Abstract. Background: It has been well documented that some organosulfur compounds (OSCs) show promise as anticancer agents. Materials and Methods: The growth inhibitory effects of six novel different synthetic sulfonate esters was evaluated on cancerous (MCF-7) and non-cancerous (MCF-10A) human breast epithelial cells. Results: We found that the most active compounds against MCF-7 breast cancer cells had a common structure of p-methoxyphenyl p-toluenesulfonate with the methoxy substituent shifted from position 4 (22) to 2 (22o) or to 3 (22m). 3-Methoxyphenyl p-toluenesulfonate (22m) showed the lowest IC50 value (89.83 μM) on breast cancer cells but was also very active on non-cancerous MCF-10A cells (IC50 value of 53.96 μM). We found that compound 22 caused a greater degree of cell cycle arrest and induced apoptosis in cancerous MCF-7 cells compared with normal breast epithelial MCF-10A cells. However, compound 22m, was less selective by significantly arresting normal cells at G2/M-phase followed by a weak induction of apoptosis. Conclusion: P-methoxyphenyl p-toluenesulfonate (22) appeared to be a more selective inhibitor of the growth of human breast cancer cells. Taken together, these results show that synthetic OSC compounds evaluated in this study can be effective antineoplastic agents and are worthy of further investigation.

Breast cancer is the second leading cause of cancer mortality in women. Surgery, radiation and chemotherapy are three common forms of treatment that have been used to treat breast cancer for a number of years. Because chemotherapy can have many unpleasant and more serious side-effects impeding the quality of life of patients and survivors, and since some breast cancers do not respond to commercially available drugs, there is an urgent need to develop new anticancer drugs.

Numerous studies have shown that organosulfur compounds (OSCs) isolated from plants such as garlic, onions or mahogany trees (e.g., allyl sulfides) or synthetically prepared inhibit the growth of different human cancer cell lines in culture, including those of prostate, breast, lung, colon, skin, uterine, esophagus, and leukemic cells (1-6), and tumor cell xenografts in animal models (7-9). Epidemiological studies have also provided evidence that the risks of developing certain types of cancer are inversely related to regular consumption of garlic products (10-12). Organosulfur compounds exert their growth inhibitory effect through various mechanisms including the inhibition of the carcinogen-activating cytochrome P450 (CYP) enzymes (13), the induction of carcinogen-detoxifying enzymes such as glutathione S-transferase (14,15), the inhibition of cell cycle progression and/or the induction of apoptosis (2, 4, 5), and the alteration of the calcium homeostasis (16).

Recently, we prepared and screened 22 synthetic aryl sulfones and aryl sulfonate esters against human skin cancer C32 cells (17). The results revealed that compound 22, p-methoxyphenyl p-toluenesulfonate, showed the most pronounced inhibitory activity against human skin cancer cell growth. In addition, the p-methoxyphenyl p-toluenesulfonate showed a good balance between effectiveness and selectivity for cancerous cells as our other biologically active synthetic organosulfur compounds did (1, 17-19).

In the present study, we wanted to investigate whether our four previously described biologically active synthetic organosulfur compounds (sulfonate esters 19, 20, 22, and 22J) had antiproliferative activity against breast cancer cells by using the human breast cancer cell line MCF-7. In
addition, we prepared and evaluated the biological effects of two novel toluenesulfonates (22a and 22m) on the growth of MCF-7 cells. Finally, we investigated the mechanism of the inhibitory activity of these compounds by evaluating their effects on the cell cycle and apoptosis.

Materials and Methods

Organosulfur compound synthesis. Sulfonate esters 19, 20, and 22 were prepared as reported earlier (17). The toluenesulfonate 22J has been described (19). Instrumentation and routine procedural details for the spectroscopic characterization of our samples have been provided previously (17, 19).

Preparation of 2-methoxyphenyl p-toluenesulfonate 22a. Part A. Catechol (0.50 g, 4.6 mmol) and dry triethylamine (0.90 g) were dissolved in dry pyridine (25 mL). The reaction mixture was cooled with an ice/water bath. Sublimed p-toluenesulfonyl chloride (1.75 g, 9.2 mmol) was added in small portions over 15 min. The reaction mixture, initially yellowish brown, turned red on stirring at ambient temperature for one week. Chloroform (150 mL) was added and the resultant solution extracted with 5% v/v hydrochloric acid (three - 100 mL aliquots). The combined organic layers were dried (MgSO4), filtered and the solvent evaporated affording crude product (0.73 g). Crude product was chromatographed on silica gel (74 g) employing 3:1 chloroform/petroleum ether (100 mL fractions). Fractions 7 - 9 were concentrated and combined affording homogencous 2-methoxyphenyl p-toluenesulfonate 22a (0.37 g, 0.67 mmol, 30%). Part B. Sodium metal (62 mg, 2.69 mmol) was dissolved in dry pyridine (30 mL). Sublimed

Preparation of compound solutions. Stock solutions (10 mg/mL) were prepared in ACS grade acetone or DMSO (Sigma-Aldrich, St. Louis, MO, USA) and were stored in the dark at 4°C. Compounds were diluted in fresh culture medium as indicated, immediately prior to the addition to cell cultures.

Cell lines and culture conditions. MCF-7 human breast cancer cell line and MCF-10A human mammary epithelial non cancerous cell line were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). MCF-7 cells were maintained in Dulbecco's Minimum Essential Medium (DME(M Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (BioMedia, Drummondville, QC, Canada), 50 ìg/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 1% FBS, 20 ng/mL of gentamicin, 2 mM of L-glutamine, 20 ng/mL of epidermal growth factor (Sigma-Aldrich), 10 ìg/mL of insulin (Sigma-Aldrich), 100 ng/mL of toxin cholera (List Biological Laboratories, Incs, Campbell, California, USA) and 1 ìg/mL of hydrocortisone (Sigma-Aldrich). Cells were cultured in 75 cm2 culture flasks at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested when they reached about 80-85% confluence using a cell scraper (MCF-7) or a 0.5% trypsin-EDTA solution (Sigma-Aldrich) diluted in phosphate buffered solution (PBS) (Sigma-Aldrich) (MCF-10A).

MTT assay. Cells were seeded in clear 96-well plates (Sarstedt, Montreal, Canada) at a density of 7,500 cells/well (MCF-10A) or 10,000 cells/well (MCF-7). After 24 hours, the culture medium was replaced by fresh medium containing various concentrations of organosulfur compounds or vehicle (acetone or DMSO). Concentrations varied from 2.5 ìg/mL to 50 ìg/mL. In some of the wells, only culture medium was added. These wells were used as negative controls and were referred as untreated control cells. Cell number was evaluated using the MTT Cell Proliferation Assay (20). Briefly, cells were rinsed twice with PBS and 50 ìl of fresh culture medium was added to each well. Next, 8 ìl of a solution

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of 4 mg/ml MTT (Sigma-Aldrich) in PBS was added to each well and cells were incubated for another 2 hours. The purple formazan crystal was solubilized by adding 120 μl of lysis buffer (1% HCl 12 N and 5% Triton X-100 in isopropanol). The absorbance was measured at 570 nm on a microplate spectrophotometer reader (Benchmark, Bio-Rad). Each compound was evaluated at least in 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 1.25x10^5 to 5x10^5/well. After 24 hours of incubation, the medium was removed and replaced by fresh culture medium containing various concentrations of OSC or the equivalent amount of removed. 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, and viable and non-viable cells were counted on a hemocytometer 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 a 6-well tissue culture plates at a density of 1.25x10^5 to 5x10^5/well. After 24 hours of incubation, the medium was removed and replaced by fresh culture medium containing various concentrations of OSC or the equivalent amount of vehicle. Cells were harvested by centrifugation (1,100 rpm, 10 min) and aliquots of 5x10^5 cells were washed and collected. After 24 hours of incubation, the medium was removed and replaced by fresh culture medium containing various concentrations of OSC or the equivalent amount of vehicle. Cells were washed 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, and viable and non-viable cells were counted on a hemocytometer plate under a microscope. The percentage of mortality was determined. This experiment was repeated three times.

Apoptosis assay. Annexin V-FITC was used as a marker of phosphatidylserine exposure and propidium iodide as a marker for dead cells (APOAF Annexin V-FITC Apoptosis Detection Kit, Sigma-Aldrich). This combination allows the differentiation among early apoptotic cells (annexin 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 vehicle as for the cell cycle analysis. After 24 h or 48 h of treatment, cells were harvested by centrifugation (1,100 rpm, 10 min) and aliquots of 5x10^5 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. About 20,000 cells were analyzed by flow cytometry using a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter). The percentage of cells in each phase of the cell cycle was calculated. The experiments were performed three times and gave similar results.

Statistical analysis. Results are expressed as the mean±SD. Statistical significance of differences between treatment groups was evaluated by Student’s t-test for unpaired observations or a Chi²-test using the Analysis Toolpak of Microsoft Excel. In all analyses, differences with p<0.05 were considered significant.

Results

Growth inhibition of MCF-7 cells. To evaluate the antiproliferative activity of the sulfonate esters under investigation, we first examined their effect on growth of MCF-7 breast cancer cells. The MTT assay was used to investigate the effect on cell viability. Exponentially dividing cells were treated with increasing concentrations (2.5 to 50 μg/mL) of each compound for 48 h as described in the Materials and Methods section. Dose-dependent cell viability curves are shown in Figure 1 and IC₅₀ values (concentration that inhibits 50% of cells) are summarized in Table I. Figure 1 shows that the OSCs tested can be divided into two groups when compared to untreated control cells and cells treated with the solvent only: Group A (compounds 19, 20, and 22j) with no or low MTT activity and Group B (compounds 22, 22m, and 22o) with high MTT activity. Compounds 22 and 22m were the most active compounds against MCF-7 cells (Figure 1B) with IC₅₀ values of 125.90 μM (35 μg/mL) and 89.83 μM (25 μg/mL), respectively, when compared to the cells treated with acetone only (>200 μg/mL) (Student’s t-test, p<0.001) (Table I).

To further investigate the effects of OSCs on MCF-7 cell growth, trypan blue exclusion assays were performed to differentiate whether the decrease in the MTT activity observed with the most active compounds was due to inhibition of cell proliferation or death. In a trypan blue exclusion assay, viable cells will exclude trypan blue and non-viable cells will retain the dye. Cells were plated at two different densities dependent on the length of the experiment. After 24 h or 48 h of treatment, cells were harvested and cultures were arrested. Table II shows the percentage of dead cells in each treatment and in untreated control cells. Cells treated with 0.2% acetone were not significantly affected (Student’s t-test) when compared to untreated cells at 24 and 48 h after treatment. In cells treated with 0.4% acetone, the number of cells was slightly reduced at 24 h compared to untreated cells (3.67x10⁵ cells/mL vs. 3.97x10⁵ cells/mL, respectively, p<0.05) but was similar to untreated control cells at 48 h. Cell mortality was not higher in cells treated with the solvent only. However, cells treated with compound 22 rapidly died as we observed, 24 h post-treatment, 25% and 27.4% mortality with 20 μg/mL and 40 μg/mL, respectively. At 48 h post-treatment, cell mortality was the same in cells treated with compound 22 at 20 μg/mL (25.9%) but strongly increased at 40 μg/mL (61.6%). A significant (p<0.001) and time-dependent decrease in cell viability was observed with compound 22 at 20 μg/mL.
corresponding to a decrease of approximately 18% at 24 h and 65% at 48 h in the number of viable cells when compared to untreated control cells ($p<0.001$). Exposure to compound 22 at 40 µg/mL resulted in a strong and significant decrease in cell viability (Table II) at both 24 and 48 h post-treatment (Student’s $t$-test, $p<0.01$ and $p<0.001$, respectively) corresponding to a decrease of approximately 56% at 24 h and 79% at 48 h in the number of viable cells when compared to untreated control cells. These data confirm the reduction of cell viability observed with the MTT assay in cells treated for 48 h with compound 22 (Figure 1B).

Compound 22m affected MCF-7 cell viability and induced cell death in a time-dependent manner at both doses: 20 and 40 µg/mL. As early as 24 h post-treatment significantly important effects on cell viability could be observed at both doses: 2.39x10^5 cells/mL at 20 µg/mL and 1.40x10^5 cells/mL at 40 µg/mL, when compared to untreated cells 3.97x10^5 cells/mL (Table II), representing a decrease in cell viability of approximately 40% and 65%, respectively. After 48 h of treatment with compound 22m, cell viability decreased by approximately 84% at 20 µg/mL and 91% at 40 µg/mL (0.5x10^5 cells/mL and 0.28x10^5 cells/mL, respectively, compared to 3.06x10^5 cells/mL in untreated cells). This confirms the reduction of cell viability measured with the MTT assay (Figure 1B). Moreover, cell mortality dramatically increased, even with the lowest dose: 74.8% of mortality at 20 µg/mL and 83.9% mortality at 40 µg/mL (Table II).

Thus, compounds 22 and 22m seem to block the proliferation of MCF-7 cells and induce cell death. However, 22m showed more potency in inhibiting MCF-7 cell proliferation than 22.

Cell cycle arrest in MCF-7 cells. The effect of compounds 22 and 22m on cell cycle progression of MCF-7 cells was determined by performing propidium iodide staining and analysis by flow cytometry 48 h after treatment. Consistent with their effect on cell growth inhibition, compounds 22 and 22m caused cell cycle arrest in the G2/M-phase in MCF-7 cells when compared to cells treated with acetone only (Figure 2). Cells treated with acetone showed a typical distribution of 68.3% in G0/G1-phase, 16.4% in the S-phase, and 13.7% in the G2/M phase (Figure 2A) similar to the untreated control culture cells (data not shown). Compound 22 at 40 µg/mL increased the proportion of cells in the G2/M phase to 21.5% whereas the number of cells in G0/G1 and the S-phase decreased to 52.1% and 11%, respectively (Figure 2B). Compound 22m at 40 µg/mL had a stronger impact on the cell cycle by increasing the G2/M populations to 40.6% and reducing G0/G1 and S populations down to 35.4% and to 9.3%, respectively (Figure 2C). Cells in sub-G1 are indicative of those undergoing apoptosis. The sub-G1 population increased to 14.8% and 14.6% when cells were treated with compound 22 and 22m, respectively (Figure 2 B and C), compared to 1.4% in cells treated with acetone 0.4% (Figure 2A).

Figure 3 shows the proportion of cells in each phase following 24 h of exposure to our organosulfur compounds at three different doses or to 0.4% acetone. A dose-dependent effect on cell cycle distribution was observed with compound 22. After 24 h of treatment, compound 22 at 20 µg/mL, significantly increased the G0/G1 population ($p<0.05$) when compared to untreated and solvent control cultures (Figure 3A). The number of cells in the S-phase were significantly
reduced ($p<0.05$) but the decrease in the G$_2$/M-phase was not found to be statistically significant. At higher doses, the number of cells in G$_0$/G$_1$- and the S-phase decreased and cells in the G$_2$/M-phase and the sub-G$_1$ population significantly increased. In cells treated with compound 22m, an important increase in the number of cells in the G$_2$/M-phase ($p<0.001$) and a reduction in the G$_0$/G$_1$- and the S-phase ($p<0.001$) was observed with all doses (Figure 3B). Cells in the sub-G$_1$ population were significantly increased at all doses.

These data demonstrated that compounds 22 and 22m induced a dose-dependent cell cycle arrest of MCF-7 cells. A maximal effect on cell cycle by compounds 22 and 22m was already observable 24 h post-treatment and with 20 $\mu$g/mL for compound 22m. In contrast, the number of cells in the sub-G$_1$ population representing apoptotic cells was maximal at 48 h post-treatment (Figure 2) for both compounds.

**Induction of apoptosis in MCF-7 cells.** In order to further determine whether compounds 22 and 22m induce apoptosis in MCF-7 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) by flow cytometry. Figures 4 and 5 show the results of the Annexin V-FITC binding and PI staining on live MCF-7 cells, following the treatment with acetone or our organosulfur compounds, at two different

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**Table I. IC$_{50}$ of sulfonate compounds.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC$_{50}$ in $\mu$M (in $\mu$g/mL)</th>
<th>MCF-7</th>
<th>MCF-10A</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td><img src="image1" alt="Structure" /></td>
<td>293.23 (78 $\mu$g/mL)</td>
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</tr>
<tr>
<td>20</td>
<td><img src="image2" alt="Structure" /></td>
<td>772.28 (156 $\mu$g/mL)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td><img src="image3" alt="Structure" /></td>
<td>125.90$^c$ (35 $\mu$g/mL)</td>
<td>$&gt;719.42$</td>
<td>($&gt;200$ $\mu$g/mL)</td>
</tr>
<tr>
<td>22J</td>
<td><img src="image4" alt="Structure" /></td>
<td>$&gt;170.07$ ($&gt;50$ $\mu$g/mL)</td>
<td>$&gt;680.27$</td>
<td>($&gt;200$ $\mu$g/mL)</td>
</tr>
<tr>
<td>22m</td>
<td><img src="image5" alt="Structure" /></td>
<td>89.93$^c$ (25 $\mu$g/mL)</td>
<td>53.96$^c$ (15 $\mu$g/mL)</td>
<td></td>
</tr>
<tr>
<td>22o</td>
<td><img src="image6" alt="Structure" /></td>
<td>143.88$^c$ (40 $\mu$g/mL)</td>
<td>$&gt;179.86$</td>
<td>($&gt;50$ $\mu$g/mL)</td>
</tr>
</tbody>
</table>

IC$_{50}$ represents the concentration of each compound that resulted in 50% inhibition of MCF-7 and MCF-10A cell growth as measured by the MTT assay after 48 h of treatment. Compounds were dissolved in DMSO (19 and 20) or acetone (22, 22J, 22m, and 22o) for testing. Solvents were themselves tested. In MCF-7 cells, DMSO had an IC$_{50}$ value of 5% by volume (the amount of DMSO in a 500 $\mu$g/mL solution), and acetone had an IC$_{50}$ value $>2\%$ by volume (the amount of acetone in a 200 $\mu$g/mL solution) in both cell lines. Hence, solutions with IC$_{50}$ values less than the IC$_{50}$ value of the corresponding solvent contained active solutes. IC$_{50}$ values greater than the IC$_{50}$ value of the corresponding solvent likely arose from the solvent in test solutions. Data shown are the means from three independent experiments performed in eight replicates. $^a p<0.05$, $^b p<0.01$, and $^c p<0.001$, comparing the indicated compound to the corresponding solvent.
doses for 48 h. The solvent control cells exhibited the same percentage as the untreated control cells (data not shown). Cells treated with 0.4% acetone exhibited 0.9% Annexin V-FITC-positive/PI-negative cells and only 0.6% Annexin V-FITC-positive/PI-positive cells, whereas cells treated with compounds 22 or 22m at 40 ìg/mL displayed more Annexin V-FITC-positive/PI-negative cells, 10.6% and 5%, respectively and contained a much larger proportion of Annexin V-FITC-positive/PI-positive cells (44.5% and 54.8%, respectively) (Figure 4). Both compounds induced apoptosis in a dose-dependent manner (Figure 5). When cells were exposed to compound 22 or 22m, a significant dose-dependent increase in early apoptotic cells and late apoptotic/necrotic cells was obtained when compared to solvent control cells (Figure 5). However, less early apoptotic cells and much later apoptotic/necrotic cells were found in cells treated with compound 22m, when compared to cells treated with compound 22. This difference was more pronounced at 20 ìg/mL for the late apoptotic/necrotic cells (Figure 5B and C).

Effect on the non-cancerous MCF-10A cell line. We next evaluated whether the inhibition of cell growth by compounds 22 and 22m was specific to cancerous cells by determining their effects on the non-cancerous but transformed MCF-10A cell line. This cell line was derived from human fibrocystic mammary tissue and was spontaneously immortalized without viral or chemical intervention from mortal diploid human breast epithelial cells after extended cultivation in medium containing low concentrations of calcium (21). MCF-10 has the characteristics of normal breast epithelium (21). MTT assays revealed that compound 22m was more active than compound 22 in inhibiting MCF-10A growth (Figure 6A) and was more active on MCF-10A than on MCF-7 (Figure 1 and Table II.

### Table II. Effects of organosulfur compounds on MCF-7 cell growth.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Number of viable cells (x10^5 cells/mL)</th>
<th>Non-viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
</tr>
<tr>
<td>Untreated</td>
<td>0 ìg/mL</td>
<td>3.97±0.33</td>
<td>3.06±0.57</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.2%</td>
<td>3.70±0.69</td>
<td>2.62±0.79</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.4%</td>
<td>3.67±0.27a</td>
<td>3.08±0.54</td>
</tr>
<tr>
<td>22</td>
<td>20 ìg/mL</td>
<td>3.24±0.06c</td>
<td>1.08±0.38c</td>
</tr>
<tr>
<td>22</td>
<td>40 ìg/mL</td>
<td>1.75±0.45b</td>
<td>0.63±0.18a</td>
</tr>
<tr>
<td>22m</td>
<td>20 ìg/mL</td>
<td>2.39±0.79a</td>
<td>0.50±0.19c</td>
</tr>
<tr>
<td>22m</td>
<td>40 ìg/mL</td>
<td>1.40±0.26c</td>
<td>0.28±0.11c</td>
</tr>
</tbody>
</table>

Viable and non-viable cells were counted by using Trypan blue exclusion assay as described in Materials and Methods. Cells were plated at a density of 2.5x10^5 cells/mL for the 24 h experiments and at a density of 1.25x10^5 cells/mL for the 48 h experiments. Percent of mortality was calculated. Data 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.

### Table III. Effect of organosulfur compounds on MCF-10A cell growth.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Number of viable cells (x10^5 cells/mL)</th>
<th>Non-viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
</tr>
<tr>
<td>Untreated</td>
<td>0 ìg/mL</td>
<td>4.10±0.77</td>
<td>4.92±1.50</td>
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<tr>
<td>Acetone</td>
<td>0.2%</td>
<td>4.05±0.96</td>
<td>4.13±0.80</td>
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<tr>
<td>Acetone</td>
<td>0.4%</td>
<td>4.13±0.74</td>
<td>4.57±1.30</td>
</tr>
<tr>
<td>22</td>
<td>20 ìg/mL</td>
<td>4.49±1.63</td>
<td>3.22±1.14a</td>
</tr>
<tr>
<td>22</td>
<td>40 ìg/mL</td>
<td>3.35±2.16</td>
<td>2.33±0.49c</td>
</tr>
<tr>
<td>22m</td>
<td>20 ìg/mL</td>
<td>1.75±0.44c</td>
<td>0.68±0.14c</td>
</tr>
<tr>
<td>22m</td>
<td>40 ìg/mL</td>
<td>0.77±0.31c</td>
<td>0.51±0.10c</td>
</tr>
</tbody>
</table>

Viable and non-viable cells were counted by using Trypan blue exclusion assay as described in Materials and Methods. Cells were plated at a density of 1.25x10^5 cells/mL for the 24 h experiments and at a density of 0.625x10^5 cells/mL for the 48 h experiments. Percent of mortality was calculated. Data 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.
6A). IC\textsubscript{50} on MCF-10A of compound 22M was 53.96 \(\mu\)M (15 \(\mu\)g/mL) compared to >719.42 \(\mu\)M (>200 \(\mu\)g/mL) for compound 22 (Table I). Compound 22m showed a higher level of MTT activity on non-cancerous MCF-10A cells than on cancerous MCF-7 cells (IC\textsubscript{50} of 53.96 \(\mu\)M compared to 89.83 \(\mu\)M, respectively). However, compound 22 was less active on non-cancerous MCF-10A cells than on MCF-7 cells (IC\textsubscript{50} >719.42 \(\mu\)M compared to 125.90 \(\mu\)M, respectively). The viability of MCF-10A cells treated with compound 22m decreased significantly in a time- and dose-dependent manner with approximately 57\% reduction as early as 24 h post-treatment with the lowest dose when compared to untreated control cells (Table III). Non-viable cells were also found to be significantly more numerous in MCF-10A cells treated with compound 22m than in untreated cells. Compound 22 did not affect MCF-10A cell growth in trypan

Figure 2. Effect of sulfonate compounds on the cell cycle of MCF-7 cells assessed by flow cytometry. MCF-7 cells were treated with A: 0.4\% acetone (solvent) or 40 \(\mu\)g/mL of B: compound 22 or C: compound 22m for 48 hours. Images are representative of three independent experiments.

Figure 3. Effect of various concentrations on cell cycle progression. MCF-7 DNA cell content was evaluated on a Beckman Coulter Cytomics FC 500 flow cytometer with CXP software. Cells were treated with 0.4\% acetone (■) or three different concentrations ( □ 20 \(\mu\)g/mL, ▬ 30 \(\mu\)g/mL, and ▾ 40 \(\mu\)g/mL) of A: compound 22 or B: 22m for 24h and then were stained with PI. 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. Effect of sulfonate compounds on apoptosis of MCF-7 cells. To investigate the ability of these compounds to induce apoptosis, MCF-7 cells were treated with A: 0.4% acetone (solvent) or 40 μg/mL of B: compound 22 or C: 22m for 48 hours. Cells were then stained with propidium iodide (PI) and Annexin V-FITC and analyzed by using a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter). Images are representative of two independent experiments.

Figure 5. Effect of various concentrations on apoptosis induction. MCF-7 cells were treated with A: 0.2% (■■) or 0.4% acetone (■) or two different concentrations of B: compound 22 or C: 22m for 48 h at a dose of 20 μg/mL (■■) or 40 μg/mL (■). 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-square-test.
blue exclusion assays when compared to untreated cells 24 h post-treatment. At 48 h a significant decrease in cell viability was observed and non-viable cells were found to be significantly more numerous only in cells treated with the highest dose (Table III).

For cell cycle progression, compound 22 (Figure 6C) induced a weak cell cycle arrest in the G0/G1-phase only at the highest concentration 40 μg/mL (Figure 6C) consistent with the effect observed in trypan blue exclusion assays. In cells treated with compound 22m, a strong dose-dependent effect on cell cycle distribution was observed. The proportion of cells in the G2/M-phase significantly increased (p<0.05 at 20 μg/mL, and p<0.001 at 30 and 40 μg/mL) and the number of cells in G0/G1 significantly decreased at all doses (p<0.01 at 20 μg/mL, and p<0.001 at 30 and 40 μg/mL). A decrease in the number of cells in the S-phase was observed at 30 and 40 μg/mL (p<0.001). Finally, compound 22m induced apoptosis in MCF-10A as measured by the number of cells in the sub-G1 population which significantly increased at 20 and 30 μg/mL, 48 h post-treatment (Figure 6C). In MCF-10A cells

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Figure 6. Effect of sulfonate compounds on non-cancerous MCF-10A cells. A: Cells were exposed to acetone (●) or OSCs (◻ 22 or ◧ 22m) for 48 h and then assayed for MTT activity. Values are expressed in terms of percent of untreated control cells set as 100 percent. Data represent means±SD of at least three independent experiments, each performed in eight replicates. All coefficients of variation were under 10%. B: Effect of various concentrations of compounds 22 or 22m on apoptosis induction. Cells were exposed to 0.4% acetone (◯) or OSCs (◻ 22 or ◧ 22m) at a concentration of 40 μg/mL for 48 h. Cells were then stained with Annexin V-FITC and PI. Values represent means±SD of two separate experiments. *p<0.05, **p<0.01, and ***p<0.001, comparing the indicated compound to untreated control cells as calculated by Chi-square test. C: Effect of various concentrations on cell cycle progression. Cells were exposed to 0.4% acetone (◯) or various concentrations of compound 22 or 22m at different concentrations (◻ 20 μg/mL, ◭ 30 μg/mL, and ◧ 40 μg/mL). 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 calculated by Student’s t-test.
treated with compound 22m at 40 μg/mL, only Annexin V-FITC-positive/PI-positive MCF-10A cells were significantly increased when compared to cells treated with acetone 0.4% (p<0.001) (Figure 6B). However, the increase observed in MCF-10A was much lower than in MCF-7 cells. Compound 22 did not induce apoptosis in MCF-10A cells.

**Discussion**

In this study we investigated the efficacy of six novel synthetic organosulfur compounds for their growth inhibition against human breast cancer cells. Compounds were classified into two groups based on their antiproliferative activity on MCF-7 cells: Group A (compounds 19, 20, and 22J) with no or low MTT activity and Group B (compounds 22, 22m, and 22o) with high MTT activity. We used the most two active compounds (22 and 22m) to investigate the effects of these compounds on cell cycle and apoptosis. We found that both compounds were acting through cell cycle arrest and induction of apoptosis. Growth inhibition observed with the OSCs used in this study was induced by cell cycle arrest in the G2/M-phase, followed by apoptosis. Similar effects of compound 22 and other synthetic OSCs on apoptosis induction and G2/M cell cycle arrest have also been reported in human leukemic cells (1). In this study, compound 22 was more selective than 22m based on their IC50 value for the non-cancerous but transformed MCF-10A cell line. Antiproliferative activity of compound 22 has also been found selective in another study (17) when its activity on cell growth was compared in human skin cancer C32 cells and in Chinese Hamster Ovary (CHO) cells.

The three most active compounds against breast cancer cells in this study had a common structure of p-methoxyphenyl p-toluenesulfonate with the methoxy substituent shifted from position 4 (compound 22) to 2 (compound 22o) or to 3 (compound 22m). 2-methoxyphenyl p-toluenesulfonate (22o) showed the highest IC50 value on breast cancer MCF-7 cells. 3-methoxyphenyl p-toluenesulfonate (22m) showed the lowest IC50 value on breast cancer cells but was also very active on non-cancerous MCF-10A cells. Overall, 4-methoxyphenyl p-toluenesulfonate (22) is the most promising potential anticancer agent we have examined for breast cancer cell growth inhibition.

Several naturally-occurring OSCs have shown to arrest the cell cycle in the G2/M-phase and induce apoptosis in cancer cell lines as the synthetic OSCs evaluated in this study. The mechanism of action of these natural OSCs is not fully understood but cell cycle arrest has been associated with an effect on protein expression of cyclin B1 (22), CDK7 (22), CDK1 (23), p21<sup>WAF1/CIP1</sup> (24, 25), MAPKs (p38) (26), an increase in acetylated histones (24, 25), and a direct binding to tubulin causing disruption of the microtubule assembly (27). Furthermore, OSCs have induced apoptosis by modulation of the MAPKs (JNK, p38 and ERK1/2) (27, 28) and by the mitochondrial dependant or independent pathway (bcl-2, bcl-x<sub>L</sub>, Bax, BAD, caspase-3, -8 and -9, cytochrome c release and cleavage of PARP) (4, 8, 28-31). These currently known mechanisms of action of other OSCs may be a good indication of the mechanism of the sulfonate esters evaluated in this study.

The synthetic OSCs evaluated in this study showed similar or lower IC50 values in breast cancer cells than the naturally occurring OSCs such as water-soluble garlic derivative S-allylmercaptocysteine (SAMC) (2, 32) or diallyl disulfide (DADS) (4-6, 24) in breast cancer and other cancerous cell lines. In addition, 3-methoxyphenyl p-toluenesulfonate (22m) showed very similar effects on breast cancer cells to those of naturally occurring OSCs by first arresting cell growth in the G2/M-phase of the cell cycle prior to inducing apoptosis. 4-Methoxyphenyl p-toluenesulfonate (22) showed a unique effect on MCF-7 cells by arresting the cell cycle at a different phase depending on the dose. At low concentration, the cell cycle was arrested at the G0/G1-phase, whereas at higher concentration the cell cycle was arrested at the G2/M-phase. Such a double effect on the cell cycle is rarely observed with OSCs. However, in a previous study with purified allicin, growth inhibition of MCF-7 cells was accompanied by an accumulation of cells in the G0/G1- and G2/M-phases of the cell cycle as in this study (33). Allicin caused a transient depletion in the intracellular glutathione (GSH) level (33). It would then be interesting to evaluate the effect of compound 22 on the GSH level in breast cancer cells.

In conclusion, we have demonstrated that synthetic OSCs can exert antiproliferative activity against breast cancer cells as potently as naturally occurring OSCs. Synthetic OSCs in this study exerted their anticancer activity through inhibition of the cell cycle and induction of apoptosis. Since these compounds are simple and inexpensive to synthesize they might become lead compounds for new therapeutic agents against breast cancer and cancer in general.
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References


