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ORIGINAL ARTICLE

Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake

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Abstract
Purpose Modulation of estrogen receptor (ER) plays a central role in selective estrogen receptor modulators (SERMs) molecular mechanism of action, although studies have indicated that additional, non-ER-mediated mechanisms exist. It has been suggested that the induction of oxidative stress by SERM could be one of the non-ER-mediated mechanisms held responsible for their pro-apoptotic role in ER-negative cells. Tumor cells are known for their high requirement of glutamine (Gln) that serves multiple functions within the cells, including nutritional and energy source, as well as one of the precursors for the synthesis of natural antioxidant glutathione (GSH). We hypothesized that one of the mechanisms responsible for ER-independent anti-neoplastic properties of SERMs and also for their adverse side effects could be dependent on the inhibition of Gln uptake.
Methods Human ER-negative MDA-MB231 breast cancer cells were treated with different doses of Tam and Ral. Gln uptake was monitored by using [^{3}H]Gln assay. The effect of Tam and Ral on Gln transporter ASCT2 expression, glutathione (GSH) levels and cellular proliferation was determined.
Results Tam and Ral inhibited Gln uptake in a dose-dependent manner through inhibition of ASCT2 Gln transporter. This effect of the anti-estrogens was associated with inhibition of GSH production and apoptosis. Treatment of cells with *N*-acetyl *L*-cysteine and 17 beta-estradiol 2 reversed the effects of Ral and Tam.
Conclusions Our results indicate that one of the mechanisms of action (and possibly some of the side effects) of TAM and RAL is associated with inhibition of cellular Gln uptake, oxidative stress and induction of apoptosis.

Keywords Tamoxifen · Raloxifene · ER-negative cancer cells · Glutamine · Glutamine transporter · Glutathione

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Introduction

The efficacy of the selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RAL) has been attributed to the induction of tumor cell growth arrest and apoptosis by inhibition of estrogen receptor (ER) signaling [1, 2]. Although SERMs have widespread clinical use, it is clear that not all of their effects can be attributed to the competitive interaction with the estrogen receptor. Experimental studies with TAM found growth inhibition and apoptosis in ER(-) cell lines [3, 4]. Responses to Tam have been observed in cancers not derived from estrogen-sensitive tissues, such as glioma [5, 6], melanoma[7], pancreatic cancer [8] and other malignancies [9]. Tam therapy has also been used for treatment of a number of other diseases such as osteoporosis [10, 11], atherosclerosis [12, 13], rheumatoid arthritis and other autoimmune diseases [14]. Tam has been shown to be effective in the treatment of mania in patients with bipolar disorder [15]. The cytotoxic effect of SERMs is believed to be due to a combination of genomic (ER-mediated) and non-genomic (non-ER-mediated or

other signaling pathways) mechanisms. These include modulation of signaling proteins such as protein kinase C (PKC), calmodulin, transforming growth factor-β (TGF-β), caspases, mitogen-activated protein kinases (MAPK) and the protooncogene c-myc [reviewed in 16]. Oxidative stress, mitochondrial permeability transition (MPT) and ceramide generation also play important roles in TAM-induced apoptosis [15].

Glutamine (Gln) is an essential nutrient for cell growth and viability [17]. In general the functions of Gln within the cell include its roles in nitrogen transport, maintaining cellular redox state, as a metabolic intermediate and as an energy source [18]. Gln via glutamate, together with glycine and cysteine is a precursor for the synthesis of glutathione (GSH), the major endogenous antioxidant in mammalian cells, which protects them from oxidative injury [19]. Gln is utilized directly for protein synthesis and serves as a precursor in the synthesis of other amino acids [20]. Some tumor cells have an absolute requirement for Gln as a growth substrate, a precursor for both DNA- and protein synthesis, as well as a respiratory substrate [21–23]. DeBerardinis et al. [24] reported that glioma cells can exhibit Gln uptake and metabolism that exceeds the cell's use of Gln for protein and nucleotide biosynthesis. This high rate of glutaminolysis was found to be beneficial because it provided the cell a high rate of NADPH production that was used to fuel lipid and nucleotide biosynthesis. In cancer patients, some tumors have been reported to consume such an abundance of Gln that they depress plasma Gln levels [25, 26].

Gln transport across cell membrane is mediated by several transport systems [27], the predominant one for human cancer cells being ASCT2 [28]. Studies have found that Gln availability regulated the expression of ASCT2 transporter in hepatoma cells [29]. The aim of the present work was to identify an ER-independent pathway that could explain, at least in part, the antineoplastic activity of TAM reported in ER-negative cells and ER-poor tumors. We hypothesized that one of the mechanisms responsible for ER-independent anti-neoplastic properties of SERMs and also for their adverse side effects could be dependent on the inhibition of Gln uptake.

Materials and methods

Cell cultures, chemicals and treatment

Human ER-negative breast cancer cell line MDA-MB-231 at passage 26 was obtained from the ATCC (Manassas, VA) and was maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin (P/S), and 2 mM glutamine. Tamoxifen citrate, raloxifene hydrochloride, 17β-estradiol 2 (E_2), sodium selenite (Se), retinyl hydrochloride (Vit A), α-tocoferol (Vit E) and *N*-acetyl-cysteine (NAC) were purchased from Sigma–Aldrich (St Louis, MO). Rabbit anti-rat ASCT2 antibody was from Chemicon. International (Temecula, CA) and anti-rabbit IgG were from Santa Cruz Biotech (Santa Cruz, CA). Radiolabeled [3H]Gln was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell proliferation/viability assay

Cells (1×10^4 cells/well) were plated in quadruplicate in a 96-well plate in 100 µl complete medium and after attachment for 24 h were cultured overnight in serum-free medium, followed by treatment with increasing concentrations of Tam or Ral (5–25 µM) for 48 h at 37°C under 5% carbon dioxide (CO_2)/95% air in a high humidity atmosphere. The effect of NAC, E2, Vit E, Vit A and selenium was determined as follows: cells were plated in 96 well plate in triplicate (1×10^4 cells/well) and incubated 24 h at 37°C and 5% CO2 in complete medium. The medium was replaced with serum-free DMEM with each of NAC (5 mM), E2 (10 nM), Vit E (100 µM), Vit A (1 µM) and selenium (30 nM) and incubated for 1 h before challenged with Ral (25 µM) or Tam (µM) for 48 h. Proliferation was assayed using a modified MTS-tetrazolium (MTS) assay with CellTiter 96 Aqueous reagent (Promega, Madison, WI) and measurement of absorbance at 490 nm, according to the manufacturer's protocol. Inhibition of proliferation was calculated as percentage of the control cultures that were not treated with Tam or Ral. All experiments were repeated at least three times. A paired *t* test with $P < 0.05$ was used to establish statistically significant differences between treatment and control.

Glutathione measurement

Cells (1×10^4 cells/well) were attached in quadruplicate in a 96-well plate in 100 µl complete medium and cultured overnight in serum-free medium before the treatment with increasing concentrations of Tam or Ral (5–25 µM) for 48 h at 37°C under 5% carbon dioxide (CO_2). The effect of NAC, E2, Vit E, Vit A and selenium was determined by preincubation the cells for 1 h with NAC (5 mM), E2 (10 nM), Vit E (100 µM), Vit A (1 µM) and selenium (30 nM), followed by Ral (25 µM) or Tam (µM) for 48 h. The amount of total GSH released in the medium was estimated by measuring the absorbance at 405 nm (GSH assay kit; Cayman Chemical, Ann Arbor, MI) using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Pure oxidized GSH (GSSG) was used to obtain a standard curve. The results were expressed as nmol/mg protein.

Glutamine uptake assay

Cells were plated in 12-well plates (1×10^5/well) and after attachment for 24 h the medium was replaced with fresh Gln-free medium and incubated overnight. The next day, the medium was replaced with medium containing 2 mM Gln, 5 μCi [3H]Gln and various concentrations of Ral and Tam (5–25 μM) and the cells were incubated at 37°C, 5% CO_2 for 45 min. The Gln uptake was terminated by three washes with cold PBS and drying at room temperature for 30 min. The cells were dissolved with 1 ml/well buffer containing 0.2N NaOH and 0.2% SDS, followed by the addition of 100 μl/well 2N HCl. The protein was measured in the lysate, and the radioactivity was measured in 5 ml scintillation cocktail with LKB Wallac 1219 liquid scintillation counter. The results are expressed as cpm/mg protein.

DNA fragmentation assay

After treatment, the cells were harvested by trypsinization and centrifuged at 1,000*g* for 5 min, washed once with PBS and the cell pellet was resuspended in a lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100. Cell lysate was left on ice for 30 min. DNA was extracted by adding an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (pH 8.0) and precipitated with 0.1 volume of 5 M sodium chloride and 2 volumes of 100% ethanol at −20°C overnight. The DNA sample was dissolved in TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) and treated with 1 mg/ml RNase at 37°C for 2 h. DNA fragments were resolved by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Western blotting

Cells (5×10^6 in 100 mm plates) were treated as indicated, harvested and sonicated in cold cell lysis buffer (Cell Signaling Tech, Danvers, MA). Protein concentration was measured using Bio-Rad protein assay (Richmond, VA). Fifty microgram of the total cytosolic protein were resolved in 10% SDS–PAGE and transferred to nitrocellulose membrane (Millipore). The membrane was blocked with 5% non-fat dry milk in TBS for 1 h at room temperature and then incubated with anti-ASCT2 antibody (1:800 in TBS-T containing 5% milk) overnight at 4°C. After washing the blots were incubated for 2 h at 4°C in ant-rabbit IgG (1:2000). The protein was visualized using enhanced chemiluminescence system (Amersham). The equal loadings were controlled by staining with Ponso S and reprobing the membranes with tubulin.

Statistical analysis

Comparisons between the groups were performed by a one-way analysis of ANOVA using statistical software StatView for Windows. All data was expressed as mean ± SE. Results with $P < 0.05$ were considered statistically significant.

Results

Cell proliferation/apoptosis and GSH synthesis

Treatment with Tam and Ral induced a dose-dependent reduction in cell survival (Fig. 1a) and down-regulation of GSH synthesis (Fig. 1b) of human ER-negative MDA-MB231 breast cancer cells. The most prominent effect of both Tam and Ral was found at a concentration of

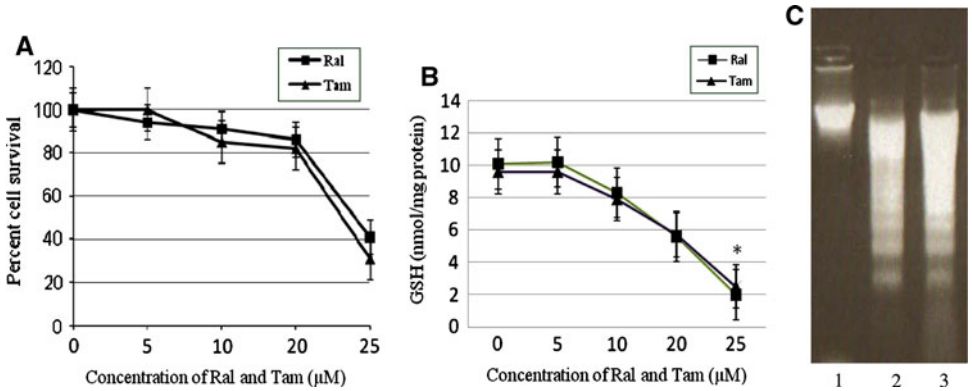
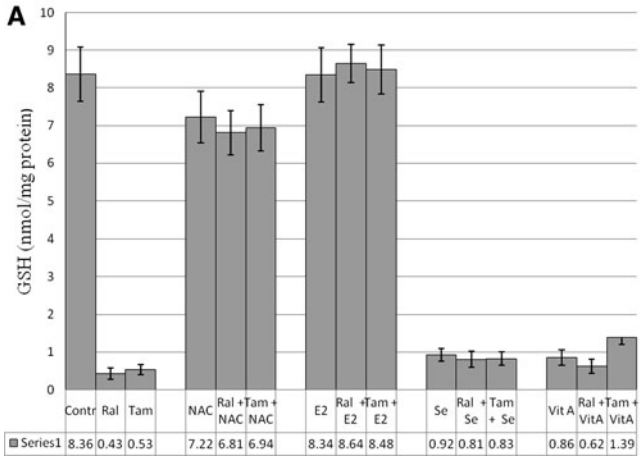


Fig. 1 Dose-dependent inhibition of cell proliferation (**a**) and GSH synthesis (**b**) and induction of apoptosis (**c**) in MDA-MB 231 cells by Ral and Tam. Cells were treated with various concentrations (5–25 μM) of Tam or Ral for 48 h as described in "Materials and methods". Cell survival was determined using MTS assay (**a**) and GSH released in the medium was determined using GSH assay (**b**). After treatment with Tam and Ral (25 μM) for 48 h, the cells were harvested, DNA was extracted and analyzed by agarose gel electrophoresis (**c**). C1, untreated control; C2, cells treated with 25 μM Tam; C3, cells treated with 25 μM Ral. Three independent experiments showed similar results. Data are mean ± SE. $P < 0.05$



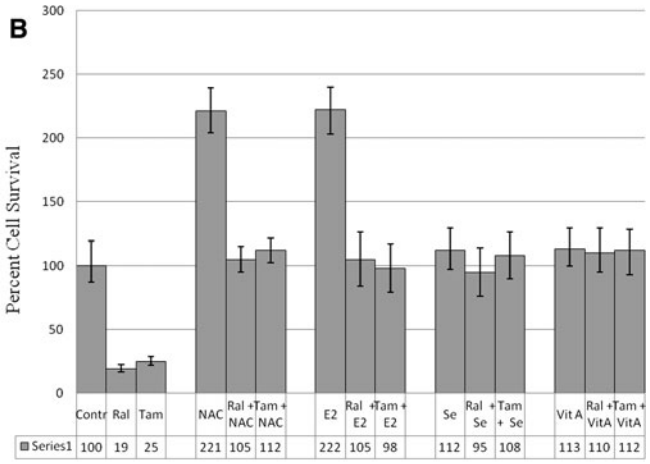


Fig. 2 The down-regulation of GSH (**a**) and suppression of cellular proliferation (**b**) of MDA-MB 231 cells by 25 μM Ral or Tam was reversed by pretreatment with NAC and E2. Cells were pretreated for 1 h with the indicated antioxidants and challenged with Ral or Tam for 48 h. Cell survival was determined by using MTS assay and GSH by a specific assay. $P < 0.05$

25 μM with a decrease in the number of live cells by more than 60%. This concentration of both Tam- and Ral (25 μM)-induced DNA fragmentation (Fig. 1c) suggestive for the occurrence of apoptosis.

Tam- and Ral-induced GSH depletion and inhibition of cell proliferation are reversed by NAC and E2

Treatment with 25 μM Tam or Ral for 48 h resulted in a significant GSH depletion (Fig. 2a) that was reversed by a pretreatment with NAC or E2, but not with Vit E, Vit A or selenium. The same effect of these antioxidants was found also on cell proliferation (Fig. 2b).

Gln uptake

In order to assess the effect of Ral and Tam (concentrations range 5–25 μM) on the uptake of Gln, MDA-MB 231 cells were incubated for 45 min in a medium containing 5 μCi [3H]Gln. The results showed that 5 μM of both Ral and Tam caused a sharp decrease in Gln uptake that continued to decrease with the increase in Ral and Tam concentration (Fig. 3.)

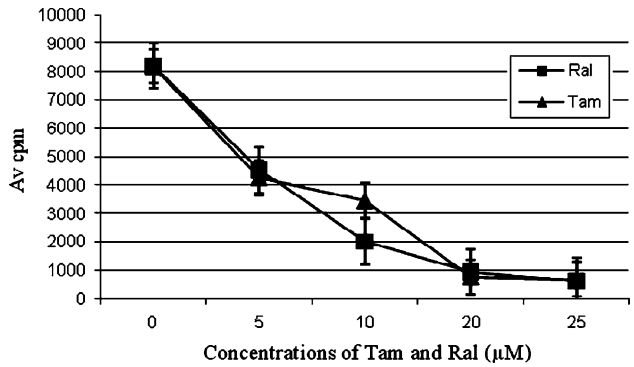


Fig. 3 Tam and Ral inhibit Gln-uptake. Cells were incubated for 45 min in a medium containing 2 mM Gln, 5 μCi [3H]Gln and various concentrations of Ral and Tam (5–25 μM). Gln uptake was assessed by measuring the radioactivity with liquid scintillation counter. $P < 0.05$

Protein expression of Gln transporter ASCT2

In order to clarify the biochemical mechanism underlying Tam- and Ral-induced inhibition of Gln uptake, we examined the protein expression of Gln transporter ASCT2. MDA-MB 231 cells were treated with Ral and Tam (25 μM), and 50 μg of the total cytosolic protein of untreated cells and cells treated with Ral and Tam were examined for the protein expression of ASCT2. The results showed that both Ral and Tam down-regulated the protein expression of ASCT2 (Fig. 4).

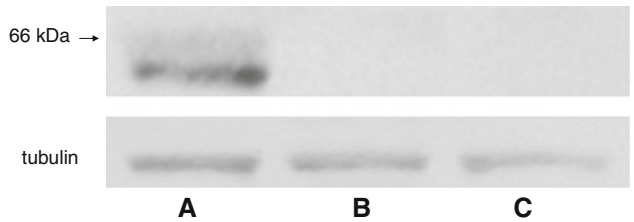


Fig. 4 Ral and Tam inhibited protein expression of Gln transporter ASCT2. The presence of ASCT2 protein was determined by western blot analysis. The equal loadings were controlled by staining with Ponso S and reprobing the membranes with tubulin. **a** Untreated cells; **b** cells treated with 25 μM Ral; **c** cells treated with 25 μM Tam

Discussion

Several large clinical trials have demonstrated the efficacy of the SERM, specifically of Tam in the adjuvant therapy of breast cancer. Although the effect of TAM in terms of

recurrence-free survival was detected in ER-dependent tumors, a significant favorable effect was detected also in ER-poor cancers [30]. The present study has shown that both anti-estrogens Tam and Ral down-regulated GSH production and induced apoptosis in ER-negative breast cancer cells grown under stressed (serum free) conditions. These effects of Tam and Ral were associated with inhibition of Gln uptake in a dose-dependent manner through an inhibition of ASCT2 Gln transporter. Treatment of cells with NAC and E2 reversed the effects of Ral and Tam.

Several in vitro properties of SERMs were suggested to be responsible for their pro-apoptotic properties in ER-negative cells including calcium channel–blocking activity, inhibition of protein kinase C [31], up-regulation of c-*myc* expression [32], ceramide generation [33], activation of caspases and JNK and p38 mitogen-activated protein kinases (MAPK) [34]. It has been suggested that Tam exerts a direct regulatory effect on the transcription of TGF-β. Studies showed that prolonged incubation of breast cancer cells with Tam resulted in accumulation of TGF-β mRNA and protein, as well as increased biological activity of TGF-β secreted in the cell culture media [35, 36]. The addition of an anti-TGF-β antibody inhibited the induction of apoptosis by TAM. Brunner et al. [37] reported that treatment with Tam significantly reduced the concentration of insulin-like growth factor II (IGF-II) in T61 human breast cancer xenografts. Reactive oxygen species (ROS) have been demonstrated to play an essential role in apoptosis induced by TAM in ER-negative human cancer cells [38, 39]. For example, Ferlini et al. [4] showed that Tam-induced apoptosis in ER-negative models resulted from ROS generation and thiol depletion in a dose-dependent manner. Pretreatment of ER-negative breast cancer cells with the antioxidant Vit E abrogated JNK activation in TAM-treated cells, suggesting the significant role of the oxidative stress in TAM-induced cellular apoptosis [24]. In fact, several studies showed that the oxidative stress induced by SERM is one of the ER-independent mechanisms of action of these drugs [40–42]. The major anti-oxidant defense system of the cell, GSH is a tripeptide (γ-glutamyl-cysteinyl-glycin) synthesized in a two-step reaction of combination of glutamate and cysteine, followed by the addition of glycine [43, 44]. It is generally accepted that the availability of cysteine [45] and Gln [46] (as a precursor of glutamate) controls the rate of this reaction. Gln supports the intracellular pool of glutamate, avoiding its depletion and the depletion of GSH. In vitro and in vivo studies have shown that NAC is chemically similar to cysteine and acts as a cysteine prodrug and a GSH precursor [47].

The results from this study showed that Ral and Tam inhibition of GSH synthesis of MDA-MB 231 cells was reversed by the addition of NAC (but not by other antioxidants), suggesting that GSH down-regulation might depend on Gln availability. The examination of the effect of Ral and Tam on Gln uptake showed a significant inhibition of Gln uptake. Studies have found that cancer cells displayed enhanced and altered channeling of amino acids into select metabolic pathways, often in concert with the aerobic glycolysis characteristic of tumors [48, 49]. Solid tumors are often poorly vascularized, so they must have efficient mechanisms for extracting plasma amino acids in order to compete with the host tissues [50]. As a result, cancer cell amino acid transporters with properties that impart growth and survival advantages are selected for and expressed at higher levels compared to the parent tissue [19]. Amino acid transport across the plasma membrane in mammalian cells is mediated by different transport systems, such as Na+-dependent systems A, ASC, Na+-independent system *L* and *N* system [51]. ASCT2 transporter has a high affinity for small neutral amino acids, such as glutamine which is avidly consumed by tumors. Current evidence suggests that ASCT2 is highly expressed in a variety of cancerous tissues, such as breast [52], colon, liver and other cancers; and therefore, it has been suggested to play an important role in the carcinogenesis [19, 37]. The importance of ASCT2 transporter in hepatoma cell proliferation has been demonstrated by Fuchs et al. [53] who showed that attenuation of ASCT2 expression by inducible antisense RNA in a number of human hepatoma cell lines led to cell death via apoptosis. Bungard and McGivan [20] found that both the expression of the glutamine transporter ASCT2 and the activity of the ASCT2 promoter in the human hepatoma cell line HepG2 were dependent upon Gln availability. The results from the present study showed that Ral- and Tam-induced inhibition of Gln uptake by MDA-MB-231 breast cancer cells were associated and probably resulted from inhibition of ASCT2 protein expression.

In our experimental model, the inhibitory effects of Ral and Tam on GSH synthesis and proliferation of ER-negative cells were reversed also by 17β-estradiol, suggesting the presence of ER-independent mechanism of action of estrogens. Recent study by Wang et al. [54] showed that 17β-, 17α- and *ent*-E_2 protected human neuroblastoma SK-N-SH cells against H_2O_2-induced oxidative stress by inhibition of lipid peroxidation, alleviated intracellular calcium elevation, attenuated ATP depletion and subsequently enhanced cell survival. Furthermore, the ER antagonist ICI 182,780 did not block the effects of 17-E_2 but increased cell survival and blunted intracellular calcium increase induced by H_2O_2.

The results from this study indicate that one of the mechanisms of action (and possibly some of the side effects) of anti-estrogens TAM and RAL is associated with inhibition of cellular GLN uptake and subsequent oxidative stress and induction of apoptosis. These data contribute to the knowledge of mechanism of action of two of the most widely used in the clinical practice SERM.

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