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]hydroxyltamoxifen ([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 comparison 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 antitumor effect since Tam is competing with estrogens for the ER and since there is some correlation between the responsiveness of breast cancer to antiestrogens 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 raise 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 (90:10) 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 hormone by culturing them for 4 days in DEM containing 5% FCS treated...
by a DCC pellet (0.25% Norit A:0.025% dextran, in 0.01 M Tris-HCl, pH 8) for 2 x 45 min at 37°C. This medium was then replaced by a serum-free medium for 1 day. The cells were then washed with Ca²⁺, Mg²⁺-free PBS and detached at 37°C in Ca²⁺, Mg²⁺-free PBS containing 1 mmol EDTA. The cells were chilled to 4°C, washed with PBS, resuspended in TET buffer, and then homogenized with a Dounce homogenizer (Kontes Co.) using the B pestle, until no more than 90% disruption of cells was checked by phase-contrast microscopy. Cytosol and nuclear extract were prepared in TET buffer and TET buffer containing 0.5 mM KCl, pH 8.5, respectively, according to Capony and Rochefort (7). Bound estradiol and Tam were determined using the DCC method (8). Specific binding was obtained after correction for nonspecific binding as evaluated with a 100-fold excess of nonradioactive estradiol or Tam. Relative binding affinities for ER were determined by competition experiments (3 to 24 hr at 0°C or 20°C) between 2 and 5 nM [³H]estradiol and increasing concentrations of unlabeled estradiol, Tam, and metabolites with lamb uterine and MCF-7 cell cytosols as described (7).

Metabolism. Cells were incubated from 3 to 72 hr at 37°C in serumfree medium containing 50 nM [³H]Tam or [³H]OH-Tam with or without 1 μM unlabeled Tam or OH-Tam. Medium was also incubated without the cells as control. The medium was then decanted, and the cells were washed and homogenized in 2 volumes of TET buffer. The homogenates were centrifuged at 105,000 x g for 1 hr. The medium and the different cell fractions were extracted and chromatographed as described previously (4).

Cell Growth Experiments. Cells (2.5 to 3 x 10⁴) growing exponentially in DEM supplemented with 10% FCS were detached with trypsin (0.05%) and EDTA (0.02%). Cells were pelleted, resuspended in growth medium, and plated in triplicate in 35-mm plastic tissue culture wells, with charcoal-treated FCS medium. After cell attachment (=12 to 16 hr), the drugs were added to the medium in ethanol solution (final concentration, 0.5%). In these conditions, we checked that =50% OH-Tam or Tam remained soluble in the culture medium from 10 nM up to 1 μM concentrations. The medium was changed every 2 days, and cells were collected after detachment by trypsin:EDTA and neutralization with growth medium. They were passed through a 0.25-mm-diameter gauge needle and counted in a Model D Coulter Counter (Coultronics, Andilly, France) using the following settings: threshold, 7; sensitivity, 0.017; volume sample, 0.5 ml. For each experiment, cell number was counted at least 3 times and in triplicate wells. The results were corrected for nonspecific binding.

RESULTS

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 MCF-7 cell extracts (Chart 1). As in other tissues, the nonspecific binding of antiestrogens was higher than that of estradiol. The Kd 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 Kd of estradiol was stable. We have not specified the serum factor responsible for the increase of ER concentration.

Competition experiments with MCF-7 extracts allowed us to study the binding specificity of the [³H]estradiol and [³H]Tam...

Table 1

<table>
<thead>
<tr>
<th>% of FCS</th>
<th>Binding sites (fmol/mg cytosol protein)</th>
<th>Kd (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Rn</td>
<td>Rc</td>
</tr>
<tr>
<td>Estradiol</td>
<td>5</td>
<td>146 ± 55</td>
</tr>
<tr>
<td>Tam</td>
<td>5</td>
<td>135 ± 91</td>
</tr>
</tbody>
</table>

a. Rc, cytosol receptor; Rn, KCl-extracted nuclear receptor.
b. Mean ± S.D.
c. Number of different experiments.
binding sites. Chart 2 shows that nonradioactive Tam and OH-Tam totally inhibited the [3H]estradiol specific binding. The competitive efficiency of OH-Tam was similar to that of estradiol and approximately 300-fold higher than that of Tam. In the calf uterus, the relative affinities of OH-Tam and Tam as evaluated by competition experiments at equilibrium (26) or by measuring the k - k+ ratio (3, 8) were found to be approximately 400 and 1, respectively. The slightly lower ratio found here might be due to the fact that the equilibrium was not completely reached after 24 hr of competition at 0° (26). We conclude that, in MCF7 as in calf uterus (3, 8, 26), 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 non-specific level with low concentrations of estradiol, diethylstilbestrol, or OH-Tam and with higher concentrations of the antiestrogens Nafodine 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 ER. Whether the interaction of antiestrogen was 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 marked 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 derivative(s) 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 method 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 concentration of DNA in 2 methods and then found a concentration of 1% charcoal-treated FCS which was then cultured in DEM without FCS for 3 days with 50 nM or 1 µM concentration of [3H]Tam and [3H]OH-Tam (a to c) or 1 day with 50 nM [3H]OH-Tam (d to f). The cell homogenate and the KCl nuclear extract were prepared as described. The media (a and d), cell homogenates (b and e), and nuclear extracts (N.E.) (c and f) were extracted with ethyl acetate and finally analyzed on thin-layer chromatography. Results are the percentage of the total radioactivity present in each extract. The positions of OH-Tam, Tam, and the front of the solvent (f) are represented.

![Chart 2](image)

**Chart 2.** Competitive binding of Tam and OH-Tam on the ER. MCF7 cytosol was incubated at 0° with [3H]estradiol (E2) (2 nM) and increasing concentrations of nonradioactive Tam (Tam), OH-Tam (OH-Tam), or estradiol (E2) (C). After 24 hr of incubation at 0°, the [3H]estradiol binding was measured by charcoal assay (2.5 hr at 0°).

![Chart 3](image)

**Chart 3.** Metabolism of Tam and OH-Tam. Cells were plated in 1% charcoal-treated FCS. They were then cultured in DEM without FCS for 3 days with 50 nM or 1 µM concentration of [3H]Tam (a to c) or 1 day with 50 nM [3H]OH-Tam (d to f). The cell homogenate and the KCl nuclear extract were prepared as described. The media (a and d), cell homogenates (b and e), and nuclear extracts (N.E.) (c and f) were extracted with ethyl acetate and finally analyzed on thin-layer chromatography. Results are the percentage of the total radioactivity present in each extract. The positions of OH-Tam, Tam, and the front of the solvent (f) are represented.

![Chart 4](image)

**Chart 4.** Measure of cell proliferation: validity of the methods. a, DNA assay: standard curve with calf thymus DNA by fluorescence (18); b, correlation between DNA content and cell number. Five x 10⁴ cells were trypsinized and increasing dilutions were made. Cell concentrations were then evaluated in a Coulter Counter, and DNA concentration was assayed as described in a.
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 MCF-7 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 MCF-7 cells. With 0.1 \( \mu \)M, both drugs were active in inhibiting the cell growth; however, OH-Tam was the most effective (Chart 5a and b). The effect was only observed after 7 days of treatment. The doubling time of the cells, as evaluated on a semilogarithmic 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 \( \mu \)M concentrations which are most likely toxic for the cells. For concentrations of 0.1 \( \mu \)M, 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 reversible 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 MCF-7 Cell Growth.** Other metabolites and
Relative efficiency of Tam and derivatives to inhibit the estradiol binding to the ER and the cell growth

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></th>
<th>% of estradiol-binding inhibition</th>
<th>% of cell growth inhibition</th>
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<tbody>
<tr>
<td></td>
<td>10⁻⁸ M</td>
<td>10⁻⁷ M</td>
</tr>
<tr>
<td>OH-Tam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb uterus</td>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>Lamc7 cells</td>
<td>55</td>
<td>85</td>
</tr>
<tr>
<td>Tam</td>
<td>&lt;5</td>
<td>10</td>
</tr>
<tr>
<td>N-desmet-Tam</td>
<td>&lt;5</td>
<td>25</td>
</tr>
<tr>
<td>Lamb uterus</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>cis-Tamoxifen</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Compound E</td>
<td>&lt;5</td>
<td>20</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Analogs 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 (300-fold higher activity) over Tam in preventing estradiol binding to ER and as cell growth inhibitor. The cis isomer of Tam was more able to compete on the ER than to prevent cell growth except at high concentrations (≥1 μM) where its effect might be nonspecific. This is consistent with the estrogenic activity of the cis isomer of Tam (14).

**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 [3H]Tam and [3H]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 KD 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 KD 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 $\approx$300-fold higher affinity for ER than Tam (26) but the same affinity for the antiestrogen-binding protein (29). Since OH-Tam is 100-fold more active than Tam in MCF$_7$ 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 $[^{3}H]$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 MCF$_7$ 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 MCF$_7$ 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 MCF$_7$ 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 (M$_2$) 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 MCF$_7$ 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 MCF$_7$ cells are more easily rescued by estradiol after Tam than after OH-Tam treatment. A similar situation might be found in vivo mostly in premenopausal 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

We are grateful to Dr. Todd and ICI Laboratories for providing us with $[^{3}H]$Tam and OH-Tam. We thank S. Ladrech for her excellent technical assistance and E. Barriè and M. Paolucci for preparing the manuscript.

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Antiestrogens and Metabolites on MCF7 Cells

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