Tamoxifen and Metabolites in MCF7 Cells: Correlation between Binding to Estrogen Receptor and Inhibition of Cell Growth

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ABSTRACT

The binding of [³H]tamoxifen ([³H]Tam), a nonsteroidal antiestrogen, and of 4-[³H]hydroxytamoxifen ([³H]OH-Tam), a metabolite accumulated in vivo in target cell nuclei, was characterized in soluble extracts of human breast cancer MCF7 cells growing in a medium depleted in estrogens. Saturation analysis indicated a much higher affinity for OH-Tam (Kd = 0.15 nm) than for Tam (Kd = 4.8 nm). The binding of [³H]Tam and [³H]estradiol was competitive and mutually exclusive, and the binding site concentration (0.16 to 0.47 pmol/mg total protein) was similar for both ligands, strongly suggesting that antiestrogens were binding to the estrogen receptor (ER) in these cells.

The ability of Tam and of some of its metabolites or derivatives to prevent the MCF7 cell growth was found to be correlated with their affinity for ER as determined by direct interaction or by binding competition with [³H]estradiol on the uterine and MCF7 cytosol ER. OH-Tam was the highest-affinity compound and was 100-fold more active than Tam. The inhibitions observed were actually due to Tam and OH-Tam, respectively, since we did not detect any significant metabolism of these two labeled compounds by the MCF7 cells. N-desmethyltamoxifen, the other Tam metabolite found in high concentration in human plasma, was as effective as Tam while cis-tamoxifen appeared less effective. Compound E, which has no lateral chain, was the only tested compound having a good affinity for ER and a poor efficiency in preventing cell growth. These results support the hypothesis that antiestrogens control the growth of breast cancer by acting directly on the ER located in cancer cells.

INTRODUCTION

The nonsteroidal antiestrogen Tam³ (15) is now currently used in the treatment of breast cancer. The mechanism by which it decreases tumor cell growth is not well understood. It is possible that its antiestrogenic property is responsible for its antitumoral effect since Tam is competing with estrogens for the ER and since there is some correlation between the responsiveness of breast cancer to antiestrogen and the ER concentration (13). However, the action of Tam on tumor cell growth may not be related to its antiestrogenic properties since Tam was described to be efficient in a chemically defined medium without estrogen (22) and 13% of ER-negative patients respond to antiestrogen therapy (13). It has therefore been proposed that antiestrogens could be acting via a receptor specific for antiestrogens but not for estrogens (28, 29).

Recently, several laboratories (1, 4, 12, 17) have shown that Tam is transformed in vivo into several metabolites. Moreover, we have found recently in rodents and chickens that hydroxylated metabolites, including OH-Tam, are formed in vivo and selectively retained at the ER sites in the nucleus (2, 4).

These results raised 2 major questions. (a) What is the biologically efficient antiestrogen in humans: Tam, a metabolite, or both? And (b) which receptor is mediating the effect of antiestrogens: the ER or another binding protein?

To answer these questions, we have decided to compare in vitro the efficiency of Tam and its metabolites in competing for binding to the ER and preventing the cell growth of an estrogen- and antiestrogen-responsive cell line. The in vitro cell culture approach is excellent, since it bypasses liver metabolism and the effect of hormones or growth factors released from other tissues; and consequently the nature and the actual concentration of drug reaching the cells are known. The MCF7 cells derived from a metastatic human breast cancer are appropriate to evaluate the effects of estrogens and antiestrogens since they contain a high level of ER (6) and they are estrogen responsive in terms of induction of the progesterone receptor (16) and other proteins (11, 32). Moreover, the growth of these cells is blocked by antiestrogens (20, 23) and the cells are protected or rescued by estrogens from the effect of antiestrogens.

In order to define the relative efficiency of Tam and its metabolites and structural derivatives, we have studied in vitro their affinity for the ER, their metabolism in MCF7 cells, and their inhibitory activity on the growth of MCF7 cells.

MATERIALS AND METHODS

Materials. [³H]Tam (15.4 Ci/mmol), Tam, and its derivatives were kindly given by Dr. J. S. Todd (ICI Laboratories, Macclesfield, United Kingdom). [³H]OH-Tam (15.4 Ci/mmol) was prepared biologically from [³H]Tam (3). [6,7-³H]estradiol (45 to 60 Ci/mmol) was purchased from CEA (Gif-sur-Yvette, France). Nonradioactive estradiol was given by Roussel Uclaf (Romainville, France). All compounds were stored at -20° in ethanol. The purity of these solutions was checked by thin-layer chromatography using benzene:ethylacetate (3:2, v/v) for estradiol; benzene:triethylamine (95:5) for Tam and derivatives; and benzene:triethylamine (85:15) for OH-Tam.

MCF7 cells were obtained from Dr. Lippman (National Cancer Institute, Bethesda, Md.). They were maintained as monolayer culture in DEM (Gibco, Paisley, Scotland) supplemented with 10% FCS from Gibco or Flow Laboratories (Asnières, France), 0.6 mg insulin (Sigma Chemical Co.) per ml, and 50 mg gentamycin (Schering Corp., Bloomington, N. J.) per ml, at 37° in a humid atmosphere containing 5% CO₂. Cells were subcultured in special plastic culture dishes (Linbro Scientific, Inc., Hamden, Conn.).

Binding Studies. The cells were withdrawn from endogenous hormones by culturing them for 4 days in DEM containing 5% FCS treated
Tam and OH-Tam Binding to the ER. \([^3H]Tam\) and \([^3H]OH-Tam\) are known to bind with high affinity to uterine ER (3, 8, 17, 24). By using the same methodology (8), we found a saturable binding of \([^3H]Tam\) and \([^3H]OH-Tam\) in the MCF7 cell extracts (Chart 1). As in other tissues, the nonspecific binding of antiestrogens was higher than that of estradiol. The \(K_d\) obtained by saturation analysis and Scatchard plot was much lower for OH-Tam (0.15 nM) than for Tam (4.8 nM). The concentration of binding sites was similar for Tam and estradiol (Table 1).

In our attempt to define the best culture conditions for assaying estradiol and Tam binding, we found that the concentration of ER sites per mg protein increased markedly with increasing concentrations of serum (Table 1) while the \(K_d\) of estradiol was stable. We have not specified the serum factor responsible for the increase of ER concentration.

**RESULTS**

**Tam and OH-Tam Binding to the ER.** \([^3H]Tam\) and \([^3H]OH-Tam\) were incubated for 4 hr at 2°, with increasing concentrations of \([^3H]Tam\) or \([^3H]OH-Tam\) with or without 1×10⁻¹⁰ M unlabeled estradiol. Binding was assayed by the DCC technique (8). In a and b, the total (\(B_t\), ○), nonspecific (\(B_n\), x), and specific (\(B_s\), •) binding are represented. In c and d, Scatchard plot representations of the specific bindings are shown. Two typical experiments are represented for Tam (a and c) and OH-Tam (b and d) obtained from 2 different preparations.

**Figure Chart 1.** Direct binding of \(^3H\)-labeled antiestrogens in MCF7 cytosol. MCF7 cell extracts were prepared in DCC buffer and centrifuged at 105,000 x g for 1 hr. The medium and the different cell fractions were extracted and chromatographed as described previously (4).

**Table 1.** Direct binding of \(^3H\)-labeled antiestrogens in MCF7 cytosol and Methods" or maintained in DEM supplemented with 1% charcoal-treated FSC for 4 to 6 months. The concentrations of binding sites were determined by charcoal assay and Scatchard plot as described (8).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>([^3H]Estradiol and [^3H]Tam binding in MCF7 cells</th>
<th>Table 1</th>
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<tbody>
<tr>
<td>% of FCS</td>
<td>Binding sites (fmol/mg cytosol protein)</td>
<td>(K_d) (nM)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>5</td>
<td>146 ± 59 (6)</td>
</tr>
<tr>
<td>Tam</td>
<td>5</td>
<td>135 ± 91 (4)</td>
</tr>
<tr>
<td>a</td>
<td>(R_c), cytosol receptor; (R_n), KCl-extracted nuclear receptor.</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>Mean ± S.D.</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Number of different experiments.</td>
<td></td>
</tr>
</tbody>
</table>
and approximative 300-fold higher than that of Tam. In the competitive efficiency of OH-Tam was similar to that of estradiol, totally inhibited the [3H]estradiol specific binding. The affinity of OH-Tam for the ER was similar to that of estradiol and at least 300-fold higher than that of Tam. Similar results were found in the KCl extract of crude nuclei which are known to contain available nuclear ER (33). The [3H]Tam-binding sites in the cytosol and KCl nuclear extract were also displaced to a same nonspecific level with low concentrations of estradiol, diethylstilbestrol, or OH-Tam and with higher concentrations of the antiestrogens Nafodixone and Tam (not shown). These results show that the binding of Tam or OH-Tam and estradiol was mutually exclusive and most likely on the same binding site as estradiol or on a distinct but functionally related site cannot be discriminated here.

Metabolism of Tam and OH-Tam. Chart 3 shows that, in our culture conditions, [3H]Tam was not markedly metabolized by the MCF7 cells. In the nuclear KCl extract, we found a minor amount of metabolite with a polarity slightly higher than that of Tam. However, in no case could we find any significant transformation into OH-Tam. Similar results were obtained with 50 nM or 1 μM concentration of [3H]Tam. Moreover, OH-Tam was also not metabolized in the MCF7 cells after 1 day of culture. Very polar derivatives were found in the culture medium both in the presence and absence of cells, suggesting that these degradation products were not due to cell metabolism.

Cell Growth Evaluation. We have measured the effect of different Tam derivatives on cell proliferation by directly counting the cells and by assaying the DNA content. The validity of these 2 methods is shown in Chart 4. The concentration of DNA was directly proportional to the increase in fluorescence (a) and to the cell number (b). The 2 methods did not give the same value for DNA content since a comparison of these methods gave a mean value of 7.5 pg DNA per cell which is low for the MCF7 cells which are known to contain 78 to 90 chromosomes. The reason for this discrepancy was not investigated. However, these 2 methods appeared valid to compare the effect of drugs on cell proliferation in the range of DNA concentration used. In order to find the best conditions for obtaining an optimal effect of antiestrogens on cell growth, we varied the percentage of the charcoal-treated FCS added for obtaining an optimal effect of antiestrogens on cell growth. The cells were more sensitive to the effect of Tam in 1% than in 3 or 5% serum, and we therefore decided to use routinely 1% FCS in all our cell growth studies. The reason why Tam was more efficient in 1% than in 3 or 5% serum, and we therefore decided to use routinely 1% FCS in all our cell growth studies.
fact, it is known that estradiol is retained in the cells for a long period of time (27). Moreover, Vignon et al. (31) have shown that the charcoal-treated serum contains estrogen sulfate which can be used as estrogen by the MCF7 cells.

**Cell Growth Inhibition by Tam and OH-Tam.** OH-Tam is a metabolite found in vivo in the nuclei of rat uterus and chicken oviduct, in addition to the nonmetabolized Tam (4). It became therefore important to specify which compound, either Tam or OH-Tam, or both, was active in the target cell. We have thus tested these 2 drugs on the growth of MCF7 cells. With 0.1 μM, both drugs were active in inhibiting the cell growth; however, OH-Tam was the most effective (Chart 5, a and b). The effect was only observed after 7 days of treatment. The doubling time of the cells, as evaluated on a semilogarithm plot, was markedly increased from 50 hr in the control to 80 and 100 hr for the Tam- and OH-Tam-treated cells, respectively. In order to evaluate the relative activity of the 2 drugs, we then varied their concentration (Chart 5c). The OH-Tam was found to be ≈100-fold more active than Tam itself. The much higher efficiency of OH-Tam appeared real, since it cannot be explained by the slightly higher solubility of OH-Tam (50%) as compared to that of Tam (30%) in the same conditions.

With both compounds, all the cells were killed and detached for 10 μM concentrations which are most likely toxic for the cells. For concentrations of 0.1 μM, the effect of Tam was thought to be specific since it was reversed by estradiol (Chart 5, a and b). However, the efficiency of estradiol to rescue the cells was found to vary according to the affinity of the antiestrogens for the ER. For concentrations >50 nM, the effect of OH-Tam was irreversible while that of Tam was clearly reversible. Conversely, when treated by 10 nM OH-Tam (Chart 6), the cells could be partially rescued with 10 nM estradiol, thus indicating that the effect of OH-Tam as that of Tam was mediated by the ER. In this experiment, OH-Tam appeared to decrease not only the growth rate of the cells but also the level of confluency.

**Relative Activity of Tam Derivatives for Competing on the ER and Inhibiting MCF7 Cell Growth.** Other metabolites and
analogons of Tam were also tested for preventing cell growth and 
[3H]estradiol binding. In MCF7 cell cytosol, as shown in Chart 2 and Table 2, OH-Tam was found approximately 100-fold more active than Tam in binding the ER and preventing cell growth. For testing the relative binding affinity of other Tam metabolites and analogs, we have used lamb uterine cytosol since the efficiency of Tam and OH-Tam in preventing [3H]-estradiol binding was similar in MCF7 cells and lamb uterine cytosols (Table 2).

For most of the tested compounds, a good correlation was found between the binding affinity for ER as determined by competition with [3H]estradiol (19) and the ability to prevent cell growth (Chart 7). When Compound E was excluded, the correlation coefficient was 0.98. Compound E which displays an apparent affinity for ER higher than that of Tam is less efficient in preventing the cell growth; the lack of side chain in its structure could explain such a result as Compound E is a weak estrogen agonist in the rat uterus. Among the antiestrogenic compounds, OH-Tam was by far the most active metabolite since the efficiency of Tam and OH-Tam in preventing [3H]-estradiol binding was similar in MCF7 cells and lamb uterine cytosols (Table 2).

The binding inhibition was determined on the cytosol ER from lamb uterus or MCF7 cells using 5 nM [3H]estradiol and increasing concentrations of competitors. The [3H]estradiol binding was assayed as described in "Materials and Methods" after incubation for 6 hr at 20° (lamb uterus) or 24 hr at 0° (MCF7 cells). The cell growth inhibition was determined as in Chart 5c (results from 3 to 10 experiments).

<table>
<thead>
<tr>
<th>% of estradiol-binding inhibition</th>
<th>% of cell growth inhibition</th>
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<tr>
<td>10^-8 M</td>
<td>10^-7 M</td>
</tr>
<tr>
<td>OH-Tam</td>
<td>Lamb uterus</td>
</tr>
<tr>
<td>Tam</td>
<td>Lamb uterus</td>
</tr>
<tr>
<td>N-desmet-Tam</td>
<td>Lamb uterus</td>
</tr>
<tr>
<td>cis-Tamoxifen</td>
<td>Lamb uterus</td>
</tr>
<tr>
<td>Compound E</td>
<td>Lamb uterus</td>
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</tbody>
</table>

* Mean ± S.D.

**DISCUSSION**

The MCF7 cells are an excellent in vitro system to specify the activity of antiestrogens in target cells. They contain high concentrations of ER (6) and their growth is specifically inhibited by antiestrogens and rescued by estradiol as shown previously by Lippman et al. (23) and confirmed by us in this paper and in Ref. 9. Moreover, the MCF7 cells do not metabolize Tam and OH-Tam which therefore act by themselves, contrary to the in vivo situation where the metabolism makes it difficult to specify the nature of the active drug.

Both antiestrogens bind with high affinity to the ER in these cells. The affinity of OH-Tam was found to be about 30-fold higher than that of Tam by direct saturation analysis with 

<table>
<thead>
<tr>
<th>[\text{ED}_{50} \text{(M)}]</th>
<th>[\text{Log apparent } K_d \text{(M)}]</th>
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<tbody>
<tr>
<td>10^-6</td>
<td>-9</td>
</tr>
<tr>
<td>10^-7</td>
<td>-8</td>
</tr>
<tr>
<td>10^-8</td>
<td>-7</td>
</tr>
<tr>
<td>10^-9</td>
<td>-6</td>
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Chart 7. Correlation between affinity for the ER and cell growth inhibition activity. The \(K_d\) values of antiestrogens were calculated according to Korenman (19) from the competitive experiments described in Table 2 by taking for the estradiol \(K_d\) value \(3 \times 10^{-12} \text{M}\) which corresponds to the ratio \(k_−:k_+\). The concentration of Tam and derivatives required to inhibit 50% of cell growth (ED50) was determined as described in Chart 5c. The same solutions of the inhibitors were taken for binding and cell growth studies.

\[\text{[3H]}\text{Tam and [3H]}\text{OH-Tam but about 300-fold higher by competitive experiments. The relative affinities of Tam, OH-Tam, and estradiol for the MCF7 cell ER calculated from competition experiments are in agreement with those found in similar experiments with calf (3, 26) and lamb uterine ER. They agree also with the \(K_d\) values calculated for each ligand by the ratio \(k_−:k_+\) of the kinetic constants (3, 8). These relative binding affinities derived from competition experiments are more reliable than those derived from direct equilibrium studies with [3H]Tam and [3H]OH-Tam since, in the case of high-affinity ligands such as estradiol and OH-Tam, the \(K_d\) value determined directly by Scatchard plot is much higher than that determined by the ratio \(k_−:k_+\) (3, 8, 30).

By comparing in vitro the relative biological efficiency of different Tam derivatives in preventing cell growth and inhibiting estradiol binding on the ER, we tried to specify the nature of the receptor and the ligands (Tam or metabolite) responsible for antiestrogen and antitumoral action. We found a good correlation between the relative binding affinity of these derivatives for the ER and their biological efficiency in preventing the growth of MCF7 cells.

In addition to the ER, there is in these cells another entity
binding specifically antiestrogens but not estrogens (29). The specificity of the antiestrogen-binding protein for the Tam metabolites is however different from that of the ER. For instance, OH-Tam has a ≈300-fold higher affinity for ER than Tam (26) but the same affinity for the antiestrogen-binding protein (29). Since OH-Tam is 100-fold more active than Tam in MCF7 cells, this strongly suggests that it is acting via the ER rather than via the antiestrogen-binding protein. This antiestrogen-binding protein was mostly observed when cells were grown in the presence of serum containing estrogens, the ER sites being occupied by hormones (29). In our conditions, the cells were cultured in an estrogen-depleted medium and the [3H]Tam was mainly interacting with the ER as indicated by its site concentration and binding specificity.

These results, supported by the phenomenon of rescue of antiestrogen-treated cells by estradiol, favor the simplest hypothesis that antiestrogen controls the growth of breast cancer by interacting directly in cancer cells with the ER rather than with a separate antiestrogen-binding protein.

The in vitro MCF7 cell system allowed us to specify more directly the relative efficiency of different Tam derivatives in inhibiting breast cancer cell growth. All antiestrogens used were found to be active by themselves in MCF7 cells. The most active compound was OH-Tam. N-desmet-Tam was as active as Tam while Compound E, lacking the lateral chain, had little efficiency, contrasting to a good affinity for ER. Except for this compound the biological activity of a ligand was directly proportional to its affinity for ER. This directly demonstrated the statement of Jordan et al. (17) that OH-Tam is a better antiestrogen than Tam as far as reaching target cells. This was also shown before by the higher activity of OH-Tam in preventing the estradiol-dependent induction of a 52 K protein which is released into the medium by MCF7 cells (32).

The nature of the compound(s) which is acting in vivo on human breast cancer would therefore depend on the local concentrations of Tam and metabolites on the one hand and on their affinity for the ER on the other hand. Contrary to the rat and chicken target organs where OH-Tam and another more polar metabolite (M2) have been characterized (3), we have no direct information concerning the drug(s) accumulated in vivo in human breast cancer tissue. We can, however, predict, from their concentration in human plasma (1, 10) and their relative affinity for the ER, that 3 identified compounds may be involved, Tam, N-desmet-Tam (both at 400 nm concentration in plasma), and OH-Tam (10 nm in plasma). From the much higher efficiency of OH-Tam in competing for ER and preventing cell growth, it is likely that this compound is at least partly responsible for the effect of Tam on breast cancer growth.

The fact that the efficiency of an antiestrogen appears to be directly correlated to its affinity for ER (26) totally disagrees with the hypothesis that the antiestrogen activity would be due to a rapid dissociation rate from ER (5). In fact, OH-Tam has a dissociation rate comparable to that of estradiol (3), slightly higher in MCF7 cells (25) but slower in the chicken oviduct where it is a full estrogen antagonist. Recently, another test has been proposed by us (25) to discriminate between estrogen agonist and antagonist. This test is based on the variation of the dissociation rate under molybdate treatment which is known to prevent receptor activation. The test is valid both for the classical antiestrogens and for their active hydroxylated metabolites like OH-Tam. These results altogether agree with an allosteric model in which a partially activated receptor is stabilized by antiestrogens. In this model, the efficiency of an antiestrogen will be directly correlated to its affinity for the ER and its ability to compete with estradiol (26). This is actually observed experimentally since the MCF7 cells are more easily rescued by estradiol after Tam than after OH-Tam treatment. A similar situation might be found in vivo mostly in postmenopausal patients where it is more likely that higher affinity metabolites such as OH-Tam rather than Tam or N-desmet-Tam are efficient in competing with estrogens and acting at the target cell level.

ACKNOWLEDGMENTS

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