Estrogen Receptor-dependent and Estrogen Receptor-independent
Pathways for Tamoxifen and 4-Hydroxytamoxifen-induced
Programmed Cell Death*

Maria Obiero, David V. Yu, and David J. Shapiro‡

From the Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

The therapeutic efficacy of tamoxifen (TAM) in cancer therapy is thought to arise primarily from its ability to compete with estrogens for binding to the estrogen receptor (ER). We show that TAM and its active metabolite, 4-hydroxytamoxifen (OHT), can actively induce programmed cell death through distinct ER-dependent and ER-independent pathways. The ER-independent pathway is activated by 10–20 μM TAM and OHT and by 10–20 μM 17β-estradiol and raloxifene, and occurs in ER-negative cells. The ER dependence of a second pathway, caused by submicromolar concentrations of TAM and OHT, was demonstrated by the ability of the ER ligands 17β-estradiol, raloxifene, and ICI 182,780 to effectively block the cell death-inducing effects of TAM and OHT. Because the p38-specific inhibitor SB203580 blocks OHT-ER-induced cell death, stress kinase pathways are likely involved. ER-independent cell death triggers classic caspase-dependent apoptosis. However, although OHT-ER triggers some hallmarks of apoptosis, including Bax translocation and cytochrome c release, the absence of poly(ADP-ribose) polymerase cleavage or DNA laddering indicates that the death pathway involved is caspase-independent. The OHT-ER-dependent cell death pathway appears to diverge from classical apoptosis at the level of caspase 9 activation. The ability to promote ER-dependent programmed cell death represents a novel activity of TAM and OHT.

Estrogens, acting through estrogen receptors (ERs), regulate the growth and differentiation of cells of the reproductive system. Binding of 17β-estradiol (E2) to the ER induces a conformational change that enables the ER to recruit transcriptional coactivators and to induce expression of estrogen-regulated genes. Several estrogen-inducible genes, including c-myc, TGF-α, and cathepsin D, are implicated in malignant transformation or tumor metastases (1–4). Tamoxifen (TAM) and its active metabolite, 4-hydroxytamoxifen (OHT), are nonsteroidal selective estrogen receptor modulators (SERMs) that compete with E2 and other estrogens for binding to ER. Structural studies and chromatin immunoprecipitations show that OHT-ER induces an ER conformation that does not recruit coactivators to target genes and in many cell and promoter contexts recruits corepressors (5, 6). The therapeutic effectiveness of TAM in treatment of hormone-dependent cancers and in preventing breast cancer in high risk women is thought to arise primarily from its ability to compete with estrogens for binding to the ER. It is thought that TAM-ER and OHT-ER are unable to effectively activate transcription of genes important for the growth and development of estrogen-dependent tumors. However, several often-conflicting studies show that TAM and OHT can actively induce programmed cell death of cancer cells (reviewed in Ref. 7). The mechanism(s) by which TAM and OHT induce programmed cell death have been quite controversial, with even the identity of the toxic agents in dispute. One group reported that high concentrations of TAM, but not OHT, induce cell death (8). Others indicated that both TAM and OHT induce cell death (9). Although our recent report was consistent with a role for ER in OHT-induced apoptosis (10), other workers suggest a number of different mechanisms for TAM-induced apoptosis. The effects of TAM might be mediated through an ER-independent increase in reactive oxygen species, resulting in caspase activation (9, 11), or through an influx of extracellular calcium (12, 13). In addition, effects of TAM on the levels of proteins important in cell growth including protein kinase C (14, 15), TGF-β (16, 17), and c-Myc (18, 19) have been reported.

Resolution of the role of ER in TAM- and OHT-induced apoptosis is complicated by the fact that available ER-positive and ER-negative breast cancer cell lines are derived from independent tumors. These cell lines therefore differ in many respects other than ER content. To simplify analysis of the role of ER in TAM- and OHT-induced apoptosis, we therefore used ER-negative HeLa, human cervical carcinoma cells, and HeLa cells stably transfected to express hERα (HeLaER6 cells) (20). These cell lines differ only in the presence or absence of ER (and the neomycin phosphotransferase gene that encodes resistance to G418).

Many compounds that are known to induce programmed cell death (PCD) work via pathways that involve mitochondria. The presence of an apoptotic stimulus triggers a rapid increase in mitochondrial permeability, leading to mitochondrial dysfunction. One of the causes of the mitochondrial permeability transition is the translocation of the proapoptotic Bax protein from the cytosol to the mitochondria, where it forms selective channels in the outer mitochondrial membrane and facilitates the release into the cytosol of cytochrome c (21, 22). In the classic apoptotic pathway, this cytosolic cytochrome c forms a complex with procaspase 9 and Apaf-1 called the apoptosome, which leads to the ATP-dependent cleavage and activation of pro-
caspase 9, the initiator caspase in mitochondrial apoptosis. Activation of pro-caspase 9 results in activation of downstream executioner caspsases, such as caspase 3 (23–25).

We find that OHT is able to induce two independent pathways of PCD. An ER-independent pathway kills ER-negative HeLa cells, requires 10–20 μM TAM or OHT, and is not TAM-specific as it is also triggered by the SERM raloxifene (RAL) and by E2. In contrast, submicromolar amounts of TAM and OHT trigger cell death only in ER-positive HeLa cells. This effect is blocked by pre-treatment with E2, RAL, and ICI 182,780, demonstrating that binding of TAM and OHT to the ER is required for this pathway of programmed cell death. The ER-dependent and ER-independent pathways both trigger a mitochondrial permeability transition and share other features of mitochondrial apoptosis, such as translocation of the pro-apoptotic Bax protein from the cytosol into the mitochondria and the release of cytochrome c into the cytosol. However, in contrast to ER-independent PCD, which displays typical apoptotic markers such as PARP cleavage, chromatin condensation, and DNA laddering, a different cell morphology, as well as the absence of those markers, indicates that the ER-dependent pathway does not involve caspase activation. The ER-dependent pathway does not result in the cleavage and activation of procaspase 9, the initiator caspase in mitochondrial apoptosis. The OHT-ER-mediated PCD pathway resembles the caspase-independent pathway referred to as necrosis-like PCD. This study describes a novel and highly specific pathway for TAM-ER- and OHT-ER-induced programmed cell death and suggests an additional mechanism to account for the therapeutic efficacy of TAM.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to PARP and cleaved caspase 9 were obtained from Cell Signaling Technologies (Beverly, MA). COX4 and cytochrome c antibodies were obtained from Clontech Laboratories (Palo Alto, CA), and actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). DiOC6(3) and propidium iodide were obtained from Molecular Probes (Eugene, OR) and R&D Systems (Minneapolis, MN), respectively. The p88 inhibitor SB203580 was obtained from Calbiochem.

Cell Culture—All cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-dextran-treated fetal bovine serum. HeLaER cells were maintained in medium supplemented with 200 μg/ml G418 (Invitrogen). HeLaER cells were plated in medium without G418 the day before experiments and maintained in G418-free medium throughout the 2–4-day time period of the experiments.

Mitochondrial Membrane Potential (Δψm) Measurement—To quantify the percentage of cells in the early stages of programmed cell death, we used a flow cytometry-based method that employs the strong cationic dye DiOC6(3). In healthy cells, the presence of a mitochondrial membrane potential (Δψm) allows DiOC6(3) to be sequestered in the mitochondria. Cells that are in the early stages of programmed cell death exhibit decreased mitochondrial retention of DiOC6(3).

Cells were seeded at 6 × 10^5 cells per 100-mm plate and incubated for 24 h. After treatment with the indicated ligands, the cells were washed once with PBS and harvested using PBS-EDTA. The cells were pelleted by centrifugation at 600 rpm for 5 min and resuspended in PBS. 40 nm DiOC6(3) was added to the resuspended pellet, and the samples were incubated at 37 °C for 15 min, after which propidium iodide (PI) was added to 5 μg/ml. Relative fluorescence intensities were measured using a Coulter XL benchtop flow cytometer with excitation at 485 nm and emission at 520 nm.

Whole Cell Extract Preparation and Western Blotting—Cells were seeded in six-well plates at a density of 1.5 × 10^5 cells/well. After treatments, the cells were washed once with PBS, and harvested with lysis buffer containing 0.02% Triton, 1% Triton X-100, 0.14 mM NaCl, 2 mM EDTA, 10 μg/ml of the protease inhibitors leupeptin, pepstatin, and aprotinin, 1 mM phenylmethanesulfonyl fluoride, and diithiothreitol. Protein content was measured using Coomassie Blue reagent (Bio-Rad, Richmond, CA). Extracts were run on SDS-polyacrylamide gels, and the proteins were electroblotted into a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk for 1 h at room temperature, probed with primary antibody overnight at 4 °C, washed three times with TBS-Tween, and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Signals were detected using the SuperSignal® West Pico chemiluminescent substrate kit (Pierce).

Transient Transfections—Transient transfections of HeLaER6 cells were performed using LipofectAMINE™ 2000 reagent (Invitrogen), using the manufacturer’s protocol. 2 × 10^5 cells per well were seeded in 12-well plates. After 24 h the cells were transfected with the indicated amounts of ATL3 reporter (26), PRLSV40, and PTZ18U. The indicated concentrations of hormone were added 4 h after transfection. After 24 h the cells were harvested, and dual luciferase assays (Promega) were performed using the manufacturer’s protocol.

Electron Microscopy—After treatment, cells were harvested by scraping and collected into a pellet by centrifugation at 600 rpm for 5 min. At the time of embedding, cells were pipetted out of the epoxy infiltration mixture and put in small BEEM capsules, which were filled up the rest of the way with fresh epoxy, spun to collect cells at the bottom, and placed in the oven overnight at 90 °C to harden. Blocks were sectioned at 60–90 nm with a Diatome diamond knife using a Richart Ultracut E ultramicrotome. The sections were picked up with 200-mesh copper grids and stained with saturated uranyl acetate, silver, and lead citrate. Sections were viewed on an H600 Hitachi transmission electron microscope (Tokyo, Japan). Images were shot with Eastman Kodak Co. electron microscopy film.

Cell Fractionation—Mitochondrial extraction was performed using the ApoAlert cell fractionation kit (Clontech). HeLa or HeLaER6 cells were seeded at 1 × 10^6 cells per sample, and various ligands were added after 24 h. After treatment, cell pellets were collected by centrifugation at 600 × g for 5 min at 4 °C, washed once, and resuspended in ice-cold fractionation buffer containing protease inhibitor mixture (Clontech) and dithiothreitol. After incubation on ice for 10 min, cells were homogenized using 2 ml of Kontes Dounce tissue grinders (Fisher). Homogenates were centrifuged at 700 × g for 10 min at 4 °C. Supernatants were centrifuged at 10,000 × g for 25 min at 4 °C. Supernatants were collected and designated as cytosolic fractions, and pellets were resuspended in fractionation buffer and designated as mitochondrial fractions. Protein concentrations were determined using the Coomassie Blue assay.

DNA Fragmentation Analysis—5 × 10^6 cells were plated in 100-mm plates. After appropriate treatments, the cells were harvested by centrifugation at 2000 rpm and 4 °C. Cell pellets were resuspended in lysis buffer containing 20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% (w/v) sodium lauryl sarcosine. Lysates were incubated with 10 μl of 1 mg/ml RNase A/RNase T1 mixture at 37 °C for 2 h and 10 μl of 20 mg/ml protease K for 16 h. Samples were run in a 1% agarose gel.

RESULTS

To facilitate comparisons between cells that lack ER and cells that contain ER, we used ER-negative, wild-type HeLa cells and our HeLaER6 cells stably transfected to express hERα (20). To minimize potential cell stress due to maintaining HeLaER6 cell lines in medium containing the selective agent G418, HeLaER6 cells were plated in medium without G418 the day before experiments and maintained in G418-free medium throughout the experiments.

Preliminary studies based on cell morphology suggested that 10–20 μM TAM and OHT were toxic to HeLa and HeLaER6 cells and that submicromolar concentrations of TAM and OHT were not visibly toxic to HeLa cells. To further compare the toxicities of TAM and OHT in HeLaER6 cells and in the parental HeLa cells, we performed dose-response experiments. Because the pathway(s) of programmed cell death induced by TAM and OHT were unknown, we elected to use a quantitative assay for cell death based on the ability of mitochondria to sequester the strong cationic dye DiOC6(3). Damaged mitochondria of apoptotic or necrotic cells exhibit decreased retention of DiOC6(3) and are visualized as a distinct subpopulation of cells in flow cytometry.

TAM and OHT Induce Programmed Cell Death in HeLaER6 Cells—To determine whether PCD induced by TAM and OHT was ER-dependent, we treated ER-negative HeLa cells and HeLaER6 cells with a range of TAM or OHT concentrations (0.1 nM–1 μM). The percentage of wild-type HeLa cells that were...
in early stage programmed cell death (low retention of DiOC₆(3) but still impermeable to the DNA-intercalating propidium iodide dye) was unaffected by concentrations of TAM or OHT from 0.1 nM to 1 μM (Fig. 1). The HeLaER6 cells exhibited an increase in the percentage of cells with decreased retention of DiOC₆(3) from 0.1 to 1 μM TAM. Lower concentrations of OHT were needed to produce mitochondrial dysfunction. The percentage of cells with low retention of DiOC₆(3) reached a plateau at 10 nM OHT with an IC₅₀ of ~1 nM OHT (Fig. 1). Much lower concentrations of OHT than TAM were also required to induce cell death in a second cell line, HeLaER5 cells (data not shown). Across a range of concentrations, ~100-fold lower amounts of OHT than TAM were required to elicit a given percentage of cell death (Fig. 1, compare 10⁻⁹ M OHT with 10⁻⁷ M TAM). OHT, the active metabolite of TAM, binds to the ER at least a 100-fold higher affinity than TAM (27). In intact cells, OHT exhibits some agonist activity and activates transcription of a reporter gene at concentrations ~100-fold lower than are required for TAM activation. These data suggested that binding to the ER was important in TAM and OHT-induced death in the HeLaER6 cells.

The Estrogen Receptor Is Involved in OHT- and TAM-induced Cell Death — Although these data were consistent with a role for ER in apoptosis induced by low concentrations of TAM and OHT, it was important to test this more directly. Because nM concentrations of OHT induce death of HeLaER6 cells, it was possible to carry out competition experiments using a large molar excess of other ER ligands over OHT and to evaluate the effect of these ligands on OHT-mediated programmed cell death. We used three structurally dissimilar compounds that are representative of the major classes of ER ligands. RAL is a well studied SERM that exhibits agonist activity in bone and antagonistic activity in many other tissues (5, 6, 28). ICI 182,780 is a nearly pure antagonist (29, 30), and E₂ is the prototypical agonist activity in many other tissues (5, 6, 28). ICI 182,780, or E₂ blocked the appearance of this subpopulation of cells (Fig. 2, A–C). The quantitative flow cytometry results were reflected in morphological changes in the cells. After 4 days in OHT-containing medium, the HeLaER6 cells were sparse, and their morphology was very different from that observed in the control, vehicle-treated cells. Control cells and cells maintained in OHT plus ICI 182,780 or OHT plus RAL were dividing actively and exhibited similar morphology (Fig. 2D).

1 μM TAM is required to induce maximal programmed cell death in HeLaER6 cells. Because two of the other ER ligands are toxic to the cells at extremely high concentrations (see Fig. 6), it was not possible to carry out traditional competition experiments in which these ER ligands were present at a 100-fold molar excess over TAM. However, because TAM binds to ER with a much lower affinity than E₂, RAL, and ICI, we elected to attempt competition experiments in the presence of equimolar concentrations of the competitors and TAM. Although they were not present in excess over TAM, 1 μM ICI 182,780, 1 μM RAL, or 1 μM E₂ were able to almost completely protect the cells from TAM-induced programmed cell death (Fig. 2D). These data provide compelling evidence for a role for ER in TAM-induced apoptosis of HeLaER6 cells. The most straightforward explanation for the ability of equimolar concentrations of E₂, ICI 182,780, and RAL to block TAM-induced apoptosis is that E₂, ICI 182,780, and RAL are known to bind to hERα with much higher affinity than TAM (31).

An Inhibitor of the p38 Pathway Protects against OHT-induced Apoptosis — The cell death response is sometimes triggered by the activation of stress-activated mitogen-activated protein kinase-based signaling pathways. It was of interest to determine whether the p38 kinase, and perhaps the JNK kinase, were involved in OHT-induced programmed cell death. Low, 0.5–2 μM, concentrations of SB203580 specifically inhibit the p38 pathway (32). At higher concentrations (5–10 μM) SB203580 also inhibits some JNK subtypes (33, 34). Pre-treatment of the HeLaER6 cells with 1 μM SB203580 reduced the percentage of cells in early stage programmed cell death by almost 40%. When the cells were pre-treated with 5 μM SB203580, this subpopulation was reduced by almost 90% (Fig. 3, C). These data suggest that activation of the p38 and perhaps JNK kinases plays a major role in OHT-induced programmed cell death.

In HeLaER6 Cells OHT Does Not Induce Transcription from the Consensus Estrogen Response Element — A potential mechanism for ER-dependent OHT cytotoxicity is binding of OHT-ER to estrogen response elements in the DNA inducing expression of pro-apoptotic genes. To determine whether OHT exhibits agonist activity in HeLaER6 cells, we performed transient transfections using a reporter gene containing four copies of the consensus estrogen response element (ERE). E₂ elicited a robust activation of the reporter, whereas 10 nM OHT, ICI 182,780, or RAL did not activate the reporter significantly (Fig. 4). Although OHT is not a classical strong agonist in these cells, these data do not exclude the possibility that OHT-ER either activates unusual EREs in specific genes or is tethered to genes containing AP-1 or SP-1 sites (35).

The ER-dependent Programmed Cell Death Pathway Is Distinct from Classical Caspase-dependent Apoptosis — When visualized by light microscopy, cells maintained in medium containing 10 nM OHT did not display typical apoptotic morphology and became elongated instead of rounded (Fig. 2B). In contrast, cells treated with 10–20 μM OHT displayed typical...
apoptotic morphology, including cell shrinkage and membrane blebbing, and detached from the surface of the plate (data not shown). These observations led us to consider the possibility that treatment with 10 nM OHT induces non-classical programmed cell death. To examine cell morphology in more detail, we performed electron microscopy. Prior to analysis the cells were treated with 10 nM OHT for 4 days or with 20 μM OHT for 2 days. Control apoptotic cells were obtained by treating the cells with etoposide, a topoisomerase inhibitor. Cells treated with etoposide or 20 μM OHT exhibited classical markers of apoptosis, most notably cell shrinkage and chromatin condensation (Fig. 5, A, B, and D). In contrast, cells treated with 10 nM OHT were significantly more elongated compared with untreated, etoposide-treated, and 20 μM OHT-treated cells (Fig. 5C). There was a significant reduction in the number of cellular organelles present compared with untreated cells, but

**Fig. 2.** Raloxifene, ICI 182,780, and E2 block TAM- and OHT-induced programmed cell death of HeLaER6 cells. HeLaER6 cells were pre-treated with 1 μM raloxifene, 1 μM ICI 182,780, or 1 μM E2 for 30 min before adding OHT to 10−8 M or TAM to 10−6 M. A, after 3 days of treatment OHT-treated cells were harvested, double-stained with DiOC6(3) and PI, and analyzed by flow cytometry, and the percentage of cells in early stage PCD was determined by flow cytometry. B, after 4 days of treatment cell images were obtained using phase-contrast microscopy at ×40. C, graphical representation of the data in panel A showing that OHT-induced death is blocked by a 100-fold molar excess of RAL, ICI 182,780, or E2. D, TAM-induced programmed cell death is blocked by equimolar concentrations of RAL, ICI 182,780, or E2.
chromatin condensation was absent, as was membrane blebbing.

One of the defining features of caspase-dependent apoptotic cell death is chromatin fragmentation (36, 37) and DNA laddering. To further clarify whether the OHT-ER death program is apoptotic and caspase-dependent, we assayed for the appearance of a DNA ladder by agarose gel electrophoresis. A DNA ladder was observed with DNA extracted from 10 μM OHT-treated cells but not with DNA from cells treated with 10 nM OHT (Fig. 5E). Another widely used marker for apoptosis is caspase-dependent cleavage of PARP into 89- and 22-kDa fragments. PARP cleavage was not detected in extracts from HeLaER6 cells maintained in medium containing 10 nM OHT (Fig. 5F). However, cells maintained in 20 μM OHT exhibit PARP cleavage. These results indicate that 10–20 μM OHT induces a caspase-dependent death pathway whereas 10 nM OHT induces a caspase-independent death pathway that shares some features with classic apoptosis, such as mitochondrial dysfunction.

The ER-independent Cell Death Pathway Involves Caspase Activation and Is Not Specific for TAM and OHT—10 μM OHT and TAM begin to initiate death of HeLa cells, whereas 20 μM OHT or Tam produces robust activation of the cell death pathway (Fig. 6A). To determine whether high concentrations of other ER ligands induce apoptosis, we tested the ability of 10–20 μM E2 and RAL to induce apoptosis. Although 1 μM E2 and RAL protect against apoptosis induced by low concentrations of OHT and TAM (Fig. 2), 2 days in 10–20 μM E2 induces apoptosis in ER-negative HeLa cells, about as well as similar concentrations of OHT and TAM (Fig. 6A). 20 μM RAL triggered apoptosis even more rapidly, with significant cell death observed less than a day after adding it to the culture medium (data not shown). These data indicate that the ER-independent death pathway is not specific for TAM and OHT. To analyze whether these diverse ER ligands activate classical caspase-dependent apoptosis, we examined the ability of 20 μM OHT, TAM, RAL, E2, and ICI 182,780 to induce PARP cleavage and cleavage of the precursor of the initiator caspase, caspase-9. In both HeLa cells and HeLaER6 cells 20 μM TAM, OHT, RAL, and E2 induced PARP cleavage and formation of activated caspase 9 (Fig. 6, B and C). ICI 182,780 did not induce significant PARP cleavage or caspase 9 activation (Fig. 6, B and C) and does not kill HeLa cells (data not shown).

10 nM OHT Induces a Cell Death Pathway That Is Dependent on Mitochondria—Depolarization of the mitochondrial membrane is widely recognized as one of the early markers of programmed cell death. Mitochondrial membrane depolarization therefore formed the basis of our initial assay for cell death. Because 10 nM OHT causes loss of mitochondrial membrane potential (ΔΨm), it was clear that mitochondria were involved in the death pathway. To determine the point at which this death pathway deviates from the classic caspase-dependent apoptosis pathway, we looked at apoptotic markers at the steps before and after mitochondrial membrane depolarization. We examined the ability of OHT-ER to induce translocation of the pro-apoptotic protein, Bax, from the cytosol into the mitochondria, the release of cytochrome c from the mitochondria into the cytosol, and the activation of the most upstream caspase involved in mitochondrial apoptosis, pro-caspase 9. Mitochondria were isolated from HeLaER6 cells (Fig. 7A) and HeLa cells (Fig. 7B) treated with 10 nM OHT or with 10 μM or 20 μM OHT. 10 nM OHT, 10 μM OHT, and 20 μM OHT elicited a large increase in mitochondrial Bax. The level of the control mitochondrial protein, COX4, remained unchanged (Fig. 7, A and B). Cytosol extracts exhibited increased cytochrome c, whereas levels of the housekeeping cytosolic protein actin remained constant. However, when we assayed for pro-caspase 9 activation, we saw that 20 μM OHT, but not 10 nM OHT, produced active, cleaved caspase 9 (Fig. 7C). These data demonstrate that the ER-dependent and ER-independent programmed cell death pathways diverge at the level of caspase 9 activation.

DISCUSSION

Two Independent Pathways for TAM- and OHT-mediated Programmed Cell Death—To help resolve the often-conflicting data on the role of ER, TAM, and OHT in the induction of programmed cell death, we developed a simple and experimentally tractable model for TAM- and OHT-induced apoptosis. We compared effects in wild-type, ER-negative, HeLa cells, and in HeLaER6 cells, which are stably transfected to express ER. Although we describe only our studies in HeLaER6 cells, our findings with a different, independent clone of HeLa cells stably transfected to express ER, HeLaER5 cells, are basically similar (data not shown).

High concentrations of TAM and/or OHT have been reported to activate caspases and trigger apoptotic cell death (7, 9, 11, 12). OHT and TAM are not the only ER ligands that induce apoptosis at high concentrations. For example, our results show that 10–20 μM RAL or E2, but not ICI 182,780, also induce apoptosis of HeLa cells. Because apoptosis of HeLa cells requires extremely high pharmacologic concentrations of TAM, OHT, RAL, and E2 and is not very structure-specific, we elected to focus primarily on programmed cell death induced by much lower concentrations of OHT and TAM. Most of our studies were carried out at 10 nM OHT, a concentration 500–1000-fold
lower than the OHT concentration that kills ER-negative, wild-type HeLa cells.

An ER-dependent Pathway for TAM- and OHT-induced Programmed Cell Death—10 nM OHT and 1 µM TAM do not induce PCD in ER-negative HeLa cells and kill ER-positive HeLaER6 cells. The dose-response curve for PCD shows that the ability of TAM and OHT to induce PCD in HeLaER6 cells is roughly proportional to their affinity for hER (27) and transactivation potential.2 These data suggested that binding of TAM and OHT to the ER was important for induction of PCD (Fig. 1). However, these data did not exclude the possibility that either traces of estrogen in the medium, or the OHT-ER complex, induce a HeLa cell protein that binds TAM and OHT and induces PCD. A recent report suggests that an estrogen-inducible G-protein, GPR30, and not binding of ligands to ER, is responsible for the growth stimulatory effects of ER ligands (38). Because all of the ER ligands these researchers tested, including E2, ICI 182,780, and TAM, produced the same effect and stimulated cell growth, and we find that submicromolar concentrations of TAM and OHT, but not E2, RAL, and ICI 182,780, stimulate PCD, it is clear that binding to GPR30 is not responsible for TAM- and OHT-induced apoptosis. It is also improbable that direct binding of TAM and OHT to an as yet unidentified TAM-ER and OHT-ER inducible protein is responsible for programmed cell death of HeLaER cells. The ability of the five different ER ligands we tested (TAM, OHT, E2, RAL, and ICI 182,780) to induce or protect against programmed cell death data roughly correlates with their binding affinities for
(31). For example, TAM exhibits a much lower affinity for hER than E2, RAL, and ICI 182,780, and equimolar concentrations of these compounds were able to protect against TAM-induced PCD (Fig. 2). The related possibility that E2, RAL, and ICI 182,780 block the ability of TAM and OHT to induce apoptosis, not because they compete with TAM and OHT for binding to the ER, but because they induce one or more anti-apoptotic proteins, seems remote. Because the nearly pure antagonist ICI 182,780, the potent agonist E2, and the SERM RAL all block TAM- and OHT-induced programmed cell death, it seems highly improbable that these three ER ligands, with their different abilities to activate transcription, would exhibit essentially equal abilities to induce an anti-apoptotic protein.

Potential Pathways of TAM- and OHT-Induced Programmed Cell Death—Activation of the p38 and JNK kinases by various cellular stresses such as reactive oxygen species and agents that damage DNA is often associated with proapoptotic events such as phosphorylation of p53 and depolarization of mitochondrial membranes (39–43). We previously used a reporter gene assay to demonstrate that high OHT concentrations activate the p38 signal transduction pathway in HeLaER cells (10). 0.5–2 μM SB203580 specifically inhibits p38 kinases, whereas 5–10 μM SB203580 can also partially block JNK kinase activity. Our finding that 1 μM SB203580 partially blocks PCD induced by 10 nM OHT and that 5 μM SB203580 almost completely blocks the onset of mitochondrial dysfunction suggests involvement of the p38 pathway and perhaps the JNK pathway in the OHT-ER-induced signaling events upstream of mitochondrial dysfunction.

ER induces transcription by binding to specific DNA sequences termed ERs or by being tethered to the DNA through proteins bound at AP-1 and SP-1 sites (35). When transcription is activated by tethering of ER to AP-1 sites, SERMS such as
TAM, OHT, ICI 182,780, and RAL act as potent agonists of ER (44). Because nM concentrations of these ligands exhibit differential effects on HeLaER6 cell viability, there is no reason to propose that tethering of ER to DNA through AP-1 or SP-1 sites plays a key role in OHT-mediated apoptosis. TAM and OHT have virtually no agonist activity on a reporter gene containing consensus EREs. However, given the great diversity of AP-1 sites, SP-1 sites, and imperfect EREs present in native genes, it remains quite possible that TAM- or OHT-activated genes could induce cell death. Although a few genes have been identified as being induced by OHT-ER, none of the known OHT-induced genes is a plausible candidate for an inducer of programmed cell death. Microarray studies that are ongoing in several laboratories will likely greatly expand the repertoire of TAM- and OHT-inducible genes.

The ER-dependent and ER-independent Pathways of Programmed Cell Death Are Different—Consistent with some earlier work suggesting that high concentrations of TAM and/or OHT induce caspase-dependent apoptosis (9, 11), we observed the hallmarks of caspase-dependent apoptosis, including DNA laddering, chromatin condensation, and PARP cleavage when HeLaER6 cells were treated with 10–20 μM OHT. In contrast, 10 nM OHT did not produce these markers, although it did elicit mitochondrial dysfunction resulting in cell death. To determine where the OHT-ER death pathway diverges from the apoptotic pathway, we examined the events occurring upstream and downstream of mitochondrial dysfunction. Our results indicated that up to the point of cytochrome c release, the mitochondrial death pathways induced by high and low OHT concentrations are parallel. However, caspase 9 activation was only triggered by 10–20 μM OHT and was not seen with 10 nM OHT even after 4 days of treatment (Fig. 7C) (data not shown). These data demonstrate that the ER-dependent and ER-independent pathways diverge at the level of pro-caspase 9 activation. Although dramatic reductions in ATP levels (45–51) and induction of heat shock proteins (52–54) reportedly interfere with the activation of caspase 9, the molecular basis for the failure of the OHT-ER PCD pathway to activate caspase 9 remains to be established.

The OHT-ER-mediated Cell Death Pathway Resembles Necrosis-like Programmed Cell Death—Three classes of mitochondrial death pathways can be triggered downstream of mitochondrial changes (23, 45): 1) the caspase-dependent pathway initiated by formation of the apotosome, 2) a caspase-independent pathway leading to necrosis-like programmed cell death, and 3) an apoptosis-like but caspase-independent pathway that results when the apoptosis-inducing factor is released from the mitochondria. The first and third pathways share some common morphological characteristics, most notably chromatin condensation and margination that are not seen in the PCD pathway induced by 10 nM OHT. The second pathway, however, is programmed cell death in the absence of chromatin condensation or at best with chromatin clustering to speckles (50, 55–54) reportedly interfere with cell morphology more characteristic of necrosis more than apoptosis. This pathway is also sometimes referred to as “aborted apoptosis,” in which the standard apoptotic program is initiated, blocked at the level of caspase activation, and then terminated by caspase-independent means (45). PCD induced by 10 nM OHT bound to ER best fits this model for programmed cell death.

In this work we describe two distinct pathways of OHT-induced programmed cell death: a caspase-dependent apoptotic cell death program inducible in the absence of ER by very high concentrations of TAM, OHT, RAL, or E2, and an ER-dependent and ligand-specific pathway induced by nanomolar concentrations of OHT and submicromolar concentrations of TAM bound to ER (Fig. 8). This novel ER-dependent cell death pathway is caspase-independent and resembles necrosis-like programmed cell death. These studies describe an activity of TAM and OHT that is distinct from their ability to compete with estrogens for binding to the ER and suggests additional potential mechanisms for the effectiveness of tamoxifen in cancer therapy and chemoprevention. When ER-positive cancer cells are exposed to extremely high concentrations of TAM or OHT, cell death likely results from a combination of the ER-dependent programmed cell death pathway we describe, the ER-independent pathway that leads to caspase-dependent apoptosis, and the ability of TAM to bind the ER and thereby block the growth-promoting and antiapoptotic effects of estrogen.

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Estrogen Receptor-dependent Programmed Cell Death

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