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European Journal of Pharmacology 481 (2003) 159–167
www.elsevier.com/locate/ejphar

Modulation of mitogen-activated protein kinases by 6-nitro-7-hydroxycoumarin mediates apoptosis in renal carcinoma cells

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Received 21 July 2003; received in revised form 10 September 2003; accepted 11 September 2003

Abstract

6-Nitro-7-hydroxycoumarin has previously been shown to be a selective anti-proliferative agent capable of activating p38, stress-activated protein kinase (SAPK) and mitogen-activated protein (MAP) kinase in the human renal cell carcinoma cell line, A-498. Here, the role of p38 MAP kinase was further investigated in relation to its participation in 6-nitro-7-hydroxycoumarin induced apoptosis. 6-Nitro-7-hydroxycoumarin was shown to alter cell cycle progression, leading to the appearance of a sub-G_1 peak, containing hypodiploid DNA, accompanied by increases in both poly(ADP-ribose)polymerase cleavage and decreased expression of cyclin D1. Drug treatment also lead to a rise in the expression in the cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$, and the appearance of inter-nucleosomal DNA cleavage and morphological changes, consistent with apoptotic cell death. Using a p38 MAP kinase inhibitor, SB203580, caused expression of $p21^{WAF1/CIP1}$ to be suppressed and both cleaved poly(ADP-ribose)polymerase and the numbers of apoptotic cells were decreased. In summary, this study shows the participation of p38 MAP kinase in 6-nitro-7-hydroxycoumarin induced apoptosis of A-498 cells and suggests that targeting of p38 may represent a novel mechanism to inhibit renal cell carcinoma and that coumarin type drugs require further investigation as potential anticancer agents directed against renal cell carcinoma.
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Keywords: 6-Nitro-7-hydroxycoumarin; Renal cell carcinoma; p38 MAP kinase; Apoptosis; Cell cycle

1. Introduction

Coumarin (1,2-Benzopyrone) is structurally the least complex member of a large class of compounds known as benzopyrones (reviewed by Egan et al., 1990). The biological activities of coumarin and related compounds are multiple and include anti-thrombotic activity (Hoult and Paya, 1996; Casley-Smith and Casley-Smith, 1997) and anti-microbial properties (Laurin et al., 1999). In addition, coumarins have been shown to inhibit *N*-methyl-*N*-nitrosourea, aflatoxin B_1 and 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis in rats (Matsunago and Hanawalt, 2000; Kelly et al., 2000). More recently, coumarin derivatives have been evaluated in the treatment of human immuno-deficiency virus, due to their ability to inhibit human immuno-deficiency virus-integrase (Kirkiacharian et al., 2002; Yu et al., 2003).

Since the late 1980s, a number of in vivo studies have investigated the possible use of coumarins in the treatment of renal cell carcinoma (Marshall et al., 1989, 1994). All of these studies have demonstrated a significant response rate following coumarin treatment alone or in combination therapy. The in vitro effects of coumarins on the growth of renal cell carcinoma derived cell lines showed that coumarin and 7-hydroxycoumarin were potent cytotoxic and cytostatic agents (Conley and Marshall, 1987; Marshall et al., 1994). A recent study carried out in our laboratory compared the anti-proliferative capability of a series of natural and synthetic coumarins including, 6-nitro-7-hydroxycoumarin, in the human renal adenocarcinoma derived cell line, A-498. This compound was shown to function by an inhibition of DNA synthesis and was non-mutagenic in the Ames assay (Finn et al., 2002). Therefore, the authors suggested that 6-nitro-7-hydroxycoumarin warranted further investigation, in order to elucidate its mechanism of action.

Our research group have previously hypothesised that the inhibitory activity of coumarins may occur through a modulation of specific intracellular signalling events (Finn

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doi:10.1016/j.ejphar.2003.09.035

et al., 2001). One such intracellular pathway worthy of investigation is mitogen-activated protein kinase (MAPK). The MAPKs are centrally involved in response to a broad and varied number of extracellular stimuli, such as growth factors, differentiation signals and cellular stress (Sawafuji et al., 2003). Members of the MAPK pathways include, the extracellular signal-related kinase (ERK), the c-jun kinase (JNK) also known as the stress-activated protein kinase (SAPK) and the p38 kinase (Cobb, 1999; Cross et al., 2000). Signalling through the MAPK cascades, regulates multiple biological activities including, cell proliferation, differentiation, transverse through cell cycle and apoptotic cell death, among others (Bourbon et al., 2000). Although exceptions occur, the majority of evidence suggests that activation of the ERK pathway is implicated in cellular proliferation, differentiation and survival. A role for p38 MAPK activation during differentiation has been reported by many authors, and in several cell types (Englaro et al., 1998; Engelman, 1999; Nebreda, 2000; Hagemann and Blank, 2001; Hu et al., 2003). In addition, activation of the SAPK and p38 kinase cascades is generally, although not exclusively, associated with inflammation, apoptosis and differentiation (Ozaki et al., 1999). Identification of the importance of the MAPK cascade has lead to the investigation of conventional and novel anti-neoplastic agents and therapies as potential modulators of MAPK activation (Barnes and Bagheri-Yarmand, 2003). In the present study, we examined the role of p38 MAP kinase in mediating the cytotoxic effects of 6-nitro-7-hydroxycoumarin in A-498 cells. Our results confirm a role for p38 MAP kinase in modulating cell cycle events and inducing cell death through an activation of regulatory proteins controlling apoptotic cell death.

2. Materials and methods

2.1. Test compounds and reagents

Dimethyl sulphoxide (DMSO), propidium iodide, RNAse A and 4-(4-fluorophenyl)-2-4-methylsulfinylphenyl (SB203580) were purchased from Sigma-Aldrich, Ireland. 6-Nitro-7-hydroxycoumarin was synthesised by direct nitration of 7-hydroxycoumarin (Finn et al., 2001). Structure and purity was confirmed by thin layer chromatography, infra red analysis, ^{1}H- and ^{13}C-NMR spectroscopy, along with elemental analysis. The chemiluminescence substrate was purchased from Pierce Laboratories (UK), while antibodies specific for cleaved poly(ADP-ribose)polymerase and cyclin D1 were purchased from New England Biolabs. Mouse monoclonal antibodies raised against the cyclin-dependant kinase inhibitor, p21$^{WAF1/CIP1}$ and the DNA fragmentation kit were purchase from Calbiochem, UK and Oncogene, UK, respectively. All cell culture reagents and media were purchased from Euroclone, UK, unless otherwise stated.

2.2. Cell lines and cell culture

A-498 cells (human kidney adenocarcinoma) were purchased from the American Type Culture Collection, Manassas, USA. These cells were maintained in Eagles Minimum Essential Medium with Earle's balanced salt solution, supplemented with 2 mM L-glutamine and 10% foetal bovine serum (Flow laboratories, Herts, UK). Cells were grown at 37 °C in a humidified atmosphere, with 5% CO_2.

2.3. Cell treatment and Western blot analysis

A-498 cells were grown in 100 mm petri dishes and treated with either DMSO alone or 6-nitro-7-hydroxycoumarin (0, 5, 10, 50, 100, 250 and 500 μM) for 48, 72 and 96 h. Cells were then washed twice in ice-cold 0.1 M phosphate buffer saline, pH 7.4 (PBS), harvested by scraping and centrifuged at 4000 rpm for 10 min. Whole cell extracts were prepared by resuspension of cells in lysis buffer [M-per lysing solution (Pierce), supplemented to contain 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonylflouride, 2 μg/ml leupeptin and 2 μg/ml aprotinin]. After 15 min on ice, mixtures were clarified by centrifugation at a speed of 8000 rpm for 8 min and at 4 °C. The resultant supernatants were collected. Total protein lysates (40 μg) were resolved on 12% sodium dodecyl sulphate polycralymide gels (SDS-PAGE) and transferred to nitrocellulose membranes. The level of protein expression was determined using specific primary antibodies, followed by peroxidase-conjugated secondary antibodies and visualisation using chemiluminescent substrate (Luminol) and exposure to X-ray film.

2.4. Cell cycle analysis

Following drug treatment, cells were washed twice in ice-cold PBS, harvested by trypsinisation and collected by centrifugation as outlined above. The effect of drug treatment on cell cycle was determined using flow cytometric analysis according to the method of Nunez (2001). Briefly, cell suspensions were fixed and permeabilised by the vigorous addition of nine volumes of ice-cold 70% ethanol and stored at −20 °C for a minimum of 24 h, prior to analysis. Cells at a density of approximately 1×10^6 were resuspended in 800 μl of propidium iodide staining solution (20 μg/ml propidium iodide and 200 μg/ml RNase A in PBS, pH 7.4), and incubated in the dark at room temperature for 30 min. Cell cycle distributions were then determined by flow cytometry using Cell Quest software™ (Becton Dickinson).

2.5. DNA fragmentation

Chromosomal DNA from drug treated cells was extracted using the Suicide-track™ DNA ladder isolation kit (Oncogene). Briefly, cells were cultured in 150 mm petri

dishes and treated with 6-nitro-7-hydroxycoumarin for 96 h. Floating or detached cells were collected by centrifuging at 1500 rpm for 5 min, while adherent cells were harvested by scraping and then combined with the previously collected floating cells. All cells were centrifuged together at 1500 rpm for 5 min. The resulting pellet consisting of approximately 5×10^6 cells was resuspended in 55 μl of lysis solution, followed by the addition of 20 μl of RNase A solution and incubated at 37 °C in a water bath for 1 h. DNA isolation solution (25 μl) was added and tubes were incubated overnight at 50 °C. Resuspension buffer (500 μl) (10 mM Tris–HCl, pH 7.5, containing 1 mM EDTA) was then added to each sample. Intact and fragmented DNA was precipitated by the addition of 2 μl of Pellet-paint™ co-precipitant followed by 60 μl of 3 M sodium acetate, pH 5.2 and 662 μl of 2-propanol. Samples were mixed by inversion and then washed once in 70% ethanol and once in 100% ethanol. Finally, DNA samples were air dried and resuspended in 50 μl of resuspension buffer (10 mM Tris–HCl, pH 6.8) before analysis on 1.8% agarose gels. Positive controls consisted of 1×10^6 HL-60 cells (Human leukaemia cells) treated with 0.5 μg/ml Actinomycin D for 24 h, both supplied by Oncogene.

Table 1
Effects of 6-nitro-7-hydroxycoumarin treatment (0–500 μM) on cell cycle distribution in A-498 cells, following incubation for 48, 72 and 96 h

	Control	10 μM	50 μM	100 μM	250 μM	500 μM
48 h						
% G_0/G_1	64 ± 4	61 ± 6	65 ± 2	63 ± 7	58 ± 2	50 ± 4
% S	28 ± 2	30 ± 4	27 ± 1	28 ± 4	34 ± 1	44 ± 7
% G_2M	8 ± 2	9 ± 4	8 ± 1	9 ± 3	8 ± 1	6 ± 2
72 h						
% Sub G_1	0	0	0	0	4 ± 1	6 ± 4
% G_0/G_1	75 ± 11	74 ± 8	79 ± 2	82 ± 4	70 ± 6	67 ± 2
% S	15 ± 5	16 ± 6	12 ± 1	10 ± 2	15 ± 5	18 ± 7
% G_2M	10 ± 1	10 ± 2	9 ± 2	8 ± 4	11 ± 4	9 ± 2
96 h						
% Sub G_1	0	0	11 ± 1	19 ± 3	26 ± 6	75 ± 14
% G_0/G_1	78 ± 7	85 ± 1	71 ± 4	68 ± 5	62 ± 2	18 ± 4
% S	12 ± 5	9 ± 3	15 ± 6	11 ± 3	8 ± 1	6 ± 2
% G_2M	10 ± 1	6 ± 3	3 ± 2	2 ± 1	4 ± 1	1 ± 3

Cells were harvested, alcohol fixed and stained with propidium iodide and analysed by flow cytometry. Results show a dose- and time-dependant disruption of phase progression, with the appearance of a sub-G1 peak, representative of hypodiploid DNA content and indicative of apoptosis.

3. Results

3.1. Inhibition of cell cycle progression

6-Nitro-7-hydroxycoumarin treatment altered cell cycle events, which became apparent following 48 h exposure and at concentrations of 250 μM and 500 μM, with an S phase accumulation and a decrease in the percentage of cells in G_0/G_1. The first observed presence of a sub-G_1 peak corresponding to hypodiploid cells appeared following 72 h exposure and at drug concentrations of 250 and 500 μM, which coincided with a decrease in the percentage of cells in G_0/G_1 (Table 1). At the longest incubation period (96 h) and at a concentration of 10 μM, 6-nitro-7-hydroxycoumarin caused a G_0/G_1 accumulation, which decreased with increasing drug concentration. However, this decrease corresponded with the appearance of a sub-G1 peak (Table 1). Induction of this sub-G_1 peak appeared to be dose- and time-dependent. In addition, following 96-h exposure, the percentage of cells entering S phase appeared to increase and subsequently decrease with increasing drug concentration. Finally, the percentage of cells in the G_2/M phase decreased over the concentration range studied (Table 1).

3.2. Modulation in cyclin D1 and $p21^{WAF1/CIP1}$ expression

The cyclin-dependant kinase inhibitor, $p21^{WAF1/CIP1}$ is regarded as an indirect marker of apoptosis in cell systems where G_1 phase blockade contributes to cell death (Chan et al., 2003; Ghosh et al., 2003). Cells were treated with 6-nitro-7-hydroxycoumarin (0–500 μM) for 96 h and the expression of both cyclin D1 and $p21^{WAF1/CIP1}$ was determined using Western blot analysis. Expression of cyclin D1 appeared to decrease in a dose-dependant manner (Fig. 1A). However, a more pronounced effect was observed for the cyclin-dependant kinase inhibitor, $p21^{WAF1/CIP1}$, since 6-nitro-7-hydroxycoumarin resulted in a dose-dependant increase in the level of expression of cellular $p21^{WAF1/CIP1}$ (Fig. 1B).

3.3. Induction of cleaved DNA and altered morphology

Cleavage of genomic DNA by endogenous endonucleases during apoptosis is an irreversible event that commits the cell to die (Blatt and Glinck, 2001). Analysis of DNA from apoptotic cells by agarose electrophoresis produces a characteristic DNA ladder that is widely regarded as the biochemical hallmark of apoptosis. The presence of sub-G_1 peaks from flow cytometry experiments indicated the possible induction of cell death by apoptosis following drug treatment (Table. 1). In order to confirm this, genomic DNA was extracted from treated cells and analysed for DNA fragmentation by electrophoresis. Fig. 2 illustrates that 6-nitro-7-hydroxycoumarin induced a dose-dependent increase in 180 base pair multimeric bands. Cytological examination of A-498 cells treated with 6-nitro-7-hydroxycoumarin (50 and 100 μM) for 96 h, resulted in a thinning of the monolayer and the appearance of vacuolated, rounded cells with progressive nuclear shrinkage and their eventual detachment from the culture dish (Fig. 3C and D). Treatment of cells with

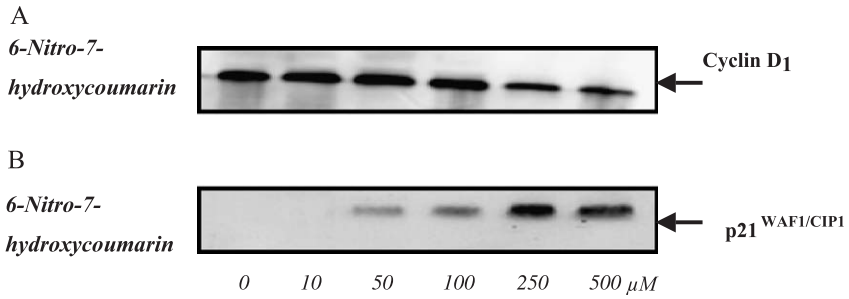

Fig. 1. Effects of 6-nitro-7-hydroxycoumarin (0–500 μM) treatment on the expression of (A) cyclin D_1 and (B) the cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ following drug treatment for 96 h. Both proteins were separated on a 12% SDS-PAGE, transferred to a nitrocellulose membrane and detected using monoclonal antibodies, HRP-conjugated secondary antibodies with ECL detection. Expression of Cyclin D_1 decreased, while $p21^{WAF1/CIP1}$ increased. Western blots are representative of three independent experiments.

culture media or vehicle alone appeared to have had no visible effect on morphology (Fig. 3A and B, respectively).

3.4. Induction of poly(ADP-ribose)polymerase cleavage

Proteolytic cleavage of the 116 kDa, poly(ADP-ribose)-polymerase protein results in the separation of the N-terminal binding domain (24 kDa) from its C-terminal catalytic domain (89 kDa) (Kartner et al., 1993). The effect of 6-nitro-7-hydroxycoumarin treatment on poly(ADP-ribose)polymerase cleavage was determined by Western blot analysis, following exposure to test agent for 96 h. Fig. 4A shows that cleaved poly(ADP-ribose)polymerase (89 kDa) increased with increased drug concentration.

3.5. The p38 inhibitor, SB203580 modulates the in vitro pro-apoptotic activity of 6-nitro-7-hydroxycoumarin

Previously, our research group has shown that exposure of A-498 cells to 6-nitro-7-hydroxycoumarin resulted in an activation of p38 MAPK (Finn et al., 2003). Consequently, in this study we examined the effect of using 6-nitro-7-hydroxycoumarin in the presence of the p38 inhibitor, SB203580 on the expression of $p21^{WAF1/CIP1}$ and poly

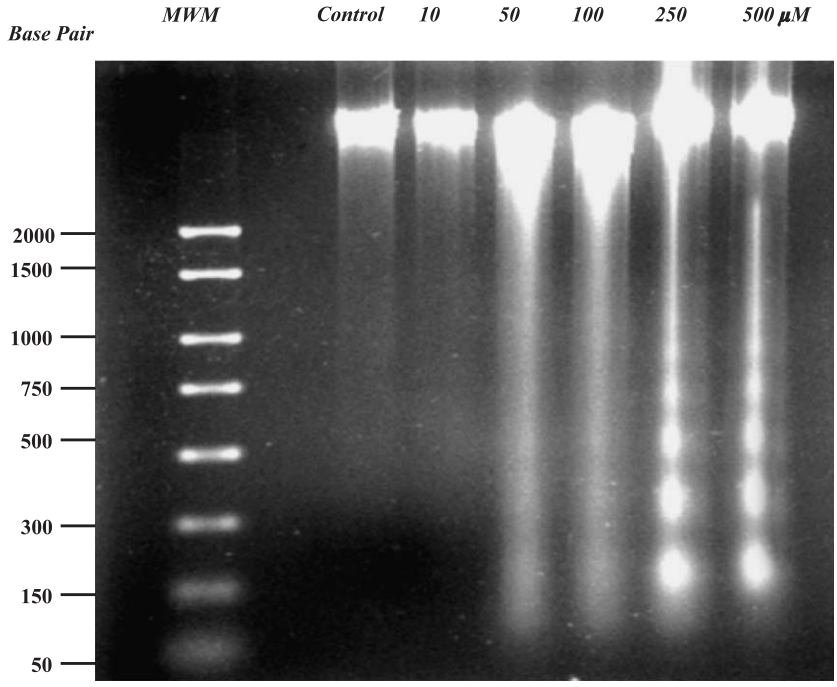

Fig. 2. Effects of 6-nitro-7-hydrocycoumarin (0–500 μM) on DNA fragmentation in A-498 cells treated for 96 h. Treated cells were collected and genomic DNA extracted using the Suicide-track™ DNA ladder isolation kit. DNA samples and molecular weight markers (MWM) were subjected to electrophoretic analysis using a 1.8% agarose gel. Bands were visualised by ethidium bromide staining and photographed under ultraviolet light. The presence of 180 base pair multimeric bands is a hallmark of apoptotic cell death, as executed by endogenous endonucleases. Gel presented represents a single experiment repeated three times.

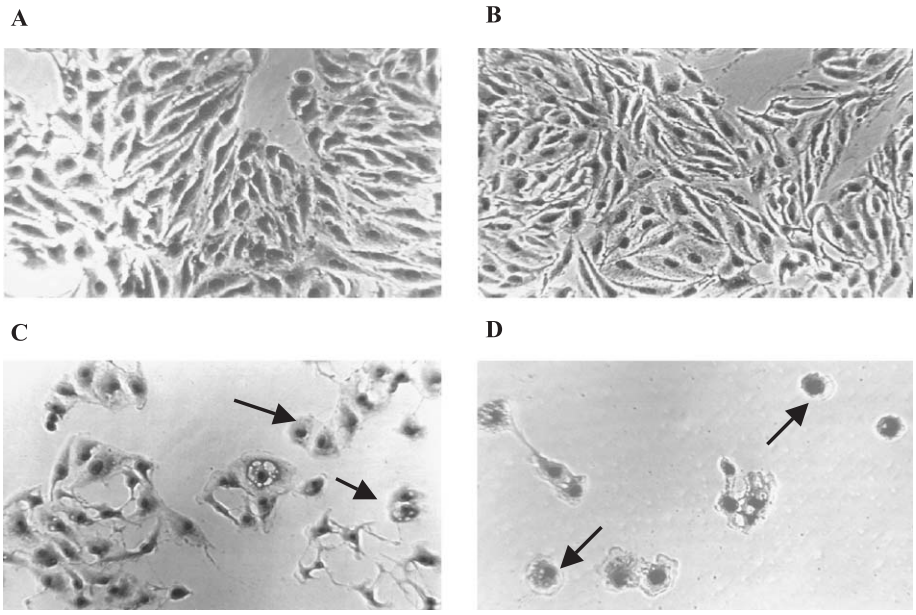

Fig. 3. Effects of 6-nitro-7-hydroxycoumarin (50 and 100 μM), culture media or solvent vehicle for 96 h on cellular morphology. Cells were stained with Giemsa and photographed using phase microscopy (250× magnification). Drug treatment resulted in a thinning of the monolayer and the appearance of rounded, vacuolated cells (arrows), with progressive nuclear shrinkage, morphology consistent with apoptosis. Cells eventually became detached from the culture dish (panels C and D). Cells cultured in growth medium alone and treated with solvent vehicle for 96 h had no effects on morphology (panels A and B,

(ADP-ribose) polymerase cleavage. 6-Nitro-7-hydroxycoumarin (0–500 μM) when combined with SB203580 (10 μM) for 96 h, resulted in the complete inhibition of 6-nitro-7-hydroxycoumarin induced expression of $p21^{WAF1/CIP1}$ (Fig. 4B) and a reduction in the amount of cleaved poly (ADP-ribose)polymerase product (89 kDa) (Fig. 4C). Fig. 5 shows the effects of co-incubating the p38 MAP kinase inhibitor (SB203580) with 6-nitro-7-hydroxycoumarin, on induction of apoptosis in A-498 cells. Those cells treated with 6-nitro-7-hydroxycoumarin (50 μM) alone for 96 h demonstrated

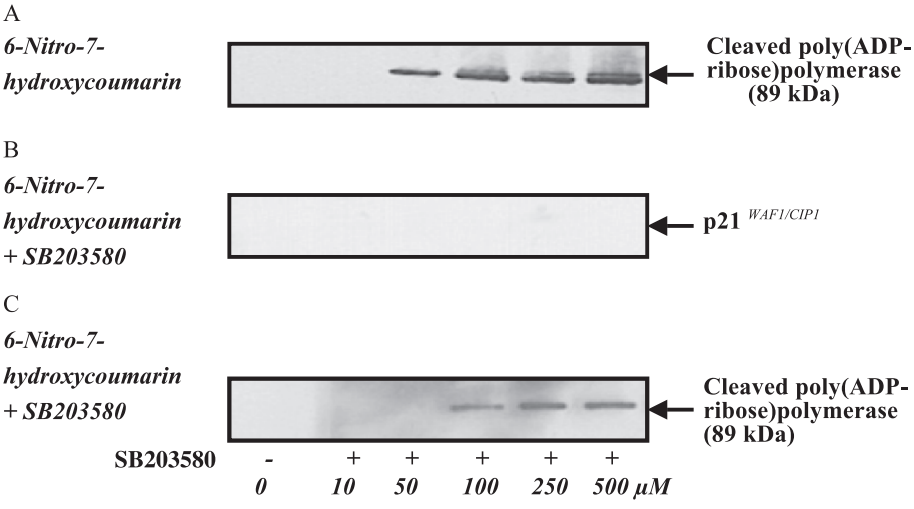

Fig. 4. Effects of 6-nitro-7-hydroxycoumarin treatment on (A) poly(ADP-ribose)polymerase cleavage in A-498 cells following 96-h incubation, (B) $p21^{WAF1/CIP1}$ expression and (C) cleaved poly(ADP-ribose)polymerase in the presence of the p38 MAPK inhibitor, SB203580 (10 μM). Treated cells were collected and lysed and proteins separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Bands corresponding to cleaved poly(ADP-ribose)polymerase (89 kDa) were detected, as shown by arrows. Cleaved poly(ADP-ribose)polymerase (89 kDa) appeared to increase with increased drug concentration. For expression of proteins in the presence of SB203580, bands corresponding to $p21^{WAF1/CIP1}$ (21 kDa) and cleaved poly(ADP-ribose)polymerase (89 kDa) were detected as described previously. Panels B and C show a complete inhibition of 6-nitro-7-hydroxycoumarin induced expression of $p21^{WAF1/CIP1}$ and a dose-dependant reduction in the amount of cleaved poly(ADP-ribose)polymerase product. Western blot shown is representative of three independent experiments.

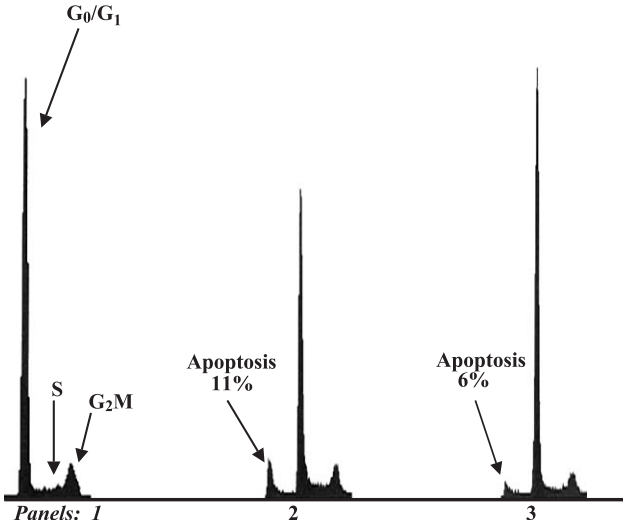

Fig. 5. Effects of 6-nitro-7-hydroxycoumarin (50 μM) treatment for 96 h in the presence of the p38 MAPK inhibitor, SB203580 (10 μM) on the percentage of A-498 cells with hypodiploid apoptotic cells, using flow cytometric analysis. Cells treated with solvent vehicle alone are shown in panel 1, where each phase of the cycle has been indicated by arrows. 6-Nitro-7-hydroxycoumarin (50 μM) treatment resulted in 11% of cells with hypodiploid DNA (panel 2), which decreased to 6% when 6-nitro-7-hydroxycoumarin (50 μM) was co-incubated with SB203580 (10 μM) (panel 3). Histograms shown are representative of three independent experiments.

11% of the cell population possessed hypodiploid DNA. However, when 6-nitro-7-hydroxycoumarin (50 μM) was combined with SB203580 (10 μM) for 96 h, it resulted in a reduction in this percentage from 11 to 6. These results indicate that p38 MAP kinase activation by 6-nitro-7-hydroxycoumarin was contributing to the transduction of cell death signals. The concentration of the MAP kinase inhibitor SB203580 (10 μM) used in these experiments was shown to have minimal cytotoxic effect in A-498 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cytotoxicity assay (data not shown).

4. Discussion

Our current study shows that 6-nitro-7-hydroxycoumarin exerts its cytotoxic effect in a renal cell carcinoma cell line, through a disturbance of cell cycle events, leading to cell death by apoptosis. Also, activation of p38 MAP kinase appears to play a role in mediating these events. In previous studies we have shown that 6-nitro-7-hydroxycoumarin is a potent and selective anti-proliferative agent to A-498 cells (Finn et al., 2002). More recently, 6-nitro-7-hydroxycoumarin was shown to alter the phosphorylation status of ERK1/ERK2, p38 MAP kinase and SAP kinase (Finn et al., 2003). 6-Nitro-7-hydroxycoumarin inhibited ERK1/ERK phosphorylation, which was constitutively active in A-498 cells. In addition, p38 MAP kinase phosphorylation was activated in a dose- and time-dependant manner, which was not constitutively active in A-498 cells. Finally, this compound was shown to activate SAP kinase. Therefore, the primary aim of the present study was to expand on the previous findings by examining further the effects of 6-nitro-7-hydroxycoumarin and p38 MAP kinase activation on A-498 cellular proliferation.

In the United States alone, the annual diagnosis of renal cell carcinoma in new patients is approximately 30,000 (Flanagan and Yonover, 2000; Broghammer and Ratliff, 2002). Currently, chemotherapy and cytokine treatments have produced insufficient response rates with complete remissions being rare (Vuky and Motzer, 2000). Oka et al. (1995) examined whether constitutive activation of the ERK MAP kinase pathway was associated with the carcinogenesis of renal cell carcinoma. They screened 25 human renal cell carcinoma tumours and normal kidney tissue. They found constitutive activation of ERK MAP kinase in 48% of cases. Furthermore, they found an overexpression of ERK kinase in 52% of cases, which correlated with ERK kinase activation, and suggested that constitutive activation of ERK MAP kinases is associated with the carcinogenesis of human renal cell carcinoma. Consequently, as 6-nitro-7-hydroxycoumarin has previously been shown to activate p38 and SAPK in A-498 cells, the relationship between this effect and specific cellular responses, including cell cycle progression and apoptosis was investigated.

All of the results, obtained and presented here, illustrate that 6-nitro-7-hydroxycoumarin is capable of inducing apoptotic cell death in A-498 cells. Results from flow cytometric studies showed that 6-nitro-7-hydroxycoumarin caused a G_1 phase accumulation (Fig. 5 and Table 1). This observation is considered to be a hallmark of apoptotic cell death, as during apoptosis, chromosomal DNA is cleaved by a caspase-activated DNase to facilitate phagocytosis (Darzynkiewicz et al., 2001). DNA isolated from treated A-498 cells showed the characteristic ladder pattern on agarose gel, due to internucleosomal DNA degradation (Fig. 2). Cytological examination of 6-nitro-7-hydroxycoumarin treated cells showed morphological characteristics consistent with apoptotic cell death (Fig. 3) (Hanahan and Weinberg, 2002). Other research groups have demonstrated that coumarin derivatives could induce apoptosis (Marshall et al., 1994; Egan et al., 1997) but only in non-epithelial-derived cell lines.

Since 6-nitro-7-hydroxycoumarin caused a G_1 phase accumulation, its effects on the G_1 phase regulator, cyclin D_1 were examined. Growth factor stimulated cells that are coming out of the G_0 phase, increase expression of cyclin D_1, which in turn binds to cyclin-dependant kinase 4 to activate the Rb/E2F pathway. 6-Nitro-7-hydroxycoumarin caused a moderate decrease in cyclin-D_1 protein expression (Fig. 1A) consistent with the observed cell cycle effects. A number of in vitro studies have examined the effects of coumarin molecules on cell cycle regulatory proteins in an attempt to explain their potential mechanism(s) of action.

Jimenez-Orozco et al. (2001) found that 7-hydroxycoumarin caused a G_1/S phase inhibition of cell cycle progression in A-427 cells, where coumarin had no such effect. They suggested that 7-hydroxycoumarin induced reductions in cyclin D1 were post-transcriptional, as messenger RNA levels for cyclin D_1 remained unchanged. Similarly, Chu et al. (2001) and Wang et al. (2002) demonstrated that 6,7-dihydroxycoumarin caused a G_1 phase arrest with reduced cyclin D1 levels in human HL-60 (human leukaemic) cells, resulting in apoptosis. Such findings are significant as increased expression of cyclin proteins, in particular G_1 related cyclins (D & E), have been found in several types of human tumours, which may in part explain the uncontrolled proliferation of cancer cells (Bartkova et al., 1997).

The effects of 6-nitro-7-hydroxycoumarin on the G1 associated cyclin-dependant kinase inhibitor, $p21^{WAF1/CIP1}$ showed that this coumarin derivative caused a dose-dependant induction in the cyclin-dependant kinase inhibitor, $p21^{WAF1/CIP1}$ (Fig. 1B). Up-regulation of p21 WAF1/CIP1 has been shown to correlate with an inhibition of cell growth, which ultimately decides the fate of the cell between differentiation and death (Goroscpe et al., 1999; Takuwa and Takuwa, 2001). Further evidence suggesting that 6-nitro-7-hydroxycoumarin induced apoptosis was provided by the dose-dependant increase in cleaved poly(ADP-ribose)polymerase (Fig. 4A), a 116-kDa nuclear enzyme, involved in genomic stability, which is proteolytically cleaved by the effector caspase-3 (Hanahan and Weinberg, 2000). Previously, 6-nitro-7-hydroxycoumarin has been shown to activate p38 and SAP kinase phosphorylation in A-498 cells (Finn et al., 2003). These results suggested that 6-nitro-7-hydroxycoumarin activated the two MAP Kinase cascades associated with cellular differentiation and death via apoptosis (Adler et al., 1999; Cross et al., 2000). To dissect the participation of p38 in the observed apoptotic response, the p38 MAP kinase inhibitor, SB203580 was co-incubated with 6-nitro-7-hydroxycoumarin. Results from the present study provide evidence to suggest that activation of p38 MAP kinase participates in 6-nitro-7-hydroxycoumarin induced apoptosis. This conclusion comes from the observation that co-treatment of A-498 cells with SB203580 and 6-nitro-7-hydroxycoumarin, resulted in a reduction in the percentage of apoptotic cells. This was evident as SB203580 suppressed 6-nitro-7-hydroxycoumarin induced poly(ADP-ribose)polymerase cleavage and the number of cells with hyopdiploid DNA (Figs. 4C and 5, respectively). The exact mechanism by which p38 MAP kinase plays a role in 6-nitro-7-hydroxycoumarin induced apoptosis remains to be defined. However, this pathway along with the SAPK pathway have been implicated in negative regulation of pro-survival members of the *Bcl* family, which may ultimately tilt cells towards a death response (She et al., 2001).

It seems likely that a dynamic down regulation of ERK1/ERK2 coupled with activation of SAP and p38 MAP kinase results in the predomination of a death rather than a survival signal. It is this event, which may allow SAPK and p38 MAP kinase to phosphorylate nuclear protein targets and transcription factors, leading to an up-regulation in the expression of pro-apoptotic effector molecules. For example, activation of SAPK and p38 MAP kinase, can lead to transcriptional activation of the tumour suppressor gene, p53, which down-regulates the expression of anti-apoptotic, *Bcl-2*. In addition, it appears unlikely that modulation of the MAP kinase pathway by 6-nitro-7-hydroxycoumarin is exclusively responsible for the death response. Instead, it is more probable that this molecule inhibits a number of pro-survival signal cascades, possibly by inhibition of catalytic function through steric inhibition of co-factor binding sites. Evidence to support this hypothesis comes from the findings of Lu et al. (1997) who have shown that coumarin displays similar electron densities distribution, around the lactone moiety, to that of guanine. Consequently, down regulation of catalytic activity of pro-survival signalling molecules could initiate stress signals, resulting in activation of p38 and SAPK MAP kinases and finally apoptosis.

Taken together, these findings suggest that 6-nitro-7-hydroxycoumarin is capable of inducing apoptosis in A-498 cells by modulation of MAP kinase signalling. This finding is particularly noteworthy as it is widely regarded that over-expression of anti-apoptotic regulators contributes to renal cell carcinoma pathogenesis (Gobe et al., 2002). In addition, this study is the first report of a coumarin derivative inducing apoptosis in a malignant epithelial cell line. Consequently, the participation of p38 MAP kinase in 6-nitro-7-hydroxycoumarin induced apoptosis of A-498 cells suggests that targeting of p38 may represent a novel mechanism to inhibit renal cell carcinoma and that coumarin type drugs require further investigation as potential anticancer agents directed against renal cell carcinoma.

Acknowledgements

This research was supported by the Graduate Training Programme, under the European Social Fund Operational Programme for Industrial Development and the Higher Education Authority, Ireland. This research was carried out in the National Centre for Sensor Research (NCSR), jointly located at Institute of Technology, Tallaght and Dublin City University, Dublin 9, Ireland.

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