Evaluating the Cytotoxic Effects of Novel Quinone Compounds

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Abstract. *Background/Aim: Quinone-containing compounds can induce cell death in cancer cells and are, therefore, promising lead compounds for the development of novel anti-cancer drugs. Materials and Methods: In the present study, we evaluated the cytotoxic effects of fifteen novel synthetic quinone-containing compounds in cell cultures in an attempt to establish structure/activity relationships for these compounds. The compounds were clustered into four groups (1, 2, 3, 4) based on common structural features. In vitro cell cultures were treated for 24 h with the compounds, after which cell viability was assessed by flow cytometry. The APOPercentage™ assay, the Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay and the caspase-3 assay was used to investigate the activation of apoptosis in the cells. Results: Compounds from groups 2 and 4 were highly toxic to the cells. The compounds induced apoptosis in some human cancer cell cultures and exhibited low toxicity towards the non-cancerous cell line, KMST-6. The induction of apoptosis in CHO cells was associated with the activation of caspase-3 cleavage, DNA fragmentation and the reactive oxygen species (ROS) generation. Conclusion: The present study demonstrates that five of the quinone-containing compounds induced apoptosis in human cancer cells and are therefore promising lead compounds for the development of novel anticancer drugs.*

The quinone moiety is comprised of an unsaturated benzene ring to which two oxygen atoms are bonded as carbonyl groups. Quinone compounds are sub-classified based on their ring structure as 1,4-benzoquinone (cyclohexadienedione), 1,2-benzoquinone (ortho-quinone), 1,4-naphthoquinone or 9,10-anthraquinone. Naturally occurring quinones are present in bacteria, fungi, lichens, gymnosperms and angiosperms (1). In the animal kingdom, quinones occur in echinoderms (*e.g.* isoprenoid quinones in sea urchins) (2) and arthropods (*e.g.* anthraquinones in insects such as cochineal) (3). Asterriquinone, mitomycin C, doxorubicin and diospyrin, are examples of naturally-occurring quinones that were isolated from *Aspergillus terreus*, *Streptomyces caespitosus*, *Streptomyces peucetius*, and *Euclea natalensis*, respectively (4-13). Various biological activities, which include anti-fungal, anti-protozoal, anti-bacterial, and anti-cancer activity, have been demonstrated for quinone-containing compounds (14).

The cytotoxicity of quinone compounds is not fully understood, but two general mechanisms of cytotoxicity have been described in the literature. One of these mechanisms is mediated through quinone redox cycling and the other through the effects these compounds have on biomolecules (DNA, RNA, lipids and proteins) (15, 16). Quinones are easily reduced to semiquinones and hydroquinones. Semiquinones can be oxidised by molecular oxygen, leading to the production of superoxide anion radicals. This process is known as quinone redox cycling (17-19) which in turn leads to the production of reactive oxygen species (ROS), in particular hydrogen peroxide and hydroxyl radicals (20). The production of ROS leads to an oxidant-antioxidant imbalance or oxidative stress. ROS can interact with lipids, proteins, RNA, and DNA causing irreversible damage to these molecules. Oxidative stress can cause DNA strand breaks, DNA intra-strand breaks and DNA-protein cross-linking (21-23). It is well-known that these DNA lesions can activate apoptosis through p53, checkpoint kinase-1, and checkpoint kinase-2 (24). ROS can also damage mitochondrial membranes, causing the release of pro-apoptotic agents (cytochrome *c* and Apoptosis Inducing Factor) from the mitochondria, with consequent activation of apoptosis (25-30). These cellular nucleophiles can be thiols on cysteine residues of cellular proteins or glutathione (GSH). High intra-cellular concentrations of quinones may deplete the levels of GSH, leading to increased alkylation of SH-dependent proteins (31-34). It was shown that arylating quinone compounds activate the pancreatic endoplasmic reticulum (ER) kinase pathway resulting in ER-stress-induced cell death (35, 36). It is thus evident that quinone compounds can activate several intra-cellular signalling pathways that trigger apoptosis.

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Apoptosis is a genetically-controlled physiological process, which prevents the proliferation of damaged cells by activating cell death (37). Apoptosis is a form of cell death that is characterised by a set of biochemical and physiological changes involving the endoplasmic reticulum cytoplasm, mitochondria, nucleus and plasma membrane. Apoptotic cells eventually breaks-up into apoptotic bodies, which are removed by macrophages. Failure to remove damaged cells can lead to the development of cancer. Compounds that are able to induce apoptosis in cancer cells are therefore promising lead compounds for the development of novel anti-cancer drugs. Quinone-based anticancer drugs such as doxorubicin, daunorubicin and mitomycin C are used extensively in the treatment of cancer (38-40). However, there are limitations associated with the use of these drugs, which include toxicity to surrounding non-cancerous cells (*e.g.* chronic cardiotoxicity associated with doxorubicin), acquired drug resistance in cancer cells, and adverse side effects (41, 42). Consequently there is a continued search for new and novel anticancer drugs displaying reduced side-effects.

We have previously described the synthesis of quinonoid analogues (43, 44). Here we describe the cytotoxicity screening of fifteen novel quinone-containing compounds. We show that some of these compounds induce apoptosis in human cancer cells and are therefore promising lead compounds for the development of novel anti-cancer drugs.

Materials and Methods

Cell culture. CHO cells were cultured in Hams F-12 medium containing 1 mM L-glutamine, 5% (v/v) foetal calf serum and 0.2% (v/v) streptomycin-penicillin. HEpG2, KMST6, MCF7, and HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium with GlutaMAX-1, 10% (v/v) foetal calf serum, and 0.2% (v/v) streptomycin-penicillin. Jurkat T cells were cultured in Roswell Park Memorial Institute (RPMI) medium with GlutaMAX-1, 10% (v/v) foetal calf serum, and 0.2% (v/v) streptomycin-penicillin. HEpG2, KMST6, MCF7, HT-29, and Jurkat T cell lines used in this study were kindly provided by Prof Denver Hendricks (Department of Clinical and Laboratory Medicine, University of Cape Town - South Africa). CHO cells were kindly provided by Dr. Jasper Rees (Sir William Dunn School of Pathology, Oxford University – United Kingdom). All cell culture reagents were supplied by Invitrogen Ltd. (Carlsbad, California, USA). All cell lines were maintained at 37˚C in an atmosphere of 5% CO_2. Cells were plated in 6-well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a cell density of 2.5×10^5 cells *per* well or in 24 well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a cell density of 1×10^5 cells *per* well. After 24 h the medium was replaced with medium containing the test compounds. The cells were treated for the indicated times, after which they were harvested and the extent of apoptosis was assessed.

APOPercentage™ assay. Cells were plated in 24 well tissue culture plates at a cell density of 1×10^5 cells *per* well. After 24 h the medium was replaced with medium containing the compounds. As a positive control, the cells were treated with 20 μM doxorubicin. All treatments were performed in triplicate. The cells were treated for 24 h, after which they were harvested and the extent of apoptosis was assessed using the APOPercentage™ assay (Biocolor Ltd., Newtonabbey, Northern Ireland, United Kingdom) as described previously (45). Briefly, the cells were removed by trypsinization, washed with PBS, and stained with APOPercentage dye for 30 min at 37˚C. The cells were washed with PBS and analysed by flow cytometry at 670 nm on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, USA). A minimum of 10,000 cells *per* sample were acquired and analysed using CELLQuest PRO software (BD Biosciences Pharmingen, San Diego, USA).

Caspase-3 assay. The activation of caspase-3 was detected using a phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody specific for the cleaved caspase-3 (BD Biosciences Pharmingen San Diego, California, USA). CHO cells were plated in 6 well tissue culture plates and treated for 24 h with 5 μM of the compounds. As a positive control, the cells were treated for 24 h with 20 μM doxorubicin. All the treatments were performed in triplicate. The cells were removed by trypsinization, washed twice with cold PBS and re-suspended in Cytofix/Cytoperm buffer (BD Biosciences Pharmingen San Diego, California, USA). Following 20 min of incubation on ice, the cells were washed twice with Perm/Wash buffer (BD Biosciences Pharmingen San Diego, California, USA). and stained for 30 min at room temperature with a phycoerythrin conjugated monoclonal antibody specific for active caspase-3 (BD Biosciences Pharmingen San Diego, California, USA). Cell staining was measured by flow cytometry at 670 nm on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen San Diego, California, USA). A minimum of 10,000 cells *per* sample were acquired and analysed using CELLQuest PRO software (BD Biosciences Pharmingen San Diego, California, USA).

Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay. To analyse the occurrence of DNA fragmentation, the TUNEL assay (BD Biosciences Pharmingen San Diego, California, USA) was used. CHO cells were plated in 6 well tissue culture plates at a cell density of 2.5×10^5 cells *per* well. The cells were treated for 24 h with the compounds. As a positive control, the cells were treated for 24 h with 20 μM doxorubicin. The cells were removed by trypsinization, washed twice with PBS and fixed for 1 h in 1% paraformaldehyde. The cells were washed twice with PBS and permeabilized for 48 h in 70% ethanol at –20˚C. Subsequently the cells were labelled with FITC-dUTP and propidium iodide (PI) as described in the manufacturer's manual (BD Biosciences Pharmingen San Diego, California, USA). Cell staining was measured by flow cytometry at 530 nm and 585 nm using a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen San Diego, California, USA). A minimum of 10,000 cells *per* sample were acquired and analyzed using CELLQuest PRO software (BD Biosciences Pharmingen San Diego, California, USA). Dual parameter analysis (side scatter on the X-axis and FITC-dUTP on the Y-axis) was used to analyze the cells.

Reactive oxygen species (ROS) assay. CHO cells were plated in 24-well tissue culture plate at a density of 1×10^5 cells *per* well. After 24 h, the medium was replaced with medium containing 5 μM of

quinone compounds or 20 μM of doxorubicin (positive control) and incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 24 h. All the treatments were performed for 24 h in triplicate. Following treatment, the cells were removed by trypsinization and gently washed with PBS. The cells were stained with 2',7'-dichlorofluorescin diacetate (DCFH-DA), and incubated for 30 min at room temperature in the dark. After the incubation, the cells were analyzed at 530 nm on Becton Dickinson FACScan instrument (BD Biosciences Pharmingen San Diego, California, USA). A minimum of 10,000 cells *per* sample was acquired and analyzed using CELLQuest PRO software (BD Biosciences Pharmingen San Diego, California, USA).

Results

Chemical structures of quinone compounds. The synthesis and characterisation of the fifteen novel quinone-containing compounds (named SK1 - SK15) were previously described (43, 46). We grouped these compounds into four clusters (Groups -1 - 4) based on structural features and oxidation levels (Figure 1). Group 1 contains SK1: 2-(2',5'-dimethoxyphenyl)naphthalene-1,4-dione), SK2: 2-(3',6'-dioxoclyclohexa-1',4'-dienyl)-7-methylnaphthalene-1,4-dione), SK3: 2-(3',6'-dioxoclyclohexa-1',4'-dienyl)-5-hydroxy-7-methylnaphthalene-1,4-dione), SK6: 2-(3',6'-dioxoclyclohexa-1',4'-dienyl)-5-methoxy-7-methylnaphthalene-1,4-dione) and SK13: 2-bromo-6-methylnaphthalene-1,4-dione. Group 2 contains SK7: 5-acetoxy-2-(2'-thianthrene) naphthalene-1,4-dione), SK8: 5-methoxy-7-methyl-2-(2'-thianthrene)naphthalene-1,4-dione) and SK11: 2-(2'-thianthrene-5',10'-doxo)naphthalene-1,4-dione, For group 3 we have SK4: 7,7'-dimethyl-2,2'-binaphthyl-1,1',4,4'-tetraone, SK5: 1',4'-dimethoxy-7,7'-dimethyl-2,2'-binaphthyl-1,4-dione, SK9: 7-methoxy2,2'-binaphthyl-1,4-dione, SK10: 6'-methoxy-2,2'-binaphthyl-1,4-dione and SK12: 5,6'-dimethoxy-7-methyl-2,2'-binaphthyl-1,4-dione. Group 4 contains SK14: 6'-methoxy-1,4-dioxo-1,4-dihydro-2,2'-binaphthyl-8-yl acetate and SK15: 5'-methoxy-1,4-dioxo-1,4-dihydro-2,2'-binaphthyl-8-yl acetate.

Assessing the pro-apoptotic activity using the APOPercentage™ assay. The cytotoxic effects of the quinone compounds were assessed using the APOPercentage™ assay. CHO cells were treated for 24 h with 5 μM of the compounds, stained with APOPercentage dye and analyzed by flow cytometry. Seven (SK1, SK7, SK8, SK11, SK12, SK14 and SK15) of the fifteen quinone-compounds screened in the present study induced significant levels (between 50% and 90%) of apoptosis in CHO cells (Figure 2). The quinone compounds induced apoptosis in a dose- and time-dependent manner (data not shown). Since the chemical stability of SK1 was not very good, this compound was subsequently removed from the study. The bioactivities of the other six compounds (SK7, SK8, SK11, SK12, SK1 and SK15) were further investigated on a panel of human cell lines, which included four human cancer cell lines (HEpG2, HT-29, Jurkat T and MCF7) and one non-cancerous human cell line (KMST6). The compounds demonstrated selectivity towards certain cancer cell lines. Two of the human cancer cell lines (Jurkat T and MCF7) were highly sensitive to the effects of the quinone compounds, with Jurkat T cells being more susceptible. Figure 3 shows a cell death rate of between 80% and 95% for Jurkat T cells treated with five (SK7, SK8, SK11, SK14 and SK15) of the six compounds. A similar cell death rate was observed for MCF7 cells, however, these cells appeared to be more resistant to SK12. HEpG2, HT-29 and the non-cancerous KMST6 cells were less susceptible to the effects of the compounds. The cell death rate observed for HEpG2, HT-29 and KMST6 cells treated with four of the compounds (SK7, SK8, SK11 and SK12) were between 10 and 40%. In general, the toxicity of SK12 was very low in all five cell lines with the rate of cell death in the least sensitive cell line (KMST6) being 10% and the most sensitive cell line (Jurkat T) was 40%. SK14 and SK15 displayed the highest toxicity, with SK15 inducing apoptosis in about 60% of the more resistant cell lines (HEpG2, HT-29 and KMST6), compared to the other compounds, which induced apoptosis in about 20% to 40% of the cells.

Assessing caspase-3 cleavage and DNA fragmentation. Two additional assays (the caspase-3 assay and DNA fragmentation assay) were used to further characterize the induction of apoptosis in CHO cells. Cells were treated for 24 h with 5 μM of the most active compounds (SK7, SK8, SK11, SK12, SK14 and SK15). The activation of caspase-3 was assessed using a phycoerythrin-conjugated anti-active caspase-3 monoclonal antibody and flow cytometry. Figure 4 shows that four of these compounds (SK7, SK11, SK14 and SK15) induce caspase-3 cleavage in 60% to 80% of CHO cells, whereas the other two compounds (SK8 and SK12) could only induce caspase-3 cleavage in 10 to 30% of the cells. The TUNEL assay (Figure 5) shows that three of the compounds (SK7, SK8 and SK11) induced DNA fragmentation in approximately 25% of CHO cells. In comparison SK15, SK14 and SK12 induced low levels of DNA fragmentation in these cells.

Evaluating production of reactive oxygen species (ROS). The reactive oxygen species assay was used to further evaluate the production of ROS in cells treated with the active quinone compounds. CHO cells were treated with 5 μM of the quinone compounds (SK7, SK8, SK11, SK12, SK14, SK15 and SK16) for 24 h. A DCFH-DA probe was used to evaluate the production of ROS. Figure 6 shows that five of the compounds (SK7, SK8, SK11, SK14 and SK15) induced the production of ROS in 62% to 83% of CHO cells, while SK12 produced little or no ROS in CHO cells.

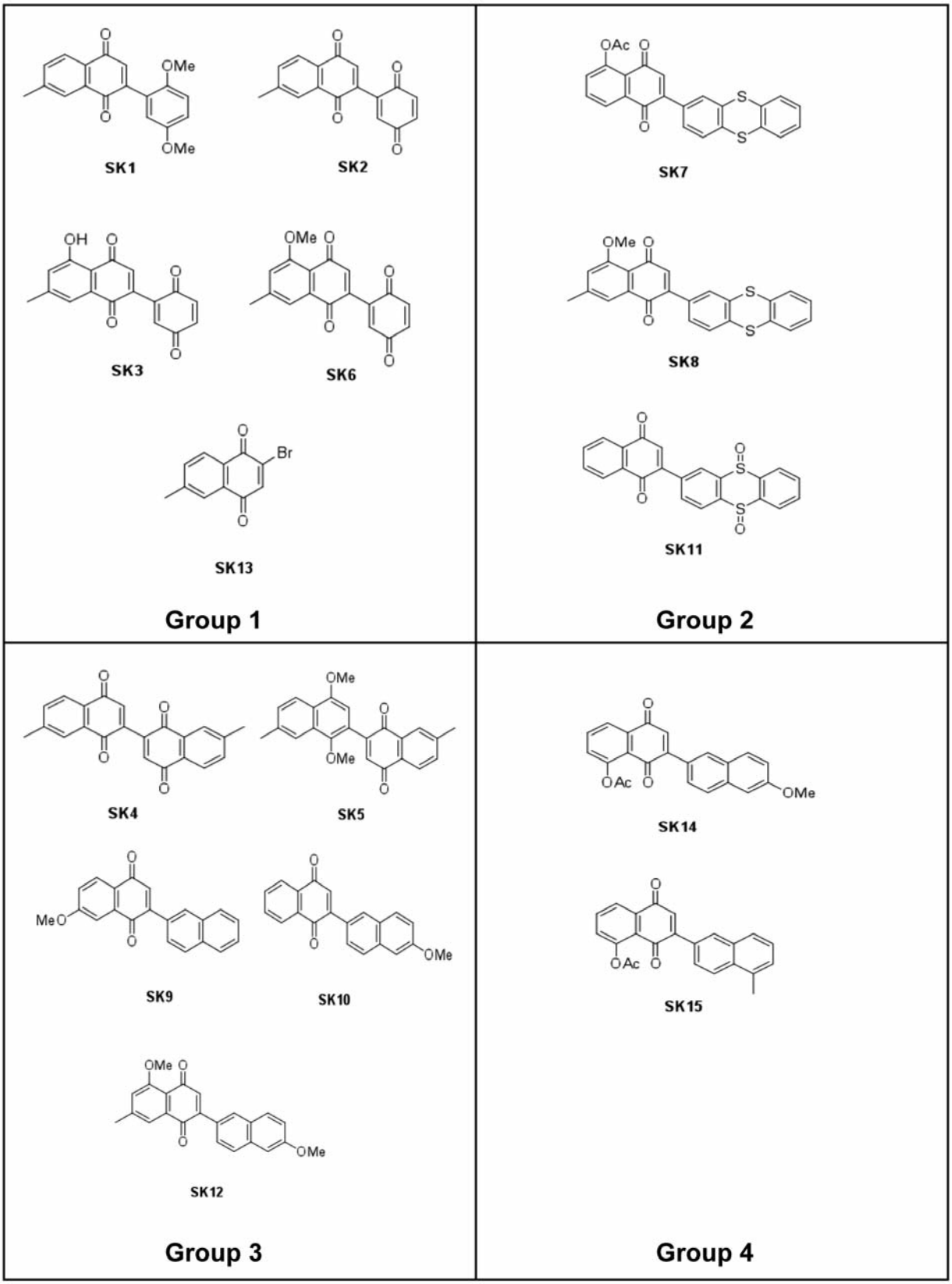

Figure 1. *Chemical structures of the synthetic quinone-containing compounds. The compounds were clustered into 4 groups based on their structure.*

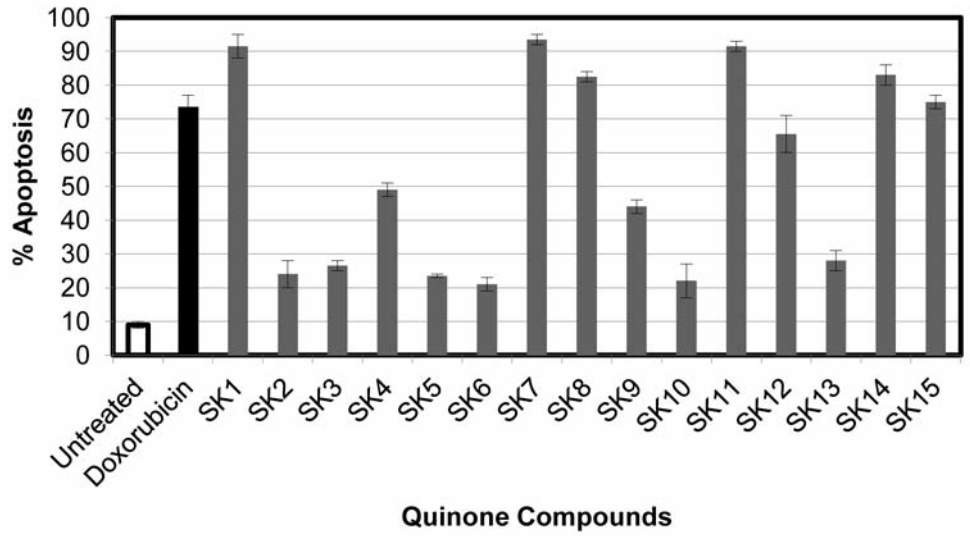

Figure 2. *The pro-apoptotic activity of synthetic quinone compounds in CHO cells. CHO cells were treated with 5 μM of the different quinone compounds. After 24 h, the cells were stained with the APOPercentage dye and analyzed by flow cytometry. The graphs indicate the percentage of cells staining positive for the presence of the dye.*

Discussion

In the present study we tested the cytotoxicity of fifteen novel quinone-containing compounds. A preliminary screen of the fifteen compounds using the APOPercentage™ assay showed that seven of these compounds (SK1, SK7, SK8, SK11, SK12, SK14 and SK15) induce significant levels of apoptosis (more than 50%) in CHO cells. The rodent cell line, CHO, is one of the best-characterized mammalian cell lines. It is often used for the production of recombinant therapeutic proteins, but has also been used in toxicology studies to evaluate the induction of apoptosis (45-47).

The APOPercentage™ assay detects apoptosis at the stage of phosphatidylserine externalization and is a specific assay for the quantification of apoptosis. Since the bioactivity of the other eight compounds (SK2, SK3, SK4, SK5, SK6, SK9, SK10 and SK13) was low, these compounds were not further studied. Even though the bioactivity of SK1 was high, this compound was unstable in solution and was consequently excluded from further investigations.

Three additional bioassays, which detect three different markers of cytotoxicity (caspase-3 cleavage, DNA fragmentation and ROS production), were used to investigate the bioactivity of the most active quinone-containing compounds (SK7, SK8, SK11, SK12, SK14, and SK15). The cleavage and activation of caspase-3 as well as DNA fragmentation are known markers of apoptosis (47). The compounds in Groups 2 and 4 were generally more active than the compounds in Groups 1 and 3. All the compounds in Groups 2 and 4 (SK7, SK8, SK11, SK14 and SK15) induced significant levels of apoptosis (as measured by the APOPercentage™ assay) and ROS production (as measured by DCFH-DA) in CHO cells. Except for SK8, all these compounds also induced caspase-3 cleavage in CHO cells. This may suggest that this compound induces apoptosis in a caspase-3-independent manner.

Interestingly, only compounds from Group 2 (SK7, SK8 and SK11) induced DNA fragmentation in CHO cells. SK14 and SK15 (Group 4 compounds) and SK12 were not able to induce DNA fragmentation in these cells. It does, therefore, appear that DNA fragmentation is a function of the presence of the dithianthrenyl ring system in Group 2, which is absent in Group 4.

The APOPercentage™ assay was also used to evaluate the pro-apoptotic activity of the most active compounds (SK7, SK8, SK11, SK12, SK14 and SK15) on a panel of five human cell lines. SK12 showed very low activity on the five cell lines. The panel of cell lines included KMST-6 (non-cancerous fibroblast cells), Jurkat T (acute T-cell leukaemia), MCF7 (human breast adenocarcinoma), HEpG2 (hepatocellular carcinoma) and HT-29 (colon adenocarinoma). Two of the cell lines, MCF7 and Jurkat T, were highly sensitive to the effects of the compounds. Between 70% and 95% cell death was observed in these two cell lines when the cells were treated for 24 h with 5 μM of the compounds (SK7, SK8, SK11, SK14 and SK15). HEpG2, HT-29 and KMST-6 cells were less sensitive to the effects of the compounds. This demonstrates selective toxicity towards MCF7 and Jurkat T cells with very low toxicity to the non-cancerous KMST-6 cells. However, more studies are required to determine the selective index for these compounds.

Although SK12 induced significant levels of apoptosis in CHO cells, the human cell lines (KMST-6, Jurkat T, MCF7,

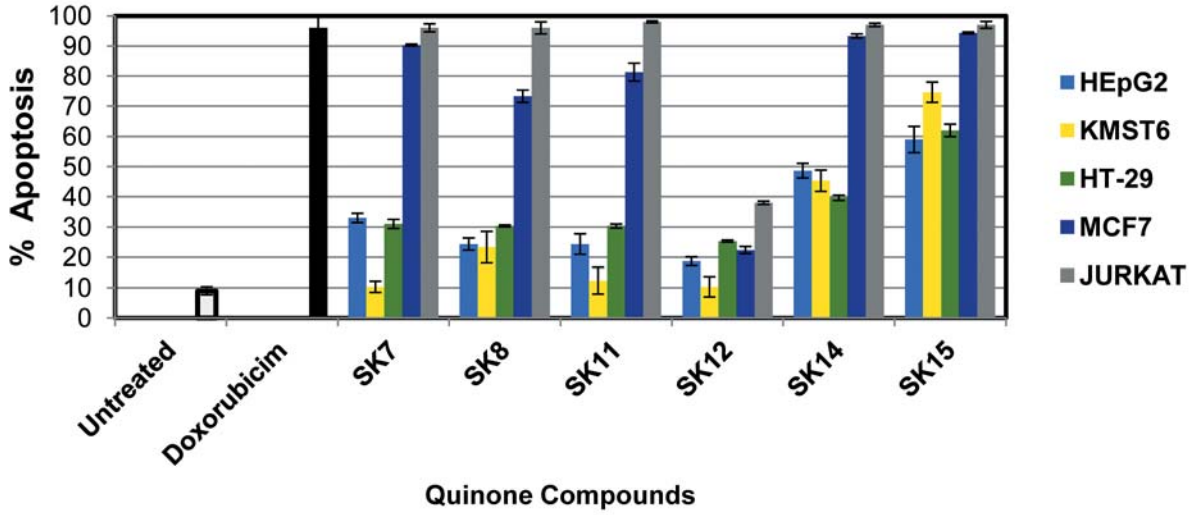

Figure 3. *Evaluating the pro-apoptotic activity of quinone compounds on a panel of human cancer cell lines. Four human cancer cell lines (HEpG2, HT-29, MCF7 and Jurkat T) and one non-cancerous human cell line (KMST6) were treated for 24 h with the compounds (5 μM). Apoptosis was assessed by flow cytometry using the APOPercentage™ assay.*

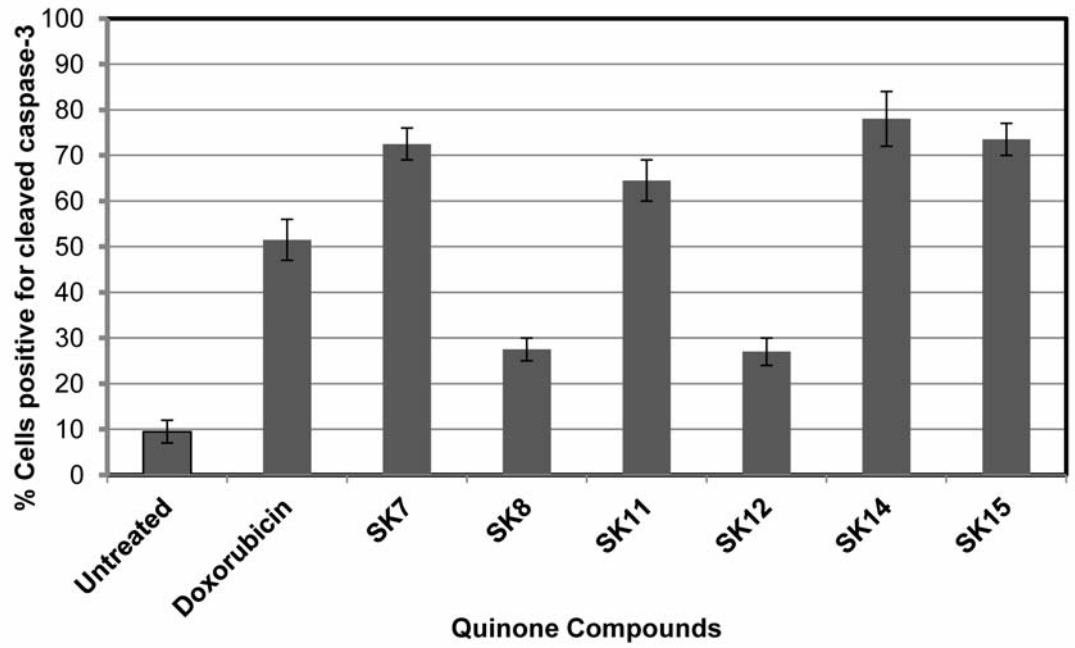

Figure 4. *Evaluating the activation of caspase-3 in CHO cells. Caspase-3 cleavage was assessed using a phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody. CHO cells were treated for 24 h with 5 μM of the compounds. Cell fluorescence was measured by flow cytometry. The bar graph indicates the percentage of cells staining positive for active caspase-3.*

HEpG2 and HT-29) were resistant to the effects of this compound. Compared to SK7, SK8, SK11, SK14 and SK15, the ability of SK12 to induce DNA fragmentation and generate ROS in CHO cells was also very low. This could in part be due to the fact that there are no free hydroxyl groups present since both oxygens are present as methyl ethers and their demethylation is not easily achieved as is the deacetylation in viz., SK14. This may also be explained by

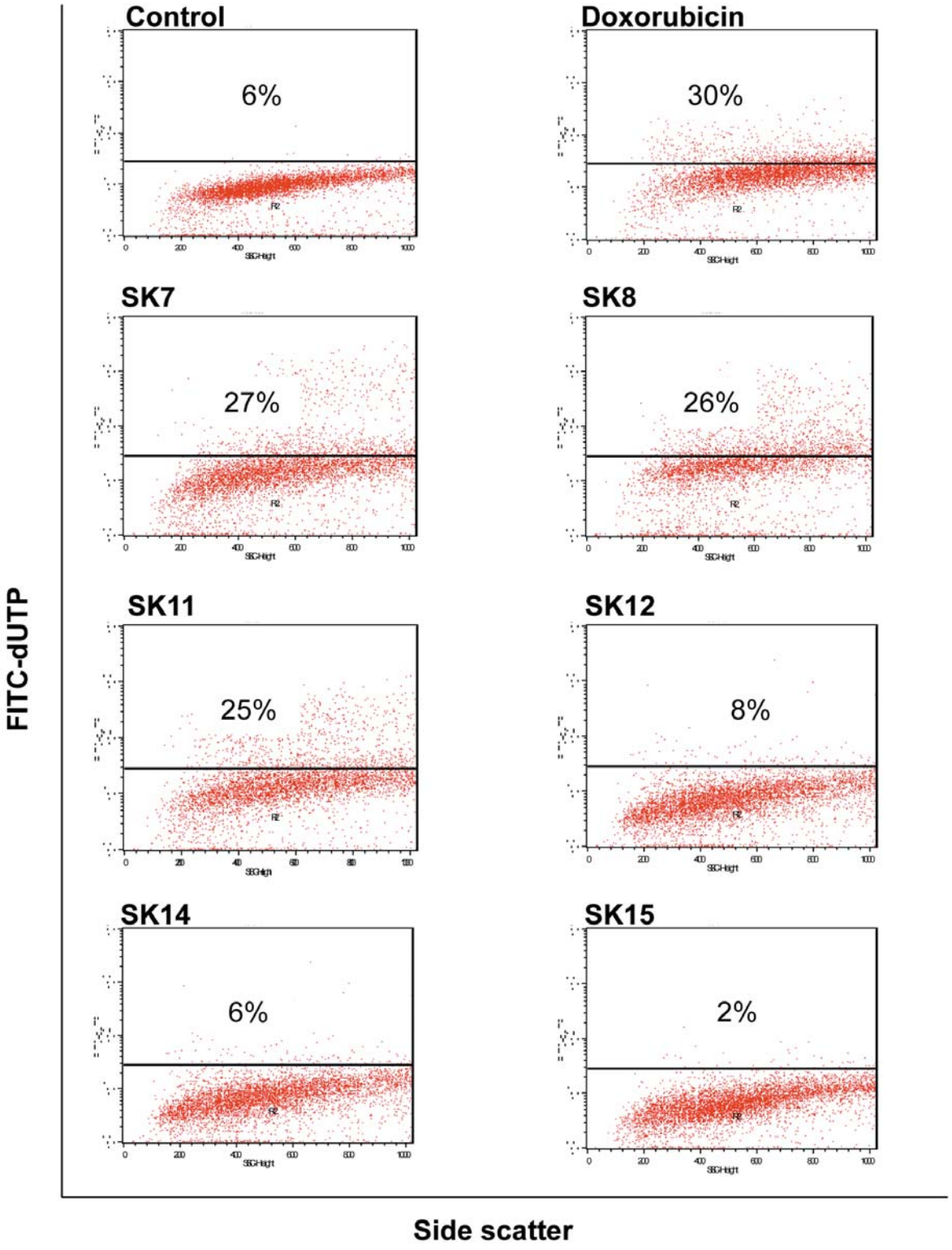

Figure 5. *Assessing DNA fragmentation in CHO cells treated with quinone compounds. CHO cells were treated for 24 h with 5 μM of the indicated compounds. DNA fragmentation was assessed using the TUNEL assay. Cell fluorescence was measured by flow cytometry. The dot plots is a comparison of the side scatter (X-axis) and FITC-dUTP (Y-axis) fluorescence detected in the cells. The numbers on the plots refer to the number of cells with fragmented DNA.*

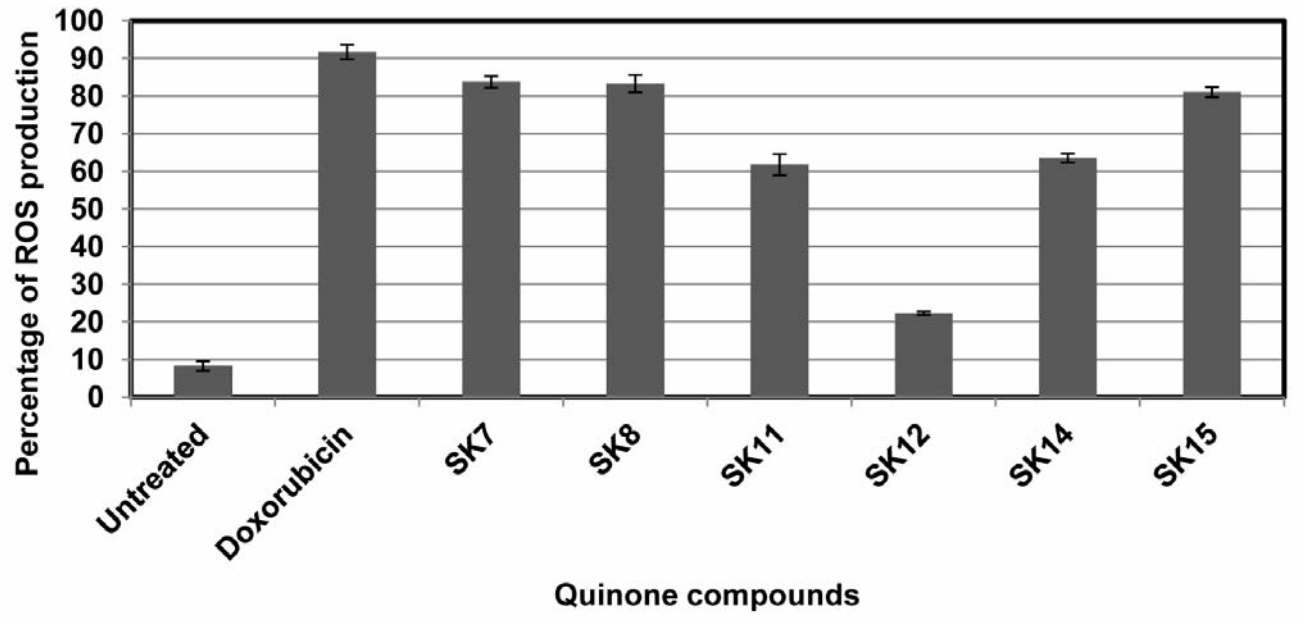

Figure 6. *Evaluating the production of ROS in CHO cells treated with quinone compounds. CHO cells were treated for 24 h with 5 μM of the indicated compounds. ROS production was measured by flow cytometry using the DCFH-DA probe.*

the fact that CHO is a rodent cell line, while the other cells lines are human. Interestingly, SK12 also failed to induce high levels of ROS in CHO cells, while the toxicity of SK7, SK8, SK11, SK14 and SK15 was associated with high levels of oxidative stress.

The quinone-containing compounds (in particular SK7, SK8, SK11, SK14 and SK15) described in this study were toxic to a number of human cancer cell lines. However, the selective cytotoxicity of these compounds towards human cancer cells must be further investigated. It was also demonstrated in CHO cells that the toxicity of these compounds was due to the activation of apoptosis through the generation of ROS, DNA fragmentation and caspase-3 cleavage. However, it is not known whether apoptosis activated by these quinone-containing compounds in human cancer cell lines are also associated with these processes. This study demonstrated that these novel quinone-containing compounds are potential anticancer agents and should therefore be subjected to further study.

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Disclosures

None.

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