Tamoxifen non-estrogen receptor mediated molecular targets

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Abstract

Recent experimental studies revealing new biological effects of tamoxifen on tumor cells both expressing and not expressing different types of estrogen receptors (ERα and ERβ) show new aspects of a seemingly well known agent. This review describes tamoxifen targets, the blocking of which leads to inhibition of tumor cell growth and angiogenesis, stimulation of programmed cell death (apoptosis, autophagia and necrosis), inhibition of multidrug resistance, invasion and metastasis. Since outcomes of tamoxifen action on cells are prognostically good from the point of view of both tumor growth/metastasis inhibition and tumor response to drug therapy, the authors believe this is an extremely important addition to tamoxifen antiestrogenic effect. Arguments are provided to consider the strategy of long-term tamoxifen treatment proposed by Professor Craig V. Jordan in the 1970s that is also applicable to the treatment of other tumors. This is, first of all, the fact that expression of estrogen receptor-beta that can also be targeted by tamoxifen therapy in solid tumors of practically all known sites and histologies. The authors believe that molecular biological screening of patients with respect to expression of tamoxifen cellular targets other than ERα and ERβ is needed to use to the full all tamoxifen biological activities other than modulation of estrogen receptors during long-term adjuvant therapy for cancers of various sites.

Introduction

Notwithstanding the rapid progress in hormonal anticancer therapies and resulting new effective agents, tamoxifen has been for many years the most outstanding and invariably effective cancer therapy. This agent is considered a gold standard in the treatment of estrogen receptor (ER)-positive breast cancer. This is the first and the oldest targeted drug, remaining a leader in breast cancer treatment.1,2

Estrogen receptors are the most important cell target in view of both control of carcinogenesis and inhibition of tumor cell growth. Results of studies over many years (since the early 1970s) of tamoxifen treatment of patients with ER-positive breast cancer are an excellent confirmation of this finding. The drug is also effective as adjuvant therapy and considerably improves follow-up outcomes of surgery by reducing the risk of disease recurrence and death. Tamoxifen antitumor effect is first of all due to the ability to selectively block the ERs found in most breast cancer patients. This explains the continuing interest in antiestrogen therapy for breast cancer.

The situation started to change gradually in 1996 when new ER-type estrogen receptors beta (ERβ) were discovered. Since then, the ERs, whose status was determined in breast tumors, were referred to as estrogen receptors alpha (ERα). As far as ERβ is concerned, they have been found in a variety of tumor sites, including carcinomas of the colon, esophagus, stomach, lung, brain, prostate, pancreas.3,4 This provided a rationale for broadening indications of tamoxifen in ERα-negative ERβ-positive tumors, including breast cancer and tumors of other sites. Furthermore, recent experimental studies have revealed new biological effects of tamoxifen on tumor cells. A large spectrum of tamoxifen targets other than estrogen receptors have been discovered that are key mediators of signal pathways activating cell proliferation, determining aggressive course of neoplastic disease or tumor sensitivity to chemotherapy. As a result, new aspects of a seemingly well known agent have become apparent, as well as prospects for its new clinical indications, in particular in the treatment of solid tumors other than breast cancer. This includes its use in combinations with current target therapies. These new targets are analyzed below.

Inhibition of protein kinase C

Protein kinase C (PKC) is serine/threonine-specific protein kinase expressed on practically all mammalian cells. It plays an extremely important role in intracellular signaling. PKC substrates in various cell types include nuclear proteins, cytoskeletal proteins, enzymes. PKC mediates transmission of a broad range of external signals including those regulating cell growth.

Tamoxifen produced cytotoxic effect on human prostate cancer cell cultures PC3 and PC3-M that at a molecular level was associated with
inhibition of PKC which was followed by induction of p21 (waf1/cip1), Rb dephosphorylation and G/S phase cell arrest. Interestingly, Ro31-8220, a PKC specific inhibitor, produced a similar effect. Tamoxifen demonstrated similar effects on cells of other tumor types, such as hepatocellular carcinoma and astrocytoma. Tamoxifen inhibiting effect on PKC was also demonstrated experimentally on malignant gliomas. As shown on breast cancer MCF-7 cell culture, tamoxifen exerts its antiproliferative activity through direct interaction with PKC-epsilon associated with tumor cell differentiation and growth.

**Suppression of metastasis**

Several authors described an important effect of tamoxifen, i.e. antimitastic activity that might take place regardless of ER expression on human cells and was associated with impairment of many cellular targets. For example, tamoxifen up-regulated expression of tissue inhibitor of metalloproteinases-1, TIMP-1, and down-regulated expression of metalloproteinase 9 in lung adenocarcinoma SPC-A-1 and breast cancer MCF-7 cells, that led to inhibition of cell invasion in matrigel. Both of these cell lines were ER-positive. Tamoxifen antimitastic activity was described on ER-positive colon cancer cells with high metastatic potential. The antiestrogen down-regulated expression of matrix metalloproteinase MMP7 and inhibited cell migration into the wound in monolayer culture. Inhibition of cell growth and migration into the wound was also seen on cultures of ER-negative human thyroid cancer cells. This effect was confirmed in vivo on FTC133 xenografts in nude mice. Tamoxifen inhibited the growth of FTC133 xenografts in nude mice. In murine melanoma B16BL6 cell culture, tamoxifen inhibited mRNA expression and protein activities of matrix metalloproteases. Experiments in vivo on a murine model demonstrated a significant inhibition of melanoma metastasis into lungs. In rat breast cancer wild-type Mat B-III cells and with overexpression of urokinase receptor uPAR tamoxifen inhibited uPAR gene transcription, mRNA expression and protein production. In in vivo experiments the antiestrogen both reduced tumor size and inhibited metastasis after tumor cell transplantation to Fisher rats.

**Activation of programmed cell death**

The tamoxifen ability to enhance programmed cell death or apoptosis contributes largely to its cytostatic effects.

A variety of non-ER mediated apoptosis enhancing mechanisms in cells of different histogenesis are described, including inhibition of phospholipase C, D- and protein kinase C-mediated pathways, that is a possible mechanism of action independent of ER expression. Tamoxifen effect on ER-positive breast cancer cells leads to activation of caspases 6, 7 and 9. Tamoxifen induced apoptosis of rat glioma C6 cells via inhibition of AKT activation and JNK transitory activation, whereas MAP kinase ERK evidenced sustained activation in response to the drug treatment. A mechanism is described for apoptosis stimulation under the tamoxifen effect on cells over-expressing antiapoptotic proteins. Tamoxifen treatment of Bcl-2 over-expressing clones was found to induce apoptosis via activation of c-Jun N-terminal kinase (JNK), p38 kinase and phosphorylation of c-Jun, in parallel with increasing LNA-binding activity of AP-1, expression of FasL and activating caspase 8. No such effects were seen in cells under-expressing this protein. Tamoxifen was also shown to induce apoptotic changes in cells both by up-regulating and keeping stable levels of Bcl-2 in cells.

From the point of view of variability of effects on cells, the influence of tamoxifen on expression of other proteins involved in apoptotic processes is of interest. Similarly to the effects on Bcl-2, tamoxifen may produce variable effects on levels of these proteins. Tamoxifen inhibited growth and induced apoptosis in cells of human bile duct carcinoma QBC939, up-regulated expression of p53 and p21 (waf1/cip1), and induced G1-G2 phase cell cycle arrest. Downregulation of cyclin D and C-Myc expression, upregulation of Bcl-2, Bax and caspases 6, 7 and 9 were also observed in tamoxifen-treated cells.

Tamoxifen can activate mitochondria-dependent apoptosis. Tamoxifen enhanced apoptosis and oxidative stress via mitochondria-dependent and nitric oxide (NO)-dependent pathways associated with increased intramitochondrial Ca2+ concentration. Tamoxifen stimulation of mitochondrial NO synthase resulted in suppression of mitochondrial respiration, reduced release of cytochrome C, increased mitochondrial lipid peroxidation, and decreased aggregation of mitochondria. All these processes finally led to tumor cell apoptosis with formation and phagocytosis of apoptotic bodies.

Type II programmed cell death is associated with autophagia program, i.e. degradation of organelles and cytoplasmatic material with intracellular structures contributing to this process. Treatment with various selective modulators of ERs (including tamoxifen) led to inhibition of type II cell death in a dose-dependent manner. Further studies discovered that autophagia activation by tamoxifen began with depolymerization of actin and degradation of intermediate filaments. Tamoxifen-induced activation of autophagia was confirmed by a series of studies on cell lines from primary and metastatic carcinomas of the breast, colon and lymphoma.

Finally, tamoxifen influences type III cell death, i.e. programmed necrosis. The necrosis may be induced by activating apoptosis via binding of ligands such as FasL (a member of tumor necrosis factor family) and TRAIL (TNF-related apoptosis-inducing ligand), and by inducing overexpression of protoapoptotic protein Bax simultaneously, or by inhibiting activity of caspases, or by inducing overexpression of anti-apoptotic proteins. Tamoxifen induces apoptosis in Fas receptor-positive cells through regulation of expression of Fas ligand. This mechanism explains selective stimulation of apoptosis in osteoclasts that prevents bone resorption and osteoporosis.

What cell cycle phases are associated with different types of cell death? Unlike apoptosis that can start in various cell cycle phases, including mitosis per se as a mitotic catastrophe, autophagic death is observed mainly in non-proliferating cells. However, if apoptotic mechanisms are inhibited in proliferating cells, e.g. through caspase inactivation, then death of the proliferating cells is by a mechanism of programmed necrosis. Combinations of types of tamoxifen-induced cell death present multiple targets for this antiestrogen.

Interesting findings were reported by a study of tamoxifen effect on expression of 36 proteins in MCF7 breast cancer cells. Tamoxifen was shown to change expression of heat shock proteins, various structural proteins, and proteins involved in apoptosis, posttranslational modifications, glycolysis, RNA processing, cell cycle progression, DNA transcription and translation, cell differentiation. A total of 9 proteins from different groups appeared up-regulated, while 7 others were down-regulated. The authors also demonstrated that tamoxifen altered functioning of E6-associated protein (E6AP, also UBE3A) which, on the one hand, was a coactivator of estrogen receptor alpha and, on the other hand, was a E3 ubiquitin-protein ligase involved in degradation of various proteins. After binding to estrogen receptors alpha, tamoxifen interfered with E6AP coactivation function followed by E6AP transfer from the nucleus into cytoplasmatic space and degradation. Biological effects in this case include G1-G2 growth arrest and appearance of cells in apoptotic state.

Tamoxifen was found to produce synergic effects if used with other apoptosis activators which might be of great importance for counteract-
ing drug resistance. For instance, co-treatment with tamoxifen and TRAIL down-regulates antiapoptotic proteins FLIP and Bcl-2, while simultaneously up-regulating proapoptotic proteins FADD, tBid, Bax, caspases 8 and 9 in breast tumors, regardless of their ER status.33

The augmentation of tamoxifen apoptotic effects was observed when the antiestrogen was used in combination with apoptosis inducer, roscovitine, that inhibited cyclin-dependent kinases mainly through effects on kinases CDK2, CDK7 and CDK9. Co-treatment with the two agents was associated with synergism of apoptotic effect in ER-positive breast cancer cells and antagonism in ER-negative cells.34-36

Therefore, stimulation of apoptosis, autophagia or necrosis depending on cell context contributes to tamoxifen effects and broadens the range of indications for tamoxifen administration, including its use in combination with other agents.

![Tamoxifen effects on specific interaction of monoclonal antibody (clone 4E3, Abcam) with P-glycoprotein (Pgp) in Jurkat human T-lymphoblastic leukemia cell line. (A) Cell distribution with respect to fluorescence intensity (flow cytometer BD FACS Canto II). x axis: fluorescence intensity (conditional units); y axis: the number of cells. Shaded bars show cell fluorescence intensity without tamoxifen treatment; blank bars show cell fluorescence intensity after incubation with tamoxifen. Figures correspond to percentages of specifically fluorescent cells with respect to isotype control. (B–E) Cell photographs after fluorescent staining with anti-Pgp monoclonal antibody made using a light fluorescent microscope Leica DMI 6000B (magnification 400x). Cells in the light and fluorescent fields: (B) and (C) free from tamoxifen treatment. (D) and (E) after tamoxifen treatment.]

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Inhibition of angiogenesis

It is currently commonly understood that angiogenesis is a necessary condition for malignant growth and metastasis. There is a vast literature on tamoxifen antiangiogenic properties similar to those of other antiangiogenic agents.37-39

The tamoxifen ability to inhibit angiogenesis has been tested on classical models of antiangiogenic studies, such as measurement of microvascular density in rat fibrosarcoma, aortic ring assay, chicken chorioallantoic membrane assay and rabbit corneal pocket assays.39

Tamoxifen angiogenesis inhibition effects were extensively studied both in vitro and in vivo, and were seen on both ER-positive and ER-negative tumor models.40,41

There are several mechanisms of tamoxifen antiangiogenic effect, such as inhibition of cyclin-dependent endothelial cell growth,42 modulation of transforming growth factor beta (TGF-β) in breast cancer cells,43 inhibition of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).44-46 VEGF is a key promoter of tumor angiogenesis. VEGF expression rises in response to hypoxia, activation of oncogenes and various cytokines. VEGF induces endothelial cell proliferation and migration, and inhibits apoptosis. The antian-
giogenic activity of tamoxifen and other antiestrogen agents (nafoxidine, clomifen and ICI 182,780) is not associated with tumor ER status and is due to direct inhibition of VEGF and bFGF, as shown in a 6-day chicken chorioallantoic membrane assay. Tamoxifen is also shown to inhibit VEGF secretion in ER-positive human breast cancer cell line MCF-7. Furthermore, tamoxifen decreased the levels of proangiogenic VEGF and angiogenin while increasing antiangiogenic angiostatin levels in the normal human breast tissue. Tamoxifen inhibitory effect on angiogenesis is also associated with increased expression of matrix metalloproteinases MMP2, MMP9 and antiangiogenic protein endostatin. Effect of tamoxifen in combination with estradiol on xenografts of breast cancer MCF7 in nude mice led to elevation in levels of endostatin and MMP2, and MMP2 and MMP9 activity. Tamoxifen produced the same effect, i.e. increased expression of the above-mentioned proteins, on MCF7 cells in vitro in the absence of estradiol. Tamoxifen effect on MCF7 cells was also associated with increased expression of tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2. Another mechanism of tamoxifen antiangiogenic effects is upregulation of interleukin receptor antagonist-1 (IL-1Ra) that interferes with IL-1α and IL-1β signal transmission. Since both IL-1α and

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**Figure 3.** Tamoxifen effects on specific interaction of monoclonal antibody (clone LMR5, Abcam) with lung resistance-related protein (LRP) in A549 human lung adenocarcinoma cell line. (A) Cell distribution with respect to fluorescence intensity (flow cytometer BD FACSCanto II). x axis: fluorescence intensity (conditional units); y axis: the number of cells. Shaded bars show cell fluorescence intensity without tamoxifen treatment; blank bars show cell fluorescence intensity after incubation with tamoxifen. Figures correspond to percentages of specifically fluorescent cells with respect to isotype control. (B-E) Cell photographs after fluorescent staining with anti-LRP monoclonal antibody made using a light fluorescent microscope Leica DMI 6000B (magnification x400). Cells in the light and fluorescent fields: (B) and (C) free from tamoxifen treatment. (D) and (E) after tamoxifen treatment. (C) and (E) clearly show granularity of the staining with monoclonal antibody against lung resistance-related protein that, unlike other multidrug resistance proteins, is found within ribonucleoprotein particles or vaults rather than in a free state. These vaults can form tubular structures that are seen as fluorescent granularity during fluorescent analysis.
IL-1β promote tumor angiogenesis, inhibition of these interleukins decreases activation of neoangiogenesis. Tamoxifen increased IL-1Ra expression in a murine model of human breast cancer and thus inhibited angiogenesis.60 Heparin-binding polypeptide, angiogenin, is the most important and a most powerful angiogenic factor. It binds to the endothelial cell surface and induces cell proliferation,60 activates endothelial cell proteases,51 and promotes tube formation by endothelial cells.52 Elevated angiogenin levels were found in tumors of various sites.53-56 Angiogenin levels were increasing in the series normal breast tissue → carcinoma in situ → invasive carcinoma and demonstrated positive correlation with high tumor grade, positive estrogen receptor status, and hypoxia-inducible factor.61 Interesting findings were recently reported concerning angiogenin regulation by estradiol and tamoxifen in breast tissue.62 Extracellular angiogenin was shown to correlate significantly with estradiol in normal breast tissue in vivo, and exposure of normal breast tissue biopsies to estradiol stimulated angiogenin secretion. In breast cancer patients, angiogenin levels are significantly higher in tumors compared with the adjacent normal breast tissue. In estrogen receptor-positive breast cancer cells, estradiol increases angiogenin secretion and endothelial cell proliferation. Tamoxifen reverses both of these estradiol effects and decreases in vivo the level of angiogenin and angiogenesis. Anti-angiogenin antibody therapy decreases growth of MCF7 tumors in nude mice. Taken together, these results suggest previously unknown mechanisms of angiogenesis regulation by estrogen and the antiestrogen.

Interaction with multidrug resistance proteins

Another tamoxifen target are proteins that throw out antitumor agents with different structure and mechanism of action from cells and are associated with multidrug resistance mechanism (MDR). These are transport proteins, members of the ABC transporter family, such as P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRP) and breast cancer resistance protein. The group also includes multidrug resistance marker major vault protein, also refereed to as lung and breast cancer resistance protein. The group also includes multidrug resistance marker major vault protein, also referred to as lung resistance-related protein (LRP).

Tamoxifen enhanced specific activity of some cytostatics against many tumors with Pgp-associated MDR phenotype in cell cultures and animal models.53,64 Furthermore, tamoxifen interacted with Pgp, while some antitumor agents competed for binding to Pgp with verapamil and tamoxifen in cell cultures of different histogenesis, which might be an explanation for the above-described phenomenon.65,66 Tamoxifen was also shown to enhance effects of adriamycin, mitomycin and vindesine in human cholangiocarcinoma cell line QBC939 with MDR phenotype. However, when specific anti-Pgp antibody was added to tamoxifen combination with antitumor drugs, this antibody blocked the tamoxifen-induced downregulation of Pgp expression.67 Therefore, tamoxifen effect may be defined as recovery of response to chemotherapy in tumors with MDR phenotype due to tamoxifen influence on Pgp.

Direct experiments to investigate tamoxifen effects on monoclonal antibody-Pgp binding in human chronic myelogenous leukemia cell line K562 discovered that the antiestrogen competed with the antibody for the binding.68 A similar result was obtained in a study of the effect of tamoxifen on the monoclonal antibody binding with MRPI in human cervical carcinoma cell line HeLa.69 In our experiments (T Bogush, E Dudko, E Bogush, B Polotsky, S Tjulandin, M Davydov, unpublished data, 2012 year) tamoxifen competitive interaction with Pgp, MRPI and LRP was visualized by fluorescent microscopy. Changes in specific fluorescent staining of cells under tamoxifen effect were confirmed by flow cytometry in all cases (Figures 1-3). Incubation with tamoxifen increased the number of specifically fluorescent cells and fluorescence intensity of some anti-Pgp antibody-stained cells in Jurkat human T-lymphoblastic leukemia cell suspension (Figure 1). On the contrary, there was a decrease in the number of specifically fluorescent cells and fluorescence intensity of some cells in response to tamoxifen treatment after HeLa and A549 lung adenocarcinoma monolayer cultures were stained with anti-MRP1 and anti-LRP monoclonal antibodies (Figures 2 and 3). These findings prove that tamoxifen interacts with MDR markers Pgp, MRPI and LRP and thus interferes in the interaction of antitumor drugs with these transporter proteins, consequently inhibiting the associated MDR mechanism. We believe that the tamoxifen interaction with Pgp, MRPI and LRP should inevitably decrease the intracellular concentration of tamoxifen available for interaction with other cell targets including ER. Therefore, tamoxifen interaction with Pgp, MRPI and LRP in MDR cells may result in a negative effect, that is a reduction in tamoxifen’s own efficacy. It is obvious that the higher the overexpression of transport proteins, the greater prognostic value (with respect to tamoxifen resistance) they have. It should be emphasized, however, that this is not the case with the MDR mechanism associated with drug throw-out from cells. This is basically a new mechanism of a reduction in tamoxifen efficacy due to intracellular inactivation as a result of binding to these proteins on its way to its own targets, rather than due to Pgp, MRPI or LRP functioning.

### Table 1. Tamoxifen biological effects independent from tumor estrogen receptor status

| Tamoxifen biological effects                          | Mechanism of tamoxifen biological effects                                      |
|--------------------------------------------------------|--------------------------------------------------------------------------------|
| Stimulation of cell programmed death: apoptosis, autophagia, necrosis | Activation of caspases 6, 7, 8 and 9  
 Activation of JNK, p38, p53, p21 and FasL  
 Downregulation of Bcl-2, E6AP                                     |
| Cell proliferation inhibition                          | Inhibition of protein kinase C  
 Inhibition of transferring growth factor-betal                                             |
| Angiogenesis inhibition                                | Inhibition of VEGF, bFGF and angiogenin  
 Stimulation of IL-1Ra  
 Activation of MMP2, MMP9 and endostatin                                      |
| Invasion and metastasis inhibition                     | Stimulation of TIMP-1  
 Inhibition of MMP7 and 9  
 Inhibition of uPAR                                                                |
| Multidrug resistance inhibition                        | Interaction with multidrug resistance-associated proteins Pgp, MRPI and LRP |

VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-1Ra, interleukin receptor antagonist-1; MMP, matrix metalloproteinases; TIMP-1, tissue inhibitor of metalloproteinases-1; uPAR, urokinase receptor; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; LRP, lung resistance-related protein.
Conclusions

In conclusion, we should mention the strategy of long-term tamoxifen treatment proposed by Professor Craig V. Jordan in the 1970s that has strongly positioned tamoxifen as an inevitably effective agent in the treatment of ER-positive breast cancer for more than four decades.1

We believe that the discovery in 1996 of a new type of tamoxifen target, ER-beta, together with data on its expression in tumors of various sites, makes the long-term adjuvant therapy with tamoxifen also applicable to tumors other than breast cancer. It is currently commonly understood that various ERs are expressed in tumors of all sites and histologies. Differences may be found only in frequency and intensity of expression of these tamoxifen cellular targets.3,4

Findings concerning key mediators of signal pathways and cellular receptor proteins targeted by tamoxifen, as reviewed in this paper, demonstrate that tamoxifen is a unique polyvalent targeted drug. Table 1 summarizes data of tamoxifen non-ER cellular targets. Tamoxifen effect on these targets activates or inhibits most important biological processes that control tumor growth and response to chemotherapy. As seen, outcomes of tamoxifen action on cells are prognostically good from the point of view of both tumor growth/metastasis inhibition and tumor response to drug therapy. This is an extremely important addition to tamoxifen antiestrogenic effect. We believe that molecular biological screening of patients with respect to expression of tamoxifen cellular targets other than different types of ERs is needed to use to the full all tamoxifen biological activities during long-term adjuvant cancer therapy. Without such a screening, one can hardly expect a good full all tamoxifen biological activities during long-term adjuvant cancer therapy.

Furthermore, assessment of tamoxifen cellular targets other than estrogen receptors will help enlarge indications of adjuvant tamoxifen therapy not only in breast cancer but also in tumors in other sites.

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