Selective Estrogen Receptor Modulators as Inhibitors of Repopulation of Human Breast Cancer Cell Lines after Chemotherapy

Wu Licun and Ian F. Tannock
Division of Experimental Therapeutics and Department of Medical Oncology and Hematology, Princess Margaret Hospital and University of Toronto, Toronto, Ontario MSG 2M9 Canada

ABSTRACT

Purpose: Repopulation of surviving tumor cells between courses of chemotherapy might lead to effective drug resistance. Here we study inhibition of repopulation of hormone-responsive human breast cancer cell lines by selective estrogen receptor (ER) modulators (SERMs) during courses of chemotherapy.

Experimental Design: Hormone responsive breast cancer cell lines MCF-7 and T47D, and the ER- cell line MDA-231, were treated with either 4-hydroxy tamoxifen (4OHT) or arzoxifene during weekly courses of treatment with 5-fluorouracil (5-FU) or methotrexate (MTX). Clonogenic assays were performed to determine the overall survival of tumor cells after treatment with the SERMs alone, after one to three doses of 5-FU or MTX alone, and after 5-FU or MTX followed by each of the SERMs.

Results: Both SERMs inhibited the growth of ER+ cells MCF-7 and T47D but had no effect on the ER- cell line MDA-231. Arzoxifene was more effective than 4OHT. Between courses of treatment with either 5-FU or MTX, repopulation of ER+ cells was specifically inhibited by the SERMs, whereas repopulation of ER- MDA-231 was not affected.

Conclusions: Arzoxifene and 4OHT can inhibit specifically the repopulation of ER+ breast cancer cells between courses of chemotherapy. Scheduling of short-acting SERMs between courses of chemotherapy has the potential to improve therapeutic index.

INTRODUCTION

Courses of chemotherapy are often given at intervals of ~3 weeks, which allows recovery (or repopulation) of the bone marrow and other proliferative tissues. Unfortunately, surviving tumor cells can also proliferate in these intervals, and this repopulation decreases the effectiveness of treatment. Repopulation during fractionated radiotherapy is recognized as a major factor that limits the ability to achieve local tumor control, and there is evidence that the rate of repopulation may increase with time (1–4). This process is likely to be even more important during the longer intervals between courses of chemotherapy but has been rather neglected as a cause of clinical resistance to chemotherapy (5). There is evidence from a few studies of experimental tumors (and spheroids) that the rate of proliferation of surviving tumor cells may increase after treatment with chemotherapy (6–10). Selective inhibition of repopulation of tumor cells between courses of chemotherapy requires the use of antiproliferative agents that are tumor specific, because repopulation of normal tissues is essential to prevent life-threatening toxicity. Antiproliferative agents should be relatively short-lived and allow tumor cells to proliferate at the time of subsequent cycles of chemotherapy, because almost all of the anticancer drugs are more active against proliferating cells.

In the present study, we have investigated the strategy of obtaining selective inhibition of repopulation of tumor cells between cycles of chemotherapy by using SERMs. We have evaluated 4OHT, the active form of tamoxifen, and arzoxifene (also known as LY353381.HCL), a potent, third generation SERM (11, 12), as inhibitors of proliferation in tissue culture of ER+ breast cancer cell lines after one to three sequential treatments with 5-FU or MTX.

MATERIALS AND METHODS

Cells and Detection of ERs. MCF-7 cells were kindly provided by Dr. Kent Osborne, University of Texas Health Science Center, San Antonio, TX. T47D and MDA-231 cells were purchased from the American Type Culture Collection. MCF-7 and MDA-231 cells were maintained routinely in α-MEM supplemented with antibiotics and 10% FCS; T47D cells were grown in RPMI 1640 with 10% FCS. Cell lines were checked routinely for the presence of Mycoplasma by using the Hoechst 33258 fluorescent detection method and were found to be negative.

Immunohistochemical staining was performed to detect ER-α using a mouse monoclonal antibody against human ER-α (Santa Cruz Biotechnology Inc., Santa Cruz, CA). This was done immediately before the present experiments.

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To whom requests for reprints should be addressed, at Princess Margaret Hospital, Suite 5-208, 610 University Avenue, Toronto, Ontario MSG 2M9, Canada. Phone: (416) 946-2245; Fax: (416) 946-2082; E-mail: ian.tannock@uhn.on.ca.

The abbreviations used are: SERM, selective estrogen receptor modulator; 4OHT, 4-hydroxy tamoxifen; ER, estrogen receptor; 5-FU, 5-fluorouracil; MTX, methotrexate.
Effects of SERMs on Cell Growth After Chemotherapy.

The effect of 4OHT (Sigma, St. Louis, MO) and arzoxifene (Lilly Corporate Center, Indianapolis, IN) alone on cell growth was determined for previously untreated cells and after treatments with 5-FU and MTX, given at 7-day intervals. 5-FU (Pharmacia & Upjohn Inc., Mississauga, Ontario, Canada) and MTX [Faulding (Canada), Inc., Vaudreuil, Quebec, Canada] were obtained from the hospital pharmacy.

To evaluate the effects of SERMs on control cells, exponentially growing cells were trypsinized, and 10^5 cells were seeded into multiple small flasks. Different doses of 4OHT or arzoxifene ranging from 0 to 10 \mu M were added immediately to each flask, and the drug-containing medium was replaced every 2 days. At 3-day intervals, one flask from each group was selected at random, and the cells were trypsinized and counted using a Coulter counter (Z Series 9914591-C; Beckman Coulter Inc., Miami, FL). After 8 days of treatment, the number of colony-forming cells was determined (see below).

In additional experiments, 10^6 exponentially growing cells were plated into eight 50 ml flasks and treated with 5 \mu M of either 5-FU or MTX for 24 h, then washed three times with PBS. One flask from each group was randomly selected and the cells were trypsinized, and cell survival was determined in a clonogenic assay as described below. Medium containing one of the SERMs or diluent was added to the other six flasks and replaced every 2 days. After 6 days, cells were washed three times with PBS to remove the SERMs, and medium was added. One day later, cells were trypsinized, and one-fifth of the resuspended cells from each group were seeded into new flasks. Cells were exposed to 5-FU or MTX for 24 h as described above, and treatment with each SERM or with diluent was continued. The same procedures were used before and after the third course of chemotherapy. Colony-forming assays were performed to determine the number of clonogenic cells before and after each treatment with chemotherapy. Growth curves after replating and treatment of cells with 5-FU or MTX were corrected for the 1:5 dilution by multiplying the cell number by 5.

Clonogenic Assays. The sensitivity of each cell line to different doses of each SERM and to the chemotherapeutic agents was determined in preliminary experiments. Exponentially growing cells were exposed to various doses of 5-FU or MTX for 24 h, or to each of the SERMs for 8 days. After this treatment, the cells were trypsinized and washed three times. Serial dilutions were plated in 50 mm Petri dishes in 5 ml of medium. The dishes were incubated for 12–14 days in an atmosphere containing 5% CO₂ at 90% humidity and 37°C. The incubation time depended on cell type: MDA-231 colonies formed after 11 days, whereas MCF-7 and T47D cells needed 14 days. At these times the plates were stained with methylene blue and colonies containing >50 cells were counted.

Assays of cell survival were performed immediately before and after one, two, or three courses of chemotherapy, with interim exposure to one of the SERMs or diluent.

Statistical Analysis. The numbers of total cells and clonogenic cells were presented as means ± SE. The t test was performed to compare means and distributions for different treatment groups. Statistical significance was based on two-tail Ps < 0.05 (α = 0.05).

RESULTS

Confirmation of ER-α Status. About 70–90% of the cultured MCF-7 and T47D cells expressed ER-α, although the intensity of staining was heterogeneous; MDA-231 cells were ER-α negative. Repeated evaluation has shown that the expression of ER-α remains quite stable.

Effects of SERMs on Cell Growth. Lower doses of 4OHT or arzoxifene had little or no effect on growth of the ER-negative MDA-231 cells; higher doses of 10 \mu M arzoxifene caused inhibition of growth, but this was probably because of
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between courses of treatment with 5-FU (Fig. 2, chemotherapy. similar to that of the cells exposed to 4OHT or arzoxifene after clonogenic cells after treatment with chemotherapy alone was
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clonogenic cells during 3 weekly courses of 5-FU or MTX, with the
numbers of clono-
genic cells after treatment with the SERMs, as compared with controls, as indicated in Table 1 (P< 0.05 for each comparison).

Influence of SERMs on Repopulation of Cells After Treatment with Anticancer Drugs. The numbers of clono-
genic cells during 3 weekly courses of 5-FU or MTX, with the SERMs or diluent present between treatments, are shown in Figs. 2 and 3, respectively. For the ER-negative MDA-231 cells, the SERMs had no effect on repopulation during weekly treatments with 5-FU (Fig. 2A) or MTX (Fig. 3A). The number of clonogenic cells after treatment with chemotherapy alone was similar to that of the cells exposed to 4OHT or arzoxifene after chemotherapy.

Repopulation of both ER+ cell lines, MCF-7 and T47D, between courses of treatment with 5-FU (Fig. 2, B and C) or MTX (Fig. 3, B and C) was inhibited by either 4OHT or arzoxifene (P< 0.05), with little difference between these two agents (P> 0.05). After two courses of treatment the number of clonogenic cells after treatment with chemotherapy alone was substantially higher (by a factor of 6–12) than when treatment with the SERMs was used to inhibit repopulation after chemotherapy (P< 0.05). There were no significant differences in the effectiveness of the two SERMs to inhibit repopulation.

DISCUSSION

About 60% of human breast cancers express ER, and most are initially responsive to hormonal treatment. Antiestrogens such as tamoxifen and arzoxifene bind to the ER, thereby blocking the transmission of estrogen-ER-initiated signals for proliferation and leading eventually to apoptotic cell death. After local treatment of primary breast cancer, both adjuvant tamoxifen and chemotherapy have been shown to improve long-term survival of patients with ER-positive breast cancers, and are used routinely as adjuvant treatment for most patients with any poor prognostic features (13, 14). A recent trial has suggested that sequential use of chemotherapy followed by tamoxifen leads to better outcome than continued use of tamoxifen from start of chemotherapy (15). Presumably this result occurred because concurrent tamoxifen had either a direct effect to decrease cell killing by chemotherapy, as has been reported in some model systems (16, 17), and/or inhibited proliferation and, hence, chemosensitivity of target tumor cells. However, delaying hormonal therapy until completion of chemotherapy may also be a suboptimal strategy, because immediate toxic effects of hormonal treatment are lost, and surviving tumor cells that are hormone sensitive can repopulate the tumor between courses of chemotherapy. A preferred strategy to maximize the killing of hormone-sensitive breast cancer cells might be to give a short acting cytostatic hormonal treatment between cycles of chemotherapy and to stop this agent before the next course to allow cells to resume proliferation.

An ideal hormonal agent that is used to inhibit repopulation between courses of chemotherapy should have a short half-life, so that its effects can be withdrawn before the next course of chemotherapy. The most commonly used agent, tamoxifen, is not very suitable, because tamoxifen (as well as toremifene and idoxifene) has a long half-life with mean values of about 6–8 days (18). Arzoxifene is a pure estrogen antagonist with a shorter half-life of ~30 h (11); therefore, it may be more suitable for inhibiting repopulation and has the added advantage of lacking estrogen-agonist effects that can lead to uterine cancers. Withdrawal should also decrease the probability of any direct effects of antiestrogens to inhibit the toxicity of chemotherapeutic drugs. However, the timing of withdrawal of a drug in vivo will represent a compromise between maintaining optimal levels of inhibition of suppression of proliferation of ER+ cells between cycles of chemotherapy and the requirement for very low levels of a SERM to be present when the next cycle of chemotherapy is given.

In the present paper, we have found that repopulation of ER-positive breast cancer cells MCF-7 and T47D was specifically inhibited by the SERMs arzoxifene and 4-hydroxy-tamoxifen, whereas there was no effect on the ER-negative MDA-231 cells. This effect was substantial leading, on average, to a 6–12-fold difference in the number of surviving clonogenic cells after only three cycles of chemotherapy in our in vitro
model. Withers (19) has estimated that the reduction in cell survival from use of a standard 6-month course of adjuvant chemotherapy in patients with breast cancer is only of the order of 10–100-fold. We recognize that effects to inhibit repopulation might be smaller for slower growing primary breast cancers, although the interval between treatments is longer, and high rates of repopulation of clonogenic cells have been estimated even for slow-growing human tumors during fractionated therapy.

Fig. 2  Effects of exposure to 1 μM doses of 4OHT or arzoxifene on the number of clonogenic cells in three cell lines between and after weekly courses of treatment with 5-FU. A, MDA-231; B, MCF-7; and C, T47D. 5-FU alone; 5-FU followed by 4OHT; 5-FU followed by arzoxifene. Means are based on three experiments; bars, ±SE. The difference in number of clonogenic cells after three courses is significantly different to controls after treatment with 4OHT (P = 0.008, MCF-7; P = 0.02, T47D) or arzoxifene (P = 0.005, MCF-7; P = 0.01, T47D).

Fig. 3  Effects of exposure to 1 μM doses of 4OHT or arzoxifene on the number of clonogenic cells in three cell lines between and after weekly courses of treatment with MTX. A, MDA-231; B, MCF-7; and C, T47D. MTX alone; MTX followed by 4OHT; MTX followed by arzoxifene. Means are based on three experiments; bars, ±SE. The difference in number of clonogenic cells after three courses is significantly different to controls after treatment with 4OHT (P = 0.014, MCF-7; P = 0.002, T47D) or arzoxifene (P = 0.01, MCF-7; P = 0.006, T47D).
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Radiotherapy (1–4). Hence, if inhibition of repopulation during courses of chemotherapy (albeit incomplete as shown in Figs. 2 and 3) can have an effect to reduce cell survival by a substantial amount, as in our experiments, there could be a profound effect on net cell kill after 6 months of adjuvant chemotherapy given to patients with ER+ tumors. Thus, we provide supporting evidence for the principle that administration of short-acting SERMs such as arzoxifene between courses of chemotherapy might be a specific approach to controlling repopulation of the surviving tumor cells for estrogen-dependent cancers, and might, therefore, lead to a substantial increase in therapeutic index.

Repopulation of surviving tumor cells between courses of chemotherapy is a potentially important and neglected cause of clinical resistance (5). Whereas our current experiments provide evidence that selective inhibition of repopulation can increase cell killing in a tissue culture model, they do not model some of the complexities of the in vivo situation, such as the declining serum levels of SERMs after their withdrawal, as opposed to abrupt removal from medium in vitro. It will be important to demonstrate therapeutic gain in animal models. Key requirements will be: (a) the inhibition of tumor cell proliferation shortly after initiating treatment with a SERM; (b) selective effects such that repopulation of critical normal tissues is not affected; and (c) scheduling of a short-acting SERM to allow re-entry of cells into cycle at the time of the next treatment with chemotherapy. Studies of repopulation and its inhibition using murine models are in progress in our laboratory.

REFERENCES

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