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5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of Tamoxifen in breast cancer cells☆

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ABSTRACT

Tamoxifen (Tam) is a selective estrogen receptor modulator (SERM) that remains one of the major drugs used in the hormonotherapy of breast cancer (BC). In addition to its SERM activity, we recently showed that the oxidative metabolism of cholesterol plays a role in its anticancer pharmacology. We established that these effects were not regulated by the ER but by the microsomal antiestrogen binding site/cholesterol-5,6-epoxide hydrolase complex (AEBS/ChEH). The present study aimed to identify the oxysterols that are produced under Tam treatment and to define their mechanisms of action. Tam and PBPE (a selective AEBS/ChEH ligand) stimulated the production and the accumulation of 5,6α-epoxy-cholesterol (5,6α-EC), 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS), 5,6β-epoxy-cholesterol (5,6β-EC) in MCF-7 cells through a ROS-dependent mechanism, by inhibiting ChEH and inducing sulfation of 5,6α-EC by SULT2B1b. We showed that only 5,6α-EC was responsible for the induction of triacylglycerol (TAG) biosynthesis by Tam and PBPE, through the modulation of the oxysterol receptor LXRβ. The cytotoxicity mediated by Tam and PBPE was triggered by 5,6β-EC through an LXRβ-independent route and by 5,6-ECS through an LXRβ-dependent mechanism. The importance of SULT2B1b was confirmed by its ectopic expression in the SULT2B1b(-) MDA-MB-231 cells, which became sensitive to 5,6α-EC, Tam or PBPE at a comparable level to MCF-7 cells. This study established that 5,6-EC metabolites contribute to the anticancer pharmacology of Tam and highlights a novel signaling pathway that points to a rationale for re-sensitizing BC cells to Tam and AEBS/ChEH ligands.

☆ This work is part of the PhD thesis of GS.

Abbreviations: AEBS, antiestrogen binding site; ChEH, cholesterol epoxide hydrolase; Tam, tamoxifen, *trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethylamine; 4OH-tam, 4-[(Z)-1-[4-(2-dimethylaminoethyloxy)phenyl]-2-phenylbut-1-enyl]phenol; Ralox, raloxifene, [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone; Clom, clomiphene, 2-[4-[(Z)-2-chloro-1,2-di(phenyl)ethenyl]phenoxy]-N,N-diethylethanamine; BZA, bazedoxifene1-[[4-[2-(azepan-1-yl)ethoxy]phenyl]methyl]-2-(4-hydroxyphenyl)-3-methylindol-5-ol; Tesm, tesmilifene (DPPE), N,N′-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl; PBPE, 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl; PCPE, 1-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)pyrrolidine; MBPE, 4-(2-(4-benzylphenoxy)ethyl)morpholine; MCPE, 4-(2-(4-(2-phenylpropan-2-yl)phenoxy)ethyl)morpholine; BD-1008, N-(3,4-dichlorophenethyl)-N-methyl-2-(pyrrolidin-1-yl)ethanemine; SR-31747A, (E)-N-(4-(3-chloro-4-cyclohexylphenyl) but-3-enyl)-N-ethylcyclohexanamine; FPT, flupenthixol, 2-[4-[(3Z)-3-[2-(trifluoromethyl)thio xanthen-9-ylidene]propyl]piperazin-1-yl]ethanol; CLP, chlorpromazine, 3-(2-chlorophenothiazin-10-yl)-N,N-dimethylpropan-1-amine; TFP, trifluoroperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)phenothiazine; U-18666A, 3-beta-(2-(diethylamino)ethoxy)androst-5-en-17-one; AY-9944, *trans*-1,4-Bis(2-chlorobenzaminomethyl)cyclohexane; Triparanol, 2-(4-chlorophenyl)-1-(4-(2-(diethylamino)ethoxy)phenyl)-1-p-tolylethanol; D8D7I, 3β-hydroxysterol-Δ^8-Δ^7-isomerase; DHCR7, 3β-hydroxysterol-Δ^7-reductase; 7-KC, 7-ketocholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 4β-HC, 4β-hydroxycholesterol; 5,6-EC, 5,6-epoxy-cholesterol; CT, cholestane-3β,5α,6β-triol; 5,6-αEC, 5,6α-epoxy-5α-cholestestan-3β-ol; 5,6β-EC, 5,6β-epoxy-5β-cholestestan-3β-ol; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; 5,6-ECS, 5,6α-epoxy-5α-cholestestan-3β-sulfate; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LXRα, Liver-X-Receptor alpha; LXRβ, Liver-X-Receptor beta; SULT2B1, Steroid sulfotransferase 2B1; SCD1, Stearoyl-CoA desaturase 1; ACC, Acetyl-CoA carboxylase; siRNA, small interfering RNA; siSC, siRNA scrambled.

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1. Introduction

Breast cancer (BC) is the most common cancer in women affecting more than 1 million women world-wide and with about 400,000 deaths due to this disease every year [1]. Tamoxifen (Tam) is one of the major drugs used as an adjuvant treatment to prevent BC recurrence and as a therapy to extend the lives of patients with metastatic disease [2]. Tam is a selective estrogen receptor modulator (SERM) that can compete with 17β-estradiol (E2) at the estrogen receptor (ER) on ER positive breast cancers and block its mitogenic action. This mechanism constitutes the rational for its clinical use [3]. It is now emerging that Tam displays a complex pharmacology and may exert additional ER-independent mechanisms [4]. Cholesterol metabolism is reportedly involved in breast cancer development in mice [5–7] and in resistance to Tam in patients [8,9]. At the molecular level, Tam has been shown to modulate cholesterol metabolism through its interaction with the microsomal antiestrogen binding site (AEBS) [4]. The AEBS binds selective estrogen modulators (SERMs) such as Tam, 4-hydroxytamoxifen, raloxifene and clomiphene [10]. Diphenylmethane (DPM) compounds such as 1-(2-(4-benzylphenoxy)ethyl)pyrrolidin-HCl (PBPE) and N,N'-diethylamino-4-(phenylmethylphenoxy)ethanamine-HCl (tesmilifene) have been developed to selectively bind to the AEBS/ChEH complex [11–14]. DPM were used for the molecular characterization the AEBS/ChEH complex [15–21] and the definition of its functional role in the pharmacology of its cognate ligands [12–14,16,22–27]. Despite a lack of a clear understanding of its mechanism of action at that time, tesmilifene was evaluated positively for the treatment of breast and prostate cancer in phase II and II clinical trials [28–31]. However, a pivotal phase III clinical trial was aborted because of the lack of a therapeutic outcome [32]. It is clear that a better understanding of its mechanism of action would have warranted a better selection of patients and an improved clinical response to tesmilifene.

We established that the AEBS is a hetero-oligomeric complex consisting of 3β-hydroxysteroid-Δ^8-Δ^7-isomerase (D8D7I, EBP) and 3β-hydroxysteroid-Δ^7-reductase (DHCR7) [16] with both enzymes being involved in the post-lanosterol cholesterol biosynthesis pathway. In addition, we showed that the AEBS carried out cholesterol-5,6-epoxide hydrolase (ChEH) activity [15]. ChEH catalyzes the *trans*-hydration of 5,6α-epoxy-cholesterol (5,6α-EC) and 5,6β-epoxy-cholesterol (5,6β-EC) into cholestane-3β,5α,6β-triol (CT) [33]. We showed that the interaction of the AEBS/ChEH complex with its cognate ligands induced: (1) the intracellular accumulation of free cholesterol precursors due to a non-competitive inhibition of cholesterogenic enzymes that are involved in the AEBS [8,16]; (2) the competitive inhibition of ChEH that could lead to the accumulation of 5,6-EC [15,33]. We established that free sterols accumulated in cells in multilamellar bodies (MLB) [23,24] and were responsible for the induction of a survival autophagy [8,10,23,34,35].

We previously showed that SERMs and other AEBS/ChEH ligands induced BC cell differentiation and cytotoxicity in a concentration- and time-dependent manner [12,23–25]. We found that these effects occurred independently of the ER through the modulation of the oxidative metabolism of cholesterol, and these effects were inhibited by the antioxidant vitamin E (Vit E, alpha-tocopherol) [10,23,24,34]. We found that the exposure of MCF-7 cells to 1–5 μM Tam or 10–20 μM PBPE for 3 days led to the appearance of BC cell characteristics of differentiation with no cytotoxicity [24], while 10 μM Tam and 40 μM PBPE triggered cell death [23,24]. Cytotoxicity required the expression of new genes and new proteins in BC cells establishing that transcription factors were involved in this process [23].

ROS include different reactive oxygen species, and some of them are known to produce different oxysterols [36]. NAD(P)H oxidase (NOX) is a ROS producing enzyme that induces the production of superoxide anion ($O_2^{\bullet -}$) which can be transformed by superoxide dismutase into H_2O_2 [37]. H_2O_2 produces 5,6-ECs as major cholesterol oxidation products [38]. We reported earlier that Tam and PBPE stimulated ROS production in MCF-7 cells [23] and other groups have reported that Tam induced ROS production in different cell lines including BC cells such as MCF-7 and MDA-MB-231 cells [39–42]. Consistent with these data, it was recently reported that the induction of TAG by Tam in MCF7 was inhibited by catalase [40], the enzyme that destroys H_2O_2, suggesting the formation of unknown endogenous mediator. We postulated that the stimulation of ROS by Tam can induce the production of 5,6-ECs. One of the major characteristics of BC cell differentiation induced by AEBS ligands is the stimulation of triacylglycerol (TAG) biosynthesis [23,24,40,43]. TAG biosynthesis is known to be under control of the oxysterol receptors Liver-X-Receptors (LXR) [44], and LXR were shown to be modulated by 5,6α-EC [45], suggesting that LXR could contribute to the oxysterol-dependent activity of Tam. The impact of Tam and AEBS/ChEH ligands on 5,6-EC metabolism in BC cells remains to be studied. 5,6-EC are known to be present in low amounts in mammals [36] and their presence in BC cells has never been studied. We designed the present study to identify the cholesterol autoxidation species that are produced under Tam- and AEBS ligand-treatment and to determine the molecular pathways involved in the induction of TAG biosynthesis by these oxysterols and Tam and PBPE and cytotoxicity in BC cells.

2. Materials and methods

2.1. Chemicals and reagents

Raloxifene, RU-39411 and SR-31747A were from Sanofi-Aventis. Triparanol was from Dr C Wolf (University Paris 06, France). BD-1008 was kindly given by Pr W D Bowen (Brown University, RI, USA). Bazedoxifene was synthesized as previously described [46] as were other compounds [11,15]. 5,6α-EC, 5,6β-EC, d7-5,6α-EC, d7-5,6β-EC, [^{14}C]-5,6α-EC and [^{14}C]-5,6β-EC were synthesized as described previously [15,47]. Other deuterated oxysterols were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2. Detection and quantification of 5,6-EC

Cells were grown to 70% confluence, and then pre-treated for 30 min with the appropriate amount of drug or solvent vehicle in the presence or absence of 500 μM Vit E. After a 48 h incubation, the cells were washed and scraped in cold PBS and the neutral lipids were extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1 v/v) as reported previously [48]. 10^8 cells were used for analysis. The organic phase was evaporated to dryness under an argon stream and the residues dissolved in 50 μl of ethanol. 100 pmol of deuterated oxysterols as internal standards (IS) were added under argon flux, the samples saponified with KOH 1 N for 1 h at 55 °C, and oxysterols extracted with chloroform/methanol/8.8% aqueous KCl (2:1:1, v/v/v). Oxysterol purification was accomplished using a 100 mg Sep-Pak Silica Vac RC Cartridge equilibrated with hexane. Samples were applied to the silica cartridge, washed with hexane (5 ml), 12% methylterbutyl ether (MTBE) in hexane (5 ml), 23% MTBE in hexane (7 ml), 40% MTBE in acetone (5 ml) and the oxysterol fraction was eluted with 5 × 2 ml of MeOH. Under these conditions of preparation, no 5,6-EC were formed as artifacts. We measured a 98% yield in 5,6-EC recovery using [^{14}C]-5,6-EC without cholesterol and vit E contaminations.