

Estrogen Receptor Expression and Estrogen Receptor-independent Cytotoxic Effects of Tamoxifen on Malignant Rhabdoid Tumor Cells *in vitro*

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Recent studies have shown that the antiestrogen tamoxifen (TAM) can be used in the treatment of malignant neoplasms other than breast cancer. In the present study, we investigated the expression of estrogen receptor (ER) in six malignant rhabdoid tumor (MRT) cell lines. Alterations in MRT cell growth in response to estrogen or antiestrogens (4-hydroxytamoxifen (4-OHT), TAM, and ICI 182 780) were also investigated. RT-PCR and western blotting showed that ER- α was expressed in three of the six MRT cell lines. While 17- β -estradiol (E2) did not significantly alter MRT cell line proliferation, the hydroxylated tamoxifen metabolite 4-OHT significantly inhibited the growth of all 6 MRT cell lines. However, the steroidal antiestrogen ICI 182 780 did not alter the proliferation of any of the MRT cell lines. 4-OHT induced apoptosis in both ER- α -negative and ER- α -positive MRT cell lines, as assessed by nuclear morphology and DNA fragmentation. Neither growth inhibition nor induction of apoptosis due to 4-OHT was blocked by the addition of excess E2. Our data suggested that 4-OHT induced cytotoxic effects against MRT cells, and that these effects were independent of ER expression.

Key words: Malignant rhabdoid tumor — Estrogen receptor — Antiestrogen — Apoptosis

Estrogens have long been recognized to play important roles in the growth and differentiation of reproductive and non-reproductive tissues.^{1,2} It has also become clear that most breast cancers are dependent on estrogens for growth and progression, and this has been the basis for continuing interest in the role of estrogen receptor (ER) in the prognosis of this devastating disease. However, some recent reports have demonstrated that ER expression may have a role in neoplasms other than breast cancer, such as colon cancer, meningioma, prostate cancer, and myeloma.^{3–6}

The antiestrogen tamoxifen (TAM) has been widely used in the treatment of breast cancer,⁷ and recently against some other cancers as well.⁸ Antiestrogens can be classified into two major groups: analogs of TAM or its metabolites (type I) that have mixed estrogenic/antiestrogenic actions, and pure antiestrogens (type II) that have no estrogen-like properties in laboratory assays.⁹ 4-Hydroxytamoxifen (4-OHT) is converted to an active metabolite of TAM, while ICI 182 780 is a pure antiestrogen that is being clinically evaluated as a useful agent following failure of TAM treatment.^{9–12} It is thought that inhibition of ER by antiestrogens may block the secretion of growth factors, such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF- α). In addition to its cytostatic effects, TAM has also been shown to be cytotoxic against tumor cells, even tumor cells lacking ER.^{13,14} Such cytotoxic effects were thought to be mediated by the induction of apoptosis.^{15,16}

Malignant rhabdoid tumors (MRT) are rare, highly aggressive neoplasms specific to early childhood, and have an extremely poor prognosis due to the high potential for distant metastasis.^{17–19} While various organs and tissues, including the kidneys, can be the primary site of MRT,^{20–24} a characteristic feature of MRT cells is the presence of large eosinophilic inclusions in the cytoplasm.²⁵ Poor response to treatment results in an extremely poor prognosis.¹⁹ Our laboratory has previously reported that expression of insulin-like growth factor receptor²⁶ and EGF receptor²⁷ was associated with MRT cell proliferation. However, very little is known of MRT cell growth and differentiation. We believe that further investigation into the mechanisms of MRT cell proliferation may help to improve the poor disease prognosis.

ER expression and the effects of antiestrogens on MRT cells are currently unknown. Therefore, we first focussed on MRT cell ER expression through examination of ER mRNA and protein levels in six MRT cell lines. We then studied the effects of antiestrogens 4-OHT, TAM, and ICI 182 780 on the MRT cell lines by analyzing alterations in cell growth. Lastly, we also studied the induction of apoptosis through the use of nuclear morphology and DNA fragmentation assays.

MATERIALS AND METHODS

Cell culture and compound MRT cell lines TM87-16, STM91-01, TTC549, TTC642 and TTC1240 were provided by Dr. Hiroyuki Shimada and Dr. Timothy J. Triche (Childrens Hospital Los Angeles, Los Angeles, CA). MRT

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Table I. Clinical History of MRT Cell Lines

Name of cell lines	Age (month) and Sex	Primary site	Outcome	Origin of cell line
TM87-16	21 M	Retroperitoneal	Died	Pleural effusion
STM91-01	8 M	Left kidney	Died	Lung metastasis
TTC549	6 F	Liver	Died	Primary site
TTC642	5 F	Neck mass	Died	Primary site
YAM-RTK1	7 M	Left kidney	Died	Ascites
TTC1240	9 F	Kidney	Died	Brain

cell line YAM-RTK1 was provided by Dr. Kanji Sugita (Yamanashi Medical University, Kofu). MCF-7 human breast cancer cell line was obtained from the Health Science Research Resources Bank, Osaka. All assays on the MRT cell lines were performed before passage 24. Clinical data on the patients whose tumors were used to establish these cell lines are summarized in Table I.

All cells were maintained in phenol red containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂, and were maintained in phenol red-free RPMI 1640 with 10% dextran-coated charcoal-treated FBS, to remove steroids, at least 48 h before each experiment. 4-OHT, TAM and 17-β-estradiol (E2) were purchased from Sigma Chemicals (St. Louis, MO). ICI 182 780 was a gift from AstraZeneca Pharmaceuticals (Macclesfield, UK). All compounds were dissolved in 100% ethanol and added to media at 1:1000 dilution.

RNA preparation and RT-PCR analyses RNA extraction and cDNA synthesis were performed as described previously.²⁷⁾ Aliquots of cDNA (1 μl) were amplified using *Taq* polymerase (Toyobo, Tokyo) in a total volume of 25 μl. For detection of ER-α mRNA, PCR was performed for 35 cycles of denaturation at 95°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 60 s, with a pre-denaturing time of 2 min, and a final extension time of 5 min. The primer sequences used for ER-α were 5'-ATATGTGTCCAGCCACCAAC-3' and 5'-CCAA-CAAGGCACTGACCATC-3' (306 bp). PCR reaction products were electrophoresed through 2% agarose gels (Nacalai Tesque, Kyoto) containing 0.2 mg/ml ethidium bromide. PCR-amplified products were directly sequenced as previously described.²⁷⁾ The *β-actin* gene was co-amplified to determine the quality and quantity of the synthesized cDNA.

Immunocytochemistry Immunocytochemical analysis was performed on untreated subconfluent cells cultured in 60 mm dishes. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Immunocytochemical staining was performed using 1:100 dilution of mouse anti-human ER-α monoclonal antibody D-12 (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were

then incubated with peroxidase-conjugated anti-mouse immunoglobulin (Envision+; Dako Co., Carpinteria, CA) for 30 min. Conjugates were visualized by incubation with 0.05% Ni-diaminobenzidine solution containing 0.01% hydrogen peroxide for 5 min at room temperature.

Western blot analysis Whole-cell extracts were prepared by direct lysis. Protein concentrations were measured using the Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of total protein (50 μg) were analyzed by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with monoclonal antibody D-12 (Santa Cruz Biotechnology). Actin antibody H-300 (Santa Cruz Biotechnology) was used to standardize loading. Complexes were detected using the ECL kit (Amersham Pharmacia, Buckinghamshire, UK) and the membranes were wrapped in plastic and exposed to Hyperfilm ECL (Amersham Pharmacia).

Cell proliferation assay Cells were seeded at 1×10⁵ per dish in estrogen-free medium and left undisturbed for one day to facilitate cell attachment to the plates. The following day (day 0), the medium was removed, and 3 ml of medium containing the indicated compounds was added. After incubation for several days, cell numbers were determined as previously described.²⁷⁾

DNA fragmentation Apoptosis was determined by DNA fragmentation assay using agarose gel electrophoresis. Genomic DNA was extracted using a Suicide-Track DNA ladder isolation kit (Oncogene Research Products, Cambridge, MA) to prevent contamination of DNA ladder fragments with high-molecular-weight DNA, and 1 μg aliquots of DNA were electrophoresed in 2% agarose gels. Ladders were visualized by SYBR Green I (TaKaRa Shuzo, Shiga) staining.

Morphological analysis of apoptotic cells Cells isolated after exposure to trypsin were washed twice with PBS, fixed in 1% glutaraldehyde for 24 h, and stained with 2.5 μg/ml Hoechst 33258 and 2.5 μg/ml propidium iodide. Cells were then examined by fluorescence microscopy. Intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei were considered as viable, early apoptotic, late apoptotic, and necrotic cells, respectively.

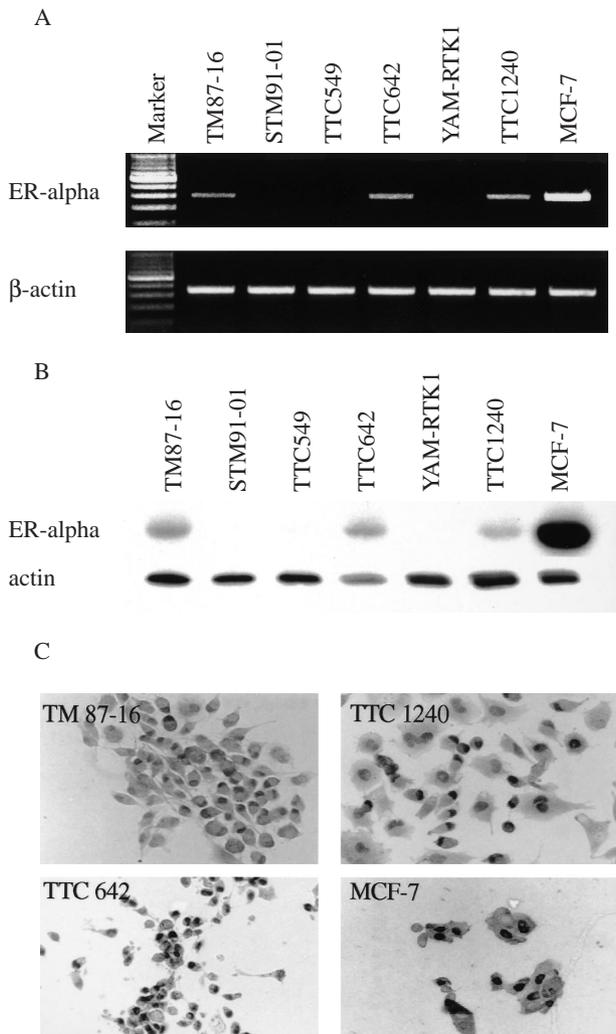


Fig. 1. Expression of ER- α on MRT cell lines. A and B, mRNA and protein expression for ER- α were examined by RT-PCR and western blotting respectively. C, Localization of ER- α protein was examined by immunocytochemistry. MCF-7 breast cancer cell line served as a positive control for ER- α expression.

Statistical analysis Data are presented as the mean \pm SD. Differences were examined by using Student's two-tailed *t* test. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Expression of ER- α Expression of ER- α mRNA was detected in TM87-16, TTC642, and TTC1240 cells, but not in STM91-01, TTC549, or YAM-RTK1 cells (Fig. 1A). Results of western blotting analysis using anti-ER- α antibody correlated well with the ER- α mRNA results, with expression of ER- α protein observed in TM87-16,

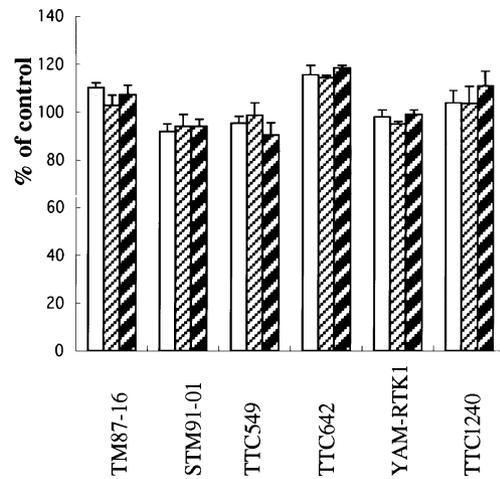


Fig. 2. Effect of E2 on growth of MRT cell lines. The cells were treated with 1 (\square), 5 (\square), and 10 (\square) nM E2 for 4 days. The cell number was compared with that of the cell in estrogen-free media alone.

TTC642, and TTC1240 cells (Fig. 1B). Immunocytochemical staining of TM87-16, TTC642, and TTC1240 cells using a monoclonal antibody demonstrated that ER- α protein was located in the nucleus, as in the case of MCF-7 cells (Fig. 1C).

Growth effect of estrogens and antiestrogens in MRT cells We first examined the effect of E2 on the six MRT cell lines. Although a slight increase in ER- α expression was observed in the MRT cell lines in response to E2, this increase was not significant (Fig. 2). We next examined whether 4-OHT could inhibit MRT cell growth. Treatment with 4-OHT (5–10 μ M) led to a dose-dependent reduction in cell growth in all 6 MRT cell lines (Fig. 3). This suppression of cell growth appeared to be independent of ER expression by the MRT cells. To clarify this, we cultured the MRT cell lines with other antiestrogens (TAM, ICI 182 780) at the same concentrations as 4-OHT. TAM also inhibited the growth of all MRT cell lines (data not shown). As shown in Fig. 4, ICI 182 780 mildly decreased the growth of YAM-RTK1 and TTC1240 cells, but this decrease was not statistically significant. IC₅₀ of the antiestrogens was estimated by regression plot analysis.²⁸⁾ It was found that 4 to 6 μ M 4-OHT inhibited MRT cell line growth by 50%. Although TAM also inhibited MRT cell line growth, slightly higher concentrations of TAM were required to achieve the same effects as 4-OHT in three cell lines (STM91-01, TTC549, and TTC1240) (Table II).

Growth effect of E2 on the 4-OHT-induced growth inhibition In order to assess whether 4-OHT inhibited the growth of ER- α -positive cells via the estrogen receptor, we cultured ER- α -positive TM87-16 cells with 1.0, 5.0, or

10.0 μM 4-OHT in the presence of equimolar or excess E2 (10.0 μM). The additional E2 did not alter the growth inhibition of TM87-16 cells (Fig. 5). This result was repeated using the other ER- α -positive cells (TTC642 and TTC1240), which were also inhibited by 4-OHT even in the presence of E2 (data not shown). E2 (10.0 μM) in the absence of 4-OHT failed to inhibit the growth of any of the ER- α -positive MRT cell lines (data not shown).

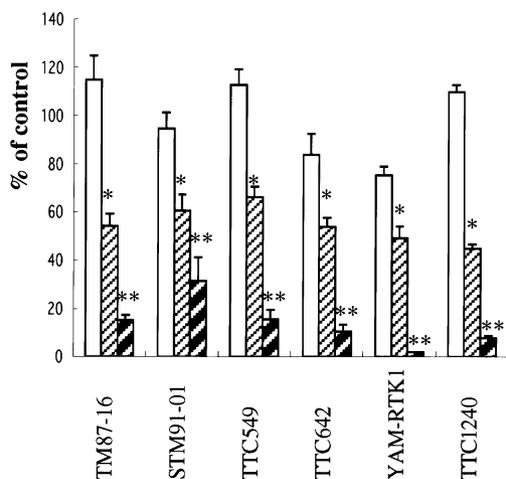


Fig. 3. Effect of 4-OHT on growth of MRT cell lines. The cells were treated with 1 (\square), 5 (\square with diagonal lines), and 10 (\blacksquare) μM 4-OHT in estrogen-free media for 4 days. The cell number was compared with that of the cell in estrogen-free media alone. * $P < 0.05$, ** $P < 0.001$.

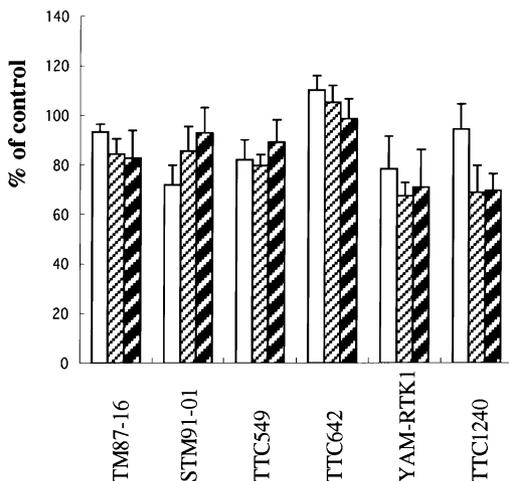


Fig. 4. Effect of ICI 182 780 on growth of MRT cell lines. The cells were treated with 1 (\square), 5 (\square with diagonal lines), and 10 (\blacksquare) μM ICI 182 780 in estrogen-free media for 4 days. The cell number was compared with that of the cell in estrogen-free media alone.

Induction of apoptosis in MRT cells treated with 4-OHT

To determine whether growth inhibition by 4-OHT was due to cell growth arrest or cell death, we assayed cell death by nuclear staining (Hoechst 33258 and propidium iodide). TM87-16 cells were treated with 10 μM 4-OHT for various times. The number of Hoechst-positive condensed/fragmented nuclei increased only after 48 h, and additional E2 did not inhibit this effect (Fig. 6A). These results suggested that 4-OHT-induced cell growth inhibition of TM87-16 cells was due to apoptosis. A DNA fragmentation assay confirmed the induction of apoptosis in TM87-16 cells (Fig. 6B). Similar results were also obtained in ER- α -negative YAM-RTK1 cells treated with 4-OHT (Fig. 7, A and B).

DISCUSSION

It has recently been demonstrated that ER expression may have a role in other neoplasms besides breast cancer.

Table II. IC_{50} of MRT Cell Lines

Cell lines	IC_{50} (μM)	
	4-OHT	TAM
TM87-16	5.3	3.6
STM91-01	6.4	9.1
TTC549	6.1	8.1
TTC642	5.2	4.1
YAM-RTK1	4.9	5.3
TTC1240	4.3	8.4

Estimation of IC_{50} values (concentration of the drug resulting in 50% inhibition of cell viability) for MRT cells. Regression plot analysis was used to calculate IC_{50} of 4-OHT and TAM.

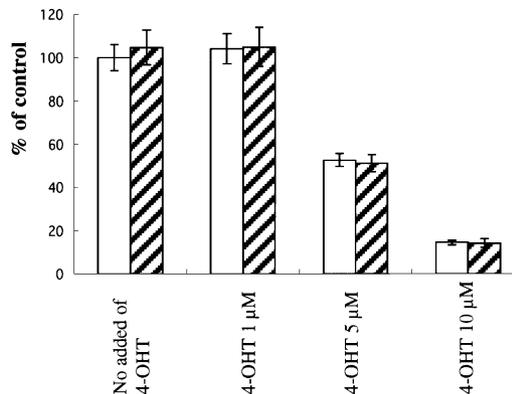


Fig. 5. Effect of E2 on the growth inhibition treated with 4-OHT. ER- α -positive TM87-16 cells were cultured with 1, 5, and 10 μM 4-OHT in the presence of 10 nM (\square with diagonal lines) E2 or without E2 (\square).

Also, TAM, an antiestrogen, has been used in the treatment of malignancies other than breast cancer. However, ER expression and the effect of antiestrogens on MRT cells remained unknown. In this study, we investigated the expression of ER- α by MRT cells, and the effects of antiestrogen treatment. We also analyzed the induction of apoptosis in MRT in response to antiestrogen treatment.

We first demonstrated by RT-PCR, immunocytochemistry, and western blot analyses that three of six MRT cell

lines (TM87-16, TTC642, and TTC1240) expressed ER- α . E2 modulates sexual development and reproductive functions, as well as affecting the central nervous system, while ER- α is distributed throughout the central nervous system.²⁹⁾ As MRT is thought to be neurogenic in origin,^{30,31)} it is reasonable to suggest that MRT cells may express ER. Our observation of ER- α expression in MRT cells suggested that estrogens may have a role in the regulation of MRT cell proliferation.

We next examined whether antiestrogen (4-OHT) could alter MRT cell growth. 4-OHT at 5.0 and 10 μ M signifi-

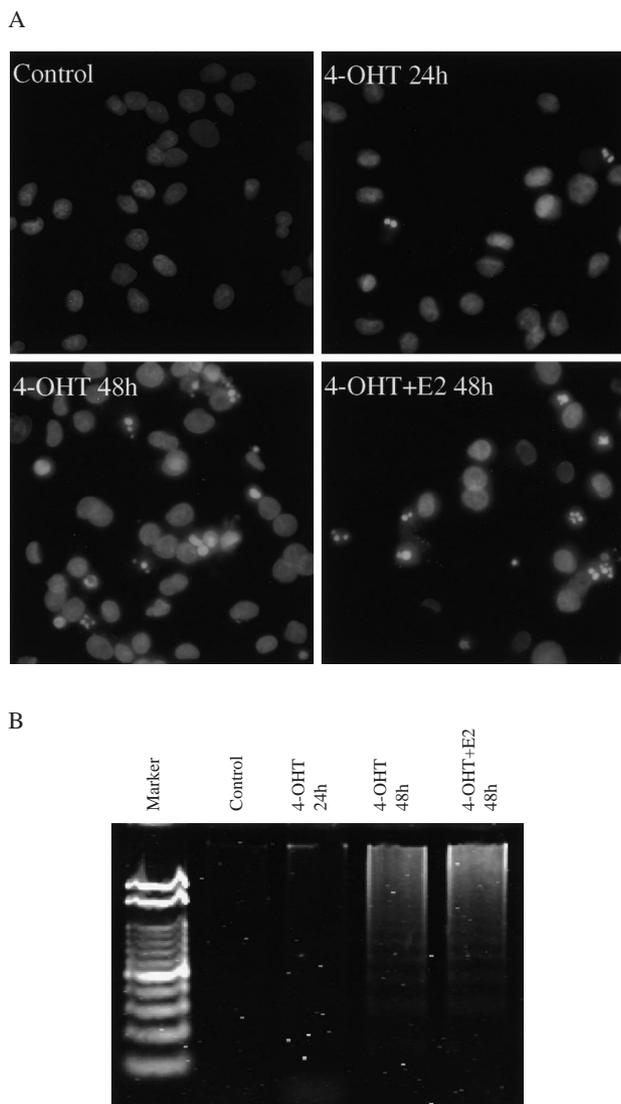


Fig. 6. Induction of apoptosis in ER- α -positive MRT cells by 4-OHT. A, Nuclear staining with Hoechst 33258. TM87-16 cells were treated with 10 μ M 4-OHT or E2 concomitantly. Cells were harvested and stained as described in "Materials and Methods." Cells were examined under a fluorescence microscope. B, DNA fragmentation. After treatment with 10 μ M 4-OHT or E2 concomitantly, genomic DNA was extracted.

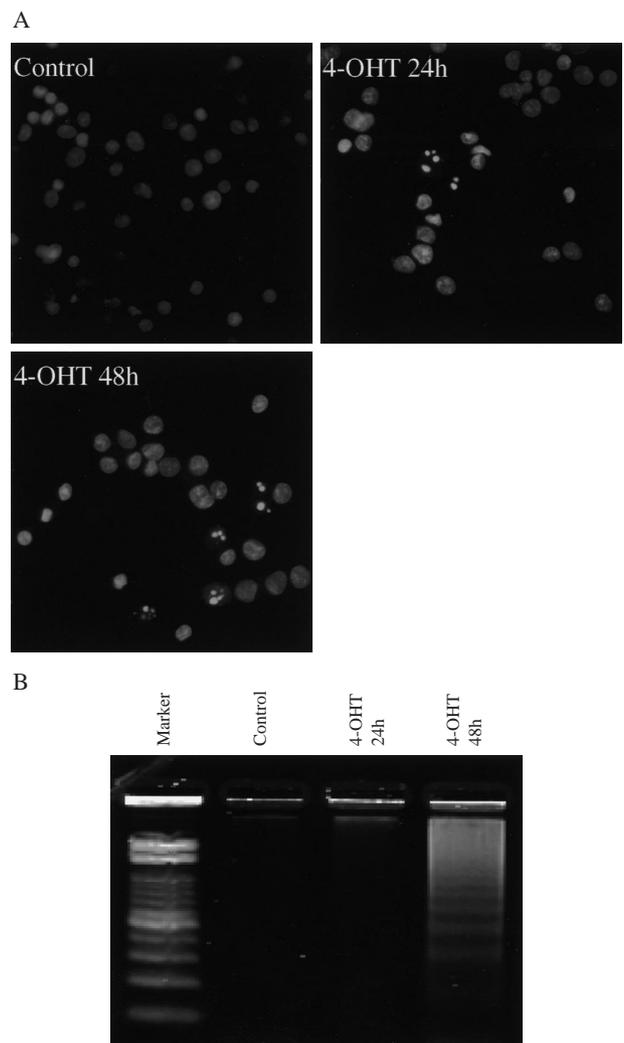


Fig. 7. Induction of apoptosis in ER- α -negative MRT cells by 4-OHT. A, Nuclear staining with Hoechst 33258. YAM-RTK1 cells were treated with 10 μ M 4-OHT. Cells were harvested and stained as described in "Materials and Methods." Cells were examined under a fluorescence microscope. B, DNA fragmentation. After treatment with 10 μ M 4-OHT, genomic DNA was extracted.

cantly suppressed the growth of not only ER- α -positive MRT cell lines, but also ER- α -negative MRT cell lines. Excess estrogen failed to alter the growth inhibition of ER- α -positive TM87-16 cells. These data suggested that the growth-inhibitory effects were not mediated via ER- α . Our finding that smaller growth-inhibitory effects were observed using pure antiestrogen ICI 182 780 supports this hypothesis.

Our results indicated that the growth inhibition of MRT cells by 4-OHT involved apoptosis. It is possible that 4-OHT antagonizes estrogen binding to ER, thereby inducing apoptosis in MRT cells, as well as in breast cancer cells.^{32,33} However, the apoptotic effect of 4-OHT on MRT cells was observed even in ER- α -negative cell lines and the effect was not inhibited by E2 in ER- α -positive cell lines. These results suggested that the apoptotic effects of 4-OHT are independent of ER- α expression. This was supported by the finding that apoptosis was not induced by ICI 182 780.

The antiestrogen TAM is commonly used to treat breast cancer, but also has therapeutic activity in several other cancer types not known to express ER.⁸ The action of TAM was initially believed to result from ER interactions. However, it is now clear that TAM has various effects that appear to be independent of steroid-related pathways. For instance, TAM inhibits protein kinase C (PKC),³⁴ binds to calmodulin,³⁵ and possesses antioxidant properties.³⁶ Therefore, we speculate that TAM also has antitumor effects against MRT cells. Various pathways have been demonstrated through which TAM could inhibit growth and induce apoptosis in ER-negative cells. Considering

that some MRTs are neurogenic in origin,^{30,31} the effect of TAM on malignant glioma cells may give us a clue to the mechanism of TAM action against MRT. Malignant glioma cells possess high PKC activity, and a PKC inhibitor, staurosporine, induces apoptosis in them, as TAM does.³⁴ However, ICI 182 780, which does not inhibit PKC, failed to induce apoptosis of MRT cells. Therefore, we speculate PKC inhibition is one possible pathway through which TAM affects MRT cells.

In conclusion, our study showed that while ER- α was expressed by some MRT cell lines, 4-OHT inhibited the cell growth of MRT cells irrespective of ER- α expression, probably through the induction of apoptosis. Our results indicate that antiestrogens might be useful in the treatment of highly aggressive MRT.

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