3-*O*-Methylfunicone, a secondary metabolite produced by *Penicillium pinophilum*, induces growth arrest and apoptosis in HeLa cells

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Abstract. 3-*O*-Methylfunicone (OMF) is a secondary metabolite produced by the soil fungus *Penicillium pinophilum* which has cytostatic properties. The aim of this study was to investigate the mechanisms by which such properties are exerted, with special reference to any anti-proliferative and apoptotic potential, on HeLa cells. OMF treatment caused about 44% inhibition of cell growth after 24 h, and modifications in the tubulin fibre organization. In addition, a significant increase in p21 mRNA expression and a decrease in cyclin D1 and Cdk4 mRNA expression resulted at the same time. Apoptosis induction was demonstrated by the annexin V assay, cytofluorimetric analysis of the DNA content of the sub-G₁ fraction and DNA laddering. Taken together, our data showed that the compound inhibits proliferation of HeLa cells by several mechanisms, such as disruption of tubulin fibres, cell cycle arrest and apoptosis induction. The capacity of the compound to affect the cell cycle and to modulate apoptosis is indicative of a potential for the development of a new agent for cancer chemotherapy.

INTRODUCTION

Penicillium pinophilum is a soil fungus known to be an antagonist of plant pathogenic fungi (Dhedhi *et al.* 1990; Alagesaboopathi 1994). Its antagonistic behaviour has been reported in relation to the production of 3-*O*-methylfunicone (OMF), a secondary metabolite which inhibits *in vitro* growth of several fungal species, including dermatophytes (De Stefano *et al.* 1999). Previous studies carried out on a human tumour cell line (Hep-2, derived from a laryngeal carcinoma) reported the cytostatic properties of the compound (Stammati *et al.* 2002).

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Figure 1. Chemical structure of 3-O-methylfunicone.

The structure of OMF (Fig. 1) is quite peculiarly idiosyncratic and at present it is difficult to advance conjecture about its mode of action at the cytological level. However, there are products evaluated and/or used as anti-tumour agents that show some common traits concerning the aryl moiety of the molecule. In fact, in compounds such as colchicine, combretastatin, steganacin, the chalcones and the podophyllotoxins, this part of the molecule is trimethoxylated, and it has been shown that the substitution of even one methoxy group consistently reduces their cytostatic activity (Edwards *et al.* 1990; Getahun *et al.* 1992; Damayanthi & Lown 1998). Moreover, the methoxy groups are known to represent the binding site for tubulin, probably occurring in correspondence of sulphhydryl residues of the protein (Edwards *et al.* 1990); this assumption may trace cytostatic activity to the inhibition of microtubule assembly.

Homeostasis in normal tissues is maintained by a balance between cell proliferation and cell death, whereas tumour growth occurs when the cellular birth rate exceeds the death rate (Barrett *et al.* 1994). Reversible controls on cell growth are important in the process of normal development and tissue homeostasis, and in pathological conditions such as neoplasia. Terminal growth arrest and cell death are also important in normal and neoplastic growth. There are several forms of reversible growth control, including terminal differentiation, cellular senescence, apoptosis and necrosis (Barrett *et al.* 1994).

Apoptosis, or programmed cell death, is characterized morphologically by cell shrinkage and chromatin condensation, loss of contact with neighbouring cells and the extracellular matrix (Frisch & Francis 1994), actin cleavage (Guenal *et al.* 1997), and biochemically by DNA laddering (Barrett *et al.* 1994). A cascade of genes is activated as a consequence of the induction of a defined genetic programme, in which *p53* has a prominent role.

The gene *Waf1/Cip1* encodes the protein p21 (Michieli *et al.* 1994), whose overexpression in mammalian cells causes growth arrest through the inhibition of cyclin/Cdk complexes which are required for cell-cycle progression. In particular, the transition from G₁ to S phase is regulated by an interaction of proteins that mediate the phosphorylation of retinoblastoma protein (pRb). The cyclin D1/Cdk4-6 complex phosphorylates pRb, while both P16^{Ink4a} and p21, Cdk inhibitors, inhibit this process (Kang *et al.* 2002a). p21 can be induced by DNA damage and during apoptosis in specific cell types as a function of wild-type *p53* activation (El Deiry *et al.* 1994). It can also be induced in a *p53*-independent manner as an immediate early gene following mitogenic stimulation of growth arrested cells (Michieli *et al.* 1994). Recently, further evidence has been attained that p21 could mediate apoptosis in a *p53*-independent manner. In fact, microarray analysis has demonstrated the involvement of a new pattern of genes, among which *Bax*, *Bcl-2*, and *caspase-3* are not induced, while *hsp70B* is activated (Wu *et al.* 2002).

In this study we demonstrate that OMF causes growth arrest and apoptosis in HeLa cells. Apoptosis occurs 48 h after OMF treatment in a p21-dependent pathway.

MATERIALS AND METHODS

OMF preparation

OMF was extracted from liquid cultures of isolate LT4 of *Penicillium pinophilum* as previously described (De Stefano *et al.* 1999). For biological assays, the compound was dissolved in absolute ethanol at a concentration of 10 mg/ml.

Cell culture and treatments

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C, 5% CO₂. A preliminary dose–response curve was performed to determine the concentration and the time by which OMF produced significant effects on cell morphology (data not shown). Cell aliquots (4×10^5) were plated in 6-well plates (35-mm diameter) with 2 ml DMEM and treated with 25 µg/ml OMF for 24, 48 and 72 h. Control HeLa cells were treated or not with 5 µl absolute ethanol.

Morphological analysis

Morphological features of HeLa cells treated with OMF were defined by phase-contrast microscopy (Olympus CDK40) at 20 × magnification.

Direct immunofluorescence microscopy

HeLa cells were grown on glass coverslips in 6-well plates (35-mm diameter). After exposure to OMF or absolute ethanol, for visualization of total microtubules in the permeabilized cells, HeLa cells were incubated for 1 h at 37 °C with mouse anti-chicken α-tubulin (clone DMIA), FITC conjugated (Sigma, St Louis, MO, USA) 1 : 50 (Osborn & Weber 1982). A negative control was included in which treated cells were not incubated with FITC-conjugated antibody (data not shown). Cells were observed at 100 × magnification using a fluorescence microscope (Zeiss).

Cell viability, apoptotic cells

Cell viability was determined by the trypan blue exclusion method, while the annexin V assay was performed to evaluate the occurrence of apoptosis. HeLa cells, grown on glass slides, were treated with 25 µg/ml OMF for 24, 48 and 72 h. To discriminate necrotic from apoptotic cells, the presence of phosphatidylserine on the outer surface of the apoptotic cells was detected by exposure of HeLa cells to 20 µl FITC annexin V and 20 µl propidium iodide (50 µg/ml) (Xiao *et al.* 2003b). After 15 min incubation at 22 °C in the dark, the slides were washed twice in phosphate-buffered saline (PBS) and analysed under a fluorescence microscope. Cells exhibiting green fluorescence on the plasma-membranes were counted and estimated as a percentage of total cells. As a negative control, HeLa cells treated with OMF were not exposed to annexin V.

FACS analysis

The apoptosis induced by OMF treatment was additionally measured by fluorescence-activated cell sorting (FACS) analysis. Forty-eight and 72 h after OMF treatment, HeLa cells were trypsinized and fixed in 70% ethanol, then treated with 10 µg/ml RNase and 50 µg/ml propidium iodide (Sigma) for 2 h. Stained cells were analysed by means of a FACS Vantage (Becton Dickinson) for determination of the relative DNA content.

DNA fragmentation assay

To evaluate the possible occurrence of internucleosomal hydrolysis of genomic DNA in OMF-treated cells, 1×10^6 HeLa cells were incubated in the standard culture medium at 37 °C, in the presence or absence of 25 µg OMF, then analysed by agarose gel electrophoresis. After 24, 48 and 72 h, the cells were harvested with a cell scraper, centrifuged at 400 g, washed in PBS and finally suspended in 100 µl TNE buffer (150 mM sodium chloride, 10 mM EDTA, 10 mM Tris-HCl, pH 8). The cell suspensions were lysed with 3 volumes of lysis buffer [0.2% sodium dodecyl sulfate (SDS), 100 µg/ml RNase in TNE], and the lysate was incubated at 37 °C. After 1 h, the lysate was added with 100 µg/ml proteinase K and the mixture was incubated for an additional 2.5 h at 56 °C. The high molecular weight genomic DNA, extracted from proteinase K-treated lysates according to a known procedure (Sambrook *et al.* 1989), was analysed by electrophoresis (2 h, 80 V) in ethidium bromide-containing 1% agarose gel in TBE (0.045 M tris-borate, 0.001 M EDTA, pH 8.0).

RT-PCR (reverse transcription-polymerase chain reaction) analysis

Total RNA, isolated by High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from HeLa cells before and after the treatment with 25 µg/ml OMF, was transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche Diagnostics) at 42 °C for 45 min according to the manufacturer's instructions. Two microlitres of complementary DNA (cDNA) were amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 µM dNTP, and 2.5 units of Taq DNA polymerase (Roche Diagnostics) in a final volume of 50 µl. For the co-amplification of p21, cyclin D1 and GAPDH, the PCR was carried out in the presence of 0.5 µM sense and antisense *p21* or *cyclin D1* primers, and 0.05 µM sense and antisense *GAPDH* primers. For the co-amplification of *Bax*, *Cdk4*, *hsp70B*, and β -actin, the PCR was carried out in the presence of 0.5 µM sense and antisense *Bax*, *Cdk4*, or *hsp70B* primers, and 0.05 µM sense and antisense β -actin primers. Conditions and size of products are reported in Table 1. For the amplification of *ICE*, *c-myc*, *Bcl-2* and *p53*, a kit for multiple gene expression was used (APO-M050G, Maxim Biotech). *GAPDH* was used as the internal control of the reaction. The reaction was carried out in a DNA thermal cycler (Perkin Elmer – 2400). β -actin RT-PCR was performed

Table 1. Human primers sense and antisense sequences and expected PCR products (bp)

Gene	Sense and antisense sequences	Conditions	Size of product
P21	5'-CTGCCCAAGCTCTACCTTCC-3' 5'-CAGGTCCACATGGTCTTCCT-3'	29 cycles at 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s	123
Cyclin D1	5'-ACCTGGATGCTGGAGGTCTG-3' 5'-GAACTTCACATCTGTGGCACA-3'	30 cycles at 95 °C for 45 s, 50 °C for 45 s, 72 °C for 45 s	402
Cdk4	5'-CATGTAGACCAGGACCTAAG-3' 5'-GGAGCTCGGTACCAGAGTG-3'	30 cycles at 95 °C for 45 s, 54 °C for 45 s, 72 °C for 45 s	297
HSP70B	5'-CTCCAGCATCCGACAAGAAGC-3' 5'-ACGGTGTGTGGGGGTTTCAGG-3'	32 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s	234
BAX	5'-TGGCAGCTGACATGTTTTCTGAC-3' 5'-CGTCCCAACCACCTGGTCT-3'	30 cycles at 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s	380
GAPDH	5'-CGGAGTCAACGGATTGGTCGTAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'		306
β -actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'		661

on mRNA extracted at 24, 48 and 72 h, to confirm that the mRNAs were suitable for RT-PCR analysis. The PCR products were analysed by electrophoresis on 1.8% agarose gel in TBE. The expected size of the multiplex PCR products was 204 bp, 233 bp, 371 bp, 658 bp and 921 bp, for *p53*, *Bcl-2*, *c-myc*, *ICE* and *GAPDH*, respectively.

Protein extraction and western blotting analysis of p21, p53 and bcl-2

HeLa cells were treated with 25 µg/ml OMF for 72 h. The cells were scraped with 1 mL PBS and the cell pellet was homogenized with 300 µl ice-cold buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1.5 mM MgCl₂, 5 mM EGTA) supplemented with 20 mM sodium pyrophosphate, 40 µg/ml aprotinin, 4 mM polyvinylidene difluoride (PMSF), 10 mM sodium orthovanadate, 25 mM NaF. Total extracts were cleared by centrifugation for 30 min at 4 °C at 11 000 g and assayed for the protein content by Bradford's method. Fifty micrograms of protein from each cell lysate were separated by a 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes and the filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Blots were attained overnight with 5% non-fat dry milk, and then incubated with anti-p21 (187), anti-p53 (DO-1), and anti-Bcl-2 (100) mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1 µg/ml, in TBS (150 mM NaCl, 20 mM Tris-HCl pH 8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filters were incubated with 1 : 2000 peroxidase-conjugated anti-mouse immunoglobulins for 1 h at 22 °C. They were extensively washed and finally analysed using the ECL system (Amersham).

RESULTS

Anti-proliferative effect and morphological changes in OMF-treated cells

As evaluated by the trypan blue exclusion test, OMF produced approximately 44% inhibition of cell growth after 24 h (Fig. 2a), in the absence of necrotic cells. At the same time, a few cells showed apparent morphological changes (Fig. 2c). Cell shrinkage and blebs on the membrane surface (typical signs of apoptotic occurrence) appeared only 48 and 72 h after treatment (Fig. 2d and e, respectively). The same pieces of evidence were not observed when cells had not been treated (Fig. 2b), or treated with absolute ethanol (Fig. 2f). In order to analyse the morphological changes exerted by OMF treatment, untreated and OMF-treated cells were stained with FITC-conjugated monoclonal antibody anti- α tubulin, to evaluate intracellular organization of tubulin fibres. Cells subjected to OMF for 24 h (Fig. 3b) were rounded and showed modification in cytoskeletal organization compared with untreated (Fig. 3a) or absolute ethanol-treated cells (Fig. 3c).

OMF induces apoptosis in HeLa cells

To demonstrate that the events observed were as a result of triggering of the apoptotic programme, DNA extraction, annexin V assay and cytofluorimetric analysis of the DNA content of the sub-G₁ fraction were performed. As shown in Fig. 4(a), DNA laddering was demonstrated on 72-h exposure to OMF, while ethanol did not produce any effect. The apoptotic potential of OMF was further investigated by fluorescence microscopy of cells stained with annexin V and propidium iodide (Fig. 4b and c) and by FACS analysis (Fig. 4d–f). Annexin V is a calcium-dependent phospholipids-binding protein with a high affinity for phosphatidylserine. This is a negatively charged phospholipid located on the inner (cytoplasmic) surface of the plasmatic membrane of living cells (Xiao *et al.* 2003b). However, phosphatidylserine is translocated to the

Figure 2. Effect of OMF treatment on cell growth proliferation and on morphology of HeLa cells determined by trypan blue dye exclusion assay (a) and by phase contrast microscopy (b–f). (b) Untreated cells (72 h), (c) cells treated for 24 h with OMF, (d) cells treated for 48 h with OMF, (e) cells treated for 72 h with OMF, (f) cells treated for 72 h with absolute ethanol. Initial magnification 20 $\times$.

outer side of the plasma membrane in cells undergoing apoptosis (Xiao *et al.* 2003b). As can be seen in Fig. 4(b), the annexin V assay was positive in OMF-treated cells for 48 h. In addition, when cells were treated for 72 h, either green or red cells (propidium iodide stained cells) were present, because of the occurrence of a late apoptotic phenomenon (Fig. 4c). Ethanol treatment did not produce any effect (data not shown). Finally, when OMF-treated cells were monitored by FACS analysis, more than 30% of the cells were observed in the sub- G_1 phase after 48 and 72 h (Fig. 4e and f, respectively), as compared with the untreated control (Fig. 4d). This result confirms the pro-apoptotic response induced by OMF on HeLa cells.

OMF treatment results in time-dependent increase of p21 mRNA expression and in cyclin D1/Cdk4 complex down-regulation. *p21* overexpression can result in G_1 arrest in many cell types (Kang *et al.* 2002a; Lodygin *et al.* 2002). To demonstrate that OMF increased *p21* expression, RT-PCR analysis was performed on cells treated or not with 25 μ g/ml OMF. As shown in Fig. 5, *p21* was overexpressed as little as 6 h after treatment. Its expression was stronger at 17 h and maintained at 24, 48 and 72 h. The result was also confirmed by western blot analysis (Fig. 6). Furthermore, as p21 protein acts by inhibiting the activity of the cyclin/Cdk complex, preventing the transition $G_1 \rightarrow S$ (Shackelford *et al.* 1999), we analysed the expression of the cyclin D1/Cdk4 complex, regulated by p21. It is known that cyclin D1 is deregulated/overexpressed in many human tumours, implying that cyclin D1 may function as a positive growth regulator (Shackelford *et al.* 1999). As shown in Fig. 7, RT-PCR demonstrated the down regulation of both *cyclin D1* and *Cdk4* at 24 h.

OMF treatment results in Bcl-2 and in Bax mRNA expression decrease and increase, respectively

To evaluate the genetic pathway triggered by OMF, we analysed some genes known to be involved in apoptosis. By multiplex RT-PCR, the expression of *caspase-1*, *c-myc*, *Bcl-2* and *p53* was

Figure 3. OMF treatment induces tubulin fibre modification. Tubulin fibres were visualized using FITC-conjugated monoclonal antibody anti- α tubulin. (a) Untreated cells (24 h), (b) cells treated for 24 h with OMF, (c) cells treated for 24 h with absolute ethanol. Initial magnification: 100 $\times$.

Figure 4. OMF treatment induces apoptosis. (a) DNA fragmentation induced in OMF-treated cells after 48 and 72 h (lanes 3 and 4, respectively). Absolute ethanol does not induce DNA fragmentation (lane 5). Lane 1, untreated cells; lane 2, cells treated for 24 h. M, 100 bp ladder (Roche Diagnostics) used as MW marker. (b, c) Annexin V assay of OMF-treated cells after 48 and 72 h, respectively. (d, e, f) DNA content of the sub- G_1 fraction. HeLa cells, stained with propidium iodide, were analysed by FACS. When OMF-treated cells were analysed after 48 h of treatment, over 30% of cells were observed in the sub- G_1 phase (e). (f) FACS analysis at 72 h of treatment. Control cells (d). The results are representative of five different experiments.

evaluated 72 h after treatment, when the apoptotic effect of OMF was clearly evident. The same analysis performed at 24 and 48 h did not produce significant modification in the gene expression (data not shown). As shown in Fig. 8, we did not find *caspase-1*, *c-myc* nor *p53* induction, while only a slight decrease was demonstrated for *Bcl-2* at 72 h after treatment. In addition, western blot analysis confirmed the result obtained by RT-PCR for *p53* and *Bcl-2* (Figs 9 and 10, respectively). It is worth pointing out that defective apoptotic programmes have been already observed. In particular, several cases of *p53* independent apoptosis have recently been reported, suggesting that this gene is not always active in apoptosis (Michieli *et al.* 1994; Wu *et al.* 2002). Because high level overexpression of the anti-apoptotic protein *Bcl-2* prevented activity of the pro-apoptotic protein *Bax*, we also analysed *Bax* expression at 72 h, when down-regulation of *Bcl-2* was demonstrated. As shown in Fig. 11 we observed a remarkable increase in *Bax* gene expression.

OMF treatment results in *hsp70B* mRNA expression increase

Several studies have demonstrated the importance of heat shock proteins in apoptosis (Sen 1992). Their expression increases when cells are exposed to a number of metabolic insults, including elevated temperature, exposure to amino acid analogues, various heavy metals and ionophores, some metabolic poisons and, finally, agents that modify protein sulphhydryls (Welch 1992). We observed that OMF was able to induce *hsp70B* expression 72 h after treatment (Fig. 12), which demonstrates the involvement of other than the commonly expected apoptotic

Figure 5. RT-PCR analysis using specific primers for p21 mRNA expression. Lane 1, untreated-cell mRNA; lanes 2–6, mRNA from HeLa cells treated with OMF for 6, 17, 24, 48 and 72 h, respectively; lane 7, mRNA from HeLa cells treated with absolute ethanol for 72 h. Densitometric analysis of ethidium-bromide-stained agarose gel (NIH image V 1.6) was performed to quantify the amount of specific mRNAs present in the total RNA prepared from the different cell samples. The p21/GAPDH FI (fluorescence intensity) ratios were as follows: lanes 1, 2, 3, 4, 5, 6 and 7, 0.7 ± 0.12 , 1.7 ± 0.25 , 1.86 ± 0.32 , 1.75 ± 0.36 , 1.42 ± 0.26 , 1.4 ± 0.37 and 0.53 ± 0.12 , respectively. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

Figure 6. Induction of p21 protein after treatment with OMF. Lane 1, untreated-cells; lanes 2–6, HeLa cells treated with OMF for 6, 17, 24, 48 and 72 h, respectively; lane 7, HeLa cells treated with absolute ethanol for 72 h. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

genes. The same analysis performed at 24 and 48 h did not produce significant modification in the gene expression (data not shown).

DISCUSSION

Results reported here demonstrate that OMF induces growth arrest and apoptosis in HeLa cells. About 44% inhibition of cell growth was demonstrated after 24 h, whereas RT-PCR showed that *p21* overexpression was already evident 6 h after treatment, reaching a peak at 17 h. Furthermore, the p21 protein product causes growth arrest through inhibition of the Cdk/cyclin D1 complex, which is required for G₁ to S transition (Michieli *et al.* 1994). Cell proliferation and

Figure 7. RT-PCR analysis using specific primers for Cdk4 and cyclin D1 mRNA expression. (a, b) Lane 1, untreated-cell mRNA; lane 2, mRNA from HeLa cells treated with OMF for 24 h; lane 3, mRNA from HeLa cells treated with absolute ethanol for 24 h. The Cdk4/GAPDH FI ratios were as follows: lanes 1, 2 and 3, 3.1 ± 0.54 , 1.97 ± 0.35 and 3.2 ± 0.65 , respectively. The cyclin D1/ β -actin FI ratios were as follows: lanes 1, 2 and 3, 3.74 ± 0.37 , 1.96 ± 0.25 and 3.67 ± 0.41 , respectively. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

differentiation are specifically controlled in the G_1 phase and the G_1 phase transition in the cell cycle (Kang *et al.* 2002b). Deregulation of genes involved in the progression from G_1 to S phase of the cell cycle is a frequent event in several tumour types (Kang *et al.* 2002b). Therefore, we decided to monitor the G_1 phase in particular. As demonstrated by RT-PCR analysis, we found a Cdk4 and cyclin D1 expression reduction after 24 h. It is reasonable to postulate that OMF may cause cell cycle arrest by inducing p21 expression and reducing the activity of the Cdk4/cyclin D1 complex. In particular, p21 overexpression was maintained until 72 h when cells had been induced into apoptosis. This finding could be explained assuming that p21 is an early gene that inhibits cell cycle when DNA is damaged. It is already known that this dampens the activity of newly formed cyclin-Cdk complexes, thereby serving as an internal control which prevents cells from entering the S phase prematurely (Michieli *et al.* 1994). As OMF causes apoptosis too, we hypothesize that cells maintain high p21 expression to avoid inheritance of the progeny of OMF-induced damage. Ultimately, HeLa cells, being unable to repair the damage, commit suicide. It is important to note that the annexin V assay identifies apoptotic (green fluorescence)

Figure 8. Multiplex RT-PCR analysis using specific primers for caspase-1, c-myc, p53, bcl2. Lane 1, untreated-cell mRNA; lane 2, mRNA from HeLa cells treated with OMF for 72 h; lane 3, mRNA from HeLa cells treated with absolute ethanol for 72 h. The caspase-1/GAPDH FI ratios were as follows: lanes 1, 2 and 3, 1.15 ± 0.07 , 1.19 ± 0.02 and 1.2 ± 0.06 , respectively. The c-myc/GAPDH FI ratios were as follows: lanes 1, 2 and 3, 0.63 ± 0.09 , 0.57 ± 0.05 and 0.61 ± 0.05 , respectively. The bcl-2/GAPDH FI ratios were as follows: lanes 1, 2 and 3, 0.85 ± 0.17 , 0.24 ± 0.08 and 0.79 ± 0.11 , respectively. The p53/GAPDH FI ratios were as follows: lanes 1, 2 and 3, 0.61 ± 0.13 , 0.65 ± 0.11 and 0.57 ± 0.09 , respectively. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

Figure 9. Western blot analysis relative to p53 analysis. Lane 1, untreated-cells; lane 2, HeLa cells treated with OMF for 72 h; lane 3, HeLa cells treated with absolute ethanol for 72 h. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

cells. In OMF-treated cells, green fluorescence and sporadic red fluorescence confirm that DNA laddering and the molecular events demonstrated were because of apoptotic induction only.

Microtubules are major cell structural components. They play important roles in the development and maintenance of cell shape, cell replication and division, cell signalling, and cellular movement (Wilson & Jordan 1995). They are composed of α and β tubulin. Polymerization and

Figure 10. Western blot analysis relative to Bcl-2 analysis. Lane 1, untreated-cells; lane 2, HeLa cells treated with OMF for 72 h; lane 3, HeLa cells treated with absolute ethanol for 72 h. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

Figure 11. RT-PCR analysis using specific primers for Bax mRNA expression. Lane 1, untreated-cell mRNA; lane 2, mRNA from HeLa cells treated with OMF for 72 h; lane 3, mRNA from HeLa cells treated with absolute ethanol for 72 h. The Bax/ β actin FI ratios were as follows: lanes 1, 2 and 3, 0.61 ± 0.24 , 1.2 ± 0.41 and 0.55 ± 0.19 , respectively. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

depolymerization of tubulin fibres can be influenced by numerous factors that disrupt the dynamics of microtubule assembly, perturbing the formation and function of the mitotic spindle apparatus and arresting cells in mitosis (Wilson & Jordan 1995). It has been reported, for several compounds, that growth inhibition, M-phase arrest and induction of apoptosis are associated with depolymerization of cellular microtubules as a consequence of their direct interaction with tubulin (Manfredi & Horwitz 1984; Xiao *et al.* 2003a). In particular, podophyllotoxin (a compound which is already used in cancer chemotherapy and presents the same trimethoxylated aryl moiety as OMF in its molecular structure) depresses cell proliferation by inhibiting tubulin polymerization during cell division (Damayanthi & Lown 1998). Our data demonstrate that OMF induces remarkable modification of tubulin fibre rearrangement. It could be inferred that in OMF-treated cells the cytoskeletal framework for chromosome separation is destroyed, thus arresting cell division. The effect of OMF on tubulin rearrangement inhibition could be the main consequence of either cell-cycle arrest or the induced apoptotic event. In particular, the disassembly of tubulin fibres at 24 h of OMF treatment is only an early stage of the apoptotic process, leading, successively, to microtubule degradation and phosphatidylserine exposure on the membrane surface.

Figure 12. RT-PCR analysis using specific primers for hsp70B mRNA expression. Lane 1, untreated-cell mRNA; lane 2 mRNA from HeLa cells treated with OMF for 72 h; lane 3, mRNA from HeLa cells treated with absolute alcohol for 72 h. The hsp70B/ β -actin FI ratios were as follows: lanes 1, 2 and 3, 0.43 ± 0.09 , 1.4 ± 0.23 and 0.58 ± 0.12 , respectively. The values are the mean of at least five independent experiments $\pm$ SD ($P < 0.05$).

Multiplex RT-PCR revealed a lack of overexpression of *caspase-1*, *c-myc* and *p53* at 72 h. Only a slight decrease of *Bcl-2* expression was evident. However, *Bax* overexpression and *hsp70B* induction were demonstrated 72 h after treatment, when OMF clearly produced its apoptotic effect. The analysis of the same genes at 24 and 48 h did not show modification in their expression. It is known that different apoptotic pathways can be triggered when elicited by different compounds (Kang *et al.* 2002a; Tang *et al.* 2002; Thorburn *et al.* 2003). The genes involved are not always the same; additional genes are often discovered, complicating the analysis of new genetic pathways, as may be in the case of OMF. In contrast, the participation of some classic apoptotic genes, such as *Bax* and *hsp70B*, has encouraged our studies. In particular, in HeLa cells the expression of E7 by HPV16/18 leads to the inactivation of pRB (Zur Hausen 2001). In the absence of functional pocket proteins (pRb, p107, p130), which are downstream effectors of p21 function, cell cycle arrest is converted to induction of apoptosis in a p53-independent manner (Kang *et al.* 2002a; Lodygin *et al.* 2002). Kang *et al.* (2002b), demonstrated the induction of apoptosis in HeLa cells by a naphthoquinone analogue in a p53-independent way, accompanied by p21 induction.

In conclusion, this study demonstrates the anti-proliferative effectiveness of OMF on HeLa cells. The anti-proliferative mechanisms of OMF seem to be triggered by microtubule disruption and cell cycle arrest; such first events are followed by induction of apoptosis. Taken together, our findings suggest that OMF possesses a potential for development as a new agent for cancer chemotherapy.

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REFERENCES

- ALAGESABOOPATHI C (1994) Biological control of damping-off disease of cotton seedling. *Curr. Sci.* **66**, 865.
- BARRETT JC, ANNAB LA, ALCORTA D, PRESTON G, VOJTA P, YIN Y (1994) Cellular senescence and cancer. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 411.
- DAMAYANTHI Y, LOWN JW (1998) Podophyllotoxins: current status and recent developments. *Curr. Med. Chem.* **5**, 205.
- DE STEFANO S, NICOLETTI R, MILONE A, ZAMBARDINO S (1999) 3-*O*-Methylfunicone, a fungitoxic metabolite produced by the fungus *Penicillium pinophilum*. *Phytochemistry* **52**, 1399.
- DHEDHI BM, GUPTA O, PATEL VA (1990) Antagonistic effect of microorganisms to *Fusarium oxysporum* f.sp. *ciceri*. *Indian J. Mycol. Plant Pathol.* **20**, 70.
- EDWARDS ML, STEMERICK DM, SUNKARA PS (1990) Chalcones: a new class of antimitotic agents. *J. Med. Chem.* **33**, 1948.
- EL DEIRY W, HARPER JW, O'CONNOR PM, VELCULESCU VE, CANMAN CE, JACKMAN J, PIETENPAL JA, BURRELL M, HILL DE, WANG Y (1994) Waf1/Cip1 is induced in p53 mediated G1 arrest and apoptosis. *Cancer Res.* **54**, 1169.
- FRISCH SM, FRANCIS H (1994) Disruption of epithelial cell-matrix interaction induces apoptosis. *J. Cell Biol.* **124**, 619.
- GETAHUN Z, JURD L, CHU PS, LIN CM, HAMEL E (1992) Synthesis of alkoxy-substituted diaryl compounds and correlation of ring separation with inhibition of tubulin polymerization: differential enhancement of inhibitory effects under suboptimal polymerization reaction conditions. *J. Med. Chem.* **35**, 1058.
- GUENAL I, RISLER Y, MIGNOTTE B (1997) Down-regulation of actin genes precedes microfilament network disruption and actin cleavage during p53-mediated apoptosis. *Br. J. Cell Sci.* **110**, 489.
- KANG KH, LEE H, KIM KC, HAM SW, KIM MY, CHOI KH (2002a) Induction of p73b by naphthoquinone analog is mediated by E2F-1 and triggers apoptosis in HeLa cells. *FEBS* **522**, 161.
- KANG Y, OZBUN LL, ANGISEN J, MOODY W, PRENTICE M, DIWAN AB, JAKOWLEW SB (2002b) Altered expression of G₁/S regulatory genes occurs early and frequently in lung carcinogenesis in transforming growth factor- β 1 heterozygous mice. *Carcinogenesis* **23**, 1217.
- LODYGIN D, MENSEN A, HERMEKING H (2002) Induction of the Cdk inhibitor p21 by LY83583 inhibits tumour cell proliferation in a p53-independent manner. *J. Clin. Invest.* **110**, 1717.
- MANFREDI JJ, HORWITZ SB (1984) Taxol: an antimitotic agent with a new mechanism of action. *Pharmacol. Ther.* **25**, 83.
- MICHEL P, CHEDID M, LIN D, PIERCE HJ, MERCER WE, GIVOL D (1994) Induction of Waf1/Cip1 by a p53-independent pathway. *Cancer Res.* **54**, 3391.
- OSBORN M, WEBER K (1982) Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin containing structures. *Meth. Cell Biol.* **24**, 97.
- SAMBROOK J, FRITSCH EF, MANIATIS T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory.
- SEN S (1992) Programmed cell death: concept, mechanism and control. *Biol. Rev.* **67**, 287.
- SHACKELFORD RE, KAUFMANN WK, PAULES RS (1999) Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ. Health Perspect.* **107**, 5.
- STAMMATI A, NICOLETTI R, DE STEFANO S, ZAMPAGLIONI F, ZUCCO F (2002) Cytostatic properties of a novel compound derived from *Penicillium pinophilum*: an *in vitro* study. *Altern. Lab. Anim.* **30**, 69.
- TANG D, WU D, HIRAO A, LAHTI JM, LIU L, MAZZA B, KIDD VJ, MAK TW, INGRAM AJ (2002) Erk activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *J. Biol. Chem.* **277**, 12710.
- THORBURN J, FRANKEL AE, THORBURN A (2003) Apoptosis by leukemia cell-targeted diptheria toxin occurs via receptor-independent activation of FAS-associated death domain protein. *Clin. Cancer Res.* **9**, 861.
- WELCH WJ (1992) Mammalian stress response: cell physiology, structure/function stress proteins, and implications for medicine and disease. *Physiol. Rev.* **72**, 1063.
- WILSON L, JORDAN MA (1995) Microtubule dynamics: taking aim at a moving target. *Chem. Biol.* **2**, 569.
- WU Q, KIRSCHMEIER P, HOCKENBERRY T, YANG TY, BRASSARDI DL, WANG L, MCCLANAHAN T, BLACK S, RIZZI G, MUSCO ML, MIRZA A, LIU S (2002) Transcriptional regulation during p21^{Waf1/Cip1}-induced apoptosis in human ovarian cancer cells. *J. Biol. Chem.* **277**, 36329.
- XIAO D, PINTO TJ, SOH JW, DEGUCHI A, GUNDERSEN GG, PALAZZO AF, YOON JT, SHIRIN H, WEINSTEIN BI (2003a) Induction of apoptosis by the garlic-derived compound S-allylmercaptocysteine (SAMC) is associated with microtubule depolymerization and c-jun NH₂-terminal kinase 1 activation. *Cancer Res.* **63**, 6825.
- XIAO D, SRIVASTAVA SK, LEW KL, ZENG Y, HERSHBERGER P, JOHNSON CS, TRUMP DL, SINGH SV (2003b) Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer by causing G₂/M arrest and inducing apoptosis. *Carcinogenesis* **24**, 891.
- ZUR HAUSEN (2001) Oncogenic DNA viruses. *Oncogene* **20**, 7820.