Cell Cycle Arrest and Apoptosis Responses of Human Breast Epithelial Cells to the Synthetic Organosulfur Compound *p*-Methoxyphenyl *p*-Toluenesulfonate

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Abstract. *Background: There are several studies documenting that organosulfur compounds show promise as anticancer agents. Although some mechanisms of the antiproliferative activity of naturally occurring organosulfur compounds have been elucidated, few studies have reported the differential response of human breast cells to these compounds. Materials and Methods: The effect of the synthetic sulfonate ester, p-methoxyphenyl p-toluenesulfonate on growth inhibitory activity depending upon the estrogen-receptor (ER), p53, bcl-2 and caspase-3 status of cells was investigated by comparing its effects on three distinct human breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) and on one normal human mammary epithelial cell line (MCF-10A). Results: This sulfonate ester selectively killed cancer cells at doses of 100 μM. Flow cytometry analysis showed that treatment with p-methoxyphenyl p-toluenesulfonate caused different cell cycle responses in the four cell lines but no clear association with p53 status was observed. Apoptosis was also induced in cells harboring different levels of Bcl-2 expression, but again independently of the p53 or ER status of the cells. Conclusion: These results suggest that p-methoxyphenyl p-toluenesulfonate acts on multiple signaling pathways leading to growth inhibition and activation of mechanisms of cell death selectively affecting survival of breast cancer cells. Thus, p-methoxyphenyl p-toluenesulfonate is the first member of a new class of tumor-specific chemotherapeutic agents for the treatment of breast cancer.*

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Key Words: Sulfonate ester, breast cancer, cell growth inhibition, cell cycle arrest and apoptosis, p53 and Bcl-2 status.

Organosulfur compounds (OSCs), whether isolated from plants such as garlic, onions and mahogany trees, or synthetically prepared, have been reported to have anticancer activity (1-7). Naturally occurring OSCs have been shown to inhibit cell proliferation of various cancer cell lines *in vitro* including human breast cancer cell lines. *S*-Allylmercaptocysteine (SAMC), found in low concentrations in aged garlic extracts, was effective in inhibiting MCF-7 human breast cancer cell growth (8), whereas diallyl disulfide (DADS), an oil-soluble OSC found in garlic, caused inhibition of estrogen receptor (ER)-positive and -negative breast cancer cell growth (9) and reduced the incidence and delayed the onset of *N*-methyl-*N*-nitrosourea-induced mammary tumors in rats (10).

Possible mechanisms for the antiproliferative property of these naturally occurring OSCs include inhibition of the carcinogen-activating cytochrome P450 (CYP) enzymes (11), the induction of carcinogen-detoxifying enzymes such as glutathione-*S*-transferase (12, 13), the inhibition of cell cycle progression and/or the induction of apoptosis (14), and the alteration of calcium homeostasis (15). OSCs caused cell cycle arrest of many different types of cells, mostly in the G_2/M phase (4, 11, 12, 14, 16-19) and induced apoptosis by the mitochondrial pathway (*e.g.* induction of the executor caspase-3) (4, 5, 9, 13-15).

We have prepared and screened various synthetic aryl sulfones and aryl sulfonate esters for their antiproliferative effects against different cancer cell lines. Some of them have shown pronounced and selective anticancer activity against leukemic (2), human skin cancer C32 (6, 20), and breast cancer (7) cells when compared to noncancerous cell lines. In addition, we reported an association between OSC structure and growth inhibitory activity in leukemic cells, by comparing the antiproliferative activity of novel relatives of dysoxysulfone, a natural OSC derived from the Fijian medicinal plant, *Dysoxylum richii* (2). We have also reported that our most active synthetic toluenesulfonate compounds evaluated for their growth inhibitory effects against MCF-7 breast cancer cells shared structures closely related to

p-methoxyphenyl *p*-toluenesulfonate with the methoxy substituent shifted from position 4 to 2 or 3 (7). The sulfonate with the 4 position methoxy group (MPTS) was identified as the most promising compound for antineoplastic activity against breast cancer (Figure 1). It caused a greater degree of cell cycle arrest and induced apoptosis in cancerous MCF-7 cells compared with normal breast epithelial MCF-10A cells.

The dependence on *p53*, or other gene status, of the mechanism of action of organosulfur compounds, in general, is poorly understood. However, to be effective as therapeutic agents against breast cancer, promising antitumor compounds such as MPTS must be able to inhibit the growth of breast tumor cells showing different cellular and molecular characteristics. A systematic, detailed study of the growth inhibitory effects of MPTS on human breast cancer cell lines in relation to hormone dependency and other gene status has yet to be performed. Thus, the primary object of this study was to investigate whether the effect of the promising synthetic OSC, MPTS, on growth inhibition would depend on the characteristics of human breast epithelial cells. Three human breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) and one normal human mammary epithelial cell line (MCF-10A) were used to investigate whether the mechanism of the inhibitory activity of MPTS was dependent on the status of *p53, bcl-2,* estrogen receptor (ER) and *caspase-3*. The effects of MPTS on mammary epithelial cells were also compared with two well known inhibitory compounds: staurosporine and 5-fluorouracil (5-FU).

Materials and Methods

Chemical synthesis. MPTS was prepared as reported earlier (20). Instrumentation and routine procedural details for the spectroscopic characterization of our samples have been provided previously (20, 21).

Cell lines and culture conditions. MCF-7, MDA-MB-231, and MDA-MB-453 human breast cancer cell lines and MCF-10A normal human mammary epithelial immortalized cell line were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). MCF-7, MDA-MB-231 and MDA-MB-453 breast cancer cells were maintained in Dulbecco's minimum essential medium (DMEM; Sigma-Aldrich, St-Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; BioMedia, Drummondville, QC, Canada), 50 μg/mL gentamicin (Invitrogen Canada Inc., Burlington, Ontario, Canada) and 2 mM L-glutamine (Sigma-Aldrich). MCF-10A cells were maintained in DMEM-F12 (Sigma-Aldrich) supplemented with 10% FBS, 50 μg/ml of gentamicin, 2 mM of L-glutamine, 20 ng/ml of epidermal growth factor (Sigma-Aldrich), 10 μg/ml of insulin (Sigma-Aldrich), 100 ng/ml of toxin cholera (List Biological Laboratories, Incs, Campbell, California, USA) and 1 μg/mL of hydrocortisone (Sigma-Aldrich). Cells were cultured in 75 cm^2 culture flasks at 37˚C in a humidified atmosphere of 5% CO_2. Cells were harvested when they reached about 80-85% confluence using a cell scraper (MCF-7, MDA-MB-231 and MDA-MB-453) or a 0.5% trypsin-EDTA solution (Sigma-Aldrich) diluted in phosphate buffered solution (PBS) (Sigma-Aldrich) (MCF-10A).

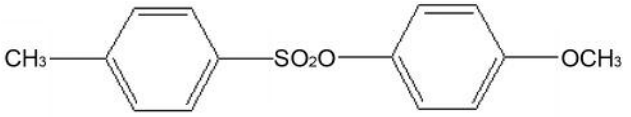

Figure 1. *Molecular structure of p-methoxyphenyl p-toluenesulfonate (MPTS).*

MTT assay. Cells were seeded in clear 96-well plates (Sarstedt, Montreal, QC, Canada) at a density of 7,500 cells/well (MCF-10A) or 10,000 cells/well (MCF-7, MDA-MB-231 and MDA-MB-453). After 24 hours, the culture medium was replaced by fresh medium containing different concentrations of MPTS or acetone (solvent). Concentrations ranged from 9 μM to 180 μM. In some of the wells, only culture medium was added. These wells were used as negative controls and were referred to as untreated control cells. Cell number was evaluated using the MTT Cell Proliferation Assay (22). The absorbance was measured at 570 nm on a microplate spectrophotometer reader (Benchmark, Bio-Rad, Hercules, CA, USA). Each compound was evaluated in at least 3 independent experiments with eight replicates in each experiment.

Trypan blue exclusion assay. Cells were seeded in 6-well tissue culture plates (Sarstedt) at a density of 1.25×10^5 to 5×10^5/well. After 24 hours of incubation, the medium was removed and replaced by fresh culture medium containing different concentrations of MPTS, staurosporine (Sigma-Aldrich), 5-FU (Sigma-Aldrich), or the equivalent amount of solvent. Cells were incubated for 24 h or 48 h, then cells were collected and an equal volume of a solution of 0.4% of Trypan blue (Sigma-Aldrich) diluted in PBS was added. Viable and nonviable cells were counted on a hematocytometer plate under a microscope. The percentage of mortality was determined. This experiment was repeated three times.

Cell cycle analysis. Propidium iodide (PI) (Sigma-Aldrich) was used to analyze DNA content. Cells were seeded in 6-well tissue culture plates at a density of 1.25×10^5 to 5×10^5/well. After 24 hours of incubation, the medium was removed and replaced by fresh culture medium containing different concentrations of MPTS, staurosporine or the equivalent amount of solvent. After 24 h and 48 h of exposure to the compounds, cells were harvested by centrifugation (1,100 rpm, 10 min) and aliquots of 5×10^5 cells were fixed in cold 70% ethanol at 4˚C for 24 h. Cells were then washed with PBS, centrifuged (3,500 rpm, 1 min) and cell pellets were resuspended with 100 μl of a solution of 100 μg/ml RNAse A (Sigma-Aldrich) in PBS at room temperature for 30 minutes. Cells were then incubated with 400 μl of a solution of 50 μg/ml PI in PBS, at room temperature in the dark for 30 min. Approximately 20,000 cells were analyzed by flow cytometry using a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter, Mississauga, ON, Canada). The apoptotic cells were considered to constitute the sub-G_1 cell population and the percentage of cells in each phase of the cell cycle was calculated. The experiments were performed three times and gave similar results.

Apoptosis assay. Annexin V-FITC was used as a marker of phosphatidylserine exposure and PI as a marker for dead cells (APOAF Annexin V-FITC Apoptosis Detection Kit; Sigma-Aldrich). This combination allows differentiation among early apoptotic cells (annexin

Table I. *Comparison of IC_{50} of p-methoxyphenyl p-toluenesulfonate (MPTS) with two chemotherapeutic agents on malignant and nonmalignant human breast cell lines.*

Cell line	Characteristic					IC_{50} (μM)		
	Malignant	ER	*p53*	*caspase-3*	*bcl-2*	MPTS	Staurosporine	5-Fluorouracil
MCF-10A	No	–	wt	+	Normal	>719	<0.005	<0.5
MCF-7	No	+	wt	–	Overexpression	126***	~0.5	>5
MDA-MB-231	Yes	–	Mutant	+	Normal	108***	< 0.05	~5
MDA-MB-453	Yes	–	Mutant	+	Down-regulation	144***	~0.5	<5, >1

IC_{50} represents the concentration of compounds that resulted in 50% inhibition of cell growth as measured by the MTT assay (MPTS) or Trypan blue assay (staurosporine and 5-FU) after 48 h of treatment. Data shown are the means from three independent experiments performed in eight replicates (MTT assays, MPTS) or at least three independent experiments (Trypan blue assays, staurosporine and 5-FU). ***$p<0.001$ compared to the noncancerous MCF-10A cells. ER: estrogen receptor, wt: wild-type.

V-positive, PI-negative), late apoptotic/necrotic cells (annexin V-positive, PI-positive), and viable cells (annexin V-negative, PI-negative). Cells were seeded and treated with the indicated compounds or solvent as for the cell cycle analysis. After 48 h of treatment, cells were harvested by centrifugation (1,100 rpm, 10 min) and aliquots of 5×10^5 cells were washed with PBS and resuspended in 500 μl of binding buffer 1X provided with the kit. A volume of 2.5 μl of Annexin V-FITC and 2 μl of PI were added and cells were incubated at room temperature in the dark for 30 min. Approximately 20,000 cells were analyzed by flow cytometry using a using a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter). The percentage of cells in each category was determined. The experiments were performed twice and gave similar results.

Statistical analysis. Results are expressed as the mean±SD. The statistical significance of differences between treatment groups was evaluated by a Student's *t*-test for unpaired observations or a Chi-test using the Analysis Toolpak of Microsoft Excel. In all analyses, differences with $p<0.05$ were considered significant.

Results

Effect of MPTS on mammary epithelial cell proliferation. To determine whether MPTS has a different growth inhibitory activity depending on the characteristics of cells, we examined its effects on the growth of three different human breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) and on one normal human breast epithelial cell line (MCF-10A). The four breast cell lines differ in their ER, *p53, bcl-2*, and *caspase-3* status (Table I). MCF-10A is a near diploid cell line that was derived from human fibrocystic mammary tissue and was immortalized after extended cultivation in medium containing low concentrations of calcium (23). We therefore used normal mammary epithelial MCF-10A cells to also investigate the selectivity of MPTS activity.

Exponentially dividing cells were treated with increasing concentrations of MPTS (9 to 180 μM) for 48 h and IC_{50} values (concentration that inhibits 50% of cells) were calculated. Results are shown in Table I and in Figure 2. The three breast cancer cell lines were much more sensitive to MPTS than normal MCF-10A cells since the IC_{50} values for all three breast cancer cell lines were in the range of 100 μM compared to >719 μM for normal MCF-10A cells (Student's *t*-test, $p<0.001$). These results suggest a selective inhibitory activity of MPTS for tumor cells without a clear association with any of the cell characteristics listed above.

To further investigate the effects of MPTS on human breast cell growth, Trypan blue exclusion assays were performed. In Trypan blue exclusion assay, viable cells will exclude Trypan blue and nonviable cells will retain the dye. Cells were plated at two different densities dependent on the length of the experiment. As shown in Table II, MCF-10A cells treated with MPTS showed less than 10% mortality whatever the dose or the time of incubation. In contrast, MPTS caused between 60% and 75% mortality on all three breast cancer cell lines at a dose close to their IC_{50} values (144 μM) 48 h post-treatment (Table II). Again these results suggest that the effects of MPTS on viability seem not to be related to the ER, *p53* or *bcl-2* status of cells.

Cell cycle arrest in response to MPTS. To determine whether MPTS induces different responses in the cell cycle depending on the *p53* status of cells, its effect on cell cycle progression of breast cancer cells was determined by performing PI staining and analysis by flow cytometry. Figure 3 shows the proportion of cells in each phase following 24 h or 48 h of exposure to MPTS at three different doses, or to 0.4% acetone. Cells treated with acetone at 0.4%, the highest concentration, showed a cell distribution similar to untreated control culture cells (Figure 3). MPTS caused significant cell cycle arrest in the G_2/M phase in wild-type *p53* MCF-7 and mutant *p53* MDA-MB-231 cancer cells. A significant dose-dependent effect on the cell cycle distribution was clearly observed in both cell lines when compared to control cultures. However, in mutant *p53* MDA-MB-453 and wild-type *p53* MCF-10A cells, very little effect on cell progression was obtained and no dose-

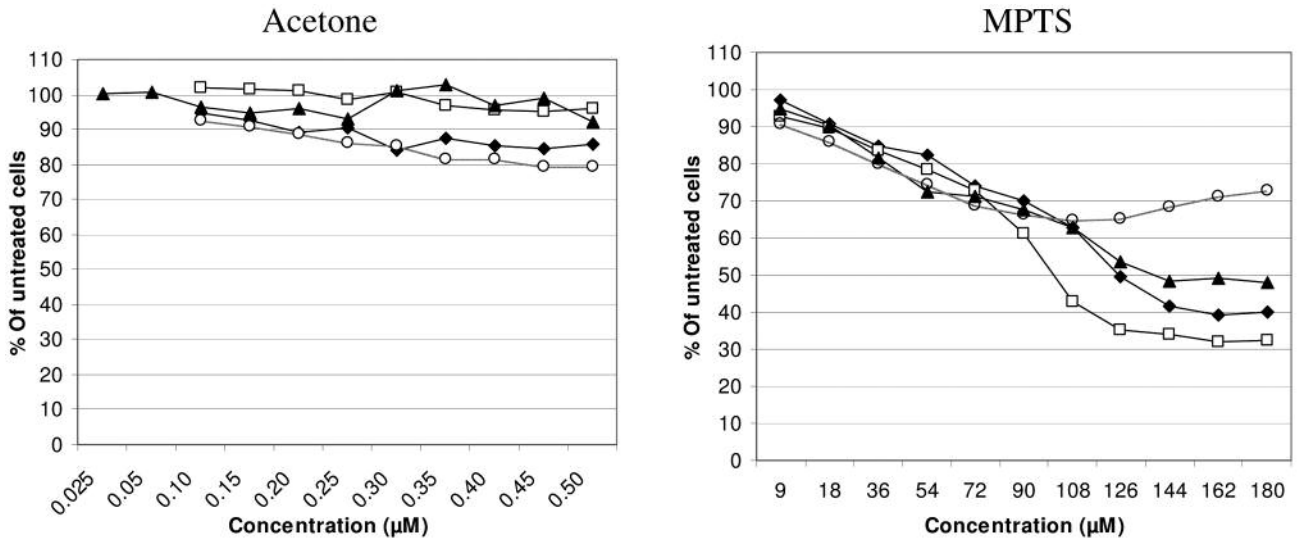

Figure 2. *Growth inhibition in the presence of the compound p-methoxyphenyl p-toluenesulfonate (MPTS). Cells were treated with acetone (A) or MPTS (B) at different doses for 48 hours after which the percentage of viable cells was determined. The maximum solvent used was 0.5% corresponding to the quantity of solvent present in the highest concentration (180 μM) of MPTS. Values are expressed as a percentage of untreated control cells set as 100%. The data represent the mean values from at least 3 independent experiments, each performed in eight replicates. All coefficients of variation were under 10%. ◆ MCF-7, □ MDA-MB-231, ▲ MDA-MB-453, ○ MCF-10A.*

dependent effect could be clearly observed on these two cell lines. Nevertheless, 48 h after exposure to MPTS at high doses, the percentage of MDA-MB-453 and MCF-10A cells in the G_0/G_1 phase slightly increased, showing a different response in these two cell lines. Even though MPTS did not block the cell cycle similarly in all four cell lines, no clear association with *p53* status could be observed.

Induction of apoptosis by MPTS. In order to further determine whether apoptosis responses to MPTS differ according to the status of *p53* or *bcl-2* of cells, we measured the percentage of annexin V-FITC-positive/PI-negative (early apoptotic cells) and of annexin V-FITC-positive/PI-positive cells (late apoptotic/necrotic cells) and also calculated the number of cells in the sub-G_1 population by flow cytometry. Figure 4 shows the results of the annexin V-FITC binding and PI staining on live cancerous and normal cells, following exposure for 48 h to MPTS at two different doses or to 0.4% acetone. The number of cells in the sub-G_1 population is shown in Figure 3. The solvent control cells exhibited the same percentage as the untreated control cells in all four cell lines at all doses (some data not shown). As shown in Figure 4, MPTS was able to induce apoptosis in a dose-dependent manner in all three breast cancer cells. However, apoptosis induction was more pronounced in mutant *p53*, normal *bcl-2* MDA-MB-231 cancer cells than in other cell lines. Apoptosis response was lower in both MCF-7 cells having wild-type *p53* and *bcl-2* overexpression and MDA-MB-453 cells with mutant *p53* and *bcl-2* down-regulation. However, in MCF-7 cells, a significant ($p<0.001$) dose-dependent increase in the proportion of early apoptotic cells was obtained when compared to solvent control cells. In annexin V-PI assay, MPTS did not induce apoptosis in normal cells at doses tested. The percentage of cells in the sub-G_1 population (Figure 3) was higher in MDA-MB-231 and very low in MCF-10A cells confirming the results obtained with annexin V-PI assays. A significant increase of cells in the sub-G_1 population was observed in MDA-MB-453 at the lowest dose, thus reducing the number of cells entering into the G_0/G_1 phase but no dose-dependent effect was obtained. As in the annexin V-PI assay, MDA-MB-453 response to apoptosis induced by higher doses of MPTS was less pronounced than in the other two breast cancer cell lines. Altogether, these results demonstrate that MPTS can induce apoptosis in cells harboring different levels of expression of Bcl-2 and independently of the p53 or ER status of the cells.

Effect of staurosporine and 5-fluorouracil on cell growth. Exponentially dividing cells were treated with increasing concentrations of staurosporine (5 to 500 nM), or 5-FU (0.5 to 5 μM) for 48 h and IC_{50} values were calculated. Results are shown in Table I. In contrast to MPTS, the cells most affected by staurosporine and 5-FU were normal MCF-10A cells (IC_{50} values <0.005 and <0.5 μM, respectively). The cells least sensitive to staurosporine were

Table II. *Percentage of dead cells*.

Incubation time (hours)	Cell line	Untreated	MPTS		Staurosporine		5-Fluorouracil	
		0 μM	72 μM	144 μM	0.005 μM	0.5 μM	0.5 μM	5 μM
24	MCF-10A	6.7±3.1	10.1±4.7	9.1±3.1	6.6±2.5	66.0±25.3	4.2±2.1	45.1±4.3
	MCF-7	6.2±1.5	25.0±1.0*	27.4±17.9	4.6±4.6	57.4±1.0	4.4±1.4	13.1±8.4*
	MDA-MB-231	5.0±0.5	15.1±1.1	6.1±2.6	2.6±0.2	6.6±2.7	7.1±6.2	13.7±5.1**
	MDA-MB-453	3.6±0.9	9.5±2.3	10.6±3.8	3.5±0.5	31.6±6.5	32.8±5.1**	32.9±5.2*
48	MCF-10A	6.3±2.9	9.7±3.3	10.2±2.0	8.5±4.2	75.3±17.9	3.9±3.6	77.2±7.0
	MCF-7	11.6±4.8	25.9±2.7***	61.6±13.8**	5.5±3.2	32.5±7.7*	10.5±2.8	40.1±14.0*
	MDA-MB-231	10.3±3.8	68.3±9.9***	74.8±13.5**	5.3±1.8	84.1±8.1	9.1±0.9	19.7±0.4**
	MDA-MB-453	8.7±4.2	48.4±10.9*	64.9±13.9**	7.0±2.7	80.2±12.1	8.3±4.4	97.5±0.7*

Nonviable cells were counted by using Trypan blue exclusion assay as described in Materials and Methods. Percentage mortality was calculated for each treatment. Data represent means±SD of at least three independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, compared to the noncancerous MCF-10A cells.

MDA-MB-453 and MCF-7 (~0.5 μM) and to 5-FU, MCF-7 (>5 μM). Moreover, when MCF-10A cells were treated with staurosporine or 5-FU, a significant dose- and time-dependent increase in the percentage of dead cells was observed (Table II). Interestingly, when MCF-10A cells were treated with staurosporine or 5-FU at concentrations near to the IC_{50} values (0.005 and 0.5 μM, respectively), the number of dead cells did not significantly increase when compared to those of untreated cells at either 24 or 48 h, indicating that staurosporine and 5-FU might exert their inhibitory effects principally by inhibiting cell proliferation instead of exerting cytotoxicity at these doses. However, on 48 h post-treatment at higher doses, staurosporine and 5-FU were as potent in killing normal MCF-10A cells (75.3% mortality at 0.5 μM compared to 77.2% at 5 μM, respectively). Percentages of dead cells were less than 50% in MCF-7 cells treated with 5-FU, suggesting that this compound might also act through an inhibition of cell proliferation in addition to cytotoxicity. In contrast, high percentages of mortality were found in MDA-MB-453 cells treated with staurosporine (80.2%) or 5-FU (97.5%) 48 h post-treatment at concentrations near to their IC_{50} values (0.5 and between 1 and 5 μM, respectively) showing the cytotoxicity of these two compounds on the MDA-MB-453 cell line. Finally, at 48 h, staurosporine showed cytotoxicity on MDA-MB-231 cells (84.1% mortality at 0.5 μM) whereas 5-FU had only a slight effect on cell viability (19.7% at 5 μM).

Staurosporine caused cell cycle arrest at the G_0/G_1 phase at the lowest dose and at the G_2/M phase at higher doses in MDA-MB-231 and MDA-MB-453 breast cancer lines and in normal cells as well (Figure 5). MCF-7 cells arrested only at G_2/M. Exposure to 50 nM staurosporine induced apoptosis in MCF-7 and MDA-MB-231 cancer cells but to a lesser extent than MPTS at 144 μM (Figures 4 and 5). Staurosporine slightly induced apoptosis in normal cells but did not induce apoptosis in MDA-MB-453 cells in this assay. However, at the highest dose (500 nM), staurosporine induced apoptosis in all cell lines as shown in Figure 5 by the increase of the sub-G_1 population. Cell cycle arrest and apoptosis induced by staurosporine was then not found to be selective for breast cancer cells in contrast to MPTS.

Discussion

This study reports the relationship between the status of the *p53* tumor suppressor, *bcl-2* expression, ER and the *caspase-3* apoptosis effector in four different human mammary cell lines and the antiproliferative activity of the synthetic OSC MPTS. MPTS inhibited growth of all three breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-453 in a similar manner, with IC_{50} values in the range of 100 μM. We have found that tumor cells were more sensitive to MPTS than normal cells. In addition, MPTS caused between 60% and 75% mortality 48 h post-treatment in breast cancer cell lines at a dose close to their IC_{50} values without increasing cell mortality in normal MCF-10A cells. These results showed that MPTS can selectively kill cancer cells at doses of 100 μM. Selective antiproliferative activity has also been found for other synthetic organosulfur compounds in human skin cancer C32 cells when compared to Chinese hamster ovary (CHO) cells (20).

Wild-type p53 is critical in regulating cell cycle arrest and apoptosis (24, 25). *p53* Gene mutation or deletion and loss of *p53* normal function lead to many types of human tumors. In the current study, we have examined the p53-dependent effects of MPTS on the cell cycle and apoptosis by comparing responses in two cell lines with mutant *p53* (MDA-MB-453 and MDA-MB-231) with two cell lines with wild-type (wt) *p53* (MCF-7 and MCF-10A). We

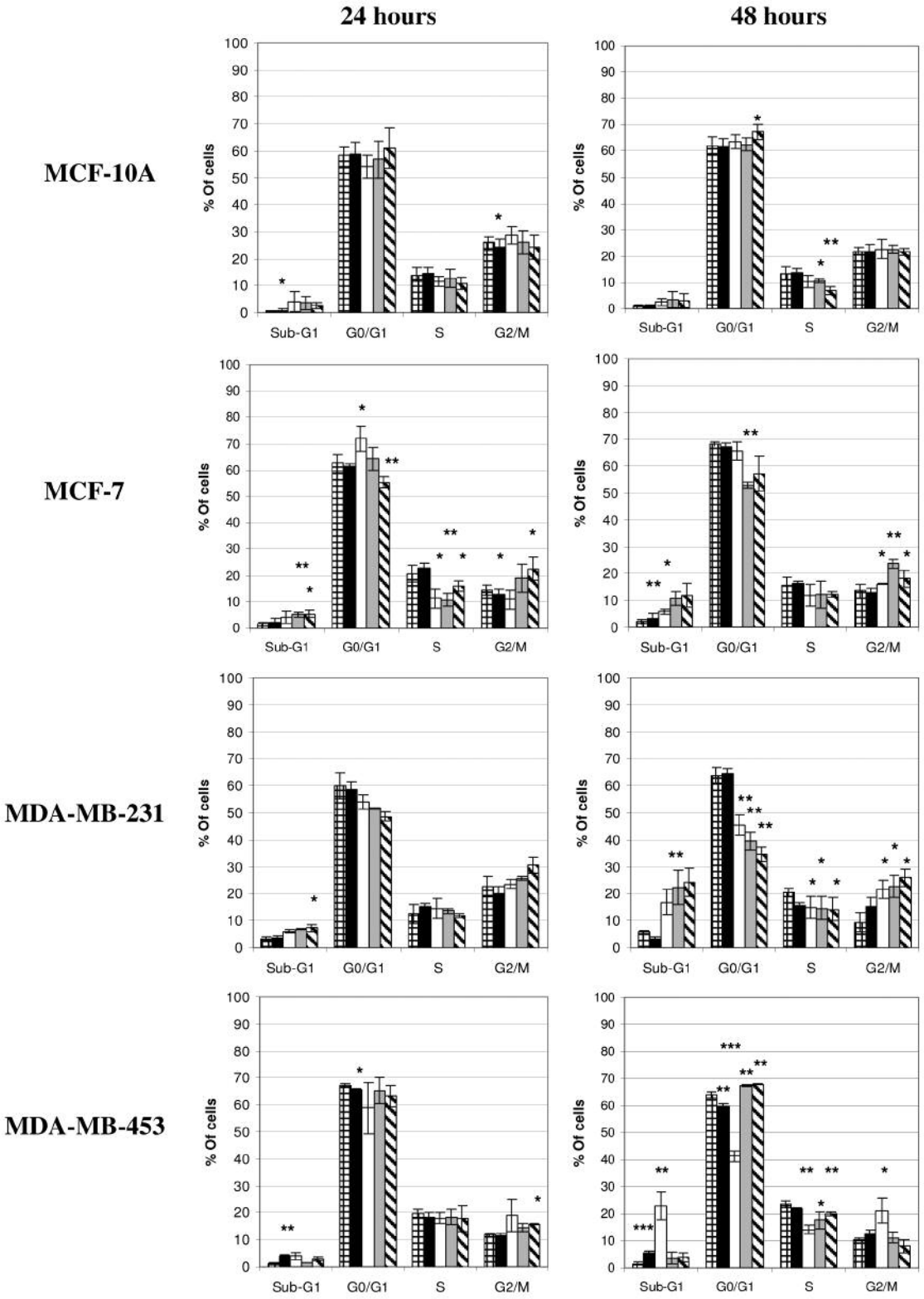

Figure 3. *Effect of MPTS on cell cycle of different human breast cells. MCF-10A (A, B), MCF-7 (C, D), MDA-MB-231 (E, F) and MDA-MB-453 (G, H) cells were treated with 0.4% acetone (■) or three different concentrations (□ 72 μM, ■ 108 μM, or ⬔ 144 μM) of MPTS for 24 (A, C, E, G) or 48 hours (B, D, F, H). Cells were stained with PI and DNA cell content was evaluated on a Beckman Coulter Cytomics FC 500 flow cytometer with CXP software. Untreated (⊞) control cultures were also analysed. Values represent means±SD of at least three independent experiments. *p<0.05, **p<0.01, and ***p<0.001, comparing the indicated compound to untreated control cells.*

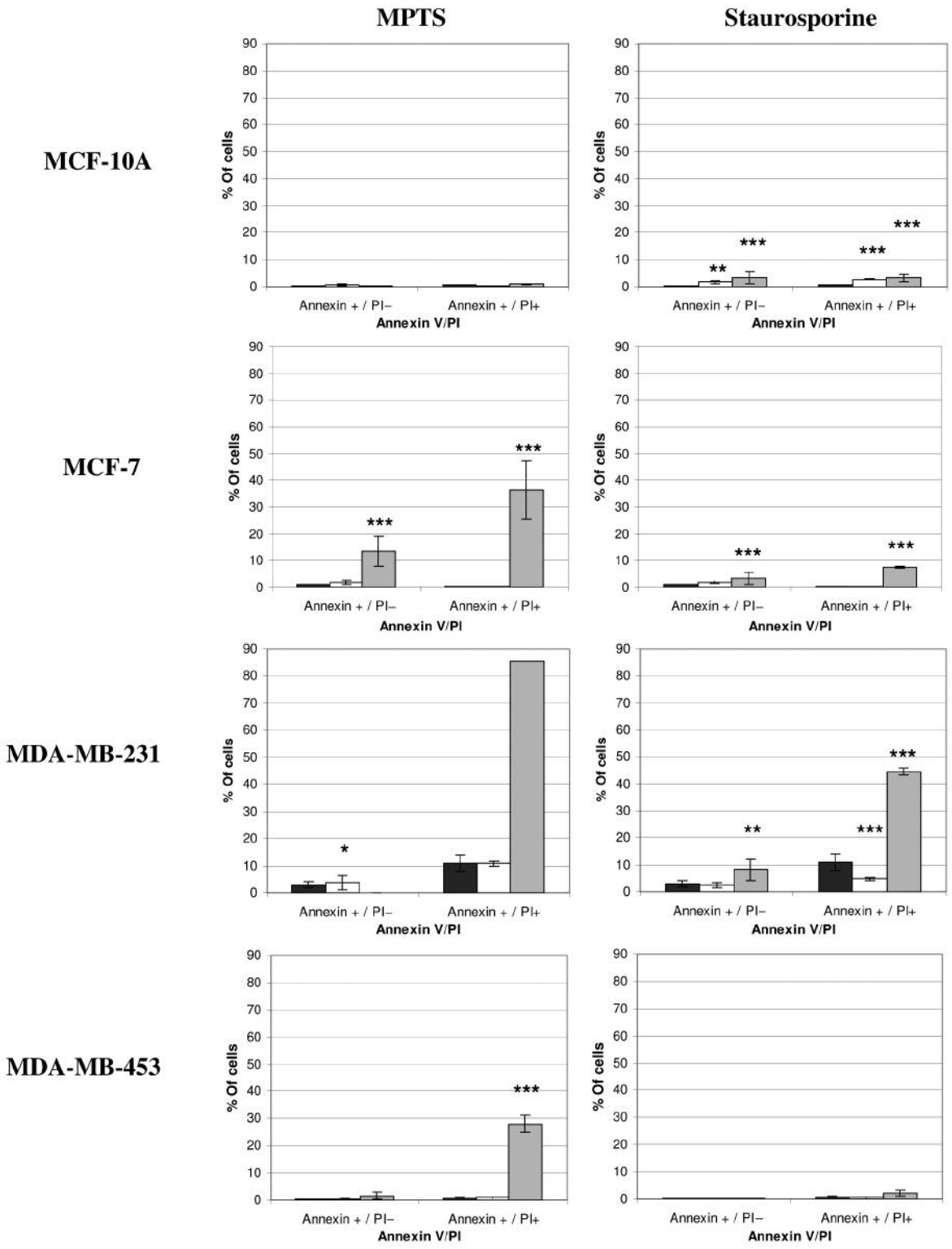

Figure 4. *Apoptosis induction in breast cells with different p53 and bcl-2 status. MCF-10A (A, B), MCF-7 (C, D), MDA-MB-231 (E, F) and MDA-MB-453 (G, H) cells were treated with two different concentrations of MPTS (A, C, E, G) or staurosporine (B, D, F, H) for 48 hours. Cells were then stained with annexin V-FITC and PI. The total apoptotic cells are the sum of Annexin V+/PI– (early apoptotic) and annexin V+/PI+ (late apoptotic/necrotic) cell populations. Values represent means±SD of two independent experiments. *p<0.05, **p<0.01, and ***p<0.001, comparing the indicated compound to untreated control cells as calculated by Chi-test. A, C, E, G: ■: untreated control cells, □: 72 μM MPTS, ■: 144 μM MPTS. B, D, F, H: ■: untreated control cells, □: 5 nM staurosporine, ■: 50 nM staurosporine.*

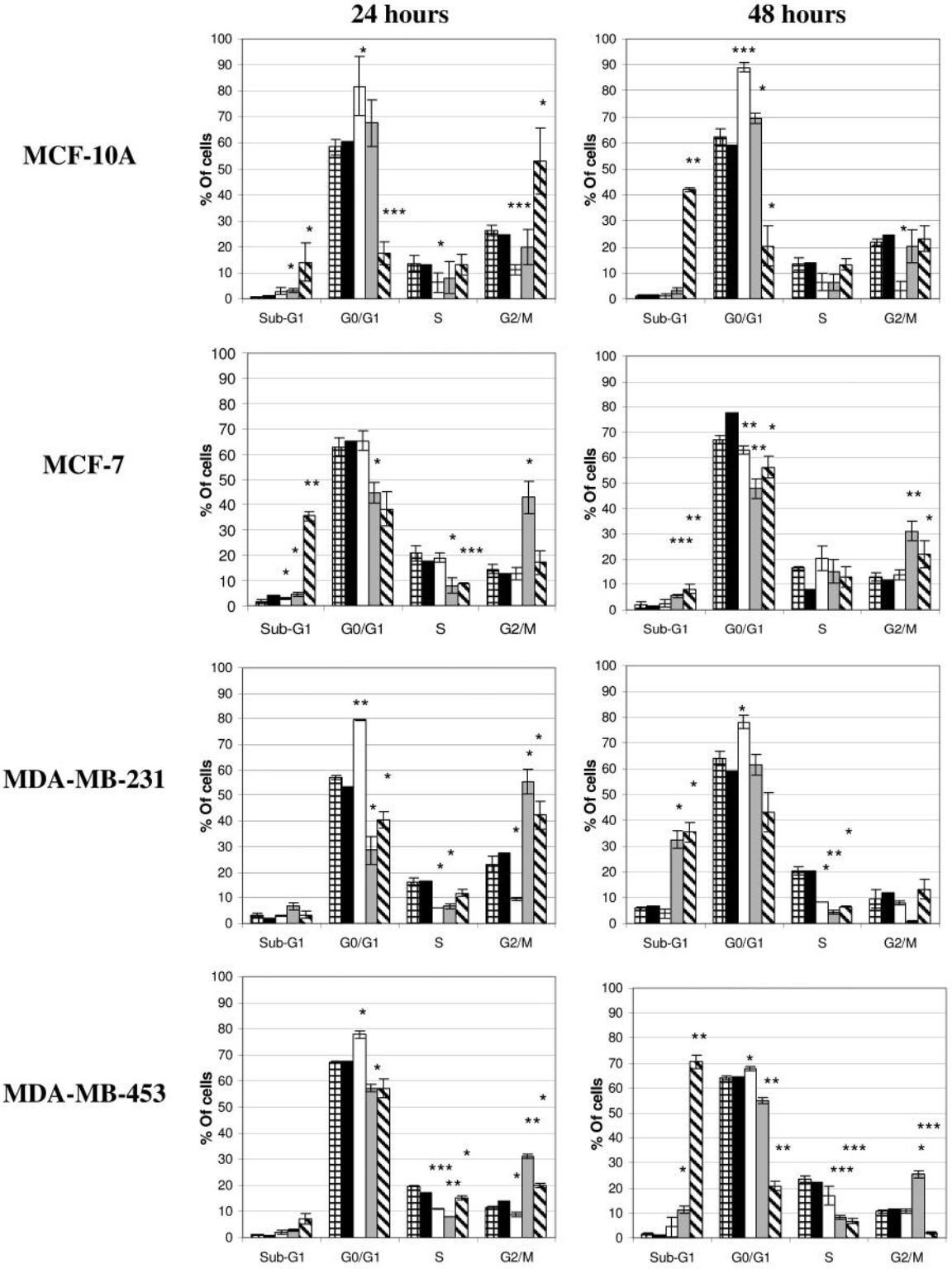

Figure 5. *Effect of staurosporine on cell cycle of different human breast cells. MCF-10A (A, B), MCF-7 (C, D), MDA-MB-231 (E, F) and MDA-MB-453 (G, H) cells were treated with 0.2% DMSO (■) or three different concentrations (□ 5 nM, ■ 50 nM, or ⬔ 500 nM) of staurosporine for 24 (A, C, E, G) or 48 hours (B, D, F, H). Cells were stained with PI and DNA cell content was evaluated on a Beckman Coulter Cytomics FC 500 flow cytometer with CXP software. Untreated (⊞) control cultures were also analysed. Values represent means±SD of at least three independent experiments. *p<0.05, **p<0.01, and ***p<0.001, comparing the indicated compound to untreated control cells.*

found different effects on cell cycle arrest and apoptosis caused by MPTS. The cell cycle arrested in the G_2/M phase in breast cancer wt *p53* MCF-7 and mutant *p53* MDA-MB-231 cells, whereas the cell cycle was slightly arrested in the G_0/G_1 phase in tumor mutant *p53* MDA-MB-453 and in normal mammary epithelial MCF-10A (wt *p53*) cells. These results suggest that *p53* status was not associated with the differential response observed in cell cycle following treatment with MPTS. We also found that normal MCF-10A cells with wt *p53* were more resistant to induction of apoptosis by MPTS than breast cancer cell lines. However, this resistance to apoptosis was not related to the presence of wt *p53* since apoptosis was also induced in MCF-7 cells that contained wt *p53*. Our results are in agreement with a previous study in which DADS, an oil-soluble organosulfur compound found in processed garlic, was shown to be an effective inhibitor of both ER-positive, wt *p53* MCF-7 and ER-negative, mutant *p53* MDA-MB-231 breast cancer cells (9).

Bcl-2 and Bcl-xL proteins are inhibitors of the mitochondrial apoptosis pathway by preventing the release of cytochrome *c* and the activation of caspases (26, 27). Overexpression of Bcl-2 and Bcl-x_L has been observed in several types of cancer (1, 17, 28) and has been implicated in resistance to a wide range of anticancer drugs (29, 30). Bcl-2 protein functions as a suppressor of apoptotic death triggered by a variety of signals (31, 32) and is negatively regulated by wt *p53*. In this study we investigated whether apoptosis responses to MPTS was dependent upon the *bcl-2* status of cells. MPTS was able to induce apoptosis in all three breast cancer cell lines having different levels of expression of *bcl-2*, but a higher level of apoptosis was found in MDA-MB-231 cells containing normal *bcl-2* when compared to the other two breast cancer cell lines having either down-regulation (MDA-MB-453) or overexpression (MCF-7) of *bcl-2*. Interestingly, no induction of apoptosis was observed in MCF-10A cells containing normal *bcl-2*. High levels of Bcl-2 protein and lack of caspase-3 in MCF-7 cells might have contributed to their higher resistance to apoptosis when compared to MDA-MB-231 cells. MDA-MB-453 cells showed the lowest apoptosis response when compared to MCF-7 and MDA-MB-231 cells despite the down-regulation of *bcl-2*. The *p53*-dependent pathway is deficient in MDA-MB-231 cells but the normal level of Bcl-2 and caspase-3 suggests a mitochondrial pathway induction of apoptosis in these cells. Therefore, our results suggest that in addition to a mitochondrial pathway, the apoptosis response to MPTS in breast cancer cells is associated with other signaling pathways and not necessarily with *bcl-2* status. These findings support previous studies that have demonstrated that organosulfur compounds can induce apoptosis by various pathways such as modulation of mitogen-activated protein kinases (MAPKs) (JNK, p38 and ERK1/2) (4, 33-36), mitochondrial-dependent or –independent pathways (9, 14, 33, 35, 36) and production of reactive oxygen species (ROS) (37, 38). Reduction of Bcl-2 or Bcl-X_L, increase of Bax or Bak, and activation of caspases 3, 8, and 9 were responses observed in different types of cells following treatment with organosulfur compounds (9, 33, 35, 36, 39-42).

In this study, induction of apoptosis was more important in the two breast cancer cell lines (MDA-MB-231 and MCF-7), where a response in the cell cycle was more pronounced by an arrest in the G_2/M phase, suggesting that MPTS may act through cell cycle arrest in G_2/M followed by induction of apoptosis in these breast cancer cells. Similar effects of MPTS and other synthetic OSCs on apoptosis induction and G_2/M cell cycle arrest have also been reported in human leukemia cells (2). In MDA-MB-231 cells, DADS induction of apoptosis has been associated with up-regulation of apoptotic Bax protein, down-regulation of anti-apoptotic Bcl-X_L protein and activation of caspase-3. It would be interesting to investigate whether the apoptosis response to MPTS would be the same in MDA-MB-231 cells that were the most sensitive cancer cells evaluated in this study.

In MDA-MB-453 cells with mutant *p53* and *bcl-2* down-regulation, MPTS seems to act mainly by cytotoxicity since the weak effects on the cell cycle and apoptosis cannot explain the high percentage of mortality observed in this cell line. MDA-MB-231 and MDA-MB-453 cells have both mutant *p53* and normal *caspase-3*, but they differ in their *bcl-2* status (normal *vs*. down-regulated, respectively) suggesting that a low level of Bcl-2 could have protected MDA-MB-453 cells from apoptosis induced by MPTS and might be responsible for cell death. Our results are in agreement with another study in which a low level of Bcl-2 was associated with necrosis rather than apoptosis following treatment with staurosporine (43).

Altogether, our results demonstrated that the inhibition of cell proliferation was not related to the status of *p53, ER, caspase-3* or *bcl-2* expression of breast cancer cells. MPTS was found to be more selective for tumor cells than staurosporine and 5-FU. Therefore our findings indicate that MPTS could be a promising anticancer agent for both hormone-dependent and -independent breast cancer and may represent a novel class of tumor-specific antiproliferative agents that could have wide application for targeted therapy of cancer.

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