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ORIGINAL ARTICLE

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Quinoxaline 1,4-dioxides induce G_2/M cell cycle arrest and apoptosis in human colon cancer cells

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Abstract We have recently shown that quinoxaline 1,4-dioxide (QdNO) derivatives, namely 2-benzoyl-3-phenyl-6,7-dichloroquinoxaline 1,4-dioxide (DCQ), 2-benzoyl-3-phenyl-quinoxaline 1,4-dioxide (BPQ) and 2-acetyl-3-methyl-quinoxaline 1,4-dioxide (AMQ), suppress the growth of T-84 human colon cancer cells. Here we show that the growth-suppressive effects of QdNOs are due to their ability to induce cell cycle arrest and/or apoptosis. While AMQ blocked more than 60% of cells at the G_2/M phase without inducing apoptosis, DCQ caused a significant increase in apoptotic cells with no noticeable effects on the cycling of cells. Treatment with BPQ resulted in G_2/M cell cycle arrest and induction of apoptosis. With regard to the effects of QdNOs on molecules that regulate apoptosis and the G_2 to M transition, both BPQ and AMQ inhibited the expression of cyclin B, while DCQ significantly decreased the levels of Bcl-2 and increased Bax expression. Next, we investigated whether transforming growth factor-beta1 (TGF-β1) and/or extracellular signal-regulated kinase (ERK) mediate the antiproliferative and apoptotic effects of QdNOs in colon cancer cells. Interestingly, the above QdNOs increased differentially total TGFβ1 mRNA expression and decreased TGFα mRNA and ERK phosphorylation. None of these QdNOs induced changes in TGFβ-2 mRNA expression. The addition of a specific inhibitor of MEK greatly enhanced apoptosis in cells treated with DCQ, suggesting that the inhibition of ERK phosphorylation may explain, to an extent, the apoptogenic effects of this compound. Taken together, these findings provide insights into possible molecular mechanisms of growth inhibition by QdNOs that could aid in their evaluation for anticancer therapy.

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Abbreviations AMQ: 2-Acetyl-3-methylquinoxaline 1,4-dioxide · BPQ: 2-Benzoyl-3-phenylquinoxaline 1,4-dioxide · DCQ: 2-Benzoyl-3-phenyl-6,7-dichloro quinoxaline 1,4-dioxide · DMEM: Dulbecco's modified Eagle's medium · DMSO: Dimethyl sulfoxide · ERK: Extracellular signal-regulated kinase · FBS: Fetal bovine serum · JNK: cJUN-N terminal kinase · MAPK: Mitogen-activated protein kinase · NGF: Neuron growth factor · PI: Propidium iodide · QdNO: Quinoxaline 1,4-dioxide · TdT: Terminal deoxynucleotide transferase · TGFα: Transforming growth factor alpha · TGFβ: Transforming growth factor beta

Introduction

Colorectal carcinoma is one of the most common visceral malignancies in the world, which accounts for over 70,000 deaths annually in the USA. Previous studies have established that colon carcinoma cells secrete and express receptors for several polypeptide growth factors, particularly transforming growth factor alpha (TGFα) and beta (TGFβ), that are involved in regulation of various cellular responses including cell growth, differentiation and apoptosis [1]. The TGF pathway is known to play an important role in both human and murine colon cancer [2]. TGFβ1 receptors are downregulated during colon cancer progression [2] and the inappropriate expression of TGFα in growth arrest is known to contribute to malignant progression of human colon cancer [3]. Given the increasing cancer mortality, it is essential to evaluate agents that can protect against colon cancer by modulating the TGF signaling pathway.

Three major mammalian mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase

(ERK), cJUN-N terminal kinase (JNK) and p38 kinase, are regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology including cell growth, differentiation and apoptosis [4]. In general, the ERK cascade is activated by growth factors, namely TGFβ1 and TGFβ2 [5]. ERK activation has been shown to correlate with the malignant progression of colorectal carcinomas in rat models of chemically induced intestinal neoplasia [6]. Controversial evidence has indicated that more complex roles of ERK cascade exist to transmit distinct cellular effects in different cell lineages. For example, ERK promotes differentiation in muscle cells [7] in contrast to transmitting mitogenic signals in fibroblasts [8]. The inhibition of ERK has been recently shown to be a key biochemical switch that is responsible for the differentiation and apoptotic pathways in intestinal cells [9]. In addition, inhibition of ERK phosphorylation by a synthetic ERK kinase 1/2 (MEK 1/2) inhibitor decreases the growth and inhibits the transformed phenotype of colon 26 cells in vitro and suppresses the in vivo growth of mouse and human colon tumor xenografts [10]. In a recent screening study of more than 100 colonic tumors, particularly high levels of ERK 1/2 phosphorylation were found in these tumors [11]. While transient ERK activation leads to proliferation, persistent activation mediates growth arrest or differentiation signals [12, 13].

The involvement of TGF and ERK signal transduction pathways in tumor promotion and malignant progression of human colon cancers [11, 14] has led to increased interest in compounds that reduce tumor cell viability and induce cell cycle arrest or apoptosis by modulating TGF [15] and/or MAPK [16] signaling as therapeutic agents against metastatic carcinomas. Quinoxaline 1,4-dioxides (QdNOs) are heterocyclic aromatic N-oxides that have been recently found in our laboratories to suppress the growth of T-84 human colon cancer cells [17]. We have tested four differently substituted QdNOs and found that small changes in the chemical structure profoundly influence the antiproliferative activities of these compounds. The aim of this study was to test the toxicity of QdNOs in normal epithelial cells and to further examine the mechanism of growth inhibition of colon cancer cells by QdNOs. In particular, we sought to determine if drug treatment induced changes in TGF or phospho-ERK (p-ERK) expression, cell cycle progression and/or programmed cell death in T-84 human colon cancer cells.

Materials and methods

Reagents and drugs

The QdNOs used were 2-benzoyl-3-phenylquinoxaline 1,4-dioxide (BPQ), 2-benzoyl-3-phenyl-6,7 dichloroquinoxaline 1,4-dioxide (DCQ), and 2-acetyl-3-methyl quinoxaline 1,4-dioxide. The QdNOs were synthesized by the Beirut reaction [18] and dissolved in dimethyl sulfoxide (DMSO) prior to treatment. Dulbecco's modified Eagle's medium (DMEM), and Ham's F-12 and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, Md.). TGF oligonucleotide primers were obtained from Interactiva Biotechnologie (Ulm, Germany). PD98059 (2′-amino-3′-methoxyflavone) was from Cell Signaling Technology (Beverly, Mass.).

Cell lines and treatment

T-84 human colon carcinoma cells, kindly supplied by Dr. Dominique Kaiserlian (Institute Pasteur de Lyon, France), were cultured in DMEM/Ham's F12. IEC-6 rat intestinal normal epithelial cells, kindly provided by Dr. Fadia Homaidan (Physiology Department, Medical School, American University of Beirut) were cultured in DMEM supplemented with 4.5 g/l glucose and 0.1 U/ml bovine insulin. Murine small intestinal epithelial cell line, Mode-K cells, were also obtained from Dr. Homaidan with the permission of Dr. P.B. Ernst (University of Texas, Medical Branch, Galveston, Tx.). Mode-K cells were maintained in DMEM (low glucose) containing 10 m*M* sodium pyruvate, and nonessential amino acids. All cells were supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 µg/ml). Cells were cultured in a humidified incubator (37°C) in an atmosphere comprising 95% air and 5% CO_2. For experiments, T-84 cells seeded on 24-well plates at a density of 1×10^5 cells per well and were left to grow for 24 h, after which they were treated with defined concentrations of the QdNOs dissolved in DMSO such that the DMSO concentration did not exceed 0.1% per well. Control plates were treated with DMSO only. The effects of QdNOs on the growth of normal epithelial cells (Mode K and IEC-6) were investigated using the trypan blue dye exclusion method. The cells were pretreated with PD98059 for 30 min prior to addition of DCQ.

DNA flow cytometric analysis

Cells were seeded in 100 mm dishes at a density of 7.5×10^5 cells per well. They were incubated and allowed to grow to 40–50% confluence after which they were treated with the QdNOs at their IC_{50} concentrations and incubated for various times. They were then harvested by trypsin release, washed twice with phosphate-buffered saline, permeabilized with 70% ethanol, treated with 1% RNase and finally stained with propidium iodide (PI) (100 µg/ml final concentration). Distribution of cell cycle phases with different DNA contents was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). The relative proportions of cells with DNA content indicative of apoptosis ($<2N$),

diploid G_0–G_1 (2N), S phase (> 2N but < 4N), and G_2/M phase (4N) were determined using CellQuest.

Apoptosis: TUNEL assay

Terminal deoxynucleotide transferase (TdT)-mediated labeling of free DNA 3′ ends with fluorescein-conjugated dUTP was accomplished using an In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) to monitor the extent of DNA fragmentation as a measure of apoptosis. Cytospin preparations were fixed and labeled according to the manufacturer's instructions. Four independent ×100 fields containing a minimum of 300 cells on each of two replicate slides were evaluated for nuclear labeling by fluorescence microscopy (Axiovert 200, Zeiss, Intermedic, Germany) for each treatment or condition.

Analysis of TGFα mRNA using RT-PCR

Total RNA was isolated from cells treated with or without QdNOs (as described above) for 24 h using the SV Total RNA isolation system (Promega, Madison, Wis.) according to the manufacturer's instruction. Precipitated nucleic acids were washed with 75% ethanol, dried, and resuspended in water. Total RNA (6 μg) was converted into cDNA by reverse transcription using 10 U avian myeloblastosis reverse transcriptase (Promega), oligo(dT)17 primer (50 p *M*) and 0.2 m*M* of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP; Promega) in reaction buffer (50 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* spermidine, 10 m*M* dithiothreitol) in a total volume of 50 μl for 60 min at 42°C. cDNA was amplified by polymerase chain reaction (PCR) using specific oligonucleotide primers (TGFα, TGFβ1 and TGFβ2) designed to detect the target cDNAs (Table 1)

PCR reactions were carried out in 100 μl of 75 m*M* Tris-HCl, pH 9, 20 m*M* $(NH_4)_2SO_4$ 0.01% Tween 20, 1 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 0.5 U DNA polymerase, 50 p *M* of each primer and 2 ml of the cDNA mixture. PCR was performed using the following conditions: denaturation for 45 s at 94°C, annealing at 50°C for 45 s, and elongation at 72°C for 45 s, followed by a final 5 min at 72°C. For each oligonucleotide pair and for every RNA sample, a preliminary analysis was conducted to define the appropriate range of cycles consistent with an exponential increase in the amount of DNA product. The PCR products (10 μl) were separated by electrophoresis on 3% agarose gels and stained with ethidium bromide; the intensity of the luminescent bands was evaluated using Molecular Analyst/PC image analysis software (BioRad Laboratories, Hercules, Calif.). Results were expressed as relative densitometric units, normalized to the values of the phosphoribosomal protein mRNA used as an internal control. Control PCR was performed using RNA samples which were not subjected to reverse transcription to check whether any amplified fragment(s) corresponding to genomic DNA could be detected.

Protein extraction and Western blot analysis

Control or treated cells were harvested at the indicated times. Total cellular protein was extracted by lysing cells in a buffer containing 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 4% protease inhibitors and 1% phosphatase inhibitors. The cell lysate was rotated at 4°C for 30 min, centrifuged at 10,000 rpm for 10 min and the precipitates discarded. Protein concentrations were determined using a DC BioRad protein assay kit (BioRad Laboratories, Hercules, Calif.) using bovine serum albumin as a standard. Cellular protein (25 μg) was loaded onto 10% SDS-polyacrylamide gels. The protein bands were then transferred electrophoretically to PVDF membranes (NEN Life Sciences Products, Boston, Mass.). The following primary antibodies were then used for immunoblotting: Bcl-2 (N-19), Bax (N-20) cyclin B, and p-ERK (E-4) (all from Santa Cruz Biotechnology, Santa Cruz, Calif.); TGFα and TGFβ1(TB21) (Chemicon International, Temecula, Calif.). Membranes were then probed with horseradish peroxidase-conjugated second antibody (Bio-Rad, Hercules, Calif.). The actin antibody (20-33) was from Sigma. Protein bands were detected using an enhanced chemiluminescence system according to the manufacturer's recommendations (Amersham, Pharmacia, Biotech).

Table 1 Synthetic oligonucleotides and experimental conditions used for RT-PCR analysis

Gene	Position	Size	Sequence	Number of cycles	T^0 hybridization
Ribosomal phosphoprotein	344–363 850–830	486	5′GTTCACCAAGGAGGACCTCA3′ 3′AGACACCTCTGCCTAATGTG5′	28	50
TGF-β1	731–752 1415–1392	661	5′GAAGTCACCCGCGTGCTAATGG3′ 3′GGATGTAAACCTCGGACCTGTGTG5′	32	50
TGF-β2	1–22 214–192	192	5′TTCGCAGGTATCGATGGCACCT3′ 3′CGTCGTATTAACGACGGAAGCGG5′	37–40	50
TGF-α	3538–3557 3930–3911	373	5′ATGTTGTTCCCTGCAAGTCC3′ 3′ACTATGGAGAGGGGTCGCTT5′	30	50

Results

Effects of QdNOs on growth, cell cycle progression and apoptosis

We have shown recently that the three QdNOs AMQ, BPQ, and DCQ show potent antiproliferative effects against T-84 human colon cancer cells [17]. The IC_{50} values of these compounds were 100, 20 and 1 μM, respectively. Thus the concentration of DCQ required for 50% growth inhibition was 20-fold lower than that of BPQ and 100-fold lower than that of AMQ. It is of special significance that these QdNOs were not cytotoxic to normal intestinal IEC-6 or Mode K cells at their IC_{50} concentrations (Fig. 1).

To dissect the mechanism for the enhanced antiproliferative effects of DCQ and to determine whether the growth-inhibitory effects of QdNOs are associated with specific changes in cell cycle progression and/or the induction of apoptosis, cell cycle analyses were carried out using DNA flow cytometry and TUNEL assays. T-84 cells were treated with each drug at its IC_{50} and harvested at 12, 24 and 48 h after treatment. Exposure of cells to AMQ resulted in more than 60% of cells in the G_2/M phase within 48 h as shown in Fig. 2, providing evidence of G_2/M arrest. Upon treatment with DCQ, the accumulation of a sub-G_1 peak of hypodiploid cells was evident and this population represented more than 35% of the total cells (Fig. 2). Exposure of T-84 cells to BPQ caused an arrest of 35% of the cells in G_2/M phase at 24 h and an accumulation of 30% of cells in sub-G_1 at 48 h (Fig. 2). To understand and confirm

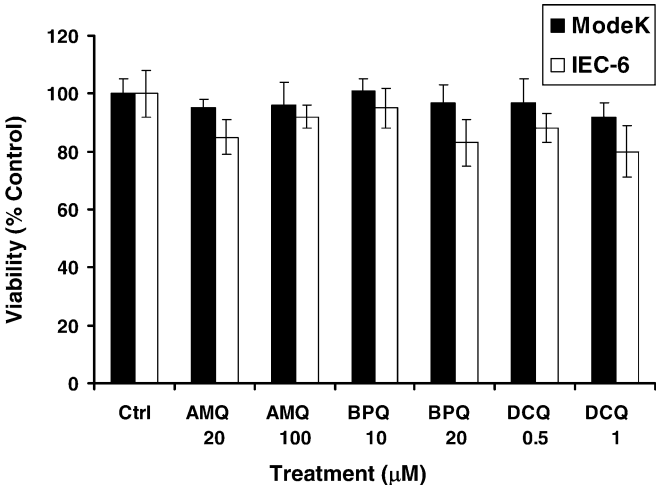


Fig. 1 The effect of QdNOs on the viability of two normal intestinal cells, IEC-6 rat intestinal epithelial cells and Mode K mouse duodenum cells. Cells were grown under standard conditions and treated with graded concentrations of the three QdNOs (AMQ, BPQ, DCQ) for 48 h. QdNOs were dissolved in DMSO and the concentration of DMSO in treated and control wells did not exceed 0.1% per well. Cell viability was determined by the trypan blue dye exclusion method. Each value is the mean ± SD of two separate experiments done in duplicate

Fig. 2 DNA content analysis following treatment with the QdNOs. T-84 cells were either treated with 0.1% DMSO or were exposed to DCQ, BPQ or AMQ at their IC_{50} values for 12, 24 or 48 h. After drug exposure, cell cycle distribution and apoptosis were determined by flow cytometry as described in "Materials and methods." Each data point represents the mean of two independent experiments

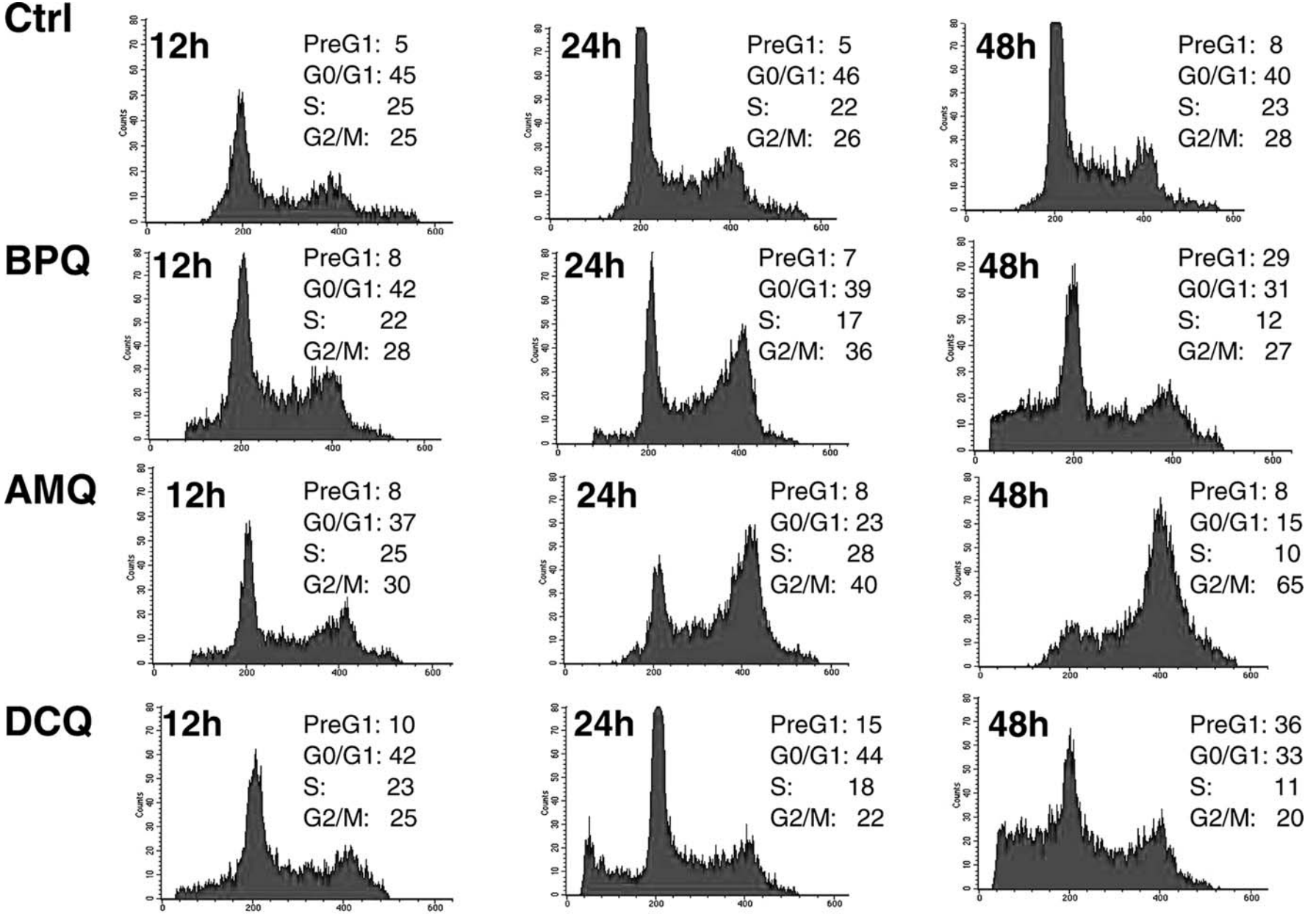
the nature of cell death, we utilized the TUNEL assay method in which FITC-conjugated dUTP is incorporated into the DNA strand breaks due to apoptosis and observed using fluorescence microscopy. The percentages of apoptotic cells were clearly increased in cultures of T-84 cells treated for 48 h with DCQ and to a lesser extent with BPQ (Fig. 3). DCQ induced apoptosis with no noticeable changes on the cycling of cells. In contrast, no induction of apoptosis was noted in AMQ-treated cells. These findings suggest that the increase in antiproliferative activity of DCQ compared to BPQ or AMQ can be attributed, to some extent, to an increase in apoptosis.

Effects of QdNOs on the expression of cell cycle and apoptosis-related proteins

In view of the above-described effects of QdNOs on G_2/M cell cycle arrest and induction of apoptosis, we examined the effects of QdNOs on the levels of expression of G_2/M-related cell cycle control and apoptosis-related proteins using Western blot analysis. T-84 cells were treated with each drug at its IC_{50}, and cells were harvested after 24 h. As shown in Fig. 4, treatment with AMQ or BPQ was associated with decreased levels of cyclin B, a protein that plays a key role in the control of the G_2/M transition of the cell cycle. However, no significant changes in the level of the cyclin B protein were noted with DCQ treatment. On the other hand, within 24 h of treatment, DCQ was the most effective at increasing the level of expression of the proapoptotic protein Bax and decreasing the level of the antiapoptotic protein Bcl-2, thus reducing the ratio of Bcl-2 to Bax. Loading of equivalent amounts of protein for all samples was confirmed by incubating the same blot with actin antibody. On the basis of quantification of the data by scanning densitometry, DCQ was found to be the most effective at reducing the Bc1-2/Bax ratio, followed by BPQ.

Effects of QdNOs on TGF expression and on the survival signaling molecule ERK

In addition to investigating the modulation of cell cycle/apoptosis-related proteins by QdNOs, we examined whether QdNOs modulate TGF expression and/or p-ERK protein levels in light of the importance of TGF and ERK in malignant progression of human colon cancers. Cells were treated with either drug at its IC_{50} for


Ctrl
12h
PreG1: 5
G0/G1: 45
S: 25
G2/M: 25
24h
PreG1: 5
G0/G1: 46
S: 22
G2/M: 26
48h
PreG1: 8
G0/G1: 40
S: 23
G2/M: 28
BPQ
12h
PreG1: 8
G0/G1: 42
S: 22
G2/M: 28
24h
PreG1: 7
G0/G1: 39
S: 17
G2/M: 36
48h
PreG1: 29
G0/G1: 31
S: 12
G2/M: 27
AMQ
12h
PreG1: 8
G0/G1: 37
S: 25
G2/M: 30
24h
PreG1: 8
G0/G1: 23
S: 28
G2/M: 40
48h
PreG1: 8
G0/G1: 15
S: 10
G2/M: 65
DCQ
12h
PreG1: 10
G0/G1: 42
S: 23
G2/M: 25
24h
PreG1: 15
G0/G1: 44
S: 18
G2/M: 22
48h
PreG1: 36
G0/G1: 33
S: 11
G2/M: 20
Counts
DNA Content (PI staining)

24 h and extracts were prepared and examined for TGF expression and p-ERK expression by RT-PCR and Western blot analysis. Culturing of colon cancer cells with DCQ resulted in marked decreases in TGFα transcript, TGFα protein and p-ERK protein levels as shown in Fig. 5. In contrast, the transcript and protein levels of TGFβ1 increased after treatment with QdNOs. Among the QdNOs tested, DCQ was the most effective at increasing TGFβ1 mRNA and protein expression. No changes in TGFβ2 expression were noted upon treatment of T-84 cells with QdNOs. After 24 h of incubation, a decrease in p-ERK protein level was observed in the DCQ-treated cells which exceeded that observed for BPQ or AMQ (Fig. 5b). These findings suggest that the enhanced apoptosis associated with DCQ treatment is perhaps due to its ability to induce apoptosis through pathways that involve TGF and/or ERK signaling. The remarkable decrease in p-ERK expression following DCQ treatment prompted us to characterize its effects on this protein.

Inhibition of ERK activation augments DCQ-induced apoptosis

Because there is evidence [19] that ERK can mediate extracellular stimulus-induced apoptosis, we sought to determine whether ERK activation could mediate DCQ-induced apoptosis. For this purpose, T-84 cells were pretreated for 30 min with graded concentrations (10–30 μM) of PD98059, a specific inhibitor of MAPK/ERK kinase (MEK). A dose of 10 μM of PD98059 was chosen for drug-treatment experiments since this concentration inhibited ERK activation but did not affect the growth or morphology of cells (data not shown). Subsequently, the inhibitor-treated cells were exposed to DCQ and

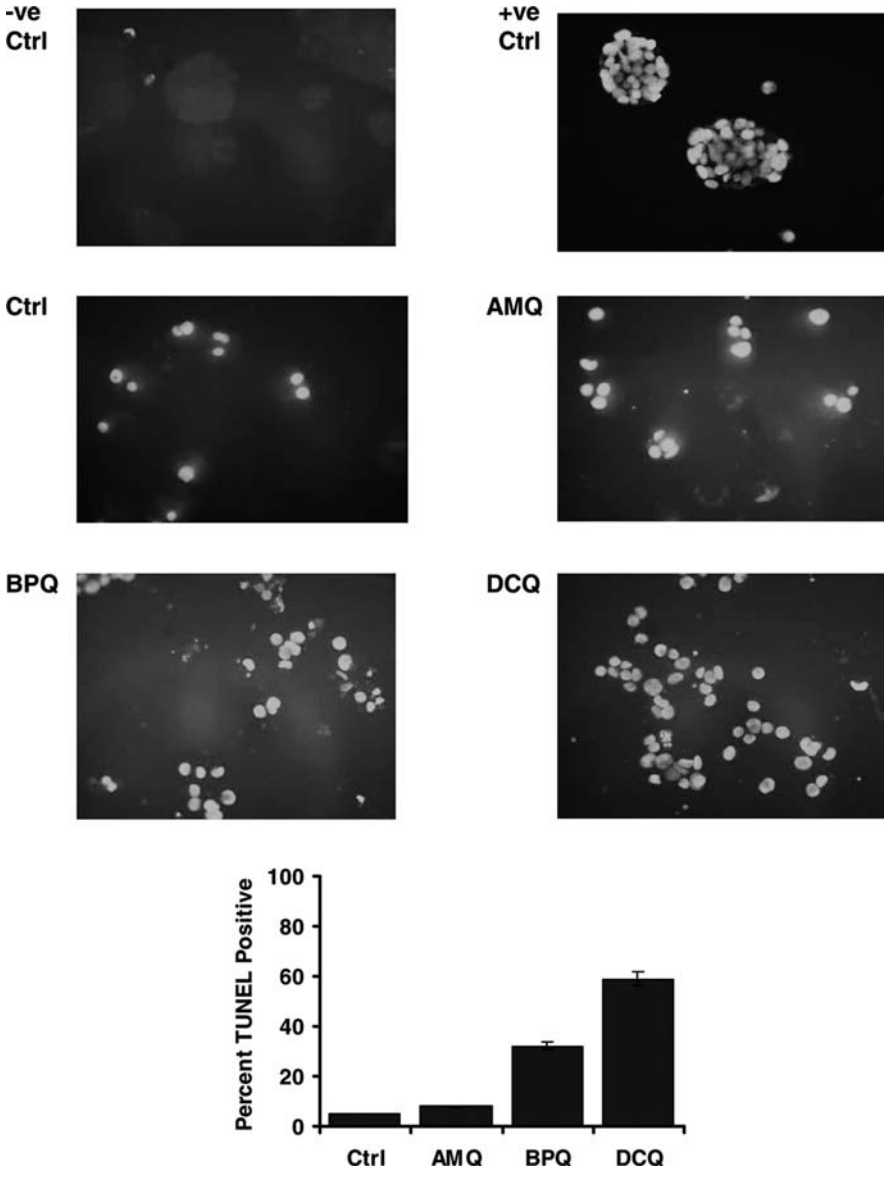


Fig. 3 Induction of apoptosis of colon cancer cells by QdNOs. T-84 cells were cultured with 0.1% DMSO (*Ctrl*) or with the various QdNOs at their IC_{50} values. The TUNEL assay was used to determine the percentage of T-84 cells with fragmented DNA after culture for 48 h with the drugs (*+ve Ctrl* cells treated with DNase I (5 U/µl), *−ve Ctrl* cells treated with nucleotide mixture in reaction buffer without TdT enzyme). Each bar represents the mean ± SD of two independent experiments

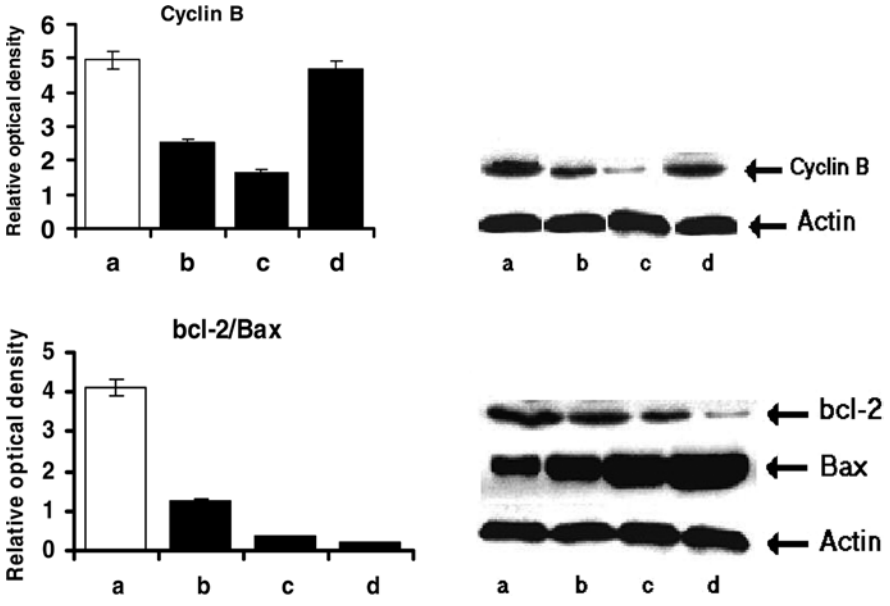


Fig. 4 Effect of QdNOs on the levels of cyclin B, Bcl-2 and Bax protein. T-84 cells were either treated with 0.1% DMSO (*a*) or were exposed to AMQ (*b*), BPQ (*c*) or DCQ (*d*) at their IC_{50} values. After 24 h of incubation, cells were lysed with lysis buffer, and the amounts of protein were determined with a Bio-Rad DC protein assay kit as described in "Materials and methods." Lysates were analyzed by SDS-PAGE/immunoblotting using antibodies specific for cyclin B or Bax/Bcl-2. The experiment was repeated three times, the quantification performed on the autoradiographs, and the means ± SD calculated. Representative autoradiographs are shown. β-Actin was used as a sample loading control

apoptosis quantitated. Further inhibition of p-ERK expression by PD98059 resulted in an increase of 40% over and above the apoptosis induced by DCQ alone as shown in Fig. 6, suggesting that ERK could modulate DCQ-induced apoptosis.

Discussion

Promising therapeutic approaches for the treatment of colon cancer include the inhibition of growth factor signaling [15], modulation of downstream signal transduction pathways [16], and induction of apoptosis [20]. The latter two approaches may be especially important in tumors that can use signals from multiple growth factor receptors for growth and survival. In a previous study, we demonstrated a dose-dependent inhibition of the proliferation of T-84 human colon cancer cells by QdNOs [17]. The present study was undertaken to explore possible molecular mechanisms of the QdNO-induced antiproliferative effects in T-84 cells. We report here that the growth-inhibitory effects of AMQ and BPQ were associated with cell cycle arrest at the G_2/M phase, while DCQ caused an increase in apoptosis. By surveying known components of the cell proliferation, cell cycle and/or apoptosis signaling pathways, we found that the QdNOs decreased TGFα and increased TGFβ1 mRNA expression, without modulating TGFβ2 mRNA levels. Cell cycle arrest at the G_2/M phase correlated with reduced levels of cyclin B protein, while DCQ's apoptotic effects were associated with the upregulation of Bax and a decrease in Bcl-2 protein levels. Interestingly, the greater apoptotic response of DCQ also correlated with its ability to markedly inhibit p-ERK protein levels.

QdNOs were approved in the late 1960s for use as feed additives in stock farming to promote animal growth and to replace the banned therapeutic antibiotics penicillin and tetracyclines [21]. There is a large body of evidence showing that quinoxaline antibiotics are inhibitors of DNA synthesis with their activity being dramatically increased under anaerobic conditions. The mechanism of action of QdNOs is believed to be via a free radical intermediate created by the transfer of an electron from an electron-rich environment to the electron-poor nitrogen centers of the 1,4-di-N-oxide moiety [22]. This transfer is more likely to occur under the reducing conditions of hypoxic cells, targeting the cytotoxicity of these compounds to hypoxic cells. We have recently examined the effect of differently substituted QdNOs on the proliferation of T-84 human colon cancer cells and evaluated their selective toxicity to hypoxic cells. The 50–100-fold greater toxicity of QdNOs to hypoxic cells as well as their antineoplastic and apoptotic effects in aerobic cells provides an additive advantage for using these drugs to target the oxic and hypoxic subpopulations of cells within a tumor and improve therapeutic effects in patients. It should be noted that concentrations of QdNOs which are able to cause cell cycle arrest and/or apoptosis in tumor cells are nontoxic to normal cells (Fig. 1), indicating that QdNOs could be used to reduce the overall tumor burden without causing additional damage in the well-oxygenated normal tissues. Upon treatment of normal epithelial cells with 1 μM DCQ, a relatively high percentage of viable cells (more than 80%) remained as compared to only 10% viable T-84 human colon cancer cells [17].

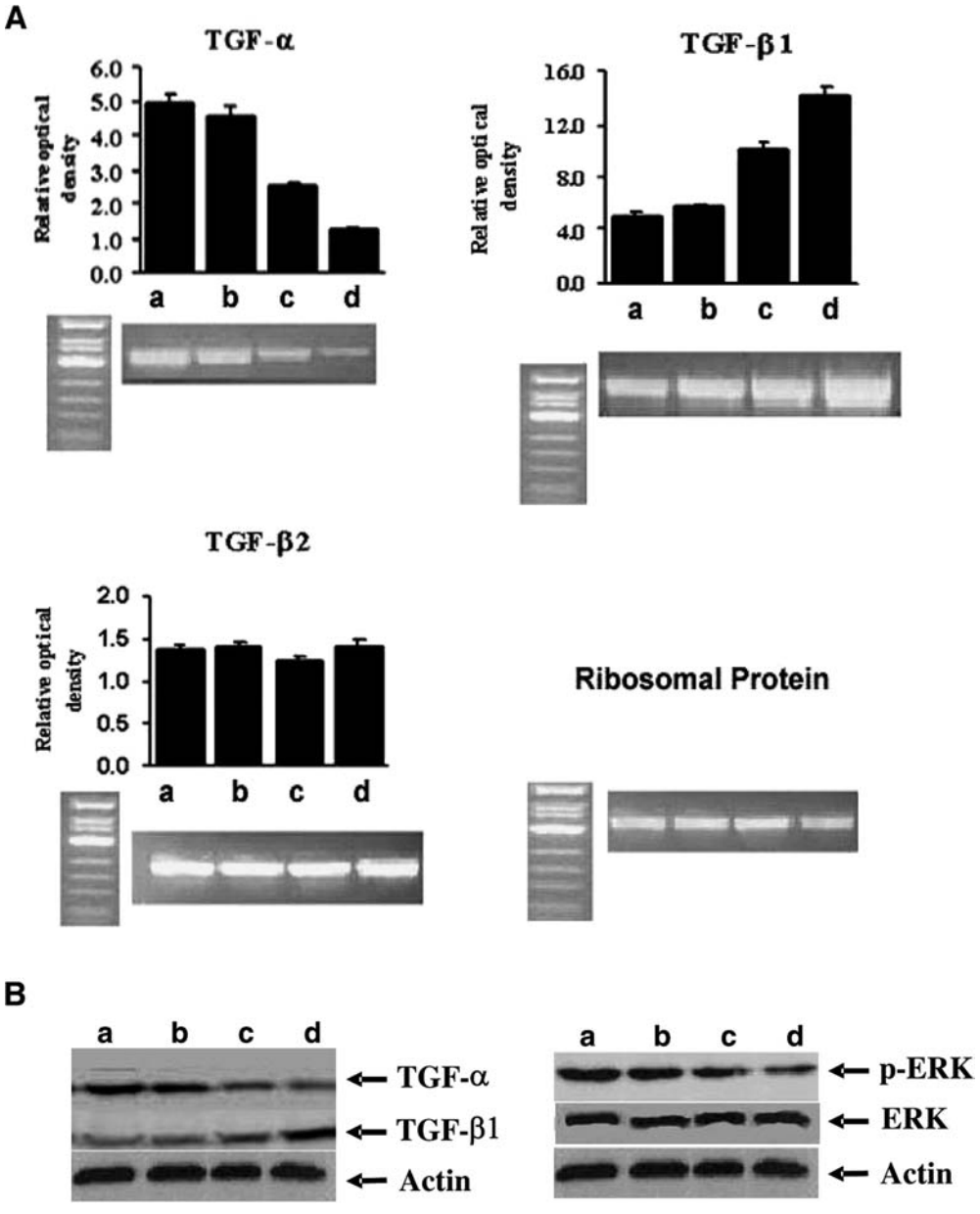


Fig. 5 Assay for (**a**) TGF and (**b**) p-ERK expression in T-84 cells treated with or without the various QdNOs. T-84 cells were cultured in the presence of 0.1% DMSO (*a*), 100 μ*M* AMQ (*b*), 20 μ*M* BPQ (*c*) or 1 μ*M* DCQ (*d*) for 24 h. After exposure, cells taken from culture were divided into two parts. One was for the determination of TGFα, *β*1 and *β*2 expression by RT-PCR, and the other was for the determination of phosphorylated ERK, ERK, TGFα and TGF*β*1 protein expression by Western blot analysis. Each data point represents the mean of two independent experiments. *β*-Actin was used as a sample loading control

Our findings that QdNOs increase TGF*β* expression are of special significance in the light of the fact that TGF*β* is a multifunctional protein with potent growth-inhibitory activities and potential to produce an apoptotic response in various cell types [23]. Moreover, the downregulation of TGFα by QdNOs has important clinical implications, since this protein is a key mediator of growth stimulation with documented mitogenic effects [3]. It is also relevant that dysfunction of TGF signaling, particularly of TGF*β*, has been observed in colon carcinoma and has been suggested to be involved in the development of the malignant phenotype and progression of colon cancer [24]. Thus, the identification of compounds that could enhance the effects of TGF*β* and/or antagonize the effects of TGFα could prove to be effective in the treatment of colon cancer.

Although the detailed mechanism by which DCQ induces apoptosis remains to be elucidated, it appears that this compound modulates multiple factors that could increase cellular susceptibility to apoptosis. Of note in this regard are the changes we observed in the proapoptotic Bax and antiapoptotic Bcl-2 proteins, which promotes cell death by modulating mitochondrial release of proapoptotic factors such as cytochrome *c* [20]. Because Bax is an important player in the mitochondrial (intrinsic) pathway, it appears that an intrinsic pathway plays a role in DCQ-induced apoptosis. The ability of DCQ to upregulate TGF*β*1, a protein that has been shown to trigger apoptosis in a variety of cells [25, 26], and to downregulate the antiapoptotic p-ERK protein, could account for its enhanced apoptotic effects.

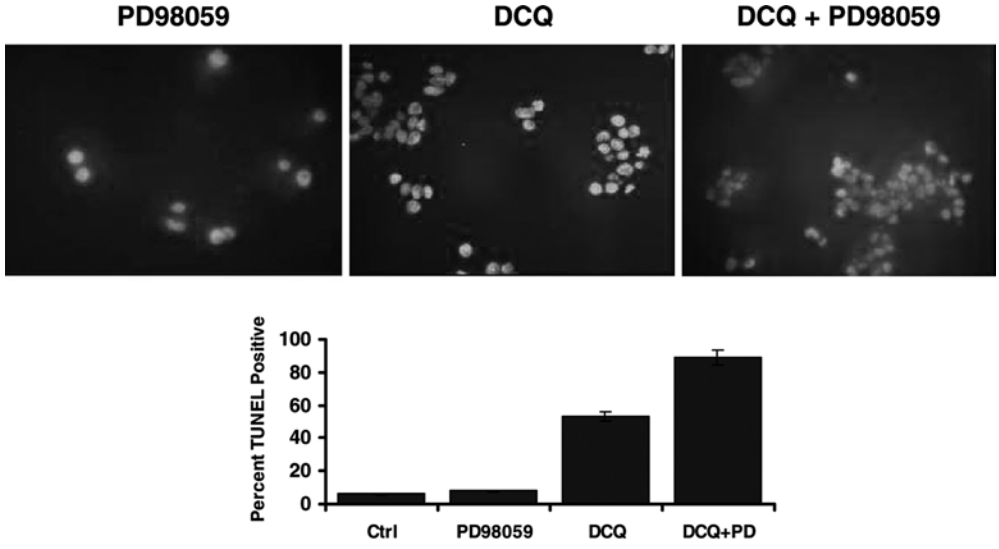


Fig. 6 Inhibition of ERK signaling enhances DCQ-induced apoptosis. T-84 cells were treated with 1 μ*M* of DCQ for 48 h after preincubation for 30 min with 10 μ*M* of PD98059. The fraction of apoptotic cells was determined using the TUNEL assay as described in "Materials and methods." Each bar represents the mean ± SD of two independent experiments

There is a precedent for ERK functioning as an antiapoptotic intermediate, and increasing evidence supports a role for ERK in cell survival [19]. In PC-12 cells, withdrawal of neuron growth factor (NGF) leads to inhibition of ERK activity and increased cell death, whereas constitutive activation of the ERK pathway in these cells inhibits the apoptosis induced by NGF withdrawal [27]. In Jurkat T cells, activation of ERK by TPA inhibits Fas-induced apoptosis [28]. A similar situation has been also observed in satratoxin-induced apoptosis [29] and deoxycholic-induced apoptosis [30], where elevated ERK activity blunts the apoptotic response. In the present study we demonstrated that DCQ-induced apoptosis was augmented following treatment of the cells with the MEK inhibitor, PD98059, suggesting that the MEK pathway is involved, to an extent, in the apoptogenic effects of DCQ. Interestingly, the combination of DCQ and PD98059 was synergistic rather than additive, suggesting that they could be affecting independent processes, which subsequently converge upon apoptosis. The signals downstream of MEK that lead to apoptosis remain undefined.

Recent studies of MAPK activity in colorectal adenomas and carcinomas from carcinogen-treated rats indicate that ERK activity is elevated, on average, 29-fold above that found in normal mucosa [6]. In addition, there is ample evidence in the literature suggesting that drug-induced ERK inhibition is an important step in inhibition of colon carcinogenesis. For example, sulindac, a nonsteroidal antiinflammatory drug, has been shown to cause inhibition of ERK phosphorylation [31], regression of precancerous adenomatous polyps, and lower mortality from colorectal cancer. Geraniol, a natural component of plant essential oils, has been found to exert potent antiproliferative effects on human colon cancer Caco-2 cells mainly decreasing the expression of p44/p42 ERK active forms [32]. Thus, the inhibition of ERK activity may be a useful biochemical target for the development of chemopreventive and chemotherapeutic drugs for human colon cancer. Studies are currently ongoing in our laboratories to characterize DCQ's effects at the level of the MAPKs (p38, MAPK, JNK) and other apoptosis signaling pathways in order to elucidate the mechanism of cell death induced by this compound. We also aim at determining whether additional targets or factors account for its antiproliferative effects. The growth-inhibitory apoptotic potential and growth factor-modulatory effects of QdNOs together with their hypoxia-selective toxicity [33] suggest that they might be developed as promising antitumor agents.

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References

1. Murthy U, Anzano MA, Greig RG (1989) Expression of TGF-alpha/EGF and TGF-beta receptors in human colon carcinoma cell lines. Int J Cancer 44:110–115
2. Biasi F, Tessitore L, Zanetti D, Cutrin JC, Zingaro B, Chiarpotto E, Zarkovic N, Serviddio G, Poli G (2002) Associated changes of lipid peroxidation and transforming growth factor beta1 levels in human colon cancer during tumour progression. Gut 50:361–367
3. Wang D, Patil S, Li W, Humphrey LE, Brattain MG, Howell GM (2002) Activation of the TGFalpha autocrine loop is downstream of IGF-I receptor activation during mitogenesis in growth factor dependent human colon carcinoma cells. Oncogene 21:2785–2796
4. Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185
5. Frey RS, Mulder KM (1997) Involvement of extracellular signal-regulated kinase 2 and stress activated protein kinase/Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells. Cancer Res 57:628–633

6. Licato LL, Keku TO, Wurzelmann JI, Murray SC, Woosley JT, Sandler RS, Brenner DA (1997) In vivo activation of mitogen-activated protein kinases in rat intestinal neoplasia. Gastroenterology 113:1589–1598
7. Gredinger E, Gerber AN, Tamir Y, Tapscott SJ, Bengal E (1998) Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. J Biol Chem 273:10436–10444
8. Plattner R, Gupta S, Khosravi-Far R, Sato KY, Perucho M, Der CJ, Stanbridge EJ (1999) Differential contribution of the ERK and JNK mitogen-activated protein kinase cascades to Ras transformation of HT1080 fibrosarcoma and DLD-1 colon carcinoma cells. Oncogene 18:1807–1817
9. Taupin D, Podolsky DK (1999) Mitogen-activated protein kinase activation regulates intestinal epithelial differentiation. Gastroenterology 116:1072–1080
10. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med 5:810–816
11. Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M (1999) Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18:813–822
12. Bennett AM, Tonks NK (1997) Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinase. Science 278:1288–1291
13. Yen A, Roberson MS, Varvayanis S, Lee AT (1998) Retinoic acid induced mitogen-activated protein [MAP]/extracellular signal-regulated kinase [ERK] kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Cancer Res 58:3163–3172
14. Yan Z, Deng X, Friedman E (2001) Oncogenic Ki-ras confers a more aggressive colon cancer phenotype through modification of transforming growth factor-beta receptor III. J Biol Chem 276:1555–1563
15. Akhurst RJ (2002) TGF-beta antagonists: why suppress a tumor suppressor? J Clin Invest 109:1533–1536
16. Sebolt-Leopold JS (2000) Development of anticancer drugs targeting the MAP kinase pathway. Oncogene 19:6594–6599
17. Gali-Muhtasib HU, Haddadin MJ, Rahhal DN, Younes I (2001) Quinoxaline 1,4-dioxides as anticancer and hypoxia-selective drugs. Oncol Rep 8:679–684
18. Haddadin MJ, Issidorides CH (1993) The Beirut reaction. Heterocycles 35:1503–1525
19. Orlowski RZ, Small GW, Shi YY (2002) Evidence that inhibition of p44/42 mitogen-activated protein kinase signaling is a factor in proteasome inhibitor-mediated apoptosis. J Biol Chem 277:27864–27871
20. Reed JC (1998) Bcl-2 family proteins. Oncogene 17:3225–3236
21. Hennessey TD, Edwards JR (1972) Antibacterial properties of quindoxin: a new growth-promoting agent. Vet Rec 90:187–191
22. Priyadarsini KI, Dennis MF, Naylor MA, Stratford MRL, Wardman P (1996) Free radical intermediates in the reduction of quinoxaline N-oxide antitumor drugs: redox and prototropic reactions. J Am Chem Soc 118:5648–5654
23. Alexandrow M, Moses H (1995) Transforming growth factor β and cell cycle regulation. Cancer Res 55:1452–1457
24. Guda K, Giardina C, Nambiar P, Cui H, Rosenberg DW (2001) Aberrant transforming growth factor-beta signaling in azoxymethane-induced mouse colon tumors. Mol Carcinog 31:204–213
25. Hagimoto N, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, Maeyama T, Hara N (2002) TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. J Immunol 168:6470–6478
26. Kim BC, Mamura M, Choi KS, Calabretta B, Kim SJ (2002) Transforming growth factor beta 1 induces apoptosis through cleavage of BAD in a Smad3-dependent mechanism in FaO hepatoma cells. Mol Cell Biol 22:1369–1378
27. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331
28. Wilson DJ, Alessandrini A, Budd RC (1999) MEK1 activation rescues Jurkat T cells from Fas-induced apoptosis. Cell Immunol 194:67–77
29. Yang GH., Jarvis BB, Chung YJ, Pestka JJ (2000) Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. Toxicol Appl Pharmacol 164:149–160
30. Qiao D, Stratagouleas ED, Martinez JD (2001) Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis. Carcinogenesis 22:35–41
31. Rice PL, Goldberg RJ, Ray EC, Driggers LJ, Ahnen DJ (2001) Inhibition of extracellular signal-regulated kinase 1/2 phosphorylation and induction of apoptosis by sulindac metabolites. Cancer Res 61:1541–1547
32. Carnesecchi S, Bradaia A, Fischer B, Coelho D, Scholler-Guinard M, Gosse F, Raul F (2002) Perturbation by geraniol of cell membrane permeability and signal transduction pathways in human colon cancer cells. J Pharmacol Exp Ther 303:711–715
33. Diab-Assaf M, Haddadin MJ, Yared P, Al-Assaad C, Gali-Muhtasib HU (2002) Quinoxaline 1,4-dioxides: hypoxia selective therapeutic agents. Mol Carcinog 33:198–205