Tamoxifen induces ultrastructural alterations in membranes of *Bacillus Stearothermophilus*

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Abstract

Tamoxifen (TAM), a non-steroid antiestrogen, is the mostly used drug for chemotherapy and chemoprevention of breast cancer. However, the mechanisms by which TAM inhibits cell proliferation in breast cancer are not fully understood. TAM strongly incorporates in biomembranes and a variety of effects have been assigned to biophysical and biochemical interactions with membranes. Therefore, a better understanding of the physicochemical basis of interaction of TAM with biomembranes is essential to elucidate the molecular mechanisms of action. A strain of *Bacillus stearothermophilus* has been used as a model to clarify the interaction of TAM with the cell membrane. TAM effects on the ultrastructure of membranes of this bacterium were evaluated by electron microscopy. Important ultrastructural alterations were observed in *B. stearothermophilus* treated with TAM, namely change in the geometry of the membrane profile from asymmetric to symmetric, disaggregation of ribosomes, coagulation of the cytoplasmic matrix, occurrence of mesossomes, appearance of fractures in membranes and the alteration of the ultrastructure of cell wall. These ultrastructural alterations confirm that TAM is a membrane-active drug and that membrane damage may be involved in molecular mechanisms of cell death induced by this drug.
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1. Introduction

Tamoxifen (TAM), a non-steroid antiestrogen, is the mostly used drug for chemotherapy and chemoprevention of breast cancer (Neven and Vernaeve, 2000; Radmacher and Simon, 2000). Although tamoxifen is usually considered an estrogen antagonist, it is now evident that the antiproliferative effects may involve mechanisms not restricted to the classical oestrogen receptor (ER) binding model (Kon, 1989). TAM has been reported to inhibit the growth of ER-negative breast cancer cells and other types of cells that lack ER (Croxtall et al., 1994; Van den Koedijke et al., 1994;

Charlier et al., 1995). These mechanisms underlying the ER-independent inhibition of tumor cell growth by TAM are not yet understood. Thus, extensive studies have been performed and multiple cellular effects have been described, namely binding to antiestrogen binding sites (Lazier, 1987; Shuterland et al., 1980), antioxidant action (Custódio et al., 1994; Wiseman et al., 1990), inhibition of protein kinase C (O'Brian, 1986; Bignon et al., 1991), inhibition of cAMP phosphodiesterase (Lam, 1984; Rowlands et al., 1990), inhibition of of a nucleoside transporter protein (Cai and Lee, 1996), antagonism of calmodulin by direct interaction with this protein (Lopes et al., 1990) and induction of apoptosis (Gelman, 1996; Ellerby et al., 1997).

TAM strongly incorporates in biomembranes (Custódio et al., 1991) interacting with lipids (Custódio et al., 1993; Luxo et al., 1996) and proteins (Lopes et al., 1990; Custódio et al., 1996). A variety of effects of TAM have

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Abbreviations: TAM; Tamoxifen; ER; Oestrogen receptor

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been assigned to biophysical and biochemical interactions with membranes. These effects include the stimulation of ATP hydrolysis (Custódio et al., 1996; Chen et al., 1999) and the decrease in the energetic efficiency of the Ca^{2+}-pump of sarcoplasmic reticulum (Custódio et al., 1996), modifications in the morphology and structure of the breast tumor cell membranes, potentially responsible for its estrogen-independent antiproliferative activity (Sica et al., 1984), hemolytic effects (Cruz Silva et al., 2000), mitochondrial swelling (Custódio et al., 1998) and proton leak across the mitochondrial inner membrane (Cardoso et al., 2002). Therefore, biomembranes are likely targets of TAM.

A better understanding of the physicochemical basis of interaction of TAM with biomembranes is essential to test the possibility of TAM cytotoxicity being dependent on its membrane interaction.

Biomembranes are common components to all living structures and the first target of contact for xenobiotics, perhaps the most important for lipophilic compounds. Mammalian membranes are undoubtly the most suitable models to study membrane interactions of pharmacological compounds, TAM included. However, due to the high complexity, other membrane models, e.g. artificial membranes composed of synthetic lipids, are usually used (Balasubramanian and Straunbinger, 1994; Canaves et al., 1991; Custódio et al., 1993; Wright and White, 1986). Also, prokaryotic membranes, with a simple phospholipid composition as compared with eukaryotic membranes, have been used as suitable models (Sikkema et al., 1995). They are similar to the actual target membranes owing to a similar lipid composition (including several phospholipids classes and species), although with a simple structure; furthermore, bacterial membranes are easy to isolate, yielding very pure preparations, since there are no intracellular compartments in most prokaryots and a single membrane involves the cytoplasm in Gram-positive *Eubacteria*. Finally, bacterial phospholipids of eubacteria are structurally similar to the eukaryotic counterparts (Ratledge and Wilkinson, 1988) and they also play similar roles to eukaryotic lipids (Russel, 1989), providing a fluid bilayer to embed integral proteins preventing hydrophobic mismatch (Bloom et al., 1991). *Bacillus stearothermophilus* has been extensively used in our laboratory to study the toxic effects of several xenobiotics (Luxo et al., 1996; Donato et al., 1997a; Rosa et al., 2000a). In addition to general advantages of prokaryotic cells as mentioned above, *B. stearothermophilus* has the ability to overcome the threatening of adverse environmental conditions, namely chemical stress, by undergoing membrane lipid composition changes (Luxo et al., 1998; Donato et al., 1997b, Rosa et al., 2000b). On the other hand, bacterial growth inhibition has shown a good relationship with other bioindicators of chemical stress in eukaryotic cells, *e.g.*, the respiratory

activity of rat liver mitochondria (Donato et al., 1997c).

Previous studies show that TAM inhibits growth of *B. stearothermophilus* and perturbs the physical behaviour of bacterial membrane lipids (Luxo et al., 1996). Additionally, *B. stearothermophilus* modifies membrane lipid composition in response to the addition of TAM to the growth medium in order to compensate the perturbing effects of this drug (Luxo et al., 1998). Although, *B. stearothermophilus* is endowed with efficient molecular mechanisms of adaptation, TAM concentrations higher than 10 µM completely inhibit growth and induce cell death (Luxo et al., 1998). Molecular mechanisms of cell death induced by this drug remain obscure, but membrane damage may putatively be involved. In our study, we evaluated TAM effects on the ultrastructure of *B. stearothermophilus* by electron microscopy attempting to clarify antiproliferative mechanisms of TAM.

Studies of TAM effects on the ultrastructure of membranes of *B. stearothermophilus* may be extrapolated to other membrane systems, namely eukaryotic, and may contribute to clarify antiproliferative mechanisms of TAM.

2. Materials and methods

2.1. Chemicals

Tamoxifen was obtained from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Cultures

The strain of *B. stearothermophilus* was isolated from discs impregnated with spores supplied by Mast Laboratoires, UK (lot no. 8879). Stock cultures were maintained at $-80\ ^{\circ}C$. Liquid cultures were started with an early stationary inoculum from a culture medium (L-Broth: tryptone 5 g, yeast extract 2.5 g, sodium chloride 1.25 g, 1 M sodium hydroxide 2 ml, 100 mM cystine in 1 M HCl 1 ml, H_2O 1000 ml) and were grown in 1 l Erlenmeyer flasks containing 200 ml of the same medium shaken at 100 rpm in a New Brunswick water-bath shaker, at $65\ ^{\circ}C$. Tamoxifen from a concentrated ethanolic solution was added to the cultures, in the exponential phase, to obtain concentrations ranging from 5 to 10 µM. The bacterial growth in media containing tamoxifen had to be performed in silanized Erlenmeyer flasks, since this drug binds strongly to glass material (Custódio et al., 1991).

Growth was measured by turbidimetry at 610 nm in a Spectronic 201 (Milton Roy Co., Rochester, NY, USA) instrument.

2.3. Electron microscopy

Two millilitres of glutaraldehyde 25% were added to aliquots (20 ml) of the different cultures (with and without TAM). After centrifugation at 10,000 g for 5 min, the cells were resuspended in glutaraldehyde at 2.5% in 0.1 M cacodylate buffer, pH 7.0, for 4 h. After, they were whashed twice with 50 mM cacodylate buffer supplemented with 10 mM $CaCl_2$ (pH 6.4) and fixed at room temperature, for 2 h, in 1% osmium tetroxide (OsO_4) in veronal-acetate buffer, supplemented with 10 mM $CaCl_2$ (Silva and Macedo, 1983). After fixation, the samples were dehydrated with ethanol and embedded in Epon. Ultrathin sections were obtained with an LKB Ultrotome III fitted with a diamond knife and stained with uranyl acetate and lead citrate (Silva et al., 1987). Observations and micrographs were made with a Zeiss EM 10C electron microscope.

3. Results

3.1. Effects of TAM on the growth of B. stearothermophilus

The strain of *Bacillus stearothermophilus* was grown at 65 °C (optimal temperature) in a culture medium (L-Broth) and TAM was added in a concentration range from 5 to 10 μM (5; 7.5; 10 μM) to the cultures in exponential phase. Addition of TAM during exponential phase induced bacterial growth inhibition as a function of concentration (Fig. 1).

3.2. Effects of TAM on the ultrastructure of Bacillus stearothermophilus

Addition of TAM to the growth medium of *B. stearothermophilus* induced important ultrastructural alterations in membranes of *B. stearothermophilus*. Aspects of control *B. stearothermophilus* are shown in Fig. 2. The cytoplasmic membrane (MC) is continuous and shows the asymmetric geometry typical of Gram-positive bacteria. The cell wall (PC) is intact and the fibrillar nucleoide (N) and abundant ribosomes in the cytoplasm are clearly visible. The treatment with TAM resulted in important ultrastructural alterations namely, change in the membrane staining pattern from the normal asymmetric to symmetric geometry, the disaggregation of ribosomes, the coagulation of the cytoplasmic matrix and formation of complex mesosome-like membranous structures (Figs. 3 and 4). The cell wall remained unchanged (Figs. 3 and 4). After longer treatment times or with high concentrations, the membrane alterations were more dramatic, with fracturing and membrane solubilization and the cell wall appears extensively damaged (Fig. 5). Cell wall breakage may occur (arrow) with leakage of cytoplasmic material (Fig. 5).

4. Discussion

It is clearly shown that TAM induces important membrane ultrastructural alterations in *B. stearothermophilus*. The change of the asymmetric geometry of cytoplasmic membranes of Gram-positives to sym-
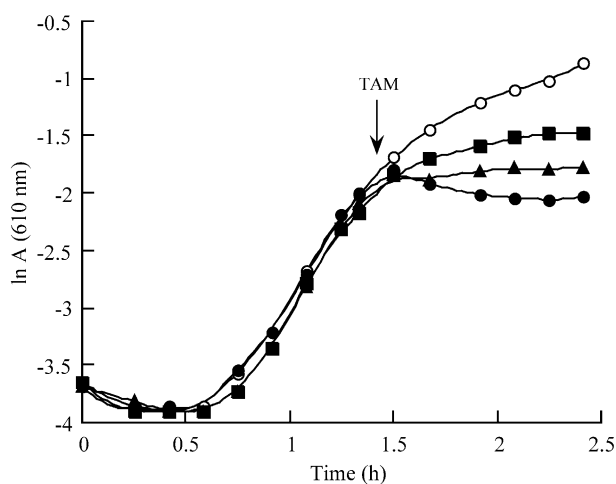

Fig. 1. Effect of tamoxifen addition on the growth of cultures of *B. stearothermophilus* at 65 °C. Growth of cultures without tamoxifen (○) addition in exponential phase or with addition of 5 μM (■), 7.5 μM (◆) or 10 μM (●) tamoxifen. Growth was measured as absorbance at 610 nm. The results shown are typical of three separate experiments.
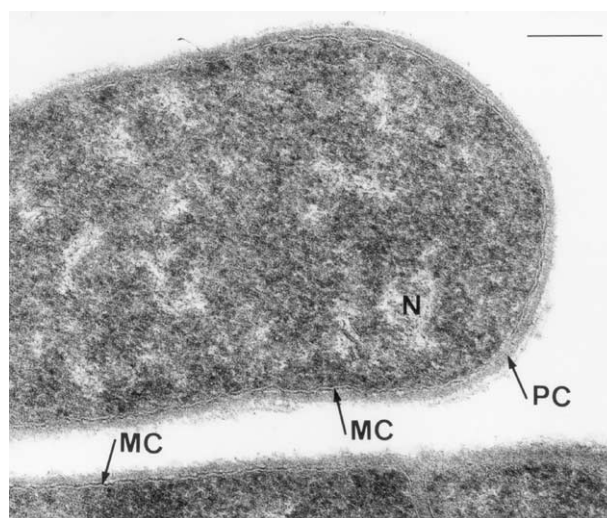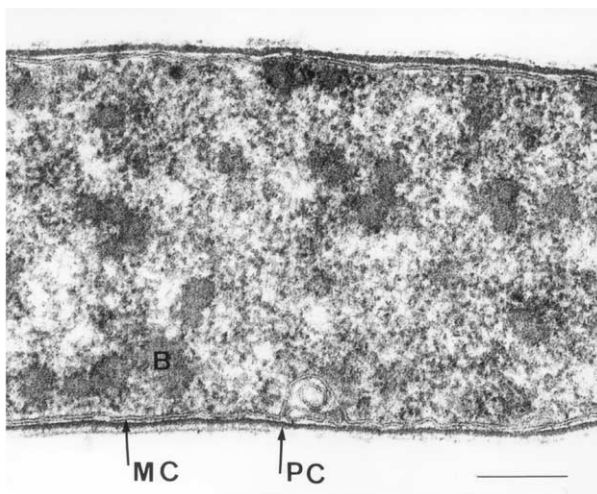

Fig. 2. Control cell of *Bacillus stearothermophilus*, showing the asymmetric and continuous membrane profile (MC) and the intact cell wall (PC). Notice the fibrillar nucleoside (N) and the abundant ribosomes. Bar = 0.2 μM.

metric geometry indicates membrane damage and occurs in several situations. This is the case in which Gram-positive bacteria are damaged by lysis (Silva, 1967) or by treatment with membrane active molecules, namely, local anesthetics (Silva et al., 1979), phenethyl alcohol (Silva et al., 1976) and organic solvents (Silva et al., 1978). This change of the membrane profile is usually a very early alteration and, therefore, it represents a useful marker for membrane damage. Bacterial ribosome disappearance has been also observed for several damaged Gram-positive bacteria (Silva and Macedo, 1983). Several mechanisms can be advanced to explain ribosome disappearance: ion leakage associated with the loss of selective permeability as a result of membrane damage at the beginning of the degradative
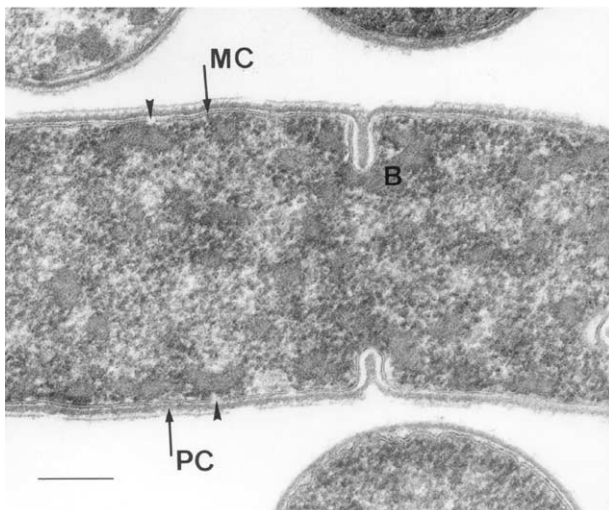

(a)

(b)

Fig. 3. *B. stearothermophilus* cells treated with TAM ((a)-7.5 μM/110 min.; (b)-10 μM/110 min). Cells with a change in the cytoplasmic membrane (MC) profile from asymmetric to symmetric and with the appearance of fractures (arrows). Notice the coagulated cytoplasm with dense blocks (B) and ribosomes partially disagregated. The cell wall (PC) remains intact. Bar = 0.2 μm.
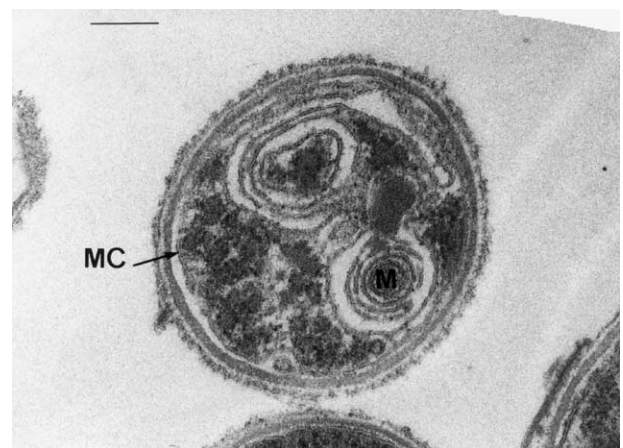
process, since ribosome stability depends on the intra-cellular ionic environment (Silva and Macedo, 1983); activation of bacterial endogenous RNases occurring in situations of membrane damage (Santos Mota et al., 1971).

Considering that biomembranes are structurally similar in eukaryotic and prokaryotic cells, it is predictable that TAM may also induce similar ultrastructural alterations in eukaryotic membranes. Sica et al. have shown that tamoxifen induces modifications in the morphology and structure of human breast cancer cell membranes (CG-5) and suggest that these alterations could be partially responsible for its oestrogen-independent antiproliferative activity (Sica et al., 1984).

Several effects of TAM have been suggested to be a consequence of membrane damage. The stimulation of ATP hydrolysis (Custódio et al., 1996; Chen et al., 1999) and the decrease energetic efficiency of sarcoplasmic reticulum Ca^{2+}-pump induced by TAM would be related with structural defects induced by this drug on membranes (Custódio et al., 1996). In the same way, mitochondrial swelling and membrane potential depolarisation induced by TAM reflect a direct action of this drug on the mitochondrial inner membrane structure (Custódio et al., 1998). Hemolytic anemia, observed in patients treated with TAM, has also been suggested to result from structural defects in the erythrocyte membrane induced by this drug (Cruz Silva et al., 2000). Moreover, TAM affects mitochondrial bioenergetics (Cardoso et al., 2001) and induces proton leak across the mitochondrial inner membrane (Cardoso et al., 2002) as a consequence of the destructive effects in the structural integrity of the mitochondrial inner membrane. Accordingly, the ultrastructural alterations observed in *B. stearothermophilus* confirm that TAM is a membrane-active drug. The disruption of the structural characteristics of biomembranes by TAM may

Fig. 4. *B. stearothermophilus* cell treated with TAM (10 μM/110 min). Transversal section of a cell showing symmetric cytoplasmic membrane profiles (MC) with zones of extensively solubilization. Notice two lamellar mesossomes (M). Bar = 0.2 μM.
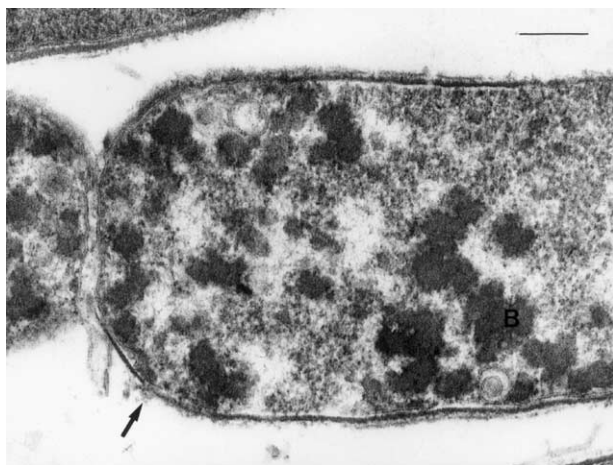
Fig. 5. *B. stearothermophilus* cell treated with TAM (10 μM/140 min). The cytoplasmic membrane is extensively solubilized. The membrane remants have a symmetric profile. Notice the blocks of compact cytoplasmic matrix (B). The cell wall is extensively damaged with cell wall disruption (arrow) and leakage of cytoplasmic material. Bar = 0.2 μM.

contribute for the multiple mechanisms of anticancer action not related to the oestrogen receptors and may be involved in molecular mechanisms of cell death induced by this drug.

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