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Substituted diaryl diselenides: Cytotoxic and apoptotic effect in human colon adenocarcinoma cells

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ABSTRACT

Aims: To investigate the effects and study the underlying cell death mechanisms of diaryl diselenides, including: diphenyl diselenide $(C_6H_5Se)_2$; 4-chlorodiphenyl diselenide $(4\text{-}ClC_6H_4Se)_2$; 3-(trifluoromethyl)-diphenyl diselenide $(3\text{-}CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide $(4\text{-}MeOC_6H_4Se)_2$, on the human colon adenocarcinoma cell line HT-29.

Main methods: The viability of HT-29 cells after exposure to the diaryl diselenides and its substituted structures was based on the MTT assay. To verify if cell death was mediated throughout apoptosis mechanisms, flow cytometry and real-time PCR (qPCR) analyses were conducted.

Key findings: The MTT assay and flow cytometry analyses showed that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ induced cytotoxicity through apoptosis mechanisms in HT-29 cells. qPCR revealed there was an up-regulation of pro-apoptotic (Bax, casapase-9, caspase-8, apoptosis-inducing factor (AIF) and Endonuclease G (EndoG)) and cell-cycle arrest genes (p53 and p21) and down-regulation of anti-apoptotic (Bcl-2 and survivin) and Myc genes.

Significance: These results demonstrate that $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)_2$ have the potential to induce apoptosis in HT-29 cells through the activation of caspase-dependent and independent pathways and through cell-cycle arrest.

Introduction

Colorectal cancer is one of the leading causes of cancer mortality (Limami et al., 2011), corresponding to 9.4% of all cases of cancer worldwide (Cantero-Muñoz et al., 2011). Fifty percent of all recently diagnosed patients ultimately develop metastatic disease. Regardless of the advances in developing new chemotherapy agents, no drug has been able to treat colorectal cancer metastasis with a non-relapsing cure rate. Currently the clinical challenge is to develop new drugs that will have a significant impact on cure rates, by reversing drug resistance, and with minimal toxicity (Miura et al., 2011).

Selenium is an essential trace element (Zeng and Combs, 2008) that has the ability to prevent cancer in several animal models and to enhance chemopreventive efficacy in human lung, colorectal, head and neck and prostate cancer (Suzuki et al., 2010). The chemopreventive role of selenium is well supported by epidemiological, preclinical, and clinical evidence (Clark et al., 1998). Furthermore, emerging evidence has indicated the potential of selenium compounds in cancer chemotherapy (Suzuki et al., 2010).

Diphenyl diselenide $(C_6H_5Se)_2$, an organic selenium compound, has raised great interest due to its antioxidant, antidepressant-like, neuroprotective and antinociceptive properties (Nogueira and Rocha, 2011; Savegnago et al., 2007, 2008a, 2008b). Recently, Posser et al. (2011) showed, for the first time, that $(C_6H_5Se)_2$ was cytotoxic to human cancer cells (SH-SY5Y) in vitro, possibly mediated by the ERK1/2 pathway (Posser et al., 2011). However, to date no study has evaluated the cytotoxic effect of $(C_6H_5Se)_2$ in other human cancer cell types.

In addition, studies have demonstrated that the introduction of a substitute (e.g., chloro, fluor or methoxyl) in the aromatic ring of (C_6H_5Se) can alter its molecular properties (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). The introduction of chloro into the aryl group of diaryl diselenide conferred a weak cytotoxic effect on V79 cells (Chinese hamster lung fibroblast cells) compared to (C_6H_5Se) (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). Although this substitute could alter the biological effects of (C_6H_5Se), their potential as cytotoxic agents for cancer chemotherapy has not yet been explored.

Therefore, our objective was to investigate the effect and the underlying cell death mechanisms of $(C_6H_5Se)_2$ and its substituted structures,

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4-chlorodiphenyl diselenide $(4\text{-}ClC_6H_4Se)_2$, 3-(trifluoromethyl)-diphenyl diselenide $(3\text{-}CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide $(4\text{-}MeOC_6H_4Se)_2$ on the human colon adenocarcinoma cell line (HT-29). In addition, we also verified whether the introduction of an electron donating (-methoxyl) or an electron withdrawing group (-chloro and -trifluoromethyl) into the aryl group of diaryl diselenide altered its biological effect. To the best of our knowledge this is the first study that demonstrates the effect of $(C_6H_5Se)_2$ and its substituted structures on HT-29 cells.

Materials and methods

Chemicals

$(C_6H_5Se)_2$, $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ (Fig. 1) were prepared according to methods in the literature. Analysis of ^1H and ^{13}C NMR spectra showed that the analytical and spectroscopic data was in full agreement with its assigned structure. The chemical purity of these compounds was determined by gas chromatography/mass spectrometry.

Cell culture

The HT-29 cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS), purchased from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA), respectively. Cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth.

Determination of cytotoxicity

The viability of the HT-29 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water insoluble formazan (Ali et al., 2010; Henn et al., 2011). Briefly, cells were seeded at a density of 2×10^4 cells per well in a volume of 100 μL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h before being used in the MTT assay. Cells were incubated with different concentrations of $(C_6H_5Se)_2$, $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ or $(4\text{-}MeOC_6H_4Se)_2$ (5–80 μM) for 24, 48 and 72 h. These compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the DMEM supplemented with 10% FBS to the desired concentrations. The final DMSO concentration in the culture medium never exceeded 0.8% and a control group exposed to an equivalent concentration of DMSO was evaluated. After incubation the media were removed and 180 μL of DMEM and 20 μL MTT (5 mg MTT/mL solution) were added to each well. The plates were incubated for an additional 3 h and the medium was discarded. 200 μL of DMSO was added to each well, and the formazan was solubilized on a shaker for 5 min at 100×*g*. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 492 nm. The percentage inhibition of cell growth was determined as follows: inhibitory rate $=(1-Abs_{4treated\ cells}/Abs_{492control\ cells})\times100$ (Zheng et al., 2011). All observations were validated by at least three independent experiments and for each experiment the analyses were performed in triplicate.

Apoptotic assay

The Guava Nexin assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, 2.0×10^4 to 1.0×10^5 of the treated HT-29 cells (100 μL) were added to 100 μL of Guava Nexin reagent. Cells were incubated in the dark at room temperature for 20 min and samples (2000 cells per well) were acquired on the flow cytometry Guava EasyCyte System. In this assay, an annexin V-negative and 7-AAD-positive result indicated nuclear debris, an annexin V-positive and 7-AAD-positive result indicated late apoptotic cells, while an annexin V-negative and 7-AAD-negative result indicated live healthy cells and annexin V-positive and 7-AAD-negative result indicated the presence of early apoptotic cells.

Gene expression evaluation by real-time PCR

The HT-29 cells were seeded in a 6-well flat bottom plate at a density of 2×10^5 per well and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Cells were then exposed to 20, 40 and 80 μM of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ or $(4\text{-}MeOC_6H_4Se)_2$ for 48 h. After this period the cells were washed with phosphate-buffered saline (PBS; Gibco) and the RNA was extracted from the cells. Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) were carried out as previously described (Campos et al., 2010). Briefly, RNA samples were isolated using TRIzol Reagent (Invitrogen) and samples were DNase-treated with a DNA-free kit (Ambion, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK)

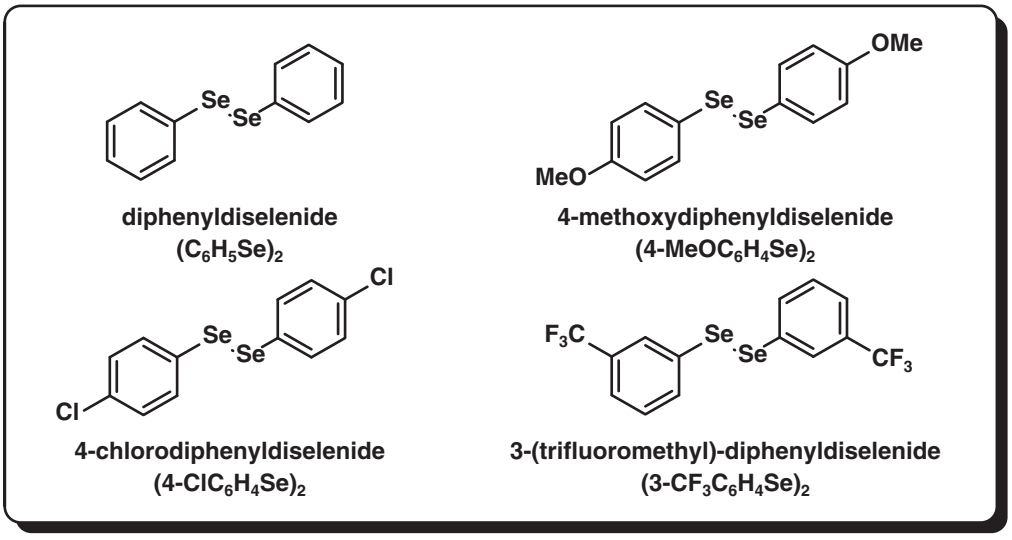

Fig. 1. Chemical structure of diaryl diselenides.

according to the manufacturer's protocol. The qPCR reactions were run on a Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK) using the primers described in Table 1.

Data analysis

Data sets from the MTT assay and qPCR were analysed using a two-way ANOVA followed by a Tukey test for multiple comparisons. Two factors were considered: the compound used (four levels) and the concentration of the compound (three levels). Significance was considered at $p<0.05$ in all analyses. The data are expressed as the means ± SEM.

Results

Determination of cytotoxicity

Both the $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$ compounds had a significant cytotoxic effect on the HT-29 cells at 80 μM and this effect improved significantly with exposure time (Fig. 2). Both the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds achieved significant cytotoxicity at a concentration of 20 μM. After 48 h exposure to 20 μM $(3\text{-}CF_3C_6H_4Se)$, cytotoxicity was 24% ($p<0.05$) and this increased significantly to 96% at 80 μM (Fig. 2). The cytotoxicity of the $(4\text{-}MeOC_6H_4Se)$ compound at 20 μM, after 24 h exposure, was 44% and further increases in the concentration of the compound resulted in a significant reduction in the viability of the HT-29 cells (62 and 75% cytotoxicity, Fig. 2). The exposure time had no significant effect on the cytotoxicity of the $(3\text{-}CF_3C_6H_4Se)$ compound. Only the $(4\text{-}MeOC_6H_4Se)$ compound showed a significant improvement with exposure time, for example, at 20 μM and after 24 and 48 h exposure, cytotoxicity increased from 44 to 65%, respectively, although there was no further improvement at 72 h (Fig. 2). The presence of 0.8% DMSO in the culture medium had no effect on cell viability, as compared to the control cells without DMSO.

Apoptosis analysis

The annexin-PE staining assay was performed to further characterize the observation that the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds could induce apoptosis in HT-29 cells after exposure for 48 h. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. The results indicated that (C_6H_5Se) induced apoptosis at a concentration of 80 μM (22.5%, Fig. 3B). The lower concentrations (20 and 40 μM) of (C_6H_5Se) were not effective in causing cell death through apoptosis, inducing similar levels of apoptosis (5.2 and 6.1%, respectively) seen in the control groups (3.0 and 6.1%, respectively). The $(3\text{-}CF_3C_6H_4Se)$ compound induced a higher percentage of apoptosis at the 40 and 80 μM concentrations (22.3 and 84.7%, respectively) compared to the controls and the (C_6H_5Se) compound. At the 20 μM concentration the percentage of apoptotic cells was 7.8%, similar to that observed in the control groups. The $(4\text{-}MeOC_6H_4Se)$ compound was able to induce significant apoptosis in the HT-29 cells at 20 μM (38.6%), this increased to 58.9% upon exposure to a concentration of 40 μM, although a further increase in concentration to 80 μM did not increase apoptosis (54.7%). Apoptosis induction from exposure of the HT-29 cells to 0.8% DMSO had no effect.

Gene expression

In order to evaluate the likely apoptosis pathways activated by $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in HT-29 cells (48 h exposure), anti-apoptotic and pro-apoptotic gene expressions were investigated. Bax mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) when compared to the control groups (Fig. 4A). However, (C_6H_5Se) had no effect on Bax mRNA levels when compared to the control groups ($p>0.05$). Bcl-2 mRNA levels decreased significantly ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) when compared to control groups. HT-29 cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (40 μM), $(4\text{-}MeOC_6H_4Se)$ (20 μM) and (C_6H_5Se) (40 and 80 μM) decreased Bcl-2 mRNA levels when compared to control groups ($p<0.05$) (Fig. 4B). Caspase 9 was up-regulated ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM), $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) (Fig. 4C). Exposure to $(3\text{-}CF_3C_6H_4Se)$ (20 and 40 μM), $(4\text{-}MeOC_6H_4Se)$ (20 μM) and (C_6H_5Se) (20, 40 and 80 μM) had no effect on caspase 9 gene expression ($p>0.05$). However, caspase 8 mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) when compared to the control groups. (C_6H_5Se), $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ (20 μM) did not affect caspase 8 gene expression ($p>0.05$) (Fig. 4D). Survivin expression was significantly down-regulated ($p<0.05$) in HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)$ (40 and 80 μM), $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and (C_6H_5Se) (80 μM) when compared to the control group (Fig. 4E). The $(3\text{-}CF_3C_6H_4Se)$ (20 μM) and (C_6H_5Se) (20 and 40 μM) compounds had no effect on survivin expression ($p>0.05$).

The mRNA levels for AIF and EndoG were also evaluated. AIF expression was significantly up-regulated ($p<0.05$) upon exposure to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) when compared to the control group (Fig. 4F). However, (C_6H_5Se) and $3\text{-}CF_3C_6H_4Se)$ (20 and 40 μM) had no effect on AIF mRNA levels when compared to control groups ($p>0.05$). EndoG mRNA expression was up-regulated ($p<0.05$) when the HT-29 cells were treated with $(C_6H_5Se)_2$ (20, 40 and 80 μM), $(3\text{-}CF_3C_6H_4Se)$ (20, 40 and 80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) compared to the control group (Fig. 4G). HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) had altered levels of cell cycle-related gene expression, p53 expression was significantly up-regulated ($p<0.05$), in comparison to the control groups. (C_6H_5Se), at all concentrations tested, had no effect on p53 mRNA levels (Fig. 5A). p21gene expression showed the same expression pattern as p53, where $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) caused significant up-regulation ($p<0.05$) and (C_6H_5Se) had no effect (Fig. 5B). MYC gene expression was significantly reduced ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM). (C_6H_5Se) had no effect on MYC gene expression (Fig. 5C). Gene

Table 1
Primer sequences used in this study.

Primers	Sequence 5′→3′	Reference
p53 for	AGCGAGCACTGCCCAACA	Gochhait et al. (2009)
p53 rev	CACGCCCACGGATCTGAA	
Bcl-2 for	GTGTGGAGAGCGTCAACC	Chen et al. (2010)
Bcl-2 rev	CTTCAGAGACAGCCAGGAG	
Bax for	ATGCGTCCACCAAGAAGC	Chen et al. (2010)
Bax rev	ACGGCGGCAATCATCCTC	
Casp9 for	CCAGAGATTCGCAAACCAGAGG	Huang et al. (2007)
Casp9 rev	GAGCACCGACATCACCAAATCC	
Survivin for	CTGTGGGCCCCTTAGCAAT	Wang et al. (2008)
Survivin rev	TAAGCCCGGGAATCAAAACA	
p21 for	CCTAATCCGCCCACAGGAA	Wang et al. (2008)
p21 rev	ACCTCCGGGAGAGAGGAAAA	
MYC for	TCAGCAACAACCGAAAATGC	Wang et al. (2008)
MYC rev	TTCCGTAGCTGTTCAAGTTTGTG	
GAPDH for	GGATTTGGTCGTATTGGG	Hu et al. (2010)
GAPDH rev	TCGCTCCTGGAAGATGG	
Casp8 for	GGATGGCCACTGTGAATAACTG	Lin et al. (2011)
Casp8 rev	TCGAGGACATCGCTCTCTCA	
AIF for	GGGAGGACTACGGCAAAGGT	Lu et al. (2010)
AIF rev	CTTCCTTGCTATTGGCATTCG	
EndG for	GTACCAGGTCATCGGCAAGAA	Lin et al. (2008)
EndG rev	CGTAGGTGCGGAGCTCAATT	

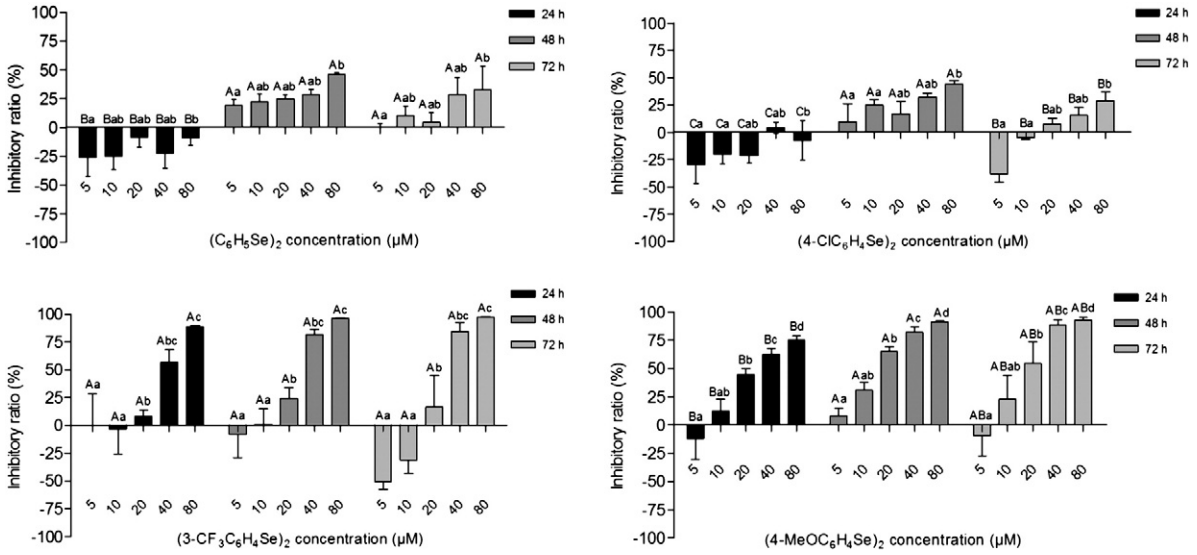

Fig. 2. Effect of the different concentration of substituted diaryl diselenides, $(C_6H_5Se)_2$ $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ following exposure for 24, 48 and 72 h on the inhibition of HT-29 cells. Data are expressed as the means ± SEM. Uppercase letters indicate significant differences between treatment times and lowercase letters indicate significant differences in the concentrations used. A p-value < 0.05 was considered significant (Tukey test).

expression upon exposure to 0.8% DMSO was similar to the control group in all experiments.

Discussion

Previous studies have confirmed that organoselenium compounds, such as (C_6H_5Se) and its substituted structures, exhibit a remarkable spectrum of pharmacological properties (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). Indeed, (C_6H_5Se) has exhibited antioxidant, antidepressant-like, neuroprotective and antinociceptive properties and recently it was demonstrated that (C_6H_5Se) had a cytotoxic effect, mediated by the ERK1/2 pathway, on SH-SY5Y cancer cells (Posser et al., 2011). Posser et al. (2011) reported that 30 μM (C_6H_5Se) significantly decreased cell viability in 50% of cells and, at a concentration of 10 μM, induced changes in cell morphology (Posser et al., 2011). To the best of our knowledge no study has evaluated the effect of $(C_6H_5Se)_2$ and the substituted diaryl diselenides $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ as cytotoxic and apoptotic agents against cancer cells in vitro or in vivo.

In the present study, (C_6H_5Se) and one of its substituted structures, $(4\text{-}ClC_6H_4Se)_2$, only presented significant cytotoxic effects against the HT-209 cells at a concentration of 80 μM. A similar study that used a neuroblastoma cell line reported cytotoxic effects at lower concentrations (10–30 μM (C_6H_5Se)). However, this discrepancy may be related to differences between the SH-SY5Y and HT-29 tumor cell lines, as they exhibit different gene profiles when exposed to potent toxic substances (Thirunavukkarasusx et al., 2011). These results suggest that (C_6H_5Se) has a selective action and therefore offers an opportunity to investigate its use as a therapeutic agent. This selectivity has been observed with other selenium compounds, where cancer cells, including lung (A549) and head and neck (HSC-3), were substantially more sensitive to selenite and prone to induction of apoptosis than the breast cancer cell line MCF-7 (Suzuki et al., 2010). The $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds induced cytotoxicity and alterations in cell morphology in HT-29 cells in a dose-dependent manner: 20 μM (24.4 vs. 65.2%), 40 μM (81.8 vs. 81.7%) and 80 μM (91.2 vs. 96.1%), respectively. A recent study evaluated the ability of different selenium compounds (selenate, selenite, MeSeA, MeSeCys and SeMet) to induce cell death in HT-29 cells (Lunøe et al., 2011). The most effective compound was selenite, an inorganic selenium, the percentage of cell death was 21 (10 μM) and 39% (100 μM), followed by two organic selenium compounds, MeSeA (methylseleninic acid) 2 (10 μM) and 14% (100 μM), and MeSeCys (Se-methylselenocysteine) 3% (100 μM). This suggests that the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds evaluated in the current study are potentially cytotoxic against human colon adenocarcinoma cells, albeit in vitro. The substitution of a hydrogen atom on the aryl group of diaryl diselenide by an electron withdrawing group (-trifluoromethyl) or an electron donating group (-methoxyl) altered the cytotoxicity when compared to diphenyl diselenide. However, these effects were independent of the nature of the aromatic ring in the diaryl diselenide. Both molecules demonstrated greater cytotoxicity compared to (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$. It has been reported that selenium can inhibit cell proliferation, inducing injury via generation of reactive oxygen species (ROS) (Rudolf et al., 2008). ROS levels can activate the JNK pathway and caspases-3 and 9 via cytochrome c, with down-regulation of Bcl-2 and up-regulation of Bax (Chen et al., 2012). Also, it has been demonstrated that (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$ present higher thiol peroxidase activity and an improved antioxidant potential than $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in vivo (Meotti et al., 2004). Since, selenium-induced apoptosis in cancer cells can be suppressed by antioxidants (Wu et al., 2010), it is possible that the higher antioxidant potential of (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$ could trigger a less effective cytotoxic effect on HT-29 cells than $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$.

Since apoptosis is thought to be the mediator of selenium anticancer activity, we verified, by an Annexin-PE staining assay, that the cytotoxicity effect caused by the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds was mediated by apoptosis. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. One pathway by which caspases can be activated involves the extrinsic death receptor pathway, where death ligands bind to death receptors, activating caspase 8 and subsequently initiating apoptosis by cleaving other downstream or executioner caspases (Wong, 2011). When $(C_6H_5Se)_2$ and its substituted structures were tested for their ability to stimulate expression of caspase-8, $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) was the only compound that induced high levels of caspase-8 mRNA. Since the upstream caspase for the extrinsic death receptor pathway is caspase-8, this suggests that $(4\text{-}MeOC_6H_4Se)$ could be activating a death receptor and therefore contributing to apoptosis in the HT-29 cells. In addition, $(4\text{-}MeOC_6H_4Se)$ could present a different biological effect from the other substituted structures due to its electron donating group (-methoxyl).

A second pathway involved in caspase activation is the mitochondrial release of cytochrome c (Wong, 2011). The cytoplasmatic release of cytochrome c activates capase-3 via the formation of a complex (apoptosome)

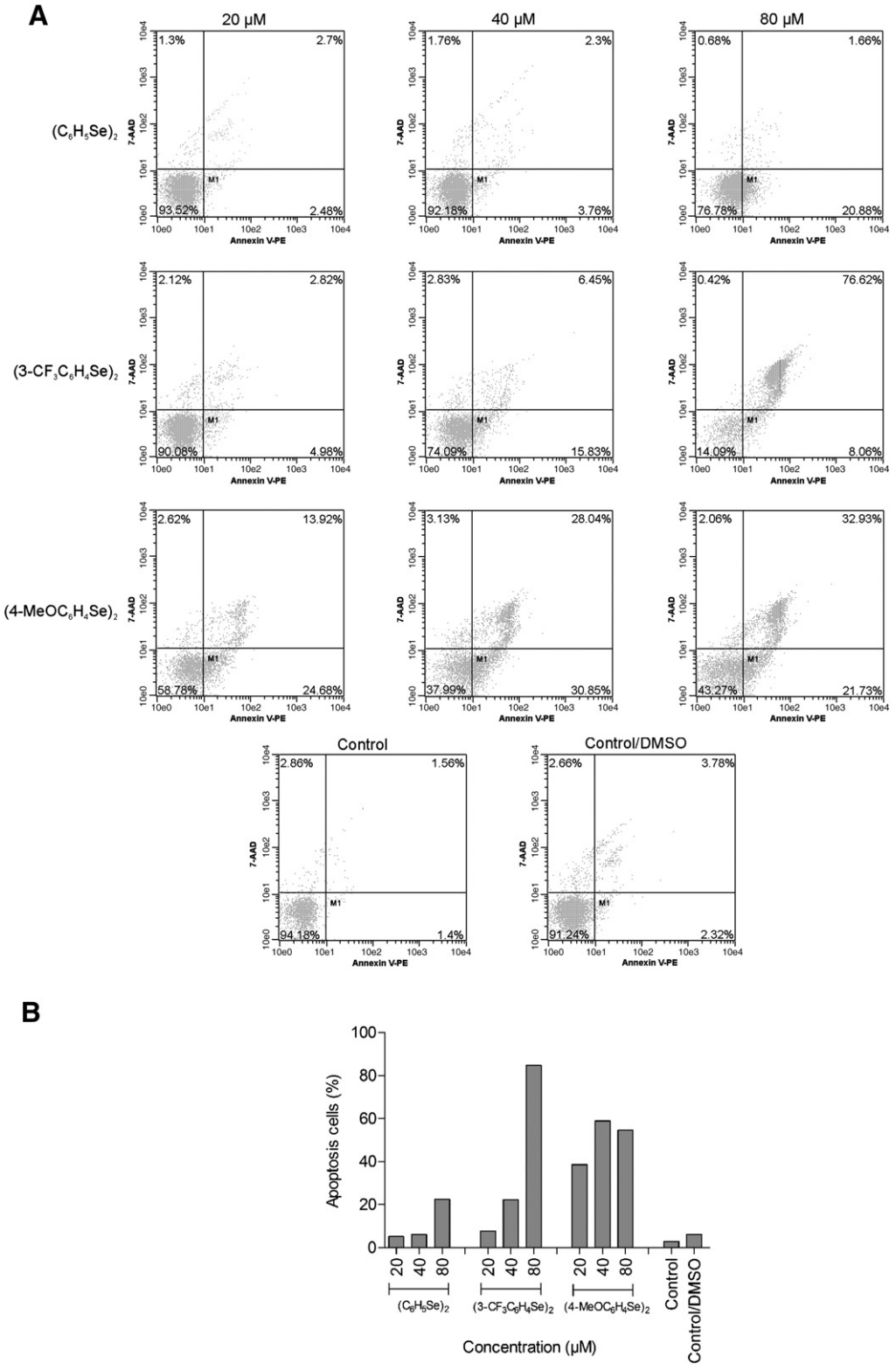

Fig. 3. Annexin V-PE analysis of HT-29 cells treated with 20, 40 and 80 μM of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, and control groups after exposure for 48 h. Panel A. Flow cytometry graphs. Panel B. Percentage of apoptotic cells.

which is made of cytochrome c, APAF-1 and caspase-9 (Jackson and Combs, 2008). Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) are closely involved in this process, an increase in Bcl-2 expression prevents cytochrome c release from the mitochondria, inhibiting the activation of caspase-9 and caspase-3, and preventing apoptosis (Santandreu et al., 2011). In the present study, Bcl-2 expression was down-regulated by $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM), whereas Bax expression was up-regulated. These findings suggest that Bax and Bcl-2 were involved in mediating the apoptotic effects associated with the cytotoxicity of $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in HT-29 cells. In addition, caspase-9 mRNA levels were significantly increased by treatment with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM)

showing that caspase-9 was involved in mediating the apoptotic effects associated with these compounds. Apoptosis induced by selenium has been reported to involve the activation of caspases. It was shown that MeSeA induced apoptosis in human prostate cancer (Yamaguchi et al., 2005) and leukemia cells (Kim et al., 2001) by the activation of multiple caspases (caspases-3, -7, -8 and -9), mitochondrial release of cytochrome

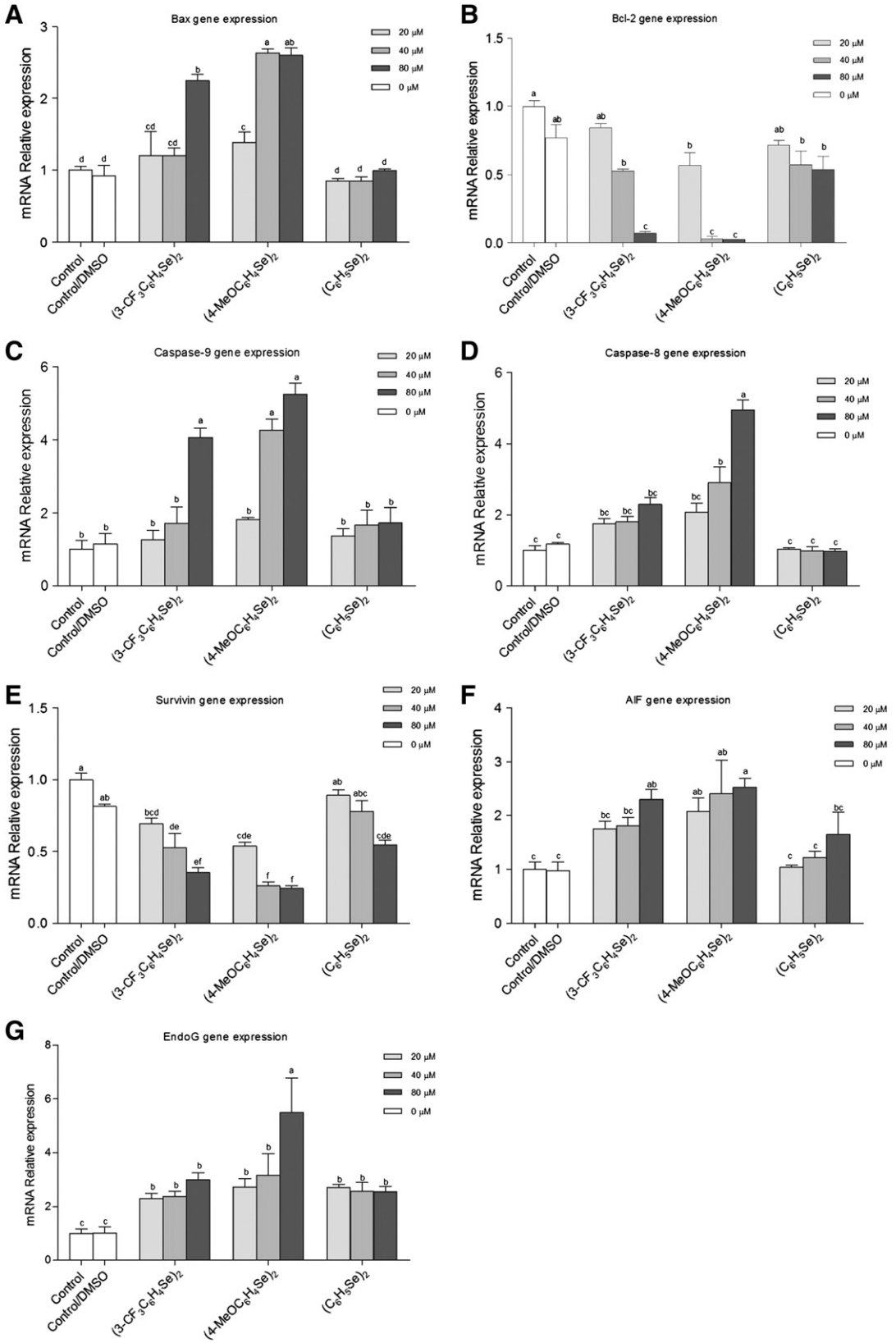

Fig. 4. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in apoptotic-related gene expression. A—Bax, B—Bcl-2, C—caspase 9, D—caspase 8, E—survivin, F—AIF and G—EndoG. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

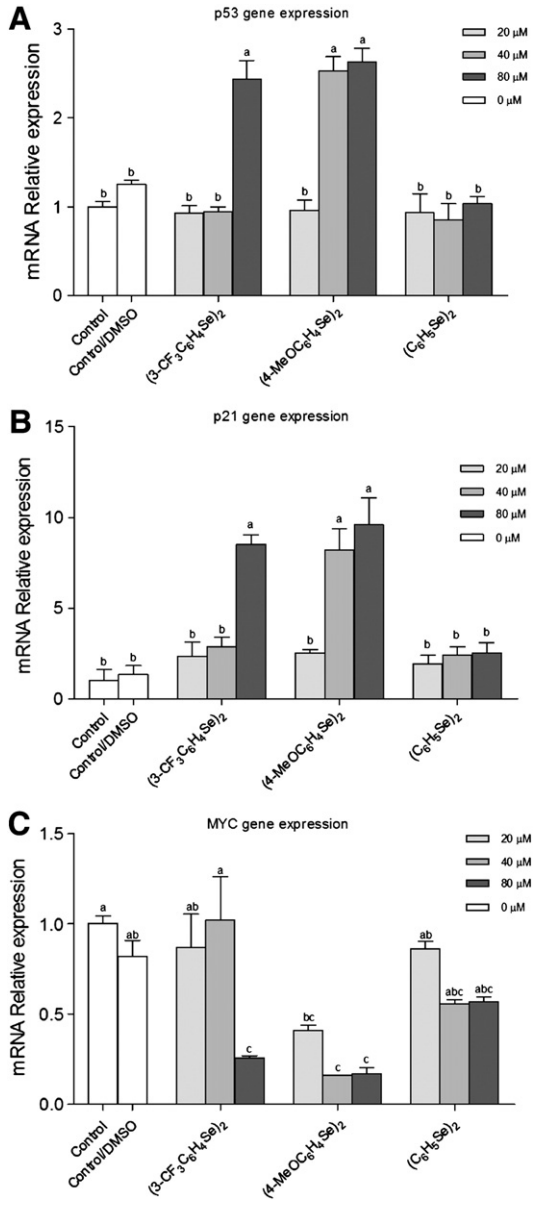

Fig. 5. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in cell-cycle arrest-related gene expression. A—p53, B—p21 and C—Myc. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

c and DNA fragmentation. Other organic and inorganic selenium compounds have been shown to induce caspase-mediated apoptosis, including MeSeCys, selenite (Suzuki et al., 2010), sodium selenite (Chen et al., 2012), and selenium dioxide (SeO_2) (Rikiishi, 2007).

Additional apoptotic factors that can be released from the mitochondrial intermembrane space into the cytosol are AIF and EndoG, which translocate to the nucleus, triggering chromatin condensation and DNA degradation in a caspase-independent manner (Vařecha et al., 2012; Wong, 2011). In the current study AIF gene expression was up-regulated by $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and EndoG was up-regulated by exposure to the two substituted diaryl diselenides as well as to $(C_6H_5Se)_2$. These results suggest time that diaryl diselenide and its substituted structures could induce apoptosis not only through the activation of multiple caspases but also through a caspase-independent pathway.

Survivin has been implicated in the inhibition of apoptosis, cell proliferation, angiogenesis, and cellular stress response. In HT-29 cells, $(3\text{-}CF_3C_6H_4Se)$ (40 and 80 μM), $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and (C_6H_5Se) (80 μM) down-regulated the gene expression of survivin. Survivin expression was down-regulated in cell lines derived from prostate cancer cells, such as LNCaP, C4-2 (Chun et al., 2007), DU145 and PC-3 (Hu et al., 2008) treated with selenium. However, when the same selenium compound was tested with a metastatic cell line derived from PC-3 (PC-3M) and two other prostate cancer cell lines (C4-2B and 22Rv1), it had no effect on survivin expression, indicating that the apoptosis induced by selenium was not mediated by decreasing survivin expression (Liu et al., 2010). These results indicated that selenium could trigger different responses depending on the type of cell. Furthermore, p53 and p21 mRNA expression levels were increased while MYC gene expression was down-regulated upon exposure to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM). The expression of p53, p21 and MYC induced by (C_6H_5Se) did not differ from that of the control groups. Investigators have shown that cells deficient in p21 escaped G2/M phase cell cycle arrest when exposed to DNA damaging agents (Rosa et al., 2007b), and that p53 arrested the cell cycle by lowering cyclin B1 levels (Rosa et al., 2007a). In addition, reduction of MYC expression was associated with cell cycle arrest in SH-SY5Y cells (Posser et al., 2011). Our results suggest that $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ influenced the expression of p53, p21 and MYC and that they could be effective as antiproliferative agents by inducing G2/M cell cycle arrest. Selenite was shown to elevate the levels of phosphorylated p53 protein at Ser-15 and concomitantly increase the expression of p21. In addition, the pro-apoptotic Bax levels were elevated and when a p53-specific inhibitor was used Bax expression was reduced by 50%, suggesting that selenium compounds could mediate tumor cell death by the p53 pathway. However, other mechanisms may also contribute to the expression of Bax. In addition, it was observed that cytochrome c, capspases-9 and -8 did not participate in the execution of apoptosis in selenite-exposed cells (Rudolf et al., 2008). In the present study, the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds appeared to mediate apoptosis in a caspase-dependent manner, since the expression of caspase-9 was significantly higher in treated HT-29 cells. However, p53 phosphorylation could also contribute to elevated Bax expression leading to apoptosis.

Of note, the role of apoptosis in the current study was determined using real-time PCR and this is a potential limitation as it is known that mRNA does not necessarily reflect protein concentration, this will be part of future work on these compounds. Furthermore, it is important to clarify that the benefit of selenium compounds is related to its bioavailability in the intestine and its ability to enter the bloodstream where it can be distributed to various organs and tissues. Of note, the bioavailability of selenium is closely related to its chemical form (Thiry et al., 2012). In this study the most cytotoxic compound, $(4\text{-}MeOC_6H_4Se)$, exhibited a significant inhibitory effect (> 40%) on HT-29 cells at a concentration of 20 μM that increased to >75% at a concentration of 80 μM following exposure for 24 h. Furthermore, these concentrations are similar to those used in other studies that reported induction of apoptosis in cancer cells with similar doses (10–100 μM) of selenium compounds (Lunøe et al., 2011; Posser et al., 2011). Further work will need to be carried out to verify the cytotoxic effects of the compounds in animal models and to confirm their bioavailability at these concentrations.

Conclusion

In summary, for the first time the cytotoxic potential of $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ was demonstrated in human colon adenocarcinoma cells and the cytotoxic effect was likely mediated through the induction of apoptosis. In addition, several molecular targets of these compounds were investigated and the evidence suggests that apoptosis was stimulated by a caspase-dependant pathway as well as by a caspase-independent pathway and that cell-cycle arrest was mediated by the p53, p21 and MYC genes. However, mRNA levels do not necessarily reflect protein concentration and further work will be required to confirm these findings.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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