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Research update

Classification:

(1) Antibiotics and chemotherapeutics or (3) metabolic disorders and endocrinology

Title:

Improving the efficacy of hormone therapy in breast cancer: the role of cholesterol metabolism in SERM-mediated autophagy, cell differentiation and death

Julie Leignadier[a], Florence Dalenc[a,b], Marc Poirot[a$],and Sandrine Silvente-Poirot [a$]

[a]*Cancer Research Center of Toulouse, UMR 1037INSERM-University of Toulouse, Toulouse, France;* [b]*Institut Claudius Regaud, Institut Universitaire du Cancer-Oncopole, Toulouse, France.*

[$]Address correspondence to these authors at the Department of Cholesterol Metabolism, Cancer Research Center of Toulouse, INSERM UMR 1037-University of Toulouse III, P.O. Box: 31017, Toulouse, France; Tel/Fax: +33-582741626; E-mail: marc.poirot@inserm.fr; sandrine.poirot@inserm.fr

Running title: Cholesterol metabolism and pharmacology of tamoxifen

Abstract: Breast cancer (BC) is one of the most common female cancers in the world, with estrogen receptor (ER)-positive BC the most frequent subtype. Tamoxifen (Tam) is an effective drug that competitively binds to the ER and is routinely used for the treatment of ER-positive BC. However, a number of ER-positive BC do not respond to Tam treatment and acquired resistance is often observed, constituting a major challenge for extending patient life expectancy. The mechanisms responsible for these treatment failures remain unclear, indicating the requirement for other targets and better predictors for patient response to Tam. One of Tam's off-targets of interest is the microsomal antiestrogen binding site (AEBS), a multiproteic complex made up of the cholesterol-5,6-epoxide hydrolase (ChEH) enzymes that are involved in the late stages of cholesterol biosynthesis. Tam and other selective ER modulators stimulate oxidative stress and inhibit the ChEH subunits at pharmacological doses, triggering the production and accumulation of cholesterol-5,6-epoxide metabolites responsible for BC cell differentiation and death. However, inhibition of the cholesterogenic activity of the AEBS subunits also induces the accumulation of sterol precursors, which triggers a survival autophagy to impair Tam's efficacy. Altogether, these studies have highlighted the involvement of cholesterol metabolism in the pharmacology of Tam that has provided new clues on how to improve its therapeutic efficacy in both BC and other cancers as well as offering a new rationale for developing more efficient drugs for BC treatment.

Keywords: Tamoxifen; AEBS; breast cancer; zymostenol; 5,6-epoxycholesterol

Chemical compounds:

Tamoxifen (PubChem CID: 2733526); toremifene (PubChem CID: 3005573); tesmilifene (PubChem CID: 108092); PBPE (PubChem CID: 71311880); 4-hydroxytamoxifen (PubChem CID: 449459); lazofoxifene (PubChem CID: 216416); raloxifene (PubChem CID: 5035); bazedoxifene (PubChem CID: 154257); clomiphene (PubChem CID: 1548953); 17β-estradiol (PubChem CID: 5757); ICI 164,684 (PubChem CID: 104772). ICI 182,780 (PubChem CID: 44307470); RU 58,668 (PubChemCID: 119604); MER29, triparanol (PubChem CID: 6536); boxidine (PubChem CID: 31742); dendrogenin A (PubChem CID: 9806490); cholesterol (PubChem CID: 5997); desmosterol: (PubChem CID: 439577); zymostenol (PubChem CID: 101770); zymosterol (PubChem CID: 92746); 7-dehydrocholesterol (PubChem CID: 439423); 5,6α-epoxycholesterol (PubChem CID: 227037); 5,6β-epoxycholesterol (PubChem CID: 108109); histamine (PubChem CID: 774).

1. INTRODUCTION

Each year, breast cancer (BC) affects more than 1 million women worldwide and about 400,000 patients die from this disease [1]. Currently, only two biomarkers are used to decide upon BC therapeutic strategy irrespective of the stage of the disease: estrogen receptor α (ER) and the Human Epidermal growth factor Receptor 2 (HER2) (NCCN 2017, https://www.nccn.org/). Great strides have been made in BC treatment, through the use of targeted therapies such as endocrine therapies like Tamoxifen (Tam) (Fig. 1A) for BC expressing ER (70 to 80% of BC), or agents targeting overexpressed HER2 (15 to 18% of BC) [2]. However, not all BC respond to these therapies, and many develop resistance despite initial beneficial effects [3]. Therefore, there is an urgent requirement to further characterize the molecular actors involved in the pharmacology of Tam to improve the efficacy of hormone therapy and provide new molecular weapons to fight resistance.

Tam was initially developed to antagonize the tumor promoter effects of 17β-estradiol (E2) by competing with it for binding to ER [4, 5], and was shown to effectively inhibit $ER^{(+)}$ BC development in animal models and patients [4]. Although first given as an adjuvant, Tam is now administered as first line treatment and has also been approved by the Food and Drug Administration (FDA) for chemopreventive use in subjects at risk [1]. Although Tam is well-tolerated compared to non-specific chemotherapy, it does produce some side effects, such as hot flushes, vaginal discharge, arthralgia, myalgia, weight gain and hypertriglyceridemia [1, 6, 7]. The binding of Tam to ER induces a specific conformational modification of the receptor, which affects the affinity of the ER-Tam complex for co-activators and co-repressors that drive the ER transcriptional response [8]. Thus, there is pool of cellular co-regulators that contribute towards driving the agonistic or antagonistic action of Tam, and this is why Tam and its analogues have been called selective estrogen receptor modulators (SERM) [8].

The estrogenic effects of Tam in bone tissue are beneficial to patients and it protects against osteoporosis in the long-term. However, the estrogenic actions of Tam on the uterus are believed to be responsible for the low but significant increase in endometrial cancer [9-11]. As a result of this, SERMs with no uterotrophic effects, such as raloxifene, lasofoxifene, and bazedoxifene were developed [12], but despite their good efficacy on $ER^{(+)}$ BC, none of them were able to supplant Tam in the clinic [12]. Studies related to the action and metabolism of Tam in terms of its control of ER have been extensively reviewed and we encourage readers to look at them [1, 4, 13-21].

Hormonal therapies for BC, such as Tam, are given after first confirming the expression of one of two hormonal receptors, ER and/or the progesterone receptor (PR). The majority of $ER^{(+)}$ BC are also $PR^{(+)}$. The expression of ER and PR in BC tumors as justification for patient eligibility for hormonotherapy has been successful, however not all patients respond to treatment and acquired resistance occurs systematically in metastatic settings and during adjuvant treatment, sometimes after 2 to 3 years or more. Aromatase inhibitors (AI) are inhibitors of 17β-estradiol biosynthesis and are commonly used as for $ER^{(+)}$ BC treatments. AI but not Tam or other SERMs, were shown to induce acquired resistance in which mutations were found on the ER [22]. In contrast, oxidative stress-neutralizing pathways and ligand-X-receptor (LXR)-driven cholesterol metabolic enzymes were found to be upregulated in animal models and patients with $ER^{(+)}$ BC treated with Tam [15, 23-25]. Along the same lines, cholesterol-lowering medication during adjuvant endocrine therapy has also been shown to improve response to Tam [26].

Despite its efficacy in $ER^{(+)}$ BC, Tam displays a complex pharmacology and several off-targets have been identified [27]. Studies of these off-targets can not only explain several side effects and thereby improve the whole clinical pharmacology of SERMs, but may also help in defining additional pathways involved in acquired and intrinsic resistance that could be

pharmacologically targeted. Treatment of patients with Tam gives a serum concentration at equilibrium ranging from 1µM to 10 µM [28], suggesting that targets of Tam with an affinity within this concentration range should be considered in its anticancer pharmacology. Protein kinase C (PKC) has been identified as a low affinity (>µM) target for Tam [27]. This enzyme is a known target for tumor-promoting phorbol esters, calmodulin (which controls calcium-dependent enzymes), and the acyl-coA-cholesterol acyl transferase (ACAT, or SOAT1 and SOAT2). ACAT controls the biosynthesis of fatty acyl-cholesteryl esters, which have recently been identified as tumor promoters, with the inhibition of cholesterol esterification appearing to be a new promising target for cancer treatment [29-33]. Other molecular targets of Tam and the SERM 4OHTam were shown to be inhibitors of phospholipase D1 and D2, respectively [34-37]. Tam and 4OHTam are also inhibitors of glycosylceramide synthase [38, 39] and of cyclin-dependent kinase 5 (CDK5) [40]. The relationship between these molecular targets and the anticancer activity of Tam remains to be established in BC.

Tam and other SERMs have also been shown to interact with high affinity (nM) to an intracellular and membranous binding site named the antiestrogen binding site (AEBS) [41], whose molecular characterization has established a link between Tam and cholesterol metabolism in its pharmacology [42]. The aim of the present article is to summarize the available data on the structure and function of the AEBS and explain how this binding site can account for the pharmacology of Tam, SERMs and AEBS ligands. We hope to shed light on a new field of investigation, which is improving our understanding of the pharmacology of Tam and will help in the development of the future generations of drugs for BC management.

2. The antiestrogen binding site (AEBS)

2.1. Pharmacological profile of the AEBS

The existence of the AEBS was first reported by Robert Sutherland and Jean-Charles Faye in the eighties [41, 43, 44]. Although initially detected in the cytosolic fractions of cells and tissues [41, 43-45], the AEBS is an intracellular microsomal Tam binding site [46, 47]. The name "antiestrogen binding site" was chosen because it was shown to bind to Tam and analogues but not 17β-estradiol, which distinguished it from ER [41]. Synthetic estrogens such as diethylstilbestrol (DES) or pure steroidal antiestrogens (also named SERDs: for selective estrogen receptor destructors) [17, 18] such as ICI 164,384, ICI 182,780 (Faslodex) or RU 58,668, do not bind to the AEBS [27, 48]. Structure-affinity studies performed on the AEBS have shown that the presence of a phenolic group in the polyphenyl part of SERMs decreases their affinity for the AEBS [27, 49, 50] but improves their affinity for ER [51]. The presence of a cationic aminoalkyl side chain in drugs and drug candidates was also found to be required for high affinity binding to the AEBS [27]. Structure-affinity studies have also revealed structural elements of Tam that are required for AEBS binding (Fig. 1B), which are different from the structural determinants required for ER binding (Fig. 1C) [27, 49]. The selective affinity binding properties in the pharmacological profile of the AEBS are thus different from those of ER [47].

2.2. Development of selective AEBS ligands

Several research groups have developed Tam analogues and found some that bound preferentially to AEBS with negligible affinity for ER, including the cis isomer of centchroman [52] and a benzofuran analog of Tam [53]. The impacts of these compounds on other targets of Tam, such as ACAT, PKC and calmodulin, have not yet been studied. The synthetic high affinity AEBS ligands have been have been based upon the diphenyl methane (DPM) series of compounds, due to the seminal work of L.J. Brandes who first identified the DPM compound DPPE (tesmilifene, Fig. 1D) as a high affinity AEBS ligand [54]. Other DPM compounds have also been chemically synthesized and among them structure-affinity

studies have shown that PBPE (Fig. 1E) is one of the highest affinity ligands for the AEBS [49]. DPPE and PBPE have been the most studied selective AEBS ligands [15, 27, 55-64], and when DPPE was brought to the clinic under the trade name of tesmilifene it showed some efficacy in cancer treatment [61, 65-69]. DPPE and PBPE were shown to be selective for the AEBS with no impact on ER [49], ACAT [48], PKC [70, 71] or calmodulin [63, 72], evidencing that DPM compounds are useful tools for defining the importance of the AEBS in the anticancer pharmacology of SERMs.

In the quest to determine the physiological significance of the AEBS, several groups have studied the lipid constituents of serum and found that 7-ketocholesterol (Fig. 1F) [73] and unsaturated fatty acids [74, 75] are AEBS ligands. Structure-affinity studies were conducted on a range of oxysterols, and only 6-ketocholestanol and/or 7-ketocholestanol were found to be high affinity AEBS ligands, while side chain-oxysterols displayed a weak or unmeasurable affinity for the AEBS [76, 77]. Two other ring B-oxysterols: 5,6α-epoxycholesterol (5,6α-EC) (Fig. 1G) and 5,6β-epoxycholesterol (Fig. 1H) (5,6β-EC) have been shown to be high affinity AEBS ligands [57]. 5,6α-EC was also shown to be a ligand for LXRα and a modulator for both the LXRα and β subtypes [78], however they have not been tested on other off-targets of Tam or other known oxysterol targets such as AhR [79, 80], ROR [81-83], EBI2 [84], Hedgehog component smoothened [85], oxysterol-binding protein-related proteins [86] or ER [87, 88]. Histamine (Fig. 1I) was shown to be an endogenous AEBS ligand [89-91], and recently a stereoselective conjugation product of the condensation of histamine and 5,6α-epoxycholesterol, named dendrogenin A (Fig. 1J), was identified and found to be an AEBS ligand [92-97].

2.3. Molecular characterization of the AEBS

2.3.1. The AEBS is made up of two enzymes involved in the late stages of cholesterol biosynthesis

Analysis of the structure of the known AEBS ligands suggested some links between AEBS and cholesterol metabolism. Tam and PBPE are both structurally related to cholesterol biosynthesis inhibitors that were developed in the sixties, MER-29 (Fig. 1K) and boxidine (Fig. 1L), which are inhibitors of the 3β-hydroxysteroid-Δ^{24}-reductase (DHCR24) [98] and the 3β-hydroxysteroid-Δ^{7}-reductase (DHCR7), respectively. The fact that MER-29 and boxidine induce the accumulation of desmosterol (Fig. 1M) (the substrate of DHCR24) and 7-dehydrocholesterol (7DHC, Fig. 1N) (the substrate of DHCR7) [99], suggested that Tam and AEBS ligands could inhibit cholesterol biosynthesis at a post-lanosterol step (Fig. 2A). Indeed, Craste de Paulet's group in Montpellier (France) was the first to show that Tam inhibited cholesterol biosynthesis in BC cell lines [100]. Miettinen's group in Helsinki (Finland) also analyzed the sterol profile in the blood of BC patients treated with Tam and observed the appearance of zymostenol (zymo) (Fig.1O), suggesting an inhibition of cholesterol biosynthesis at the D8D7I (EBP, 3β-hydroxysteroid-Δ^{8},Δ^{7}-isomerase) step (Fig. 2B) [101]. Tests were conducted on breast cancer cell lines *in vitro* and the analysis of the sterol profile confirmed that Tam induced the accumulation of zymo, showing an inhibition of the EBP/D8D7I enzyme [42, 55, 59]. A subsequent evaluation of selective AEBS ligands from the DPM series showed that, in addition to zymo, DPPE and PBPE induced the accumulation of 7DHC suggesting that DHCR7 could be associated to the AEBS [42, 55, 59]. In line with this, 7-ketocholesterol, and 6- and 7-ketocholestanol were reported to inhibit EBP/D8D7I and induced zymo accumulation [42, 59]. However, no correlation was found between binding affinity to the AEBS and inhibition of cholesterogenic enzymes [42]. In addition, it was observed that AEBS ligands were not obligatory inhibitors of these cholesterogenic enzymes, and a shift between affinity for the AEBS and the doses required to inhibit these enzymes was found [42]. For example, weak affinity AEBS ligands such tBuPE did not inhibit cholesterol

biosynthesis in BC [42]. Phenolic SERMS that are ligands of the AEBS, such as 4OHTam, also induced the accumulation of desmosterol reflecting the inhibition of DHCR24 with no impact on D8D7I, while raloxifene induced the accumulation of zymosterol (Fig. 1P) reflecting a dual inhibition of D8D7I and DHCR24 [42, 55, 59, 60]. SERMs were reported to induce the accumulation of cholesterol precursors in cancer cell lines of different tissue origins, such as melanoma cells, leukemia cells and neuroblastoma cells [42, 55, 102-106].

Molecular reconstitution of the AEBS complex was done in Cos cells by transfection of plasmids encoding human D8D7I and DHCR7. It was found that the expression of both enzymes was required to reconstitute a high affinity Tam binding site displaying the pharmacological profile of the AEBS [42]. Co-immunoprecipitation experiments showed that EBP/D8D7I physically interacted with DHCR7 and bound Tam [42]. DHCR7 was also recently reported to interact physically and functionally with DHCR24 [107]. The AEBS is a multiproteic complex that involved EBP/D8D7I, DHCR7 and also DHCR24 and possibly other enzymes from the post-lanosterol cholesterol biosynthesis (Fig. 2A-C).

2.3.2. The accumulation of cholesterol precursors by Tam and AEBS ligands in BC cells triggered a survival autophagy.

Among the AEBS ligands, those that are cholesterol biosynthesis inhibitors were shown to induce a huge accumulation of free sterols in cells, which led to the appearance of cytoplasmic multilamellar bodies (MLB), observed through transmission electron microcopy (Fig. 2D). The accumulation of free sterols was visualized by fluorescence microscopy after labeling with the free sterol chelating agent filipin (Fig. 2D) [55, 59]. Although the significance of MLB formation has not yet been identified, it is possible that they are aggregates of free sterols and sphingolipids such as sphingomyelin, since an increase in sphingomyelin accumulation was measured in BC cells [60, 108]. MLB formation has been

observed in response to every AEBS ligand that inhibits cholesterol biosynthesis, independently of the nature of the accumulated cholesterol precursor [55, 59, 60].

Cholesterol intermediates have different physicochemical properties and may accumulate in different compartments of cells. Several cholesterol precursors restricted to FF-Mas [109, 110], desmosterol and zymosterol [111], were shown to display modulatory activities on the nuclear receptor LXR. Desmosterol was also shown to be an LXRα and LXRβ ligand [111]. Other sterol intermediates, such as lanosterol, lathosterol and 7-dehydrocholesterol, did not display LXR modulatory activities [110, 111], and the impact of zymo on LXR has not been investigated to date. These observations suggest that the nature of the sterol intermediate that accumulate may have different consequences on cancer cells. For example, it was shown that 4OHTam, which induced desmosterol accumulation, triggered acquired resistance in MCF7 cells [112, 113] suggesting that LXR activation could be related to acquired resistance to Tam. 7DHC accumulation could reflect teratogenic risks or neurological disorders [105, 106]. The determination of a pharmacophore to predict the selectivity in the inhibition of cholesterogenic enzyme by AEBS ligands is thus important to study and will deserves further investigation.

Importantly, sterol intermediates are prone to oxygenation through radical chain oxidation and lipoperoxidation [114-116]. The oxygenation products of 7DHC have been studied in depth [114, 116], but other lipoperoxidation products of cholesterol biosynthesis intermediates remain to be chemically characterized and their biological properties defined. The positioning and the nature of the oxygenation group is thought to control the molecular targeting and biological properties of these compounds. Oxysterol binding proteins and receptors have been reported and, interestingly, display a high selectivity towards oxysterol subtypes [117].

The accumulation of sterols in cells treated with Tam has been linked to the induction of autophagy, a self-digestion cellular mechanism [55, 58, 59, 118, 119]. It was initially proposed that Tam induces a lethal autophagy [120, 121], however other studies have since concluded that Tam and its analogues in fact induce a survival autophagy (Fig.2D), which constitutes a mechanism of resistance to Tam [55, 58, 59, 112, 113, 122-125].

2.3.3. The AEBS carries out the cholesterol-5,6-epoxide hydrolase (ChEH) activity

The natural high affinity AEBS ligands from the oxysterol series were also known as extremely potent inhibitors of the microsomal cholesterol-5,6-epoxide hydrolase (ChEH) [126], which catalyzes the hydration of 5,6α-epoxycholesterols (5,6-EC) into cholestane-3β,5α,6β-triol (CT) (Fig.3A) [127]. It was found that 5,6-EC were high affinity and competitive inhibitors of Tam at the AEBS [57]. AEBS ligands belonging to different chemical families with affinities ranging from nM to μM were tested alongside ChEH substrates and inhibitors in our AEBS binding assays. Substrates of ChEH were potent and competitive inhibitors of Tam binding on the AEBS, and a positive correlation between AEBS affinity and inhibition of ChEH activity by the different tested compounds was established. Consequently, compounds with no affinity for the AEBS did not inhibit ChEH. This showed that ChEH and AEBS are pharmacologically identical [57]. However, although the binding of a given compound to the AEBS can predict its inhibition of ChEH, and the inhibition of ChEH can predict the AEBS affinity of a compound, the inhibition of ChEH cannot predict post-lanosterol cholesterol biosynthesis inhibition. Knockdown of each AEBS subunit (EBP/D8D7I an DHCR7) in the breast cancer cell line MCF-7 was also shown to lead to a dual loss of AEBS and ChEH activities, and, conversely, overexpression of EBP/D8D7I an DHCR7 in COS cells led to the reconstitution of ChEH activity with the expected

pharmacological profile [128]. Altogether, these results established that ChEH is molecularly identical to the AEBS (Fig. 3A).

2.4. The AEBS plays a role in the anticancer pharmacology of Tam

The AEBS was proposed to play a role in the anticancer activity of Tam even before its molecular identification was done. Several studies suggested that the AEBS might be of interest since an increase or decrease in AEBS expression was associated with a loss of sensitivity to Tam [45, 129]. Another study contradicted these findings [130], but this was mainly based on the comparison of Tam with tBuPE, a selective but weak affinity AEBS ligand [131], which was later established as not potent enough to induce similar effects to Tam in terms of cholesterol metabolism perturbation [42]. Further studies evaluating selective AEBS ligands showed that if they had no direct ER modulatory activity they were cytotoxic and induced cancer cell differentiation [56, 59, 60, 63, 64, 71, 132-134].

2.5. Selective AEBS ligands control cancer cell death and differentiation

High affinity AEBS ligands such as Tam and PBPE were shown to induce cytotoxicity in $ER^{(+)}$ and $ER^{(-)}$ cancer cells of various tumor origins. From a number of BC cell lines, the $ER^{(+)}$ MCF-7 cell line was found to display the highest sensitivity to DPM compounds while triple negative ($ER^{(-)}$, $PR^{(-)}$, $HER2^{(-)}$) MDA-MB-231 cells were 10 times less sensitive to these compounds and to Tam [56]. More specifically, analyses of the effects of Tam and AEBS ligands showed that all of these compounds induced cell synchronization in the G0-G1 phase of the cell cycle and characteristics of ductal cell re-differentiation, as observed with either the histone deacetylase (HDAC) inhibitor SAHA [135] or *all trans*-retinoic acid [136, 137]. Similar observations, although requiring higher doses of AEBS ligands, were observed in the $ER^{(-)}$ SKBR3 BC cells and MDA-MB-231 cells, suggesting that AEBS ligands induced trans-differentiation in cancer cells [56, 60]. These effects were found to be completely blocked in

the presence of antioxidants such as vitamin E [56, 122]. Antioxidants such as vitamin E and C are known to inhibit the cytotoxicity of cancer cells induced by Tam [58-60, 138-144], suggesting possible adverse effects in patients who are being treated with Tam and taking vitamin C or E as dietary complements.

2.6. Cholesterol epoxidation is involved in BC cell differentiation and death induced by Tam and AEBS ligands

Analyses of the oxysterol profiles of BC cells treated with Tam, SERMs and AEBS ligands have revealed that these compounds stimulate the appearance and accumulation of 5,6α-EC and 5,6β-EC, which are not normally present in cancer cells (Fig. 3C). Co-treatment of cells with Tam or AEBS ligands and vitamin E inhibited both their pharmacological effects and the accumulation of 5,6-EC, strongly suggesting that 5,6-EC could be second messengers in Tam-induced BC cell death and differentiation (Fig. 3C). It was also shown that Tam and AEBS stimulated lipoperoxidation [55, 56, 60, 122], which is responsible for cholesterol epoxidation [96], and that their inhibition of ChEH blocked 5,6-EC metabolism to CT in cancer cells [128]. Hydrogen peroxide and lipoperoxidation are involved in cholesterol epoxidation and are therefore also responsible for the production of the second messengers that are responsible for the induction of BC cell differentiation and death by Tam and AEBS ligands. Indeed, several studies have identified a dysregulation in the expression of hydrogen peroxide metabolic enzymes, both in a mouse BC model and in human BC tumors, which is consistent with the involvement of 5,6-EC in the pharmacology of Tam [23, 25].

This mechanism of ROS-induced 5,6-EC production and its inhibition by vitamin E provides potential routes by which cancer cells can protect themselves against the cytotoxicity induced by Tam, other SERMS and AEBS-selective ligands (Fig. 3C-D) [58-60, 138-146].

2.7. Importance of the sulfotransferase SULT2B1b and the nuclear receptor LXR in cytotoxicity induced by Tam and AEBS ligands

Analyses of 5,6-EC biological properties and metabolism in BC cells have revealed that the diastereoisomers 5,6α-EC and 5,6β-EC both contribute to cell death induced by Tam or AEBS ligands but through different mechanisms (Fig. 3C). 5,6β-EC induced a cytotoxicity linked with mitochondrial impairment (cytotoxicity route 1:CD1, Fig. 3C) and is 5,6α-EC is responsible for the induction of BC cell differentiation and death, which was found to be mediated by the nuclear receptor LXRβ and defined the cytotoxicity route 2 (CD2, Fig 3C) [56]. Accordingly, invalidation of LXRβ expression induced a loss in BC cell differentiation and decreased the amplitude of cell death triggered by AEBS ligands. 5,6α-EC is sulfated by the sulfotransferase SULT2B1b to give 5,6α-epoxycholesterol-3β-sulfate (5,6α-ECS) (Fig. 3C and 4A) [56]. 5,6α-ECS has been proposed to be a LXR antagonist [147] but it has now been accepted that in fact it is a modulator of LXR and activates the expression of certain LXR-responsive genes, leading to TG biosynthesis [56]. Under basal conditions (in the absence of oxidative stress and 5,6-EC), SULT2B1b gene invalidation reportedly blocked LXRα signaling [148, 149] and activated cell proliferation [150-154]. The effects of 5,6α-ECS are thus peculiar among those reported for other sulfated sterols. Side chain-oxysterols were reported to stimulate cell proliferation [148, 150-152, 154] and to accumulate in prostate cancer, suggesting a link between this accumulation and carcinogenesis [155]. It should be noted that SULT2B1b displays different subcellular localizations and functions in prostate versus breast cancer cells, suggesting different biochemical properties under different intercellular conditions [156]. Knockdown of LXRβ in MCF-7 cells was found to induce a loss of sensitivity to Tam, AEBS ligands and 5,6α-ECS (Fig. 4B), establishing its importance in the pharmacology of these compounds. In the absence of the cytotoxic route 2 (CD2), the remaining sensitivity to Tam is due to the activation of the cytotoxic route 2 (CD2) and reach

the magnitude of that found in MDA-MB-231 in which only CD1 is possible (Fig. 4D). Similarly, knockdown of SULT2B1b in MCF-7 cells induced a loss of sensitivity to Tam and AEBS ligands due to the absence of CD2, however these cells remained sensitive to 5,6α-ECS, which can reactivate CD2 (Fig. 4C). This established that SULT2B1b contributes to the sensitivity of cancer cells to Tam and AEBS ligands. Together, these observations suggest that 5,6-EC metabolism, LXRβ and SULT2B1b may represent new markers of sensitivity to Tam and AEBS ligands. MDA-MB-231 are triple negative BC cells that are not sensitive to the proliferative action of 17β-estradiol. These cells are 10 times less sensitive than $ER^{(+)}$ MCF-7 cells to Tam-induced cytotoxicity and are considered intrinsically resistant to Tam (Tam^R, Fig. 4D), and only CD1 can be activated. They are also less sensitive to other AEBS ligands compared to MCF-7 cells [56]. MDA-MB-231 cells express the AEBS and LXRβ but not SULT2B1b, thus they produce 5,6-EC but not 5,6α-ECS following Tam treatment. Their sensitivity to Tam is similar to that of MCF-7/LXRβ- (Fig. 4B) which reflects the cytotoxicity induced by 5,6β-EC (CD2, Fig. 3C). These cells are as sensitive as MCF-7 cells to 5,6α-ECS, which can activate CD2 [56]. Interestingly, the ectopic expression of SULT2B1b in these cells was shown to activate 5,6α-ECS biosynthesis and sensitized cells to Tam and AEBS ligands to the same level as MCF-7 cells (Fig. 4E) [56], confirming that 5,6-EC metabolism actively contributes to the response to Tam and AEBS ligands in BC cells. In these cells, the CD2 (Fig. 4E) involving 5,6α-ECS and LXR is activated.

3. Conclusion

This update on Tam highlights the importance of cholesterol metabolism and the AEBS in the anticancer pharmacology of Tam, other SERMs and AEBS ligands. The molecular identification of the AEBS has opened up new research avenues and has identified a new signaling pathway that is involved in the control of BC differentiation and death by Tam and

is centered on cholesterol metabolism. The identification of cholesterogenic enzymes as targets of Tam and AEBS ligands is interesting since their inhibition, and the accumulation of sterol precursors, have been associated with the induction of a protective autophagy, leading to new hypotheses on the appearance of mechanisms of Tam resistance. Overexpression of the AEBS was found to be predictive of acquired resistance to Tam in patients with Muc1$^{(+)}$ ER$^{(+)}$ BC [15, 24], however it is not known whether zymo levels in the blood of patients may represent a predictive marker of response to Tam. Zymo has been shown to be prone to rapid oxidation with a short half life (less than one week at minus 80°C) and it is not inhibited by commonly used antioxidants [42, 157]. Thus, its sterol profile determined in biological materials (cancer cells, blood) must be analyzed extemporaneously, otherwise the zymo signal in analytical methods will be lost. This makes it difficult to analyze the blood sterol profile and to quantify zymo in large cohorts of patients for which sera have been stored for long periods of time. Identification of the end-product(s) of zymo oxidative metabolism may represent an alternative that needs further exploration because these compounds will be stable and measurable. As observed for zymo, 5,6-EC levels in the circulating blood of patients may also represent a marker of Tam activity, and a feasibility study investigating this has very recently been published by Dalenc et al. (OXYTAM, clinical Trial NCT 1553903) [158]. The results from this study, although preliminary, indicate that Tam treatment modifies the oxysterol profile in the blood of patients, with a tendency for increased 5,6-EC levels to be associated with changes in levels of oxysterol markers, which are indicative of LXR transcriptional activity. This study requires further investigation to validate it and to determine whether the modification of oxysterol profiles in the blood of patients can be correlated with clinical outcome, but it offers much potential, especially given that LXR was shown to be central to the transcriptional signature associated with acquired resistance to Tam [24]. The identification of new sterols (such as 5,6α-ECS and dendrogenins) with specific properties

related to cancer also supports the future use of sterolomics approaches, which allow the quantification of these species [96, 159]. Finally, since LXR, cholesterol metabolism and oxysterols have been shown to play a role in cancer [160, 161] and in modulating both the immune system and the tumor microenvironment [162-164], and since they are involved in the anticancer pharmacology of Tam, further studies are also required to analyze the incidence of Tam treatment on these parameters.

Another important point for future investigations arises from reports that vitamin E and vitamin C inhibit Tam-induced BC cell death and/or differentiation and. Thus, considering that BC patients are prone to automedication with "natural" products [165-167], this point deserves further investigation in order to determine whether these may interfere with Tam treatment in the clinic.

Altogether, this review has highlighted the unprecedented characterization of the importance of cholesterol metabolism in the anticancer pharmacology of Tam in tumor tissues, which has opened up new avenues for improving its clinical efficacy and testing alternative approaches to fight against mechanisms of resistance to Tam.

LIST OF ABBREVIATIONS

AEBS, antiestrogen binding site; ChEH, cholesterol-5,6-epoxide hydrolase; Tam, tamoxifen, ICI 46,474, nolvadex: (Z)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N-dimethylethanamine; DPM, diphenylmethane; tesmilifene/DPPE, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanemine; PBPE, 1-[2-[4-(phenylmethyl)phenoxy]ethyl]-pyrrolidine; 4OHTam, 4-hydroxytamoxifen; lazofoxifene, (5R,6S)-6-phenyl-5-[4-(2-pyrrolidin-1-ylethoxy)phenyl]-5,6,7,8-tetrahydronaphthalen-2-ol; raloxifene/keoxifen, [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone; bazedoxifene, 1-{4-[2-(Azepan-1-yl)ethoxy]benzyl}-2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol; clomiphene, 2-[4-[(E)-2-chloro-1,2-diphenylethe nyl]phenoxy]-N,N-diethylethanamine; triparanol/Mer-29, metasqualene,2-(4-chlorophenyl)-1-[4-[2-(diethylamino)ethoxy]phenyl]-1-(4-methylphenyl)etanol; boxidine, (1-[2-[[4'-(Trifluoromethyl)-4-biphenylyl]oxy]ethyl]pyrrolidine); tBuPE, t-butylphenoxyethyl diethylamine; dendrogenin A/DDA, 5α-hydroxy-6β-[2-(1H-imidazol-4-yl)-ethylamino]-cholestan-3β-ol; DHCR24, 3β-hydroxysteroid-Δ^{24}-reductase; DHCR7, 3β-hydroxysteroid-Δ^{7}-

reductase; D8D7I/EBP, 3β-hydroxysteroid-Δ^8,Δ^7-isomerase; SC5D, 3β-hydroxysteroid-Δ^5-desaturase; cholesterol, cholest-5-en-3β-ol; desmosterol, cholest-5,24-dien-3β-ol; zymostenol, 5α-cholest-8-ene-3β-ol; zymosterol, 5α-cholesta-8,24-dien-3β-ol; 7-dehydrocholesterol, cholest-5,7-dien-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol; 5,6α-EC, 5,6α-epoxycholesterol; 5,6β-EC, 5,6β-epoxycholesterol; 5,6α-ECS, 5,6α-epoxycholesterol-3β-sulfate; HA, histamine; ER, estrogen receptor; BC, breast cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Chemical structure of some synthetic and endogenous AEBS ligands and pharmacophores from the tamoxifen series. A) Tamoxifen. Molecular features that are necessary for tamoxifen to recognize its two high affinity targets: B) the microsomal antiestrogen binding site (AEBS) and C) ER. D-E) Selective AEBS ligands with no affinity to ER. F-J) natural endogenous AEBS ligands. K) Mer29 is an inhibitor of DHCR24. L) Boxidine is an inhibitor of DHCR7. M-P) Structure of cholesterol biosynthesis intermediates that could accumulate in cancer cells treated with AEBS ligands.

Figure 2: The AEBS is made up of two cholesterogenic enzymes, and AEBS ligands induced the accumulation of cholesterol precursors, which triggered a survival autophagy in cancer cells. A) The AEBS consists of an association between D8D7I and DHCR7 linked to DHCR24. B) Binding of Tam to the AEBS led to the inhibition of D8D7I and to the accumulation of zymostenol (zymo). Binding of PBPE led to the accumulation of zymo and 7-dehydrocholesterol (7DHC). C) Binding of 4OHtam induced the accumulation of desmosterol (desmo). D) Treatment of BC cells with Tam induced the accumulation of zymo in intracellular organelles. The accumulation of free sterols is revealed by filipin labeling under fluorescence microscopy. Ultrastructure analyses of cells through transmission electron microscopy (TEM) showed an accumulation of lysosomes (Lys) and the appearance of autophagosomes (AutoPh). These cellular events are associated with the induction of a survival autophagy in cancer cells. N: nucleus; C: cytopasm.

Figure 3: The AEBS carries out cholesterol-5,6-epoxide hydrolase (ChEH) activity and 5,6-ECs are second messengers of tam that are responsible for the induction of differentiation and death of breast cancer cells. A) ChEH catalyzes the hydration of cholesterol 5,6-epoxides (5,6αEC and 5,6β-EC) into cholestane-3β,5α,6β-triol. B) Tam inhibits ChEH and induces the accumulation of the 5,6-ECs. C) AEBS ligands induced the biosynthesis and the accumulation of 5,6-EC. 5,6β-EC contributes to route 1 cell death (CD1) through impairing mitochondrial activity. 5,6α-EC is metabolized into 5,6α-ECS by SULT2B1b and induces LXRβ-dependent characteristics of differentiation and cell death (route 2 cell death, CD2). D) Vitamin E (Vit E) inhibits cholesterol epoxidation and blocks the induction of cancer cell differentiation and cancer cell death (CD1). N: nucleus; C: cytoplasm; Mito: mitochondria; TG: triacylglycerol; Lys: lysosome; Chol: cholesterol; ROS: reactive oxygen species; zymo: zymostenol.

Figure 4: The importance of SULT2B1b and LXRβ on the sensitivity of BC cells to Tam. Molecular mechanisms of BC cell death induced by Tam and AEBS ligands: A) MCF-7 cells are sensitive to Tam (Tam^S) and 5,6-ECS ($5,6\text{-}ECS^S$); both the route 1 (CD1) and route 2 (CD2) are observable. B) MCF-7 cells invalidated for the expression of LXRβ (MCF-7/LXRβ-) became less sensitive to Tam (Tam^R) and 5,6-ECS ($5,6\text{-}ECS^R$); only route 1 (CD1) is observable. C) MCF-7 cells invalidated for the expression of SULT2B1b (MCF-7/SULT2B1b-) are Tam^R and $5,6\text{-}ECS^S$; only CD1 is observable with Tam but 5,6-ECS can activate CD2. D) MDA-MB-231 cells are Tam^R and $5,6\text{-}ECS^S$. MDA-MB-231 are less sensitive than MCF-7 to tam cytotoxicity; only CD1 is observable. E) Transgenic MDA-MB-231 cells expressing SULT2B1b (MDA-MB-231/SULT2B1b+) are Tam^S and $5,6\text{-}ECS^S$; both CD1 and CD2 occurred, these cells are as sensitive to Tam than MCF-7 cells. N: nucleus; C: cytoplasm; Mito: mitochondria; TG: triacylglycerol; Lys: lysosome; Chol: cholesterol; ROS: reactive oxygen species; zymo: zymostenol; CD1: cytotoxic route 1; CD2: cytotoxic route 2.

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Figure 1R1

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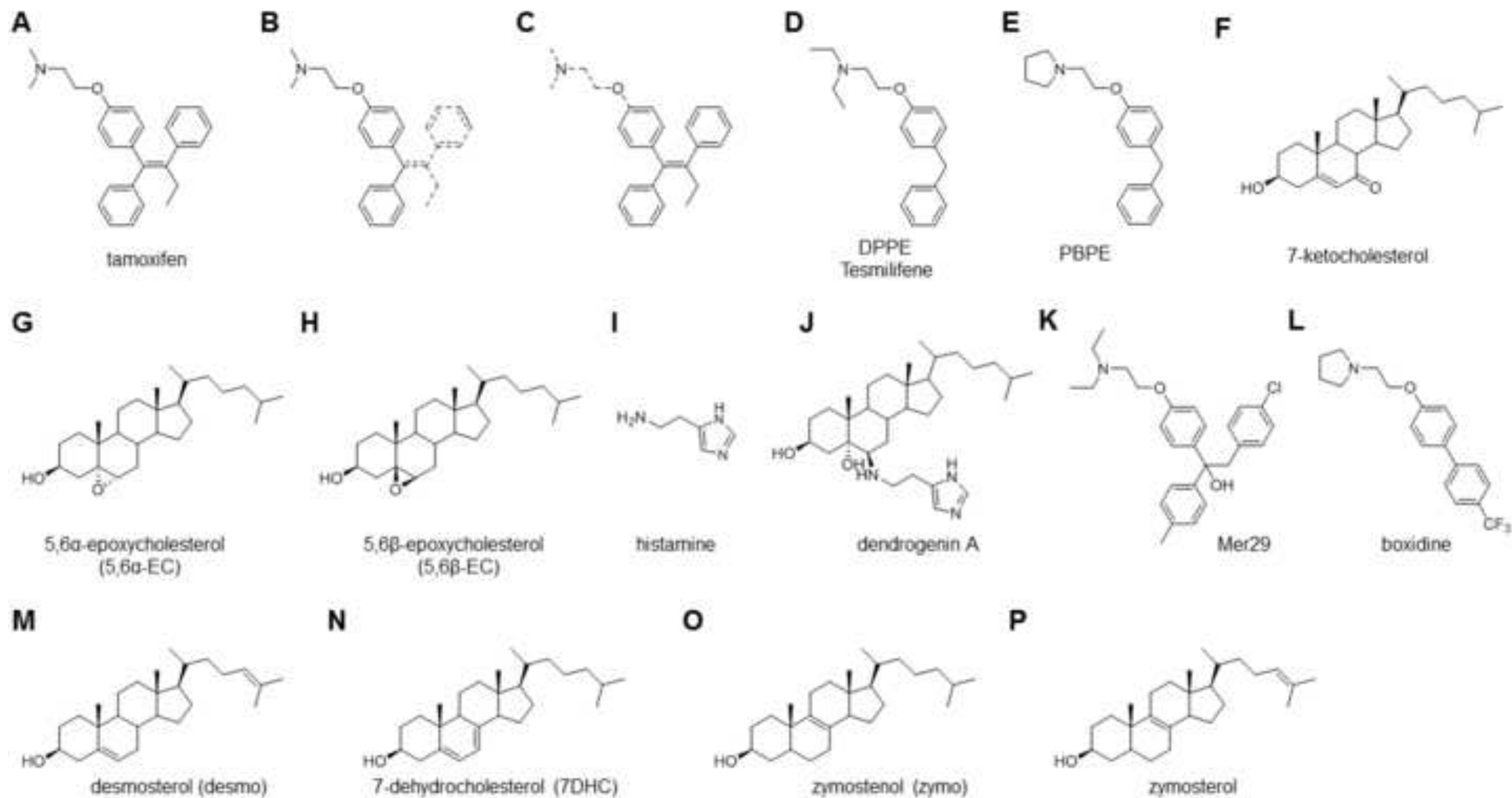

Figure 2R1

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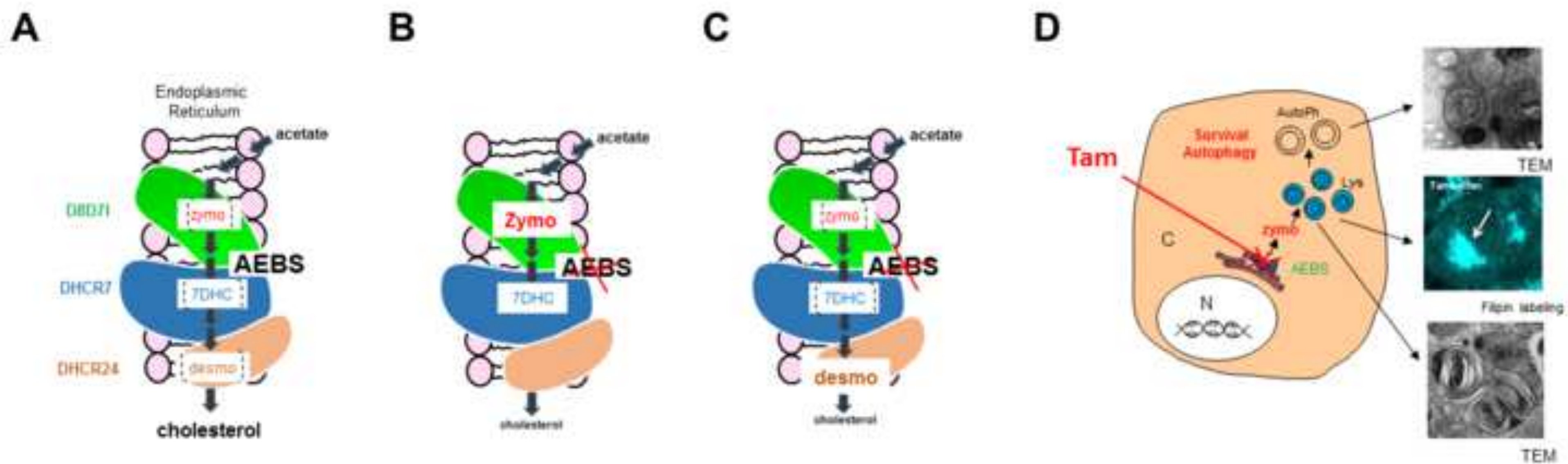

Figure 3R1

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Figure 4R1

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A MCF-7 (ER+, AEBS+, LXRβ+, SULT2B1b+)

B MCF-7/LXRβ- (ER+, AEBS+, LXRβ-, SULT2B1b+)

C MCF-7/SULT2B1b- (ER+, AEBS+, LXRβ+, SULT2B1b-)

D MDA-MB-231 (ER-,AEBS+, LXRβ+, SULT2B1b-)

E MDA-MB-231/SULT2B1b+ (ER-,AEBS+, LXRβ+, SULT2B1b+)

*Graphical Abstract (for review)
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