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Inhibition of angiogenesis by selective estrogen receptor modulators through blockade of cholesterol trafficking rather than estrogen receptor antagonism

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Abstract

Selective estrogen receptor modulators (SERM) including tamoxifen are known to inhibit angiogenesis. However, the underlying mechanism, which is independent of their action on the estrogen receptor (ER), has remained largely unknown. In the present study, we found that tamoxifen and other SERM inhibited cholesterol trafficking in endothelial cells, causing a hyper-accumulation of cholesterol in late endosomes/lysosomes. Inhibition of cholesterol trafficking by tamoxifen was accompanied by abnormal subcellular distribution of vascular endothelial growth factor receptor-2 (VEGFR2) and inhibition of the terminal glycosylation of the receptor. Tamoxifen also caused perinuclear positioning of lysosomes, which in turn trapped the mammalian target of rapamycin (mTOR) in perinuclear region in endothelial cells. Abnormal distribution of VEGFR2 and mTOR and inhibition of VEGFR2 and mTOR activities by tamoxifen were significantly reversed by addition of cholesterol-cyclodextrin complex to the culture media of endothelial cells. Moreover, high concentrations of tamoxifen inhibited endothelial and breast cancer cell proliferation in a cholesterol-dependent, but ER-independent, manner. Together, these results unraveled a previously unrecognized mechanism of angiogenesis inhibition by tamoxifen and other SERM, implicating cholesterol trafficking as an attractive therapeutic target for cancer treatment.

Keywords

Angiogenesis; selective estrogen receptor modulator; tamoxifen; cholesterol trafficking

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Conflict of Interest

Authors have declared that no conflict of interest exists.

1. Introduction

Tamoxifen and selective estrogen receptor modulators (SERM) have been used to treat hormone responsive, estrogen receptor (ER)-positive breast cancers since the 1980s. It has generally been accepted that the anticancer activity of tamoxifen is mainly attributable to its competitive antagonism to ER, thereby inhibiting the proliferation of ER-positive breast cancer cells [1]. However, whether this is the only mechanism of action underlying the anticancer activity of SERM has been questioned since tamoxifen and other SERM also showed anticancer activity in ER-negative breast cancers [2-4]. Since the 1990s, several groups have found that tamoxifen and SERM strongly inhibited angiogenesis by mechanisms independent of ER [5-7]. Based on these findings, tamoxifen and other SERM are now being actively investigated as anti-angiogenic agents in clinical trials for cancer treatment [8-10]. However, the underlying molecular mechanism by which tamoxifen inhibits angiogenesis has remained largely unknown.

Cholesterol is an essential component of cellular membranes and plays a key role in membrane permeability and fluidity. In addition to a structural role, it also functions in intracellular transport and cell signaling [11, 12]. Serum cholesterol is delivered throughout the body in the form of low-density lipoprotein (LDL) and transported into cells through receptor-mediated endocytosis [13]. Endocytosed LDL is transported to the late endosomes and lysosomes (endolysosomes) where cholesteryl esters are hydrolyzed and free cholesterol is released from the endosomal system for delivery to other compartments, including the plasma membrane and endoplasmic reticulum [14]. One of the most important machineries of cholesterol trafficking in the endolysosomes is the Niemann-Pick, type C (NPC) proteins (NPC1 and NPC2), which help acid lipase-mediated hydrolysis of cholesteryl esters and deliver free cholesterol out of the endolysosomes [15]. Inhibition of NPC1 or 2 causes accumulation of cholesterol and glycolipids in the endolysosomes, a phenotype called NPC after the genetic disease of the same name [16].

We have previously reported that a newly identified anti-angiogenic drug itraconazole inhibited cholesterol trafficking and induced NPC-like phenotype in endothelial cells [17]. Inhibition of cholesterol trafficking by itraconazole is accompanied by inhibition of mTOR signaling and VEGFR2 glycosylation, both of which are essential signaling components for endothelial cell proliferation [17, 18]. Recently, Fang et al. showed that upon over-expression, apoA-I binding protein (AIBP), which is responsible for cholesterol efflux from endothelial cells, inhibited angiogenesis by depleting cholesterol from the plasma membrane, thereby inhibiting the VEGFR2 signaling pathway in endothelial cells and animal models [19]. Similar to AIBP over-expression, cells with NPC phenotype induced by small molecules showed accumulation of cholesterol in the endolysosomes leading to cholesterol depletion in plasma membrane and inhibition of VEGFR2 signaling pathway [17, 18]. These results strongly suggest that cholesterol trafficking in endothelial cells is critical for proper angiogenesis.

In the present study, we found that tamoxifen and other SERM inhibited cholesterol trafficking in endothelial cells. Blockade of cholesterol trafficking by SERM led to an

abnormal subcellular distribution of mTOR and VEGFR2 and caused inhibition of their signaling pathways in a cholesterol-dependent manner. These data suggest that tamoxifen and other SERM inhibit angiogenesis by interfering with cholesterol trafficking in endothelial cells and that cholesterol trafficking is a novel target for anti-angiogenesis therapy.

2. Materials and methods

Cells and reagents

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Allendale, NJ) and were grown in endothelial cell growth medium-2 (EGM-2) using the EGM-2 bullet kit (Lonza). MCF-7 (ER-positive) breast cancer cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY) and 1% antibiotics (penicillin and streptomycin) solution (Life Technologies). MDA-MB-231 (triple negative) breast cancer cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS (Life Technologies) and 1% antibiotics. All the cells were maintained in a humidified incubator at 37°C adjusted to 5% CO₂. Methyl- β -cyclodextrin, cholesterol and filipin were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human VEGF-165 was purchased from R&D systems (Minneapolis, MN).

Filipin staining

Filipin staining was performed as described with slight modifications [17]. HUVEC were cultured in a Nunc Lab-Tek II 8-Chamber Slide (Thermo Scientific, Rockford, IL) at 1×10^4 cells/well. Cells were treated with SERM with or without cholesterol and cyclodextrin complex for 24 h. Cells were then fixed with 4% paraformaldehyde for 20 min at room temperature and stained with filipin at a final concentration of 50 μ g/ml in the dark for 1 h at room temperature. Cells were washed with PBS, mounted with Immu-mount (Thermo Scientific), and observed under a Zeiss 510 Meta multiphoton confocal microscope (Carl Zeiss, Thornwood, NY).

Immunofluorescence imaging

For co-staining of proteins and cholesterol, HUVEC (1×10^4 cells/well) grown in a Nunc Lab-Tek II 8-Chamber Slide were treated with compounds for 24 h, fixed with 4% paraformaldehyde for 20 min at room temperature and stained with filipin (50 μ g/ml) for 1 h at room temperature. Cells were then permeabilized with 0.2% saponin supplemented with 50 μ g/ml filipin and 5% bovine serum albumin (BSA) in PBS for 30 min. Cells were incubated with primary antibodies in PBS together with 50 μ g/ml filipin, 0.05% saponin and 5% BSA overnight at 4°C. Cells were then incubated with secondary antibodies in PBS with 50 μ g/ml filipin, 0.05% saponin and 5% BSA at room temperature for 1 h. Cells were washed with PBS, mounted with Immu-mount, and observed under a Zeiss 510 Meta multiphoton confocal microscope. For general immunofluorescence, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 10 min and washed with PBS prior to blocking in 1% BSA in PBS containing 0.1% tween 20 (PBST) for 1 h. Cells were then incubated with primary antibodies including anti-VEGFR2 (Cell

Signaling Technology, Danvers, MA), anti-LAMP1 (SantaCruz Biotechnology, Santa Cruz, CA), anti-mTOR (Cell Signaling Technology) and anti-GM130 (BD Biosciences, San Jose, CA) in the blocking solution overnight at 4 °C, and then incubated with secondary antibodies conjugated with Alexa-Fluo488 or Alexa-Fluo594 for 1 h. The cellular nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) and actin cytoskeleton was stained with rhodamine-phalloidin (Life Technologies). The immunofluorescence images were obtained using the Zeiss 510 Meta multiphoton confocal microscope.

[³H]-thymidine DNA incorporation assay

HUVEC, MCF-7 or MDA-MB-231 were seeded at 3×10^3 cells/well in 96-well plates and allowed to adhere at 37 °C for 24 h. Cells were then treated with compounds for 24 h prior to being pulsed with 0.5 µCi [³H]-thymidine (PerkinElmer, Waltham, MA) for 16 h and then trypsinized. The cells were harvested onto FilterMat A glass fiber filters (Wallac, Turku, Finland) using a Harvester 96 cell harvester (Tomtec, Hamden, CT), and the radioactivity of [³H]-thymidine incorporated into DNA was counted using a MicroBeta liquid scintillation plate reader (Perkin Elmer). The IC₅₀ values and 95% confidence intervals were calculated using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

AlamarBlue cell viability assay

HUVEC, MCF-7 or MDA-MB-231 were seeded at 3×10^3 cells/well in 96-well plates and allowed to adhere at 37°C for 24 h. The cells were treated with compounds in the presence or absence of cholesterol or cyclodextrin, or both for 24 h. AlamarBlue reagent (Life Technologies) was added to the media at a final concentration of 10% and the incubation was continued for an additional 2 h. The fluorescence signal was read with an excitation wavelength of 570 nm and an emission wavelength of 590 nm using a SpectraMax M5 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Western blot analysis

HUVEC (2×10^5 cells/well) were seeded in 6-well plates and allowed to adhere overnight. Following drug treatment for 24 h, cells were lysed by adding 2× Laemmli buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125M Tris-HCl, pH 6.8 and the lysates were boiled for 10 min and vortexed. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% non-fat dried milk at room temperature for 1 h, incubated with the primary antibodies including anti-VEGFR2 (Cell Signaling Technology), anti-phospho-VEGFR2 (Tyr1175, Cell Signaling Technology), anti-FGFR1 (Cell Signaling Technology), anti-mTOR (Cell Signaling Technology), anti-phospho-mTOR (Ser2448, Cell Signaling Technology) anti-S6K (Cell Signaling Technology), anti-phospho-S6K (Thr389, Cell Signaling Technology), anti-PDGFRβ (Santa Cruz Biotechnologies), anti-actin (Santa Cruz Biotechnologies) or anti-α-tubulin (Santa Cruz Biotechnologies) antibodies overnight at 4°C and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The immune-complexes were detected using enhanced chemiluminescence (ECL) detection reagent (GE Healthcare, Pittsburgh, PA).

Endothelial cell tube formation assay

A 96-well plate was coated with 50 μ l Matrigel (BD Biosciences) and was incubated at 37 °C for 1 h to allow for polymerization. HUVEC were mixed with appropriate compounds and seeded (2×10^4 cells/well) on the Matrigel-coated wells, followed by incubation in a CO₂ incubator for 16 h. Cells were washed carefully with PBS once and Calcein-AM (2 μ M in PBS, Life Technologies) solution was added to the cells. After incubation at 37 °C for 30 minutes, the cells were washed gently with PBS and the fluorescence-labeled tubular structures were observed under a Nikon Eclipse TS100 fluorescence microscope with an excitation wavelength of 485 nm and an emission wavelength of 520 nm at magnification $\times 100$. The total tube lengths, sizes and number of junctions from the fluorescence images were quantified using the AngioQuant v1.33 software (The MathWorks, Natick, MA) and plotted using the GraphPad Prism 5.0 software.

Statistical analysis

Statistical significance of the data between control and test groups was determined by two-sided Student's *t*-test using the GraphPad Prism 5.0 software. The *P* values less than 0.05 were considered significant.

3. Results

Tamoxifen and SERM induce NPC-like phenotype in HUVEC

We tested four FDA-approved SERM including tamoxifen, toremifene, clomifene and raloxifene (Supplementary Fig. 1) for effects on cholesterol trafficking in HUVEC. Intracellular cholesterol was visualized by staining fixed cells with filipin [20]. All four SERM induced accumulation of cholesterol in the perinuclear region of HUVEC, a phenotype similar to NPC and that induced by itraconazole (Fig. 1A and Supplementary Fig. 2A). The cell morphology was also changed from large flat morphology into a partially shrunken form. These effects, however, were reversed upon addition of exogenous cholesterol and its carrier methyl- β -cyclodextrin (CD) (Fig. 1B and Supplementary Fig. 2B), suggesting that they are mediated largely through inhibition of cholesterol trafficking.

Tamoxifen traps VEGFR2 in Golgi and induces perinuclear positioning of lysosomes in HUVEC

We have previously reported that a cholesterol trafficking inhibitor, itraconazole, inhibited the VEGFR2 and mTOR signaling pathways, both of which have been shown to be critical for angiogenesis [17, 18]. To determine the effect of tamoxifen on VEGFR2 and mTOR signaling, we first analyzed the subcellular localization of those two proteins. Immunofluorescence labeling showed that VEGFR2 was localized largely in the plasma membrane and some in intracellular regions in control HUVEC (Fig. 2A). Treatment of cells with tamoxifen caused an accumulation of a significant amount of VEGFR2 in the perinuclear region, which colocalized with the Golgi marker GM130 (Fig. 2A and B). To determine if the abnormal subcellular distribution of VEGFR2 caused by tamoxifen is due to the inhibition of cholesterol trafficking, we added cholesterol/CD complex to the cell culture media. VEGFR2 mislocalization by tamoxifen was clearly reversed by the cholesterol/CD

complex (Fig. 2B). On the other hand, mTOR was mainly located in peripheral cytoplasm in control HUVEC and was well co-localized with the endolysosomal marker, LAMP1 (Fig. 2C). Tamoxifen treatment caused cholesterol accumulation in the perinuclear region overlapping with the LAMP1-positive endolysosomes. Though tamoxifen did not alter mTOR association with LAMP1-positive endolysosomes, the localization of both mTOR and lysosomes was confined to the perinuclear region in the tamoxifen-treated HUVEC, whereas both proteins were more evenly distributed in the cytoplasm in control HUVEC. Staining of the actin cytoskeleton clearly showed that mTOR localization was confined to the perinuclear region by tamoxifen treatment (Fig. 2D). This effect was reversed by cholesterol/CD complex (Fig. 2D). These results indicated that tamoxifen caused subcellular redistribution of VEGFR2 and mTOR in endothelial cells in a cholesterol-dependent manner.

Tamoxifen inhibits VEGFR2 glycosylation and mTOR activity in HUVEC

Proper subcellular localization of proteins is critical for their proper function. Like other cell surface proteins, VEGFR2 is highly glycosylated and is expressed on the cell surface upon completion of glycosylation. Protein glycosylation occurs co-translationally in the endoplasmic reticulum and subsequently in the Golgi by a series of glycosidases and glycosyltransferases located in each organelle [21]. Abnormal subcellular localization of glycoproteins will cause improper glycosylation and, therefore, affect their functions. As tamoxifen caused abnormal subcellular distribution of VEGFR2, we determined the effect of tamoxifen on the glycosylation pattern and the activity of VEGFR2. Control HUVEC showed two glycosylated forms of VEGFR2 (200 and 230 kD) (Fig. 3A). The 230 kD (mature terminal glycosylated form) protein band was dominant over the 200 kD (intermediate glycosylated form) band. Tunicamycin is a glycosyltransferase inhibitor that acts in an initial step of glycosylation in the endoplasmic reticulum. It completely inhibited VEGFR2 glycosylation, reducing the protein's mass to 180 kD as expected.

Deoxymannojirimycin (dMM), an inhibitor of α -mannosidases, and itraconazole are known to inhibit terminal glycosylation, thus shifting the VEGFR2 from 230 to 200 kD form. Similar to dMM and itraconazole, tamoxifen and other SERM caused a shift in apparent molecular mass of VEGFR2 from 230 to 200 kD, suggesting that they inhibited terminal glycosylation which mainly occurs in the Golgi. Inhibition of terminal glycosylation of VEGFR2 by SERM occurred in a concentration-dependent manner (Fig. 3B-D).

Subcellular localization of mTOR is also important for its activity. Recently, it was reported that nutrient starvation induced abnormal lysosomal positioning (increase in perinuclear positioning) [22]. This perinuclear lysosomal positioning was accompanied by mTOR Complex-1 (mTORC1) redistribution and inhibited the activity of mTOR. Tamoxifen and other SERM also caused perinuclear lysosomal positioning (Fig. 2C and D) and inhibited the phosphorylation of S6 kinase (S6K), a substrate of mTORC1, in HUVEC (Fig. 3B-D). To further validate the inhibitory effect of tamoxifen on mTORC1 activity, phosphorylation status of mTOR at Ser2448 was assessed. Like the direct mTOR inhibitor rapamycin, tamoxifen dose-dependently inhibited mTOR phosphorylation at Ser2448 in HUVEC (Fig. 4A). The phosphorylation of S6K was also inhibited by tamoxifen in parallel to the inhibition of mTOR phosphorylation.

We next examined glycosylation patterns of other receptor tyrosine kinases related to angiogenesis, including fibroblast growth factor receptor 1 (FGFR1) and platelet-derived growth factor receptor β (PDGFR β), to see if the tamoxifen effect was specific to VEGFR2. Similar to the effect observed on VEGFR2, tamoxifen caused a shift in the apparent molecular mass of PDGFR β and FGFR1 (Fig. 4B). Sorafenib, a kinase inhibitor, had no effect on the receptor molecular masses. However dMM caused a shift in the receptors' molecular masses in a manner similar to that of tamoxifen, suggesting that tamoxifen inhibited terminal glycosylation of the receptor tyrosine kinases. These results suggested that tamoxifen inhibits a common pathway in terminal glycosylation of receptor tyrosine kinases in endothelial cells.

Cholesterol reverses the inhibitory effects of tamoxifen on VEGFR2 and mTOR signaling in HUVEC

We next determined the effect of cholesterol on the inhibition of VEGFR2 glycosylation by tamoxifen in HUVEC. Inhibition of terminal glycosylation by either tamoxifen or toremifene was completely reversed by addition of cholesterol/CD complex (Fig. 5A and B). To see if the inhibition of terminal glycosylation by tamoxifen affected VEGFR2 activity/signaling, tyrosine phosphorylation status of VEGFR2 was assessed. Inhibition of terminal glycosylation of VEGFR2 by tamoxifen led to the inhibition of VEGFR2 phosphorylation which was completely reversed by cholesterol/CD complex (Fig. 5C). A VEGFR2 tyrosine kinase inhibitor sunitinib also inhibited VEGFR2 phosphorylation. But, this effect was not reversed by cholesterol/CD complex (Fig. 5C). These data suggested that the effect of tamoxifen on VEGFR2 was mediated through inhibition of cholesterol trafficking in endothelial cells. We further investigated the effect of cholesterol on inhibition of mTOR signaling by tamoxifen in HUVEC. Inhibition of S6K phosphorylation by either tamoxifen or toremifene was completely reversed by cholesterol/CD complex (Fig. 5D). CD alone could partially reverse tamoxifen activity. This was presumably due to the reversal effect of CD on NPC phenotype by releasing free cholesterol from endolysosomes [23]. Inhibition of mTOR signaling by tamoxifen and its reversal by cholesterol were further confirmed by examining 4E-BP1 phosphorylation, which was also decreased (Supplementary Fig. 3).

Cholesterol reverses the inhibitory effects of tamoxifen on HUVEC proliferation

To assess the relationship between VEGFR2 phosphorylation, mTOR activity and cell proliferation, we examined the effect of SERM on HUVEC proliferation. Half maximal inhibitory concentrations (IC₅₀) of tamoxifen, toremifene and raloxifene for HUVEC proliferation were determined to be 0.98, 1.2, and 1.42 μ M, respectively (Fig. 6A and Supplementary Table 1). We then determined if cholesterol could reverse the inhibition of HUVEC proliferation by SERM. Tamoxifen and toremifene strongly inhibited the growth of HUVEC at 2 and 4 μ M, respectively. The inhibitions were partially reversed by CD and were fully reversed by cholesterol/CD complex (Fig. 6B). Cholesterol alone has no reversal effect on the cell growth inhibition by tamoxifen or toremifene. These data corroborated the effects of SERM on VEGFR2 and mTOR (Fig. 5A-D).

Inhibition of cholesterol trafficking by tamoxifen is independent of ER

SERM are potent antagonists of ER, with K_d values ranging from picomolar to single-digit nanomolar concentrations, hence showing anti-proliferative effects on ER-positive breast cancer cells [24]. We thus tested whether SERM have different sensitivity on cell proliferation and cholesterol trafficking in cells with different ER expression statuses. In an ER-positive cell line (MCF-7), SERM showed a biphasic cell growth inhibition; marginal inhibition at lower concentrations (from nanomolar to single-digit micromolar, dotted arrows) and strong/complete inhibition at higher concentrations (from single- to double-digit micromolar, solid arrows) (Fig. 7A). In an ER-negative cell line (MDA-MB-231), SERM showed a typical dose-response curve with growth inhibition at higher concentrations (from single- to double-digit micromolar) (Fig. 7B). These data suggested that SERM have at least two independent targets for growth inhibition in MCF-7 cells. It could be postulated that the more sensitive target is ER while the less sensitive target is cholesterol trafficking in ER-positive MCF-7 cells. We further tested the effect of a high concentration of tamoxifen (10 μ M) on cholesterol trafficking in both MCF-7 and MDA-MB-231. Tamoxifen strongly inhibited cholesterol trafficking in both cells and the inhibition was reversed by cholesterol/CD complex (Fig. 7C). A high concentration of tamoxifen strongly inhibited the cell growth of both MCF-7 and MDA-MB-231 and the inhibition was reversed by cholesterol/CD complex (Fig. 7D). These data demonstrated that inhibition of cholesterol trafficking by tamoxifen is independent of its action on ER.

Cholesterol reverses the inhibitory effect of tamoxifen on HUVEC tube formation in Matrigel

We determined the effect of SERM on endothelial cell tube formation, a well-established *in vitro* assessment of angiogenesis. As expected from previous reports [5, 6], both tamoxifen and toremifene inhibited the tube formation of HUVEC on Matrigel (Fig. 8A). The inhibition of tube formation by SERM, however, was significantly reversed by an addition of cholesterol/CD complex, while cholesterol/CD itself did not affect the tube structures (Fig. 8A and B). Taken together, these results suggested that anti-angiogenic activity of tamoxifen is mainly mediated by its effect on cholesterol trafficking in endothelial cells.

4. Discussion

SERM are mixed agonists/antagonists of ER, which act differently depending on cell and tissue types. As they act as antagonists of ER in breast tissue, SERM, especially tamoxifen, have long been used to treat ER-positive breast cancer [25]. However, it has been questioned whether the anticancer effect of SERM is solely due to the ER antagonism, since a number of reports have shown therapeutic effects of tamoxifen on ER-negative breast cancer [2-4]. The anti-angiogenic activity of SERM was first reported by Gagliardi and Collins in 1993 [6]. SERM including clomiphene and tamoxifen significantly inhibited angiogenesis in the chorioallantoic membrane in growing chick embryos. Addition of excessive amount of 17 β -estradiol did not alter anti-angiogenic activity of SERM, suggesting that angiogenesis inhibition by SERM was independent of the blockade of ER. Subsequent studies have demonstrated that SERM are effective anti-angiogenic agents using several animal models, including ER-negative rat models [5, 7]. Based on these observations, SERM are under

review in several clinical studies as an anti-angiogenic monotherapy or an adjuvant therapy with chemotherapy drugs in a broad range of cancer types [8-10]. All the evidence strongly suggests that inhibition of angiogenesis is one of the major mechanisms underlying the anticancer activity of SERM in addition to ER. However, the mechanism underlying the anti-angiogenic activity of SERM has remained elusive.

In the present study, we showed that SERM inhibited cholesterol trafficking in endothelial cells, as evidenced by abnormal accumulation of free cholesterol in the endolysosomes. This effect was accompanied by aberrant subcellular localization of two major players in angiogenesis, VEGFR2 and mTOR, in endothelial cells. VEGFR2 undergoes glycosylation upon translation and the glycosylation is required for receptor auto-phosphorylation and activation [26]. Tamoxifen treatment caused trapping of the VEGFR2 in the Golgi, inhibited the terminal glycosylation and depleted the receptor in the plasma membrane. Consequently, tamoxifen inhibited the VEGF-induced phosphorylation of VEGFR2 in endothelial cells. Tamoxifen also inhibited terminal glycosylation of other receptor tyrosine kinases related to angiogenesis such as FGFR1 and PDGFR β in HUVEC. These data suggest that tamoxifen inhibits a common terminal glycosylation pathway in endothelial cells leading to the inhibition of maturation of the receptor tyrosine kinases. On the other hand, mTORC1 is recruited to the lysosome surface and regulates lysosomal functions when cells are in normal nutrient-rich state [27]. Conversely, mTOR activity is also regulated by lysosomes by altering subcellular lysosomal positioning [22]. The lysosomal positioning is critical for mTOR activity [22, 28, 29]. Under starvation, intracellular pH (pHi) was increased and this pHi change in turn caused perinuclear clustering of lysosomes. The perinuclear clustering of lysosomes led to an inhibition of mTOR activity. Forced movement of lysosomes to the cell periphery by overexpressing kinesin family of proteins could restore mTOR activity, suggesting a critical role of lysosomal positioning in mTOR signaling pathway [22]. In our study, we found that tamoxifen did not alter the association of mTOR with the LAMP1-positive endolysosomes. Instead, it switched lysosomal positioning from the cell periphery to perinuclear region leading to the inhibition of mTOR activity. Although the mechanism by which lysosomal positioning influences mTOR activity remains to be elucidated, it has been proposed that lysosomes in the cell periphery would enable mTOR to access to its upstream signaling molecules such as activated Akt at the cell surface membrane. Confining lysosomes to the perinuclear region would prevent mTOR from accessing its upstream signaling molecules at the cell membrane, leading to the blockade of its activation [22]. Further studies are necessary to elucidate the causal relationship between SERM-induced lysosomal positioning and mTOR activity.

We further showed that addition of extracellular cholesterol could significantly reverse the abnormal localization of VEGFR2 and inhibition of terminal glycosylation and receptor phosphorylation caused by SERM. Cholesterol also could reverse SERM-induced perinuclear lysosomal positioning and inhibition of mTOR activity. These data strongly suggest that cholesterol trafficking lies upstream of VEGFR2 trafficking and glycosylation and lysosomal positioning by SERM. In addition, inhibition of endothelial cell proliferation and tube formation by SERM was markedly reversed by the addition of extracellular cholesterol, implying that inhibition of cholesterol trafficking led to inhibition of two major

signaling pathways, VEGFR2 and mTOR, which is likely the main mechanism mediating the anti-angiogenic activity of SERM.

In this study, we have not identified the molecular target responsible for inhibition of endothelial cell cholesterol trafficking by SERM. ER could be a candidate, but it was ruled out by testing two different breast cancer cell lines, MCF-7 (ER-positive) and MDA-MB-231 (ER-negative). SERM at high concentrations caused abnormal cholesterol accumulation in both cell lines regardless of ER status and inhibited cell proliferation in a cholesterol-dependent manner. One possible mechanism of cholesterol trafficking inhibition by SERM could be that SERM, especially tamoxifen, act as a lipophilic weak base and increase the pH in acidic organelles such as lysosomes [30]. Changes in organellar pH could inhibit enzyme activities in the organelles including NPC proteins and glycosylation enzymes, which could potentially affect cholesterol and receptor tyrosine kinases trafficking in the cells. Ongoing studies are focused on lysosomal pH change in endothelial cells and the results will be presented in the near future.

The vascular endothelium is the first layer of cells that are in contact with the full circulating lipoproteins from blood plasma, and is responsible for the uptake of LDL-cholesterol from the plasma and efflux of cellular cholesterol to HDL in plasma. Several recent reports showed that cholesterol uptake and efflux in endothelial cells are important regulators of angiogenesis [19, 31, 32]. Usui et al. showed that LDL alone could activate VEGFR1 signaling in the absence of VEGF in macrophages [31]. The activation of VEGFR1 signaling was mediated by recruitment of the LDL receptor to VEGFR1 by LDL during its uptake. Conversely, Fang et al. demonstrated that AIBP mediates cholesterol efflux from endothelial cells, and its overexpression suppresses angiogenesis in animal models through inhibition of VEGFR2 signaling [19]. In our previous studies, itraconazole was found to inhibit cholesterol trafficking in endothelial cells [17]. Itraconazole is undergoing multiple clinical studies as an anti-angiogenic agent. Several positive clinical results have been reported recently from Phase II studies for cancer treatment, including metastatic and castration-resistant prostate cancer, non-small cell lung cancer and basal cell carcinoma [33-35]. These studies together with our current results suggest that endothelial cell cholesterol trafficking can serve as a novel therapeutic target for angiogenesis-related diseases including cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Selective estrogen receptor modulators (SERM) inhibited cholesterol trafficking in endothelial cells.
- SERM caused abnormal subcellular localizations of VEGFR2 and mTOR in endothelial cells.
- SERM inhibited VEGFR2 and mTOR signaling pathways in endothelial cells.
- Inhibition of angiogenesis by SERM was cholesterol-dependent, but ER-independent.
- Cholesterol trafficking in endothelial cells is a novel target for inhibition of angiogenesis and cancer.

Figure 1.

Effect of SERM on cholesterol trafficking in HUVEC. (A) HUVEC were treated with tamoxifen (TMX), toremifene (TRM), clomifene (CLM) and raloxifene (RLX) for 24 h, and intracellular cholesterol was visualized by filipin. (B) HUVEC were treated with 1 μM tamoxifen (TMX, *upper panel*) or 1 μM toremifene (TRM, *lower panel*) in the presence or absence of cholesterol (5 μg/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 24 h, and intracellular cholesterol was visualized by filipin. Representative confocal images from four independent experiments are shown.

Figure 2.

Effects of SERM and cholesterol on the subcellular localization of VEGFR2 and mTOR in HUVEC. (A) HUVEC were treated with or without 1 μ M tamoxifen (TMX) for 24 h and subcellular localization of VEGFR2 was assessed under a confocal microscope. Arrows indicate VEGFR2. (B) HUVEC were treated with or without 1 μ M tamoxifen (TMX) in the presence or absence of cholesterol (5 μ g/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 24 h and subcellular localization of VEGFR2 and Golgi (GM130) was assessed. (C) HUVEC were treated with or without 1 μ M tamoxifen (TMX) for 24 h and subcellular localization of cholesterol (Filipin), lysosomes (LAMP1), and mTOR was assessed. (D) HUVEC were treated with 1 μ M tamoxifen (TMX) in the presence or absence of cholesterol (5 μ g/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 24 h and subcellular localization of mTOR and actin was assessed. Arrows indicate that tamoxifen induced a change in mTOR localization from peripheral cytoplasm to perinuclear region and this was reversed by cholesterol-cyclodextrin complex. Representative confocal images from four independent experiments are shown.

Figure 3.

Effect of SERM on VEGFR2 glycosylation and mTORC1 pathway in HUVEC. (A) HUVEC were treated with SERM including tamoxifen (TMX, 5 μ M), toremifene (TRM, 5 μ M) and clomifene (CLM, 5 μ M) for 24 h and VEGFR2 glycosylation was assessed by Western blotting. Glycosylation inhibitors including deoxymannojirimycin (dMM, 500 μ M) and tunicamycin (TUM, 2 μ g/ml), and a cholesterol trafficking inhibitor itraconazole (ITRA, 1 μ M) were used as positive controls. Three different glycosylated forms of VEGFR2 (a: 230 kD hyper-glycosylated form, b: 200 kD intermediate glycosylated form and c: 180 kD unglycosylated form) are shown. (B), (C), and (D) Effect of various concentrations of SERM on VEGFR2 glycosylation and mTORC1 pathway – indicated by the level of phosphorylated S6K (pS6K) – are shown. Representative Western blot images from three independent experiments are shown.

Figure 4.

Effect of tamoxifen on the phosphorylation of mTOR and the glycosylation of receptor tyrosine kinases in HUVEC. (A) HUVEC were treated with tamoxifen (TMX) or rapamycin (Rapa) at indicated concentrations for 24 h and the phosphorylation of mTOR at Ser2448 as well as phosphorylated S6K (pS6K) and total S6K were analyzed. (B) HUVEC were treated with tamoxifen (TMX), sorafenib (Soraf) or deoxymannojirimycin (dMM) at indicated concentrations for 24 h. The terminal glycosylation of the receptor tyrosine kinases was assessed by Western blotting using specific antibodies against each receptor tyrosine kinase in the presence of the known glycosylation inhibitor dMM. Representative Western blot images from three independent experiments are shown.

Figure 5.

Reversal effect of cholesterol on the inhibition of VEGFR2 and mTOR activities by SERM. (A) and (B) HUVEC were treated with tamoxifen (TMX) or toremifene (TRM) with or without cholesterol (5 μg/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 24 h, and VEGFR2 glycosylation was assessed by Western blotting. (C) HUVEC were grown in low serum media (0.1% FBS without additional growth factor supplements) and treated with drugs including tamoxifen (TMX) and sunitinib (Sunit, 100 nM) for 24 h in the presence or absence of cholesterol (5 μg/ml)/cyclodextrin (0.1%) complex (Chol/CD). Cells were then stimulated with 50 ng/ml of VEGF-165 for 5 min and the levels of total and phosphorylated VEGFR2 were assessed by Western blotting. (D) HUVEC were treated with tamoxifen (TMX, 5 μM) or toremifene (TRM, 5 μM) with or without cholesterol (Chol, 5 μg/ml) and cyclodextrin (CD, 0.1%) for 24 h, and mTOR activity was assessed by Western blotting of phosphorylated S6- kinase (pS6K). Representative Western blot images from three independent experiments are shown.

Figure 6.

Effects of SERM and cholesterol on HUVEC proliferation. (A) HUVEC were treated with various concentrations of tamoxifen, toremifene or raloxifene for 24 h and cell proliferation was assessed through the [3H]-thymidine uptake assay. (B) HUVEC were treated with tamoxifen (TMX, 2 μM) or toremifene (TRM, 4 μM) for 24 h and were observed under a phase contrast microscope. Cholesterol (Chol, 5 μg/ml) and/or cyclodextrin (CD, 0.1%) were added together to assess reversibility on the anti-proliferative effect of SERM. NT denotes Not Treated with Chol/CD. Representative phase-contrast images from four independent experiments are shown. The cell viability was quantified by AlamarBlue staining and was plotted using the GraphPad Prism 5.0 software (*right panel*). Data represent mean ± standard deviation (SD) from four independent experiments. ** $P < 0.01$ between two indicated groups.

Figure 7.

Effects of SERM and cholesterol on ER-positive or ER-negative breast cancer cell proliferation. (A) and (B) MCF-7 (ER-positive) or MDA-MB-231 (ER-negative) cells were treated with various concentrations of tamoxifen, toremifene or raloxifene for 24 h and cell proliferation was assessed through the [3H]-thymidine uptake assay. SERM showed a biphasic growth inhibition in MCF-7 cells. Dotted arrows indicate concentration ranges that show marginal cell growth inhibition, whereas solid arrows represent concentration ranges with strong cell growth inhibition. (C) MCF-7 or MDA-MB-231 cells were treated with 10 μM tamoxifen (TMX) in the presence or absence of cholesterol (5 μg/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 24 h and intracellular cholesterol was labeled with filipin staining. Representative confocal images from four independent experiments are shown. (D) MCF-7 or MDA-MB-231 cells were treated with 10 μM tamoxifen (TMX) in the presence or absence of cholesterol (Chol, 5 μg/ml)/cyclodextrin (CD, 0.1%) complex (Chol/CD) for 24 h. The cell viability was quantified by AlamarBlue staining and was plotted using the GraphPad Prism 5.0 software. Data represent mean ± standard deviation (SD) from four independent experiments. ** $P < 0.01$ between two indicated groups.

Figure 8.

Effects of SERM and cholesterol on HUVEC tube formation. (A) HUVEC were seeded onto a Matrigel-coated plate to promote tube formation. Cells were treated with 5 µM tamoxifen (TMX) or 5 µM toremifene (TRM) in the presence or absence (NT) of cholesterol (5 µg/ml)/cyclodextrin (0.1%) complex (Chol/CD) for 18 h. The tube formation was visualized by staining with Calcein-AM under a fluorescence microscope. Representative fluorescence images from six independent experiments are shown. (B) Total tube lengths, sizes and number of junctions from the fluorescence images from six experiments were quantified using the AngioQuant software. $**P < 0.01$ between two indicated groups.