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RESEARCH PAPER

A novel quinone-based derivative (DTNQ-Pro) induces apoptotic death via modulation of heat shock protein expression in Caco-2 cells

Isabel Gomez-Monterrey[1], Pietro Campiglia[2], Alessia Bertamino[2], Claudio Aquino[1], Marina Sala[2], Paolo Grieco[1], Alessandra Dicitore[3], Daniela Vanacore[3], Amalia Porta[2], Bruno Maresca[2], Ettore Novellino[1] and Paola Stiuso[3]

[1]*Department of Pharmaceutical and Toxicological Chemistry, University of Naples Federico II, Naples, Italy,* [2]*Department of Pharmaceutical Sciences, University of Salerno, Fisciano, Salerno, Italy, and* [3]*Department of Biochemistry and Biophysics, Second University of Naples, Naples, Italy*

Background and purpose: The resistance of human colon adenocarcinoma cells to antineoplastic agents may be related to the high endogenous expression of stress proteins, including the family of heat shock proteins (HSPs). Recently, a quinone-based pentacyclic derivative, DTNQ-Pro, showed high cytotoxic activity in human colon carcinoma cell lines. The aim of the present study was to determine the precise cellular mechanisms of this cytotoxic action of DTNQ-Pro.
Experimental approach: Using human colorectal carcinoma-derived Caco-2 cells as a model, we studied the effects of DTNQ-Pro on cellular viability and oxidative stress; HSP70 and HSP27 accumulation; and cell cycle, differentiation and apoptosis.
Key results: Incubation of Caco-2 cells with DTNQ-Pro reduced cell growth and increased the levels of reactive oxygen species in mitochondria. After 48 h of treatment, cells surviving showed an increased expression of Mn-superoxide dismutase (SOD), nitric oxide production and membrane lipid peroxidation. Treatment with DTNQ-Pro decreased HSP70 expression, and redistributed HSP27 and vimentin within the cell. DTNQ-Pro down-regulated the expression of A and B cyclins with arrest of the cell cycle in S phase and increased cellular differentiation. A second treatment of Caco-2 cells with DTNQ-Pro induced cellular death by activation of the apoptotic pathway.
Conclusions and implications: DTNQ-Pro causes Caco-2 cell death by induction of apoptosis via inhibition of HSP70 accumulation and the intracellular redistribution of HSP27. These findings suggest the potential use of DTNQ-Pro in combination chemotherapy for colon cancer.
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Keywords: cellular differentiation; apoptosis; Caco-2 cell line; heat shock proteins; quinone-based anti-tumour agents

Abbreviations: ALP, alkaline phosphatase; Bax, BCL2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; DMEM, Dulbecco's modified Eagle's medium; DTNQ-Pro, (3*S*,3′*R*)spiro[(hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione)-6,3′-(2′,3′-dihydrothieno-[2,3*b*]naphtho-4′,9′-dione; HE, hydroethidine; HSP27, heat shock protein 27; HSP70, heat shock protein 70; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; TBA, thiobarbituric acid

Introduction

Adenocarcinoma cells, such as colorectal tumour cells, are remarkably resistant to damage induced by radiation and systemic, immunological and chemotherapeutic agents. As a consequence, the tumours are hard to treat and often proliferate rapidly, even under conditions that may adversely affect normal cells. The mechanisms underlying its survival advantage may be related in part to the high endogenous expression of stress proteins. In contrast to normal cells, the basal level of inducible heat shock proteins (HSPs) are frequently higher in tumour cells (Jolly and Morimoto, 2000; Calderwwod *et al.*, 2006). HSPs are highly conserved throughout evolution: they may be expressed constitutively and after stressful conditions, such as heat shock, anoxia, anti-neoplasic drugs, etc. (Parsell and Lindquist, 1994). Although the role of HSPs is a key

Correspondence: Paola Stiuso, Department of Biochemistry and Biophysics, Second University of Naples, Via Costantinopoli 16, 80138 Naples, Italy. E-mail: paola.stiuso@unina2.it
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biological problem, it is generally believed that the primary function of these proteins is to maintain cell homeostasis and mediate cytoprotective effects (Jäättelä, 1996). Recent data provide direct evidence that, among mammalian HSPs, the expression of the small, 27 kDa HSP27 is related to the acquisition of drug resistance to commonly used therapeutic drugs, particularly to anthracyclines (Garrido *et al*., 1997; Charette *et al*., 2000). HSP27 has been implicated also in a wide spectrum of cell functions other than cell protection and particularly in differentiation and cell proliferation processes (Mehlen *et al*., 1997; Ciocca *et al*., 1999). The high expression of members of the HSP70 (70 kDa) family in colorectal cancer cells has been associated with metastasis and resistance to chemotherapy. However, in experimental models, HSP27 and HSP70 have been shown to increase tumourigenicity of cancer cells, and HSP depletion can induce a spontaneous regression of the tumour (Garrido *et al*., 1998; Gurbuxani *et al*., 2001). Indeed, Rashmi *et al*. (2004), using a SW480 human colon cancer cell line, showed that HSP70 protects against the induction of apoptosis by curcumin. In addition, HSPs can associate either with specific lipids or with areas of particular membrane topology (such as lipid rafts), and changes of membrane physical state alter HSP gene expression (Vigh *et al*., 1998; Török *et al*., 2003; Nagy *et al*., 2007). Thus, development of specific inducers of HSP expression could be desirable either to shed further light on the functional roles of these important proteins in cell survival and in cell death, or to further develop new clinically useful anti-tumour drugs.

In this regard, a quinone-based pentacyclic derivative (3*S*,3′*R*) spiro[(hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione)-6,3′-(2′,3′-dihydrothieno [2,3-*b*]naphtho-4′,9′-dione)] (DTNQ-Pro) was identified in our laboratory as a novel cytotoxic agent with a broad spectrum of activity against a number of tumour cell lines. In addition, cross-resistance to this compound was not observed in doxorubicin and cisplatin-resistant cell lines (Gomez-Monterrey *et al*., 2007). However, although this derivative has many of the characteristics of classical quinone-based DNA intercalators, it did not inhibit topoisomerase II at equicytotoxic concentrations. Taken together, these data imply that other factors such as differences in cellular uptake, cell distribution and/or an additional target within the cell might affect the cytotoxicity of this derivative.

The work presented here aimed at understanding the biochemical events elicited by DTNQ-Pro in Caco-2 human colon adenocarcinoma cells. This cell line, which is not particularly sensitive to doxorubicin treatment, was chosen specifically as a model of colon tumour cells, based on our previous results demonstrating the sensitivity of this cell line to DTNQ-Pro (Gomez-Monterrey *et al*., 2007). Moreover, as differentiated Caco2 cells is a well-accepted model for human enterocytes, they have been utilized to characterize a safety profile of compounds in terms of cell selectivity (Cotter *et al*., 2003; Kamath *et al*., 2003). We investigated the effects of DTNQ-Pro on colon adenocarcinoma cell lines in terms of growth and apoptosis, modulation of oxidative stress and HSP70 expression, HSP27 and vimentin distribution.

Methods

Cell culture

Caco-2 (American Type Culture Collection, Rockville, MD, USA), HT-29 (human colon adenocarcinoma grade II), SW260 (human colon carcinoma) cells were grown at 37°C in h-glucose MEM containing: 1% (by vol) non-essential amino acids and supplemented with 10% (by vol) de-complemented fetal bovine serum (FBS) (Flow, McLean, VA, USA), 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 1% L-glutamine and 1% sodium pyruvate. Cells were grown (17–21 passages) in a humidified atmosphere of 95% air/5% CO_2 at 37°C, and in six multi-well plates at different cell densities. After incubation for 4 h in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, the cells were washed with 1% phosphate-buffered saline (PBS) to remove unattached dead cells, and were incubated with different concentration of DTNQ-Pro. All experiments were performed in triplicate.

Cell proliferation analysis

For cell proliferation experiments, Caco-2 cells (1.0×10^5 control and treated cells) were seeded in 12 multi-well plates, and incubated at 37°C. After 12, 24 and 48 h, the cell number was determined with a haemocytometric counter, and cell proliferation was determined by CyQuant cell proliferation assay kit (Invitrogen, Milan, Italy) with dye fluorescence measurement at 480 nm excitation maximum and 520 nm emission maximum. Cell proliferation was expressed as percentage of cell proliferation compared with control. All data are the mean ± SD of at least three experiments.

Sensitivity of the cell lines to DTNQ-Pro

We assessed the sensitivity of the cell lines tested to DTNQ-Pro using a microplate colorimetric assay that measures the ability of viable cells to transform a soluble tetrazolium salt (MTT) to an insoluble purple formazan precipitate. Cells were plated at the appropriate density (5×10^3 HT-29 or SW620, or undifferentiated Caco-2 cells per well and 20×10^3 differentiated Caco-2 cells per well) in 96-well microtitre plates. After 4 h, cells were exposed to various concentrations of DTNQ-Pro for 48 h. Then, 50 µL of MTT ($1 mg \cdot mL^{-1}$) and 200 µL of medium were added to the cells in each well. After a 4 h incubation at 37°C, the medium was removed, then the formazan crystals were solubilized by adding 150 µL of DMSO and by mixing it in an orbital shaker for 5 min. Absorbance at 550 nm was measured using a plate reader. Experiments were performed in triplicate. As a control, 0.5% DMSO was added to untreated cells.

Flow cytometry analysis

Caco-2 cells were seeded in six multi-well plates at the density of 25×10^5 cells per plate. After 12, 24 and 48 h of incubation with different concentrations of DTNQ-Pro, cells were washed in PBS, centrifuged and directly stained in a propidium iodide (PI) solution (50 mg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 min at 4°C in the dark. Flow cytometric analysis

was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). To evaluate cell cycle, PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, not less than 20 000 events for each point were analysed in at least three separate experiments giving a SD less than 5%.

Superoxide anion production in mitochondria was determined by hydroethidine (HE) staining. The treated and untreated cells were incubated for 1 h with 20 $ng \cdot mL^{-1}$ HE, and were scraped, washed twice with PBS and the cell pellet was added to 1 mL PBS. HE–superoxide anion (HE-O) accumulation was measured by FACScan flow cytometer (FACScan, Becton Dickinson) using CellQuest software. For each sample 2×10^4 events were acquired. Analysis was carried out in triplicate in at least three separate experiments.

Alkaline phosphatase (ALP) activity

ALP activity was used as marker of the degree of differentiation of Caco-2 cells. Attached and floating cells were washed and lysed with 0.25% sodium deoxycholate, essentially as described by Herz *et al*. (1981). ALP activity was determined using Sigma Diagnostics ALP reagent (no. 245, Sigma Diagnostics Inc., St Louis, MO, USA). Total cellular protein content of the samples was determined in a microassay procedure as described by Bradford (1976) using the Coomassie protein assay reagent kit (Pierce, Rockford, IL, USA). ALP activity was calculated as units of activity per milligram of protein.

Immunofluorescence staining

Cells grown on coverslips were gently rinsed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were first incubated for 2 h at 4°C with the two primary antibodies, and then for 1 h with the specific secondary FITC- or TRITC-conjugated antibodies at room temperature. The primary antibodies used were: a monoclonal anti-vimentin antibody (Sigma), diluted 1/1000 and a polyclonal anti-HSP27 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1/800. The immunolabelled cells were examined with a confocal laser scanning microscope (LSM 510, Carl Zeiss Microimaging GmBH, Berlin, Germany).

Terminal deoxynucleotidyl transferase-mediated dUPT-biotin nick end-labelling (TUNEL) assay

TUNEL assay was performed using ApoAlert DNA fragmentation kit (Clontech Laboratories, Inc., Saint-Germain-en-Laye, France). Caco-2 cells were grown in multi-well plates and stimulated with different concentrations of DTNQ-Pro up to 72 h. DNA fragmentation was detected according to the manufacturer's recommendations. Apoptosis was quantified by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany).

Western blot assay

The effects of DTNQ-Pro on expression of Bcl-2, Bax, HSP70, HSP27, poly(ADP-ribose)polymerase (PARP), caspase-9, caspase-8 cyclin A and cyclin B1 were determined by Western blots. DTNQ-Pro stimulated and unstimulated (control) cell lysates were prepared using an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors containing antipain, bestatin, chymostatin, leupeptin, pepstatin, phosphoramidon, pefabloc, EDTA and aprotinin (Boehringer, Mannheim, Germany). Equivalent protein samples were resolved on 8–12% sodium dodecyl sulphate–polyacrylamide gels, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Segrate, Milan, Italy). For immunodetection, membranes were incubated overnight with specific antibodies at the concentrations recommended by the manufacturer. All antibodies were diluted in Tris-buffered saline/Tween 20–1% milk powder. This step was followed by incubation with the corresponding horseradish peroxidase conjugated antibody (anti-mouse IgG 1:2000, anti-rabbit IgG 1:6000; BioSource International, Camarillo, CA, USA). Bands were analysed by enhanced chemiluminescence (ECL kit, Amersham, Buckinghamshire, UK; GE Healthcare Europe GmbH, Milan, Italy).

Nitrite assays

NO is rapidly converted into the stable end products nitrite and nitrate. Nitrite was measured by the Griess reaction as reported by Green *et al*. (1982). Briefly, 100 µL of culture supernatant was mixed with an equal volume of Griess reagent (0.5% sulphanilamide, 2.5% H_3PO_4 and 0.05% naphthylethylene diamine in H_2O), and incubated for 10 min at room temperature. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrite.

Lipid peroxidation assay

Lipid peroxidation was evaluated using an analytical quantitative methodology. It relies upon the formation of a coloured adduct produced by the stoichiometric reaction of aldehydes with thiobarbituric acid (TBA). The TBARs assay was performed on membranes extracted from cells treated with DTNQ-Pro, and from untreated, control cells, using an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors. The homogenate was centrifuged at 1200× *g* for 10 min in order to separate cytosol (supernatants) from membranes (pellet). The pellet was dissolved in 50 mM Tris, 150 mM NaCl and 10 mM EDTA, and the protein content of the samples was determined by Bio-Rad assay (Bio-Rad Laboratories, San Diego, CA, USA). Aliquots (10 µL) of the menbrane preparation were added to 2 mL of TBA–trichloroacetic acid (TCA) (15% TCA, 0.3% TBA in 0.12 N HCl) solution at 100°C for 30 min. The reaction was stopped by cooling the sample in cold water, and, after a centrifugation at 15 000× *g* for 10 min, the chromogen (TBARs) was quantified by spectrophotometry at a wavelength of 532 nm. The amount of TBARs was expressed as $\mu M \cdot \mu g^{-1}$ proteins. All data are the mean ± SD of three experiments.

Statistical analysis

Values are expressed as the mean ± SE. The significance of the difference between the control and each experimental test

condition was analysed by unpaired Student's *t*-test, and $P < 0.05$ was considered statistically significant.

Materials

DMEM, PBS, MEM non-essential amino acids, streptomycin, penicillin, L-glutamine and FBS were purchased from Gibco-BRL (Grand Island, NY, USA). Tissue culture plasticware was purchased from Becton Dickinson (Lincoln Park, NJ, USA). HE was purchased from Invitrogen SRL, 2-TBA and TCA from Sigma Chemical Co. (St Louis, MO, USA). DTNQ-Pro used in this study was synthesized as described (Gomez-Monterrey *et al*., 2007).

The drug and molecular target nomenclature used in this paper follows Alexander *et al*. (2009).

Results

DNTQ-Pro exhibits cytotoxicity, modulates reactive oxygen species (ROS) and NO productions and increases the expression of MnSOD in Caco-2 cells

Exposure of pre-confluent Caco-2 cells to DTNQ-Pro caused a concentration- and time-dependent reduction of cell number (Figure 1A). A toxic effect was clearly observed after 12 h incubation with DTNQ-Pro (500 nM) resulting in 56% cell death, but this toxicity decreased after 24 or 48 h exposure to DTNQ-Pro to 42 and 17% cell death respectively. To investigate whether we had selected a drug-resistant cell population, fresh medium with 50 and 500 nM DTNQ-Pro was added. We observed a net decrease in the total number of cells (52% cell death) and accumulation of floating Caco-2 cells in the culture after a second treatment with 500 nM DTNQ-Pro for 24 h.

As free radical production is one of the main cytotoxic mechanisms elicited by quinone-based anti-tumour agents (Muller *et al*., 1998), such as doxorubicin, we examined mitochondrial superoxide anion production induced by DTNQ-Pro. Figure 1B shows the percentage mean fluorescence intensity of HE-superoxide anion (HE-O) obtained after incubation of Caco-2 cells with 50 and 500 nM of DTNQ-Pro for different periods. HE-O production after 12 h of treatment with DTNQ-Pro was increased 1.7-fold, compared to control cells, while at 48 h, production was decreased by 1.6-fold. These data suggest that cell growth inhibition after 12 h of DTNQ-Pro treatment was related to an initially increased production of ROS. To demonstrate that the initial cytotoxic effect of DTNQ-Pro was related to

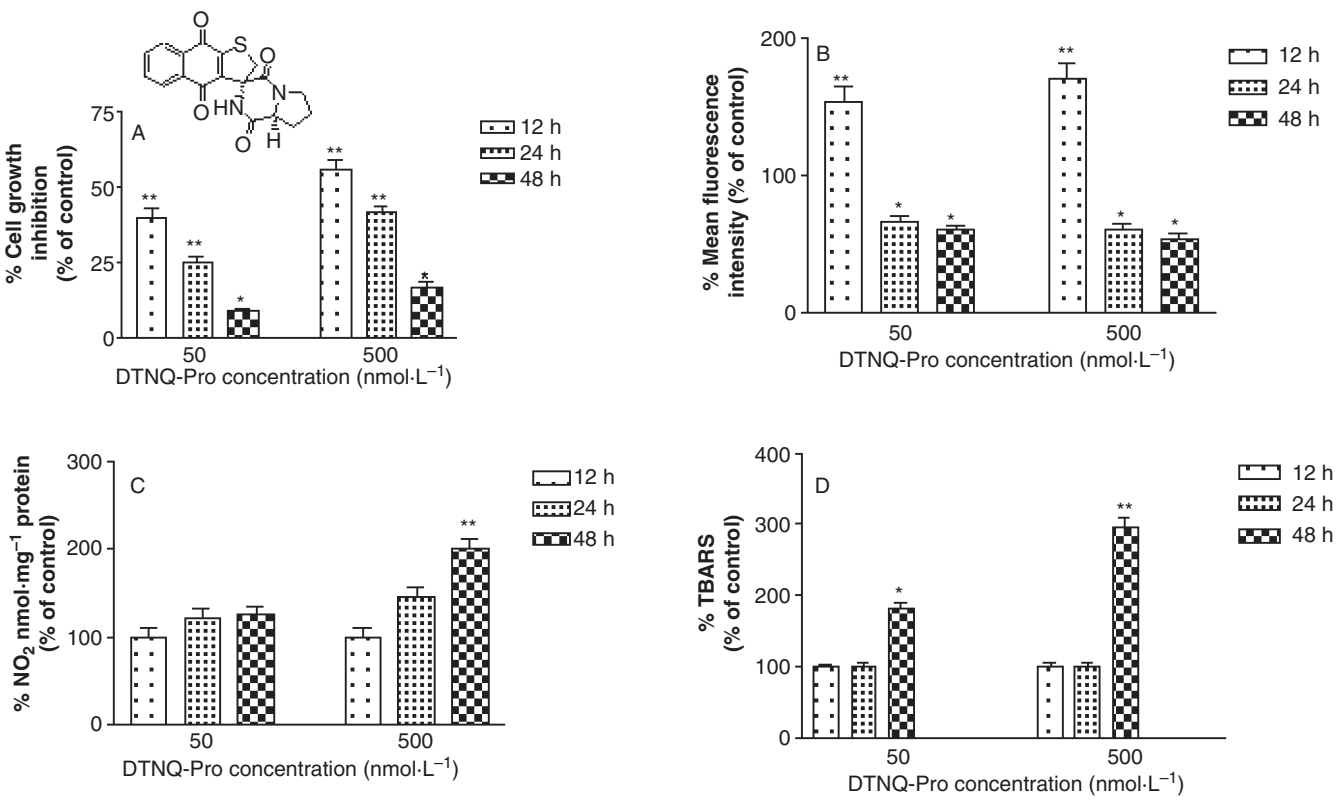

Figure 1 Structure of DTNQ-Pro. Effect of 50 and 500 nM DTNQ-Pro treatment on growth, anion superoxide, nitric oxide and TBARS production in Caco-2 cells. (A) Caco-2 cells were treated with DTNQ-Pro at 50 and 500 nM for 12, 24 and 48 h. After 12, 24 and 48 h, the cell growth inhibition was assessed by MTT assay. Cell proliferation was expressed as percent control. (B) Caco-2 cells were seeded in six multi-well plates at the density of 25×10^4 cells/plate. After 12, 24 and 48 h of the treatment with DTNQ-Pro (50 and 500 nM) at 37°C, the mitochondrial superoxide anion production was analysed by HE (20 ng·mL^{-1}) staining. Dye accumulation was analysed by FACScan flow cytometer (FACScan, Becton Dickinson) by the CellQuest software, and the intensities of the bands were expressed as percent control. For each sample, 2×10^4 events were acquired. Analysis was carried out in triplicate from at least three separate experiments. (C) Nitric oxide was measured in medium. (D) TBARS levels in Caco-2 cells after 12, 24 and 48 h of incubation with DTNQ-Pro. Results are expressed as TBARS as percent control. The bars represent means ± SEM of three independent experiments. Asterisks indicate significant difference between the Caco-2-treated samples compared with control value $^{**}P < 0.003$; $^{*}P < 0.05$; n.s. not significant.

Table 1 Growth inhibition of Caco-2 cells pre-incubated with ascorbic acid (aa-Caco-2) after treatment with DTNQ-Pro (50 or 500 nM) for 12 or 24 h

	12 h treatment		*24 h treatment*	
	DTNQ-Pro 50 nM	*DTNQ-Pro 500 nM*	*DTNQ-Pro 50 nM*	*DTNQ-Pro 500 nM*
aa-Caco-2 1 μM ascorbic acid	9 ± 0.2	30 ± 0.5	11 ± 0.3	21 ± 0.2
aa-Caco-2 10 μM ascorbic acid	5 ± 0.1 (40 ± 3)	25 ± 0.8(56 ± 3.5)	11 ± 0.2 (25 ± 1.9)	18 ± 0.5 (42 ± 2)

Data in the table are expressed as % inhibition of growth in aa-Caco-2 cells not treated with DNTQ-Pro (0% inhibition). Data in parentheses show inhibition by DNTQ-Pro in cells without pre-incubation with ascorbic acid and are taken from Figure 1A.

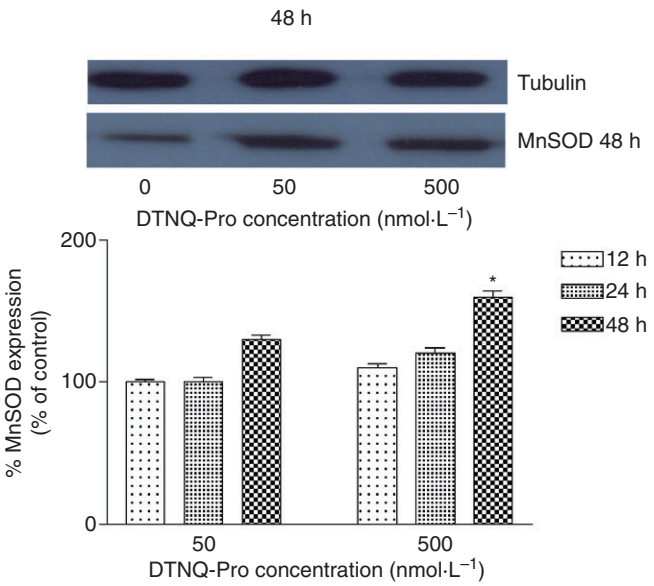

Figure 2 Time-course of MnSOD expression. The upper half of the figure shows a Western blot for MnSOD was normalized against the house-keeping protein γ-tubulin. Caco-2 cells were treated with 50 or 500 nM DTNQ-Pro for 48 h. All the experiments were performed at least three times with similar results. Summary data shown (lower half) are means ± SEM ($n = 4$; $^*P < 0.05$).

increased ROS production, we assessed cell death after treatment for 12 and 24 h of DTNQ-Pro in Caco-2 cells pre-incubated for 4 h with ascorbic acid (1 and 10 μM; aa-Caco-2 cells). Table 1 shows the inhibition of growth in Caco-2 cells under these conditions. The inhibition of cell growth after 12 h exposure to 500 nM DTNQ-Pro, in the aa-Caco-2 cells (pre-incubated with 10 μM ascorbic acid), was less than 50% of the inhibition in Caco-2 cells, without pre-incubation with ascorbic acid.

We have also studied the effect of DTNQ-Pro on endogenous nitric oxide (as NO_2^-) production and TBARS as lipid peroxidation markers (Wink and Mitchell, 1998). As shown in Figure 1, we observed an increase of both NO production (C) in the medium of Caco-2 cells and TBARS levels (D), but only after 48 h of treatment with 500 nM DTNQ-Pro. Figure 2 shows the time-course of MnSOD expression in Caco-2 cells, with and without treatment with DTNQ-Pro (50 or 500 nM). A 48 h treatment with 500 nM DTNQ-Pro induced a 60% increase in the expression of MnSOD, compared to untreated cells.

Cytotoxic activity of DTNQ-Pro in human colon cell lines

In order to characterize a safety profile of DTNQ-Pro, we evaluated its cytotoxic activity in undifferentiated and differentiated Caco2, HT29 and SW620 human colon cell lines. DTNQ-Pro presented a similar and potent cytotoxic activity against the undifferentiated Caco-2, HT29 and SW620, colon tumour cell lines, whereas this compound was 23-fold less potent in differentiated Caco-2 cells (Table 2).

DTNQ-Pro treatment decreases HSP70 accumulation, and redistributes HSP27 and vimentin in Caco-2 cell line

Figure 3A,B shows the time-course of accumulation of HSP70 and HSP27 in Caco-2 cell with and without DTNQ-Pro. After treatment with DTNQ-Pro (500 nM, 48 h), the surviving cell population showed a significant decrease (about 75%) of HSP70 accumulation, as compared with control cells not exposed to DTNQ-Pro. In contrast, HSP27 accumulation, in the same condition, increased 1.8-fold, compared to the control. Under our experimental conditions, vimentin and HSP27 protein were co-localized in the cytoplasm with a strong perinuclear staining in untreated (control) Caco-2 cells (Figure 4). Treatment for 48 h with 500 nM DTNQ-Pro induced a redistribution of perinuclear vimentin and HSP27 to the cell periphery, and a morphological re-arrangement of vimentin filaments.

DTNQ-Pro induces cell cycle arrest and enhances pre-confluent Caco-2 differentiation

To elucidate whether DTNQ-Pro was involved in cell cycle regulation, we analysed the percentage of G1, G2 and S-phase cells by FACS (Figure 5A). After DNTQ-Pro treatment (500 nM, 48 h), Caco-2 cells arrested in the S and G2 phases, while the G1 populations decreased. About 48% of Caco-2 cells were in S phase. This finding was further confirmed by a decrease in expression of cyclin A and B (Nigg, 2001) in Caco-2 cells treated with 50 nM DTNQ-Pro for 48 h (90 and 75%, respectively; Figure 5B).

As cell division arrest is one of the prerequisites for cell differentiation (Ding *et al*., 1998), we determined the effect of our molecule on Caco-2 differentiation. In Figure 6, the ALP activity is shown, as a marker of differentiation into enterocytes, correlated to post-confluent phase (Matsumoto *et al*., 1990). Treatment of pre-confluent Caco-2 with 500 nM DTNQ-Pro for 48 h increased ALP activity by 65%.

Apoptotic effect of DTNQ-Pro in Caco-2 cells

Treatment of Caco-2 cells surviving a first exposure to DTNQ-Pro, with a second exposure to DTNQ-Pro, induced apoptotic

Table 2 Cytotoxic activity of DTNQ-Pro in human colon cell lines

	Cell lines			
	Caco-2			
	Undifferentiated	*Differentiated**	*HT-29*	*SW620*
IC_{50} (μM)	0.52 ± 0.03	13 ± 1	0.43 ± 0.05	0.12 ± 0.03

*The differentiated Caco-2 cells were plated at 20×10^3 cells per well in 96-well microtitre plates.
IC_{50} (μM) values were measured in human colonic carcinoma cell lines after 48 h of treatment with different concentrations of DTNQ-Pro, using the MTT assay. Data shown are means ± SEM; ($n = 8$).

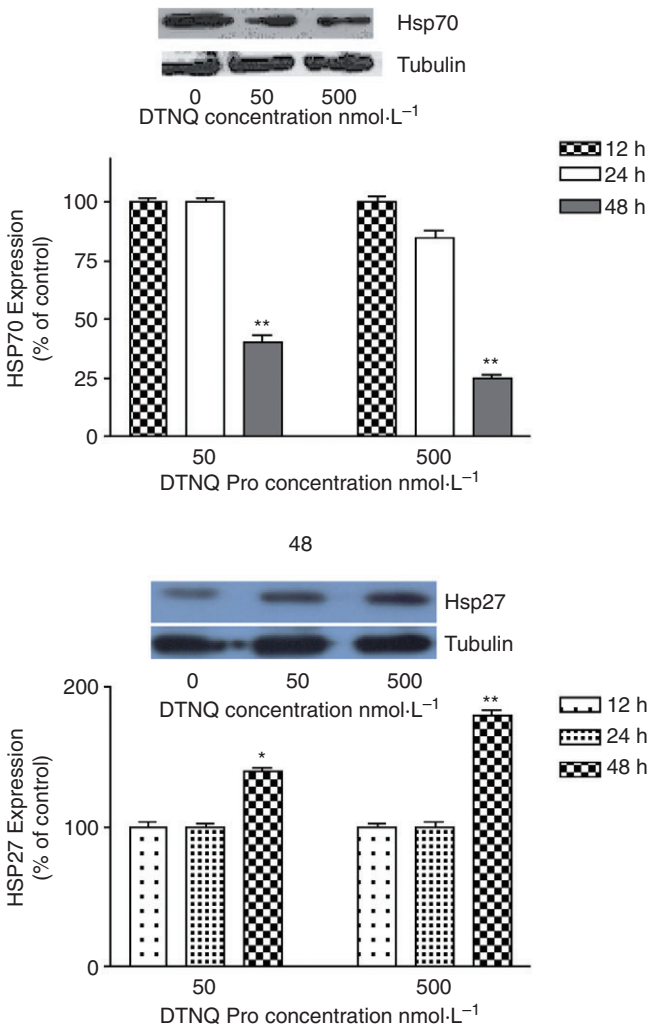

Figure 3 Expression of HSP70 and 27 in Caco-2 cells treated for 12, 24 and 48 h with DTNQ-Pro. The cells were incubated with 50 and 500 nM of DTNQ-Pro, and the HSP70 and 27 evaluated by Western blotting. All the experiments were performed at least three times with similar results. The graphs show the summary data (as % of expression in untreated cells), normalized to γ-tubulin expression after 48 h of treatment with DTNQ-Pro. Data shown are means + SEM ($n = 4$; *$P < 0.05$, **$P < 0.003$).

death after a further 24 h incubation. To confirm that cell death was induced via a programmed apoptotic pathway, we measured caspase-3 activity (Jaanicke *et al*., 1998). After 72 h treatment with 500 nM DTNQ-Pro, caspase-3 activity showed a threefold increase (Figure 7A). We also evaluated both PARP and caspase-9 cleavage as indicators of apoptosis (Germain *et al*., 1999; Los *et al*., 2002). Western blot analysis revealed that our molecule caused a dose-dependent increase of PARP and caspase-9 cleavage (Figure 7B). In addition, 500 nM DTNQ-Pro induced a higher expression of Bax pro-apoptotic factor (Figure 7C), further suggesting that the apoptotic pathway plays a role in the cytotoxicity induced by DTNQ-Pro. Furthermore, the percentage of apoptosis was evaluated labelling the cells for DNA fragmentation by TUNEL (FITC) assay and analysed using flow cytometry. After 24 h of a second exposure to DTNQ-Pro, this compound induced 24% cell death by apoptosis (data not shown).

Discussion

The present study was designed to test how DTNQ-Pro, a quinone-based pentacyclic derivative, exerts its anti-proliferative effect in the intestinal tumour cell line Caco-2. We showed that DTNQ-Pro had three major time-dependent consequences *in vitro* on Caco-2 cells.This compound modulated cellular redox status; it induced cell cycle arrest and differentiation, and it drove cells to programmed cell death, after a second treatment.

Exposure of Caco-2 cells to DTNQ-Pro up to 12 h increased the rate of both mitochondrial superoxide anions and non-apoptotic cell death. It has been reported that the pathological effects of ROS production, also caused by other quinone-based anti-tumour compounds, were related to their ability to cause oxidative damage to nuclear and mitochondrial DNA (Serrano *et al*., 1999). ROS reduction after 24 and 48 h correlated with increased expression of a major mitochondrial antioxidant scavenger, manganese superoxide dismutase (MnSOD), that directly catalyses superoxide conversion to hydrogen peroxide (H_2O_2). The decreased level of mitochondrial ROS paralleled the increase of free NO production, suggesting a potential involvement of MnSOD in regulating the balance between NO and peroxynitrite. A protective effect of NO has also been observed in endothelial cells and cardiomyocytes (Santucci *et al*., 2006), HT-29 human colon carcinoma cell line (Wenzel *et al*., 2003), urinary bladder mucosa (Andersson *et al*., 2008), inflammatory cells (Ronchetti *et al*., 2009) and cells of the CNS (Chiueh, 1999). When MnSOD is over-expressed, more superoxide radicals are converted to H_2O_2, itself also a cytotoxic agent, and therefore are removed from the physiological equilibrium, causing an increased pro-

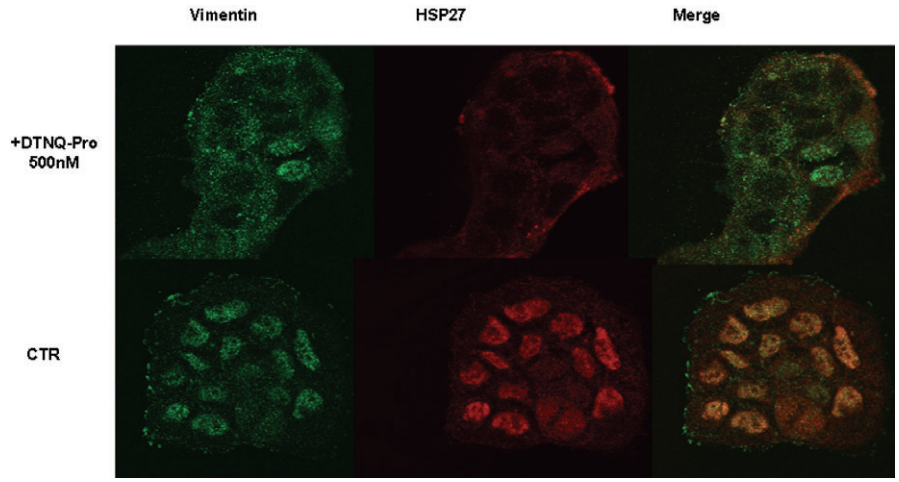

Figure 4 Subcellular co-localization of HSP27 with vimentin filaments in DTNQ-Pro-treated Caco-2 cells. The cells were incubated with anti-vimentin and anti-HSP27 antibodies. Images were obtained by confocal microscopy.

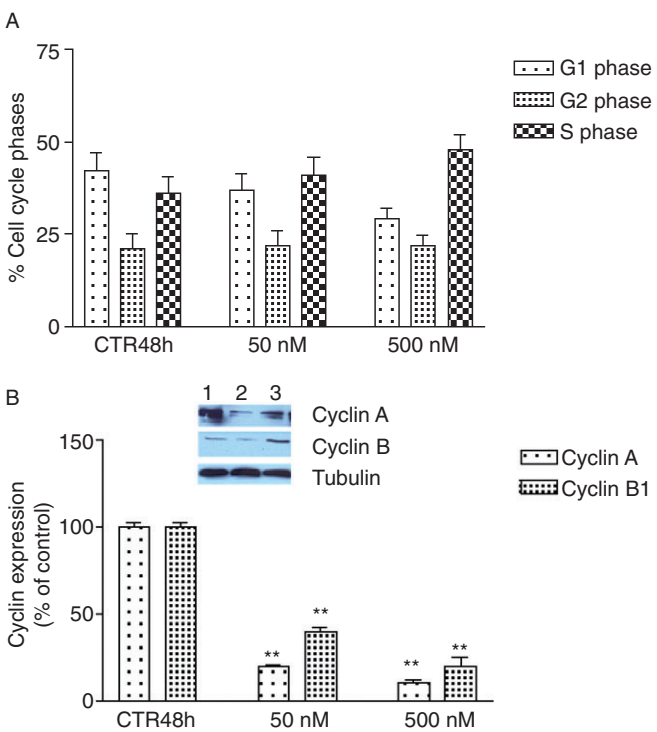

Figure 5 (A) Effects of DTNQ-Pro on the distribution of Caco-2 cell populations. Data represent the percentage of cells in each phase of the cell cycle. Cell cycle distribution was determined by DNA flow cytometric analysis. Samples from pre-confluent Caco-2 cells were analysed after 48 h of treatment with 500 or 50 nM DTNQ-Pro. Numbers indicate percentage of cells in G0/G1, S and G2/M phases. Data are representative of four separate analyses. (B) Western blot analysis of cyclin A, cyclin B and tubulin expression in untreated Caco-2 cells (lane 1), and treated with 500 and 50 nM DTNQ-Pro (lines 2 and 3 respectively). Summary data are shown in the graph, as % control (untreated Caco-2 cells) in arbitrary units;. means ± SEM; $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.003$.

duction of membrane lipid peroxidation. Higher level of membrane lipid peroxidation induced an increased expression of HSP27. Small HSPs are involved in a variety of cellular processes, including suppression of protein aggregation (Liberek *et al.*, 2008), dynamics of cytoskeleton (Kumarapeli and Wang, 2004) and cell growth and differentiation (Davidson *et al.*, 2002). Our finding suggests that the interaction of HSP27 with the intermediate filaments may help to maintain the structure and integrity of vimentin filaments under stress conditions (Mehlen *et al.*, 1997; Charette *et al.*, 2000; Concannon *et al.*, 2003). Our data show that the cell population surviving after a single treatment with DTNQ-Pro (500 nM, 48 h) had higher levels of HSP27 and arrest in S-phase, along with pre-confluent Caco-2 cell differentiation.

Moreover, it has been reported that in the Caco-2 cell line, HSP72 was expressed constitutively at high level (Musch *et al.*, 2001), and exposure to low levels of H_2O_2 and/or accumulation of HSP70 produced cells resistant to the lethal effects of a subsequent exposure to high levels of H_2O_2 (Mosser *et al.*, 2000; Aghdassi *et al.*, 2007). It has also been shown that association of HSPs with the cell membrane may result in the inactivation of membrane-perturbing signals, thereby switching off the response of HSP (Horváth *et al.*, 2008). Moreover, either the inhibition of HSP70 accumulation or depletion of inducible HSP70 caused death of tumour cancer cells (Gurbuxani *et al.*, 2001; Phillips *et al.*, 2007). Caco-2 cells after 48 h of DTNQ-Pro treatment presented a down-regulation of HSP70. Further, when the surviving differentiated cell population was subjected to a second treatment with DTNQ-Pro, cell growth rate was affected as shown by cell death determined by the mitochondrial apoptotic pathway triggered by cleavage of caspase 9 (Garrido *et al.*, 2001).

These findings show that DTNQ-Pro exerts cytotoxic activity through mechanisms of action different from other structurally related agents, such as anthracyclines (daunorubicin and doxorubicin), anthracenediones (mitoxantrone) (Gewirtz, 1999) and 2,3-disubstituted naphthoquinones (quinoclamine) (Chen *et al.*, 2009). We found that the DTNQ-Pro induced apoptosis in Caco-2 cells as a secondary event, following the modulation of oxidative stress. The decrease of mithocondrial superoxide anion by MnSOD induced a decreased expression of HSP70, with concomitantly increased membrane lipid peroxidation. Oxidative damage to the membrane induced a redistribution of both HSP27 and vimentin,

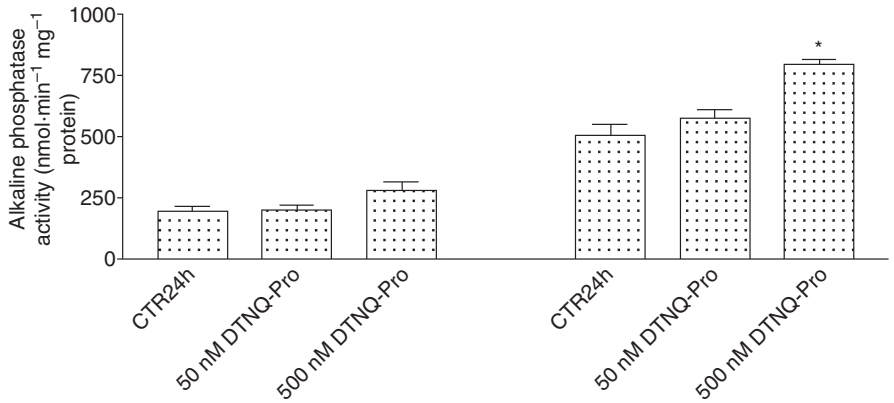

Figure 6 Differentiation of Caco-2 cells assessed by measurement of ALP activity after 24 and 48 h of culture with 0, 50 and 500 nM of DTNQ-Pro. Summary data shown are means ± SEM; (n = 4; *P < 0.05).

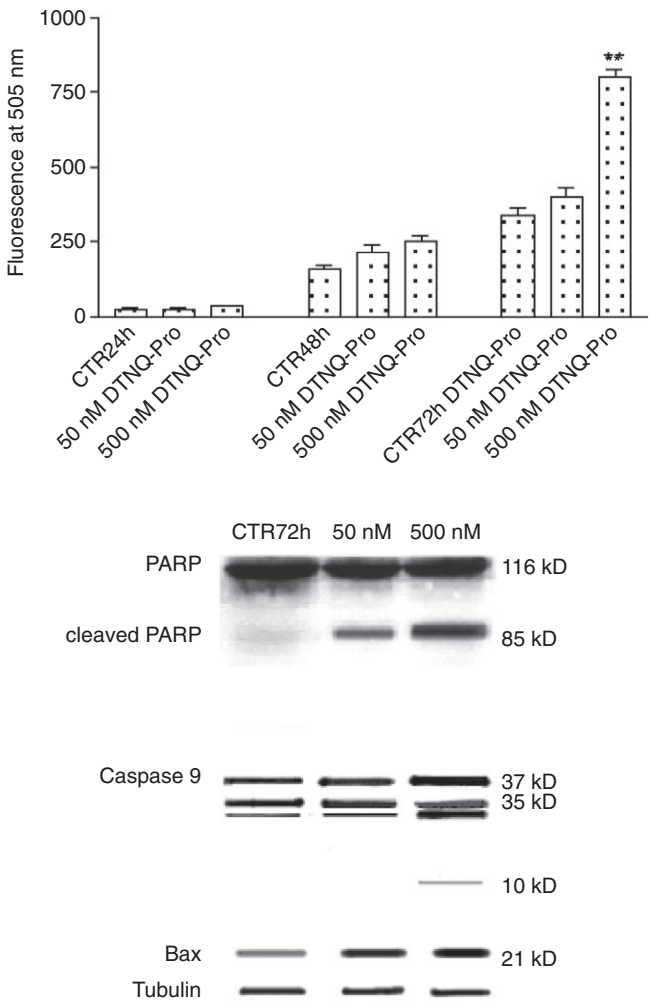

Figure 7 DTNQ-Pro induces apoptosis in Caco-2 cells. Caco-2 cells were treated with 500 or 50 nM of DTNQ-Pro for 24, 48 and with a second dose of DTNQ-Pro for 24 h followed by assay of cell-free caspase-3 activity. Caspase-3 activity was determined by incubating whole-cell extracts with 40 μM caspase-3 substrate and measuring production of hydrolysed 7-amido-4-methyl-coumarin (AMC) groups using a multi-label plate reader. The results are representative of four separate experiments; summary data shown are means ± SEM; **P < 0.003. PARP and caspase-9 cleavage as result of apoptotic cell death. Expression of Bax, after 72 h treatment of Caco-2 cells with 50 or 500 nM DTNQ-Pro.

and caused the undifferentiated Caco-2 cells to differentiate into enterocytes. Only the second treatment with DTNQ-Pro activated the apoptotic pathway.

It has been proposed that differentiated Caco-2 cell line may be considered an appropriate model for normal colon cells due to its ability to acquire the phenotype of mature small intestinal cells (Rousset, 1986; Stierum *et al*., 2003). We have found that DTNQ-Pro has a good profile in terms of cell selectivity. DTNQ-Pro presented a similar and potent cytotoxic activity against the colon tumour cell lines, while it was 23-fold less potent in the differentiated Caco-2 cells.

The ability of DTNQ-Pro to shift the undifferentiated Caco-2 cells to differentiated enterocytes and then undergo a process of programmed cell death, strongly suggests that this compound should be further investigated for its potential use in new combination chemotherapy for colon cancer.

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Conflicts of interest

None.

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