2′-Nitroflavone induces apoptosis and modulates mitogen-activated protein kinase pathways in human leukaemia cells

Mariano G. Cárdenas, Viviana C. Blank, Mariel N. Marder and Leonor P. Roguin

The cytotoxic activity of 2′-nitroflavone was evaluated in different haematological cancer cell lines and its mechanism of action was further studied in HL-60 cells. 2′-Nitroflavone arrested the cell cycle at the G_2/M phase and induced an apoptotic response characterized by an increase in the sub-G_1 fraction of cells, a typical DNA ladder fragmentation, chromatin condensation and the detection of cells stained with Annexin V. Apoptosis was dependent on the activation of at least caspase-8, caspase-9 and caspase-3. The involvement of the death receptor pathway was indicated by the upregulation of both the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptor (DR5). We also showed that 2′-nitroflavone increased the expression levels of Bax and induced the release of cytochrome C to cytosol, suggesting the participation of the mitochondria-dependent pathway. When mitogen-activated protein kinases pathways were studied, it was found that p38 and c-Jun NH_2-terminal kinase (JNK) pathways were activated by 2′-nitroflavone in HL-60 cells, whereas the phosphorylation levels of extracellular signal-regulated kinases (ERK) 1/2 decreased significantly. In addition, whereas both pharmacological inhibition of JNK and downregulation of JNK expression by RNA interference reduced the nitroflavone growth-inhibitory activity and the apoptotic effect, contrasting results were obtained when the ERK1/2 pathway was inhibited, and no effect was observed in the presence of a specific inhibitor of p38 mitogen-activated protein kinase. These findings show for the first time the antitumour action of 2′-nitroflavone in haematological cancer cell lines and suggest that both JNK and ERK1/2 cascades are involved in the apoptotic response induced by 2′-nitroflavone in HL-60 cells. *Anti-Cancer Drugs* 23:815–826 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:815–826

Keywords: apoptosis, caspases, human promyelocytic leukaemia cells, mitogen-activated protein kinases, 2′-nitroflavone

Institute of Biochemistry and Biophysics (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

Correspondence to Leonor P. Roguin, PhD, Institute of Biochemistry and Biophysics (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956-C1113AAD, Buenos Aires, Argentina
Tel: +54 114 964 8290; fax: +54 114 962 5457;
e-mail: rvroguin@qb.ffyb.uba.ar

Received 12 January 2012 **Revised form accepted** 22 March 2012

Introduction

Flavonoids, a group of polyphenolic compounds found in fruits, vegetables, tea and red wine [1,2], have been considered as promising antitumour and chemopreventive agents for cancer treatment [3–8]. Cancer is a multistep disease reflecting a succession of genetic alterations that lead to the progressive conversion of normal into malignant cells [9]. Apoptosis, the most common form of regulated cell death, can be induced by several anticancer drugs potentially useful to prevent tumour progression [10–14]. This highly organized cell death process is characterized by the activation of complex intracellular pathways leading to a series of typical morphological and biochemical changes [15,16]. The inhibition of apoptosis may be involved in the pathogenesis of proliferative diseases, such as autoimmune disorders or cancer [16]. In particular, the ability of several flavonoids to inhibit cancer cell growth and induce an apoptotic response has been considered a relevant mechanism for the elimination of cancer cells [5–8,17–20]. Different studies have shown that flavonoids can modulate various cellular transduction pathways related to growth regulation [21,22]. In mammalian systems, the family of mitogen-activated protein kinases (MAPKs) mediates multiple cellular responses that affect cell proliferation, differentiation, migration, survival and death [23–25]. As the balance between cell survival and death signals determines whether the cell lives or dies, flavonoids could control the fate of cancer cells by acting on different intracellular cascades, including the MAPK pathway, such as the extracellular signal-regulated kinases (ERK) 1/2, the c-Jun NH_2-terminal kinase (JNK) and the p38 MAPK.

We have previously examined the in-vitro antitumour activity of several natural and synthetic flavonoids in different human and murine adenocarcinoma cell lines [26]. The synthetic compound prepared in our laboratory, 2′-nitroflavone, showed the most potent and selective antiproliferative effect, without disturbing the proliferation of nontumour epithelial cells [26]. When the mechanism responsible for the antimitogenic action of this synthetic flavone was studied in a human cervix adenocarcinoma cell line, it was found that 2′-nitroflavone induced both cell cycle arrest and apoptosis [27]. The *in vivo* effectiveness of the nitroderivative was also determined in a murine mammary adenocarcinoma model [28]. In the

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328353f947

present study, we aimed to evaluate the cytotoxic activity of 2′-nitroflavone against several haematological cancer cell lines. HL-60 cells, derived from an acute promyelocytic leukaemia, were then used to elucidate the molecular mechanism underlying the growth-inhibitory action of 2′-nitroflavone. The ability of this derivative to activate the MAPKs signalling pathway and the contribution of some signals in the induction of an apoptotic response were also explored.

Materials and methods

Chemicals and antibodies

The synthesis of 2′-nitroflavone was performed as described previously [29,30]. The flavonoid was dissolved in dimethyl sulfoxide as 10 mmol/l stock solutions and stored at −70°C. Before use, the compound was diluted 1 : 10 in ethanol and added at the indicated concentrations to the culture medium. A final concentration of 10 µl vehicle/ml of medium was used in all experiments. The caspase-3 substrate Ac-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin (Ac-DEVD-AMC) was obtained from Upstate, EMD Millipore (Billerica, Massachusetts, USA), caspase-8 substrate Z-Ile-Glu-Thr-Asp 7-amino-4-trifluoromethyl coumarin (Z-IETD-AFC) was from Calbiochem (San Diego, California, USA) and caspase-9 substrate Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) was from Peptide Institute Inc. (Osaka, Japan). Caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), monoclonal antiactive Bax, anti-Fas and anti-Fas-L antibodies and rabbit polyclonal anti-Bcl-2, anti-Bcl-X_L and antitumour necrosis factor-related apoptosis-inducing ligand (anti-TRAIL) antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Monoclonal anticytochrome C antibody was from BD Biosciences Pharmingen (San Jose, California, USA). Rabbit polyclonal antibodies anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-JNK, anti-phospho-JNK (Thr183/Tyr185) and anti-death receptor 5 (DR5) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). SB203580 was from Santa Cruz Biotechnology, SP600125 was from Cayman Chemical (Ann Arbor, Minnesota, USA), and PD98059 was from Promega Corporation (Madison, Wisconsin, USA). The efficacy of SB203580, SP600125 and PD98059 as specific chemical inhibitors of p38, JNK and ERK pathways was checked properly (data not shown).

Cell lines and culture conditions

Human cell lines L-363 (plasma cell leukaemia, DSMZ ACC-49), K-562 (chronic myelogenous leukaemia, ATCC CCL-243), RPMI-8226 (multiple myeloma, ATCC CCL-155), HL-60 (acute promyelocytic leukaemia, ATCC CCL-240) and Jurkat (acute T cell leukaemia, DSMZ ACC-282) were grown at 37°C in RPMI-1640 medium (Gibco BRL, Gaithersburg, Maryland, USA) supplemented with 10% foetal bovine serum (FBS), 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. The same condition, except with a supplement of 10 mmol/l HEPES and 1 mmol/l sodium pyruvate, was used to maintain human cell lines U-937 (histiocytic lymphoma, ATCC CRL-1593.2) and Daudi (Burkitt's lymphoma, ATCC CCL-213). NFS-60 cells (mouse myelogenous leukaemia, CLS NFS-60) were cultured in RPMI-1640 medium supplemented with 4 mmol/l L-glutamine, 0.05 ng/ml granulocyte colony-stimulating factor (G-CSF) and 5% FBS. LB02 cells (murine T cell leukaemia), kindly provided by Dr Elida Álvarez, Instituto de Estudios de la Inmunidad Humoral (Buenos Aires, Argentina), were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/l L-glutamine, 50 mmol/l 2-mercaptoethanol, 50 U/ml penicillin and 50 µg/ml streptomycin.

Collection and processing of primary cells

Peripheral blood samples were obtained from healthy donors through previous informed consent. Human peripheral blood mononuclear cells (PBMCs) were isolated from a density Ficoll-Hypaque separation (Histopaque-1077; Sigma Aldrich, St Louis, Missouri, USA). Primary cells were cultured in RPMI-1640 containing 10% FBS at 37°C under a 5% CO_2 atmosphere. Proliferation assay was performed by adding 5 µg/ml of phytohaemagglutinin to the culture medium.

Proliferation assay

Cells were placed in 96-well microplates at a density of 2×10^4 cells/well (L-363, K-562, RPMI-8226), 5×10^4 cells/well (Jurkat, Daudi, U-937, HL-60, NFS-60, LB02) or 2×10^5 cells/well (PBMC) and incubated for 72 h at 37°C in the presence or absence of 20 µmol/l of 2′-nitroflavone in a total volume of 0.2 ml of culture medium. In some experiments, HL-60 cells were preincubated for 1 h with 10 µmol/l SB203580, 5 µmol/l SP600125 or 10 µmol/l PD98059 for blocking p38, JNK and ERK1/2 MAPKs, respectively, and then incubated for 48 h with or without 2 µmol/l 2′-nitroflavone. Alternatively, HL-60 cells transfected with control or JNK small interfering RNA (siRNA) were also used. Cell proliferation was determined using the CellTiter 96-AQueous Non-radioactive Cell Proliferation Assay (Promega Corporation) according to the manufacturer's instructions. Briefly, after incubation with 2′-nitroflavone, 20 µl of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and an electron-coupling reagent (phenazine methosulfate) were added to the medium. The plates were incubated at 37°C under a 5% CO_2 atmosphere for 2–4 h according to the cell line used. Absorbance at 492 nm corresponding to the conversion of MTS into a soluble formazan product was measured using a microtitre plate reader (Biotrak II; Amersham Biosciences, Piscataway, New York, USA). The molar drug concentration required to cause 50% growth inhibition

(IC_{50}) was determined from dose–response curves ranging from 0.5 to 80 μmol/l.

RNA interference

JNK1 siRNA, control siRNA, transfection reagent and medium were obtained from Santa Cruz Biotechnology. To specifically inhibit JNK1 expression, HL-60 cells were transfected and after 24 h a new transfection was performed according to the manufacturer's instruction. Following another 24 h, cells were harvested and used for western blot or were treated in the presence or absence of 2′-nitroflavone for proliferation assays and flow cytometry DNA analysis.

Flow cytometry DNA analysis

To evaluate either cell cycle-phase distribution or the proportion of hypodiploid cells, HL-60 cells were incubated for different times in the presence or absence of 20 μmol/l of 2′-nitroflavone. In some experiments, cells were preincubated for 1 h with 20 μmol/l caspase-8 or caspase-9 inhibitor, 10 μmol/l SB203580, 5 μmol/l SP600125 or 10 μmol/l PD98059, or were transfected with control siRNA or JNK siRNA. After treatment, cells were harvested, washed with cold PBS and then 1×10^6 cells were fixed overnight with 1 ml of 70% cold ethanol and kept at 4°C. The fixed cells were washed twice with PBS and resuspended in 500 μl of 0.1% sodium citrate buffer, pH 8.4, 0.1% Triton X-100 and 50 μg/ml propidium iodide (PI). After incubating overnight at 4°C, the cell cycle-phase distribution or the hypodiploid DNA content was analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, California, USA).

DNA ladder fragmentation

The apoptotic response was also evaluated by measuring DNA fragmentation. After treating HL-60 cells in the presence or absence of 20 μmol/l of 2′-nitroflavone for 24 h, cells were washed twice with PBS and lysed for 3 h at 56°C with agitation in lysis buffer (50 mmol/l Tris/HCl, pH 8.0, 10 mmol/l EDTA, 0.5% SDS, 250 μg/ml proteinase K). Subsequently, RNAse A (0.5 mg/ml) was added and incubated for 1 h. DNA was extracted by the usual phenol : chloroform : isoamilic (25 : 24 : 1) method and precipitated by adding 40 μl of sodium acetate 3 mol/l, pH 5.3 and 1 ml of absolute ethanol and kept overnight at –20°C. Precipitated DNA was washed with 70% cold ethanol and resuspended with 20 μl of 10 mmol/l Tris/HCl and 1 mmol/l EDTA at 50°C for 30 min with agitation. After electrophoresis in 2% agarose gels, DNA laddering was visualized by ethidium bromide staining.

Morphological changes visualization

HL-60 cells (5×10^5 cells/ml) were incubated for 16 and 24 h at 37°C in the presence or absence of 20 μmol/l of 2′-nitroflavone. After washing, cells were stained with 50 μg/ml ethidium bromide and 50 μg/ml acridine orange, and visualized using a fluorescence Olympus BX50 microscope (Olympus America Inc., Center Valley, Pennsylvania, USA) with the corresponding filters (ethidium bromide: 510–550 nm excitation and 590 nm emission wavelengths; acridine orange: 470–490 nm excitation and 515 nm emission wavelengths). Images obtained were then merged and analysed using the software Image Pro Plus 5.1 (MediaCybernetics Inc., Bethesda, Maryland, USA).

Annexin V apoptosis assay

HL-60 cells were incubated for 16 h in the presence or absence of 20 μmol/l of 2′-nitroflavone. After harvesting and washing the cells with cold PBS, phosphatidylserine externalization was assessed by Annexin V/PI double staining, according to the manufacturer's instructions (ApoAlert Annexin V-FITC Apoptosis Kit; BD Biosciences Clontech, Mountain View, California, USA). The results were analysed using a FACScan with WinMDi software (Becton Dickinson).

Caspase activity assays

HL-60 cells were incubated for different times in the presence or absence of 20 μmol/l of 2′-nitroflavone and after treatment cells were washed twice with cold PBS. Then, 1×10^6 cells were lysed for 30 min at 4°C in 50 μl of lysis buffer (10 mmol/l HEPES, pH 7.4, 50 mmol/l NaCl, 2 mmol/l $MgCl_2$, 5 mmol/l EGTA, 1 mmol/l phenylmethanesulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin), followed by three cycles of rapid freezing and thawing. Cell lysates were centrifuged at 17 000*g* for 15 min, and the total protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, California, USA). Aliquots containing 100 μg of protein were diluted in assay buffer [20 mmol/l HEPES, 132 mmol/l NaCl, 6 mmol/l KCl, 1 mmol/l $MgSO_4$, 1.2 mmol/l K_2HPO_4, pH 7.4, 20% glycerol and 5 mmol/l dithiothreitol (DTT)] and incubated for 2 h at 37°C with 50 μmol/l of the corresponding fluorogenic substrates (Ac-DEVD-AMC: caspase-3, Z-IETD-AFC: caspase-8, Ac-LEHD-AMC: caspase-9). Cleavage of the substrates was monitored in an SFM25 Konton Fluorometer. The fluorescence emitted by the 7-amino-4-methyl coumarin (AMC) was measured at 355 nm excitation and 460 nm emission wavelengths, whereas 7-amino-4-trifluoromethyl coumarin (AFC) was monitored at 400 nm excitation and 505 nm emission. Results were expressed as the change in fluorescence units (per μg of protein) relative to the control.

Western blot analysis

HL-60 cells were incubated for different times in the presence or absence of 20 μmol/l of 2′-nitroflavone and washed twice with cold PBS. Then, 1×10^6cells were lysed for 30 min at 4°C in 10 μl of lysis buffer (10% glycerol, 0.5% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml trypsin inhibitor, 1 μg/ml leupeptin, 10 mmol/l $Na_4P_2O_7$, 10 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l EDTA, 1 mmol/l PMSF, 150 mmol/l NaCl and 50 mmol/l Tris, pH 7.4). Clear cell lysate supernatants were prepared by

centrifugation and aliquots containing 100 µg of protein were resuspended in 0.063 mol/l Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% 2-mercaptoethanol, subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto nitrocellulose membranes (Amersham Biosciences) for 1 h at 100 V in 25 mmol/l Tris, 195 mmol/l glycine and 20% methanol, pH 8.2. After blocking, membranes were treated as in the usual western blotting. The secondary antibodies applied were anti-mouse IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology) or anti-rabbit IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology). Immunoreactive proteins were visualized using the ECL Plus detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. For the quantification of band intensity, western blots were scanned using a densitometer (Gel Pro Analyzer software; MediaCybernetics Inc.). Equal protein loading was confirmed by reprobing membranes with a rabbit antiactin antibody (Sigma Aldrich).

Immunodetection of cytosolic cytochrome C

After incubating HL-60 cells for different times in the presence or absence of 20 µmol/l of 2′-nitroflavone, 1×10^6cells were resuspended in 30 µl of sucrose buffer (250 mmol/l sucrose, 20 mmol/l HEPES, pH 7.5, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 0.1 mmol/l PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) and incubated on ice for 15 min. Cells were then homogenized, cytosolic fractions were prepared by differential centrifugation and 30 µg of protein/lane was loaded onto 16% SDS-PAGE and then transferred onto nitrocellulose membranes as indicated above.

Statistical analysis

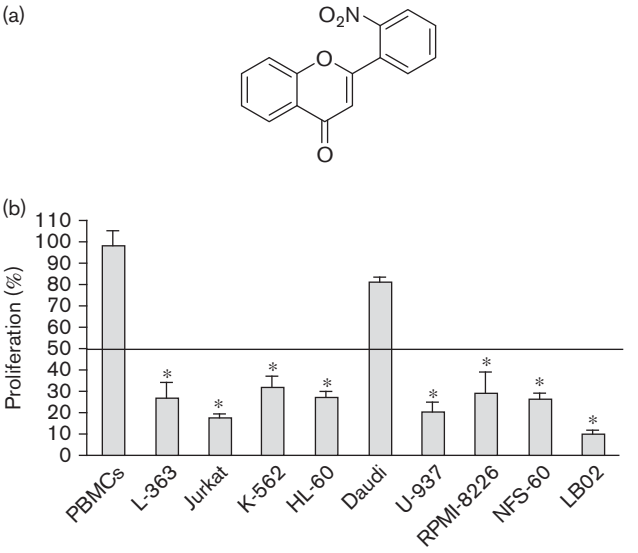
The values are expressed as mean ± SEM. Statistical analysis of the data was performed using Student's *t*-test. A *P* value less than 0.05 denotes a statistically significant difference.

Results

Cytotoxic activity of 2′-nitroflavone on different haematological cancer cell lines

The effect of 2′-nitroflavone (chemical structure shown in Fig. 1a) on cell proliferation was evaluated *in vitro* in various human and murine haematological cancer cells. Cell viability was initially tested at a 20 µmol/l concentration of 2′-nitroflavone in four human leukaemia cell lines (L-363, Jurkat, K-562, HL-60), two human lymphomas (Daudi, U-937), one human myeloma cell line (RPMI-8226) and two murine leukaemia cell lines (NFS-60, LB02). PBMCs extracted from healthy donors were also used as selectivity controls. As shown in Fig. 1b, 2′-nitroflavone significantly inhibited the growth of the majority of the cancer cell lines tested, the human Burkitt's lymphoma Daudi being the most resistant to growth inhibition. In addition, 2′-nitroflavone did not affect the proliferation of PBMC, suggesting a selective action against tumour cells. Cytotoxic activities, expressed as IC_{50} values, were then evaluated in tumour cells that inhibited greater than 50% at 20 µmol/l 2′-nitroflavone and also in nontumour cells (Table 1). Remarkably, the nitroflavone derivative showed a potent cytotoxic effect in the cancer cells tested (IC_{50} values <10 µmol/l), but not in PBMC (IC_{50} value >80 µmol/l). On the basis of these results, the mechanism of antitumour action was further studied on HL-60 cells, the most susceptible cell line to the effect of 2′-nitroflavone (IC_{50} 1 ± 0.5 µmol/l).

Fig. 1



(a) Chemical structure of 2′-nitroflavone. (b) Effect of 2′-nitroflavone on the proliferation of haematological cancer and nontumour cells. After incubating 2×10^4 cells/well (L-363, K-562, RPMI-8226), 5×10^4 cells/well (Jurkat, Daudi, U-937, HL-60, NFS-60, LB02) or 2×10^5 cells/well (PBMC) in the absence (control) or presence of 20 µmol/l 2′-nitroflavone for 72 h at 37°C, cell proliferation was determined using the MTS method. Results are expressed as the percentage of proliferation obtained with respect to the control cells and represent the mean ± SEM of three independent experiments (*$P<0.001$). PBMC, peripheral blood mononuclear cells.

2′-Nitroflavone induces cell cycle arrest on HL-60 cells

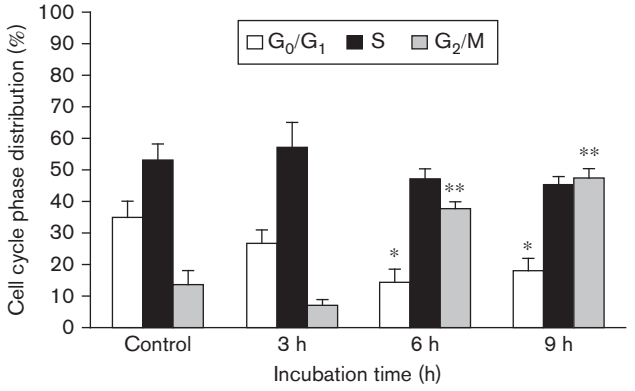
To examine whether 2′-nitroflavone affects cell cycle progression, HL-60 cells were incubated in the presence or absence of a 20 µmol/l concentration of the synthetic flavone for different times and then analysed by flow cytometry. As shown in Fig. 2, 2′-nitroflavone treatment for up to 9 h led to a progressive increase in the population of cells in G_2/M and a decrease in cells in the G_0/G_1 phase. Thus, the percentage of cells in G_0/G_1 decreased from 35 ± 5% (control) to 18 ± 4% after 9 h of incubation with 2′-nitroflavone, whereas a significant increase from 14 ± 4% (control) to 47 ± 4% was observed in the G_2/M phase.

Table 1 Cytotoxic activity of 2′-nitroflavone on haematological cancer cell lines

Cell line	IC_{50} (μmol/l)[a]
L-363	4±0.5
Jurkat	3±1
K-562	2±0.5
HL-60	1±0.5
U-937	9±1.4
RPMI-8226	4±2
NFS-60	8±0.8
LB02	8±3
PBMCs	>80

PBMC, peripheral blood mononuclear cells.
[a]The drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose–response curves. Results represent means±SEM of at least three different experiments.

Fig. 2



Effect of 2′-nitroflavone on the cell cycle-phase distribution in HL-60 cells. Cells (2.5×10^5 cells/ml) were incubated for different times in the absence (control) or presence of 20 μmol/l 2′-nitroflavone and then analysed by flow cytometry. Control bars correspond to cells incubated without 2′-nitroflavone for 3 h and similar results were obtained after 6 and 9 h of incubation. Analysis of the DNA content was carried out excluding the sub-G1 cell population. Results are mean values ± SEM of three different experiments. Statistical significance in comparison with the corresponding control values is indicated by $*P<0.01$; $**P<0.001$.

2′-Nitroflavone induces apoptosis on HL-60 cells

Different experimental approaches were used to evaluate the induction of an apoptotic response by 2′-nitroflavone on HL-60 cells. Thus, an increase in the sub-G_1 fraction of cells, representing an apoptotic state, was observed by flow cytometry. The DNA histograms shown in Fig. 3a showed that after incubating HL-60 cells with 2′-nitroflavone for up to 24 h, the percentage of hypodiploid cells increased significantly in a time-dependent manner. In addition, a typical DNA ladder fragmentation pattern was obtained when HL-60 cells were treated with 2′-nitroflavone for 24 h (lane 4) and no DNA fragmentation was observed in control cells (lane 2) or cells incubated in the presence of vehicle (lane 3) (Fig. 3b). The apoptotic effect of 2′-nitroflavone was also evaluated by determining the phosphatidylserine exposition on the outer side of the plasma membrane with Annexin V. When HL-60 cells were treated with 2′-nitroflavone for 16 h, a considerable increase in the population of early apoptotic cells was observed (Fig. 3c). Thus, the percentage of Annexin $V^+ PI^-$ cells increased from 7±3 to 21±3%, whereas no significant change was observed in the population of late apoptotic cells (Annexin $V^+ PI^+$).

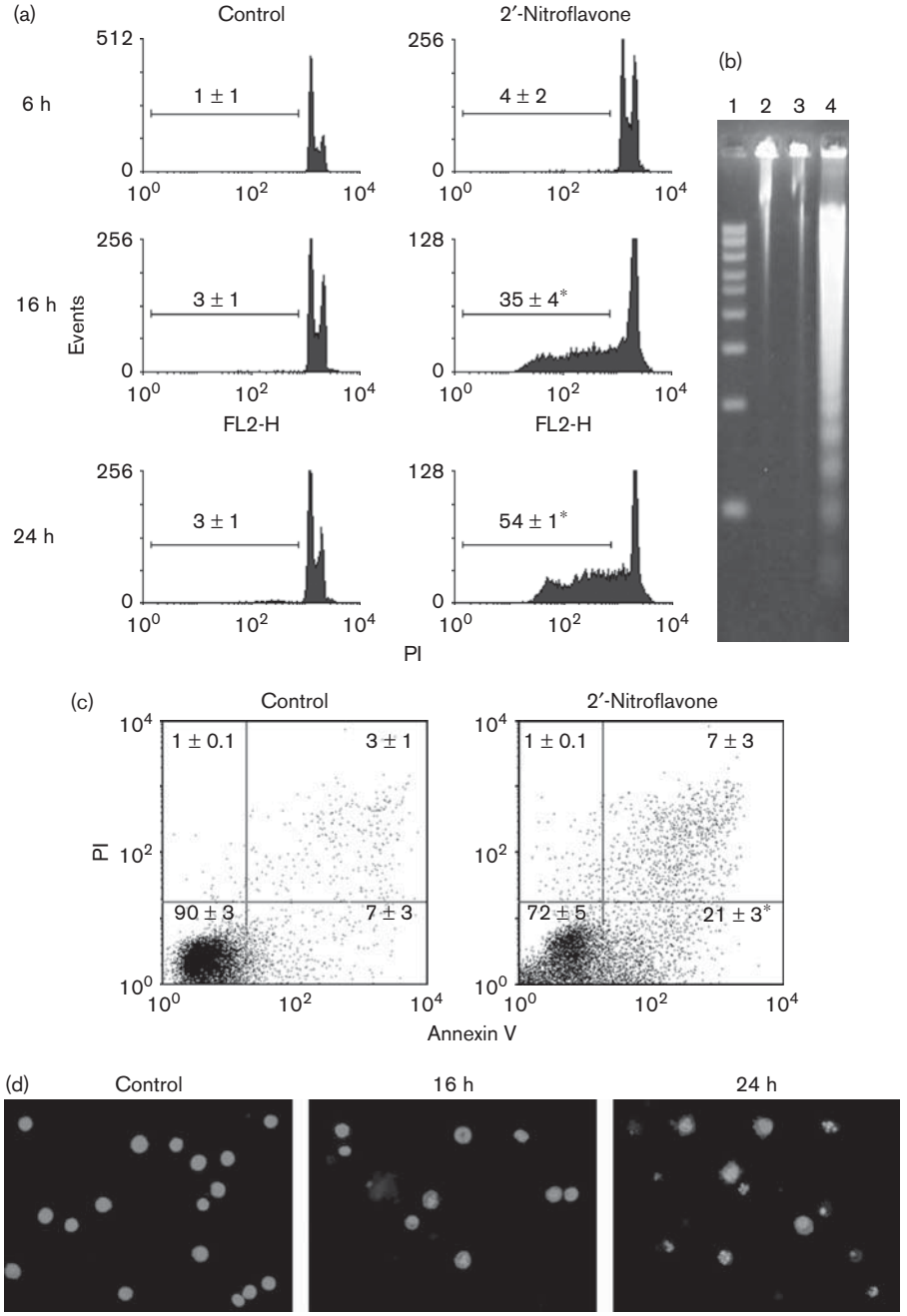
To further characterize the apoptotic effect induced by 2′-nitroflavone, HL-60 cells were stained with ethidium bromide and acridine orange and visualized using a fluorescence microscope. The characteristic morphological changes in viable apoptotic cells (green condensed or fragmented chromatin) were observed after 16 h of incubation, whereas both viable and nonviable (orange fragmented DNA) apoptotic cells were distinguished after 24 h (Fig. 3d).

Death receptor and mitochondrial pathways are involved in the apoptosis induced by 2′-nitroflavone

To examine whether 2′-nitroflavone-induced cell death is dependent on caspase activation, we measured the proteolytic activity of the executioner caspase-3 and the initiator caspase-8 and caspase-9. As shown in Fig. 4, a significant increase in caspase-3 activity was observed after 6 h of exposure to 2′-nitroflavone, the activity being higher and persistent between 9 and 24 h. The activation of caspase-8 was evident after 16 and 24 h of incubation, whereas caspase-9 activity increased 9 h after treatment and remained elevated for at least 24 h. The contribution of caspases was also examined after preincubating 2′-nitroflavone-treated cells with the specific inhibitors of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK). Incubation with 2′-nitroflavone for 24 h increased the sub-G_1 population of HL-60 cells from 6±2 (control) to 53±4%, whereas a lower percentage of hypodiploid cells was obtained in the presence of Z-IETD-FMK (44±3%, $P < 0.005$) or Z-LEHD-FMK (39±1%, $P < 0.005$).

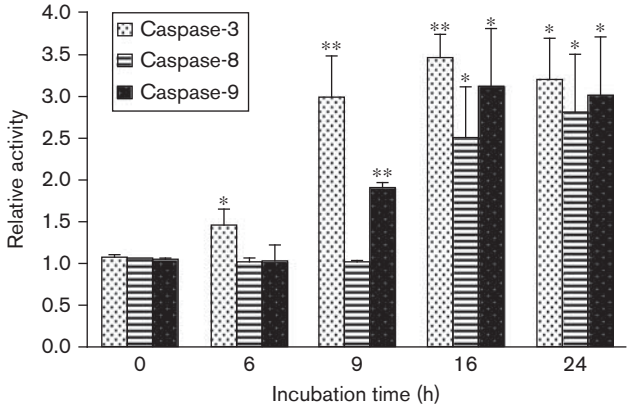
As caspase-8 activation suggested the contribution of the death receptor pathway, we used western blot to evaluate whether there was any change in the expression of either death receptors or their corresponding ligands. It was found that the TRAIL and DR5 levels increased almost two-fold as early as 6 h after treatment with 2′-nitroflavone, whereas the Fas and Fas-L levels remained constant (Fig. 5a and d). We further evaluated whether the apoptotic cell death induced by 2′-nitroflavone induced a change in the expression of Bcl-2 family proteins. When HL-60 cells were incubated for different times with the nitroflavone derivative, no change was found in the amount of the antiapoptotic proteins Bcl-2 and Bcl-X_L, whereas a significant increase in the expression of the proapoptotic Bax protein was observed (Fig. 5b and d). Thus, Bax levels increased approximately 50% after 6 h of treatment and continued to increase for at least 16 h. As it has been established that mitochondrial activation is followed by the release of cytochrome C from the inter-

Fig. 3



Apoptotic action of 2′-nitroflavone in HL-60 cells. (a) Cells (2.5×10^5 cells/ml) were incubated for different times in the absence (control) or presence of 20 μmol/l 2′-nitroflavone and then the hypodiploid DNA content was evaluated by flow cytometry after propidium iodide (PI) staining. The percentage of apoptotic cells ± SEM of three different experiments is shown in each histogram. Statistical significance in comparison with the corresponding control values is indicated by $*P<0.001$. (b) Cells (2.5×10^5 cells/ml) were incubated for 24 h in the absence (control) or presence of 20 μmol/l 2′-nitroflavone. Genomic DNA was extracted, electrophoresed on a 2% agarose gel and then visualized by ethidium bromide staining. (1) Molecular weight markers; (2) control cells; (3) cells with 10 μl vehicle/ml of culture medium; (4) 2′-nitroflavone. (c) Cells (2.5×10^5 cells/ml) were incubated for 16 h in the absence (control) or presence of 20 μmol/l 2′-nitroflavone. After double staining with Annexin V/PI, the apoptotic cell population was evaluated by flow cytometry. Results represent mean values ± SEM of three different experiments. Statistical significance in comparison with the corresponding control values is indicated by $*P<0.005$. (d) Fluorescence microscopy images of HL-60 cells. Cells were incubated in the absence (control) or presence of 20 μmol/l 2′-nitroflavone for 16 and 24 h and then stained with a mixture of ethidium bromide and acridine orange (magnification, × 400).

Fig. 4



Caspases-mediated apoptosis induced by 2′-nitroflavone. HL-60 cells (2.5×10^5 cells/ml) were incubated for different times in the absence (control) or presence of 20 μmol/l 2′-nitroflavone and then the activities of caspase-3, caspase-8 and caspase-9 were determined as indicated in the Materials and methods section. Enzymatic activities are expressed as the ratio of the fluorescence per μg of protein of the treated sample with respect to the fluorescence per μg of protein of the control and represent mean values ± SEM of three different experiments. Statistical significance in comparison with the corresponding control values is indicated by $*P<0.05$; $**P<0.005$.

membrane space, we decided to evaluate cytochrome C levels in the cytosol after 2′-nitroflavone treatment. As shown in Fig. 5c and d, a significant increase in the amount of cytochrome C was found at 9, 16 and 24 h of incubation with 2′-nitroflavone.

Role of the mitogen-activated protein kinases pathway in the apoptotic action induced by 2′-nitroflavone

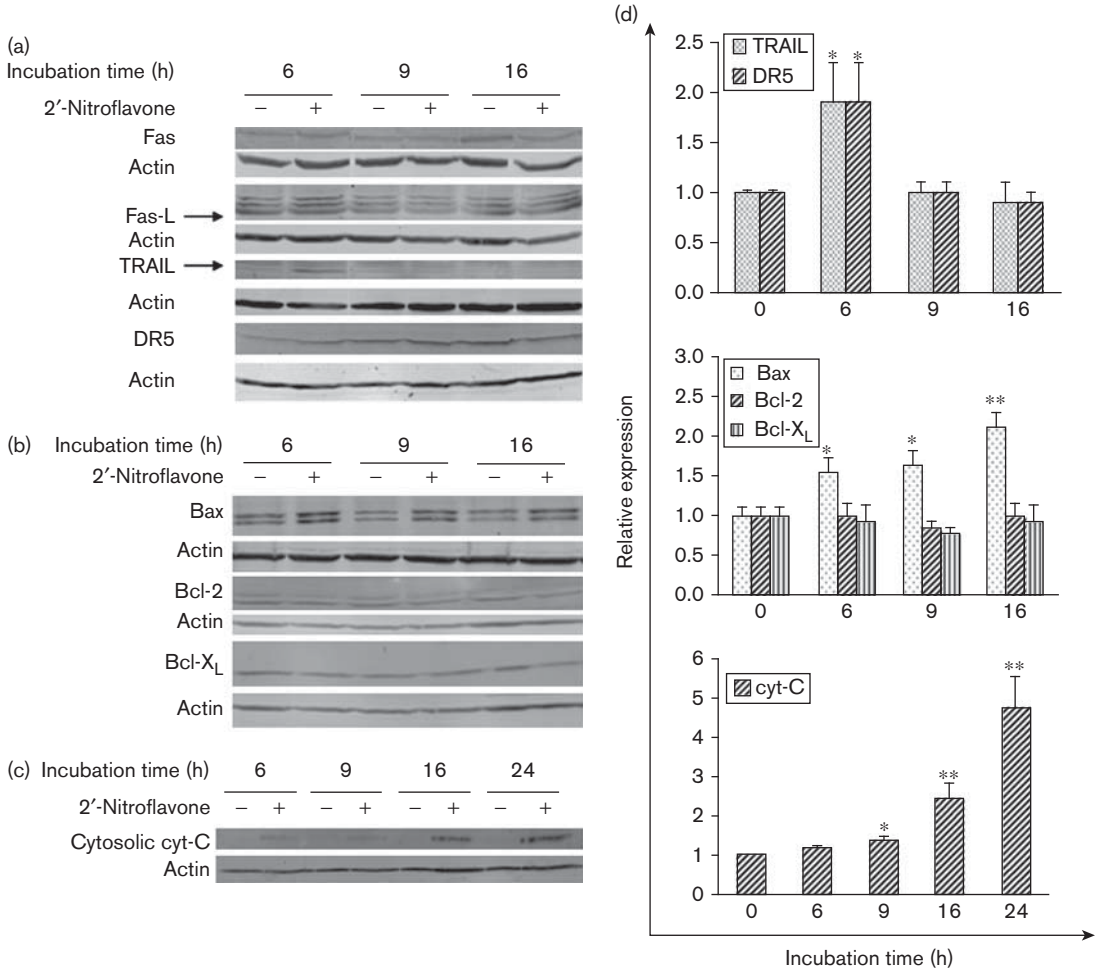
Mammalian MAPKs include three main groups: ERK1/2, p38 and JNK kinases [23–25,31–33]. The ERK1/2 pathway has mainly been associated with mitogenic and survival signals [31–34]. In contrast, p38 and JNK cascades have frequently been involved in the induction of apoptotic responses [31–33]. To explore whether 2′-nitroflavone affects the activation of these kinases in HL-60 cells, the phosphorylation kinetics of ERK1/2, p38 and JNK were evaluated by western blot analyses with phospho-specific antibodies. A significant decrease in ERK1/2 phosphorylation was found in HL-60 cells 15 min after the addition of 2′-nitroflavone and this reduction was maintained for up to 120 min (Fig. 6a and b). Activation of p38 was observed after 15 min and phosphorylation levels remained elevated for at least 120 min (Fig. 6a and b). In addition, JNK was strongly activated and high levels of phosphorylated JNK were obtained at 30, 60 and 120 min after treatment (Fig. 6a and b). The contribution of p38, JNK and ERK1/2 pathways towards the growth-inhibitory and apoptotic responses induced by 2′-nitroflavone was further studied using the corresponding selective inhibitors SB203580, SP600125 and PD98059. When HL-60 cells incubated with the nitroflavone derivative were pretreated with SB203580, no difference in cell growth was found after 48 h of incubation, whereas a significant increase was observed in the presence of SP600125 and a greater growth inhibition was achieved when cells were incubated with PD98059 (Fig. 7a). In addition, SP600125 but not SB203580 reduced the amount of apoptotic cells obtained after 2′-nitroflavone treatment (Fig. 7b). Thus, when cells incubated with the synthetic flavone were pretreated with the JNK inhibitor, the percentage of hypodiploid cells decreased from 27 ± 4 to 13 ± 1%, suggesting that JNK activation is involved in the apoptotic cell death mediated by 2′-nitroflavone (Fig. 7b). Furthermore, a higher amount of hypodiploid cells (42 ± 2%) was obtained for cells pretreated with PD98059 and exposed to 2′-nitroflavone, indicating that the ERK1/2 pathway contributes to proliferation and survival signals.

To confirm that JNK signalling is required for 2′-nitroflavone-induced cell death, JNK expression was inhibited using specific siRNAs. Densitometric analysis of western blots assays showed that the JNK levels decreased approximately 30% after blocking with the corresponding JNK siRNA (Fig. 8a). When JNK knockdown cells were incubated with 2′-nitroflavone, a significant increase in cell proliferation was observed (Fig. 8b). Furthermore, a lower percentage of apoptotic cells was obtained for specific siRNA-treated cells with respect to control siRNA cells incubated in the presence of the flavone derivative (Fig. 8c). Thus, after transfection with JNK siRNA, the sub-G_1 fraction of cells decreased from 21 ± 1 to 16 ± 1% for 2′-nitroflavone-treated cells.

Discussion

We have previously shown the in-vitro and in-vivo antitumour action of the synthetic flavonoid 2′-nitroflavone in different human and murine adenocarcinoma cell lines [26–28]. In this study, we showed for the first time that 2′-nitroflavone also shows a potent and selective cytotoxic activity in several haematological cancer cell lines, without affecting the proliferation of human PBMC isolated from healthy donors. The molecular pathways involved in growth inhibition were further studied in HL-60 cells, the most susceptible cell line to the effect of 2′-nitroflavone (IC_{50} 1 ± 0.5 μmol/l). In comparison with the cytotoxicity shown by different natural flavonoids in HL-60 cells, 2′-nitroflavone showed stronger potency. Thus, when the effect of some natural flavonoids, such as apigenin, luteolin, quercetin and chrysin, was tested on the proliferation of HL-60 cells, IC_{50} values around 10–20 μmol/l were reported [35]. A comparative study between these hydroxylated flavonoids and their fully methylated counterparts showed that although both types of flavonoids were able to inhibit cellular proliferation, in general, the unmethylated compounds showed higher effectiveness in human leukaemia HL-60 cells [36]. However, a greater activity

Fig. 5



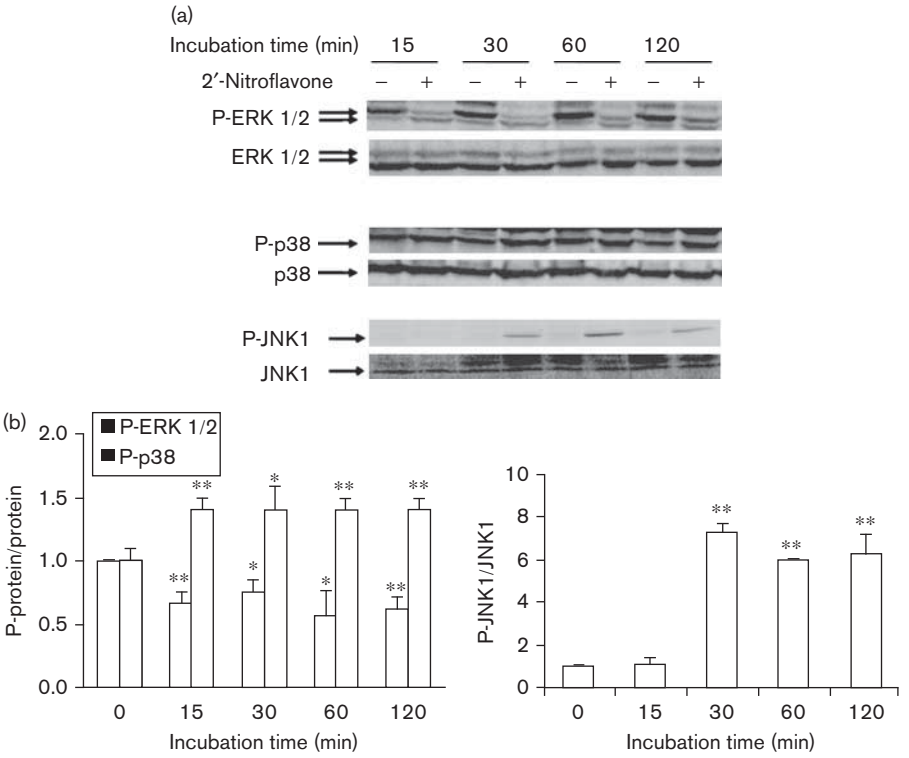
Levels of Fas and DR5 receptors, TRAIL and Fas-L, Bcl-2 family proteins and cytochrome C in HL-60 cells treated with 2′-nitroflavone. Cells were incubated for different times with 20 μmol/l 2′-nitroflavone. Total cell lysates for (a) Fas, Fas-L, DR5 and TRAIL and (b) Bcl-2 family protein, and cytosolic fraction for (c) cytochrome C, were processed for western blot analysis as described in the Materials and methods section; equal loading was confirmed by stripping and reprobing each blot for actin. The results from one representative experiment are shown. (d) Protein quantification was performed by densitometric analysis using Gel Pro Analyzer software. Results are expressed as means ± SEM of three different experiments (*$P<0.05$; **$P<0.005$).

has been reported for some partially methylated compounds [37,38]. In this respect, a derivative of quercetin, 5,7,3′-trihydroxy-3,4′-dimethoxyflavone, has been found that has cytotoxic potency in human leukaemia cells similar to that determined here for 2′-nitroflavone [38].

As it has been shown that the cell cycle arrest mediated by some flavonoids is dependent on the particular compound structure [39], we evaluated the effect of 2′-nitroflavone on HL-60 cell cycle-phase distribution. We showed that the nitroderivative produced an increase in the population of cells in the G_2/M phase and a decrease in G_0/G_1 in a time-dependent manner. A slightly different cell phase distribution, with an increase in the G_2/M and S phases, was observed previously when the same compound was tested in HeLa cells [27], indicating a cell type-specific 2′-nitroflavone effect.

The induction of an apoptotic response was assessed by the increase in the population of HL-60 cells with a sub-G_1 DNA content, the detection of a typical pattern of DNA fragmentation, the appearance of early apoptotic cells stained with Annexin V and the identification of cells with condensed or fragmented chromatin. It is known that the binding of death ligands, such as Fas-L, TNF or TRAIL, to their corresponding cell surface receptors promotes the formation of a death-inducing signalling complex that activates caspase-8, whereas caspase-9 is activated after the release of cytochrome C from the mitochondria and the formation of the apoptosome, a cytosolic death signalling complex. Activation of the initiator caspases leads to the cleavage of caspase-3 and other caspases that are responsible for the morphological and biochemical changes in the apoptotic process [16,40–42]. We found that 2′-nitroflavone-induced

Fig. 6



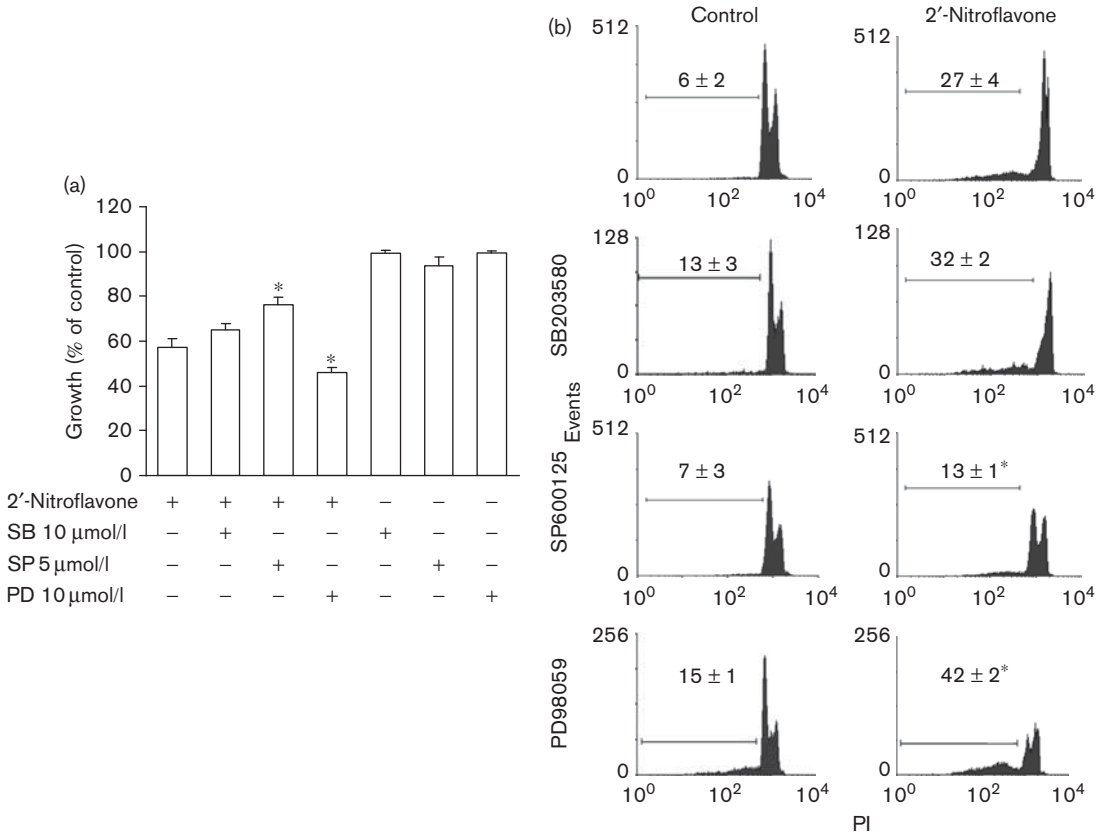
Effect of 2′-nitroflavone on the phosphorylation of mitogen-activated protein kinases. HL-60 cells were maintained in the absence of FBS for 24 h and then incubated for different times with 20 μmol/l 2′-nitroflavone. Cells lysates were subjected to SDS-polyacrylamide gel electrophoresis under the conditions described in the Materials and methods section. (a) Western blot assays were performed with antibodies against anti-phospho-ERK1/2 (P-ERK), anti-phospho-p38 (P-p38) and anti-phospho-JNK (P-JNK). Membranes were stripped and reprobed with anti-ERK, anti-p38 and anti-JNK antibodies, as loading controls. The results from one representative experiment are shown. (b) Quantification of the western blots was performed by densitometric analysis. Results represent the mean ± SEM of three independent experiments (*$P<0.05$; **$P<0.005$).

cell death was dependent on the activation of caspases, caspase-8 being related to the involvement of the death receptor pathway and caspase-9 to the mitochondria-mediated pathway [16,41,42]. In agreement with the results obtained previously in HeLa cells [27], these pathways would be independently activated, as the apoptotic effect of 2′-nitroflavone in HL-60 cells was partially inhibited in the presence of caspase-8-specific or caspase-9-specific inhibitors. The increase in the expression levels of the death ligand TRAIL and the upregulation of its specific cell surface receptor DR5 [43] would suggest the participation of the death receptor pathway. The activation of caspase-9, together with the change observed in Bax protein expression and the release of cytochrome C to the cytosol, would support the involvement of the mitochondrial pathway in the apoptotic cell death induced by 2′-nitroflavone in HL-60 cells.

As MAPKs regulate various cellular functions, including proliferation, differentiation and apoptosis, the role of p38, JNK and ERK1/2 in the 2′-nitroflavone-induced cytotoxicity was explored. The p38 pathway mediates proapoptotic signals and regulates cell cycle arrest as well as cell differentiation [23,33]. However, this pathway may also regulate cell proliferation and antiapoptotic responses [23,33,44,45]. In this study, we found that the activation of p38 would not be involved in the apoptotic effect induced by 2′-nitroflavone, as neither cell proliferation nor the amount of hypodiploid cells were affected in the presence of the specific inhibitor SB203580. A similar behaviour of p38 MAPK has been reported for other natural and synthetic flavonoids that inhibit the proliferation of leukaemia cells and activate the three main MAPKs pathways, p38 activation not being related to the apoptotic response [38,46].

Our results also showed that either after incubating HL-60 cells with the specific JNK inhibitor or after blocking JNK expression with siRNA, a significant increase in cell growth and a lower apoptotic effect was observed, indicating that JNK phosphorylation may be associated with 2′-nitroflavone-induced cell death. The activation of JNK has also been linked to apoptosis triggered by some natural and synthetic flavonoids in different solid tumour cell lines and leukaemia cells [38,46–49]. Despite these findings, it should be considered that some studies have established a role for JNK in cell survival [23,50]. Thus, it has been shown that the inhibition of this cascade by

Fig. 7



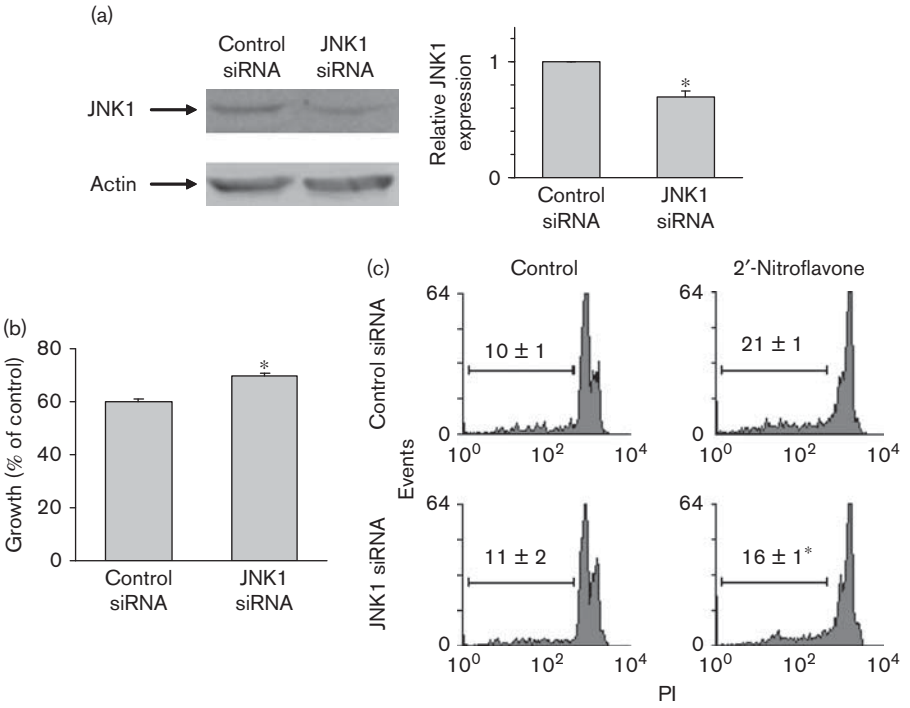
Effect of p38, JNK and ERK1/2 inhibitors on the antiproliferative and apoptotic effect induced by 2′-nitroflavone in HL-60 cells. (a) HL-60 cells were pretreated for 1 h at 37°C with or without 10 μmol/l SB203580 (SB), 5 μmol/l SP600125 (SP) or 10 μmol/l PD98059 (PD), and then incubated for 48 h in the absence or presence of 2 μmol/l 2′-nitroflavone. Cell proliferation was evaluated as described in the Materials and methods section. Results represent the mean±SEM of three different experiments. Statistical significance in comparison with the corresponding control value is indicated by $*P<0.01$. (b) After pretreatment with or without the specific inhibitors as described above, HL-60 cells were incubated for 18 h in the absence (control) or presence of 20 μmol/l 2′-nitroflavone. Hypodiploid DNA content was evaluated by flow cytometry after propidium iodide (PI) staining. The percentage of apoptotic cells ± SEM of three different experiments is shown in each histogram. Statistical significance in comparison with 2′-nitroflavone-treated cells incubated in the absence of specific inhibitors is indicated by $*P<0.005$.

SP600125 increases the apoptotic response in adenocarcinoma cells [51,52]. In addition, although the flavonoid derivative trifolin acetate induces phosphorylation of JNK in HL-60 cells, the selective inhibition of this pathway also enhances the apoptotic cell death [53].

In agreement with the general role of ERK1/2 in mediating proliferation signals and survival [31–34], it has been reported that the inhibition of this cascade in leukaemia cells with pharmacological inhibitors induces apoptosis and cell cycle arrest [54,55]. The antiproliferative effect of eupatilin, a flavone derived from Artemisia plants, on human breast cells as well as the growth-inhibitory effect of the plant compound diallyl disulfide on HL-60 cells have also been associated with the inhibition of ERK1/2 signalling [56,57]. Similarly, we found that 2′-nitroflavone treatment inhibited the ERK1/2 pathway, suggesting that this blockage would contribute to a higher susceptibility of HL-60 cells to the apoptotic response induced by the nitroderivative. In this respect, we showed that the inhibition of this cascade with the specific ERK1/2 inhibitor decreased cell proliferation and increased the amount of hypodiploid cells. Although the impairment in a survival pathway seems to favour a cell death process, the apoptotic effects of some flavonoids, such as the trifolin acetate, the betuletol 3-methyl ether and a derivative of quercetin in human leukaemia cell lines [38,46,53], and different natural flavonoids, such as quercetin, baicalin and baicalein in adenocarcinoma cells [58,59], have been closely linked to the activation of ERK phosphorylation. Thus, in general, different lines of evidence indicate that the specific roles of p38, JNK and ERK1/2 appear to be dependent on the cellular context, the kind of the stimulus, and even the kinetics of the activation.

In summary, our study shows that 2′-nitroflavone inhibits the proliferation of HL-60 cells by arresting cell cycle in the G_2/M phase and by inducing apoptosis through the activation of both death receptor and mitochondrial

Fig. 8



Effect of JNK depletion on the 2′-nitroflavone-induced antiproliferative and apoptotic effect. (a) HL-60 cells were incubated in the presence of either specific JNK small interfering RNA (siRNA) or control siRNA according to the manufacturer's instructions. Then, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and western blot. Equal loading was confirmed by stripping and reprobing each blot for actin. The results from one representative experiment are shown (left panel). Quantification was performed by densitometric analysis (right panel). Results are expressed as mean ± SEM of three independent experiments (*$P<0.01$). (b) HL-60 cells transfected with specific siRNA or control siRNA were incubated for 48 h in the absence or presence of 2 μmol/l 2′-nitroflavone. Cell proliferation was evaluated as described in the Materials and methods section. Results represent the mean ± SEM of three different experiments. Statistical significance in comparison with the corresponding control value is indicated by *$P<0.001$. (c) After transfection, HL-60 cells were incubated for 18 h in the absence (control) or presence of 20 μmol/l 2′-nitroflavone. Hypodiploid DNA content was evaluated by flow cytometry after propidium iodide (PI) staining. The percentage of apoptotic cells ± SEM of three different experiments is shown in each histogram. Statistical significance in comparison with the corresponding control value (2′-nitroflavone-treated cells transfected with control siRNA) is indicated by *$P<0.01$.

pathways. Phosphorylation of JNK and inhibition of ERK1/2 pathways would also contribute to the apoptotic response induced by the nitro derivative. Although the cytotoxic potency of 2′-nitroflavone is lower than that reported for flavopiridol in HL-60 cells, its usefulness as an antitumour compound remains to be determined [60,61]. Thus, additional preclinical studies with primary tumour cells *ex vivo* and/or animal models *in vivo* have to be developed to consider 2′-nitroflavone as a potential therapeutic agent for the treatment of human leukaemia.

Acknowledgements

The authors are indebted to Dr Verónica J. Marino and Verónica A. Furmento (IQUIFIB, Buenos Aires, Argentina) for helpful discussions and critical revision of the manuscript.

This work was supported by grants from the National Research Council (PIP 1261, CONICET) and the University of Buenos Aires (#20020100100081), Argentina.

Conflicts of interest

There are no conflicts of interest.

References

1 Harborne JB.*The Flavonoids: advances in research since 1986*. London: Chapman and Hall; 1999.
2 Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000; **52**:673–751.
3 Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol Ther* 2001; **90**:157–177.
4 Le Marchand L. Cancer preventive effects of flavonoids – a review. *Biomed Pharmacother* 2002; **56**:296–301.
5 Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* 2001; **21**:381–406.
6 Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *J Nutr Biochem* 2007; **18**:427–442.
7 Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. *Med Res Rev* 2003; **23**:519–534.
8 Manthey JA, Grohmann K, Guthrie N. Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Curr Med Chem* 2001; **8**:135–153.
9 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**:57–70.
10 Bold RJ, Termuhlen PM, McConkey DJ. Apoptosis, cancer and cancer therapy. *Surg Oncol* 1997; **6**:133–142.
11 Delmas D, Lançon A, Colin D, Jannin B, Latruffe N. Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* 2006; **7**:423–442.

12 Quintás-Cardama A, Cortes J. Homoharringtonine for the treatment of chronic myelogenous leukemia. *Expert Opin Pharmacother* 2008; **9**: 1029–1037.
13 Sun Y, Xun K, Wang Y, Chen X. A systematic review of the anticancer properties of berberine, a natural product from Chinese herbs. *Anticancer Drugs* 2009; **20**:757–769.
14 Mullauer FB, Kessler JH, Medema JP. Betulinic acid, a natural compound with potent anticancer effects. *Anticancer Drugs* 2010; **21**:215–227.
15 Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; **407**:770–776.
16 Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* 2001; **11**:526–534.
17 Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. *Cancer Lett* 2008; **269**:226–242.
18 Lin Y, Shi R, Wang X, Shen HM. Luteolin, a flavonoid with potential for cancer prevention and therapy. *Curr Cancer Drug Targets* 2008; **8**: 634–646.
19 Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep* 2009; **26**:1001–1043.
20 Fathi AT, Karp JE. New agents in acute myeloid leukemia: beyond cytarabine and anthracyclines. *Curr Oncol Rep* 2009; **11**:346–352.
21 Ramos S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol Nutr Food Res* 2008; **52**:507–526.
22 Sarkar FH, Li Y, Wang Z, Kong D. Cellular signaling perturbation by natural products. *Cell Signal* 2009; **21**:1541–1547.
23 Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 2009; **9**:537–549.
24 Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* 2010; **1802**:396–405.
25 Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. *Oncogene* 2007; **14**:3100–3112.
26 Cárdenas M, Marder M, Blank VC, Roguin LP. Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Bioorg Med Chem* 2006; **14**:2966–2971.
27 Cárdenas MG, Blank VC, Marder M, Roguin LP. 2′-Nitroflavone induces cell cycle arrest and apoptosis in human cervical carcinoma cells. *Cancer Lett* 2008; **268**:146–157.
28 Cárdenas MG, Zotta E, Marder M, Roguin LP. *In vitro* induction of apoptosis and *in vivo* effects of a flavone nitroderivative in murine mammary adenocarcinoma cells. *Int J Cancer* 2009; **125**:222–228.
29 Marder M, Zinczuk J, Colombo MI, Wasowski C, Viola H, Wolfman C, *et al*. Synthesis of halogenated/nitrated flavone derivatives and evaluation of their affinity for the central benzodiazepine receptor. *Bioorg Med Chem Lett* 1997; **7**:2003–2008.
30 Marder M, Viola H, Bacigaluppo JA, Colombo MI, Wasowski C, Wolfman C, *et al*. Detection of benzodiazepine receptor ligands in small libraries of flavone derivatives synthesized by solution phase combinatorial chemistry. *Biochem Biophys Res Commun* 1998; **249**:481–485.
31 Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 1999; **19**: 2435–2444.
32 Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; **410**:37–40.
33 Platanias LC. Map kinase signaling pathways and hematologic malignancies. *Blood* 2003; **101**:4667–4679.
34 Balmanno K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ* 2009; **16**:368–377.
35 Chang H, Mi MT, Gu YY, Yuan JL, Ling WH, Lin H. Effects of flavonoids with different structures on proliferation of leukemia cell line HL-60. *Ai Zheng* 2007; **26**:1309–1314.
36 Landis-Piwowar KR, Milacic V, Dou QP. Relationship between the methylation status of dietary flavonoids and their growth-inhibitory and apoptosis-inducing activities in human cancer cells. *J Cell Biochem* 2008; **105**:514–523.
37 Rubio S, Quintana J, López M, Eiroa JL, Triana J, Estévez F. Phenylbenzopyrones structure–activity studies identify betuletol derivatives as potential antitumoral agents. *Eur J Pharmacol* 2006; **548**:9–20.
38 Torres F, Quintana J, Estévez F. 5,7,3′-trihydroxy-3,4′-dimethoxyflavone-induced cell death in human leukemia cells is dependent on caspases and activates the MAPK pathway. *Mol Carcinog* 2010; **49**:464–475.
39 Rao YK, Fang SH, Tzeng YM. Synthesis, growth inhibition, and cell cycle evaluations of novel flavonoid derivatives. *Bioorg Med Chem* 2005; **13**:6850–6855.
40 Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; **407**:770–776.
41 Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**:1309–1312.
42 Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; **116**:205–219.
43 Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 1999; **11**:255–260.
44 Halawani D, Mondeh R, Stanton LA, Beier F. p38 MAP kinase signaling is necessary for rat chondrosarcoma cell proliferation. *Oncogene* 2004; **23**:3726–3731.
45 Ricote M, García-Tuñón I, Bethencourt F, Fraile B, Onsurbe P, Paniagua R, *et al*. The p38 transduction pathway in prostatic neoplasia. *J Pathol* 2006; **208**:401–407.
46 Rubio S, Quintana J, Eiroa JL, Triana J, Estévez F. Betuletol 3-methyl ether induces G(2)-M phase arrest and activates the sphingomyelin and MAPK pathways in human leukemia cells. *Mol Carcinog* 2010; **49**:32–43.
47 Chang HL, Wu YC, Su JH, Yeh YT, Yuan SS. Protoapigenone, a novel flavonoid, induces apoptosis in human prostate cancer cells through activation of p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase 1/2. *J Pharmacol Exp Ther* 2008; **325**:841–849.
48 Bhattacharya U, Halder B, Mukhopadhyay S, Giri AK. Role of oxidation-triggered activation of JNK and p38 MAPK in black tea polyphenols induced apoptotic death of A375 cells. *Cancer Sci* 2009; **100**:1971–1978.
49 Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S. Quercetin modulates NF-kappa B and AP-1/JNK pathways to induce cell death in human hepatoma cells. *Nutr Cancer* 2010; **62**:390–401.
50 Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* 2005; **15**:36–42.
51 Kuntzen C, Sonuc N, De Toni EN, Opelz C, Mucha SR, Gerbes AL, *et al*. Inhibition of c-Jun-N-terminal-kinase sensitizes tumor cells to CD95-induced apoptosis and induces G2/M cell cycle arrest. *Cancer Res* 2005; **65**: 6780–6788.
52 Xia HH, He H, De Wang J, Gu Q, Lin MC, Zou B, *et al*. Induction of apoptosis and cell cycle arrest by a specific c-Jun NH2-terminal kinase (JNK) inhibitor, SP-600125, in gastrointestinal cancers. *Cancer Lett* 2006; **241**:268–274.
53 Torres F, Quintana J, Díaz JG, Carmona AJ, Estévez F. Trifolin acetate-induced cell death in human leukemia cells is dependent on caspase-6 and activates the MAPK pathway. *Apoptosis* 2008; **13**:716–728.
54 Champelovier P, El Atifi M, Pautre V, Rostaing B, Berger F, Seigneurin D. Specific inhibition of basal mitogen-activated protein kinases and phosphatidylinositol 3 kinase activities in leukemia cells: a possible therapeutic role for the kinase inhibitors. *Exp Hematol* 2008; **36**:28–36.
55 Moon DO, Park C, Heo MS, Park YM, Choi YH, Kim GY. PD98059 triggers G1 arrest and apoptosis in human leukemic U937 cells through downregulation of Akt signal pathway. *Int Immunopharmacol* 2007; **7**: 36–45.
56 Kim DH, Na HK, Oh TY, Kim WB, Surh YJ. Eupatilin, a pharmacologically active flavone derived from Artemisia plants, induces cell cycle arrest in ras-transformed human mammary epithelial cells. *Biochem Pharmacol* 2004; **68**:1081–1087.
57 Tan H, Ling H, He J, Yi L, Zhou J, Lin M, *et al*. Inhibition of ERK and activation of p38 are involved in diallyl disulfide induced apoptosis of leukemia HL-60 cells. *Arch Pharm Res* 2008; **31**:786–793.
58 Zhou QM, Wang S, Zhang H, Lu YY, Wang XF, Motoo Y, *et al*. The combination of baicalin and baicalein enhances apoptosis via the ERK/p38 MAPK pathway in human breast cancer cells. *Acta Pharmacol Sin* 2009; **30**:1648–1658.
59 Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH, Huynh H. The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* 2004; **25**: 647–659.
60 Rapoport AP, Simons-Evelyn E, Chen T, Sidell R, Luhowskyj S, Rosell K, *et al*. Flavopiridol induces apoptosis and caspase-3 activation of a newly characterized Burkitt's Lymphoma cell line containing mutant p53 genes. *Blood Cells Mol Dis* 2001; **27**:610–624.
61 Takada Y, Sethi G, Sung B, Aggarwal BB. Flavopiridol suppresses tumor necrosis factor-induced activation of activator protein-1, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK, and Akt, inhibits expression of antiapoptotic gene products, and enhances apoptosis through cytochrome c release and caspase activation in human myeloid cells. *Mol Pharmacol* 2008; **73**:1549–1557.