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Daphnetin induced differentiation of human renal carcinoma cells and its mediation by p38 mitogen-activated protein kinase

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Abstract

Daphnetin has been shown to be a potent in vitro anti-proliferative agent to the human renal cell carcinoma (RCC) cell line, A-498. In the present study, we investigated its effects on mitogen-activated protein kinase (MAPK) signalling along with cell cycle events and cellular differentiation. Daphnetin-activated p38, however, higher concentrations were required to inhibit ERK1/ERK2. In addition, it did not activate SAPK or induce apoptosis, but instead inhibited S phase cell cycle transition of A-498 cells at low concentrations and time of exposure. In addition, a late G_1, early S phase inhibition was observed at higher concentrations and time of exposure, indicating that the mechanism of daphnetin-induced differentiation was concentration dependent. Increased expression of the epithelial differentiation markers cytokeratins 8 and 18, correlated with increasing concentrations of daphnetin, while pre-treatment with a specific p38-inhibitor, served to limit this effect. There was no evidence that P-glycoprotein (P-gp) mediated multi-drug resistance (MDR) played a role in the anti-proliferative activity of daphnetin. Consequently, we concluded that p38 MAP kinase is intrinsically involved in mediating the effect of daphnetin in A-498 cells, suggesting that this drug may act by promotion of cellular maturation, and consequently may represent a novel low toxic approach for the treatment of poorly differentiated RCCs.

Keywords: Coumarin; Daphnetin; Renal cell carcinoma; MAP kinase; Differentiation; P-glycoprotein

1. Introduction

The coumarins comprise a large class of phenolic phytochemical compounds known as benzopyrones, all of which consist of a benzene ring fused to an alpha (α) or gamma (γ) ring containing one oxygen and five carbon atoms. Coumarin (1,2-benzopyrone) belongs to the benzo-α-pyrones, while flavanoids contain the γ-pyrone ring [1,2]. Coumarins and related compounds have been used clinically in the treatment of thrombotic disorders, chronic infections thermal injuries and immunological disorders and have also been evaluated in the treatment of various malignancies including, renal cell carcinoma (RCC) and malignant melanoma [3–9]. In the United States alone, the annual diagnosis of RCC in new patients runs to approximately 30,000 and accounts for almost 12,000 deaths [10–12]. Approximately 85% of RCCs are adenocarcinomas, being predominately proximal tubular in origin. Currently, chemotherapy and cytokine treatments of patients with RCC have produced insufficient response rates with complete remissions being rare [11].

The mitogen-activated protein kinases (MAPKs) are fundamental components of the signalling pathways transducing extracellular stimuli into a variety of cellular responses [13]. Members of the MAPK pathways include the extracellular signal related kinase (ERK), the c-jun kinase (JNK), also referred to as the stress-activated protein kinase (SAPK), and the p38 kinase modules [14,15]. Although exceptions occur, the majority of research suggests that activation of the ERK pathway is implicated in cellular proliferation, differentiation and survival. Conversely, activation of the SAPK and p38 kinase cascades is generally, although not exclusively, associated with inflammation, apoptosis and cellular differentiation [16,17]. Activation of the *Ras*/ERK/MAPK and other signalling

Abbreviations: RCC, renal cell carcinoma; MAPK, mitogen-activated protein kinase; ERK, extracellular signal related kinase; SAPK, stress-activated protein kinase; P-gp, P-glycoprotein; MDR, multi-drug resistence; DMSO, dimethylsulphoxide; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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cascades, through their translocation to the nucleus, ultimately activate MAPKs, and lead to activation of transcription factors. In turn these transcription factors regulate gene expression of molecules involved in homeostatic responses such as cell growth, proliferation and differentiation and may therefore represent potential novel targets for the prevention and/or treatment of cancers including RCC.

A recent study in our laboratory demonstrated the anti-proliferative effects of the coumarin derivative, daphnetin (7,8-dihydroxycoumarin), using in vitro cytotoxicity assays with two human renal cell lines. The first cell line, A-498 was a human RCC line, while the second was a non-carcinoma proximal tubular cell line termed, HK-2. Results clearly demonstrated that daphnetin was a potent anti-proliferative agent in the RCC cell line, and was significantly less toxic to the HK-2 cells, suggesting that this compound might be capable of selectively inhibiting A-498 rather than HK-2 cells. In addition, mobility shift and BrdU incorporation assays showed that daphnetin did not intercalate DNA but had a concentration-dependent inhibitory effect on its synthesis. Genotoxicity testing using the standard Ames test showed that this compound was not a mutagen either with or without a mammalian metabolic activation system [18]. Taken together, these results suggested that daphnetin warranted further investigation as an agent in the treatment of RCC.

In an attempt to explore the possible therapeutic potential of daphnetin in the treatment of RCC, we have focused our attention on elucidating its effects on a number of key cellular targets. These targets include; MAPK activation, cell cycle and key differentiation parameters along with the possibility that daphnetin may act as a substrate for P-gp mediated MDR. The selection of these molecular targets was based on the knowledge that they are centrally involved in the cause and development of RCC. Results from previous studies have shown that constitutive activation of the *Ras*/ERK/MAPK pathway has been implicated in the progression of and often the de-differentiated phenotype of RCC [19–21]. It is thought to be this factor may lead to the almost complete failure of systemic drug therapy regimes for RCC [22]. This may, in the main, be due to the highly drug resistant phenotype and slow growth kinetics observed for RCC. This theory is supported by the observation that RCC, with the exception of adrenal carcinomas, expresses the highest levels of the MDR-1 gene product, P-gp [23–25]. It has been established that over-expression of P-glycoproteins (P-gp) is responsible for the ''classical'' type of MDR. The drugs affected by this mode of resistance include: anthracyclines, epipodophyllotoxins (VP-16 and VP-26) and *Vinca* alkaloids, among others. The multi-drug transporter associated with MDR-1, P-gp-170 prevents the intracellular accumulation and as a consequence inhibits the cytotoxic effects of chemotherapeutic drugs by actively removing them from the cell before they reach their intra-cellular targets. Consequently, as the MDR phenotype of RCC greatly affects clinical prognosis, it should prove valuable to determine the potential of any novel drugs directed against RCC to act as an MDR substrate. Finally, the aim of the current study was to elucidate key aspects of the mechanism of action of daphnetin and provide evidence that this compound may offer a significant advantage in the treatment of RCC.

2. Materials and methods

2.1. Test compounds and reagents

Vinblastine, melphalan, dimethyl sulphoxide (DMSO) and SB203580 were purchased from Sigma–Aldrich, Ltd., while 7,8-dihyroxycoumarin (7,8-OHC, Daphnetin) was purchased from Lancaster Synthesis. The chemiluminescence substrate was purchased from Pierce Laboratories Ltd. All phosphorylation state specific antibodies against ERK, p38 and SAPK were purchased from New England Biolabs, Inc. Mouse monoclonal antibodies against cytokeratin 8 and cytokeratin 18 were purchase from Calbiochem. Jsb-1, the antibody specific for P-gp was purchased from Cappell. All cell culture reagents and media were purchased from Euroclon unless otherwise stated.

2.2. Cell lines and cell culture

A-498 cells (human kidney adenocarcinoma) were purchased from the American Type Culture Collection and maintained in EMEM with Earles balanced salt solution, supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS; Flow Laboratories). Chinese Hamster ovary cells (CHO-K1 and CH^RC5) were kindly provided by Dr. V. Ling, Ontario, Canada. CHO-K1 cells were maintained in Nutrient Hams F-12, containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) FCS. CH^RC5 cells were maintained in EMEM, Alpha modification (without ribonucleosides and deoxynucleosides), containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) FCS. All cell lines were grown at 37 °C in a humidified atmosphere and in the presence of 5% CO_2.

2.3. Immunoblot analysis

Daphnetin was dissolved in DMSO and diluted in culture media. A-498 cells were grown in 100 mm dishes and treated with either DMSO alone or daphnetin at concentrations of 5, 10, 50, 100, 250 or 500 μM. Following 48, 72 and 96 h exposure, cells were washed twice with ice-cold 0.1 M phosphate buffered saline, pH 7.4 (PBS) (Sigma) and harvested by scraping with a rubber policeman. Cells were then centrifuged at 3000 × *g* for 5 min. Whole cell extracts were prepared by resuspension in lysis buffer (M-per lysing solution (Pierce)), supplemented to

contain 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonylflouride (PMSF), 2 μg/ml leupeptin and 2 μg/ml aprotinin. After 15 min on ice, the mixture was clarified by centrifugation for 5 min at 10,000 × *g* and the resultant supernatant collected. Protein concentrations were determined using the method described by Bradford [26], and bovine serum albumin was used as a protein standard. Forty micrograms of total protein lysates were resolved using electrophoresis on a 12% polyacrylamide gel (PAGE) and transferred to nitrocellulose membranes. The levels of phospho- and native proteins were determined using specific primary antibodies, followed by a peroxidase-conjugated secondary antibody and visualisation by chemiluminescence. For SAPK immunodetection in A-498 cells, a commercial positive control (New England Biolabs), consisting of total cell extracts from 293 cells treated with UV light was loaded to ensure that the detection system was functioning correctly as SAPK was not constitutively active in these cells.

2.4. Confirmation of MDR-1 phenotype

The pleotrophic drug-resistant cell line (CH^RC5) has previously been shown to over-express the MDR-1 gene product, P-gp 170 and exhibit cross-resistance to structurally dissimilar drugs, while the P-gp negative CHO-K1 fails to exhibit an MDR-1 phenotype [27]. To confirm these drug phenotypes both CH^RC5 and CHO-K1 cells were assessed for chemosensitivity by continuous exposure to two anti-cancer drugs, vinblastine and melaphalan. The viability of CH^RC5 and CHO-K1 cell lines were assessed in parallel using a 96-well format with increasing concentrations of vinblastine and melaphalan (0–10 μM). Briefly, cell density was adjusted to 5×10^4 cells/ml for CHO-K1 and CH^RC5 and 100 μl of cell suspensions was added per well to a 96-well plate (Falcon). Following overnight incubation, a concentrated stock solution of each of the test compounds in DMSO stored at −20 °C was thawed directly prior to the assay and the required dilutions were prepared by serial dilution in complete culture medium. The maximum concentration of DMSO present in all wells was 0.05% (v/v), a concentration which exhibited no effect on cell morphology or growth. Viability was determined using the MTT assay [18]. The relative degree of resistance for each cell line was determined by comparison of the IC_{50} values [28].

2.5. Cell membrane preparation for P-gp

The reduced permeability of CH^RC5 cells to anti-cancer drugs has been attributed to the presence of the integral membrane glycoprotein with an apparent molecular weight of 170 kDa (P-gp 170). A membrane preparation was undertaken to confirm the presence of the ATP-binding cassette in CH^RC5 cells, and its absence in CHO-K1 cells. Cell membranes were prepared according to the method of Doige and Sharom [29]. Briefly, CH^RC5 and CHO-K1 cells were cultured in 150 mm culture dishes, washed in ice-cold PBS and harvested by scraping as before. Cells were then centrifuged at 2500 × *g* for 10 min. Cell pellets were resuspended in 5 ml of ice-cold homogenisation buffer (10 mM Tris–HCl, 250 mM sucrose, 0.2 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 7.5, containing 1 μg/ml of PMSF, leupeptin and pepstatin A). Cells were ruptured with a sonicating probe (Sonix) for 10 s and maintained on ice throughout. Homogenates were clarified by centrifugation at 6000 × *g* for 60 min at 4 °C, in order to resolve the membrane vesicles. The pellet was resuspended in 5 ml of solubilisation buffer (25 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM Tris–HCl, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.02% (w/v) NaN_3, pH 7.5) and incubated at 4 °C for 5 h. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was collected and 40 μg of total protein lysates were resolved using a 7.5% PAGE gel and detected by immunoblot analysis, with a primary mouse monoclonal antibody JSB-1, as described previously.

2.6. Cell cycle analysis

A-498 cells were grown in 100 mm dishes and treated with either DMSO alone or daphnetin for 48, 72 or 96 h. For inhibitor studies, cells were incubated with daphnetin alone or co-incubated with daphnetin (50 and 500 μM) and SB203580 (50 μM) for 96 h. Cells were then washed twice with ice-cold PBS and harvested by trypsinisation and centrifugation. A suspension of cells were fixed and permeabilised by the vigorous addition of 9 ml of ice-cold 70% (v/v) ethanol and then stored at −20 °C for a minimum of 24 h prior to analysis. Cells were resuspended at a density of 1×10^6 in 800 μl of propidium iodide (PI) staining solution (20 μg/ml PI and 200 μg/ml RNase A in PBS, pH 7.4). Cells were then incubated in the dark at room temperature for 30 min and cell cycle distributions were determined by flow cytometry using Cell Quest™ Software and ModFit LT™ Software (Verity Software) by gating on the G_0/G_1/S/G_2M singlet population to exclude aggregates and estimate cell cycle phase distributions. All samples were analysed in triplicate.

3. Results

3.1. Role of MAPK phosphorylation

Since ERKs, SAPK and p38 modules are the functional convergence points for these three distinct signalling pathways of the MAPK family, the effect of daphnetin on their phosphorylation state was investigated [30]. As shown in Fig. 1A, treatment of A-498 cells with daphnetin over the concentration range 0–500 μM did not inhibit constitutively active ERK1/ERK2 phosphorylation levels at the

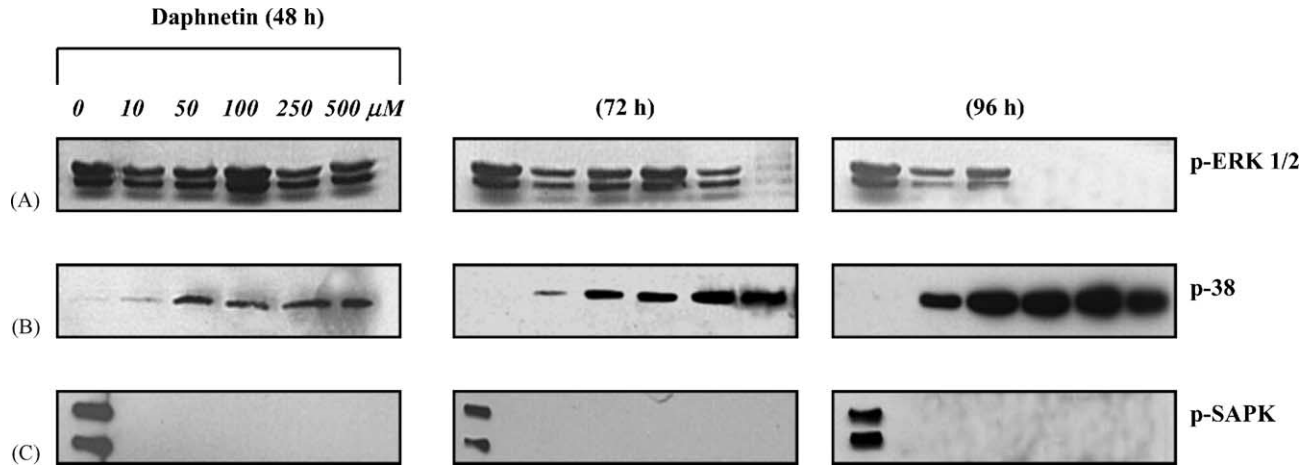


Fig. 1. Time-course activation of (A) ERK1/ERK2, (B) p38 and (C) SAPK MAPK by daphnetin in A-498 cells. Cells were incubated with either DMSO (vehicle: 0.1%, v/v) or increasing concentrations of daphnetin for periods of 48, 72 and 96 h. Activation levels of members of the MAPK family was analysed by immunoblot with their corresponding phospho-specific antibodies. For SAPK immunodetection in A-498 cells, a commercial positive control (New England Biolabs), consisting of total cell extracts from 293 cells treated with UV light was loaded to ensure that the detection system was functioning correctly. The results are representative of three independent experiments.

48 h time point, when compared to control cells that received drug vehicle alone. However, phosphorylation was inhibited at the higher concentration points and at the longer incubation times of 72 and 96 h. Also, there was no active ERK1/ERK2 detectable at concentrations above 50 μM and 96 h, suggesting that daphnetin inhibits the mitogenic signalling of ERK1/ERK2. The fact that daphnetin inhibited ERK1/ERK2 phosphorylation conclusively at 96 h and not at 48 and 72 h is interesting as this suggests that a longer treatment with a lower dose produces greater inhibition than shorter treatment times with higher doses.

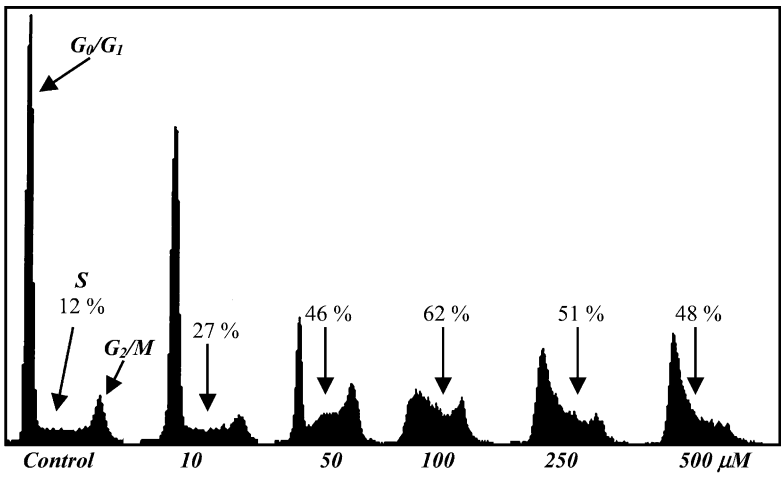
With regard to p38 MAPK, daphnetin caused a dose- and time-dependent activation of p38 MAP kinase, which was not constitutively active in A-498 cells (Fig. 1B). The observed effect of daphnetin on p38 MAPK was much stronger than that observed for ERK1/ERK2, where p38 activation was observed at all drug concentrations and as early as 48 h following treatment. Also, this activation continued through 72 and 96 h and was dose-dependent. Unlike its effects on ERK1/ERK2 and p38 activation, daphnetin did not show any effect on the phosphorylation status of SAPK, which was not constitutively active in A-498 cells (Fig. 1C). Taken together, these findings suggest that treatment of A-498 cells with daphnetin resulted in moderate inhibition of ERK1/ERK2 and considerable activation of p38, the MAPKs which have largely been associated with cell growth and differentiation, respectively [31–35].

3.2. Inhibition of cell cycle progression

The effect of daphnetin on cell cycle progression was determined using flow cytometric analysis. As illustrated in Fig. 2A and B cell cycle analysis of A-498 cells which had previously been treated with daphnetin, resulted in an S phase accumulation at all of the test concentrations, and following 48 and 72 h incubation. The observed S phase accumulation was accompanied by a significant decrease in the portion of cells in the G_1 phase. However, at concentrations of 250 and 500 μM and following 96 h incubation, daphnetin appeared to cause a significant late G_1/early S phase accumulation (Fig. 2A). Examination of the histograms indicated that there was no sub-G_1, hypodiploid cells evident in DNA histograms indicating apoptosis was not occurring. In addition, further evidence for this hypothesis arose from immunoblot analysis of PARP (poly-ADP-ribose polymerase), which did not show cleavage of the 116 kDa isoform to 89 kDa, the fragment resulting from caspase-3 cleavage (data not shown). Finally, to examine whether the cell cycle effects observed for daphnetin correlated with the observed changes in phosphorylation status of p38, A-498 cells were co-treated with daphnetin and the specific p38 MAPK inhibitor, SB203580 for 96 h. The resultant effect on cell cycle events was determined using flow cytometry. Fig. 3 shows that SB203580 treatment (50 μM) inhibited the late S phase accumulation caused by daphnetin, as less cells accumulated in this phase of the cell cycle. In addition, SB203580 (50 μM) also abrogated the late G_1/early S phase inhibition observed for daphnetin (500 μM), as more cells completed the S phase transition as indicated by a decrease in the S phase and an increase the G2/M phase peak signals (Fig. 3). Both of these findings indicate that p38 MAPK appears to at least participate in the signalling response mediating the activity of daphnetin in A-498 cells.

3.3. Expression of cellular differentiation markers

Results from MAPK studies, together with the absence of apoptotic cells, lead us to investigate if a response



(A)

Daphnetin	Control	10 μM	50 μM	100 μM	250 μM	500 μM
48 h						
% G_0/G_1	67 ± 3	60 ± 4	58 ± 6	50 ± 2	32 ± 4	15 ± 2
% S	24 ± 3	28 ± 2	34 ± 4	40 ± 4	61 ± 5	75 ± 9
% G_2M	9 ± 1	12 ± 2	8 ± 2	10 ± 2	7 ± 2	9 ± 3
72 h						
% G_0/G_1	74 ± 4	67 ± 1	61 ± 4	46 ± 7	39 ± 1	18 ± 2
% S	18 ± 3	23 ± 5	27 ± 6	42 ± 6	49 ± 1	68 ± 4
% G_2M	8 ± 1	10 ± 2	12 ± 2	12 ± 2	12 ± 2	13 ± 1

(B)

Fig. 2. Flow cytometric analysis of daphnetin on cell cycle progression in A-498 cells. DNA histograms are presented showing the effects of drug exposure following (A) 96 h and (B) 48 and 72 h, and over a concentration range of 0–500 μM. The results are representative of three independent experiments.

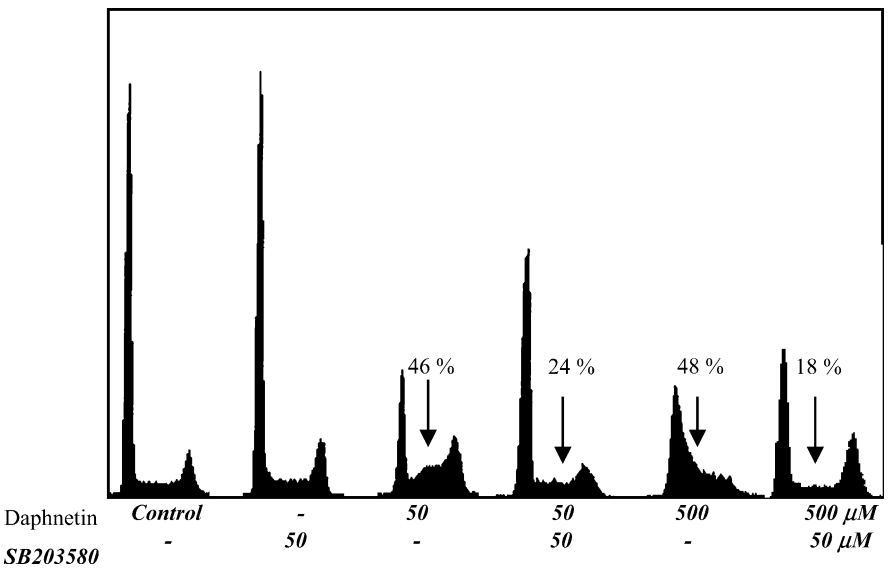


Fig. 3. Effects of SB203580 on daphnetin induced cell cycle inhibition in A-498 cells. DNA histograms are presented showing the effects of daphnetin exposure following 96 h alone (50 and 500 μM) and in combination with the p38 inhibitor, SB203580 (50 μM). Results presented are representative of three independent experiments.

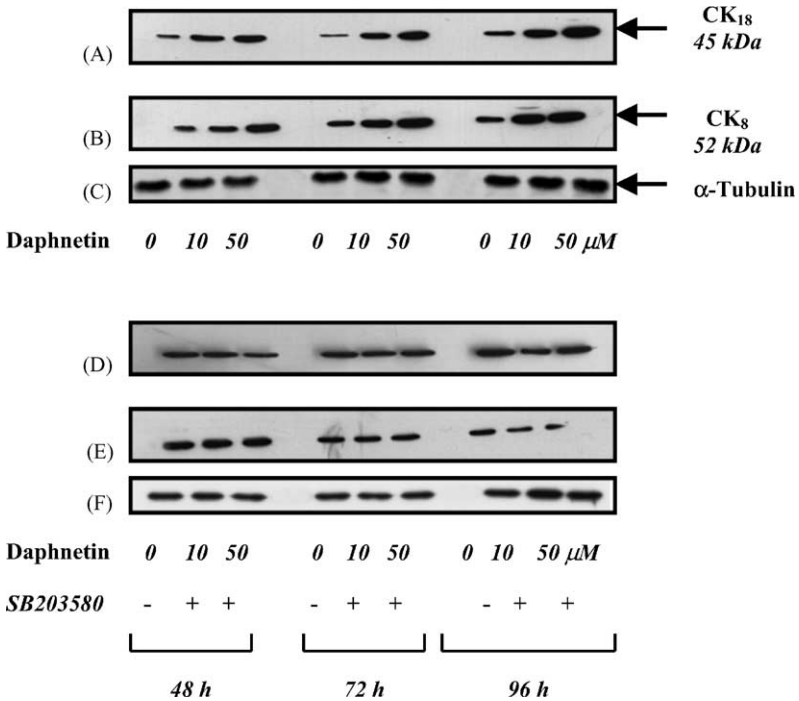


Fig. 4. Time-course analysis of the effects of daphnetin on epithelial differentiation markers cytokeratins 18 (A) and 8 (B) in A-498 cells along with the effects of the specific p38 MAPK inhibitor, SB203580, on daphnetin induced increased expression of cytokeratins 18 (D) and 8 (E). Cells were incubated with daphnetin (0, 10 and 50 μM) or SB203580 (10 and 50 μM) for 48, 72 and 96 h and expression levels of cytokeratins 8 and 18 were determined by immunoblot analysis. Panels C and F represent α-tubulin loading control. Results shown are representative of three independent experiments.

independent of apoptosis was responsible for the antiproliferative effect of this compound. Daphnetin treatment for 48, 72 and 96 h resulted in a dose- and time-dependent increase in the expression of cytokeratins 18 (45 kDa) and 8 (52 kDa), when compared to solvent treated control cells (Fig. 4A and B). This increase was apparent as early as 48 h and continued through the duration of study (96 h). p38 activation has been implicated in differentiation responses in a variety of cell types [40,41]. Therefore, we examined the effect of the specific p38 inhibitor, SB203580, on daphnetin induced p38 activation. This approach allowed us to determine if daphnetin in the presence of inhibitor could increase the expression of cytokeratins 8 and 18. As shown in Fig. 4C and D, co-treatment of A-498 cells with SB203580 and daphnetin at concentrations of 10 and 50 μM, for 48, 72 and 96 h appeared to inhibit the effect of daphnetin on expression of cytokeratins 8 and 18. These results again indicate that p38 may play a central role in the ability of daphnetin to alter the differentiation status of RCCs.

3.4. P-gp mediated MDR

It has been established that over-expression of P-gp is responsible for the ''classical'' type of MDR. This has been defined as the simultaneous resistance of a cell against a variety of structurally dissimilar cytotoxic drugs [42,43]. Also, since RCC exhibits extremely high levels of this protein cassette, we decided to evaluate the ability of P-gp to inhibit the growth inhibitory effects of daphnetin. Using a cellular model of MDR-1, we confirmed the MDR phenotype by immunological detection of P-gp along with chemosensitivity testing using vinblastine and melphalan. This method allows the MDR phenotype to be assessed by detection of the protein effector molecule and by comparison of the relative toxicities (IC_{50}) in the MDR positive and negative cell lines. Consequently, drugs that display less or equal effects in the MDR positive cell line could be potential MDR substrates. Furthermore, comparison of the relative experimental toxicities allows the calculation of a resistance factor. This factor was calculated by dividing the drug toxicity (IC_{50} value) observed in the MDR positive cells by the drug toxicity in the MDR negative cells. Fig. 5 shows that there was no detectable P-gp in the MDR negative cells (CHO-K1), while there is considerable P-gp expression in the MDR positive cells (CH^RC5). In

Table 1
Comparative toxicity data and calculated resistance factors used for confirming MDR phenotype and demonstrating whether daphnetin was a substrate for P-gp

Compound	Toxicities (IC_{50}) (μM)		Resistance factor
	$MDR^{(+)}$ CH^RC5	$MDR^{(-)}$ CHO-K1	
Vinblastine	0.490	0.012	40
Melphalan	0.298	0.041	7
Daphnetin	137	453	0

IC_{50} values were calculated from triplicate cytotoxicity assays using MTT. Resistance factors were calculated by division of $MDR^{(+)}$ IC_{50} data by $MDR^{(-)}$ IC_{50} data.

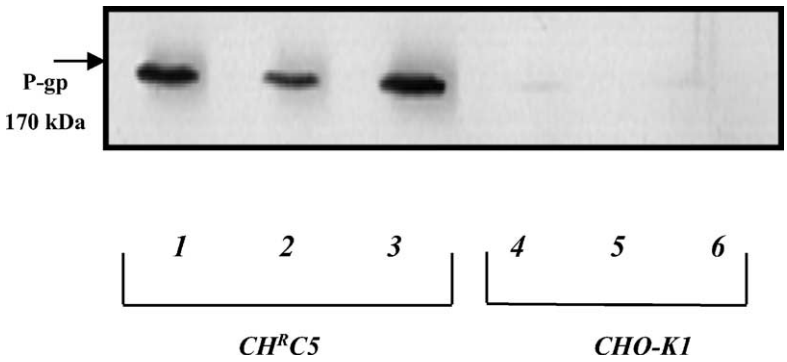


Fig. 5. Immunoblot analysis of P-gp in membrane isolates of the pleiotrophic MDR positive cell line (CH^RC5) and the MDR negative cells (CHO-K1). Cell membrane proteins were isolated and separated on a 7.5% PAGE gel. The proteins were transferred to nitrocellulose and probed with mouse monoclonal anti-human P-gp antibody (JSB-1) and a HRP-conjugated secondary antibody. A band corresponding to P-gp at a molecular weight of 170 kDa (indicated by an arrow) was detected in the CH^RC5 cell membranes (lanes 1–3) but was absent in CHO-K1 (lanes 4–6). Results presented are representative of three independent experiments.

addition, Table 1 shows the data obtained for the cytotoxicity observed with vinblastine, melphalan and daphnetin. These results indicate that the MDR phenotype of the CH^RC5 cells were retained, as P-gp was detectable in CH^RC5 cells and that vinblastine and melphalan demonstrated resistance factors of 40 and 7, respectively. However, data obtained for daphnetin illustrated that it was not a potential MDR-1 substrate, since it exhibited greater antiproliferative effect in the MDR positive cell line (Table 1).

4. Discussion

Normal cellular growth, proliferation, differentiation and apoptosis is governed by cellular signalling that functions to transduce signals by either allowing or halting progression of a cell through cycle check points ultimately toward their cellular fate [36,37]. Development of RCC from normal renal epithelium involves alterations in genes whose products control cell division. These include genes that participate directly in controlling cell cycle, such as Rb, the tumour suppressor gene *p53*, and the *Ras* gene, with products from the latter controlling mitogenic signalling [44]. *p53* mutations in RCC are generally infrequent but may be involved in the development of metastatic disease [45]. Mutations in other cell cycle related genes such as cyclin dependant kinase inhibitors (CDKIs) and *Ras* are also infrequent in RCC, but constitutive activation of down stream mediators of the *Ras* pathway may be involved in disease progression. For example, Oka et al. [20] examined whether constitutive activation of the MAP kinase pathways was associated in the carcinogenesis of RCC. They screened 25 human RCC tumours and their corresponding normal kidney tissues and found constitutive activation of MAP kinases in 48% of cases studied. Furthermore, they found an over-expression of MEK in 52% of cases, which correlated with ERK activation. They suggested that constitutive activation of ERK is associated with carcinogenesis of human RCC. In addition, this type of activation in kidney epithelial cells has been shown to destabilise epithelial architecture, which disrupts morphogenesis and induces an invasive phenotype [16]. Furthermore, the relevance of aberrant MAP kinase signalling has been demonstrated in pathophysiological conditions of the kidney including, toxic renal injury, inflammation, hypertonic stress and polycystic kidney disease [14].

In this study, we show that daphnetin inhibited constitutively active ERK1/ERK2 in A-498 cells only at elevated concentrations and longer incubation times in comparison to the results observed for p38 activation, which suggested that its major mechanism of action was p38 mediated. Also, daphnetin-activated p38 but failed to demonstrate a parallel activation of SAPK, which implies a possible differentiation response, rather than apoptotic cell death. Indeed, a role for p38 MAP kinase activation during differentiation has been reported by many authors and in several cell types [40,41,46–53].

Differentiation is often regarded as an exit from the G_1 phase of cell cycle, where proliferation has an inverse relationship to differentiation status [38]. Consequently, we examined the effects of daphnetin on the cell cycle progression of A-498 cells. We found that daphnetin caused a significant S phase accumulation at the lower concentration and time points of 48 and 72 h. However, at the longer time point of 96 h and higher concentrations daphnetin appeared to inhibit the cell cycle late in the G_1-S phase transition. These findings indicate that daphnetin, appears to have different effects at short time and low drug concentration combinations in comparison to longer incubation times and higher drug concentration combinations. The inhibition of S phase cycling observed at 48 and 72 h are consistent with previous findings where daphnetin inhibited DNA synthesis [18]. In this case, it is possible that the S phase accumulation of A-498 cells is associated with a slowing of replication at the DNA synthesis phase. Consequently, it is possible to hypothesise that at the lower concentrations and time points examined in this study,

daphnetin may be inhibiting a key component of the cell cycle machinery that functions principally during the S phase transition, such as CDKs 2, 4, or 6. In contrast, the mechanism of action of daphnetin at the higher concentrations and longer times, where a late G_1 to early S phase inhibition was observed, requires further investigation. To establish if p38 MAPK was participating in mediating the effects of daphnetin on cell cycle transition in A-498 cells, we used the specific p38 inhibitor, SB203580 to inhibit p38 during daphnetin treatment. Our results showed that SB203580 treatment modulated the cell cycle inhibitory effects observed for daphnetin, indicating that p38 does infact participate in governing the activity of daphnetin. However, it is unlikely the p38 is the sole regulator of daphnetin, as SB203580 did not totally abrogate the effects of daphnetin on cell cycle inhibition in A-498 cells. Consequently, we attempted to establish if there was a correlation between the observed effects of daphnetin on MAPK activity and cell cycle transition and up-regulation of differentiation markers.

Differentiation is essentially the process by which immature, unspecialised cells move towards a more mature state in order to perform specialised functions. Consequently, normal cells have a limited proliferative life span, after which they loose the ability to divide [55]. Alternatively, some cancer sub-types are said to display alterations in normal programs of differentiation and growth control, such that they display differing phenotypic properties associated with the mature cell [56]. In particular, leukemia's arise from increased self-renewal at the expense of differentiation or maturation and are considered blocked at a point where they continue to proliferate [57]. In some cases, cells can be treated with differentiating agents such as phorbol esters and retinoic acid which promote differentiation and maturation, leading to a down-regulation in proliferation [54,58]. Altered differentiation properties are also found in solid tumours and recently the effect of differentiating agents has been investigated in numerous solid tumour types including urological neoplasms [39].

To establish whether the observed activation of p38 MAPK was linked to a definitive differentiation response, we next examined the effect of daphnetin on expression of cytokeratins 8 and 18. Cytokeratins are intermediate filament proteins, which are specific lineage markers of epithelial cells and their expression is widely regarded as a marker of epithelial differentiation [59]. Consequently, immunohistochemical profiling of tumours for their cytokeratin profile not only allows lineage determination but also the differentiation status of a particular epithelial neoplasia to be evaluated [60]. Results from our studies showed that treatment of A-498 cells with daphnetin resulted in increased expression of cytokeratins 8 and 18 in a dose- and time-dependent manner. In addition, inhibition of daphnetin-induced activation of p38 MAP kinase with SB203580 modulated increased cytokeratin expression. These results, in addition to those findings observed for daphnetin on cell cycle transition suggest that p38 actively participates in the differentiation response elicited by daphnetin. Increased expression of cytoskeletal proteins such as the cytokertains have been shown in tumours of epithelial origin previously treated with differentiating agents. For example, Jing et al. [61] demonstrated an up-regulation in cytokeratins 8 and 18 in T47D breast cancer cells treated with trans retinoic acid, which was retinoic acid receptor-dependent. In addition, Agarwal et al. [62] describe the anti-proliferative effects of procyanidin phytochemicals in the MDA-MB468 breast carcinoma cell line. They established that these agents elicited a differentiation response, which was characterised by up-regulation of cytokeratin 8, and marked morphological alterations. Furthermore, these effects were accompanied by alterations in ERK1/ERK2 and p38 activity but not SAPK MAP kinase. Also, they did not find any evidence of apoptotic cell death. Consequently, it is possible that daphnetin modulates the differentiation status of A-498 cells by activation of the p38 MAP kinase pathway, which may in part explain the anti-proliferative effects observed following treatment with this coumarin compound.

Further evidence to substantiate this hypothesis arises from a recent publication by Mertins et al. [24] who describe a study undertaken to identify novel agents directed specifically at RCC. They surveyed cytotoxicity data from the National Cancer Institutes (USA) drug screen database for compounds that displayed selective toxicity to RCC. From 70,000 experimental compounds, they identified 16 potential candidates one of which, NSC 72151, was a dimethane sulfonate coumarin derivative. Further analysis of this compound in 10 RCC cell lines including, A-498, indicated statistical phenotypic correlations between inhibitory activity and cytokeratin 8 expression. Therefore, they proposed that NSC 72151 and possibly other related coumarins represented potential pre-clinical candidates for de-differentiated RCC sub-types.

RCCs exhibit a number of distinct characteristics which have combined to render it refractory to not only drug treatments but also in many cases, radiation therapies [13]. Included in this repertoire of characteristics is MDR, where RCC is reported to express, with the exception of adrenocortical cancers, the highest levels of MDR associated P-gp. It is for this reason that they are among the most intrinsically resistant tumour types [19,63]. Therefore, it should prove useful to screen prospective novel agents directed against RCC as potential substrates for P-gp mediated MDR. With this in mind, an in vitro model of MDR-1 was used to screen daphnetin as a potential P-gp substrate. We found no evidence to suggest that daphnetin acted as a substrate for P-gp, unlike compounds such as vinblastin and melphalan. This finding implies that daphnetin is not readily removed from cells by this mechanism and so may persist in cancer cells, which not only increases the duration of exposure, but may also serve to enhance its antiproliferative effect. Taken together, our findings

suggest that daphnetin exhibits significant pharmacological characteristics in A-498 cells and warrants further investigation.

In summary, our results indicate that daphnetin inhibits human RCC proliferation by induction of a differentiation response that is at least partly mediated by p38 MAPK signalling. However, it is unlikely that the modulation of ERK1/ERK2 and p38 MAP kinase signalling by daphnetin is the single contributory mechanism affecting the differentiation status of A-498 cells. This hypothesis comes from the fact that daphnetin exhibited what appeared to be different mechanisms of action at low concentrations and short time points compared to higher concentrations and longer times. In addition, further co-operative evidence for this hypothesis comes from the finding that daphnetin, has been shown to be a potent inhibitor of PKA, PKC and EGFR tyrosine kinase [64]. Furthermore, EGFR overexpression has been found frequently in RCC [17,19,65]. Therefore, it is possible to hypothesise that treatment with daphnetin could cause inhibition of EGFR signalling, coupled with a modulation of PKC and PKA signalling, mechanisms separate to those described in the current paper. This may then lead to a down-regulation of proliferative signals and an associated up-regulation of differentiation signals, including p38 MAP kinase. In conclusion, our findings indicate that daphnetin or structurally similar compounds, which activate p38 signalling, may represent novel therapeutic agents for the treatment of poorly differentiated RCC.

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