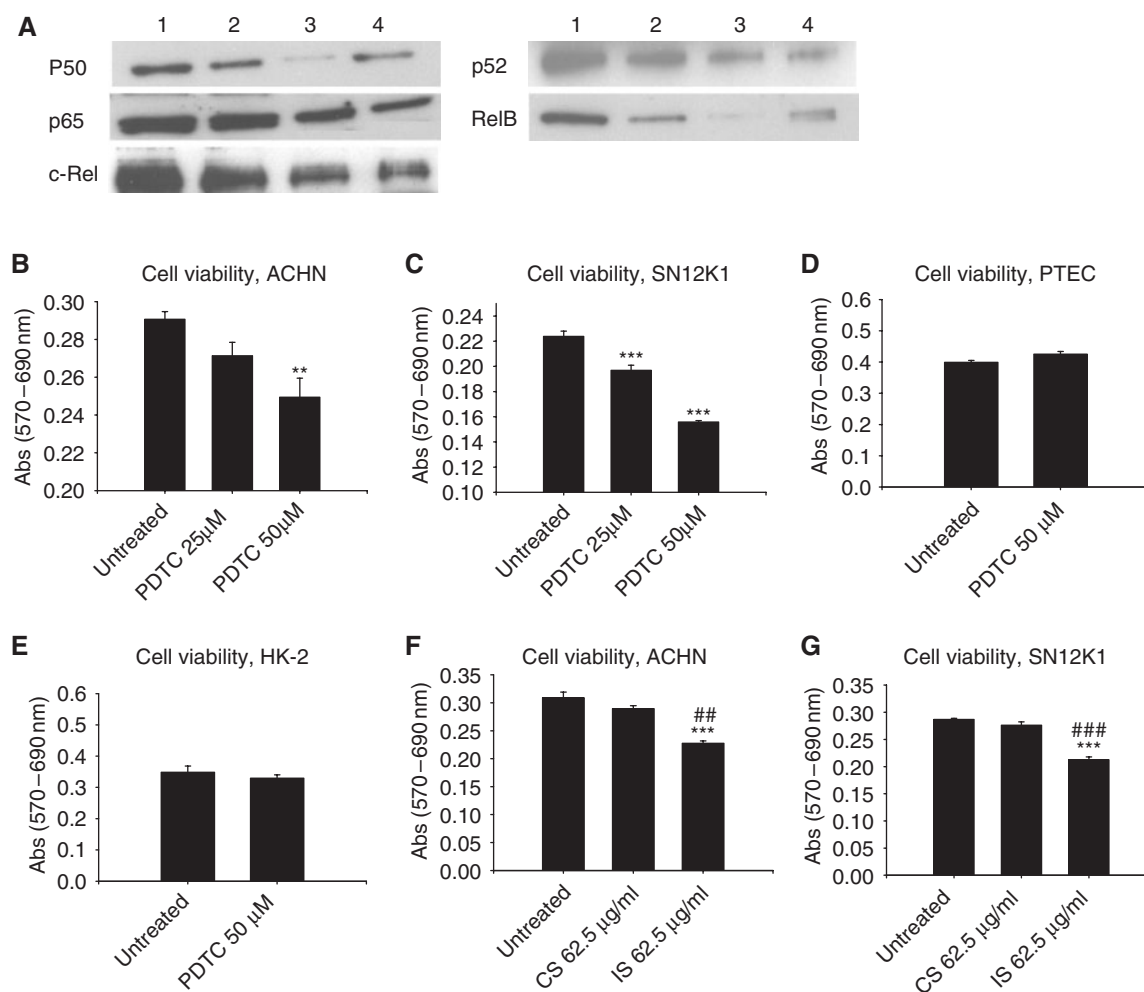
HK-2 cells (Figure 1A). Of the two RCC cell lines, ACHN showed a higher level of expression than SN12K1 cells. ACHN cells were more resistant to PDTC with only 50  $\mu$ M inducing a significant decrease in cell viability whereas 25  $\mu$ M failed to induce any significant changes (Figure 1B). SN12K1 cells were more sensitive to PDTC with both 25 and 50  $\mu$ M inducing significant decrease in cell viability (Figure 1C). PDTC at a concentration of 50  $\mu$ M did not induce any significant decrease in viability of PTEC (Figure 1D) or HK-2 cells (Figure 1E). In order to verify the decreased cell viability observed in the RCC cell lines, due to the inhibition of NF- $\kappa$ B by PDTC, we exposed ACHN and SN12K1 to various concentrations of specific NF- $\kappa$ B inhibitor sequence AAVALLPAVLLALLAPVQRKRQKLMP (sc-3060) or inactive control NF- $\kappa$ B sequence AAVALLPAVLLALLAPVQRDQGQKLMP (sc-3061) from Santa Cruz Biotechnology Inc.(CA, USA). A significant decrease in viability was observed in ACHN (Figure 1F) and SN12K1 cells (Figure 1G) that were exposed to the inhibitor sequence, whereas such significant changes were not observed in cells exposed to the inactive control sequence.

### *PDTC decreases the proliferation of RCC cell lines but not normal cells*

BrdU incorporation assay also showed that 50  $\mu$ M of PDTC induced a significant decrease in proliferation in ACHN (Figure 2A), whereas both 25  $\mu$ M and 50  $\mu$ M were effective in SN12K1 cells (Figure 2B). PDTC failed to induce any significant reduction in proliferation of PTEC (Figure 2C) or HK-2 cells (Figure 2D).

### *PDTC induces apoptosis in RCC cell lines and down-regulates anti-apoptotic proteins*

Apoptosis measurement by flow cytometry showed that 50  $\mu$ M of PDTC induced a significant increase in apoptosis in both ACHN (Figure 3A) and SN12K1 cells (Figure 3B). This assay also showed that 25  $\mu$ M of PDTC induced significant apoptosis in SN12K1 cells, without such changes in ACHN cells. As 50  $\mu$ M of PDTC was effective in both cell lines, we used this concentration for further studies. Apoptosis was further analysed using TUNEL/morphology method. Quantitative analysis showed that PDTC (50  $\mu$ M) induced a significant increase in apoptosis in both ACHN (Figure 3C) and SN12K1 cells (Figure 3D). Apoptotic nuclei showed one or more of the following changes: condensation, hyperchromasia, crescent nuclei and apoptotic bodies (Figure 3E). PDTC induced a decrease in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> without any changes in the expression of the pro-apoptotic protein Bax (Figure 3F).



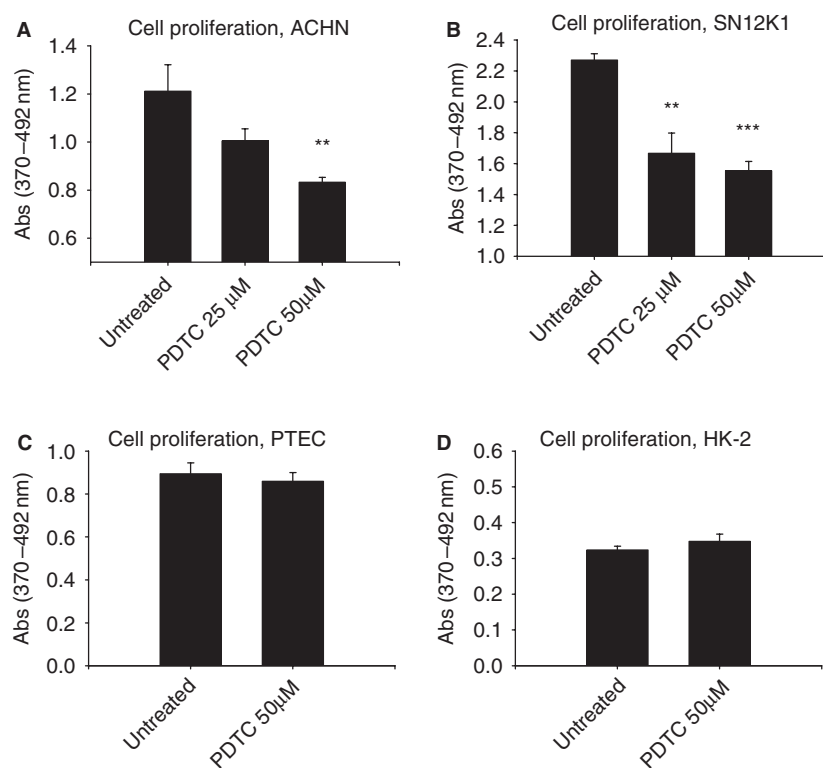
**Fig. 1.** Western blots (A) of whole cell lysates (5  $\mu$ g protein) showing a higher basal level expression of all the five subunits of NF- $\kappa$ B in RCC cell lines than normal tubular cells. Lane 1, ACHN; lane 2, SN12K1; lane 3, PTEC; lane 4, HK-2 cells. PDTC induced a significant decrease in viability of both RCC cell lines. ACHN cells (B) were more resistant to PDTC treatment with only the 50  $\mu$ M of PDTC inducing cell death whereas both 25 and 50  $\mu$ M were effective in SN12K1 cells (C). PDTC did not induce any significant cell death either in PTEC (D) or HK-2 cells (E). Treatment of RCC cell lines with a specific NF- $\kappa$ B inhibitory sequence induced a significant decrease in viability of ACHN (F) and SN12K1 (G) cells, whereas the inactive control sequence failed to induce any significant changes in cell viability CS, inactive control NF- $\kappa$ B sequence; IS, specific NF- $\kappa$ B inhibitory sequence. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 vs respective untreated groups; ### $P$  < 0.01 and #### $P$  < 0.001 vs respective inactive control NF- $\kappa$ B sequence.

#### *PDTC has a dual role in the expression of NF- $\kappa$ B subunits*

Western blots of whole cell and cytoplasmic lysates showed that PDTC markedly reduced the expression of all the subunits of NF- $\kappa$ B (Figure 4A). However, such a pattern was not observed in the nuclear lysates. While SN12K1 cells showed a decreased nuclear expression similar to that of the whole cell and cytoplasmic lysates, ACHN cells showed an unexpected increase in expression of all the five subunits of NF- $\kappa$ B (Figure 4A). These observations prompted us to investigate the *in situ* expression of the NF- $\kappa$ B subunits by immunofluorescence.

In untreated cells, the cytoplasm rather than the nucleus showed a higher level of expression of all the subunits of NF- $\kappa$ B (Figure 4B). In cells treated with PDTC, we observed three different populations.

The first group displayed normal morphology with less or similar overall expression of NF- $\kappa$ B to that of untreated cells. The second group of cells appeared hypertrophic with markedly decreased or undetectable levels of NF- $\kappa$ B subunits both in the cytoplasm and the nucleus. The third group of cells was found mostly in ACHN cells. These cells were also hypertrophic, showed a very weak cytoplasmic expression, but a strong expression in the nucleus and/or the perinuclear membrane (Figure 4B). In general, such expression pattern was stronger than the untreated cells, offering a possible explanation for the increased expression observed in nuclear lysates. In SN12K1 such a predominance of cells with a higher expression of NF- $\kappa$ B in the nucleus or perinuclear membrane in response to PDTC treatment was not observed (Figure 4B).



**Fig. 2.** Cell proliferation assay also showed that ACHN was more resistant to PDTC (A) than SN12K1 cells (B). No significant changes in proliferation of PTEC (C) or HK-2 (D) were observed in response to 50 µM of PDTC. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs respective untreated groups.

#### *PDTC decreases the nuclear binding activity of all NF- $\kappa$ B subunits*

We then analysed the DNA-binding capacity of all the NF- $\kappa$ B subunits, which is an essential step for transcription. This assay is non-radioactive, specific for NF- $\kappa$ B, 10-fold more sensitive and 40-fold faster than the traditional electro mobility shift assay (EMSA) [25]. In general, nuclear proteins in untreated ACHN cells showed a higher basal level DNA-binding activity than the untreated SN12K1 cells (Figure 5). PDTC treatment significantly reduced the nuclear binding capacity of all the subunits of NF- $\kappa$ B (Figure 5).

#### *PDTC decreases the expression of IKK complex*

The activation of IKK is crucial for the degradation of I $\kappa$ Bs and the release of active NF- $\kappa$ B. Therefore, we analysed the status of the IKK complex (IKK- $\alpha$ , IKK- $\beta$  and IKK- $\gamma$ ) in response to PDTC. Both western blotting (Figure 6A) and immunofluorescent assays (Figure 6B) showed that PDTC markedly reduced the expression of all the three subunits of the IKK complex in RCC cell lines.

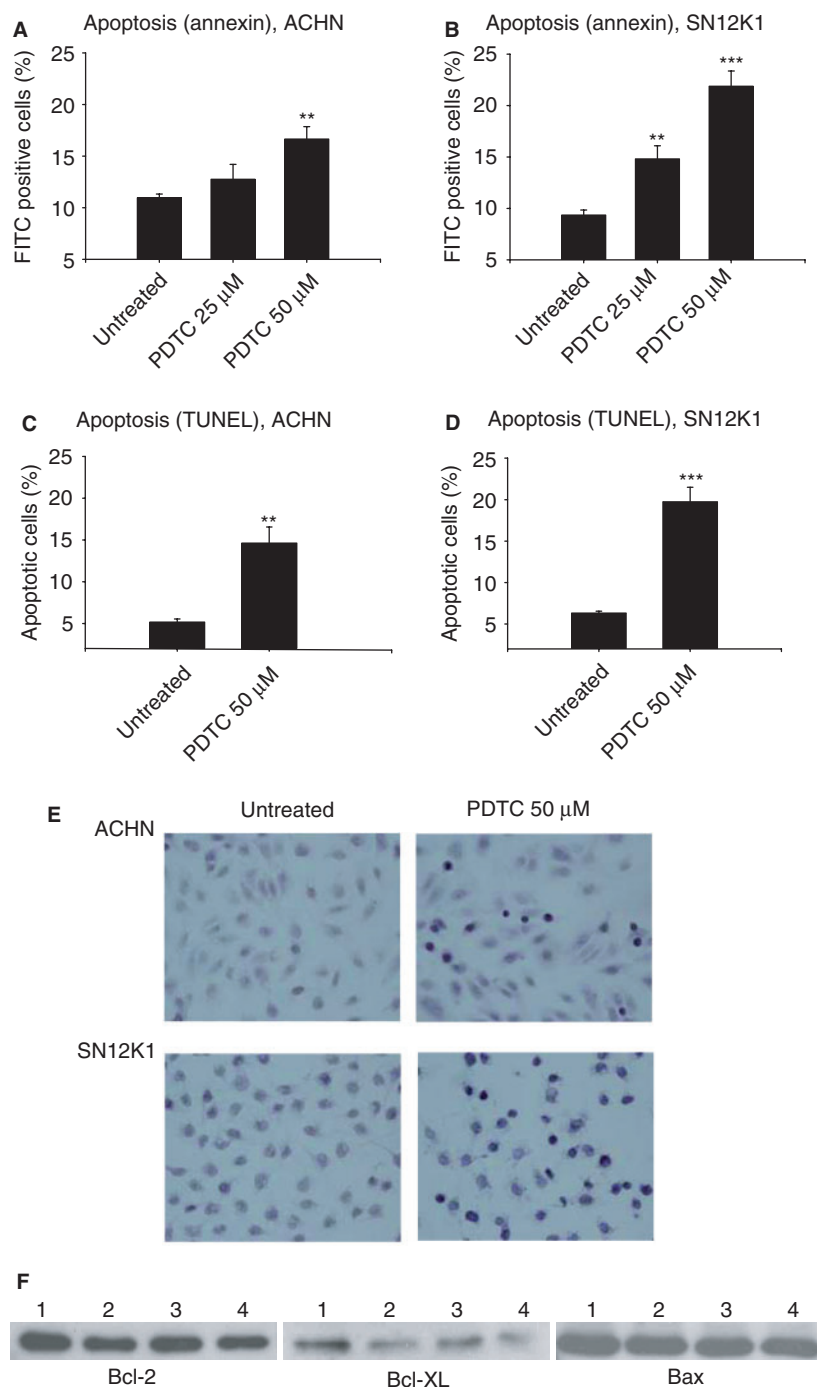
#### *PDTC decreases the expression of I $\kappa$ B- $\alpha$ and I $\kappa$ B- $\beta$*

Western blotting showed that SN12K1 had a higher basal level expression of I $\kappa$ B- $\alpha$ , but not I $\kappa$ B- $\beta$  than

ACHN cells. Contrary to what we expected, both western blotting (Figure 7A) and immunofluorescent assay (Figure 7B) showed that PDTC markedly reduced the expression of I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$ .

## Discussion

To our knowledge, this is the first report to show the increased activation of all the five subunits of NF- $\kappa$ B in RCC cell lines compared with normal tubular cells. The over expression of p50 and the p65 subunits in human RCC and in some RCC cell lines has been reported [11–13]. But the status of other subunits in RCC in relation to normal cells is largely unknown. Enhanced activation of NF- $\kappa$ B can induce chemoresistance in cancer cells either through the up-regulation of multi drug resistance genes or through the inhibition of the tumour suppressor gene p53 [32]. Our findings of higher basal-level expression and DNA-binding activity of all the five NF- $\kappa$ B subunits in ACHN compared with SN12K1, and the subsequent relative resistance of ACHN to PDTC, further support the role of NF- $\kappa$ B in drug resistance. Interestingly, normal cells, with lower NF- $\kappa$ B expression, compared with RCC cell lines, did not show significant changes in cell death and proliferation when treated with PDTC. The reason for this is unclear. However, PDTC has been shown to induce



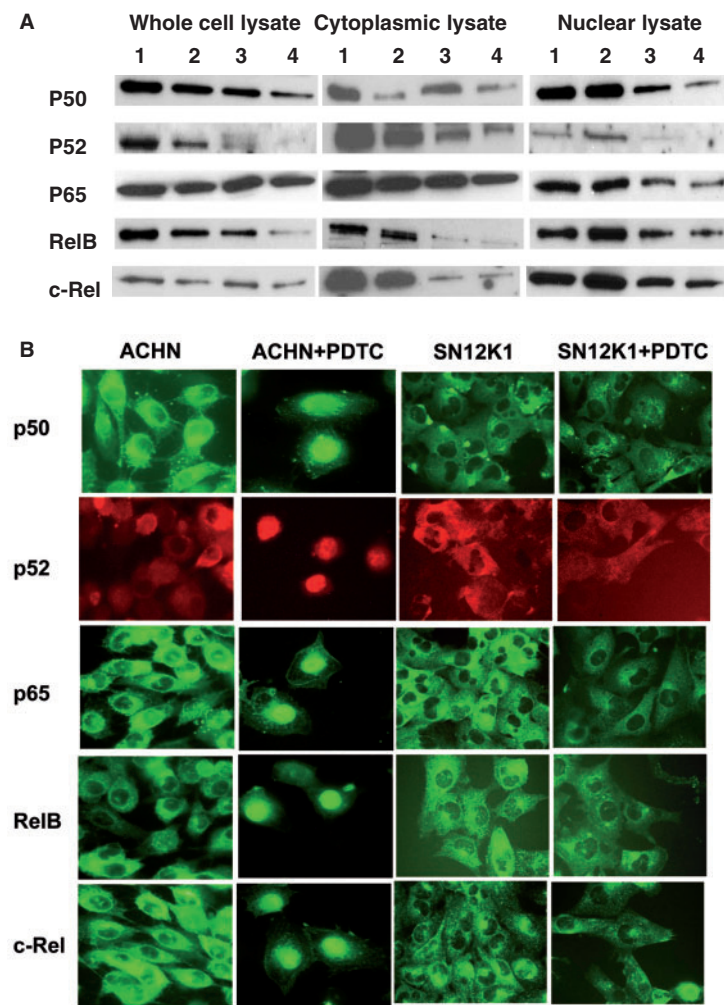
**Fig. 3.** Apoptosis measurement by flow cytometry revealed that PDTC induced a significant increase in apoptosis in both ACHN (A) and SN12K1 cells (B). This assay also showed that ACHN cells were more resistant to PDTC than SN12K1 cells. Apoptosis was further confirmed by TUNEL/morphology. PDTC (50  $\mu$ M) induced a significant increase in apoptosis in ACHN (C) and SN12K1 cells (D). Panel (E) is a representative area from untreated cells and cells treated with PDTC. The dark cells are apoptotic cells. Western blotting (whole cell lysates, 10  $\mu$ g protein) showed that PDTC (50  $\mu$ M) induced a decrease in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> without such changes in the pro-apoptotic protein Bax (F). Lane 1, ACHN untreated; lane 2, ACHN+PDTC; lane 3, SN12K1 untreated; lane 4, SN12K1+PDTC. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 vs respective untreated groups.

cell death in leukaemic CD34<sup>+</sup> cells but not in normal cells, but the effect was independent of NF- $\kappa$ B inhibition [24].

NF- $\kappa$ B activates many anti-apoptotic genes especially of the Bcl-2 family [7,33]. Bcl-2 has multiple

putative NF- $\kappa$ B binding sites [34] in its promoter region and the binding of the p50 homodimer to these sites has been shown to activate the Bcl-2 gene. The transcriptional activation of these genes by NF- $\kappa$ B is one of the mechanisms by which cancer cells gain





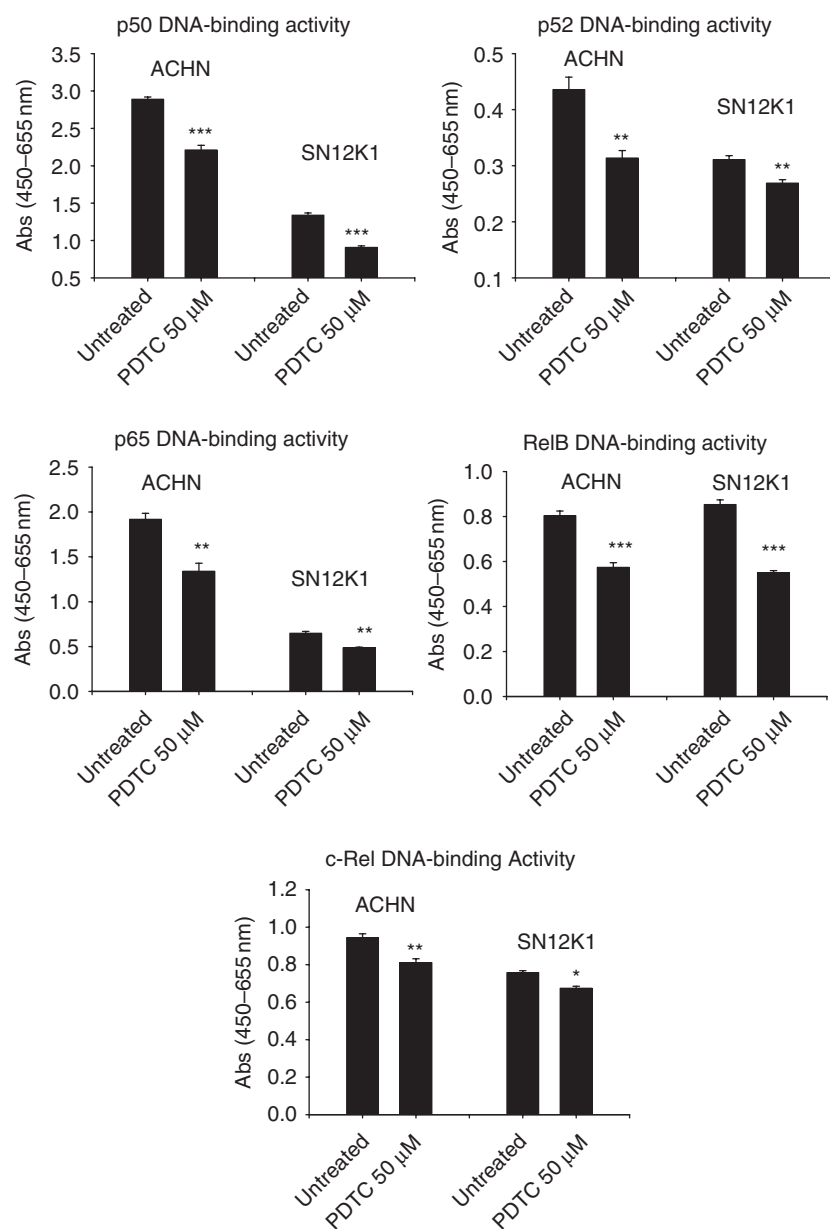
**Fig. 4.** Western blots of whole cell and cytoplasmic lysates (10  $\mu$ g protein) showed that PDTC (50  $\mu$ M) induced a decrease in the expression of all the five subunits of NF- $\kappa$ B (A). However, PDTC induced an increase in the nuclear expression of NF- $\kappa$ B subunits in ACHN cells whereas it decreased their expression in SN12K1 cells (A). Lane 1, ACHN untreated; lane 2, ACHN+PDTC; lane 3, SN12K1 untreated; lane 4, SN12K1+PDTC. Immunofluorescence assay showed that cells exposed to PDTC were hypertrophic with differential expression pattern. In ACHN cells, a subset of cells showed a decreased cytoplasmic expression with an increased nuclear and/or perinuclear expression. In (B) emphasis is given to cells that show increased expression of NF $\kappa$ B in the nucleus indicating translocation. Such subset of cells with an increased perinuclear/nuclear expression was not observed in SN12K1 cells (B). Rather the decrease was uniform in SN12K1 cells.

hyper-proliferative and anti-apoptotic characteristics. In addition, a direct correlation between Bcl-2 expression and the metastatic behaviour of RCC has been reported [35]. In our study, PDTC induced a reduction in the expression of Bcl-2 and Bcl- $\text{x}_L$ , leading to a significant increase in apoptosis and a decrease in proliferation. The inhibition of DNA-binding of all the five subunits of NF- $\kappa$ B by PDTC could have resulted in the decreased transcription of Bcl-2 and Bcl- $\text{x}_L$  and hence increased apoptosis and decreased proliferation. The inhibitory effect of PDTC on Bcl-2 expression has been reported previously [36].

While PDTC induced an overall reduction of all the NF- $\kappa$ B subunits, compartmental analysis of expression in the cytoplasm and the nucleus revealed a dual role of PDTC in ACHN and SN12K1 cells. A marked reduction of all the five subunits of NF- $\kappa$ B was observed in the nucleus of SN12K1 cells. In contrast, ACHN cells showed an increased expression of the

subunits (an example of heterogeneity of RCC). Although unexpected, this is not surprising because certain chemotherapeutic agents such as daunorubicin, cisplatin and taxol and the gamma radiation commonly used to treat cancer have been shown to up-regulate NF- $\kappa$ B as well as induce apoptosis [37]. Increased nuclear expression is a concern because it can lead to drug resistance. Despite increased nuclear expression, we found that PDTC decreased the DNA-binding activity of NF- $\kappa$ B, in line with previous reports [25,38]. Therefore even in cases where NF- $\kappa$ B-mediated drug resistance is observed, PDTC can down-regulate the DNA-binding activity thereby demonstrating its potential to be an adjunct therapeutic. To this effect, PDTC has been shown to enhance the cytotoxicity of the chemotherapeutic agent, 5-Fluorouracil in animal models of colorectal cancer [19,20]. Whether the decreased DNA-binding of the NF- $\kappa$ B subunits observed in this study is the outcome of protein



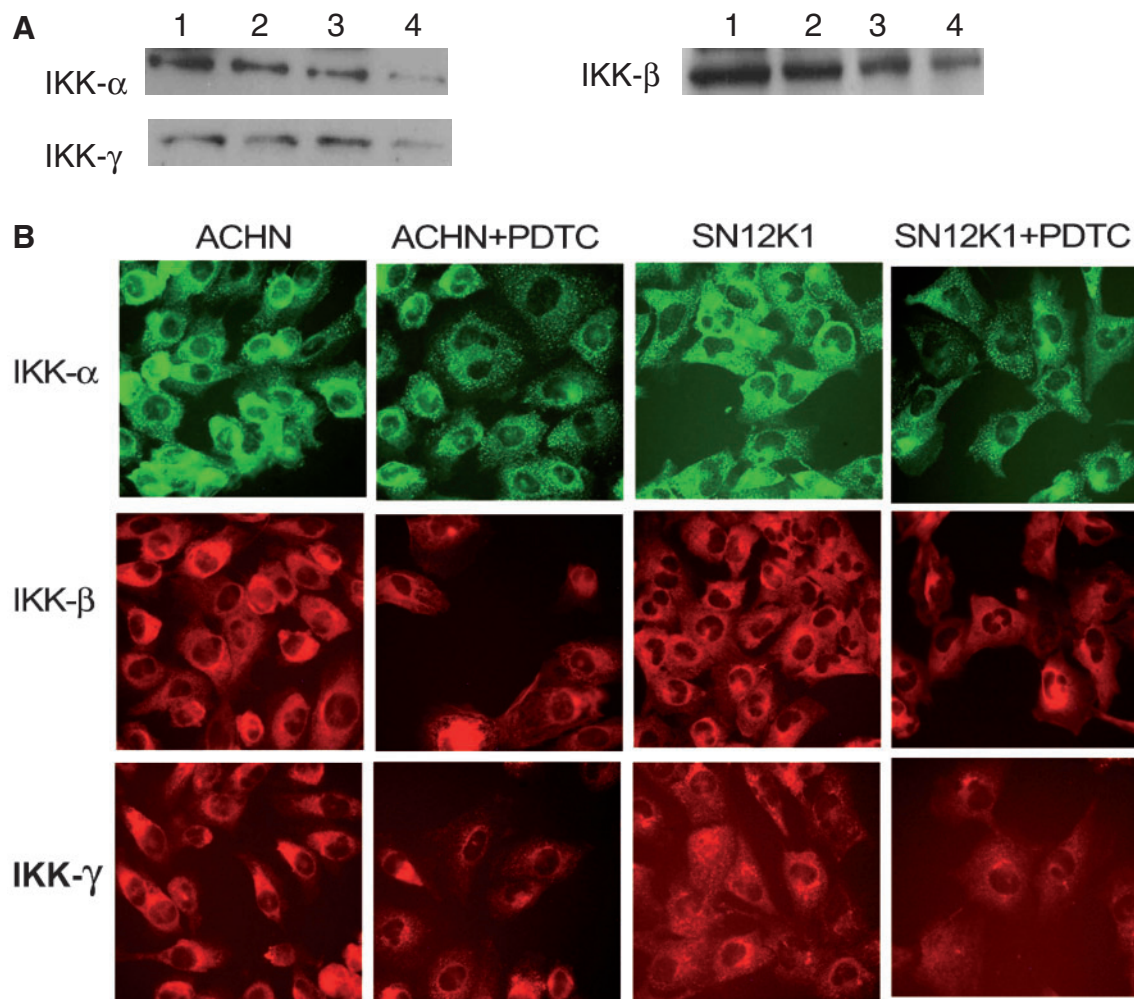


**Fig. 5.** RCC cells treated with 50  $\mu$ M of PDTC showed a significant decrease in DNA-binding activity (nuclear proteins 5  $\mu$ g). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs respective untreated groups.

concentration (i.e. decreased NF- $\kappa$ B protein content in the treated cells) or is actually an effect of PDTC on DNA-binding is not clear. However, there are reports that suggest that PDTC has an independent effect on DNA-binding [25,38].

The release of the active NF- $\kappa$ B from I $\kappa$ Bs is mediated by the activation of IKK complex, either through the classical or alternative pathway. In the classical pathway, the IKK- $\beta$  is activated, which in turn phosphorylates I $\kappa$ B- $\alpha$  at Ser 32 and Ser 36 and I $\kappa$ B- $\beta$  at Ser 19 and Ser 23 located on the N-terminal [6,8]. The phosphorylated I $\kappa$ Bs are subsequently ubiquitinated and degraded via the proteasome pathway leading to the release of the active NF- $\kappa$ B to the nucleus. In the alternative pathway, IKK- $\alpha$  is

activated, which phosphorylates the inhibitory molecule p100 at specific serine residues located both in the N- and C-terminal regions [9]. Phosphorylated p100 is ubiquitinated and cleaved to generate p52 subunit, which is translocated to the nucleus [9]. Both classical and alternative pathways are 'inducible' pathways initiated in response to various stimuli, such as TNF- $\alpha$ , lymphotoxin- $\beta$ , B-cell activating factor, CD40 ligand, human T-cell leukaemia virus and Epstein-Barr virus [6,8,9]. But what stimulates the constitutive expression of NF- $\kappa$ B in cancer cells is not fully understood. Aberrations in genes encoding for NF- $\kappa$ B, mutations in I $\kappa$ B or unrestricted activation of IKK can cause the uncoupling of the NF- $\kappa$ B from their regulators [7,10]. In addition, oncoproteins, chronic

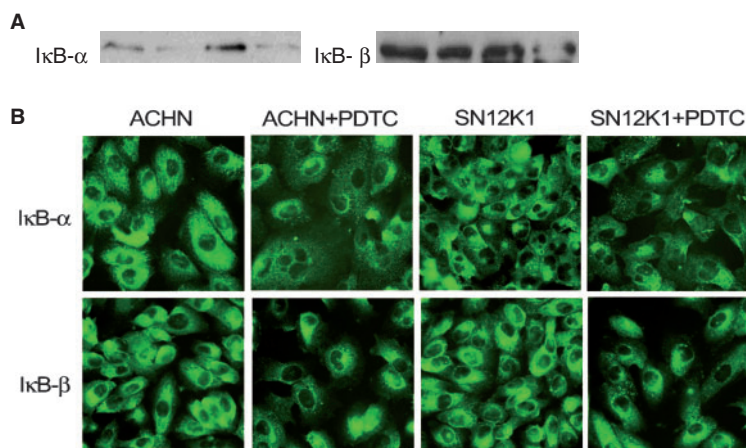


**Fig. 6.** Western blots of whole cell lysates (A) showed that PDTC (50  $\mu$ M) decreased the expression of all the three subunits of the IKK complex (10  $\mu$ g protein). Lane 1, ACHN untreated; lane 2, ACHN+PDTC; lane 3, SN12K1 untreated; lane 4, SN12K1+PDTC. Immunofluorescence (B) also showed a decrease in expression of the IKK- $\alpha$ , IKK- $\beta$  and IKK- $\gamma$  in response to PDTC.

infection and paracrine and autocrine production of pro-inflammatory cytokines by cancer cells can also persistently stimulate IKK activity, leading to the activation of NF- $\kappa$ B [7,10]. Taken together, be it 'induced' or 'constitutive' expression, IKK activation and subsequent degradation of I $\kappa$ B are essential for NF- $\kappa$ B activation. Therefore, inhibition of IKK might potentially inhibit the activation of NF- $\kappa$ B. The inhibitory effect of PDTC on IKK- $\alpha$  and IKK- $\beta$  in some experimental models has been reported [26,27]. Similar to these findings, in our study, PDTC decreased the expression of IKK- $\alpha$  and IKK- $\beta$ . In addition, PDTC also decreased the expression of IKK- $\gamma$ . This could potentially down-regulate the assembly of the IKK complex.

However, in line with the classical or alternative pathway of NF- $\kappa$ B activation, a decrease in IKK should have led to the stabilization of I $\kappa$ B leading to either an increase or at least the same level of expression of I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  in the cytoplasm [39]. To further support this view, PDTC itself has been shown to reduce the degradation of I $\kappa$ B- $\alpha$  in

experimental models of chronic inflammation and cerebral ischaemia [38,40]. We observed a decrease in the expression of I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$ . The reason for this paradox is not clear. However, there is a third type of NF- $\kappa$ B activation, the atypical pathway, which is generally initiated in response to UV radiation, anoxia or Daunorubicin [9,41,42]. This pathway is independent of IKK, yet involves the degradation of I $\kappa$ B- $\alpha$  [9,41]. Therefore, it can be speculated that PDTC has an IKK-complex independent effect on the degradation of I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$ . To support this view a recent study showed that PDTC can inhibit the synthesis of I $\kappa$ B- $\alpha$  [28]. Alternatively, decreased expression of I $\kappa$ B- $\alpha$  may be simply an indication of reduced NF- $\kappa$ B and its activity. It has been reported that NF- $\kappa$ B positively regulates and re-synthesizes I $\kappa$ B- $\alpha$  [43]. Taken together, be it classical, alternative, atypical or constitutive mode of activation, PDTC can play a role in inhibiting NF- $\kappa$ B expression and its transcription at multiple points of the NF- $\kappa$ B-IKK-I $\kappa$ B cascade. Given that PDTC does not induce toxicity in normal kidney cells and is well tolerated clinically [17], the anti cancer



**Fig. 7.** Western blots of whole cell lysates (A) showing a decrease in the expression of IκB- $\alpha$  and IκB- $\beta$  in response to PDTC (50  $\mu$ M). Lane 1, ACHN untreated; lane 2, ACHN+PDTC; lane 3, SN12K1 untreated; lane 4, SN12K1+PDTC. Immunofluorescence (B) also showed that PDTC induced a decrease in the expression of IκB- $\alpha$  and IκB- $\beta$ .

potential of this compound in RCC warrants further research.

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