Coenzyme Q_0 induces apoptosis and modulates the cell cycle in estrogen receptor negative breast cancer cells

Tiffany J. Somers-Edgar and Rhonda J. Rosengren

We postulated that methoxy-substituted cyclic compounds could inhibit estrogen receptor (ER) negative breast cancer growth *in vitro*. Therefore, this study assessed the cytotoxic potential of various methoxy-substituted cyclic compounds [7,8-dimethoxyflavone, 4-methoxyphenylacetic acid, 2-methoxyphenylacetic acid, 4-methoxybenzophenone, 5-methoxy-1-indanone, and coenzyme Q_0 (CoQ_0)] toward ER-negative human breast cancer cells (MDA-MB-231 and SKBr3). Cytotoxicity was assessed using the sulforhodamine B assay. CoQ_0 demonstrated the strongest cytotoxicity toward MDA-MB-231 and SKBr3 cells with IC_{50} values of 1.7 μmol/l and 3.1 μmol/l, respectively, whereas the other compounds were either much less potent or completely lacked cytotoxicity toward both breast cancer cell lines. Therefore, only CoQ_0 was examined for its ability to modulate cell cycle progression and induce apoptosis. Cell cycle experiments, using propidium iodide staining and flow cytometry, demonstrated that CoQ_0 at 7.5 μmol/l increased the proportion of MDA-MB-231 cells in G_1/G_0-phase by 16.6 ± 0.6% of control ($P < 0.05$), and increased in the proportion of S-phase SKBr3 cells by 37.8 ± 5.8% over control ($P < 0.05$). Induction of apoptosis was determined using propidium iodide/Annexin-V-FLUOS staining followed by flow cytometry. The results demonstrated that treatment with CoQ_0 (7.5 μmol/l) increased the proportion of apoptotic MDA-MB-231 and SKBr3 cells by 12-fold and 4-fold over control ($P < 0.05$), respectively. Thus, CoQ_0 is a potent cytotoxic drug that induces apoptosis and modulates cell cycle progression in ER-negative breast cancer cells. Therefore, CoQ_0 is an appropriate candidate for further study and development as a potential drug for ER-negative breast cancer. *Anti-Cancer Drugs* 20:33–40 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Tamoxifen, a selective estrogen receptor (ER) modulator, is currently the preferred adjuvant therapy for breast cancer. For women with ER-positive breast cancer the response rate is ~70%, whereas it is only 30% for women with ER-negative breast cancer [1,2]. As ~30% of all diagnosed breast tumors are ER-negative [3], and approximately two-thirds of breast cancers diagnosed in premenopausal women are ER-negative [4,5], there is an urgent need to develop drug therapies that target ER-negative breast cancer.

In recent years, a large number of polyphenolic compounds, such as apigenin, resveratrol, epigallocatechin gallate (EGCG), curcumin, and genistein, have received attention for their anticancer properties both *in vitro* and *in vivo*. For example, EGCG, a flavonoid contained in green tea, inhibits the growth of various ER-negative human breast cancer cell lines [6–10], and inhibits ER-negative tumor growth *in vivo* [11,12]. Although in-vivo studies with these polyphenols have demonstrated some efficacy against tumor growth, clinical studies have been less compelling. This is primarily owing to their low bioavailability that results from the high rate of glucuronidation and sulfation of hydroxyl functional groups present on these compounds [13]. However, recent studies have revealed that certain alterations to the chemical structures of such compounds can significantly improve their bioavailability. In particular, methoxylated analogs appear to have improved bioavailability compared with their parent compounds [14,15]. For example, a peak plasma concentration of 2.5 ± 0.8 μmol/l was demonstrated 1 h after oral administration of 5,7-dimethoxyflavone (5 mg/kg), whereas its unmethoxylated analog (chrysin) was not detected following an equivalent oral dose [14]. Additionally, the cytotoxic effect of 5,7-dimethoxyflavone toward human oral cancer cells (SCC-9) was 10 times greater than that produced by chrysin [15].

Based on these findings, the current study aimed to assess the cytotoxicity of a range of methoxy-containing cyclic compounds toward ER-negative breast cancer cells *in vitro*. The specific compounds investigated were 7, 8-dimethoxyflavone (7,8-DMF), 4-methoxyphenylacetic acid

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(4-MPA), 2-methoxyphenylacetic acid (2-MPA), 4-methoxybenzophenone (4-MBP), 5-methoxy-1-indanone (5-MI), and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q_0; CoQ_0) (Fig. 1). Each compound was screened for cytotoxicity using two ER-negative human breast cancer cell lines (MDA-MB-231 and SKBr3). Those compounds that demonstrated marked cytotoxicity toward ER-negative breast cancer cells were further analyzed for changes in cell cycle distribution and the induction of apoptosis as potential mechanisms of growth inhibition.

Methods

Chemicals and reagents

4-MPA, 2-MPA, 4-MBP, and 5-MI were purchased from Merck-Schuchardt (Hohenbrunn, Germany). CoQ_0 was a gift from Professor Rob Smith (Department of Chemistry, University of Otago, Dunedin, New Zealand). 7,8-DMF, antibiotic/antimycotic solution, minimum essential medium, Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F-12), sodium bicarbonate, sulforhodamine B (SRB), propidium iodide, Triton-X 100, trypsin, and trypan blue were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Acetic acid, disodium hydrogen orthophosphate anhydrous, EDTA, dimethylsulfoxide (DMSO), potassium dihydrogen orthophosphate, sodium citrate, and trichloroacetic acid were purchased from BDH Laboratory Supplies (Poole, England). Fetal bovine serum (FBS) was purchased from Life Technologies Ltd (Auckland, New Zealand). Annexin-V-FLUOS was purchased from Roche (Mannheim, Germany).

Cell Culture

MDA-MB-231 and SKBr3 human breast cancer cells were purchased from ATCC (Manassas, Virginia, USA). Cells were maintained in minimum essential medium media supplemented with 10% FBS, 1% antibiotic/antimycotic solution, and 0.2% $NaHCO_3$ at 37°C in a 5% CO_2/95% humidified air incubator.

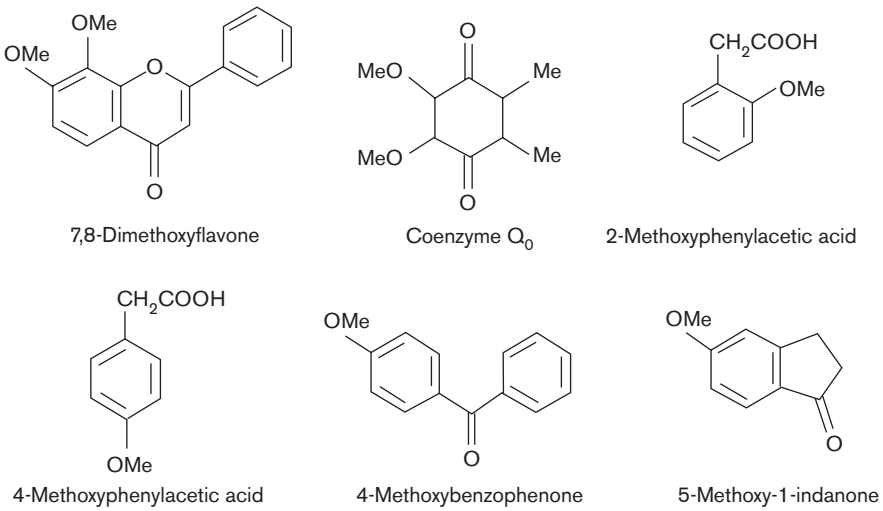
Cytotoxicity assay

MDA-MB-231 and SKBr3 human breast cancer cells were plated in 6-well plates (70 000 cells per well) in 5 ml DMEM/F-12 media supplemented with 5% FBS, 1% antibiotic/antimycotic solution, and 0.2% $NaHCO_3$. After 24 h, cells were treated with 0.1–50 µmol/l of 7,8-DMF, 4-MPA, 2-MPA, 4-MBP, 5-MI, or 0.1–10 µmol/l CoQ_0 for 5 days. Vehicle control cells were treated with DMSO alone (0.1%). Cell number was then determined using the SRB assay as previously described [16], as this dye-binding assay detects live cells and has better linearity, increased sensitivity, and a more stable end point compared with the microtetrazolium assay. Data are expressed as the mean ± SEM of cell number (% of control) from five independent experiments conducted in duplicate.

Cell cycle analysis

MDA-MB-231 and SKBr3 cells were seeded in 6-well culture plates (200 000 cells per well) in 3 ml of DMEM supplemented with 5% FBS, 1% antibiotic/antimycotic solution, and 0.2% $NaHCO_3$. After 24 h, cells were treated with 7.5 µmol/l of CoQ_0 or vehicle control (0.1% DMSO) for 6, 12, 18, or 24 h. Cell cycle distribution was assessed using propidium iodide staining, as described [17,18]. Samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey) and the proportion of cells in each of G_0/G_1-phases, S-phases, and G_2/M-phases were determined using CellQuest Pro software (Becton Dickinson). Data

Fig. 1



Structures of methoxy-substituted cyclic compounds.

are expressed as the mean ± SEM of the proportion of cells in each of the above cell cycle phases (% of control) from four independent determinations performed in duplicate.

Apoptosis analysis

MDA-MB-231 cells were seeded in 6-well culture plates (200 000 cells per well) in 3 ml of DMEM supplemented with 5% FBS, 1% antibiotic/antimycotic solution, and 0.2% $NaHCO_3$. After 24 h, cells were treated with 7.5 μmol/l of CoQ_0 or vehicle control (0.1% DMSO) for 12, 24, or 36 h. The presence of apoptosis was assessed using Annexin-V-FLUOS/propidium iodide staining, as described [9]. Samples were analyzed using a FACS calibur flow cytometer (Becton Dickinson) and the proportion of apoptotic cells (as a percent of total cells) was determined using CellQuest Pro software. Data are expressed as the mean ± SEM of the percentage of cells undergoing apoptosis, from four independent determinations performed in duplicate.

Statistical analysis

Cell cytotoxicity data was analyzed using a one-way analysis of variance coupled with a Student–Newman–Keuls post-hoc test. Time-course experiments were analyzed using a two-way analysis of variance and a Bonferroni post-hoc test. In all cases, $P < 0.05$ was the minimum requirement for a statistically significant difference.

Results

Cytotoxicity of methoxy-substituted compounds

The cytotoxic potential of six methoxy-substituted compounds (2-MPA, 4-MPA, 5-MI, 4-MBP, 7,8-DMF, and CoQ_0) toward two ER-negative (MDA-MB-231 and SKBr3) human breast cancer cells was assessed using the SRB assay. Although 2-MPA (Fig. 2a) and 4-MPA (Fig. 2b) did not significantly alter cell number in either cell line, a decrease in cell number was demonstrated following treatment with the four remaining compounds. Although 5-MI did not significantly alter MDA-MB-231 cell number (Fig. 2c), the number of SKBr3 cells was decreased by approximately 13.9 ± 1.7% of control ($P < 0.001$) following 5 days of treatment with 5-MI at 50 μmol/l. Similarly, 5 days of 4-MBP treatment (50 μmol/l) (Fig. 2d) decreased MDA-MB-231 and SKBr3 cell number by 18.2 ± 2.4% and 35.4 ± 2.8% of control, respectively ($P < 0.01$). Treatment of MDA-MB-231 and SKBr3 cells with 7,8-DMF resulted in a significant cytotoxicity (Fig. 2e). Specifically, 5 days of treatment with 10 μmol/l of 7,8-DMF decreased MDA-MB-231 and SKBr3 cell number by 74.4 ± 1.3% and 64.0 ± 1.4% of control, respectively ($P < 0.001$). However, higher concentrations of 7,8-DMF were less cytotoxic. For example, 7,8-DMF at 50 μmol/l decreased MDA-MB-231 and SKBr3 cell number by only 47.4 ± 2.2 and 49.3 ± 1.5% of control, respectively ($P < 0.001$). Of all the compounds examined, CoQ_0 was the most potent inhibitor of cell growth. Treatment with 0.1–5 μmol/l CoQ_0 resulted in a concentration-dependent decrease in MDA-MB-231 and SKBr3 cell number with IC_{50} values of 1.7 and 3.1 μmol/l, respectively (Fig. 2f), and thus CoQ_0 was the only compound to show dose-dependent cytotoxicity and low IC_{50} values in both cell lines.

Coenzyme Q_0-mediated changes in cell cycle progression

Although 7,8-DMF was cytotoxic to both MDA-MB-231 and SKBr3 cells, the observed biphasic dose–response relationship indicates that this compound is not a good candidate for further development as an ER-negative breast cancer drug. Although treatment with 4-MBP and 5-MI produced some cytotoxicity, these effects were much weaker than those produced by CoQ_0. Therefore, the only compound from the cytotoxicity screen that warranted further examination was CoQ_0. To determine whether CoQ_0 altered the progression of ER-negative breast cancer cells though the cell cycle, SKBr3 and MDA-MB-231 cells were treated with 7.5 μmol/l of CoQ_0 for 6, 12, 18, and 24 h. CoQ_0 treatment resulted in G_0/G_1-phase cell cycle arrest in MDA-MB-231 cells, and S-phase cell cycle arrest in SKBr3 cells (Table 1). Specifically, a 16.6 ± 0.9% increase in the proportion of MDA-MB-231 cells in the G_0/G_1-phase was observed following 24 h of treatment ($P < 0.05$ compared with control), whereas there was a 37.8 ± 5.8% increase in the proportion of SKBr3 cells in the S-phase cells following 12 h of treatment ($P < 0.05$ compared with control) (Table 1).

Induction of apoptosis by coenzyme Q_0

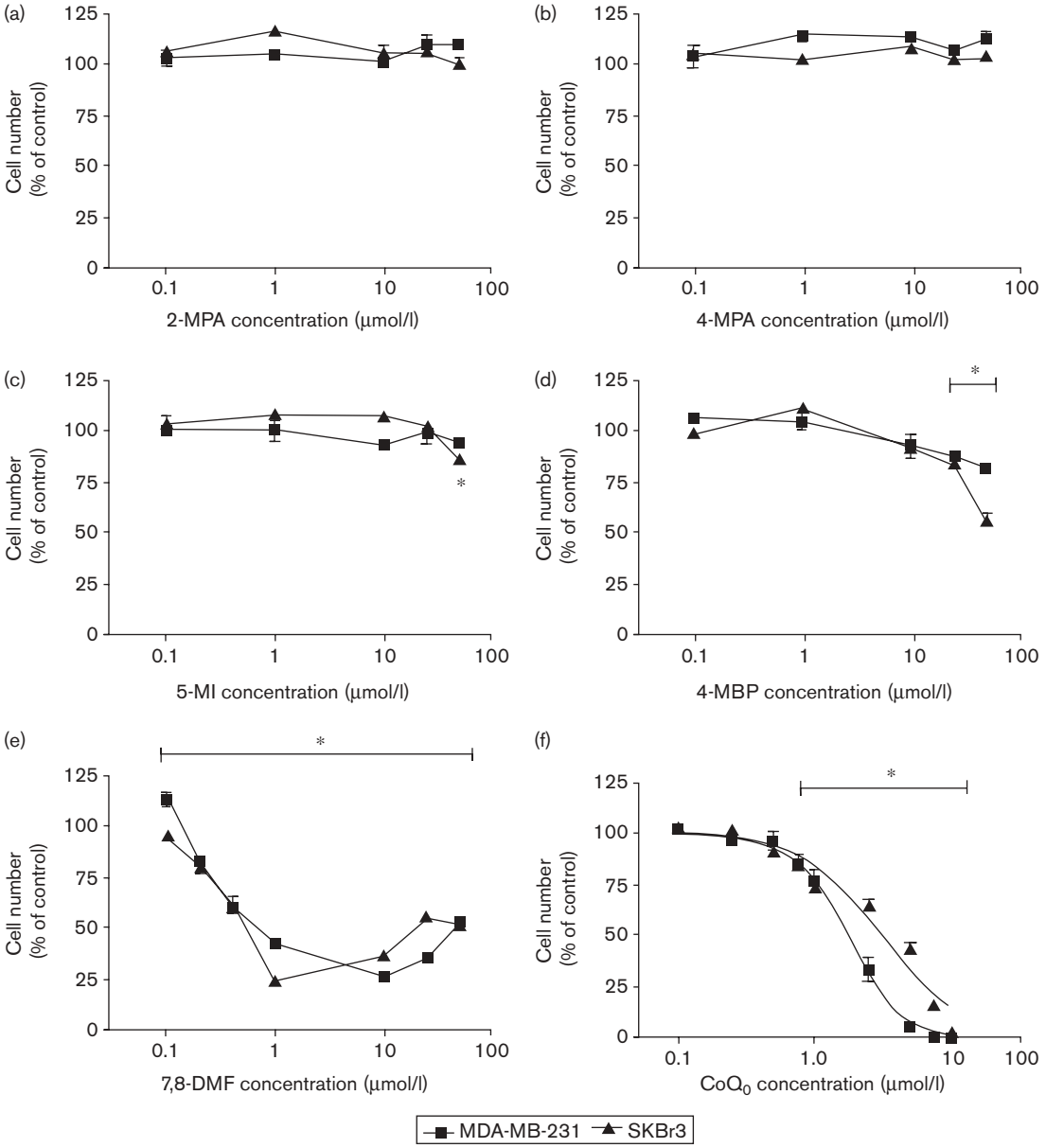
To determine whether apoptosis was induced by CoQ_0, MDA-MB-231 and SKBr3 cells were treated with 7.5 μmol/l of CoQ_0 for 12, 24, or 36 h. Determination of

Table 1 Effect of CoQ_0 treatment on cell cycle progression in ER-negative human breast cancer cells

	MDA-MB-231 cells			SKBr3 cells		
CoQ_0 (7.5 μmol/l)	G_0/G_1-phase	G_2/M-phase	S-phase	G_0/G_1-phase	G_2/M-phase	S-phase
6 h	103.6 ± 0.9%	97.0 ± 1.8%	102.5 ± 1.2%	92.4 ± 2.4%	117.4 ± 5.5%	112.9 ± 8.4%
12 h	99.0 ± 1.4%	98.0 ± 0.8%	88.0 ± 1.6%[a]	81.2 ± 3.0%[a]	96.9 ± 7.6%	137.8 ± 5.8%[a]
18 h	98.4 ± 1.4%	103.5 ± 2.1%	101.9 ± 1.7%	88.5 ± 2.6%	100.9 ± 8.1%	87.9 ± 9.5%
24 h	116.6 ± 0.9%[a]	63.3 ± 1.6%[a]	94.3 ± 1.4%	94.8 ± 0.9%	89 ± 2.0%	106.6 ± 1.9%

Values are the mean proportion of cells in each cell cycle phase (% of control) ± SEM ($n = 4$).
[a]Significantly different from control ($P < 0.05$).

Fig. 2



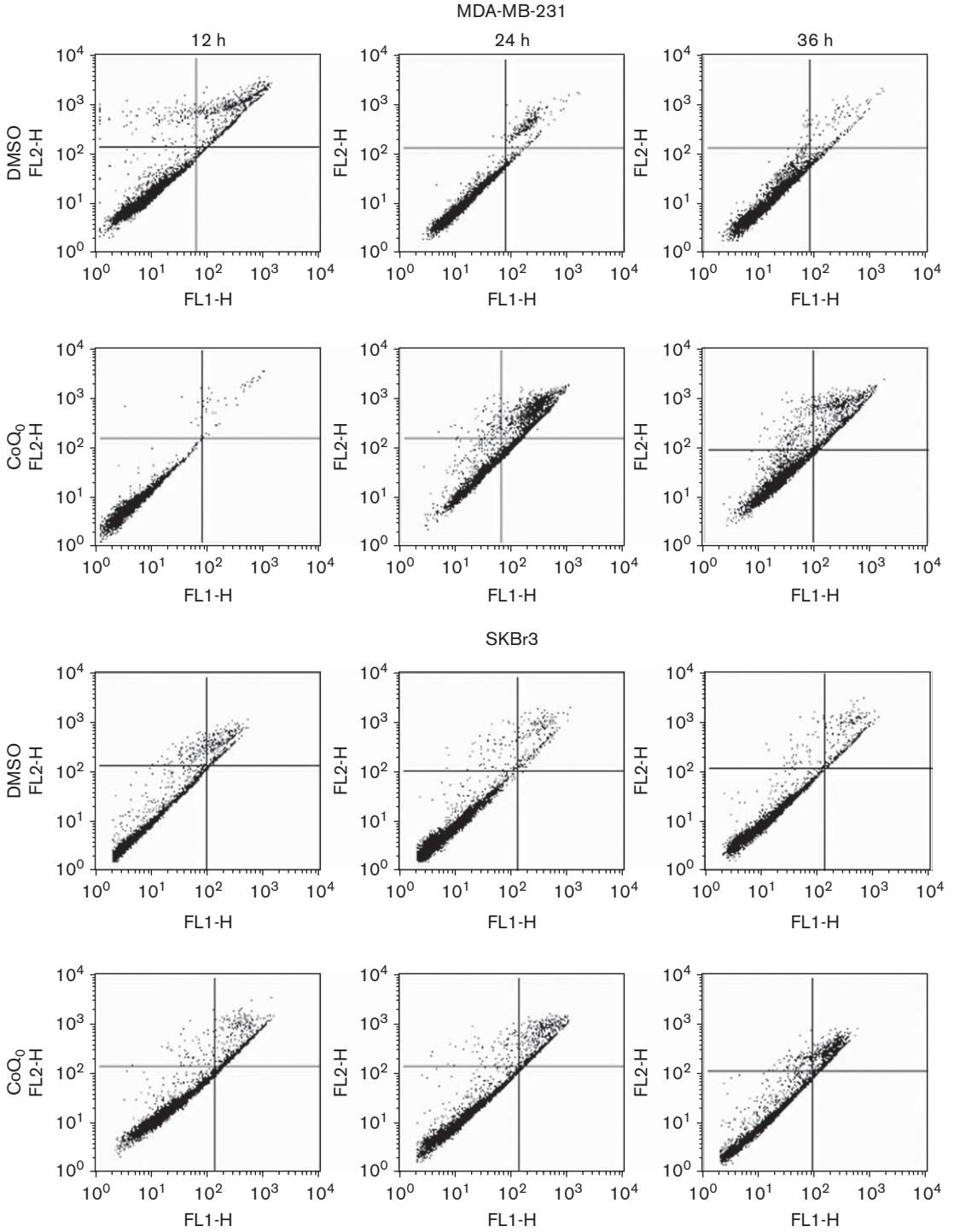
Effects of methoxy-substituted cyclic compounds on ER-negative human breast cancer cell viability. MDA-MB-231 (■) and SKBr3 (▲) cells were seeded at 7×10^4 cells per well and treated 24 h later with (a) 2-MPA, (b) 4-MPA, (c) 5-MI, (d) 4-MBP, (e) 7,8-DMF, or (f) coenzyme Q_0 (CoQ_0) for 5 days. Vehicle control cells were treated with 0.1% dimethylsulfoxide. At the end of treatment, the cell number in each well was determined using the sulforhodamine B assay. Each data point represents mean ± SEM of five independent determinations conducted in duplicate. Data were analyzed using a one-way analysis of variance coupled with a Student–Newman–Keuls post-hoc test. IC_{50} curves were generated using nonlinear regression on Prism software (GraphPad Software, San Diego, California). *Significantly different from vehicle control ($P<0.05$). DMF, dimethoxyflavone; MBP, methoxybenzophenone; MI, methoxy-1-indanone; MPA, methoxyphenylacetic.

apoptosis by flow cytometry demonstrated that CoQ_0 significantly increased the proportion of MDA-MB-231 and SkBr3 cells undergoing apoptosis as shown by dot-plots (Fig. 3). Further quantification of the flow cytometry data demonstrated that a four-fold increase in the percentage of apoptotic SKBr3 cells occurred at 36 h ($P < 0.05$, compared with control), whereas in MDA-MB-231 cells there was a 12-fold increase in the percentage of apoptotic cells at 24 h ($P < 0.05$, compared with control) (Fig. 4).

Discussion

Although advances are continually being made in the field of breast cancer research, the currently available therapies

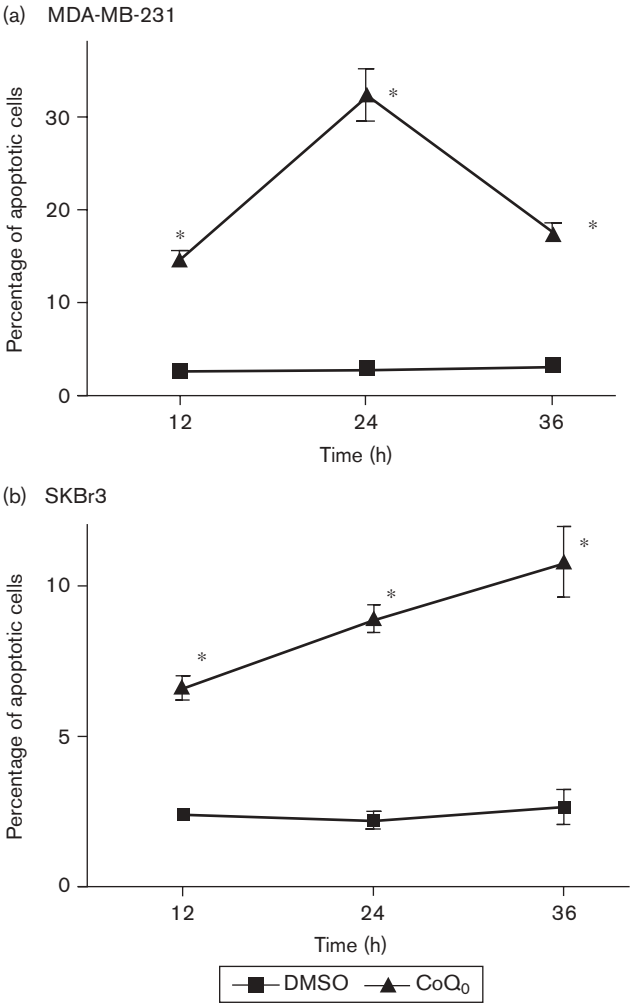
Fig. 3



Representative apoptosis dot-plots. MDA-MB-231 and SKBr3 cells were seeded at 2×10^5 cells per well and treated 24 h later with 7.5 μmol/l of coenzyme Q_0 (CoQ_0) or vehicle control [0.1% dimethylsulfoxide (DMSO)] for 12 to 36 h. Cells undergoing apoptosis were determined using propidium iodide/Annexin V-fluorescein isothiocyanate (FITC) labeling and flow cytometry. Dot-plots were generated using CellQuest Pro software.

are largely ineffective in the treatment of ER-negative breast cancer. Therefore, the goal of this study was to specifically screen a range of compounds for cytotoxic effects on this subtype of breast cancer using ER-negative human breast cancer cell lines. The most potent cytotoxic compound identified was CoQ_0, which

Fig. 4



Quantification of apoptosis by coenzyme Q_0 (CoQ_0) in ER-negative human breast cancer cells. (a) MDA-MB-231 and (b) SKBr3 cells were seeded at 2×10^5 cells per well and treated 24 h later with 7.5 μmol/l of CoQ_0 (▲) or vehicle control (0.1% DMSO; ■) for 12 to 36 h. Cells undergoing apoptosis were determined using propidium iodide/Annexin V-fluorescein isothiocyanate (FITC) labeling and flow cytometry. Each data point represents the mean ± SEM of four independent determinations conducted in duplicate. Data were analyzed with a two-way analysis of variance coupled with a Bonferroni post-hoc test. *Significantly different from vehicle control ($P < 0.05$). DMSO, dimethylsulfoxide.

significantly inhibited the growth of MDA-MB-231 and SKBr3 human breast cancer cells. Specifically, IC_{50} values of 1.7 and 3.1 μmol/l were obtained following treatment for 5 days with CoQ_0 in MDA-MB-231 and SKBr3 cells, respectively. The duration of cell treatment was consistent with our earlier work examining the cytotoxic potential of the combination of EGCG and curcumin, and thus allowed for a direct comparison between CoQ_0 and the combination therapy. Importantly, the cytotoxic potency of CoQ_0 was seven-fold greater than curcumin [19], thus further demonstrating that CoQ_0 has a high potency in ER-negative breast cancer cells. To assess the mechanisms contributing to this cytotoxic effect, cell cycle progression and induction of apoptosis were determined. CoQ_0 treatment produced G_0/G_1-phase and S-phase cell cycle arrest in MDA-MB-231 and SKBr3 cells, respectively. Furthermore, significant increases in the proportion of apoptotic cells were observed in both MDA-MB-231 and SKBr3 cells following treatment with CoQ_0.

Earlier studies have demonstrated that quinones with structural similarities to CoQ_0 can induce apoptosis [20–24]. For example, mitoQ is a quinone drug that is specifically targeted to mitochondria via its triphenylphosphonium cation moiety, and was initially developed for the protection of mitochondria against oxidative damage [25]. However, recent studies have suggested that mitoQ may produce cytotoxicity in some cell types through the generation of reactive oxygen species (ROS) [21,26,27]. For example, treatment of bovine aortic endothelial cells with 5–1000 nmol/l mitoQ was shown to significantly increase the production of intracellular superoxide radical and hydrogen peroxide [21]. This increase in ROS production was associated with caspase-3 cleavage and apoptosis induction.

In recent years, a number of groups have demonstrated that certain methoxy-containing analogs of coenzyme Q (CoQ) produce cytotoxic effects in human cancer cell lines [22–24]. CoQ is composed of a quinone nucleus and a side chain containing a variable number of *trans*-isoprenoid units. Coenzyme Q_{10} contains 10 of these side-chain units, and is the major naturally occurring form of CoQ found in primates [28]. On the other hand, CoQ_0 completely lacks isoprenoid side chains.

Recent studies have examined the importance of isoprenoid side-chain length in terms of anticancer efficacy. For example, treatment of BALL-1 human leukemia cells with 10–100 μmol/l of CoQ_2, CoQ_4, or CoQ_6 for 24 h decreased cell viability with IC_{50} values of approximately 20, 40, and 100 μmol/l, respectively, whereas treatment with 10–100 μmol/l CoQ_{10} did not affect cell proliferation [22]. A similar pattern was observed for apoptosis induction, in which DNA fragmentation and caspase-3 activity (indicators of apoptosis induction) were significantly increased by treatment with CoQ_2 and CoQ_4 but were not induced by CoQ_6 or CoQ_{10}. These findings are supported by a further study that examined the potential of a range of CoQ structures (CoQ_1, CoQ_2, CoQ_4, CoQ_6, and CoQ_{10}) to decrease the proliferation of HL60 human leukemia cells [24]. The authors demonstrated that, although CoQ_6 and CoQ_{10} inhibited HL60 cell proliferation only at very high concentrations (> 200 μmol/l), treatment with CoQ_1, CoQ_2, and CoQ_4 for 24 h significantly inhibited HL60 cell proliferation with respective IC_{50} values of approximately 14, 56.5, and 168 μmol/l.

The observed inhibition of HL60 cell proliferation was accompanied by initial S-phase arrest, followed by G_0/G_1-phase arrest at later time points, and induction of apoptosis [24]. The anticancer properties and structure–activity relationships of a range of CoQ analogs containing two or more isoprenyl subunits and altered methoxy substitution patterns have also been determined. The authors demonstrated that CoQ_2 analogs inhibited epidermal growth factor-induced and 12-*O*-tetradecanoylphorbol-13-acetate-induced cell transformation and induced apoptosis in the JB6 P^+ Cl41 mouse epidermal cell line [23]. Results demonstrated that the cytotoxic potency of the CoQ analogs depended on the length of the isoprenyl side chain, and on the position of the methoxy-substitutions on the quinone nucleus. Taken together, these observations indicate that decreasing the length of CoQ's isoprenyl side chain increases its antiproliferative activity. Our work with CoQ_0 in ER-negative breast cancer cells supports this theory.

The mechanisms by which CoQ produces these effects are still unclear. However, it has been suggested that the biochemical mechanisms that underlie the anticancer properties of certain quinones may involve ROS production [21,22]. CoQ_0 is also known to inhibit activity of complex I of the mitochondrial respiratory chain and prevent opening of the mitochondrial permeability transition pore [29]. Results from further studies have suggested that this effect is likely to involve direct binding of CoQ_0 to dihydrolipoamide components of the α-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes [30]. Although these findings may indicate that CoQ_0 treatment *in vivo* could produce general toxicity, a number of studies have utilized CoQ_0, without demonstrating any deleterious effects [31–33]. These studies all involved the administration of diets containing different combinations of nutrients including CoQ_{10}, CoQ_0 (100 mg/kg), vitamin E, vitamin C, and selenium, among others. When given as a mixture, these nutrient combinations inhibited oxidative damage to normal tissues, including the heart, liver, kidney, and spleen. However, it is important to note that these studies did not provide specific information pertaining to the in-vivo safety of CoQ_0 as a single treatment. Therefore, the in-vivo safety profile of CoQ_0 administration as a sole agent should be investigated.

Conclusion

This study assessed the effects of a range of methoxy-substituted cyclic compounds on ER-negative breast cancer cells, and demonstrated for the first time that CoQ_0 inhibits ER-negative breast cancer cell growth *in vitro*. Inhibition of cell cycle progression and induction of apoptosis were shown to underlie the growth suppression produced by CoQ_0. Overall, CoQ_0 seems to be a good candidate for further development as a treatment drug for ER-negative breast cancer. Therefore, further studies investigating both the mechanism of action and in-vivo safety of CoQ_0 are warranted.

Acknowledgements

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