Mitochondria-targeted triphenylphosphonium-based compounds do not affect estrogen receptor α

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Background: Targeting negatively charged mitochondria is often achieved using triphenylphosphonium (TPP) cations. These cationic vehicles may possess biological activity, and a docking study indicates that TPP-moieties may act as modulators of signaling through the estrogen receptor α (ERα). Moreover, *in vivo* and *in vitro* experiments revealed the estrogen-like effects of TPP-based compounds. Here, we tested the hypothesis that TPP-based compounds regulate the activity of ERα.

Methods: We used ERa-positive and ERα-negative human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-231, respectively). Cell proliferation was measured using a resazurin cell growth assay and a real-time cell analyzer assay. Cell cycle progression was analyzed using flow cytometry. Real-time PCR was used to measure transcription driven by estrogen-responsive promoters in cells transfected with an estrogen response element (ERE)$_3$-luciferase expression vector.

Results: The TPP-based molecules SkQ1 and C$_{12}$TPP, as well as the rhodamine-based SkQR1, did not increase the proliferation or alter the cell cycle progression of MCF-7 cells. In contrast, 17β estradiol increased the proliferation of MCF-7 cells and the proportion of cells in the S/G2/M-phases of the cell cycle. TPP-based compounds did not affect the induction of transcription of an ERE-luciferase expression vector *in vitro*, and SkQ1 did not alter the levels of expression of estrogen-dependent genes encoding GREB1, TFF1, COX6, and IGFBP4.

Conclusion: TPP-based compounds do not possess properties typical of ERα agonists.

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20

21 Abstract

22 **Background:** Targeting negatively charged mitochondria is often achieved using
23 triphenylphosphonium (TPP) cations. These cationic vehicles may possess biological activity,
24 and a docking study indicates that TPP-moieties may act as modulators of signaling through the
25 estrogen receptor α (ERα). Moreover, *in vivo* and *in vitro* experiments revealed the estrogen-like
26 effects of TPP-based compounds. Here, we tested the hypothesis that TPP-based compounds
27 regulate the activity of ERα.
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29 (MCF-7 and MDA-MB-231, respectively). Cell proliferation was measured using a resazurin cell
30 growth assay and a real-time cell analyzer assay. Cell cycle progression was analyzed using flow
31 cytometry. Real-time PCR was used to measure transcription driven by estrogen-responsive
32 promoters in cells transfected with an estrogen response element (ERE)$_3$-luciferase expression
33 vector.
34 **Results:** The TPP-based molecules SkQ1 and C_{12}TPP, as well as the rhodamine-based SkQR1,
35 did not increase the proliferation or alter the cell cycle progression of MCF-7 cells. In contrast,
36 17β estradiol increased the proliferation of MCF-7 cells and the proportion of cells in the
37 S/G2/M-phases of the cell cycle. TPP-based compounds did not affect the induction of
38 transcription of an ERE-luciferase expression vector *in vitro*, and SkQ1 did not alter the levels of
39 expression of estrogen-dependent genes encoding GREB1, TFF1, COX6, and IGFBP4.

40   **Conclusion:** TPP-based compounds do not possess properties typical of ERα agonists.
41

Introduction

43   Mitochondria are important target for drug development (Zielonka et al., 2017). Mitochondria-
44   targeted molecules include drug candidates, intracellular probes and sensors (Zinovkin &
45   Zamyatnin, 2019). The drug of interest is usually linked via a carbon linker to the
46   triphenylphosphonium (TPP) cation. The latter effectively confers mitochondria-targeting
47   activity. Lipophilic TPP-based molecules are readily transported across the membranes of cells
48   and mitochondria, and cationic TPP ensures targeting to negatively charged mitochondria
49   through electrostatic attraction. The widely used mitochondria-targeted antioxidants MitoQ and
50   SkQ1 exploit the TPP cation as a vehicle. Despite a wealth of experimental data obtained using
51   TPP-based compounds, numerous molecular features of intracellular signaling induced by TPP-
52   based moieties are unknown.
53   The TPP cation may possess biological activity. For example, the results of a docking study
54   suggest that TPP-moieties may act as modulators of estrogen receptor α (ERα) (Salisbury &
55   Williams, 2009). Estrogens are implicated in numerous physiological functions of females and
56   males. The major female sex hormone 17β estradiol (E2) contributes to human development and
57   reproduction. ERs include ERα, ERβ, and G protein-coupled estrogen receptor alpha. Multiple
58   isoforms of ERα and ERβ function as transcription factors. ERs dimerize upon binding to E2,
59   and the complex translocates to the nucleus where it regulates the expression of estrogen-
60   dependent genes. Furthermore, indirect mechanisms operate through multiple co-regulatory
61   proteins (Yasar et al., 2017).
62   Indirect evidence supports the possibility that the TPP-based antioxidants MitoQ and SkQ1 act
63   through estrogen-like action as follows:
64   • The mitochondria of MCF-7 and endothelial cells contain functional high-affinity ERs
65   (Pedram et al., 2006). Activation of these receptors by E2 inhibits UV radiation-induced
66   cytochrome C release, decreased mitochondrial membrane potential, and apoptosis via
67   the formation of mitochondrial reactive oxygen species (mROS) (Pedram et al., 2006).
68   MitoQ exhibits the same activities. Moreover, MitoQ and SkQ1 inhibit mROS generation
69   to prevent subsequent apoptosis (Feniouk & Skulachev, 2017), suggesting they directly
70   interact with mitochondrial ERs.
71   • Estrogen-like activity of SkQ1 is detected in female outbred SHR mice, and long-term
72   oral administration of SkQ1 inhibits the age-related decline of the estrous function
73   (Anisimov et al., 2008).
74   • SkQ1 consumption by female rats prolongs proestrus duration, typical of E2 (Chistyakov
75   et al., 2012).
76   • ERα regulates multiple NF-κB pathway components to control inflammatory responses
77   (Kovats, 2015), and SkQ1 regulates NF-κB activity in endothelial cells (Zinovkin et al.,
78   2014).

79   • E2 (Brotfain et al., 2016), MitoQ (Zhou et al., 2018), and SkQ1 (Silachev et al., 2015)
80   exert neuroprotective effects in a model of traumatic brain injury.
81 Experimental and clinical evidence link sustained exposure to estrogens with increased risk of
82 developing breast cancer (Russo & Russo, 2006). Certain mitochondria-targeted antioxidants are
83 the subject of clinical trials (Zinovkin & Zamyatnin, 2019). Eye drops containing SkQ1 are used
84 to cure dry eye syndrome (Visomitin) and MitoQ is freely available as an antioxidant
85 supplement.
86 TPP-based mitochondria-targeted compounds may possess estrogen-like activities, and it is
87 critically important to investigate this possibility. Here we tested the hypothesis that TPP-based
88 compounds such as SkQ1 regulate the activity of ERα. For this purpose, we tested the TPP-
89 based compounds C_{12}TPP and SkQ1 (plastoquinone conjugated to C_{12}TPP). The mitochondria-
90 targeted antioxidant SkQR1, which lacks the TPP moiety, served as a negative control. The
91 structures of these cations are shown in *Figure 1*.
92
93

Materials & Methods

94

Reagents

95

96 E2 and its inhibitor were 17β-Estradiol (E2758; Sigma) and fulvestrant (I4409; Sigma),
97 respectively. The protonophore was 2,4-dinitrophenol (DNP; D198501; Sigma). The cell culture
98 reagents included phenol-red-free DMEM media (Paneco, Russia); charcoal-stripped fetal bovine
99 serum (FBS; F6765; Sigma); FBS (SV30160.03; HyClone) and DMEM medium (Gibco).
100 AlamarBlue reagent (DAL1025) was purchased from Biosource, and propidium iodide (PI) was
101 purchased from MP Biomedicals, France. SkQ1, SkQR1, and C_{12}TPP were synthesized as
102 bromide salts at the Belozersky Institute of Physico-Chemical Biology.
103

Cell culture

104

105 The human breast adenocarcinoma cell lines (ERα-positive MCF-7 and ER-, progesterone
106 receptor-, and erb-b2 receptor tyrosine kinase 2-negative [triple-negative] MDA-MB-231) were
107 obtained from the Russian Collection of Cell Cultures (Institute of Cytology, St Petersburg,
108 Russia). Cells were maintained in DMEM with 10% FBS at 37°C in an atmosphere containing
109 5% CO_2. The media was replaced at least 6 days before an experiment, with phenol-red-free
110 DMEM with 5% charcoal stripped FBS to exclude activation of ERs by phenol red and serum
111 components. The images of MCF-7 cells treated with test compounds were acquired by phase-
112 contrast microscopy using Axioplan (Zeiss) microscope.
113

Resazurin cell growth assay

114

115 Cells (approximately 5,000 cells per well) were added to the wells of 96-well plates. After 2
116 days, the medium was replaced with fresh medium with or without the test compound. After 48
117 h, cell viability was determined using a resazurin assay with AlamarBlue reagent (2.75 mM).

118   Fluorescence was measured after 3.5 h at 544/590 nm. Each experiment was repeated five or six
119   times.
120
121   *Real-time cell analyzer (RTCA) assay*
122   The xCELLigence system (Roche Applied Sciences, Basel, Switzerland) was used to evaluate
123   cell growth rate and viability. The system calculates a Cell Index (CI) value defined as (Rn-Rb) /
124   Rb, where Rn is the impedance in the well at time *n*, and Rb is the background impedance. The
125   cells (20,000 cells/well) were cultured in E-plates for 24 h, and the medium was replaced with
126   fresh medium supplemented with 10 nM E2, 20 nM SkQ1, 20 nM $C_{12}TPP$ or 20 nM SkQR1. For
127   the first 2 h, CI values were determined every 5 min, and thereafter at 15 min intervals.
128
129   *Cell cycle analysis*
130   PI was used to analyze the cell cycle distribution (Galkin et al., 2014). Briefly, cells (200,000
131   cells per well) were added to the wells of 6-well plates, incubated for 24 h and then for 48 h with
132   the test compounds or controls, harvested using free-EDTA trypsin, washed with PBS, and fixed
133   in cold 70% ethanol overnight at 4°C. The cells were subsequently stained with PI containing
134   RNAse A and the samples were analyzed using a flow cytometer (Beckman Coulter FC500).
135
136   *Transfection and luciferase reporter assay*.
137   The estrogen-responsive element $(ERE)_3$–Luc reporter construct (Hall & McDonnell, 1999)
138   containing three copies of the vitellogenin ERE was purchased from Addgene (plasmid no.
139   11354). The plasmid was purified using a Plasmid midi kit (QIAGEN, USA). Cells were grown
140   to 80%–90% confluence and transiently transfected with TransIT-LT1 Transfection Reagent
141   (Mirus Bio, USA). Firefly luciferase activity was measured using a Luciferase Assay System
142   (Promega, USA), and luminescence was measured using a LKB 1251 Luminometer.
143   Luminescence was normalized to the total protein content measured using the Bradford assay.
144
145   *RNA purification and real-time PCR*.
146   Total RNA was purified from MCF-7 cells using a Quick-RNA MiniPrep Kit according to the
147   manufacturer's protocol (Zymo Research, USA). Purified RNA was reverse transcribed using
148   SuperScript III reverse transcriptase (Thermo Fisher, USA) and used for real-time PCR
149   performed using an iCycler iQ amplifier (Bio-Rad, USA) with EVA Green Master Mix (Syntol,
150   Russia) according to the manufacturer's instructions. Primer sequences are listed in Table S1.
151   The efficiencies of the primers were within 88%–100%. The relative expression levels of the
152   target genes was determined using the ΔΔCt method with the calculated primer efficiencies.
153   *RPL32* mRNA encoding ribosomal protein L32 served as a reference.
154
155   *Statistical analysis*
156   Data were analyzed using the one-way ANOVA with post-hoc Tukey HSD, or with an unpaired
157   Student's *t*-test. P < 0.05 was considered to indicate a significant difference.

158

Results

159

160   *Effect of TPP-based compounds on the growth of an estrogen-dependent human breast cancer*
161   *cell line.*
162   We measured cell proliferation in the presence of 0.2 to 20 nM C_{12}TPP and SkQ1. SkQR1 served
163   as a negative control (Fig. 1). Higher concentrations (e.g., $\geq$200 nM) significantly inhibited cell
164   growth (Supplementary Fig. 1). E2 (10 nM) served as a positive control, and fulvestrant (10 nM)
165   served as an ERα antagonist. ER-positive MCF-7 cells were incubated for 48 h with the test
166   compounds with or without fulvestrant, and cell growth was measured using the resazurin test.
167   Treatment of MCF-7 cells with 10 nM E2 significantly ($p < 0.02$ [ANOVA]) enhanced cell
168   proliferation (Fig. 2). The TPP-based compounds and SkQR1 did not significantly affect cell
169   proliferation. However, 20 nM SkQ1, 0.2 nM C_{12}TPP, 20 nM C_{12}TPP and 20 nM SkQR1
170   increased resazurin fluorescence by approximately 10% compared with untreated cells (Fig. 2).
171   This increase was not significant and concentration-independent. Fulvestrant significantly
172   ($p<0.04$ [ANOVA]) lowered resazurin fluorescence in cells treated with 10 nM E2, and the
173   fluorescence of most samples decreased, although the differences were not significant.
174   The same experiments employing ERα-negative MDA-MB-231 cells did not reveal a significant
175   effect of E2 or the test compounds (Fig. 3). However, resazurin fluorescence increased after
176   treatment with 2 nM SkQ1, 20 nM SkQ1, 20 nM C_{12}TPP, or 20 nM SkQR1 but not with E2 (Fig.
177   3). These data suggest that resazurin fluorescence may not correlate with the number of cells but
178   with a slight change in mitochondrial metabolic activities potentially induced by TPP cations. In
179   agreement with this supposition, resazurin fluorescence was slightly enhanced by the
180   mitochondrial protonophore 2,4-dinitrophenol (12 μM DNP) when added to MCF-7 or MDA-
181   MB-231 cells (Fig. 2C, 3C). We concluded therefore that the resazurin assay is inapplicable to
182   the estimation of cell proliferation in the presence of mitochondria-targeted compounds. Thus,
183   other assays are required to unambiguously measure the effect of the TPP-based compound on
184   cell proliferation.
185   The potential of TPP-based compounds to affect the proliferation of ER-positive MCF-7 cells
186   was tested using an RTCA assay (Fig. 4). The test compounds, except E2, did not significantly
187   affect the CI value. E2 first increased the CI value of MCF-7 cells approximately 36 h after its
188   addition.

189

190   *Effect of TPP-based compounds on the cell cycle distribution of estrogen-dependent breast*
191   *cancer cells.*
192   Cell proliferation is characterized by increases in the numbers of cells in the S, G2, and M
193   phases. To determine the distribution of cells among these phases, we performed flow cytometric
194   analysis of MCF-7 cells treated for 48 h with the test compounds and then with PI. E2 treatment
195   increased the numbers of cells in the S, G2, and M phases (Fig. 5) There were no detectable
196   changes in cell cycle distribution after treatment of the MCF-7 cells with 20 nM SkQ1, 20 nM
197   C_{12}TPP, and 20 nM SkQR1 (Fig. 5).

198
199 *Effect of TPP-based compounds on the transcription driven by an estrogen-responsive promoter*
200 Direct binding of an agonist to ERα activates ERE-dependent transcription. To assess the effect
201 of TPP-based cations on ERE-dependent transcription, we transfected MCF-7 cells with the
202 luciferase reporter construct ERE_3–Luc, followed by incubation for 24 h with the test
203 compounds. Only E2 increased ERE-dependent transcription and corresponding luciferase-
204 induced luminescence (approximately 5-fold, p = 0.04 [Student's *t*-test]; Fig. 6). The E2
205 antagonist fulvestrant partially inhibited this effect (p = 0.21 [Student's *t*-test]). Treatment of the
206 transfected cells with 20 nM SkQ1, 20 nM C_{12}TPP, and 20 nM SkQR1 revealed no significant
207 change in luminescence (Fig.6).
208 To further evaluate the possible effect of SkQ1 on the transcriptional activity of the estrogen-
209 responsive promoters, we measured the expression in MCF-7 cells of E2 target genes encoding
210 the following proteins: trefoil factor 1 (TFF1); growth regulating estrogen receptor binding 1
211 (GREB1); cytochrome c oxidase subunit VI (COX6); insulin-like growth factor binding protein 4
212 (IGFBP4). These genes were chosen using available mRNA expression data for MCF-7 cells
213 treated with E2 (Yamaga et al., 2013) and relative mRNA expression was estimated using real-
214 time PCR.
215 E2 treatment of MCF-7 cells increased the levels of the transcripts of all target genes (Fig. 7).
216 The simultaneous addition of the estrogen antagonist fulvestrant with E2 partially prevented the
217 increase in the expression of *TFF1* and *GREB1*. Interestingly, the same effect of fulvestrant on
218 the expressions of *TFF1* and *GREB1* was observed in vehicle and SkQ1-treated samples,
219 indicating the presence of a small amount of estrogen mimetics in the culture medium.
220 Treatment with any concentration of SkQ1 did not significantly influence the mRNA levels of all
221 E2 target genes. Nevertheless, in some experiments 0.2 nM and 20 nM SkQ1 increased *IGFBP4*
222 expression (p = 0.047 and p = 0.13, respectively [Student's *t*-test]). However, *IGFBP4*
223 expression was not decreased in E2-treated cells treated with fulvestrant, and *IGFBP4* mRNA
224 levels therefore cannot be considered to reliably indicate signaling downstream ERα.
225

226 **Discussion**
227 Testing the effects of any compound on the proliferation of ERα-positive cells is commonly
228 employed to evaluate estrogenic properties. There are several methods available to measure cell
229 proliferation rates. The most popular methods, the MTT assay and the resazurin test, rely on the
230 measurement of activity of mitochondrial enzymes. The resazurin assay measures the activity of
231 mitochondrial dehydrogenases, which convert the nonfluorescent dye resazurin to the strongly-
232 fluorescent dye resorufin. However, the mitochondrial function greatly affects the NAD(P)H-
233 based viability assays, including the resazurin test (Aleshin et al., 2015). Mitochondrial
234 uncoupling, in particular, leads to the overestimation of the MTT test results (Maioli et al.,
235 2009). Moreover, numerous penetrating cations such as SkQ1, C_{12}TPP, and SkQR1 induce
236 mitochondrial uncoupling mediated by free fatty acids (Severin et al., 2010; Antonenko et al.,
237 2011). In an agreement with these data, we observed a moderate statistically insignificant

238   increase in the resazurin fluorescence in MCF-7 cells treated with the mitochondrial penetrating
239   cations or with the "classical" protonophore DNP (Fig. 2C; Fig.3C).
240   We found it interesting that the test compounds induced slightly higher resazurin fluorescence in
241   MCF-7 than in triple-negative MDA-MB-231 cells (Figs. 2 and 3). We speculate that the
242   uncoupling of oxidative phosphorylation in mitochondria and corresponding activity of
243   mitochondrial dehydrogenases may differ between these cell lines because the basal oxygen
244   consumption rate and proton leakage significantly differs between these cells (Radde et al.,
245   2016). Generally, the MTT and resazurin tests are suitable for the compounds that do not
246   influence mitochondrial function. Therefore, these tests cannot be applied to the study of TPP-
247   based cations with mitochondrial uncoupling activities.
248   We therefore used the RTCA assay to measure the effects of TPP-based compounds on the
249   proliferation of MCF-7 cells. We found that these compounds did not significantly influence the
250   CI value, which is directly proportional to the quantity, size, and attachment forces of the cell
251   (Fig.4). Further, microscopic examination did not reveal morphological changes induced by E2
252   or the test compounds (Supplementary Fig. 2). Moreover, the CI value is proportional to the cell
253   number measured using the RTCA assay, and TPP cations therefore did not significantly
254   influence the proliferation of MCF-7 cells. Furthermore, we did not observe a detectable effect of
255   TPP-based compounds on cell cycle distribution (Fig. 5), showing that TPP cations did not
256   enhance the proliferation of ERα-positive MCF-7 cells.
257   Despite the absence of detectable stimulatory effects of TPP on the proliferation of MCF-7 cells,
258   we cannot exclude the direct binding of TPP to ERα induces downstream signaling that regulates
259   ERE-dependent gene expression. Specifically, we found no detectable effect of TPP cations on
260   the luciferase activity of MCF-7 cells transfected with the ERE$_3$-luciferase reporter (Fig.6).
261   However, the structures of the promoter regions of ERE-dependent genes is much more complex
262   than the three ERE of the reporter. Further, E2 may indirectly modulate gene expression via the
263   interaction of ER with transcription factors such as NF-κB, AP-1, and Sp-1, which, in turn, bind
264   their cognate DNA regulatory elements (Marino, Galluzzo & Ascenzi, 2006). We therefore
265   tested SkQ1 as a representative TPP-based compound for its ability to regulate the expression of
266   the ER-dependent genes GREB1, TFF1, COX6, and IGFBP4. We found that SkQ1, at any
267   concentration tested, did not significantly upregulate the expression of these genes (Fig.7).
268   Nevertheless, the expression of *IGFBP4* mRNA varied, and the effect of SkQ1 on its expression
269   requires further investigation. Our present data therefore lead us to conclude that SkQ1 did not
270   directly affect ERα and its associated downstream signaling activity related to the expression of
271   ER-dependent genes.
272   In agreement with this conclusion, several lines of evidence, described below, indicate that TPP-
273   based compounds do not possess proestrogenic effects:
274   • SkQ1 enhances human neutrophils apoptosis (Andreev-Andrievskiy et al., 2016;
275   Vorobjeva et al., 2017), and E2 has the opposite effect (Molloy et al., 2003).
276   • Though global gene expression data for TPP-based compounds are scarce, available data
277   on oral administration of MitoQ to wild-type mice indicate that MitoQ treatment does not

278   affect a specific process or signaling pathway mediated by sex hormones (Rodriguez-
279   Cuenca et al., 2010).

280   • In mouse and rat strains, estrogen increases the incidence of tumorigenesis in several
281   organs (Vollmer, 2003). In three mouse strains studied, prolonged SkQ1 treatment does
282   not affect spontaneous carcinogenesis (Yurova et al., 2011). Moreover, SkQ1 does not
283   increase the development of spontaneous tumors in BALB/c mice (Manskikh et al.,
284   2014).

285 Theoretically, mitochondria-targeted compounds may possess estrogen-like activities that are not
286 due to TPP cation. For example, certain plant quinones are phytoestrogens (Davis, 2002), and
287 plastoquinone and ubiquinone are essential components of SkQ1 and MitoQ antioxidants,
288 respectively. There are no reports, to our knowledge, about the ER-related activities of these
289 quinones or the rhodamine 19 moiety of SkQR1. Our data further confirm the absence of
290 estrogen-like activities of plastoquinone and the quinone of rhodamine 19.

291 The present study has the following limitations: first, we did not investigate the direct binding of
292 the compounds to ERα. However, our data strongly suggest low or no binding of the test
293 compounds to ERα. Second, we only used cell lines derived from breast cancers. Thus, the
294 corresponding primary cells may react in a different way. Third, we cannot exclude the
295 possibility that *in vivo* environmental conditions may greatly differ from the *in vitro*
296 experimental conditions used here. Hypothetical coregulatory factors in tissues and organs may
297 be involved in indirect estrogen-dependent signaling pathways together with TPP cations. Thus,
298 indirect involvement of TPP-based compounds in estrogen signaling cannot be fully excluded.

299

Conclusions

301 For the first time, to our knowledge, we show here that TPP-based cations do not possess
302 properties of typical ERα agonists. Our finding therefore opens new possibilities for safe use of
303 TPP-based compounds in preclinical and clinical applications.

304

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Figure 1

Mitochondria-penetrating cations used in the present study.

(A) C$_{12}$TPP. (B) SkQ1. (C) SkQR1.
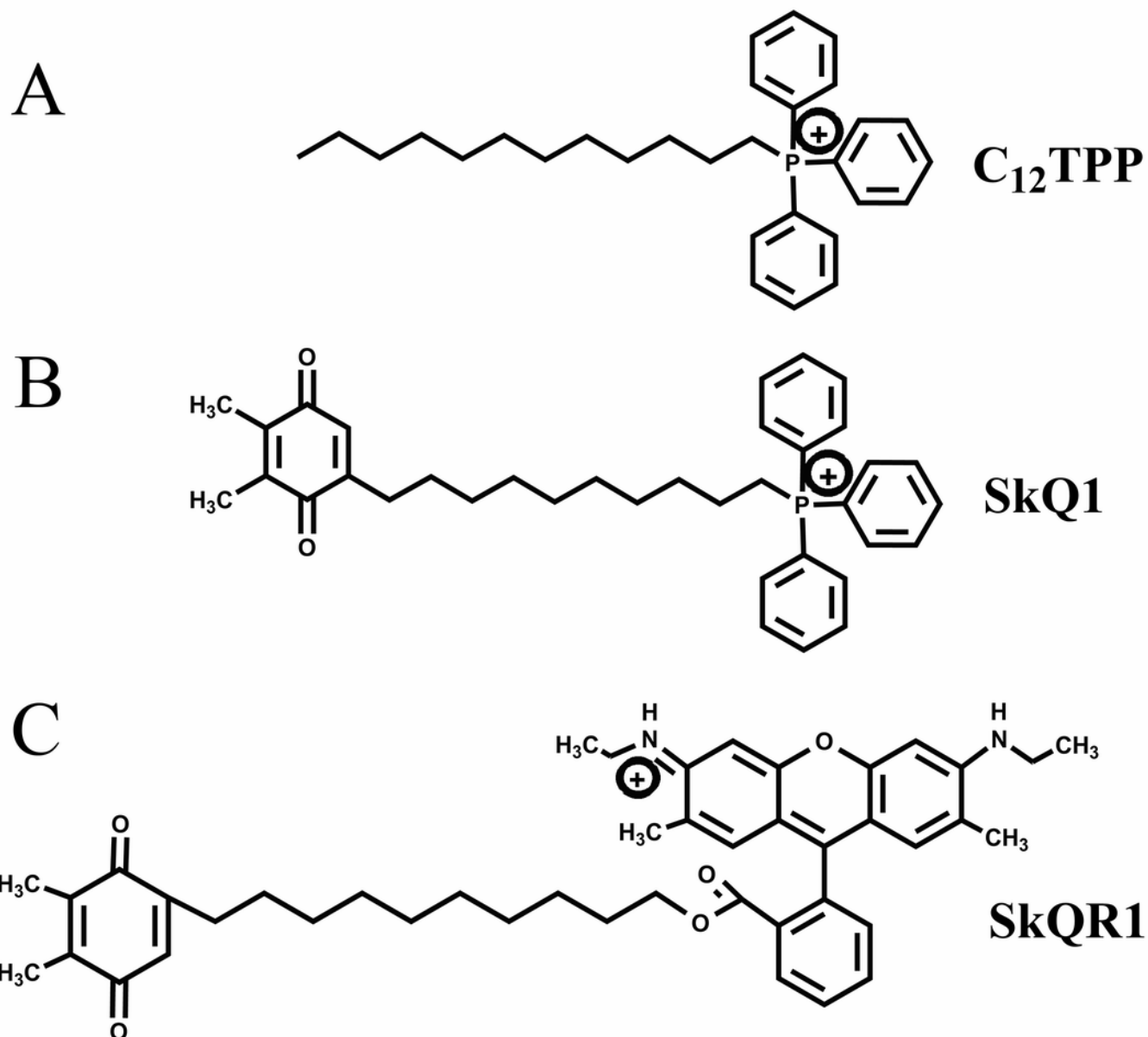
Figure 2

Effect of mitochondria-targeted compounds on the proliferation of ER-positive MCF-7 cells.

Cells (approximately 5,000 cells per well) were incubated for 48 h with (A) SkQ1; (B) $C_{12}TPP$; (C) SkQR1, and DNP. Data are expressed as the percentage of the control cells +/− standard deviation (SD), n = 5–6. P values were determined using the one-way ANOVA with post-hoc Tukey HSD. *p, E2 compared with the control; #p, E2 and E2 + fulvestrant.
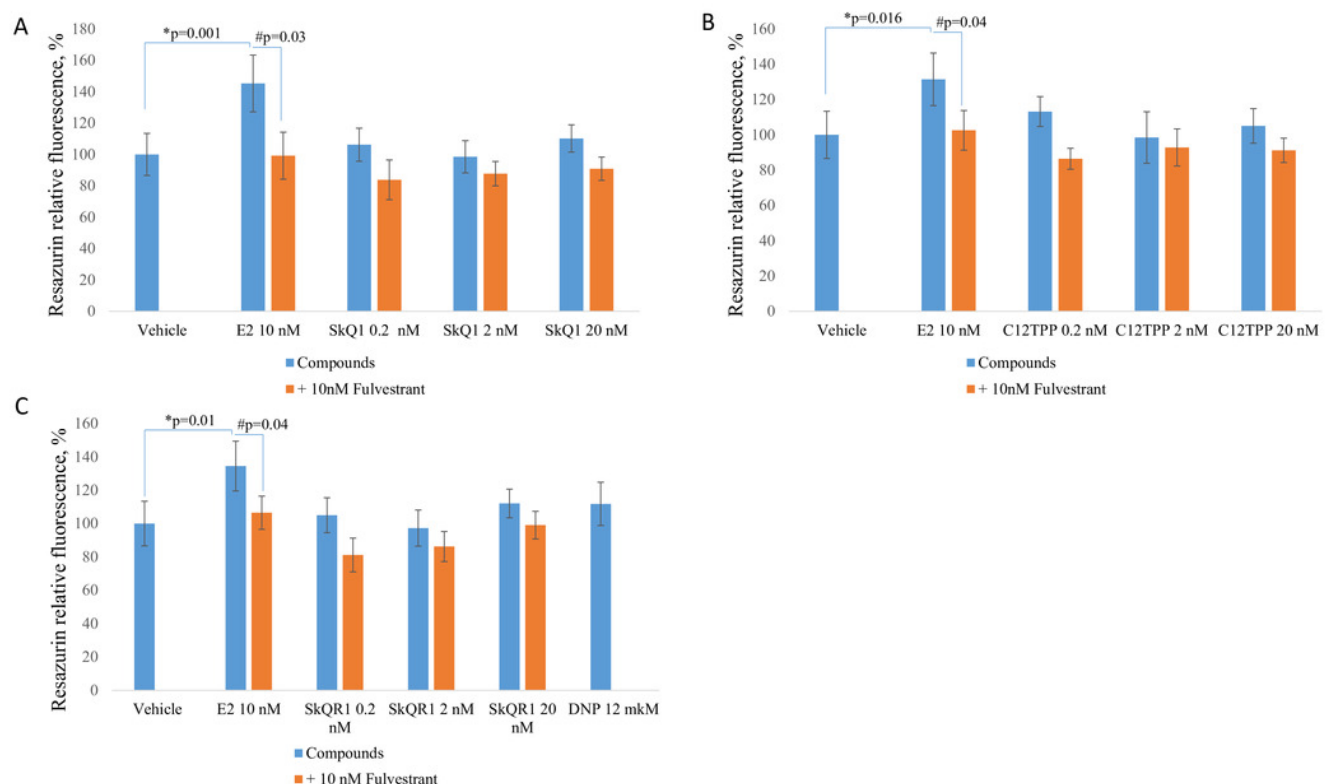
Figure 3

Effect of mitochondria-targeted compounds on the proliferation of ER-negative MDA-MB-231 cells.

The cells were cultured and treated as shown in Fig. 2 and proliferation was analyzed using a resazurin assay. (A) SkQ1; (B) C_{12}TPP; (C) SkQR1, and DNP. Data are expressed as a percentage of the control cell cultures +/− SD, n = 4.
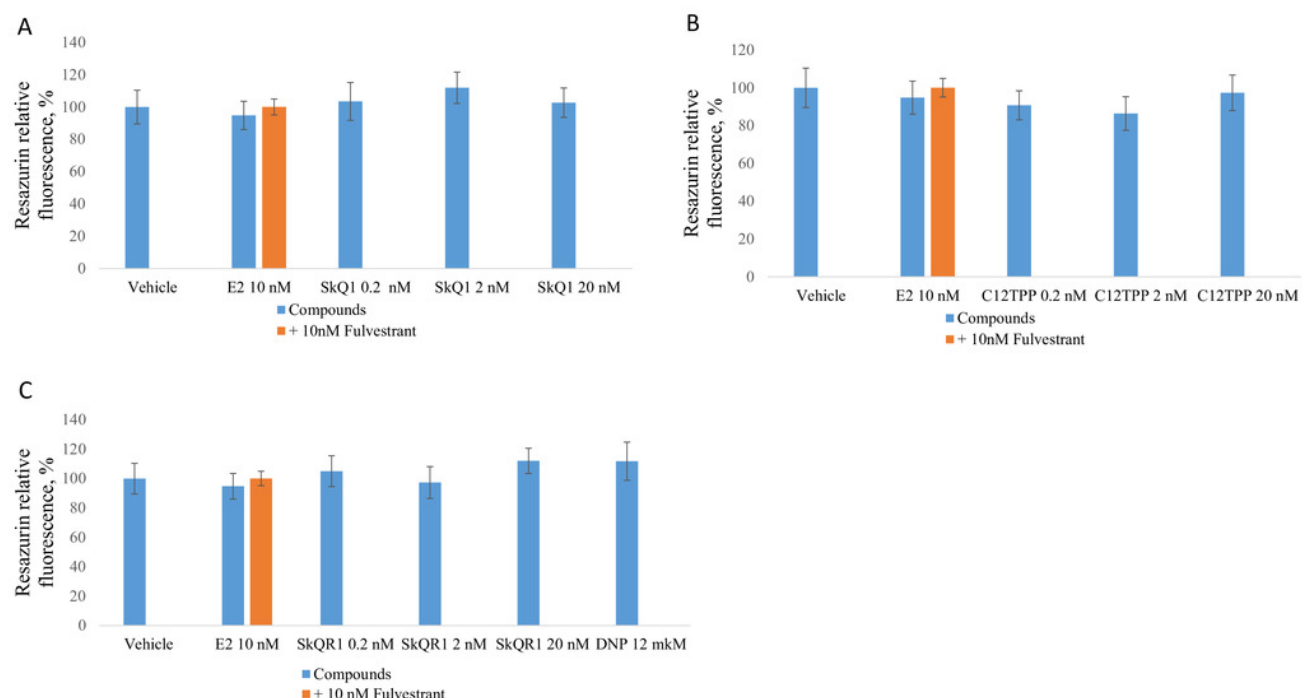
Figure 4

Proliferation of ER-positive MCF-7 cells treated with mitochondria-targeted compounds.

CI values were dynamically monitored using a xCELLigence system after the administration (arrow) of 10 nM E2 (Estrogen), 20 nM SkQ1 (SkQ1), 20 nM C_{12}TPP (C12TPP), or 20 nM SkQR1 (SkQR1). The average values of triplicate experiments are plotted against treatment time.
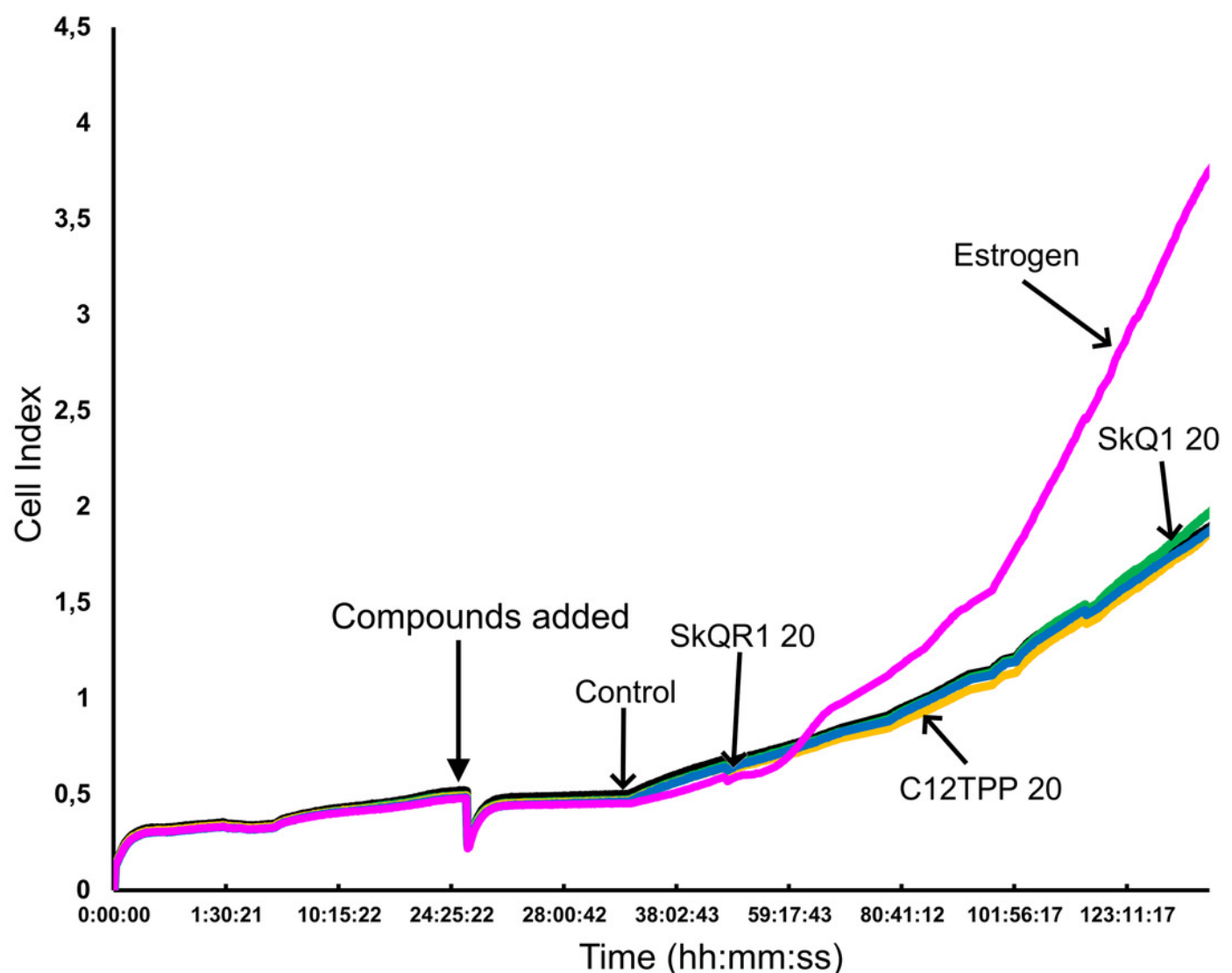
Figure 5

Cell cycle analysis of ER-positive MCF-7 cells.

Cells were treated as shown in Fig. 2. The cell cycle was analyzed using flow cytometry of PI-stained cells. (A-E) Percentages of cells in S/G2/M phases. (A) "Control," untreated cells. (B) "Estrogen," 10 nM E2. (C) "SkQ1," 20 nM SkQ1. (D) "C12TPP," 20 nM C_{12}TPP. (E) "SkQR1," 20 nM SkQR1. (F) Distributions of cells in G1/G0, S, and G2/M phases.

	SubG1	G0/G1	S	G2/M	Polyploidy
Control	1.3	70.5	5.2	15.6	6.4
Estrogen	0.3	38.7	14.4	26.3	20.2
SkQ1	2.2	66.6	3.7	18.8	8.9
C_{12}TPP	2.1	65.1	4.5	18.1	10.5
SkQR1	1.7	64.3	4.4	18.5	10.1
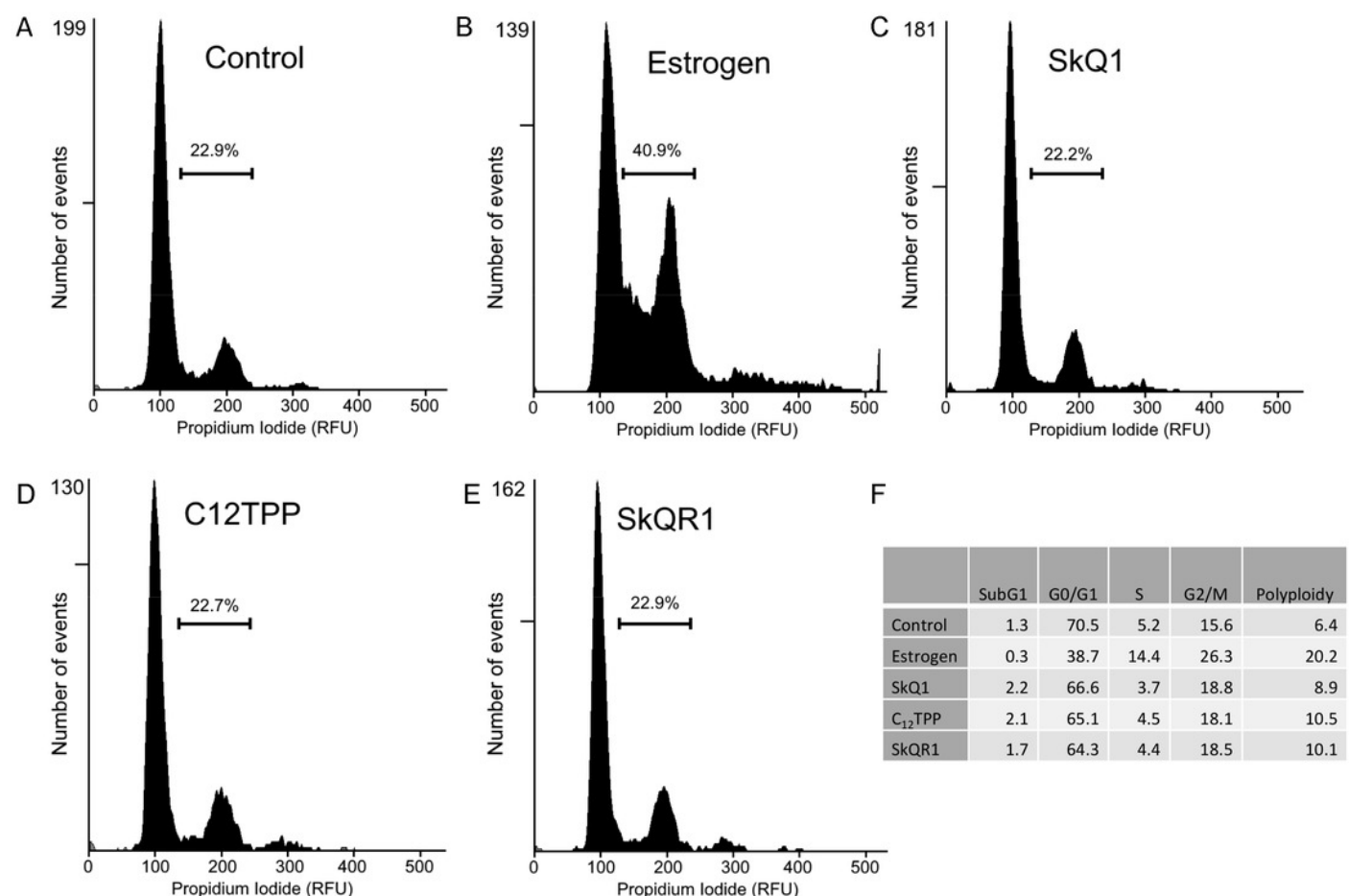
Figure 6

TPP-based compounds do not enhance expression driven by estrogen-responsive promoters in MCF-7 cells.

Cells were transfected with an ERE$_3$–Luc reporter construct. Test compounds were added 24 h after transfection followed by measurement of luminescence 24 h later. Luminescence was normalized to the total protein content. Data are expressed as the percentage of untreated transfected cells +/− SD, n = 3; *p = 0.04, untreated control vs E2-treated cells; #p = 0.04, untreated vs cells treated with both E2 and fulvestrant (unpaired Student's t-test).
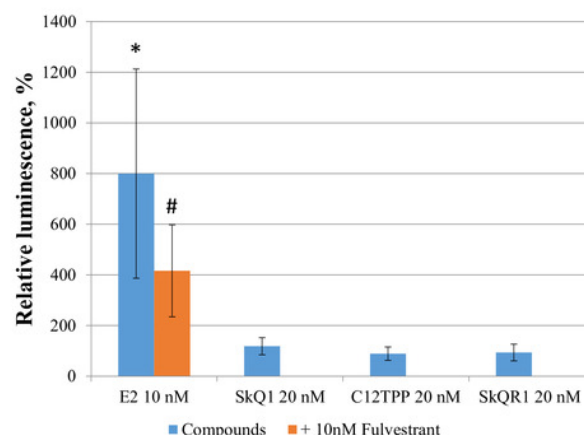
Figure 7

Analysis of gene expression in MCF-7 cells.

MCF-7 cells were treated as shown in Fig. 2, and the levels of mRNAs encoding GREB1, IGFBP4, COX6, and TFF1 were normalized to the reference *RPL32* mRNA. The relative expression of the target genes in the untreated control cells is expressed as 100%. The values represent the mean +/− SD, n = 4. P-values were calculated using the unpaired Student's *t*-test; #p, fulvestrant (Fulv) vs corresponding sample without fulvestrant; *p, samples without fulvestrant vs control.