4-Hydroxytamoxifen Inhibits Proliferation of Multiple Myeloma Cells 

*In vitro* through Down-Regulation of c-Myc, Up-Regulation of p27Kip1, and Modulation of Bcl-2 Family Members

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**ABSTRACT**

**Purpose:** Multiple myeloma is an incurable B-cell malignancy requiring new therapeutic strategies. Our approach was to analyze the *in vitro* effects of a selective estrogen receptor modulator, 4-hydroxytamoxifen (4-OHT), on six multiple myeloma cell lines.

**Experimental Design:** Cultured multiple myeloma cells were treated with various 4-OHT concentrations and the cellular response was studied: cell proliferation, cell viability, induction of apoptosis, caspase activities, and expression of signaling proteins.

**Results:** We found that pharmacologic concentrations of 4-OHT inhibit cell proliferation (4 of 6 cell lines). This inhibition is achieved by two independent events: a block at the G1 phase of the cell cycle and the induction of apoptotic death. The cellular response to 4-OHT depends on the presence of functional estrogen receptors. 4-OHT treatment activates an intrinsic mitochondrial caspase-9-dependent pathway but not the Fas/FasL death pathway. Signaling pathways known to be involved in the survival and/or proliferation of multiple myeloma cells are not affected by 4-OHT treatment. 4-OHT-induced G1 arrest is accompanied by the up-regulation of the cell cycle inhibitor p27Kip1 and the down-regulation of c-Myc. Among the Bcl-2 family members tested, the proapoptotic BimS protein is induced whereas the antiapoptotic protein Mcl-1 is decreased.

**Conclusions:** Although the effects of 4-OHT are observed at micromolar concentrations, cellular mechanisms responsible for G1 arrest, as well as apoptosis induction, are similar to those observed in breast cancer cells. Our data support the concept that 4-OHT may represent an alternative approach to inhibit proliferation and induce apoptosis of multiple myeloma cells.

**INTRODUCTION**

Multiple myeloma is a B-cell malignancy characterized by the clonal expansion of tumoral plasma cells in the bone marrow. This accumulation of malignant cells synthesizing immunoglobulins results in hyperproteinemia, renal dysfunction, bone lesions, and immunodeficiency (1). This pathology, which accounts for 2% of all cancer deaths per year in occidental countries, remains largely incurable despite novel therapeutic approaches involving the targeting of both multiple myeloma cells and bone marrow environment (2).

Selective estrogen receptor modulators (SERM), also called antiestrogens, may provide a new strategy in myeloma therapy. Indeed, the presence of estrogen receptor (ER) in various multiple myeloma cells has been suspected for some time (3) and both α and β isotypes have been detected in some multiple myeloma primary cells and cell lines (4, 5). Moreover, it has been reported that tamoxifen and, to a lesser extent, toremifene inhibit multiple myeloma cell proliferation and in rare cases induce multiple myeloma cell apoptosis (4, 5). Tamoxifen is widely used in the treatment and prevention of breast cancer where it acts by impairing the estradiol-induced mitogenic activity through binding to ER (6). 4-Hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen, is a nonsteroidal SERM able to bind ER and to convert it in an inactive form (7). In the well-studied model of breast cancer, it has been shown that tamoxifen and 4-OHT compete with 17β-estradiol (E2) to inhibit ER-mediated transcription of genes necessary for the proliferation of mammary tumor cells (8).

Moreover, tamoxifen and 4-OHT are also able to induce apoptosis through an ER-dependent mechanism. ER-independent pathways eliciting apoptosis have also been described but the detailed molecular mechanisms of the latter effects are poorly understood. The addition of tamoxifen or 4-OHT to breast cancer cell lines in culture elicits the activation of effector caspases (9, 10). In breast cancer cells and in HeLa cells constitutively expressing ER, tamoxifen- or 4-OHT-induced apoptosis recruits the p38 mitogen-activated protein kinase (MAPK) or the c-jun NH2-terminal kinase-1 (JNK) signaling pathways (10, 11).

These observations prompted us to study the effects of 4-OHT on multiple myeloma cells. We have analyzed its *in vitro* effects on the proliferation and survival of six bona fide myeloma cell lines. We report here that the 4-OHT reduces cell proliferation of ER-expressing multiple myeloma...
cell lines by two mechanisms: a block in the G1 phase of the cell cycle and the induction of apoptosis. The induction of apoptosis requires the mitochondrial intrinsic pathway but not the death receptor Fas/FasL pathway nor modifications of signaling pathways known to be essential for multiple myeloma cells survival: phosphatidylinositol 3-kinase/AKT (12, 13), p70S6K (S6K; ref. 13), and Janus-activated kinase/signal transducer and activator of transcription-3 (STAT3; ref. 14). The G1 arrest is accompanied by the up-regulation of p27^Kip1 inhibitor as well as by the down-regulation of c-Myc. The apoptosis is controlled by the induction of the BimS proapoptotic protein as well as the down-regulation of the antiapoptotic Mcl-1 protein. Moreover, the effects of 4-OHT are mediated by the ER because the introduction of either ERα or ERβ in the ER-negative Karpas 620 cell line renders the cells sensitive to 4-OHT.

MATERIALS AND METHODS

Reagents. E2 and 4-OHT were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole] and SB 202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole] were purchased from Alexis Biochemicals (Lausen, Switzerland) and Calbiochem (Darmstadt, Germany), respectively. Stock solutions were prepared in DMSO at 10 mmol/L and stock E2 and 4-OHT solutions at 10 mmol/L in absolute ethanol. Cells were treated with freshly prepared solutions. For E2, 4-OHT, or SB treatments, ethanol or DMSO vehicle controls were added at the same concentration. Antibodies raised against the following phosphorylated proteins: anti-P-AKT (Ser473), anti-P-STAT3, anti-P-extracellular signal-regulator kinase (ERK), anti-P-S6K, and anti-P-JNK as well as anti-ERK and anti-JNK antibodies, were purchased from Cell Signaling, Inc. (Beverly, MA); anti-p27^Kip1, anti-S6K, anti-AKT, anti-Mcl-1, and anti-β-tubulin antibodies were from Santa Cruz (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-Bim antibody was from R&D Systems (R&D Systems Europe, Lille, France) and anti-STAT3 antibody was from Pharmingen (BD Biosciences Europe, Erembodegem, Belgium). A monoclonal antibody specific for ERα (D12, Tebu-bio, Le Pernay and Yvelines, France) and an antibody specific for ERβ (a gift from Dr. J.-C. Faye) were used for receptors detection.

Cell Cultures, Treatments, and Cell Proliferation Determination. LP-1, OPM-2, NCI-H929, RPMI 8226, U266, and Karpas 620 multiple myeloma cell lines have been described previously (15). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L l-glutamine. In experiments with E2 or 4-OHT, cells were cultured in phenol red–free RPMI 1640 supplemented with charcoal-treated inactivated FCS to eliminate estrogen agonistic or antagonistic activities. For cell proliferation and viability studies, exponentially growing 2 × 10^5 cells were seeded into six-well plates, in triplicate, in medium containing either ethanol (vehicle) or various concentrations of 4-OHT. Viable cells, excluding trypan blue, were counted in a hemocytometer at different time intervals. Normal B-lymphocytes were isolated from a spleen (after the informed consent of patient). Cells were purified through Ficoll-Hypaque gradient then cultured in complete RPMI 1640 containing pokeweed mitogen (5 µg/mL) and in vitro activated 2 hours before antiestrogen treatment.

Cell Viability Determination [2,3-bis(2-Methoxy-4-Nitro-5-Sulphonyl)-2H-Tetrazolium-5-Carboxanilide Inner Salt Assay]. Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega, Charbonnières, France). According to the supplier, 10^4 cells were seeded into 96-well plates, incubated with vehicle or various concentrations of reagents for 48 or 72 hours. Each culture condition was analyzed in triplicate. The absorbance values at 492 nm were corrected by subtracting the average absorbance from the control wells containing “no cells”.

Cell Cycle Analysis and Apoptosis Determination. For each culture condition, 5 × 10^5 cells were washed twice in PBS, pelleted, and suspended in ice-cold ethanol (80% in PBS). Fixed cells were then centrifuged and suspended in PBS containing 100 µg/mL RNase A (Roche Molecular Biochemicals, Meylan, France) and 20 µg/mL propidium iodide (Sigma-Aldrich) for 30 minutes at 37°C. Cells were analyzed with a FACScalibur (Becton Dickinson, Le Pont de Chaix, France) and data were obtained with the CellQuest 1.2.2 and the ModFit LT 1.01 softwares (Becton Dickinson).

Fluorocytometric Examination of Fas Expression. Multiple myeloma cells were treated with 10 µmol/L 4-OHT or vehicle for 48 hours, then washed and suspended in PBS. They were incubated with phycoerythrin-conjugated anti-Fas antibody (Clone UB2, Immunotech, Marseille, France) or mouse IgG1 isotype as control for 25 minutes at 4°C. Cytofluorometric analysis was done (FACScalibur cytometer) and the percentage of Fas-positive cells as well as the mean fluorescence intensity were evaluated by the CellQuest 1.2.2. software on 10,000 acquired events.

Mitochondrial Membrane Potential Measurement. The reduction of mitochondrial membrane potential (ΔΨ_m) is an early event and obligatory step of the apoptotic process. The fluorochrome 3,3-dihexyloxacarbocyanide iodide (DiOC6) incorporates into internal mitochondria membrane of healthy cells. The reduction of ΔΨ_m representative of apoptosis induction can be quantified by the decrease of DiOC6 retardation (16). Multiple myeloma cells were treated with 10 µmol/L 4-OHT or ethanol for 24 hours, washed, and costained with 20 µg/mL propidium iodide and 40 nmol/L DiOC6 for 30 and 15 minutes, respectively. Flow cytometry was done as described above. To control ΔΨ_m reduction, cells were first treated with 150 µmol/L carbonyl cyanide m-chlorophenylhydrazone (Calbiochem) for 15 minutes, then with propidium iodide and DiOC6. Mitochondrial retention of DiOC6 was measured by gating propidium iodide–negative viable cells. Acquisition of events (10,000 gated) and analysis were achieved using the CellQuest software.

Caspase Activity Determination. Caspase activation was determined using caspase-3 and caspase-9 colorimetric assay kits as recommended by the manufacturer (R&D Systems). Briefly, 300 µg of whole cell lysates (bicinchoninic acid protein assay, Pierce Biotechnology, Inc., Rockford, IL) were incubated for 2 hours at 37°C with 5 µL of 4 mmol/L LEHD-pNA substrate (for caspase-9) or 5 µL of 4 mmol/L
DEVD-pNA substrate (for caspase-3) in triplicate. Absorbance of the samples was read at 405 nm and specific absorbance was deduced from background controls (reactions with no substrate). Changes in caspase activity were deduced from the comparison of vehicle-treated and 4-OHT-treated cells.

Transfection of Karpas 620 Cell Line. Transient transfections of Karpas 620 cells were done by electroporation (250 V, 950 μF, GenePulser II, Bio-Rad, Marnes la Coquette, France). Cells were washed, seeded at 10^7 cells in 400 μL of phenol red-serum-free medium for 2 hours at 37°C, submitted to electrical field, and cultured in complete medium (with charcoal-treated FCS) for 48 hours. Cells were treated with various concentrations of 4-OHT or vehicle and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays were done as described. Plasmids expressing ERα and ERβ cDNAs were obtained from P. Chambon and B. Haendler, respectively.

Western Blots. NP40 lysates were obtained by resuspending the cells in the following buffer: 1% NP40, 50 mM/L Tris (pH 7.5), 10% glycerol, 150 mM/L NaCl, 1 mM/L EDTA, 100 mM/L Na_2VO_4, 0.5 mM/L phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (Roche Diagnostics). For the preparation of nuclear extracts, cells were first incubated with a buffer containing 10 mM/L HEPES (pH 7.6), 0.1% NP40, 0.1 mM/L EDTA, and protease inhibitors as before. Lysates were centrifuged at 12,000 × g, 4°C for 15 minutes. Pellets were then incubated in radioimmunoprecipitation assay buffer [10 mM/L Tris (pH 7.5), 150 mM/L NaCl, 1% NP40, 0.1% SDS, 1% deoxycholic acid, protease inhibitors] for 30 minutes on ice and centrifuged at 15,000 × g, 4°C for 15 minutes. For ER detection, total cell extracts were prepared by addition of lysis buffer [50 mM/L HEPES (pH 7.5), 150 mM/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM/L MgCl_2, 1 mM/L EDTA, protease inhibitors]. Cells were lysed for 30 minutes at 4°C followed by 10-minute boiling in Laemmli sample buffer. Proteins (15-100 μg) were separated by electrophoresis on SDS/PAGE and blotted onto cellulose membranes (Hybond-C super, Amersham Biosciences, Les Ulis, France). Membranes were immunoblotted with the indicated antibodies, then exposed after incubation with the enhanced chemiluminescence Western blotting detection reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Perbio Science France, Brebières). Densitometric analyses were realized with a FluorSimager (Bio-Rad) using the QuantityOne software (Bio-Rad).

Statistical Analysis. The Student t test was used to determine the significance of differences between two experimental groups. Mean of triplicates was analyzed by a two-sided test and P <0.05 was considered significant.

RESULTS

Treatment with 4-Hydroxytamoxifen Reduced Multiple Myeloma Cell Proliferation. We have previously reported that treatment with 4-OHT inhibited the proliferation of three multiple myeloma cell lines, LP-1, NCI-H929, and U266, in a dose- and time-dependent fashion (17). In the same experimental conditions, 4-OHT had no effect on proliferation of Karpas 620 multiple myeloma cell line (17). We now extend these observations to two other lines: RPMI 8226 was sensitive to 4-OHT treatment whereas OPM-2 showed no dose response (data not shown and see below).

Inhibition of Multiple Myeloma Cell Proliferation Was due to G_1 Arrest and Apoptosis Induction. To elucidate the mechanisms of multiple myeloma cell inhibition of proliferation, we did cell cycle analysis after propidium iodide–staining and subsequent flow cytometry sorting. 4-OHT-sensitive cells were treated with the same antiestrogen concentrations, 1, 5 and 10 μM/L, for 48 hours. As an indicator of 4-OHT toxicity, we tested under the same conditions the response of normal mature B-lymphocytes isolated from human spleen and stimulated in vitro with pokeweed mitogen. As depicted in Fig. 1A, 4-OHT treatment induced a G_1 arrest in the four responsive cell lines but at different concentrations. RPMI 8226 cells were blocked after 1 μM/L 4-OHT treatment (+7%), LP-1 and U266 cells were blocked after 5 μM/L 4-OHT treatment (+13% and +11%, respectively), whereas NCI-H929 cells were more resistant (+7% for 10 μM/L 4-OHT). The appearance of a sub-G_1 fraction in a sub-G_1 fraction and triggers apoptosis in multiple myeloma cells. OPM-2 cells were assayed under the same experimental conditions. 4-OHT treatment did not modify the percentage of OPM-2 cells within the G_0/G_1 and sub-G_1 fractions, confirming their resistance to SERS (data not shown). Similarly, 4-OHT had no effects on normal mitogen-stimulated B-cells. Indeed, when normal B-lymphocytes were exposed to 5 or 10 μM/L 4-OHT for 48 hours, no major effects on cell cycle distribution were evident and apoptosis was not triggered (Table 1). The examination of sensitivity towards G_1 arrest or apoptosis of each cell line indicates that the two events are independent. Moreover, the sensitivity of multiple myeloma cells to 4-OHT-induced apoptosis was unrelated to the percentage of cells in S phase. The two cell lines possessing the highest percentage of cells in S phase, RPMI 8226 and LP-1 (46% and 43%; Fig. 1A), did not display the highest percentage of sub-G_1 (23% and 10%) after SERS treatment. The apoptotic nature of cell death after 4-OHT treatment was confirmed by microscopic morphologic examination following 4',6-diamidino-2-phenylindole staining (data not shown) and by the quantitation of downstream caspase-3 activity (Fig. 1B). Caspase-3 is believed to be the major effector (or executioner) caspase and functions downstream of all apoptosis pathways (18). Activation of caspase-3 was observed in the four 4-OHT-responsive cells, in good agreement with the cell cycle results.

The Response of Multiple Myeloma Cells to 4-Hydroxytamoxifen Is Dependent on Estrogen Receptor Expression. We next determined if the 4-OHT inhibitory effect on multiple myeloma cell proliferation requires the presence of ER (α or β). Expression of both ER isotypes in multiple myeloma cell lines was evaluated by immunoblotting (Fig. 2A). All multiple myeloma cell lines except Karpas 620 cells expressed both ERα and ERβ forms to various levels. The levels of ERα and ERβ were much weaker in multiple
myeloma cells than in human breast cancer MCF-7 cells in contrast to previous published data (4). Karpas 620 cells did not express any isotype of ER and did not respond to 4-OHT treatment. This suggests that ER is necessary for the cellular response to 4-OHT. In order to verify the functionality of ER expressed on multiple myeloma cells, they were treated with 25 nmol/L E2 for 72 hours and XTT assays were done. As shown in Fig. 2B, U266, NCI-H929, RPMI 8226, and LP-1 cells showed a decrease in cell viability. By contrast, OPM-2, although expressing both isotypes, did not show any response to E2 treatment, suggesting that in those cells ERs are not functional. Together with previous results, which indicated that OPM-2 cells were resistant to 4-OHT, we propose that the biological effects elicited by 4-OHT require functional ER molecules. To verify this hypothesis, we introduced ERα or ERβ expression vectors into Karpas 620 cells by transient transfection. Transfected cells were expanded for 48 hours before addition of 10 nmol/L 4-OHT and XTT assays were next done. Cell viability of ER-expressing cells and that of cells transfected with the empty vector (pcDNA3) were compared (Fig. 2C). The presence of ERα or ERβ in Karpas 620 cells renders cells sensitive, at least partially, to 4-OHT.

**4-Hydroxytamoxifen-Mediated Apoptosis in Multiple Myeloma Cells Requires a Mitochondrial-Dependent Death Pathway.** It has been suggested that for one multiple myeloma cell line tamoxifen treatment induces an up-regulation of Fas expression and that tamoxifen-induced apoptosis requires the death receptor extrinsic pathway (5). We therefore compared, by flow cytometry, the expression of Fas receptor in vehicle-treated multiple myeloma cells and in cells treated with...
10 μM 4-OHT for 48 hours. As shown in Fig. 3A, 92% to 99% of LP-1, RPMI 8226, and U266 cells expressed Fas, whereas NCI-H929 cells did not. No modifications of the percentage of Fas-positive cells or of mean fluorescent intensity were detected after SERM treatment (Fig. 3A, right).

To investigate whether or not a mitochondrial-dependent process is involved in the 4-OHT-induced apoptosis, multiple myeloma cells were treated with 10 μM 4-OHT or with vehicle for 24 or 36 hours and costained with propidium iodide and the fluorescent DiOC6. As presented in Fig. 3B for RPMI 8226 and U266 cells, mitochondria of vehicle-treated propidium iodide–negative cells displayed a high DiOC6 retention (74.9% and 88.7%, respectively). By contrast, mitochondria of 4-OHT-treated propidium iodide–negative cells exhibited a decrease of DiOC6 retention (~29.1% for RPMI 8226 and ~15.2% for U266). Our results indicate that 4-OHT causes the disruption of ΔΨm, a reflection of the activation of the intrinsic apoptotic mitochondrial pathway. Caspase-9 is an initiator caspase within the mitochondrial death pathway (18). In order to detect activation of caspase-9 subsequent to the mitochondrial membrane permeabilization, we analyzed the activity of whole cell extracts on the caspase-9 substrate, LEDH-pNA. As depicted in Fig. 3C, in the three cell lines studied, 4-OHT treatment led to the activation of caspase-9.

Alterations of Survival Pathways Are Not Associated with 4-Hydroxytamoxifen-Mediated Apoptosis. Regulation of multiple myeloma cell survival or proliferation is dependent on different signal transduction pathways such as MAPK, phosphatidylinositol 3-kinase/AKT, S6K, and STAT3 pathways. Some of these pathways are constitutively active in multiple myeloma cells (10–12). We next analyzed a possible alteration of these pathways following the treatment of multiple myeloma cells with 4-OHT. Extracts prepared from cells treated with various concentrations of 4-OHT (1-10 μM) for 48 hours were analyzed by immunoblotting with antiphosphospecific antibodies raised against signaling molecules. Results obtained with 5 μM 4-OHT are presented in Fig. 4A; the levels of signaling molecules analyzed were similar following treatment with 10 μM 4-OHT (data not shown). Phosphorylation of STAT3, AKT, ERK, S6K, and JNK proteins was of varying...
degrees in some, but not all, multiple myeloma cell lines, reflecting a high degree of heterogeneity. Following 4-OHT treatment, we observed a decrease of AKT phosphorylation in NCI-H29 cells and of S6K and ERK phosphorylation in OPM-2 cells, but these effects were cell line specific. These results suggest that 4-OHT does not modify significantly the phosphorylation status of STAT3, AKT, S6K, ERK1/2, and JNK proteins although the latter have been shown to play a role in estrogen and antiestrogen signal transduction (10, 11).

4-OHT activates the p38 MAPK pathway to induce apoptosis in HeLa cells stably transfected with ER (11). We therefore evaluated the contribution of this pathway in 4-OHT-mediated apoptosis of multiple myeloma cells. XTT assays were done on multiple myeloma cell lines treated with two p38 MAPK inhibitors: SB 202190 and SB 203580 (concentration range: 0-100 μmol/L) in the presence or absence of 4-OHT (5 μmol/L). Increasing concentrations (1 to 50 μmol/L) of SB 202190 or SB 203580 slightly inhibited cell proliferation of LP-1 and NCI-H929 cells but had no effects on RPMI 8226 and U266 cells (Fig. 4B). At 100 μmol/L both compounds had an inhibitory effect on cell viability (Fig. 4B). Neither synergy nor potentiation was evident when SB 203580 (10 or 50 μmol/L) and 4-OHT (5 μmol/L) were added together (Fig. 4C). These data indicate that the effects of 4-OHT on multiple myeloma are not mediated through changes in survival signaling pathway activities.

Up-Regulation of **p27**kip1 and Down-Regulation of c-Myc Are Associated with a Block of Cell Proliferation. The effects of 4-OHT on G1 arrest prompted us to investigate the expression of cell cycle regulators involved in progression within the G1 phase progression and the G1-to-S transition. As previously reported (15), NCI-H929 and RPMI 8226 express high to moderate levels of cyclin D1 whereas LP-1 does not. 4-OHT treatment did not affect cyclin D1, cyclin D2, or cyclin D3 levels in all multiple myeloma cells (data not shown). In addition, we analyzed the expression of two cell cycle inhibitors, p21**cip1** and p27**kip1**. Importantly, p27**kip1**, expressed at various basal levels, was up-regulated in a dose-dependent fashion after 4-OHT treatment in NCI-H929, RPMI 8226, and LP-1 cells.
Down-regulation of c-Myc also occurred when breast cancer cells were treated with pure antiestrogens (19), a phenomenon also observed in NCI-H929, RPMI 8226, U266, and LP-1 cells after SERM treatment (Fig. 5B). Our data suggest that p27Kip1 and c-Myc are essential mediators of cell cycle blockade in G1 phase following 4-OHT treatment.

**Up-Regulation of BimS and Down-Regulation of Mcl-1 Are Both Associated with Antiestrogen-Induced Apoptosis.** We also addressed the issue of whether some members of the Bcl-2 family were implicated in 4-OHT-induced apoptosis. The expression of Bcl-2, Bcl-XL, and Bax was unaffected by antiestrogen treatment (17). However, we observed an induction of the short form of Bim (BimS) in multiple myeloma cells treated with 10 μmol/L 4-OHT. Besides BimS induction, we also observed the transient induction of Mcl-1 followed by its down-regulation (see 5 μmol/L treatment versus 10 μmol/L). These results are presented in Fig. 5C along with a densitometric analysis of Mcl-1/β-tubulin ratios.

**DISCUSSION**

We show here that all ER-expressing multiple myeloma cells are blocked in G1 phase of the cell cycle and undergo apoptosis when treated with pharmacologic concentrations (5-10 μmol/L) of the SERM 4-OHT. OPM-2 cells are resistant to 4-OHT treatment possibly because expressed ERs are not functional. Karpas 620 cells are insensitive to 4-OHT treatment unless they are transiently transfected with Erα or Erβ. Thus, in multiple myeloma cells, as in breast cancer cells, 4-OHT inhibits ER-responsive genes regulating cell proliferation. Treon et al. (4) have reported that tamoxifen and, to a lesser extent, toremifene inhibit the proliferation of multiple myeloma cell lines (5 of 5 tested). Moreover, of 5 multiple myeloma cell lines tested, only U266 cells undergo apoptosis after tamoxifen treatment (5 and 50 μmol/L). Another group analyzed the effects of tamoxifen and toremifene on multiple myeloma cells and concluded that both antiestrogens have inhibitory effects on cell proliferation (5 of 7 cell lines tested, concentrations: 1 and 10 μmol/L; ref. 5) and trigger apoptosis in 2 cell lines (of 7 cell lines tested, concentration: 100 μmol/L). In the same experimental conditions, we have studied the effects of two selective estrogen receptor down-regulators (SERDs) or pure antiestrogens: RU 58668 and ICI 182780. Both induced either a G1 arrest or apoptosis depending on the cell line (data not shown). Together, these results confirm the potential of SERDs and SERMs as antiproliferative and/or proapoptotic agents on multiple myeloma cells.

The inhibition of cell proliferation in multiple myeloma cells treated with 4-OHT is manifest at micromolar concentrations (>1 μmol/L) but these are much lower than the concentrations of tamoxifen used in previous studies (50-100 μmol/L; refs. 4, 5). This probably reflects the known higher affinity of 4-OHT for ER (7). At physiologic concentrations (1, 10, and 100 nmol/L), E2 had no effects on multiple myeloma cell proliferation block or apoptosis (data not shown). By contrast, in breast cancer cells, the effects on cell cycle proteins and cell proliferation are obtained with nanomolar concentrations of these compounds. This work and that of others (4, 5) suggest that a nonclassic genomic action of ER is triggered by SERMs in multiple myeloma cells. Whether a
nongenomic action (20, 21) or an unexpected action of 4-OHT/ER is possible, it has been proposed that the antiestrogen binding sites could be responsible for the growth inhibition of multiple myeloma cells (4). Antiestrogen binding sites could mediate the binding and in turn the inhibitory effects of 4-OHT in human cancer cells. In multiple myeloma cells, 4-OHT-like effects on cell proliferation were observed also with micromolar concentrations of the pure antiestrogens ICI 182780 or RU 58668. However, 4-OHT was always described as a high-affinity ligand for both ER and antiestrogen binding sites (22, 23) whereas pure antiestrogens have no affinity for antiestrogen binding sites (24) and high affinity for ER (see ref. 7 for a review). Indeed, antiestrogen binding sites result from the association between two enzymes involved in the synthesis of cholesterol precursors (the 3β-hydroxysterol-Δ8-Δ7-isomerase and the 3β-hydroxysterol-Δ7-reductase) without affinity for pure antiestrogens (24). Therefore, it is unlikely that a mechanism involving antiestrogen binding sites could be responsible for the antiproliferative action of 4-OHT. It is obvious that further clarification is required to explain the activity of SERMs in multiple myeloma cells. Although the mechanism is not fully defined, these effects seem dependent on the presence of ER.

The most relevant function for estrogens in breast cancers is the promotion of cell proliferation through cyclin D1 gene (CCND1) activation. Therefore, it is not surprising that in those cells, SERM-mediated cell cycle arrest is associated with the decrease of cyclin D1 gene expression (25, 26). U266, RPMI 8226, and NCI-H929 cells constitutively express cyclin D1 although at various levels whereas in the LP-1 cell line, the CCND1 gene encoding cyclin D1 is transcriptionally silent (15). This indicates that in multiple myeloma cells, CCND1 is not the key target of 4-OHT. It has to be noticed that normal breast epithelial cells express physiologically cyclin D1 whereas CCND1 expression in B-lymphocytes is restricted to tumor cells including multiple myeloma cells. The cell cycle p27Kip1 inhibitor is also an essential mediator of antiestrogen-induced cell cycle arrest in breast cancer (19). p27Kip1 is expressed in NCI-H929, LP-1, and RPMI 8226 cells and is up-regulated after SERM treatment. Whereas the pRb pathway is defective in multiple myeloma cells, p27Kip1 is a target of 4-OHT and the accumulation of p27Kip1 is likely responsible for the G1 arrest observed in multiple myeloma cells. p27Kip1 is a major regulator of proliferation in most cells, its inhibitory activity being mediated by its binding to cyclin E/Cdk2 complexes (27). Our preliminary data indicate that in 4-OHT-treated cells inhibition of cyclin E/Cdk2 enzymatic activity is achieved through the down-regulation of CDK2. The other major event associated with antiestrogen-mediated G1 arrest in breast cancer cells is the
acute down-regulation of c-Myc (28). Although probably controlled by different pathways (here this is a late event), the down-regulation of c-myc is also associated with 4-OHT-induced cell cycle blockade in multiple myeloma cells.

Bcl-2, Bcl-XL, and Bax proteins are expressed in multiple myeloma cells; their levels were unchanged after 4-OHT treatment (17), in good agreement with studies in breast cancer cells in which Bcl-2, Bcl-XL, and Bax showed only marginal changes in the presence of antiestrogen (29). But interestingly, Mcl-1, essential for the survival of multiple myeloma cells (30–32), is down-regulated after 4-OHT treatment. The prosurvival role of Mcl-1 is required for the development and the maintenance of B-lymphocytes and its down-regulation has been previously reported in apoptotic multiple myeloma cells treated with flavopyridol (33). These data indicate that Mcl-1 is an important target of antitumoral compounds in multiple myeloma cells. The BH3-only proapoptotic proteins BimEL, BimL, and BimS are generated by alternative splicing. Unlike BimEL and BimL, which are present in normal cells but inactivated by their interactions with the cytoskeleton, BimS is transiently expressed in apoptotic cells (34). In good agreement with that, we found an up-regulation of BimS in cells treated with 10 μmol/L antiestrogen (i.e., in apoptotic cells). Mitochondrial apoptosis is regulated by the heterodimerization between prosurvival and proapoptotic Bcl-2 family members (including Mcl-1 and Bim; ref. 35). In multiple myeloma cells induced towards apoptosis by the antiestrogen treatment, the first response against the apoptotic stress is the up-regulation of Mcl-1 survival protein. This is followed by the induction of Bim and the concomitant down-regulation of Mcl-1 at the time of apoptosis completion. The possible sequestration of Mcl-1 by Bim should be verified in the near future.

We have shown here that 4-OHT is able to induce apoptosis through the mitochondrial intrinsic pathway. Our results are consistent with the data in the literature showing that tamoxifen and 4-OHT induce a mitochondrial- and caspase-dependent apoptosis in breast cancer cell lines and primary tumors (10). 4-OHT also triggers apoptosis of ER-transfected HeLa cells through a similar process. Our results are also consistent with literature concerning the mechanism of apoptosis induction in multiple myeloma. Therapeutic drugs such as dexamethasone and 2-methoxyestradiol induce apoptotic cell death, which is mediated through the dissipation of mitochondrial membrane potential. This phenomenon is accompanied by the cytosolic release of various mitochondrial proteins as cytochrome c or Smac/DIABLO (36, 37). Analysis of the subcellular distribution of these mitochondrial proteins has to be envisaged to complete the elucidation of the mechanisms of 4-OHT-induced apoptosis in multiple myeloma cells.

Cross-talks between the ER and phosphatidylinositol 3-kinase, STAT3, or ERKs have been described in various cell types (38). In breast cancer cells and in ER-transfected HeLa cells, induction of apoptosis is related to the activation of p38 MAPK and JNK survival pathway members (10, 11). However, our data indicate that the apoptosis induced by 4-OHT in multiple myeloma cells is probably not mediated through activation of these proteins. Our data show that 4-OHT does not dramatically nor significantly affect activation of these signaling pathways, all constitutively activated in multiple myeloma cells. However, it remains possible that 4-OHT inhibits the transcriptional activities of STAT3 and/or other transcription factors acting downstream the phosphatidylinositol 3-kinase or the MAPK pathways. This inhibitory mechanism is now under investigation.

The 4-OHT-mediated apoptosis does not proceed through the activation of the extrinsic death pathway. In leukemic cells, the Fas/FasL death pathway seems to be involved in drug-induced cell death but conflicting results have been reported (39, 40). Interestingly, in myeloma cells, Landowski et al. (41) reported that Fas and chemotherapeutic drugs (anthracycins, doxorubicin, and mitoxantrone) trigger independent mechanisms to activate downstream caspases. Our findings are in good agreement with this suggestion.

The induction of apoptosis is required for a chemotherapeutic agent to be active. In that respect, apoptotic cell death can be obtained by the treatment of multiple myeloma cells with 4-OHT. Importantly, in the same experimental conditions, normal B-lymphocytes are not affected by the antiestrogen treatment. Moreover, the apoptosis-inducing effect of 4-OHT is not mediated through inhibition of survival pathways associated with growth of multiple myeloma cells. Because bone marrow environment confers resistance to drug treatment through the activation of such survival pathways (2), the use of 4-OHT, which targets exclusively the intrinsic death pathway, is of major interest. Interestingly, induction of multiple myeloma apoptosis is obtained with 4-OHT concentrations (5–10 μmol/L) already evaluated clinically and compatible with anticancer treatment (42). Moreover, although our experiments must be repeated in the future with primary myeloma cells, we are confident that 4-OHT does not exert its antiproliferative and proapoptotic effects on S phase. Thus, myeloma cells, which possess a low labeling index with less than 0.5% of cells in S phase, could be the target of 4-OHT. Finally, the effects of 4-OHT are observed on cell lines exhibiting various degrees of resistance to chemotherapeutic agents. RPMI 8226, NCI-H929, and U266 are resistant to glucocorticoids and/or DNA-damaging agents (see for example refs. 33, 34). Resistance towards conventional anticancer drugs frequently appears de novo during the treatment of multiple myeloma disease. Combined association of 4-OHT with conventional drugs could potentially overcome such resistance.

ACKNOWLEDGMENTS

We thank Anne Barbaras and Aline Régnier for skillful technical assistance. We acknowledge Véronique Salalin and Brigitte Cauquelin for their help with the cytofluorometric analysis and Dr. J.C. Faye (Institut Claudius Régaud, Toulouse, France) for the gift of anti-ERβ antibody.

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4-Hydroxytamoxifen Inhibits Proliferation of Multiple Myeloma Cells \textit{In vitro} through Down-Regulation of c-Myc, Up-Regulation of p27^{Kip1}, and Modulation of Bcl-2 Family Members
