Anti-thrombotic effects of selective estrogen receptor modulator tamoxifen

Manasa K. Nayak1; Sunil K. Singh1; Arnab Roy1; Vivek Prakash2; Anand Kumar2; Debabrata Dash1

1Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India; 2Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Summary
Tamoxifen is a known anti-cancer drug and established estrogen receptor modulator. Few clinical studies have earlier implicated the drug in thrombotic complications attributable to lower anti-thrombin and protein S levels in plasma. However, action of tamoxifen on platelet signalling machinery has not been elucidated in detail. In the present report we show that tamoxifen is endowed with significant inhibitory property against human platelet aggregation. From a series of in vivo and in vitro studies tamoxifen was found to inhibit almost all platelet functions, prolong tail bleeding time in mouse and profoundly prevent thrombus formation at injured arterial wall in mice, as well as on collagen matrix perfused with platelet-rich plasma under arterial shear against the vehicle dimethylsulfoxide (DMSO). These findings strongly suggest that tamoxifen significantly downregulates platelet responses and holds potential as a promising anti-platelet / anti-thrombotic agent.

Keywords
Antiplatelet drug, estrogen receptor, platelet aggregation, selective estrogen receptor modulators (SERM), thrombosis

Introduction
Tamoxifen is a member of the triphenylethylene class of drugs with anti-estrogen properties and is widely used for the treatment and prophylaxis of breast cancers (1–6). Tamoxifen has estrogen-like activities in some tissues, while estrogen antagonistic effects in others. This has led to the reclassification of this family of drugs as selective estrogen receptor modulators (SERM) (7, 8). Tamoxifen is an effective antioxidant and protects membranes and low-density lipoprotein particles against oxidative changes (9) with potential benefits in atherosclerosis (10). The drug has also potential therapeutic applications against neurological diseases like bipolar disorder (11) and brain tumours (12–14) at doses considerably higher than that administered in breast cancer. As platelet activation plays critical role in the etio-pathogenesis of thrombosis (15–17), it is essential to study the effect of tamoxifen on platelet reactivity both under in vivo and in vitro conditions. For example, the drug has been reported to have inhibitory effect on platelets (18) and a strong haemodilution effect, resulting in reduced platelet counts (19). On the contrary, tamoxifen has also been shown to induce production of reactive oxygen species by isolated platelets, thus contributing to enhanced platelet reactivity (20). It was therefore unclear whether the net effect of reduced platelet counts, opposed by increased platelet function, will result in a procoagulant or anticoagulant phenotype, but the impact on platelet function cannot be ignored.

Materials and methods
Materials and reagents
Mouse monoclonal antibody against phosphotyrosine (clone 4G10) and horseradish peroxidase-labelled anti-mouse secondary antibody were procured from Millipore (Billerica, MA, USA) and Bangalore Genei (Bangalore, India), respectively. PE-labelled antibody against P-selectin and FITC-labelled PAC-1 were from BD Pharmingen, Fura-2 AM was from Molecular Probes (Invitrogen) and Calcein-AM was from Invitrogen (Carlsbad, CA, USA). Super

Correspondence to:
Debabrata Dash
Department of Biochemistry, Institute of Medical Sciences
Banaras Hindu University, Varanasi 221005, India
Tel.: +91 9336910665, Fax: +91 542 2367568
E-mail: ddass@satyam.net.in

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Till date, studies on effect of tamoxifen in cardiovascular diseases have mostly emerged from clinical trials on women at low-solute risk of myocardial infarction. The present study was conducted to determine whether tamoxifen and 4-OH tamoxifen (a metabolite) might influence platelet reactivity and signalling. Here we report that tamoxifen and 4-OH tamoxifen have identically potential anti-platelet efficiency, as both can inhibit platelet functions like aggregation, secretion, clot retraction, adhesion and spreading in a dose-dependent manner. Furthermore, tamoxifen prolonged tail bleeding time in mice and profoundly prevented thrombus formation at injured arterial wall in mice, as well as on collagen matrix perfused with platelet-rich plasma under arterial shear.
Signal West Pico chemiluminescent substrate and PVDF membrane were the products from Pierce (Rockford, IL, USA) and Millipore, respectively. Tamoxifen citrate, 4-OH tamoxifen, phalldin-FITC, apyrase, EGTA (ethylene glycol tetraacetic acid), sodium orthovanadate, acetylsalicylic acid, bovine serum albumin (fraction V), Triton X-100, protease inhibitors, dimethylsulfoxide (DMSO), 2,2,2-Tribromoethanol (Avertin) and other reagents were procured from Sigma (St. Louis, MO, USA). Estrogen receptor antagonist (ICI-182,780) was product of Tocris Biosciences (Bristol, UK). Thrombin, collagen, ADP and ChronoLume reagent were purchased from Chronolog Corporation (Havertown, PA, USA). Reagents for electrophoresis were products of Merck (Whitehouse Station, NJ, USA). All other reagents were of analytical grade. Milli-Q grade deionised water (Millipore) was used for preparation of solutions.

**Platelet preparation**

Blood was collected from healthy volunteers (majority male), under informed consent, who were not on any anti-platelet medication in the past two weeks. Platelets were isolated from fresh human blood by differential centrifugation in the presence of aspirin and apyrase, as already described (21). The final cell suspension did not have apyrase and was adjusted to 0.5–0.8 x 10⁹ platelets/ml. All steps were carried out under sterile conditions and precautions were taken to maintain the cells in resting condition. The study was approved by the Ethical Committee of Banaras Hindu University.

**Platelet aggregation and adenosine triphosphate (ATP) secretion**

Platelets were stirred (1,200 rpm) at 37°C for 2 minutes (min) in a Chrono-log Whole Blood/Optical Lumi-Aggregometer (model 700–2) prior to the addition of agonists. Wherever indicated, cells were stimulated without stirring to prevent aggregation. Aggregation was induced with different agonists (thrombin, 1 μM; adenosine diphosphate [ADP], 10 μM; and collagen, 10 μg/ml). For experiments using ADP as the agonist platelets were prepared without exposure to acetylsalicylic acid. Aggregation was measured as percent change in light transmission, where 100% refers to transmittance through blank sample. ATP secretion was measured with ChronoLume reagent (Stock concentration, 0.2 μM luciferase/luciferin). Luminescence generated by platelet-secreted ATP was monitored using Lumi-Aggregometer in parallel with aggregation measurement. Finally, cells were boiled in Laemmli lysis buffer and stored at –20°C till further analysis.

In order to examine the effect of tamoxifen on platelet aggregation and secretion, cells were incubated for 2 min at 37°C in presence of different concentrations of tamoxifen or 0.5% DMSO prior to the addition of agonist.

**Platelet adhesion and spreading studies**

Control cells as well as cells pre-treated with tamoxifen were charged onto slides coated either with poly-L-lysine (0.01% w/v) or fibrinogen (100 μg/ml) or collagen (50 μg/ml) and platelet adhesion and spreading on immobilised matrix was studied as described (22). Time-lapse events were captured by Leica DFC 320 CCD camera using IM50 software (Leica) and analysed by NIS-Elements AR imaging software (Nikon).

**Clot retraction studies**

Fibrinogen (2 mg/ml) was incubated with washed platelets (either control, or pretreated with varying concentrations of tamoxifen) in presence of calcium (2 mM). Clot formation was induced by addition of thrombin (1 U/ml) and retraction of platelets was studied as described (22).

**Flow cytometry**

Platelets (1 x 10⁸ cells in 100 μl) were incubated at 37°C for 10 min without stirring in presence of thrombin (1 U/ml), either in presence or absence of tamoxifen (25 μM), followed by addition of equal amount of 4% paraformaldehyde for 30 min. Post fixation, resuspended platelets were labelled with 5 μl FITC-labelled anti-CD61 antibody, and either 5 μl PE conjugated anti-CD62P antibody or 10 μl FITC-labelled PAC-1 antibody (23, 24). The samples were incubated for 30 min at room temperature in dark and analysed on the flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). Forward and side scatter voltages were set at E00 and 273, respectively, with threshold of 52V. An amorphous region (gate) was drawn to encompass the platelets to differentiate from noise and multi-platelet particles. After compensation for FITC and PE, all fluorescence data were collected using four-quadrant logarithmic amplification. CD61-positive 10,000 events were collected for each sample.

**Immunoblotting studies**

Immunoblotting for tyrosine phosphorylated proteins was carried out as previously described (22).

**Measurement of intracellular free calcium**

Intracellular calcium measurements were carried out in control platelets as well as platelets pretreated with either tamoxifen or ICI-182,780 using Fura-2 AM dye as previously described. Fluor-
escence was recorded using Hitachi fluorescence spectrophotometer (model F-2500) and intracellular calcium levels were obtained using FL Solutions software (Florida software Solutions, Chennai, India) (22).

**ANS (1-anilino-8-naphthalene sulfonate) binding**

Effect of tamoxifen on platelet membrane microenvironment was studied by labelling the cells with anionic fluorescent probe ANS. Labelling was done by ANS (5 μM final concentration) to platelet suspension (0.5 x 10⁹/ml) at room temperature (25). Fluorescence emission spectra were recorded at 30°C at excitation of 380 nm using FL Solutions software.

**In vitro model of thrombosis by dynamic flow chamber assay**

Washed human platelets were rendered fluorescent by incubation with Calcein-AM (2 μg/ml) at 37°C for 15 min and resuspended in homologous platelet-poor plasma (PPP). Type I collagen-coated glass coverslips were assembled in a parallel plate flow chamber (GlycoTech, Raleigh, NC, USA). Chamber was mounted on the stage of an inverted epifluorescence videomicroscope (Nikon, model Eclipse Ti-E) equipped with monochrome CCD cooled camera. Platelets were perfused with help of a syringe pump (Harvard Apparatus, Holliston, MA, USA, Infusion/Withdraw Pump 22) through the chamber at a constant flow rate to yield wall shear rate of 1,500 sec⁻¹ (15 dynes/cm²). Images were digitised with DS-Qi1MC digital camera using NIS-Elements AR imaging software (Nikon). Under high shear conditions control platelets adhered to collagen fibres and formed aggregates within 2 min, which consistently grew into larger thrombi by the end of the perfusion period of 6 min. Movie data were converted into sequential photo images and thrombus growth was evaluated in two dimensions by measuring the percentage of area covered by adherent fluorescent platelets at 6 min using NIS-Elements AR imaging software (Nikon).

**Tail bleeding assay**

Three millimeter segments of the tail tip were cut off with a scalpel in anaesthetised mice following 30 min of intravenous injections of tamoxifen (2.5 mg/kg) or vehicle (DMSO). Tail bleeding was monitored by gently absorbing the bead of blood with a filter paper at 15 second (sec) intervals, without touching the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. The experiment was stopped after 15 min. Animal studies were carried out strictly as per the recommendations of the “Laboratory Animals Division”, Central Drug Research Institute, India, and the “Laboratory Animal Welfare Committee” of the University.

**Intravital microscopy of thrombus formation in FeCl₃-injured mesenteric arterioles**

Intravital microscopy was performed as described previously (26). Platelets were isolated from mice blood and fluorescently labelled with Calcein-AM (2.5 μg/ml). Recipient mice (intravenously administered with either DMSO or 2.5 mg/kg tamoxifen 10 min prior to experiment) were anesthetised with Avertin (300 mg/kg). Labelled platelets were infused through the retro-orbital plexus and the mesentery was exteriorised through a midline abdominal incision. Mesenteric arteriole was visualised at 10X with inverted epifluorescence videomicroscope (Nikon, model Eclipse Ti-E) equipped with monochrome CCD cooled camera (DS-Qi1MC). Injury was induced to arteriole by topical application of 20% FeCl₃-saturated filter paper for 5 min Digital images of adhesion and aggregation of fluorescently labelled platelets were recorded for 20 min and analysed using NIS-Elements AR imaging software (Nikon).

**Electron microscopy**

Different platelet samples, either with or without tamoxifen pre-treatment, were fixed in Karnovsky fixative followed by postfixation in osmium tetroxide (1% solution) and dehydrated in ascending grades of acetone. For SEM, dehydrated samples were critical point dried followed by mounting on an aluminium stub with adhesive tape and sputter-coated with colloidal gold. Specimens were viewed under a Leo 435 VP scanning electron microscope at an operating voltage of 15 kV. For TEM, blocks were prepared as previously described (27, 28). Ultrathin sections (60-70 nm thick) were made with an ultramicrotome (Leica, EM UC6). Sections were contrasted with uranyl acetate and alkaline lead citrate. Specimens were mounted on Formvar-coated grids and viewed under Fei Morgagni 268 (D) digital transmission electron microscope at 120 kV using image analysis software from Soft Imaging System GmbH (Münster, Germany). The final magnifications were derived from the photo micrographs and the scale bars determined.

**Statistical methods**

Standard statistical methods were used. Parametric methods (t-test) were used for evaluation and significance tests were considered significant at p-value of less than 0.05 (two-tailed tests). Data are presented as means ± standard deviation (SD) of at least five individual experiments from different blood donors. Immun-
Results

Tamoxifen inhibits platelet aggregation and secretion

Thrombin-induced platelet aggregation was inhibited by 2 min pre-incubation with either tamoxifen (Fig. 1A) or 4-OH tamoxifen (Fig. 1B) in a concentration-dependent manner. Effect of tamoxifen and 4-OH tamoxifen was studied at concentrations of the drug ranging from 0.1 to 100 μM. Tamoxifen at 5 μM inhibited platelet aggregation by nearly 13%, while aggregation was completely inhibited by 75 μM drug (IC50 = 25 μM) and 4-OH tamoxifen inhibited the platelet aggregation with an IC50 of 18 μM (Fig. 1C). Inhibition was observed irrespective of duration of incubation with drugs (up to 30 min), or whether platelets were aspirinised or not. As less than 10% of administered tamoxifen is metabolised to its 4-hydroxy derivative (29), platelets were subsequently incubated with 2.5 μM 4-OH tamoxifen and an inhibition by 10% (n=10) was observed. Aggregation induced by other physiological agonists like ADP or collagen was also found to be significantly retarded by the drug (data not shown). Similar observation was recorded when resuspended platelets were substituted with platelet-rich plasma (PRP) (not shown).

Further to check whether the inhibitory effect of tamoxifen was through its interaction with platelet-specific estrogen receptors (ER), we incubated the cells with 10 μM ICI-182,780, a specific antagonist of the receptor (30) followed by treatment with tamoxifen or metabolite. Extent of tamoxifen-mediated inhibition was further enhanced, rather than suppressed, in presence of ICI-182,780, indicating that effect of tamoxifen was either independent of or antagonistic to ER (Fig. 1D). ICI-182,780 itself did not have any effect on agonist-induced platelet aggregation even when used at five-fold higher concentration.

Platelet activation is known to be associated with secretion of contents of platelet granules. Parallel to aggregation release of adenine nucleotides from platelet dense bodies was also found to be

Figure 1: Effect of tamoxifen and its metabolite on thrombin (1 U/ml)-stimulated platelet aggregation and secretion. Platelet aggregation was measured as percent change in light transmission, where 100% refers to transmittance through blank. ATP secretion was measured with luciferase/luciferin-based reaction. Luminescence generated by platelet-secreted ATP was monitored using Lumi-Aggregometer in parallel with aggregation measurement. A) Tracings 1–4 denote aggregation in presence of DMSO (vehicle) or 10, 25 and 50 μM tamoxifen, respectively. Tracings 1’–4’ denote corresponding secretion. B) Tracings 1–4 represent aggregation in presence of DMSO, or 4-OH tamoxifen (2. 5, 25 and 50 μM), respectively. Tracings 1’–4’ denote corresponding secretion. C) Derivation of IC50 of tamoxifen and 4-OH tamoxifen for thrombin stimulated platelet aggregation. X-axis, logarithm of tamoxifen and 4-OH tamoxifen concentrations used in the study (0.1, 1, 5, 10, 25, 50, 75 and 100 μM); Y-axis, percentage of inhibition in aggregation. D) Tracings 1–4 represent aggregation in presence of DMSO, 10 μM ICI-182,780, 25 μM tamoxifen and combination of ICI-182,780 (10 μM) and tamoxifen (25 μM), respectively.
inhibited by tamoxifen (Fig. 1A) and 4-OH tamoxifen (Fig. 1B) in a concentration-dependent manner in thrombin-stimulated platelets. At 50 μM tamoxifen caused almost complete inhibition of ATP secretion while a partial inhibition was observed at 10 μM.

As tamoxifen inhibited dense body secretion, we subsequently asked whether it could also prevent release of platelet alpha granule contents. P-selectin (CD62P) is a transmembrane glycoprotein found in platelet alpha granules, which translocates to surface membrane upon platelet stimulation. As expected a significant increment in P-selectin exposure was observed in thrombin-treated platelets in comparison to resting cells. However, when cells were stimulated with thrombin in presence of tamoxifen (25 μM), P-selectin expression was significantly impaired (by 73 ± 3%, n=5) compared to untreated cells (Fig. 2A). A similar effect was also observed on P-selectin expression in 4-OH tamoxifen (2.5 μM) pre-treated thrombin-activated platelets (n=5) (Fig. 2A).

A useful tool for detecting activated integrin αIIbβ3 is mAb PAC-1, which selectively recognises the high-affinity conformation of the integrin (31). As expected, thrombin stimulation significantly enhanced binding of PAC-1 to platelet surface (Fig. 2B). Preincubation with tamoxifen (25 μM) led to significant drop (by 67 ± 4%, n=5) in PAC-1 binding (Fig. 2B). The metabolite 4-OH tamoxifen (2.5 μM) had similar effect on PAC-1 binding in thrombin-activated platelets (n=5) (Fig. 2B). Thus, inhibition of alpha / dense granule secretion, and prevention of integrin conformational changes leading to lack of ligand (fibrinogen and PAC-1) binding were consistent with tamoxifen-mediated modulation of inside-out signalling in platelets in a concentration-dependent manner.

Further to check whether tamoxifen also affects post-integrin αIIbβ3 outside-in signalling, we investigated its effect on platelet-mediated retraction of fibrin clot and platelet spreading on immobilised matrix. Clot was induced to form by addition of thrombin to platelet suspension in presence of fibrinogen. Contractile apparatus in activated platelets progressively compacted bulk of the clot through interaction between integrin αIIbβ3 and fibrin (32–34). Pretreatment of platelets with increasing concentration of tamoxifen led to progressive inhibition in the extent of retraction. Up to 50% (n=5) inhibition was observed in presence of 25 μM tamoxifen, whereas 5% (n=5) inhibition was induced by 2.5 μM 4-OH tamoxifen (Fig. 3A and B).

Tamoxifen inhibits static adhesion and spreading

Adhesion of platelets to immobilised fibrinogen or collagen leads to extensive cytoskeletal reorganisation, resulting in extension of filopodia and lamellipodia and cells progressively assuming spread morphology. We next investigated the effect of tamoxifen (25 μM) and 4-OH tamoxifen (2.5 μM) on platelets charged on to different immobilised matrices (fibrinogen, collagen and poly-L-lysine). Control cells were found to adhere to the matrices, followed by spreading (studied after 30 min). Tamoxifen pretreatment significantly reduced (by 76 ± 7%, p=0.003, n=5) the number of cells adhered on to fibrinogen (Fig. 3C and D) or collagen (not shown) and inhibited spreading of platelets on either matrix. Similar results were obtained when 4-OH tamoxifen-pretreated
platelets were allowed to adhere (data not shown). Adhesion and spreading of platelets to poly-L-lysine was unaffected irrespective of drug treatment (data not shown).

In order to study organisation of actin-based cytoskeleton platelets were labelled with phalloidin-FITC for 1 hour at room temperature and allowed to adhere and spread on immobilised fibrinogen. As expected, there was increase in F-actin expression in spread platelets, which exhibited greater staining with fluorescently labelled phalloidin. Tamoxifen (25 μM) was found to significantly suppress the rise in platelet F-actin (by 85 ± 4%, n=5) (Fig. 3B).

**Effect of tamoxifen on platelet tyrosine phosphoproteome**

Phosphorylation of specific proteins on tyrosine is a hallmark of platelet activation. We studied profile of tyrosine phosphorylated proteins in tamoxifen (25 μM)-pretreated cells. As expected, several phosphorylated protein bands were observed in platelets aggregated with thrombin under stirring condition (Fig. 4A, lane 1). Tamoxifen pretreatment partially prevented aggregation of platelets (Fig. 1A). In parallel, it elicited significant phosphorylation at 50 and 70 kDa regions, associated with dephosphorylation of 65, 90, 100 and 110 kDa peptides (Fig. 4A, lane 2). Tamoxifen was found to have no significant effect on tyrosine phosphoproteome in resting platelets (Fig. 4B, lanes 1 and 2), or in platelets stimulated in absence of stirring (Fig. 4B, lanes 3 and 4).

**Tamoxifen potentiates thrombin-induced rise in platelet intracellular calcium**

As Ca²⁺ is a critical regulator of intracellular signalling, we next studied the effect of tamoxifen on level of platelet cytosolic Ca²⁺. Thrombin evoked an initial rise in [Ca²⁺], followed by a plateau. Pretreatment of platelets with tamoxifen (25 μM) further potentiated the increase in [Ca²⁺] (Fig. 4C). In subsequent experiments...
we pretreated platelets with the ER antagonist, ICI-182,780, followed by incubation with tamoxifen and then stimulation with thrombin. ICI-182,780 alone did not affect thrombin-induced changes in intracellular Ca\(^{2+}\). When treated in combination with tamoxifen ICI-182,780 did not inhibit, rather marginally potentiated, the rise in [Ca\(^{2+}\)], suggesting that effect of tamoxifen was either independent of or antagonistic to ER. 4-OH tamoxifen (2.5 \(\mu\)M) elicited changes similar to tamoxifen (not shown).

**ANS binding**

To check the effect of tamoxifen on physical microenvironment of platelet membrane, we studied affinity of ANS, an anionic fluorescent probe, to platelet membrane in presence and absence of the drug. Upon binding to the membrane emission peak of ANS was blue-shifted from 520 to 480 nm accompanied by intensity enhancement. Pretreatment of platelets with tamoxifen elicited progressive increase in fluorescence intensity in a dose-dependent manner, emission maximum remaining unchanged (►Fig. 4D).

**Tamoxifen inhibits stable thrombus formation under flow**

Control human platelets adhered to collagen fibres within 2 min under high shear conditions resembling arterial flow (1,500 sec\(^{-1}\)). Adhered cells progressively recruited free circulating platelets from surrounding to form larger aggregates by end of 6 min. In marked contrast, tamoxifen-treated platelets were consistently unable to attach firmly to the matrix, which resulted in release of adhered cells within few seconds of attachment. Drug-treated platelets thus exhibited significantly less adhesion (by 70 ± 5%, n=5) to immobilised collagen under flow (►Fig. 5A).
Figure 5: Effect of tamoxifen on platelet thrombus formation. A) Effect of tamoxifen on platelet thrombus formation under high shear arterial flow condition. Calcein-AM-stained platelets were treated either with vehicle, DMSO (control) (a-c) or 25 μM tamoxifen (d-f) and thrombus formation was observed on immobilised collagen at different time points as indicated. B) Mice were intravenously administered with either DMSO (a-c) or tamoxifen (2.5 mg/kg) (d-f) 10 min prior to the experiment. Mesenteric arterioles were injured with FeCl₃. Thrombus formation of fluorescently labelled platelets was monitored in vivo by intravital fluorescence microscopy at different time points as indicated. Scale bars, 50 μm. Data are representative of five different experiments.

Tamoxifen enhances bleeding time in mice

To investigate effect of tamoxifen on primary haemostasis in vivo, we analysed tail bleeding time in mice following intravenous administration of tamoxifen (2.5 mg/kg). In control animals bleeding ceased within 180 sec, whereas it was extended up to 600 sec in tamoxifen-administered mice. Blood platelet counts before and after administration of tamoxifen remained the same.

Tamoxifen inhibits in vivo thrombus formation

To investigate the effect of tamoxifen on thrombus formation in vivo, we monitored platelet accumulation at sites of FeCl₃-induced mesenteric arteriole injury using intravital fluorescence microscopy. In control mice platelets rapidly adhered to the site of endothelial desquamation and formed small thrombi within 10 min, which increased progressively leading to vessel occlusion up to 60% within 20 min. Remarkably, thrombus formation in tamoxifen-administered mice was completely prevented during 20 min period of observation (Fig. 5B). Although platelets transiently adhered to endothelium, there were no stable thrombi, reflective of significant antithrombotic effect of tamoxifen under physiological milieu.

Electron microscopy

Effect of tamoxifen on ultrastructural details of platelets was subsequently investigated. As examined under transmission electron microscope, spherical resting platelets were characterised by centrally localised dense granules, α-granules and scattered vacuoles. Thrombin induced activation of platelets with characteristic well developed hyaloplasmic processes (pseudopods), highly scattered dense granules, and constricted vacuolar spaces. In tamoxifen-pre-treated thrombin-activated platelets most of the granular and va-
cional spaces were retained with fewer hyaloplasmic processes reflective of reduced level of activation (Fig. 6A).

Scanning electron microscopy demonstrated protrusion of actin-rich filopodial and lamellipodial extensions (E) from surface of thrombin-stimulated platelets attributable to extensive cytoskeletal reorganisation in cells. Tamoxifen pretreatment resulted in marked inhibition in platelet morphological changes and less developed extensions (Fig. 6B). However, only tamoxifen treatment induced minor shape change in platelets, as observed from both scanning as well as transmission electron microscopy.

**Discussion**

Platelets express both ER-α and -β on their surface membranes (35, 36). The receptors consists of five functional domains including the conserved ligand-binding domain (LBD) that interacts with estrogen, tamoxifen and other structural isoforms. ER-α and -β differ markedly in respective ligand affinities (37) and structure of constituent AF-1 and D domains (38, 39). Platelets being enucleate cells, expression of these receptors is consistent with possible non-genomic effects of estrogen. Several downstream signalling pathways are known to be linked to ER activation. Besides interacting with transcription factors, activator protein-1 and Sp-1 (40), ER links to nitric oxide synthase in endothelial cells through striatin protein and caveolin-1 (41). The endogenous membrane receptor assembles as part of a large signosome complex that includes G proteins, receptor tyrosine kinases, and non-receptor tyrosine kinases, such as Src (42). In a rodent model Leng et al. showed that the effect of estrogen on platelet activation is influenced by the type of estrogen, route of administration and the platelet agonist used for the experiment (43). The major estrogen 17β-estradiol was shown to potentiate thrombin-induced platelet stimulation through ER-βand activation of Src kinase (44). SERMs comprise a heterogeneous group of agents, which preferentially bind to LBD of either of α- or β-subtypes of the receptor and modulate their functions. Different combinations of ER may respond differentially to various ligands that may translate into tissue-specific agonistic or antagonistic effects (45). ICI 182,780, a non-SERM ER antagonist, inhibits ER by binding to its LBD. There are also reports to suggest a direct effect of ICI 182,780 on cells independent of ER ligation (46–48). Contrary to a recent report by Chang et al. (18), we observed marginal potentiation of inhibitory effect of tamoxifen when platelets were pretreated with ICI 182,780, suggesting that the effect of tamoxifen was either independent of or antagonistic to ER. Contradiction between the two reports may be explained from different agonists used in these studies and/or estrogen-independent effects of ICI 182,780.

Tamoxifen belongs to the groups of SERMs, which has widespread use in the treatment and prophylaxis of hormone-dependent cancers (49, 50). The drug has also been proposed to have cardiovascular benefits (51) and at higher doses it has potential therapeutic applications against neurological disorders (11–14). Contrarily, infrequent adverse effects like retinopathy, optic neuritis (52) and induction of endometrial cancer (53) have been reported with high dose tamoxifen therapy administered for longer duration. Tamoxifen treatment, when combined with adjuvant chemotherapy, has been reported to occasionally associate with the risk of venous thrombosis (54), attributable to lower antithrombin and protein S levels in plasma (55). The drug had no significant effect on platelet aggregation at 1 μM, the concentration achievable in blood upon therapeutic administration, whereas its metabolite 4-OH tamoxifen was proaggregatory at 100 nm (20). In the

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**Figure 6:** Electron microscopy of tamoxifen treated platelets. A) Transmission electron micrographs through sections of resting, tamoxifen (25 μM)-treated, thrombin-activated and tamoxifen (25 μM)-pretreated thrombin-activated platelets. (a) Resting platelets showing dense granules (DG) (black bold arrows) and vacuoles (V) (white arrows). (b) Tamoxifen (25 μM)-treated platelets showed the similar ultra-structural organisation as resting platelets. (c) Thrombin-activated platelets with hyaloplasmic processes (HP) (black thin arrows), highly scattered dense granules (DG), and constricted vacuoles (V). (d) Thrombin-activated platelets pretreated with tamoxifen showed the retained vacuolar spaces (white arrow) with less hyaloplasmic processes. B) Scanning electron micrographs of platelets. (a) Resting platelets, (b) Tamoxifen (25 μM)-treated platelets (c) Thrombin-activated platelets, and (d) Tamoxifen (25 μM)-pretreated thrombin-activated platelets. E (black arrows) refers to filopodial extensions.
present study we have reported significant inhibition of platelet aggregation by tamoxifen in a concentration-dependent manner, with 13% inhibition recorded at drug concentration as low as 5 μM (IC₅₀ = 25 μM) (Fig. 1C). The metabolite 4-OH tamoxifen, too, effectively attenuated agonist-induced platelet aggregation. In agreement with these observations, release of contents of platelet alpha granules and dense bodies, as well as binding of PAC-1 to platelet surface integrin αIIbβ₃, were prevented by tamoxifen at similar concentrations. Platelet adhesion and spreading on immobilised matrices (collagen and fibrinogen) and retraction of fibrin clot were also suppressed by tamoxifen, indicating that the drug can attenuate both inside-out and outside-in signalling in platelets leading to platelet inhibition. Enhanced binding of ANS to platelet membrane was further suggestive of perturbation of membrane microenvironment in presence of drug. As 17 β-estradiol has been reported to potentiate thrombin-induced platelet activation, tamoxifen could attenuate agonist-mediated platelet responses by inhibiting ER. In a recent study inhibition of collagen-induced platelets by tamoxifen was attributed to ER-independent modulation of PLC-γ1- Ca²⁺-PKC-MAPK pathway (18). In different cells tamoxifen has been shown to modulate signalling parameters ranging from regulation of cAMP (56), inhibition of activities of protein kinase C (57), phospholipase D (58) and PI3K/Akt (59), to alkalinisation of cells and regulation of cholesterol metabolism through microsomal anti-estrogen binding sites (46). Possibility of such parallel signalling inputs could, too, contribute to the observed anti-platelet effect of tamoxifen.

Consistent with platelet inhibition by the drug from in vitro studies, intravenous administration of tamoxifen was found to be associated with prolonged tail bleeding in mice, indicating that the drug adversely affected primary haemostasis in absence of any effect on platelet count. Adhesion of platelets to collagen under flow is a process driven mainly by glycoprotein (GP)Ibα, GPVI and integrins αIIbβ₃ and α(v)β₃. Tamoxifen significantly delayed thrombus formation under physiological arterial shear measured ex vivo by microscopy real-time imaging. Arterial thrombosis is frequently initiated by abrupt disruption of the vascular endothelium, followed by activation and aggregation of platelets on subendothelial collagens. Tamoxifen dramatically inhibited the process of thrombus formation at the site of arterial injury by FeCl₃, visualised by intravital fluorescence microscopy, which could be attributed to modulation of platelet-collagen interaction in flow in presence of the drug. Ultrastructural studies by scanning and transmission electron microscopy supported the inhibitory impact of tamoxifen on thrombin-stimulated platelets. Thus, tamoxifen appeared to inhibit all major platelet functions in vitro, ex vivo, and in vivo, and may find potential therapeutic and/or preventive applications in arterial thrombotic disorders.

We observed potentiation of thrombin-induced rise in platelet [Ca²⁺] by the drug. In agreement with this, earlier studies have demonstrated enhanced [Ca²⁺] mobilisation by tamoxifen in platelets stimulated with thrombin (30), Chinese hamster ovary cells (60) and cancer cell lines (61). Although inhibitory action of tamoxifen on platelet aggregation contradicts rise in [Ca²⁺], similar observation has also been documented with plant estrogen ferulic acid, which mobilised intracellular calcium in platelets without inducing platelet aggregation (62). Contrarily, a recent report by Chang et al. (18) observed suppression of platelet cytosolic Ca²⁺ by tamoxifen, which could be attributed to collagen, a different agonist, used by these authors. Tamoxifen has been reported to inhibit activity of protein kinase C (PKC) (57), which is a critical regulator of platelet functions. Tamoxifen also increases level of cAMP by inhibiting calmodulin-dependent cAMP phosphodiesterase (63). cAMP is known to antagonise agonist-mediated platelet activation by maintaining the cells in resting, unstimulated state. As both these enzymes function downstream of intracellular Ca²⁺, this could explain the inhibitory effect of drug on platelet functions despite rise in cytosolic calcium. Pretreatment with tamoxifen altered the profile of tyrosine phosphorylated proteins in aggregated platelets. This phosphoproteome remains to be characterised and is a subject of future research.

The therapeutic dose of tamoxifen administered in patients of breast cancer is within the range 20–40 mg/day. Higher doses of tamoxifen have been found to be effective against brain tumours in various clinical trials (12–14), where vomiting, anorexia and thromboembolism were the only reported adverse events. Incidence of deep-vein thrombosis in these studies was lower than that in the brain tumour patients not receiving tamoxifen (13). Furthermore, 200 mg/m² of tamoxifen has also been effectively used against melanoma (64) and 160 mg/m² for metastatic breast cancer (65) in clinical trials without any aggravation of thrombotic risk. These observations led us to reason that the relatively higher dose of tamoxifen might in fact prevent thrombotic events, as established in the present study. Dosage as high as 400 mg/m² of tamoxifen with peak plasma concentrations close to 10 μM have been successfully administered for treatment of epithelial tumours with negligible side effects (66). We have observed significant inhibition of platelet aggregation at this drug concentration in vitro. Intracellular calcium concentration ([Ca²+]i) measured by Fura-2, and calcium induced calcium mobilization ([Ca²+]i release) measured by Fluo-3, are reported in the text.
venous tamoxifen at 2.5 mg/kg in mice, which yields an approximate peak serum concentration of 1.4 μM (67), showed significant in vivo anti-thrombotic activity in our study. Thus, concentrations of tamoxifen with significant anti-platelet and anti-thrombotic activity can be achieved inside the human body with minimal and no major side effects.

Taken together, the results suggest that tamoxifen can modulate intracellular signalling in platelets to bring about significant inhibition in cell reactivity and thrombus generation and thus holds promise as a potential antiplatelet/antithrombotic drug or supplement to the existing anti-thrombotic drug regimes. Reports of occasional thrombocytopenia with tamoxifen therapy may be attributable to shortening of platelet life span (68). Thus, possibility of bleeding complications with use of the drug should be critically evaluated. Incidence of cardiovascular disorders is higher among postmenopausal women than premenopausal women (69). As endometrial cancer is less common among postmenopausal women and does not exist in men, these populations can be ideal candidates for preventive or therapeutic use of tamoxifen in clinical situations associated with arterial thrombosis.

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Conflict of interest

None declared.

References

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Abbreviations

SERM, selective estrogen receptor modulators; DMSO, dimethylsulfoxide; ADP, adenosine diphosphate; PRP, platelet-rich plasma; ANS, 1-anilino-8-naphthalene sulfonate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid.
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