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Selective Estrogen Receptor Modulator Delivery of Quinone Warheads to DNA Triggering Apoptosis in Breast Cancer Cells

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ABSTRACT Estrogen exposure is a risk factor for breast cancer, and estrogen oxidative metabolites have been implicated in chemical carcinogenesis. Oxidation of the catechol metabolite of estrone (4-OHE) and the β-naphthohydroquinone metabolite of equilenin (4-OHEN) gives *o*-quinones that produce ROS and damage DNA by adduction and oxidation. To differentiate hormonal and chemical carcinogensis pathways in estrogen receptor positive ER(+) cells, catechol or β-naphthohydroquinone warheads were conjugated to the selective estrogen receptor modulator (SERM) desmethylarzoxifene (DMA). ER binding was retained in the DMA conjugates; both were antiestrogens with submicromolar potency in mammary and endometrial cells. Cytotoxicity, apoptosis, and caspase-3/7 activation were compared in ER(+) and ER(−)MDA-MB-231 cells, and production of ROS was detected using a fluorescent reporter. Comparison was made to DMA, isolated warheads, and a DMA-mustard. Conjugation of warheads to DMA increased cytotoxicity accompanied by induction of apoptosis and activation of caspase-3/7. Activation of caspase-3/7, induction of apoptosis, and cytotoxicity were all increased significantly in ER(+) cells for the DMA conjugates. ROS production was localized in the nucleus for conjugates in ER(+) cells. Observations are compatible with β-naphthohydroquinone and catechol groups being concentrated in the nucleus by ER binding, where oxidation and ROS production result, concomitant with caspase-dependent apoptosis. The results suggest that DNA damage induced by catechol estrogen metabolites can be amplified in ER(+) cells independent of hormonal activity. The novel conjugation of quinone warheads to an ER-targeting SERM gives ER-dependent, enhanced apoptosis in mammary cancer cells of potential application in cancer therapy.

Observations from clinical trials and epidemiological studies support the hypothesis that estrogen contributes to breast cancer and may be a causative agent (*1, 2*). The large prospective Women's Health Initiative Study confirmed that the increased risk of breast cancer in postmenopausal women is strongly linked to exposure to estrogen replacement therapy (ERT) agents that contain conjugated endogenous and equine estrogens (*3, 4*). The collective evidence supports the concept of: (1) an hormonal, proliferative, antiapoptotic contribution to breast cancer from estrogens (*5, 6*) together with (2), a contribution from chemical carcinogenesis resulting from the genotoxic and mutagenic actions of estrogen oxidative metabolites (*7, 8*). In human breast tissue, DNA adducts of equine estrogens have been reported (*9*). The 4-hydroxy catechol metabolite (4-hydroxyestrone, 4-OHE) of endogenous estrogen oxidation by cytochrome P450 has been proposed to be carcinogenic via formation of DNA-reactive, electrophilic *o*-quinones that also generate DNA-damaging, reactive oxygen species (ROS) (Figure 1, panel a) (*10–12*).

The relative importance of contributions from hormonal carcinogenesis versus chemical carcinogenesis pathways remains unknown, as do the relative contributions to chemical carcinogenesis from DNA alkylation versus DNA oxidation. A further related question is the contribution of the equine estrogen oxidative metabolite, 4-hydroxyequilenin (4-OHEN), since autoxidation and facile redox cycling differentiates 4-OHEN from 4-OHE (Figure 1, panel a) (*13, 14*). Autoxidation of 4-OHEN yields a naphthoquinone that induces DNA damage including single strand breaks, oxidized bases, apurinic sites, and formation of cyclic DNA adducts, both *in vitro* and *in vivo* (*9, 15–18*). Furthermore,

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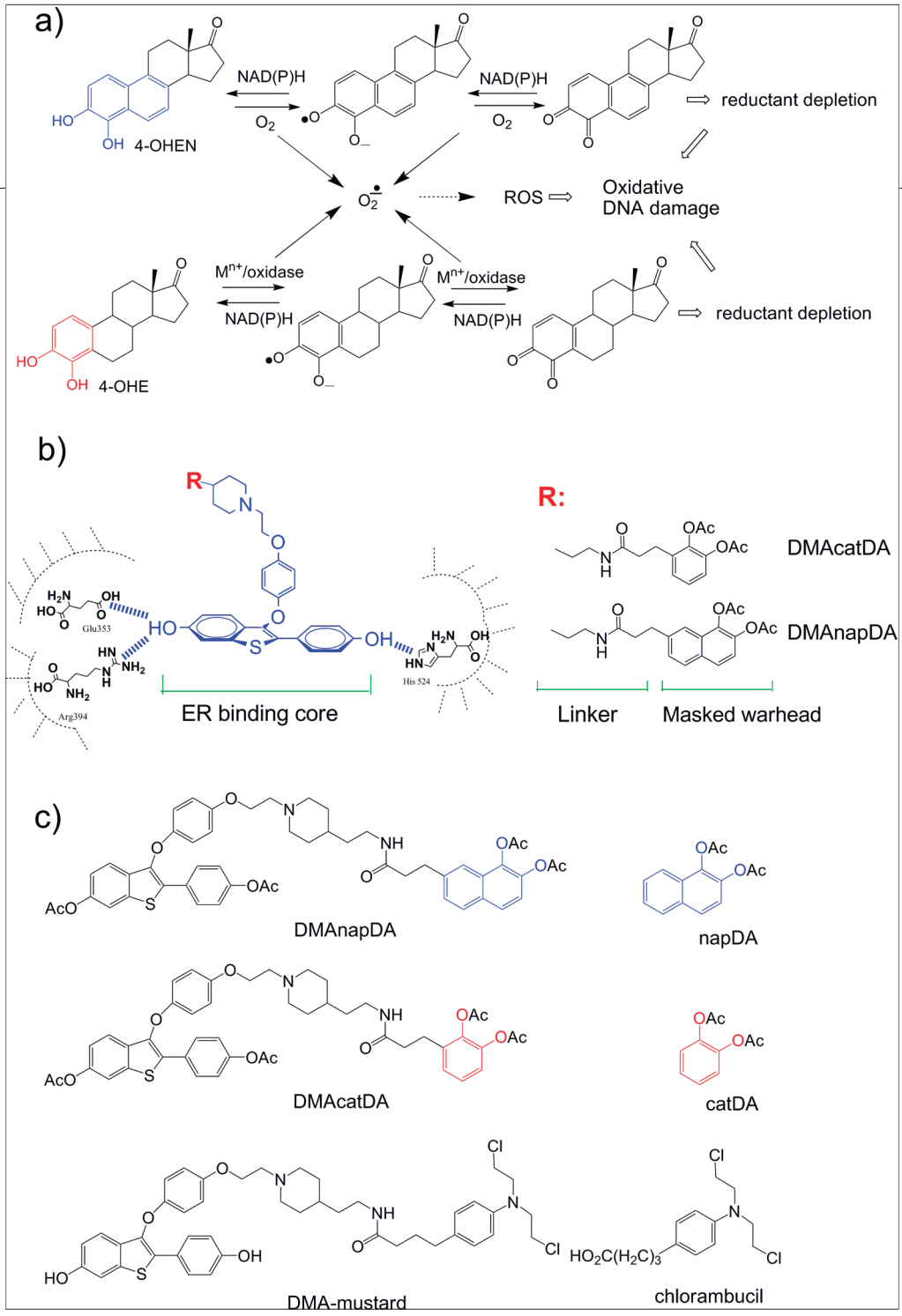

Figure 1. Design of QPEDs (quinone prodruxgs with ER delivery) based upon the reactive quinone metabolites of equine and human estrogens. (a) The human estrogen oxidative metabolite 4-OHE and the equine estrogen oxidative metabolite 4-OHEN are further oxidized to *o*-quinones generating superoxide and ROS. 4-OHEN autoxidizes and redox cycles, whereas 4-OHE requires metal ion or oxidase catalysis of oxidation. (b) Figurative scheme of QPED, showing DMA delivery unit bound to ERa; the R group is the payload containing the linker and warhead; the warhead requires bioactivation by (i) non-specific esterase activity, and (ii) oxidation to an *o*-quinone. (c) Detailed chemical structures of study compounds: NapDA is the diacetate masked warhead of DMAnapDA, which mimics 4-OHEN; whereas catDA is the masked warhead of DMAcatDA, which mimics 4-OHE.

4-OHEN rapidly induces DNA oxidation and DNA strand breaks with selectivity for ER(+) cells, compatible with a "Trojan horse" mechanism whereby estrogen receptor (ER) binds and concentrates 4-OHEN in the nucleus proximal to ER-binding DNA sequences (*17*).

Any approach to these unknowns is hindered by the difficulty of differentiating hormonal from chemical effects in ER(+) cells, which may be further confounded by the estrogenic regulation of cellular antioxidant systems (*19*). To circumvent this obstacle, a selective estrogen receptor modulator (SERM) was conjugated via a chemical linker to the reactive chemical moieties of 4-OHE and 4-OHEN, a catechol and a β-naphthohydroquinone, respectively (Figure 1, panels b and c). Owing to the known autoxidation of 4-OHEN, both of these *o*-quinone-delivery warheads were protected as diacetates to facilitate cell permeation and enhance intracellular bioavailability. The delivery unit selected was desmethylarzoxifene (DMA), a benzothiophene SERM with high ER binding affinity and well-established antiestrogenic activity in ER(+) breast cancer cells (*20, 21*). As depicted in Figure 2, these quinone prodrug with ER delivery (QPED) conjugates are anticipated to reveal the capacity of *o*-quinone warheads to damage DNA selectively in ER(+) cells, in the absence of confounding estrogenic activity, and hence to provide information on potential contributions to chemical carcinogenesis of 4-OHE and 4-OHEN.

Because QPEDs are ER-targeted agents of DNA damage, they may represent lead compounds for selective chemotherapy of estrogen-sensitive tumors including breast, endometrial, and ovarian cancers. In addition to providing DNA damage, QPEDs are expected to retain the antiestrogenic activity of the parent SERM. To minimize side effects on healthy cells, chemotherapeutics can be targeted to molecular abnormalities needed for tumor growth and carcinogenesis. In breast cancer, such targets include growth factor receptors and the ER: 75% of tumors in postmenopausal women and 50% in premenopausal women express ER (*22, 23*). Traditional chemotherapeutics that damage DNA by adduct formation include platinum complexes and mustards, both of which have been conjugated to estrogen itself (*23–25*). Examples of quinones with anticancer activity that form DNA adducts are known, mostly derived from natural products, for example, β-lapachone (*26, 27*).

To our knowledge, this is the first report of synthetic conjugation of quinone warheads to an ER-targeting SERM leading to DNA damage. To provide comparison with the QPEDs, a third DMA conjugate was prepared containing a nitrogen mustard, in addition to both masked catechol warheads without DMA delivery units (Figure 1, panel c). Antiestrogenic activity of QPEDs was retained, cytotoxicity was selective toward ER(+) cells via induction of apoptosis, and ROS production was observed to be amplified in the nucleus of ER(+) cells.

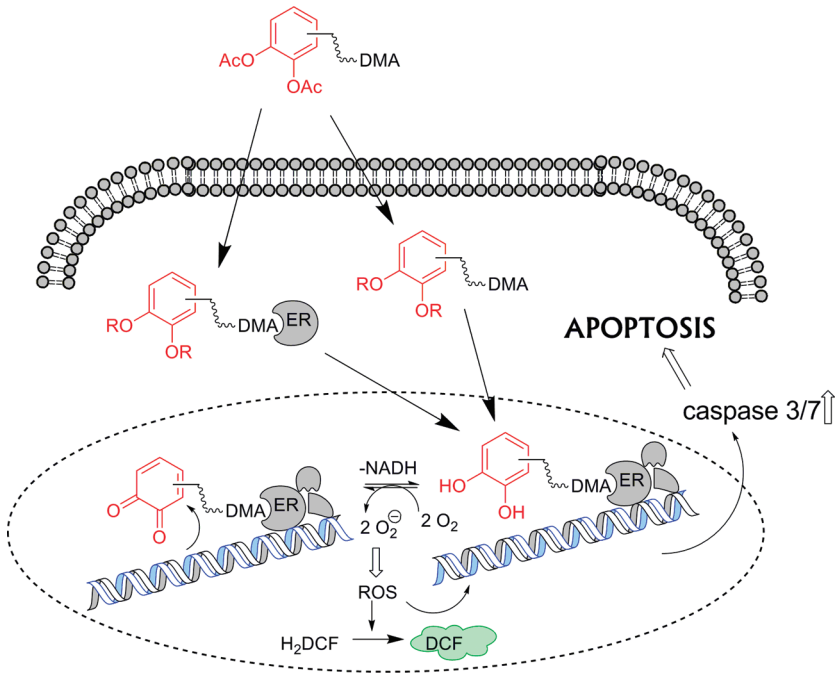

Figure 2. DMAcatDA and DMAnapDA molecules are designed as quinone prodrugs with ER delivery (QPEDs). QPEDs are anticipated to be unmasked by esterase activity in the cytoplasm, to bind to ERα, and to be concentrated in the nucleus proximal to ERE sequences of DNA. Oxidation of the unmasked catechol to a quinone will lead to ROS generation (detected by DCF fluorescence), redox cycling, and DNA damage via electrophilic (covalent) and oxidative mechanisms. The evidence presented herein shows that the consequence is apoptosis via caspase activation. R = H, Ac.

RESULTS AND DISCUSSION

ER Binding and Antiestrogenic Activity. SERMs provide excellent scaffolds for bioconjugation, because the extended side chain that disrupts ER helix 12, responsible for antiestrogenic activity, extends to the extremity of the ER ligand binding site. DMA is an active metabolite of the preclinical benzothiophene SERM ([6-hydroxy-3-[4-[2-(1-piperidinyl)-ethoxy]phenoxy]-2-(4-methoxyphenyl)]benzo[*b*]thiophene) (arzoxifene) that is itself an improved version of the SERM raloxifene, used clinically for postmenopausal indications and breast cancer chemoprevention (*28, 29*). DMA is a potent SERM: in the standard estradiol radioligand competitive binding assay, the measured IC_{50} for ERα and ERβ is <10 nM (*21*). Thus, extension of the 3-position side chain of DMA has been reported to produce conjugates that retain antiestrogenic activity; for example, conjugation of DMA with azide, a large biotin linker group, and a fluorophore linker group yielded compounds with IC_{50} for ERα of 14.8, 9.4, and 28.7 nM, respectively (*30, 31*). Hence, a similar strategy was adopted for 3-(3-(2-(1-(2-(4-(6-acetoxy-2-(4-acetoxyphenyl)benzo[*b*]thiophen-3-yloxy)phenoxy)ethyl)piperidin-4-yl)ethylamino)-3-oxopropyl)-1,2-phenylene diacetate (DMAcatDA), 7-(3-(2-(1-(2-(4-(6-acetoxy-2-(4-acetoxyphenyl)benzo[*b*]thiophen-3-yloxy)phenoxy)ethyl)piperidin-4-yl)ethylamino)-3-oxopropyl)naphthalene-1,2-diyl diacetate (DMAnapDA), and 4-(4-(bis(2-chloroethyl) amino) phenyl)-*N*-(2-(1-(2-(4-(6-hydroxy-2-(4-hydroxyphenyl) benzo[*b*]thiophen-3-yloxy)phenoxy)ethyl)piperidin-4-yl)ethyl)butanamide (DMA-mustard) (Figure 1, panel c). In the competitive binding assay, IC_{50} for the DMA-mustard was measured as 37 ± 11 and 67 ± 20 nM, for ERα and ERβ, respectively (Table 1).

TABLE 1. Antiestrogenic and cytotoxic potency of QPEDs and controls in cell culture[a]

	ERα-binding IC_{50} (nM)	MCF-7 cell antiestrogenic IC_{50} (nM)	Ishikawa cell antiestrogenic IC_{50} (nM)	S30 cell cytotoxicity LC_{50} (μM)	MDA-MB-231 cytotoxicity LC_{50} (μM)
DMA	7.8 ± 1.9	1.1 ± 0.1	0.1 ± 0.1	23.0 ± 0.8	23.5 ± 0.5
DMAcatDA	N.D.	129 ± 23	10.3 ± 1.6	1.6 ± 0.5	7.8 ± 0.3
DMAnapDA	N.D.	502 ± 95	101 ± 5.9	3.2 ± 0.7	8.2 ± 2.5
DMA-mustard	37 ± 11	103 ± 8	17.6 ± 2.6	>40	>40
catDA	N.D.	>10 000	>10 000	>40	>40
napDA	N.D.	>10 000	>10 000	8.8 ± 1.4	8.9 ± 0.9
chlorambucil	N.D.	>10 000	>10 000	>40	>40

[a]Data show means and standard deviation. N.D., not determined.

Gene expression elicited by ligand binding to ER is dependent on receptor dimerization, formation of complexes with coregulator proteins, and binding of these complexes to DNA. Therefore, cell-based assays provide a more complete picture of estrogenic/antiestrogenic activity, in addition to revealing cell membrane permeation. Two human ERα(+) cell systems routinely used for profiling classical estrogenic activity are MCF-7 breast cancer cells transiently transfected with an estrogen response element (ERE)-luciferase reporter and the endometrial Ishikawa cell line that incorporates an engineered estrogen sensitive alkaline phosphatase activity. For comparison, DMA-biotin and DMA-fluorophore conjugates demonstrated IC_{50} values of 9 and 29 nM in MCF-7 cells and 7 and 23 nM in Ishikawa cells, respectively (*32*). Expectedly, estrogenic activity was not observed for QPEDs and these compounds antagonized the effects of estradiol (E_2, 1 nM) in both cell systems (Table 1). Given the different cellular contexts and ER coregulators, relative and absolute potency can vary with different ligands. In MCF-7 cells, QPEDs were less potent antiestrogens with IC_{50} for DMAcatDA, DMAnapDA, and DMA-mustard measured as 129 nM, 502 nM, and 103 nM respectively; whereas in Ishikawa cells, IC_{50} for DMAcatDA, DMAnapDMA, and DMA-mustard was 10.3, 101, and 17.6 nM, respectively (Figure 3; Table 1). Tethering of a catechol diacetate (catDA) or a β-naphthohydroquinone diacetate (napDA) warhead to the DMA SERM scaffold reduced antiestrogenic activity relative to DMA, but the QPEDs retained submicromolar potency in cell culture. The control compounds, catDA and napDA, did not show any antiestrogenic activity at 1 μM.

Cytotoxicity in ER(+) versus ER(−) Cells. To examine the selective toxicity of QPEDs toward ER(+) cells compared to ER(−) cells, cytotoxicity was evaluated by the sulforhodamine B (SRB) colorimetric assay, a widely used method for *in vitro* cytotoxicity screening using cell density determination based on the measurement of cellular protein content (*33*). Although comparisons are often made between two breast cancer cell lines, ER(+) MCF-7 cells and ER(−) MDA-MB-231 cells, the comparison of ER(+) S30 cells with MDA-MB-231 cells provides an identical cellular background, because S30 cells are MDA-MB-231 cells stably transfected with wild type ERα. The most dramatic observation of cytotoxicity toward these human mammary epithelial cancer cells was in the comparison of catDA with DMAcatDA (Figure 4, panels a and c): the catechol warhead showed no significant toxicity at the concentrations studied (≤20 μM), whereas the corresponding QPED was cytotoxic; in ER(+) cells, DMAcatDA was a relatively potent cytotoxin with LC_{50} = 1.6 μM. In contrast, the napDA warhead was cytotoxic in S30 cells, and DMAnapDA was only a 4-fold more potent cytotoxin than the napDA (Figure 4, panel a; Table 1). Both QPEDs were equally potent cytotoxins in S30 cells. DMA itself was cytotoxic at higher concentrations; however, no cytotoxicity was observed for the DMA-mustard below solubility limits.

DMA was toxic at higher concentrations comparable to those reported previously for benzothiophene SERMs; however, cytotoxicity in breast cancer cells was not dependent on ER status (Figure 4, panel a; Table 1). Similarly, LC_{50} values obtained for napDA were also independent of ER status (Figure 4, panel c; Table 1). However, the cytotoxicity of both QPEDs was enhanced in ER(+) S30 cells relative to the ER(−) background MDA-MB-231 cells; DMAcatDA was 5-fold more toxic, and DMAnapDA toxicity was enhanced 2.5-fold.

Localization of Cellular ROS Production. The cell permeant fluorescence reporter, 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydrofluorescein diacetate (CM-H_2DCFDA), was used to identify and localize ROS generation in S30 and MDA-MB-231 cells. The same method was reported previously to demonstrate the rapid generation of ROS by 4-OHEN, which was observed to be localized in the nucleus of the ER(+) cell line but not localized to a cell compartment in MDA-MB-231 cells (*17*). CM-H_2DCF is sensitive to oxidation by oxidizing oxygen radicals including hydroxyl radicals formed from Fenton reactions of H_2O_2 (*34*) and is useful for localization of these cellular ROS, in combination with nuclear dyes, using confocal fluorescence microscopy. In ER(−) MDA-MB-231 cells, after incubation for 1 h with QPEDs and the catechol warheads, a general increase in cellular DCF fluorescence was observed, but this was not localized to the nucleus (Figure 5, panel b). This experiment

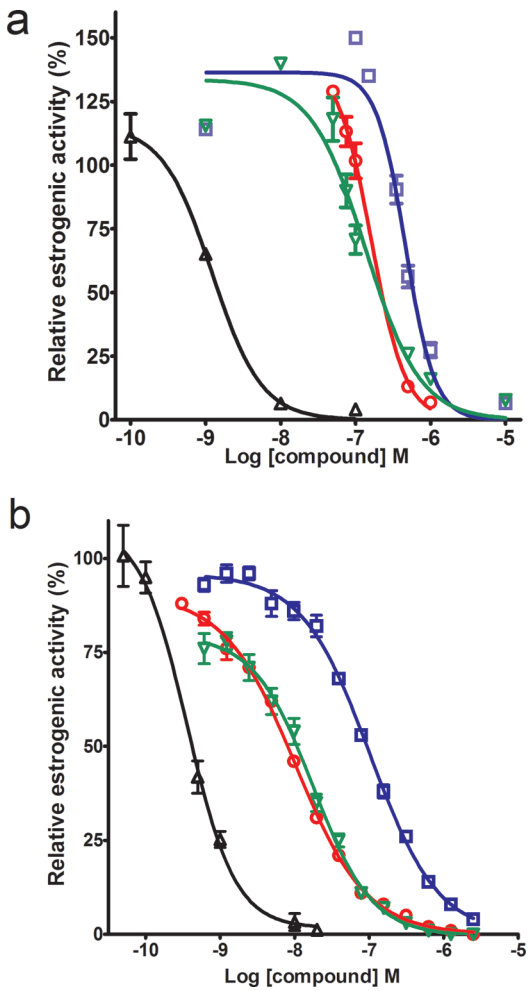

Figure 3. Antiestrogenic activity of QPEDs compared to DMA-mustard and DMA in MCF-7 and Ishikawa cells (blue squares, DMAnapDA; red circles, DMAcatDA; green inverted triangles, DMA-mustard; black triangles, DMA). (a) Concentration−response curves for inhibition of estrogen-induced ERE-luciferase activity in MCF-7 cells. Cells were transfected with ERE-luciferase plasmid for 24 h and then treated with different concentrations of drug in the presence of E_2 (1 nM) for 18 h. (b) Concentration−response curve for inhibition of estrogen-induced alkaline phosphatase activity in Ishikawa cells. Cells were seeded in 96-well plates overnight in estrogen-free medium prior to treatment with compounds in the presence of E_2 (1 nM) for 4 d. Data showing mean and sem for three separate cultures was fitted to a sigmoidal curve.

was designed to observe ROS localization, rather than quantify ROS production; therefore, it is unsafe to speculate on relative amounts of ROS from each agent. However, the same experiment carried out in ER(+) S30 cells definitively demonstrated selective increases in DCF fluorescence in the nuclei of QPED treated cells (Figure 5, panel a). In these cells, ROS detected from treatment with napDA and catDA were not localized, but ROS production from DMAnapDA and DMAcatDA was clearly localized in the nuclei as shown by overlay of images with stained nuclei. Curiously, cells treated with DMAcatDA produced a more intense localization within nuclear organelles, possibly nucleoli. These observations clearly support the ability of the ER to bind and concentrate QPEDs in the cell nucleus, leading to enhanced ROS generation in the nucleus.

Induction of Apoptosis. Oxidative stress is implicated in numerous cellular events including apoptosis, providing a cytotoxic pathway that has been proposed to be triggered by numerous chemotherapeutic agents, including ROS-generating flavonoids, such as the experimental anticancer agent aminoflavone (*35*). Because QPEDs were shown to have enhanced cytotoxicity in ER(+) cells and to produce ROS selectively in the nucleus of these cells, it was reasonable to investigate apoptosis as the cause of cell death. In apoptotic cells, the membrane phospholipid phosphatidyserine (PS) is translocated from the inner to the outer surface of the plasma membrane; exposed PS binds to the phospholipid-binding protein annexin V, providing a reliable method for quantifying the percentage of cells undergoing apoptosis using flow cytometry. Cells were analyzed in this way after treatment with compound (5 μM) or vehicle for 18 h (Figure 6, panel a). A higher concentration of napDA (9 μM, corresponding to LC_{50}) was also studied because this compound was cytotoxic at higher concentration in both cell types. The data was analyzed to quantify the total apopototic cells (viable and nonviable) as a percentage of cell count (Figure 6, panel b). An excellent correlation was observed between percentage cells in apoptosis and cytotoxicity for treatment groups, in that all compounds cytotoxic at 5 or 9 μM induced apoptosis. More importantly, napDA induced apoptosis with no dependence on cellular ER status, whereas both QPEDs induced significantly more apoptosis in ER(+) cells compared to ER(−) cells: DMAcatDA and DMAnapDA induced 48 and 24% apoptosis, respectively, in S30 cells compared to 18 and 10% apoptosis in MDA-MB-231 cells. The control compounds, catDA and napDA, at 5 μM showed no significant apop-

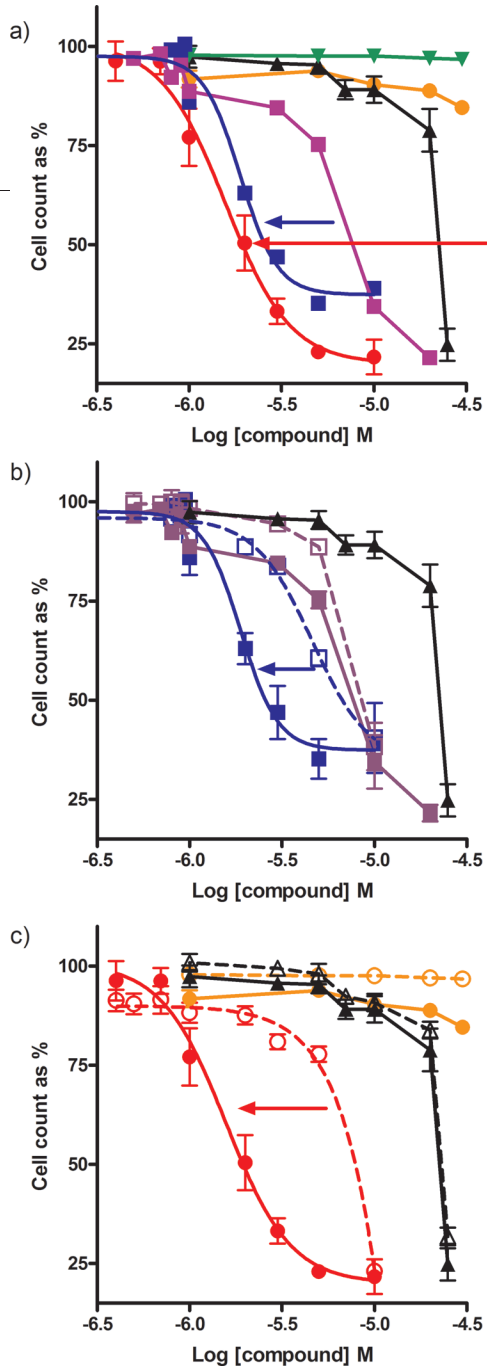

Figure 4. ER(+) selective cytotoxicity of QPEDs in breast cancer cells. (a) Comparison in ER(+) S30 cells of DMAnapDA (blue squares); napDA (purple squares); DMAcatDA (red circles); catDA (orange circles); DMA (black triangles); DMA-mustard (green inverted triangles); blue arrow shows increased toxicity of napDA conjugated to DMA; red arrow shows enhanced toxicity of catDA when conjugated to DMA. (b) Comparison of DMAnapDA (blue squares); napDA (purple squares); DMA (black triangles); S30 cells (solid symbols); ER(−) MDA-MB231 cells (open symbols); the blue arrow shows left shift of DMAnapDA toxicity in ER(+) versus ER(−) cells. (c) Comparison of DMAcatDA (red circles); catDA (orange circles); DMA (black triangles); S30 cells (solid symbols); MDA-MB231 cells (open symbols); the red arrow shows left shift of DMAcatDA toxicity in ER(+) versus ER(−) cells. Cells were seeded in 96-well plates for 24 h then treated with compounds for 18 h before cell count was measured as a percentage from the ratio of cells in compound-treated versus DMSO-treated wells using SRB assay. Data show mean and sem for three separate cultures.

tosis; however, napDA at 9 μM induced apoptosis significantly in both ERα(+) and ER(−) cells.

Induction of Caspase-3/7 Activity. Induction of cellular oxidative stress can lead to activation of caspase 3 and 7 as mediators of apoptotic cell death (*36*). Oxidative DNA damage is one trigger for caspase activation, although not the only mechanism by which oxidative stress can induce apoptosis. Caspase-3/7 activity was measured using Z-DEVD-aminoluciferin as substrate after incubation of cells with compounds under the same conditions used for detection of apoptosis (Figure 6, panel c). After 18 h incubation, caspase activity for all QPED treated cells was observed to be significantly elevated. DMAcatDA and DMA-napDA (5 μM) induced increases of 9-fold and 5-fold in caspase activity, respectively, in S30 cells, compared to 7-fold and 4-fold activation in MDA-MB-231 cells, respectively. To confirm these results, the caspase-3/7 selective inhibitor, Ac-DEVD-CHO, was shown to block activation by both QPEDs. Interestingly at the single time point studied, napDA (9 μM) induced apoptosis but did not show induction of caspase-3/7 activity. Importantly, caspase-3/7 activation by both QPEDs was significantly greater in ER(+) cells compared to ER(−) cells.

QPEDs as Chemotherapeutic Agents. The traditional DNA alkylating agents, platinum complexes and nitrogen mustards, form DNA cross-links and are applied widely in chemotherapy. In part to improve upon the activity of cisplatin in breast cancer, both platins and mustards have been conjugated to estrogens to exploit ER affinity to broaden and enhance chemotherapeutic activity (*37*). A number of groups have tethered platin warheads at the 3, 16, and 17 positions of the steroid backbone of estradiol/estrone, showing weaker antiproliferative activity in MCF-7 cells than parent platin and/or a lack of selective cytotoxicity between ER(+) and ER(−) cells (*38, 39*). For example, a recent study by Berubé, varying linker length in such conjugates, reported for the most potent compound, IC_{50} values of 21 and 14 μM in MCF-7 and MDA-MB-231 cells, respectively, compared to 18 μM for cisplatin itself (*40*). The same group has linked a platin to a benzopyran SERM scaffold yielding interesting conjugates with highly variable toxicity but no selectivity for MCF-7 cells over MDA-MB-231 cell killing. Essigmann and Croy have reported conjugates in which the 7α position of estradiol is tethered to ethylenediamine platinum dichloride ($Pt(en)Cl_2$) or a nitrogen mustard, retaining ER binding affinity

(*23–25*). The most recent estradiol-(Pt(en)Cl_2) conjugate reported by this group showed modest selectivity against MCF-7 ($LC_{50} \approx 12$ μM) versus MDA-MB-231 cells ($LC_{50} \approx 16$ μM), and more impressively the conjugate was able to kill ER(+) human ovarian cancer cells ($LC_{50} \approx 25$ μM) that did not respond to a control warhead (*25*). Reported selective cytotoxicity for the estradiol-7α-mustard conjugate was 2.6-fold higher in MCF-7 cells compared to MDA-MB-231 cells (*23, 24*).

Three models have been proposed to justify the development of platin and mustard conjugates as nuclear receptor targeted chemotherapeutic agents. First, the preferential uptake of these conjugates by the nuclear receptor and sequestration in the case of ER in ER-positive cells and tissues enhances ER-positive cancer selectivity. Second, the formation of DNA adducts that entrap the large nuclear receptor complex is proposed to physically block DNA repair enzyme function. Third, "hijacking" of the nuclear receptor might prevent receptor mediated transcription, which in the case of ER would require very high affinity conjugates to compete with endogenous estrogens. The QPEDs target and bind the ER carrying a payload of masked *o*-quinone warheads that would be anticipated to give DNA-adducts *in simile* with the known capacity of 4-OHE, 4-OHEN, and other *o*-quinone forming agents to covalently modify DNA nucleobases and form adducts (*16, 41, 42*). The relative contributions of DNA oxidation versus DNA adduction toward the cytotoxic actions QPED in breast cancer cells remains to be determined. However, the observations herein show that QPEDs are selective cytotoxins toward ER(+) cells and in these cells selectively generate ROS in the nucleus. The potency of QPEDs and the selectivity toward ER(+) cells both compare favorably with platin and mustard conjugates described above, which are in development as chemotherapeutic agents.

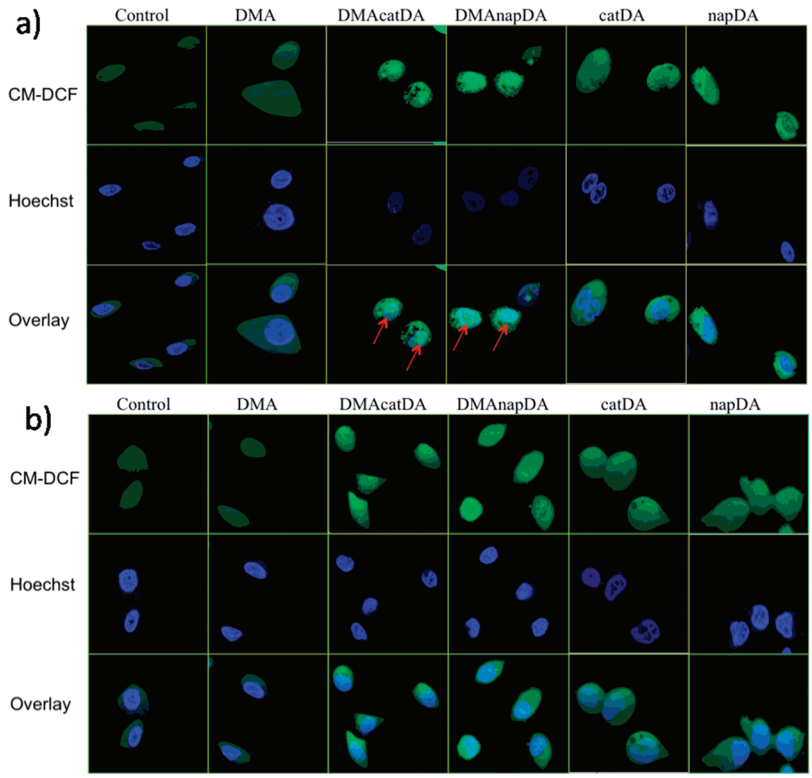

Figure 5. Nuclear localization of CM-DCF fluorescence after treatment of (a) S30 and (b) MDA-MB231 cells with QPEDs and control compounds. Cells were incubated with compounds for 1 h and followed by treatment with H_2DCFDA and Hoechst nuclear dye for 10 min before confocal fluorescence microscopy images were taken. CM-DCF fluorescence is indicative of ROS production and overlay with nuclear staining shows localization of ROS production; arrows indicate colocalization of ROS and nuclear staining. Images are representative of several experiments.

Mechanism of Action. Both QPEDs studied were cytotoxic toward human breast cancer cells in which these agents induced production of ROS, apoptosis, and executor caspase activity. These observations support a mechanism whereby induction of cellular oxidative stress by QPEDs induced apoptotic cell death mediated by caspase activation, a pathway for which there is substantial precedent. QPEDs deliver a quinone warhead after oxidative bioactivation of the catechol that is itself unmasked by nonspecific esterase bioactivation. Protection of the warhead as a catechol diacetate both enhances membrane permeation and prevents quinone formation in the cell culture medium, which would lead to reduced intracellular bioavailability. Several well-studied anti-infective and anticancer agents are quinones that induce apoptosis; these are considered to act as DNA alkylating agents, and also to generate semiquinones and ROS that can react with DNA, in addition to depleting glutathione and perturbing cellular energy and calcium homeostasis (*43*). The cytotoxicity of such quinones in hypoxic cells and a general requirement for reductive bioactivation has led to the suggestion that DNA alkylation is the dominant contributor (*43, 44*). Nevertheless, the mechanism by which the quinone anticancer agents induce apoptosis is not fully defined

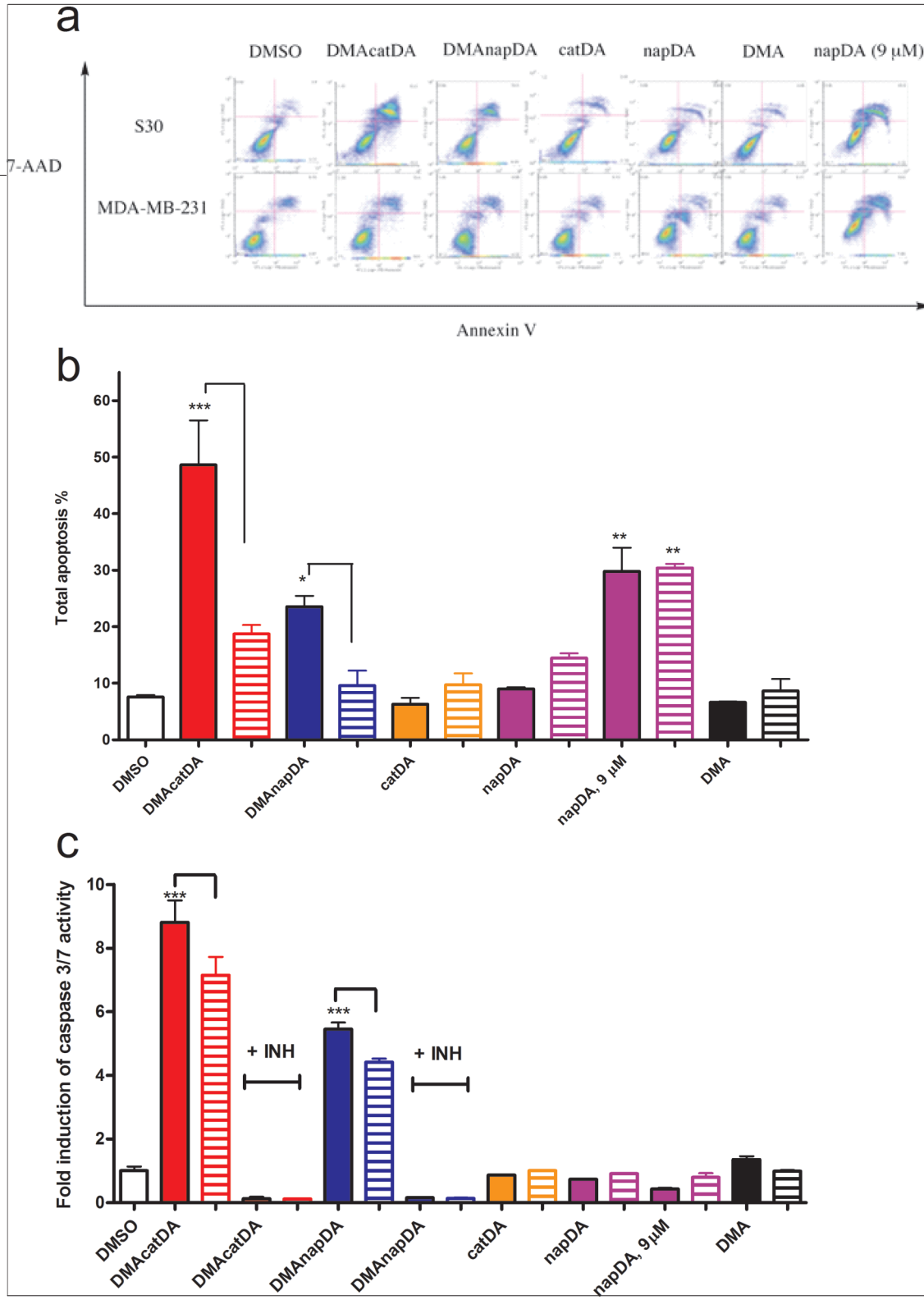

Figure 6. Apoptosis and caspase activity induced by QPEDs and control compounds in human breast cancer cells. (a) Analysis of S30 cells and MDA-MB231 cells treated with DMSO vehicle or compounds (5 μM or as indicated) for 18 h by flow cytometry (FACS) using annexin V labeling. Data shown are representative results. (b) Annexin V positive cells were scored as apoptotic and expressed as percentage of total cells: S30 cells (solid columns); MDA-MB231 cells (striped columns). (c) Caspase 3/7 activity induced in S30 (solid columns) and MDA-MB231 (striped columns) after treatment with DMSO vehicle or compounds (5 μM unless stated) for 18 h, followed by 30 min incubation with caspase-3/7 substrate (Z-DEVD-aminoluciferin) with or without caspase-3/7 inhibitor (INH, Ac-DEVE-CHO, 10 μM). Data presented as fold induction compared to DMSO. In (b) and (c), data show mean and s.d. of three independent experiments analyzed by ANOVA with Newman-Keuls post test (* $p < 0.001$; * $p < 0.05$; ** $p < 0.01$ vs DMSO control).**

and may involve triggering of mitochondrial pathways or gene regulation of pro-apoptotic signals; one study on a series of such compounds in MCF-7 cells reported that cytotoxicity correlated with induction of apoptosis and elevation of Bcl-xL/Bcl-2-associated death protein (BAD) (*45*).

Cellular GSH depletion represents one mechanism by which quinone anticancer agents may induce apoptosis. Total cellular GSH was measured in ER(+) and ER(−) cells as a function of time, after incubation with QPEDs and control compounds, by a kinetic assay using glutathione reductase/NADPH as a GSSG reducing system in concert with 5,5′-dithiobis(2-nitrobenzoic acid). The pro-apoptotic QPEDs and napDA were observed significantly to deplete cellular GSH by at least 50% after 2 h incubation, whereas catDA gave less than 20% depletion at this time point, and no significant depletion was observed for DMA, DMA-mustard, nor mustard (all agents 5 μM; Supplemental Figure 1). Furthermore, GSH depletion measured for all compounds at the 2 h time point showed concentration dependence (Supplemental Figure 2). The observed GSH depletion correlated qualitiatively with apoptosis; however, no dependence was observed on cellular ER status. That is: (i) efficacy and potency for GSH depletion by QPEDs were not significantly different in S30 versus MDA-MB-231 cells; and (ii) DMAnapDA was not significantly more potent than napDA in either cell line. Therefore, whereas GSH depletion may contribute to apoptosis, it is not the mechanism that mediates selective apoptosis of ER(+) cells.

The differences in activity of the monocyclic quinone producing agents, catDA and DMAcatDA, are most dramatic. Oxidative bioactivation of DMAcatDA may involve chelation of chromatin-bound Cu ions by the catechol; however, further studies are needed, in particular to vary the QPED linker length. Nevertheless, the results presented herein do validate the QPED design concept and encourage further research toward ER-targeted anticancer agents.

Implications for Estrogen Carcinogenesis. Evidence supporting estrogens as mammary carcinogens derives from clinical trials and epidemiological studies (*1–3*). In the hamster kidney tumor model, a variety of estrogens, including estrone and equilenin, were full carcinogens (*4*). Both hormonal and chemical carcinogenesis pathways are expected to contribute to cancer in estrogen-responsive tissues (*5–8, 46*). Despite the significant differences in the oxidative metabolism of human and equine estrogens, there has been sparse attention to the consequences of these differences for cancer in women. The extended conjugation of the naphtho-*o*-quinone of 4-OHEN provides stabilization of the oxidized form in comparison to the benzo-*o*-quinone of 4-OHE. Because 4-OHE and 4-OHEN are both estrogenic, separating hormonal from chemical pathways in ER(+) cells requires development of compounds such as the QPEDs. ER-dependent activity was observed only for the QPEDS, not for any constituent parts, neither DMA nor warheads. ROS production in the cell nucleus, activation of caspase-3/7, induction of apoptosis, and cytotoxicity were all increased significantly in ER(+) cells. This observation indicates that DNA damage induced by catechol estrogen metabolites can be amplified in ER(+) cells independent of hormonal activity. Further studies will examine differences between the types of DNA damage induced by the β-naphthoquinone and benzoquinone groups of QPEDs, which model the reactive functional groups of 4-OHEN and 4-OHE, respectively.

METHODS

Synthesis. Synthetic details and characterization are described in the Supporting Information.

Transfection and ERE-Luciferase Assays. Cells were cultured in estrogen-free media for 4 days before transfection. Cells were transfected with 2 μg of the pERE-luciferae plasmid, which contains three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of fire fly luciferase (a gift from Dr. V. C. Jordan). To normalize transfection efficiency, pRL-TK plasmid (1 μg, Promega) was cotransfected. Cells (5×10^6) in serum-free media were transfected by electroporation in a 0.4-cm cuvette (Bio-Rad Laboratories) at a voltage of 0.320 kV and a high capacitance of 950 μF in a GenePulser X-cell (Bio-Rad Laboratories). The cells were resuspended in estrogen-free media, transferred to 12-well plates immediately after electroporation, and incubated overnight. The cells were treated with the appropriate compounds with 1 nM E_2 for 18 h and ERE activation measured as described previously (*47*). Briefly, the luciferase activities in cell lysates were measured using Dual Luciferase Assay system (Promega) with a FLUOstar OPTIMA (BMG LABTECH) and data were calculated as relative luciferase activity which is the firefly luciferase reading divided by the *Renilla* luciferase reading.

Antiestrogenic Activity in Ishikawa Cells. The procedure of Pisha and Pezzuto was used as described previously (*47*). Briefly, Ishikawa cells (5×10^4 cells mL^{-1}) were incubated overnight with estrogen-free media in 96-well plates. Test samples (with 1 nM 17β-estradiol) and appropriate controls were added. The day 0 control did not contain any additional estradiol. The

cells were incubated with a total volume of 200 μL well^{-1} at 37 °C for 4 days. The cells were washed three times with PBS and lysed by freeze−thawing in the presence of 0.1 M Tris, pH 8.0. Enzyme activity was measured by reading the liberation of *p*-nitrophenol from 1 μM *p*-nitrophenylphosphate at 340 nm every 15 s for 16−20 readings with an ELISA reader (Bio-Tek Instrument). The maximum slope of the lines generated by the kinetic readings were calculated using a Kinecalc computer program (Bio-Tek Instrument). For antiestrogenic activity, the reduction in percent induction as compared to the DMSO control was determined as follows: [(slope sample − slope cells)/(slope DMSO − slope cells)] × 100.

Cytotoxicity Assay. Cells were plated (7 × 10^4 cells mL^{-1}) in 96-well plates. The following day, cells were treated with the compound for 18 h. After the incubation period, cells were fixed to the plastic substratum by the addition of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4 °C for 1 h, washed with H_2O, and air-dried. The trichloroacetic acid-fixed cells were stained by the addition of 0.4% (w/v) SRB, dissolved in 1% acetic acid for 30 min. Free SRB solution was removed by washing with 1% aqueous acetic acid. The plates were air-dried, and the bound dye was solubilized by the addition of 10 mM unbuffered Tris base, pH 10. The plates were placed on a shaker for 5 min, and the absorption was determined at 515 nm. Finally, the absorbance obtained with each of the treatment procedures was averaged and was expressed as a percentage, relative to the 0 h control (*33*).

Localization of ROS by CM-H_2DCFDA Oxidation. Both S30 and MDA-MB-231 cells (4.5 × 10^4 cells well^{-1}) were grown on a sterile Nunc chambered cover glass and incubated for 48 h at 37 °C with 5% CO_2 in phenol red free-MEME medium supplemented with 10% stripped FBS medium. These cells were then treated with 1 μM QPEDs, control compounds, or 0.5% DMSO for 1 h. After treatment for 30 min, S30 and MDA-MB-231 cells were labeled with 10 μM CM-H_2DCFDA for 30 min at 37 °C with 5% CO_2. CM-H_2DCFDA treated cells were rinsed with phosphate buffered saline (PBS) to remove the unincorporated dye and 10 mg mL^{-1} Hoechst-33258 was added to the cells to see nuclear staining. Imaging was performed with a Zeiss LSM510 laser-scanning confocal microscope with the detector gain adjusted to eliminate the background autofluorescence. The fluorescence signal from CM-DCF was monitored with a 488 nm argon/krypton laser and a 530 nm band-pass filter. Hoechst nuclear staining signal was monitored with a 345 nm UV laser and 420 nm band-pass filter. Water immersion objective (A × 63−1.2 numerical aperture) was used for all experiments. Images were analyzed using the analysis tool provided in Zeiss biophysical software package.

Apoptosis Analysis. S30 and MDA-MB-231 cells (5 × 10^5) were seeded in 6-well plates for 24 h before treatment. Cells were treated with DMSO, QPEDs, or control compounds for 18 h. Cell death was measured by flow cytometry using the Annexin-PE Apoptosis Detection Kit I (BD Bioscience) according to the manufacturer's protocol. Cells were washed twice with cold PBS and then suspended in 1× binding buffer. Cells were stained using 5 μL of PE annexin V (for apoptotic status) and 5 μL of 7-amino-actinomycin (7-AAD; for cell viability status) for 15 min at RT in the dark. After adding 200 μL of 1× binding buffer, cells were analyzed by flow cytometry within 1 h. Annexin V-positive cells were scored as total apoptotic cells as a percentage of all cells.

Measurement of Caspase-3/7 Activity. The activities of caspase-3/7 in S30 cells were measured as recommended by the kit manufacturer (Promega). Briefly, the DMSO vehicle or compound treated cells (7 × 10^4 cells mL^{-1}) were incubated in a 96-well plate for 18 h at 37 °C and then incubated with 100 μL of Caspase-Glo 3/7 reagent or with a combination of caspase-3/7 substrate and 10 μM inhibitor (Ac-DEVD-CHO) at RT for 30 min. The luminescence of each well was measured at using a FLUOstar OPTIMA (BMG LABTECH).

Determination of GSH in Cells. Experimental details and two figures presenting data observed are provided in the Supporting Information.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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