A Permanently Charged Tamoxifen Derivative Displays Anticancer Activity and Improved Tissue Selectivity in Rodents

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Abstract

A quaternized form of tamoxifen (TAM), tamoxifen methiodide (TMI), was shown to demonstrate very low brain uptake compared to TAM and, unexpectedly, was considerably less estrogenic than TAM in the uterus. The agonist activity of TMI in the bone was similar to that of TAM. TMI manifested significant dose-dependent tumoricidal activity with a rapid onset of action against MCF-7 human breast cancer implants in nude mice and a mean reduction in tumor size of 60% over six weeks.

Introduction

TAM is a triphenylbutylene derivative that binds to the estrogen receptor and exerts both antiestrogenic and estrogenic effects, depending upon the ambient estrogen concentration, the tissue, and the species (2). In humans, TAM seems to be mainly an antagonist in the brain and breast cancer and estrogen-like in the bone, uterus, and cardiovascular system (3-5). The activity of TAM against breast cancer is thought to be mediated through competition for the estrogen receptor. In addition, there is growing evidence for other mechanisms involving the cell membrane (see Ref. 4 for a review). The side effects of TAM, when compared to those of other chemotherapeutic agents, are relatively minor. However, TAM is currently being assessed as a prophylactic agent in healthy women at increased risk for the disease (7). The side effects most often cited by patients are hot flashes, sometimes accompanied by other CNS-related symptoms (depression, irritability, and memory problems) thought to reflect the antiestrogenic activity of the drug in the brain (see Ref. 4 for a review). Estrogen is important for the maintenance of short-term memory and hippocampal dendritic spines in rodents (8), and estrogen deprivation (or blockade) has been recently shown to be associated with short-term memory deficits and increased risk of Alzheimer’s disease in women (9, 10). Because quaternization of amines significantly reduces their brain penetration, conversion of TAM to a suitable quaternary ammonium salt may reduce or eliminate these adverse effects. Another problem of TAM therapy is an increased susceptibility to endometrial cancer as a result of uterine hyperplasia, due to the agonist activity of the drug in the uterus (11). These observations led us to consider the potential use of permanently charged TAM derivatives in breast cancer therapy and prevention (12). Herein, we report the brain uptake, tissue selectivity, and anti-breast cancer action of one such derivative, TMI, in mice and rats.

Materials and Methods

Chemistry and Materials. TAM-free base and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, MO. TMI was prepared as described previously (12). Briefly, TAM was treated with neat methyl iodide, generating the object compound. Chemical and isotopic purity were assessed using HPLC and were found to be >99%. Other spectrophotometric (UV and infrared), spectroscopic (1H and 13C nuclear magnetic resonance), and spectrometric (mass spectrometry) data were consistent with the assigned structure. Two formulations were used for animal experiments. Biodegradable pellets (estradiol, placebo, TMI, and TAM) were custom-made by Innovation Research of America (Sarasota, FL). The treatment pellets contained either 5 mg TAM, 6.8 mg TMI, or no drug (placebo). The pellets contained, in addition to the active ingredient, cholesterol, microcrystalline cellulose, α-lactose, di- and tricalcium phosphate, calcium and magnesium stearate, and steric acid and were designed to release the incorporated drugs at a constant rate over a 21-day period through bioerosion. Drug loading was confirmed by HPLC. The target plasma level after establishment of the zero-order release profile was 5 ng/ml.

The second formulation was a parenteral system designed for i.p. administration of TAM. In this prototype dosage form, TAM was solubilized in a 50% aqueous 2-hydroxypropyl-β-cyclodextrin (Roquette, Paris, France) solution to a concentration of 1.0 mg/ml.

Brain and Blood Distribution. TAM (0.36 mg/kg) or equimolar TMI (0.5 mg/kg) was solubilized in DMSO and injected into the tail vein of male Sprague-Dawley rats (175-200 g) at a vehicle dose of 0.5 ml/kg. At 0.25, 1, 2, and 4 h after drug administration, groups of animals (n = 6) were sacrificed. Brain and blood samples were collected, weighed, and homogenized in one volume of deionized water. To this system were then added 4.0 ml of ice-cold acetonitrile and 1.0 ml of saturated saline. The organic phase, which separated under these conditions, was collected for HPLC analysis. The system configuration included a Waters Model 510 pump, a Thermo Separation Products FL2000 spectrofluorimetric detector, a SPEC-Physics Model SP 8880 Autosampler, and a Spectra-Physics Model SP 4270 integrator. The assay used an Alltech, Inc. Altima C8 5-μm particle size column protected with a guard column and a mobile phase containing 70% acetonitrile, 15% isopropyl alcohol, and 15% of an aqueous triethyl ammonium acetate buffer (pH 4.5). The flow rate of 1.5 ml/min and all determinations were performed at ambient temperature. TAM and TMI were converted to their phenanthrene photoconversion products on-line (post-column) by passage through a 5-m nitrogen teflon reaction coil exposed to a 15 W low-pressure Hg lamp (254 nm). The products were quantitated fluorimetrically (excitation at 256 nm, emission at 380 nm).

CK Determinations. Prepubertal female Wistar-derived rats (~70 g) were obtained from the breeding colony at the Weizmann Institute of Science and were injected i.p with various doses of TAM or TAMI, either alone or concomitantly with 10 nmol 17β-estradiol. Rats were killed 24 h later, and the diaphyseal bone (tibia) and uterus were excised for determination of CK activity as described previously (13). Ten to 15 animals/dose were used in the TAM and TMI groups (2-3 separate experiments on groups of 5), and n = 60 in the estradiol alone group because the same dose of estrogen was given in all experiments.

In Vivo Antitumor Action. CDl-1 nu athymic female mice, eight weeks old, were obtained from the Weizmann Institute Science Department of Animal Services. MCF-7 human breast cancer cells (originally obtained from the laboratory of Prof. M. Lippman at the NIH, Bethesda, MD) were cultured in DMEM with 6% FCS in 5% CO2 at 37°C (14). Cells were detached from their...
flasks with 0.03% EDTA in PBS and were implanted in the flank of mice at a concentration of 10 \times 10^6 \text{cells}/0.5 \text{ml}. All animals were implanted with slow release pellets of 17\beta-estradiol (0.72 mg/pellet, 60-day release; Innovation Research of America). When tumors reached an average size of 1 cm$^3$ (1-2 months after implantation), the estrogen pellets were replaced by either a placebo pellet ($n = 9$), a 5-mg 21-day release TAM pellet ($n = 9$), or a 6.8-mg (equimolar to 5 mg TAM) 21-day release TMI pellet ($n = 10$). Animals were observed for six weeks, and tumor volumes were measured one to two times per week, starting from the day of pellet replacement. The length, width, and height of the tumors were measured using calipers and multiplied to achieve a volume estimate. The percentage change in tumor volume relative to day 0 was computed for each animal at each time point. In a second study, animals were prepared as indicated above, i.e., estrogen-stimulated tumors were allowed to reach a size of 1 cm$^3$, after which the estrogen pellets were removed. Mice were then treated with daily i.p. injections of vehicle (50% aqueous hydroxypropyl-\beta-cyclodextrin) or TMI at doses of 2, 5, and 10 mg/kg/day for 21 days. Tumors were measured weekly as described above. Significant differences in means were assessed using ANOVA with post hoc Scheffé test in which $\alpha$ was preset to 0.05.

Results and Discussion

Because quaternization greatly curtails the ability of small lipophilic molecules to cross the blood-brain barrier, we proposed that CNS side effects of TAM could be prevented by limiting its access to the brain, hopefully without affecting the anticancer activity. This hypothesis was tested using TMI (Fig. 1).

The i.v. dosing of TAM to rats provided brain and blood concentration profiles over time in accordance with the lipophilic character of the drug. Initial brain levels were very high, whereas blood levels were relatively low (Fig. 2A).

The brain: blood ratio, as measured by areas under the concentration curves from 0-4 h, was 18. Conversely, administration of TMI generated high blood concentrations and relatively low brain levels, which resulted in a brain: blood ratio of 0.5. Thus, as reflected by the respective brain: blood ratios (Fig. 2B), the exposure of the brain to TAM was significantly reduced through quaternization of the parent compound.

TMI was tested for estrogenic as well as antiestrogenic activity in vivo in two peripheral tissues, uterus and bone. Estrogen produces a reliable dose-dependent increase in CK-specific activity in both tissues (13) that can be used as a marker. In the diaphyseal bone, TMI was comparable to TAM as an agonist when tested alone or as an antagonist in the presence of injected estrogen (Fig. 3A). Significant effects were attained early in the dose-response study (100 nmol). The antiestrogenic potency of the two drugs in the uterus was also comparable; however, the agonist activity of TMI in this tissue was several-fold lower than that of TAM (Fig. 3A) over an order of magnitude of drug concentrations (Fig. 3B). In addition, the data indicate that both the agonist effect of estrogen is countered by the applied antiestrogens and the estrogenic action of TAM and its methiodide salt is negated by coadministration of an estrogen. These results demonstrate a complementary extinction of activity that cannot be explained by a simple analysis of the estrogen receptor. The mechanism of this mutual diminution of activity may be related to ancillary proteins including steroid receptor cofactors.

Anti-breast cancer activity of TMI and TAM was examined in a nude mouse model relative to a placebo control (14). Sham-treated animals (blank pellets) showed continued growth of the tumor during the first month of observation. The animals treated with TMI showed evidence of antitumor activity as early as one week after implantation. The tumors regressed in 9 of 10 mice. The remaining animal showed arrest but no regression. By the end of the six-week observation
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was identical to that of TAM when isolated estrogen receptors were examined, but that there was minimal binding when whole cells were assayed. In addition, incubation of human breast cancer-derived cells in culture with the methiodide did not affect growth at concentrations in which TAM was effective. These authors concluded that the charged molecule was hindered, relative to TAM, in its passage through the cell membrane (a 5% penetration was measured), consistent with its lack of in vitro activity (12). However, in vivo, we found that TMI demonstrated estrogenic as well as antiestrogenic activity in peripheral tissues and was efficacious as a tumoricidal period, a 60% reduction of mean tumor volume was seen in the TMI-treated group (Fig. 4A).

In four of the animals, the tumor was no longer measurable at the last time point. Under the same conditions, TAM treatment resulted in significant inhibition of the growth rate at two to three weeks. Published literature suggest that TAM gives some degree of variability in this assay with slowed tumor growth, tumoristasis, and tumor regression reported (15). At time points later than 21 days (the lifetime of the slow release pellet), the growth-inhibitory effect of TAM was no longer significant (Fig. 4A). Thus, TMI had a faster onset, more persistent action, and a more potent effect than TAM in this experiment (Fig. 4A).

Daily injections of TMI (2, 5, or 10 mg/kg/day, given i.p. over 21 days) demonstrated that the effect of the drug is dose-dependent. The 2 mg/kg dose had no effect; an intermediate (tumorstatic) effect was achieved with the intermediate dose. The 10 mg/kg dose, chosen to replicate the pellet dose, produced a significant decrease in the tumor volume, similar to that observed in the pellet study at the same time period of 21 days (Fig. 4B).

The quaternization of TAM was initially undertaken to prevent the passage of the drug through the blood-brain barrier, thus preventing CNS-related side effects. As expected, the charged molecule was, indeed, largely excluded from the brain. In addition, this manipulation seems to have changed the tissue selectivity of the molecule without eliminating its antitumor activity. Earlier studies with TMI in vitro (12) showed that its affinity ($K_a$) for the cytoplasmic estrogen receptor

![Fig. 3. Agonist and antagonist effects of TAM and TMI in uterus and bone. A, comparison of the effects of TAM and TMI in uterus and bone at a single (0.1-μmol) dose. B, dose response in the uterus. ■, TAM; □, TMI.](image)

![Fig. 4. Inhibition by TAM and TMI of growth of human breast cancer implanted in nude mice. A, antitumor effects of s.c. pellets of TAM and TMI. •, blank pellets (n = 9); X, TAM pellets (n = 9); Δ, TMI pellets (n = 10). *, significantly different from control; **, significantly different from control and TAM. B, dose dependence of the effect of TMI administered i.p. daily on estrogen-dependent human breast cancer growth in nude mice. A significant overall drug effect was due to the two higher doses, 5 and 10 mg/kg, which were significantly better than 2 mg/kg and vehicle but not significantly different from each other (post hoc Scheffé analysis, $\alpha = 0.05$).](image)
agent in an animal model of human breast cancer. The discrepancy between in vitro and in vivo findings may be related to the dynamics of TMI distribution. It is possible that TMI was excluded from cells in the whole cell receptor binding assay described by Jarman et al. (12), but this procedure required less than 1 h to complete. In whole animal studies, TMI was present for longer times, providing ample opportunity for equilibration into accessible tissues.

In addition, it is possible that TMI acts by additional mechanisms that contribute to its antitumor activity in vivo but not in vitro. TAM and other steroids, for example, have been shown to affect angiogenesis (14, 16). This is an efficient antitumor mechanism that is not possible to assay in cultures of tumor cells but that may contribute to the antitumor activity of TMI in vivo. Other membranal sites have also been suggested as possible mediators of TAM antitumor activity in estrogen-independent or estrogen receptor-negative tumors (17). These include enzymes involved in signal transduction, such as protein kinase C and phospholipases, both of which are inhibited by TAM (6). TMI is likely to inhibit such enzymes even more efficiently than TAM because the presence of a positive charge has been shown to increase the potency of various lipophilic amines as protein kinase C inhibitors (18). Indeed, TMI seems to be at least five times more potent than TAM as an inhibitor of membranal phospholipase C activity (19). These hypotheses need to be further tested experimentally.

The modified peripheral tissue selectivity of TMI was an unexpected finding, although in a clinically desirable direction. We do not know why TAM is a less potent agonist in the uterus while preserving high estrogenic activity in the bone. However, it is known that chemical modifications of antiestrogens affect their tissue selectivity profile (20), and apparently quaternization in the case of TAM had this result. The purpose of the present study was to develop a TAM derivative that preserves the beneficial antiestrogenic as well as estrogenic activities of the parent drug while having limited access to the brain to prevent hot flashes, depression, and memory problems in healthy premenopausal women taking the drug prophylactically. Breast cancer patients could also benefit from a better quality of life if these side effects are prevented. Moreover, TMI had significantly less estrogenic activity in the uterus than TAM. Because estrogenic activity is thought to underlie the increased risk of endometrial cancer in TAM-treated women, the lower activity of the methiodide may translate into a reduced risk of endometrial cancer. Thus, if the pattern of activities for TMI reported here for rodents translates to the human, TMI may offer a breast cancer treatment and prevention alternative with the benefits, but without some of the drawbacks, of TAM.

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References

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