UNFOLDED PROTEIN RESPONSE IS REQUIRED IN NU/NU MICE MICROVASCULATURE FOR TREATING BREAST TUMOR WITH TUNICAMYCIN


§Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, San Juan, PR 00936-5067; ‡Department of Molecular and Cellular Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030; †Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University and Hospital, Taiching, Taiwan 404; *Molecular Gastroenterology and Pancreatic Cancer Research Unit, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS 66160-7350; #Department of Anatomy and Cell Biology, School of Medicine, Universidad Central del Caribe, Bayamon, PR 00960-3001; and ¶Institute of Functional Nanomaterials, University of Puerto Rico, Rio Piedras Campus, San Juan, PR00931-1907. USA

1To whom correspondence should be addressed: Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, San Juan, PR 00936-5067. USA; Tel.: (787) 758-2525 Ext. 1624; Fax: (787) 274-8724; Email: dipak.banerjee@upr.edu

Running Title: Tunicamycin Inhibits Breast Tumor Growth
ABSTRACT

Upregulation of dolichol-pathway, a “hallmark” of asparagine-linked protein glycosylation enhances angiogenesis in vitro. The dynamic relationship between these two processes is now evaluated with Tunicamycin. Capillary endothelial cells treated with Tunicamycin were growth inhibited and could not be reversed with exogenous VEGF165. Inhibition of angiogenesis is supported by down-regulation of (i) phosphorylated VEGFR1 & VEGFR2 receptors; (ii) VEGF165-specific phosphotyrosine kinase activity; and (iii) Matrigel™ invasion and chemotaxis. In vivo, Tunicamycin prevented the vessel development in Matrigel™ implants in athymic Balb/c (nu/nu) mice. Immunohistochemical analysis of CD34 (p<0.001) and CD144 (p<0.001) exhibited reduced vascularization. A 3.8-fold increased expression of TSP-1, an endogenous angiogenesis inhibitor in Matrigel™ implants correlated with that in Tunicamycin (32 h) treated capillary endothelial cells. Intravenous injection of Tunicamycin (0.5 mg/Kg to 1.0 mg/Kg) per week slowed down a double negative (MDA-MB-435) grade III breast adenocarcinoma growth by ~50-60% in three weeks. Histopathological analysis of the paraffin sections indicated significant reduction in vessel size, the microvascular density and tumor mitotic index. Ki-67 and VEGF expression in tumor tissue were also reduced. A significant reduction of N-glycan expression in tumor microvesSEL was also observed. High expression of GRP-78 in CD144 positive cells supported unfolded protein response-mediated ER stress in tumor microvasculature. ~65% reduction of a triple negative (MDA-MB-231) breast tumor xenograft in one week with Tunicamycin (0.25 mg/Kg) given orally and absence of systemic and/or organ failure strongly supported Tunicamycin’s potential for a powerful glycotherapeutic treating breast cancer in the clinic.

INTRODUCTION

Breast cancer is becoming an important public health issue globally. The etiology of breast cancer is complex but this hyperproliferative disorder involves morphological and cellular transformation, dysregulation of apoptosis, uncontrolled cellular proliferation, invasion, metastasis, and angiogenesis (1-3). In malignant breast tissue, the intratumoral endothelial cell proliferation rate is higher than that of the surrounding breast (4). The central importance of angiogenesis and the current understanding of blood vessel formation have led to therapies to interrupt this process. Unfortunately, none of these therapies targeting different areas of vasculature could cure breast cancer and, therefore, the need for developing new therapeutics has emerged.

Asparagine-linked (N-linked) glycoproteins have been found to play an important role in angiogenesis (5-11). We have observed that proliferation of capillary endothelial cells as well as the capillary lumen formation is upregulated when the cells are treated with 8Br-cAMP (9,12,13). Such treatment increases mannosylphospho dolichol synthase (DPMS) activity increasing the synthesis and turnover of Glc₃Man₉GlcNAc₂-PP-Dol (lipid-linked oligosaccharide, LLO) and consequently the N-glycosylation of Factor VIIIC (eFVIIIc); enhancing angiogenesis (14). cDNA cloning of the Dpm1 gene identifies a cAMP-dependent protein kinase (PKA) mediated phosphorylation motif in the capillary endothelial cell DPMS (15, GenBank #GQ367549) as reported earlier for Dpm1 from yeast (16) and other organisms (17). Tunicamycin, a glucosamine-containing pyrimidine nucleoside (and an antibiotic) inhibits protein N-glycosylation by blocking the LLO synthesis in the endoplasmic reticulum (ER; 18). Consequently, the glycoproteins are not folded and caused an accumulation of mis- or unfolded glycoproteins in the ER. This saturation of the ER folding capacity induces ER stress and activates ER-specific adaptive response, the unfolded protein response (upr; 19). The underlying mechanism of upr-induced ER stress could indirectly impede cell cycle progression by interfering with the proper maturation of growth factor receptors or other modulators of mitogenic signaling. Alternatively, ER stress may directly induce checkpoint response and prevent cells from completing their cell division cycle (20). Recently, we have observed that Tunicamycin treatment causes cell cycle arrest in G1 and induces apoptosis of capillary endothelial cells while the cells are still in G1 (6). The present study demonstrates that...
Tunicamycin (i) inhibits angiogenesis in vivo and reduces a double (ER/PR/EGFR) and a triple-negative (ER/PR/EGFR) breast tumor growth in nude mice; and (ii) the inhibition of angiogenesis is stable under tumor microenvironment as it is irreversible by exogenous VEGF165.

**EXPERIMENTAL PROCEDURES**

**Materials** - Hydroxyurea, dimethylsulfoxide, nystatin, Tunicamycin and heparin were obtained from Sigma Aldrich (St. Louis, MO). Matrigel™, mouse monoclonal antibody for CD144 and actin were from BD-Bioscience (San-Diego, CA). Mouse monoclonal antibody for VEGF, rabbit polyclonal phospho VEGFR1 and goat polyclonal total VEGFR1 antibodies were from Oncogene (San Diego, CA). Recombinant VEGF165, rabbit polyclonal phospho VEGFR2 and total VEGFR2 antibodies were from Calbiochem (EMD Biosciences; La Jolla, CA). Mouse monoclonal antibody for CD34 was from Cell Science (Canton, MA) and mouse monoclonal antibodies for Ki-67 and thrombospondin were from Abcam (San Francisco, CA). Rabbit polyclonal antibody for GRP-78 was from Santa Cruz Biotechnology (Santa Cruz, CA). WGA (Texas-Red conjugated) was from EY Laboratories (San Mateo, CA). HRP-conjugated goat anti-rabbit IgG/anti-mouse IgG, streptavidin and ECL chemiluminescence detection kit were from GE Healthcare (Piscataway, NJ). TRIzol was from Invitrogen Life Technologies (Carlsbad, CA). PCR grade water and DNA decontamination kit were from Applied Biosystems (Ambion Inc; Austin, TX), iScript c-DNA synthesis kit, biotinylated protein molecular weight markers, DNA markers and all electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals and solvents used were of the highest purity available. All cell culture wares were from Sarstedt (Newton, NC) and fetal bovine serum was purchased from HyClone Laboratories (Logan, UT).

**Cell line and Animals** - Human breast carcinoma cells MDA-MB-231 and MDA-MB-435 were from the American Type Culture Collection (ATCC; Manassas, VA). The capillary endothelial cells were from the laboratory stock of a non-transformed endothelial cell line (21). Athymic Balb/c female (nu/nu) mice were from Charles River Laboratories (Wilmington, MA).

**Culturing of Capillary Endothelial Cells and Proliferation Assay** - The capillary endothelial cell stock was maintained as previously described (21, 22). For proliferation assay 1x10⁴ cells/ml/well were plated in triplicate in 24-well plates and synchronized (13) followed by culturing in EMEM containing 2% fetal bovine serum (heat inactivated) in the presence or absence of Tunicamycin (1 µg/ml). Viable cells were counted every 24 h.

**Analysis of Phosphotyrosine Kinase (PTK) Activity by Enzyme Linked Immunosorbent Assay (ELISA)** – Protein tyrosine kinase activity (23) was measured in a Ray Bio® (RayBiotech; Norcross, GA) cell based phosphotyrosine ELISA kit in synchronized cells treated with Tunicamycin (1 µg/ml) for 3 h in triplicate and following incubation with VEGF165 (10 ng/well) for 10 min prior to Tunicamycin addition. CBO-II (50 µM), a VEGF receptor inhibitor was added 30 min prior to the addition of VEGF165.

**SDS-PAGE and Western Blot Analyses** - Performed as before (13) with 7.5% gel concentration.

**Quantitative Real-time PCR (qRT-PCR)** - (a) Isolation of RNA: Total RNA was isolated in TRIzol, treated with DNase and quantified in a NanoDrop Bioanalyzer. The integrity of RNA was verified by agarose gel electrophoresis followed by ethidium bromide staining. (b) qRT-PCR: Total RNA (2 µg) was used to generate cDNA using the iScript™ cDNA synthesis kit. qRT-PCR was performed in an iCycler (Bio-Rad Laboratories) using iQ™ SYBR® Green Supermix for the double-stranded DNA. After optimizing the PCR conditions, reactions with SYBR® Green PCR master mix, forward/reverse primers (10 µM) and cDNA (100 ng) were performed. PCR parameters used were 95°C for 3 min (required for iTaq™ DNA polymerase activation), 40 cycles at 95°C for 10 sec and 1 min at 50°C (annealing). The PCR products were confirmed by agarose gel electrophoresis. The quantification of the target gene expression relative to the total DNA was carried out by melting curve analysis and calculated by ΔCt = Ct_housekeeping – Ct_target gene. The changes of target gene expression in Tunicamycin treated cells compared with the control are reported as ΔΔCt = ΔCt_control – ΔCt_Tunicamycin where the Ct_target and Ct_GAPDH are the fractional cycle
number at which fluorescence generated by reporter dye exceeded the fixed level above base line for target and house keeping genes. The relative expression of the target gene in the control and Tunicamycin treated samples was calculated as $2^{(\Delta \Delta \text{Ct})}$. Each sample was run three times.

The PCR primer pairs used were: mouse thrombospandin (forward) 5′-TGCGATGATGACGATGAC-3′; (reverse) 5′-CAGGGTTTGTTGTAGG-3′; mouse GAPDH (forward) 5′-GCCTTCCGTGTGGTTGTAGG-3′; (reverse) 5′-TCTTGCTCAGTGTCTTGC-3′; bovine thrombospandin (forward) 5′-GCCGATTCCAGATGATTC-3′; (reverse) 5′-CATAACCTACAGCAAGTCC-3′; and bovine GAPDH (forward) 5′-TGACCCCTTCATTGACCTTC-3′; (reverse) 5′-GATCTCGCTCCTGGAAGATG-3′.

Matrigel™ Invasion Assay and Chemotaxis - BD BioCoat Matrigel™ invasion chambers were used according to the manufacturer’s instruction. The conditioned media from MCF7 cells containing 10% fetal bovine serum and VEGF165 (10 ng) were placed in lower chambers as a chemoattractant. Tunicamycin (1 μg/ml) treated or untreated capillary endothelial cells (1 x 10⁵) in serum-free medium containing 0.1% bovine serum albumin were seeded in the upper chamber of the Matrigel™-coated (growth factor reduced) transwell. After incubating for 24 h at 37°C in a CO₂ incubator, non-migrating cells from the upper surface of the membrane were removed with a cotton swab. The cells invaded to the lower side of the membrane were fixed, stained with H & E and quantified by counting in five fields under a Zeiss optical microscope (200x). The chemotaxis was performed as described before (24).

In Vivo Matrigel™ Invasion Assay and Chemotaxis - BD BioCoat Matrigel™ invasion chambers were used according to the manufacturer’s instruction. The conditioned media from MCF7 cells containing 10% fetal bovine serum and VEGF165 (10 ng) were placed in lower chambers as a chemoattractant. Tunicamycin (1 μg/ml) treated or untreated capillary endothelial cells (1 x 10⁵) in serum-free medium containing 0.1% bovine serum albumin were seeded in the upper chamber of the Matrigel™-coated (growth factor reduced) transwell. After incubating for 24 h at 37°C in a CO₂ incubator, non-migrating cells from the upper surface of the membrane were removed with a cotton swab. The cells invaded to the lower side of the membrane were fixed, stained with H & E and quantified by counting in five fields under a Zeiss optical microscope (200x). The chemotaxis was performed as described before (24).

Histological Staining - The mice were killed 10 days post implantation. Matrigel™ plugs were removed, fixed in 10% neutral formalin, and embedded in paraffin. Breast tumor tissue from nude mice was also processed similarly. 5 μm sections were collected on glass slides, stained with Haematoxylin and Eosin (H & E), and analyzed microscopically (Axioskop 2, Carl Zeiss, Germany).

Immunohistochemistry and Immunofluorescence Microscopy - For microvessel counting, the Matrigel™ sections were stained immunohistochemically with anti-CD34 and anti-CD144 antibodies using Histomouse™ Max kit (Zymed Laboratories). A similar protocol was used to detect Ki-67 and VEGF expression in breast tumor tissue sections. De-paraffinized sections were treated with 0.3% hydrogen peroxide (10 min) at room temperature, washed with PBS, pH 7.4 (3 x 3 min each) and blocked with solutions A and B for 30 min and 10 min, respectively. The sections were incubated at room temperature with anti-CD34, anti-CD144, anti-Ki-67, anti-VEGF and anti-GRP-78 antibodies, respectively, in a moist chamber for 3 h. After rinsing with PBS, pH 7.4 (3 x 2 min each), the sections were incubated with 100 μl HRP-conjugated rabbit anti-mouse IgG for 10 min at room temperature. The sections were rinsed three times with PBS, pH 7.4, and incubated with a mixture of chromogenic substrate (cold) for 15 min. After washing with distilled water all sections were counter stained with hematoxylin before mounting and analysis. In some experiments GRP-78 and CD144 were detected with fluorescently labeled secondary antibodies. N-glycans were detected with Texas-Red conjugated WGA.

Microvessel Counts (MVC) - MVC were analyzed using the hot spot area. The sections stained with anti-CD34 and anti-CD144 antibodies were analyzed in 3 hot spots (i.e., the highest number of vessel areas under 200x magnification). Microscopic images from each area were collected (100x/0.25 ph1 A plan) in Axioskop 2 fitted with AxioCam MRc5 camera and Axion Vision Rel 4.6 software, and recorded as the MVC (25).

Development of Breast Tumor in Nude Mice - (a) Orthotopic model: 4 x 10⁶ MDA-MB-435 (a double negative) cells (25, 26) were mixed with 0.3 ml of Matrigel™ and injected into mammary fat pads of six weeks old athymic Balb/c (nu/nu) female mice. One
week later, mice bearing the tumor were divided into five groups (10 mice per group). Mice in the treatment group received Tunicamycin intravenously (i.v.) at doses of 0, 0.1, 0.5 and 1.0 mg/Kg per week, while the mice in the control group received i.v. injection of the vehicle only. Taxol (15 mg/Kg) was used as an internal control. Tumor growth was monitored by caliper measurements of length and width for 23 days after initiating the treatment and the tumor volume was calculated. At the end, the mice were sacrificed, the excised tumor was weighed and fixed in formalin. Paraffin embedded and frozen tumor sections were analyzed histologically and immunohistochemically.

(b) Xenograft Model - 4 x 10^6 MDA-MB-231 (a triple negative breast cancer) cells mixed with 0.3 ml of Matrigel™ and injected subcutaneously (s.c.) in the hind region of the mouse. One week later, the mice bearing tumor were divided into two groups. The mice in the treatment group received Tunicamycin (5 μg/mouse ≈ 0.25 mg/Kg) orally twice a week for four weeks, while the mice in the control group received the vehicle only. The tumor growth was monitored as above.

Quantification of fluorescence images of the tumor microvessels - Tumor tissue was immunostained with Texas-red conjugated WGA and photographed at 200x magnification. The fluorescence intensity of each vessel was quantified by ImageJ version 1.39 (NIH, USA: http://rsbweb.nih.gov/ij/download.html). RGB composite images from control and Tunicamycin treated vessels were created using Axion Vision rel. 4.6 and analyzed. Vessels from five different areas were used for statistical analysis.

Statistical Analysis - Statistical analysis was carried out with Graph Pad Prism 4 software (Graph Pad Software Inc., San Diego, CA). Quantitative data are presented as mean plus standard error (SE). Mean ± SE was calculated by one-way analysis of variance (ANOVA). Significance between groups was further analyzed using the post hoc Tukey’s test.

RESULTS

Tunicamycin inhibition of capillary endothelial cell proliferation is not reversible by VEGF_{165} -
cells, i.e., “0 h”. These values for VEGFR2 were ~110.3%, ~91.8% and ~68.3% respectively for the same period. The level of phosphorylated VEGFR1 when analyzed, it was found down regulated by ~30.5%, ~33.5% and ~75.7% in cells treated with Tunicamycin for 3 h, 12 h and 32 h, respectively compared to their respective controls. The phosphorylated VEGFR2 was down-regulated by ~44.4%, and ~72.6% in cells treated with Tunicamycin for 3 h and 32 h, respectively but increased by ~3.3% in cells treated with Tunicamycin for 12 h (Fig 1C and D) compared to their controls. Interestingly, the reduction of phosphorylated VEGFR1 (i.e., ~30.5%) and VEGFR2 (i.e., ~44.4%) is serendipitously very similar to ~46.6% reduction of protein tyrosine kinase activity following VEGF stimulation in cells treated with Tunicamycin for 3 h. There was also a 1.6-2.4 fold reduction of phosphorylated VEGFR1 and VEGFR2 in cells treated with Tunicamycin for 32 h over that of 3 h. On the other hand, there was a 2.7-2.9 fold difference between the ratio of down-regulated phosphoVEGF receptors (i.e., VEGFR1 and VEGFR2) and the total VEGFR 1 and VEGFR2 at 3 h and 32 h of Tunicamycin treatment (Table 1).

Tunicamycin inhibits angiogenesis in vivo - To study the anti-angiogenic effect of Tunicamycin in vivo, we have used Matrigel™ plug assay in nude mice. Angiogenesis was induced by injecting Matrigel™ containing VEGF165. Intense vascularization was observed ten days post-implantation in Matrigel™ plugs containing VEGF165. In contrast, the plugs with Matrigel™ alone appeared pale. Tunicamycin inhibited neo-vascularization and the plugs appeared pale as well even when VEGF165 was present (Fig. 2A, right). Histological analysis of H & E stained paraffin sections also supported an increased number of vessels in the plugs containing VEGF165, as opposed to the plugs with Matrigel™ alone. Tunicamycin treatment caused considerable reduction of neo-vascularization in the plugs containing VEGF165 (Fig. 2B). Quantitative analysis supported ~75% reduction in the vessel density in Tunicamycin treated Matrigel™ plugs (Fig. 2B far right; p<0.001).

Decreased CD34 and CD144 expression in Tunicamycin treated Matrigel™ plugs - Microvessel density is a vital prognostic biomarker correlating the growth and development of breast cancer (27) and is often used to evaluate tumor growth (28). To assess the Tunicamycin-mediated inhibition of vessel formation in Matrigel™ plugs, the microvessel density in the plugs was examined immunohistochemically after staining for CD34 and CD144. Microscopic examination revealed numerous CD34 and CD144 stained vessels in the positive control group (VEGF165 + heparin) (Fig. 2C and 2D, middle), but only a few vessels in the group treated with Tunicamycin (Fig. 2C, right and Fig. 2D, far right). 87 ± 4.04 vessels stained for CD34 and 78 ± 3.4 vessels for CD144 in the positive control group as opposed to only 12 ± 1.2 vessels in CD34 or 8 ± 1.5 vessels in CD144 in the group treated with Tunicamycin, respectively. Quantification (Fig. 2C and 2D, far right) indicates ~87.5% decrease (p=0.001) in microvessel density due to Tunicamycin treatment.

Increased expression of thrombospondin-1 gene in Tunicamycin treated Matrigel™ plugs - Angiogenesis is a balance between the expression of pro- and anti-angiogenic molecules (29). Thrombospondin-1 (Tsp-1) is an endogenous inhibitor of angiogenesis (30). To get an insight into the Tunicamycin-induced inhibition of neo-vascularization in Matrigel™ plugs, Tsp-1 gene expression was monitored in the plugs. qRT-PCR analysis reveals ~3.8-fold (p<0.001) upregulation of Tsp-1 gene expression (Fig. 3A). Upregulation of TSP-1 mRNA as well as protein was also observed in capillary endothelial cells treated with Tunicamycin for 32 h (Fig. 3B and 3C).

Tunicamycin inhibits breast tumor growth in athymic nude mice - In vitro cell culture and in vivo Matrigel™ implant models raised a question if Tunicamycin could treat breast tumors. To answer, we took the following approaches: (i) orthotopic breast tumor in nude mice were developed with MDA-MB-435 (ER-/PR-/EGFR+) cells and treated with Tunicamycin by intravenous injection (group a; Fig. 4A); and (ii) breast tumor xenografts in nude mice were developed with MDA-MB-231 (ER-/PR-/EGFR+) cells and treated orally with Tunicamycin (group b; Fig. 5A). In group a, Tunicamycin treatment was started after the tumor size reached to ~3mm in diameter in 6 days at doses 0.1, 0.5, and
Tunicamycin is anti-angiogenic and treats breast tumor after Tunicamycin treatment - To correlate the tumor growth with the cellular markers, expression of Ki-67 and VEGF was analyzed in tumor tissue immunohistochemically. Ki-67 is a cellular proliferation marker and VEGF is pro-angiogenic. The tumor we have examined here was a grade III adenocarcinoma, and Ki-67 or VEGF expression in tumors from Tunicamycin treated group of mice (1.0 mg/Kg) was reduced significantly (Fig. 4D and E). This paralleled the reduction of microvessel count and the mitotic index (Fig. 4B and C), respectively. Accumulating evidence thus supports Tunicamycin is anti-angiogenic and treats breast tumor in nude mice.

Decreased invasion and chemotaxis of Tunicamycin treated capillary endothelial cells - Capillary proliferation and tissue invasion are important for breast tumor progression. We have studied the Matrigel™ invasion of capillary endothelial cells as well as their chemotactic activity in the presence or absence of Tunicamycin. Invasion through the transwell membrane in control plate (Fig. 6A) remained almost the same whereas those migrated through the Matrigel™ reduced significantly in the presence of Tunicamycin (Fig. 6B and Fig. 6B far right; p<0.001). This supported that Tunicamycin makes capillaries less capable of invading the extracellular matrix. In addition to measuring the invasion character, the cells were also tested for their chemotaxis, i.e., the migratory activity on a wounded surface. The rate of migration was ~75% higher in the presence of VEGF_{165}. In the presence of Tunicamycin it was only ~60% of the control and was further reduced (i.e., ~25%) when both Tunicamycin and VEGF_{165} were present (Fig. 6C, 6C far right; p<0.001).

Surface expression of N-glycans on tumor microvasculature - Tunicamycin inhibits LLO biosynthesis and consequently the glycosylation of N-linked glycoproteins. To verify the status of the N-glycans on the breast tumor microvascular endothelial cell surface, the tissue sections were stained with Texas-Red conjugated WGA and examined under a fluorescence microscope. Tumor microvessels in untreated controls were stained markedly but the staining intensity per vessel was reduced almost 50% upon treating with Tunicamycin (Fig. 7A). This was further confirmed by quantifying these images with NIH-IMAGE J program (Fig. 8). Tumor cells from untreated control (Fig. 7B) exhibited positive WGA staining but the intensity was much less than the endothelial cells. Tunicamycin treatment caused a morphological change on the tumor cells and the WGA staining was not only reduced but also appeared amorphous.

Development of unfolded protein response in tumor microvasculature during Tunicamycin treatment - Inhibition of N-glycan biosynthesis with Tunicamycin develops upr mediated by ER stress (19). This has led us to investigate whether ER stress-mediated upr exists in breast tumor microvasculature of the mice receiving Tunicamycin treatment. Tumor tissue is heterogeneous. To answer this question, the tumor microvascular endothelial cells were first identified by staining for CD144 (green fluorescence; a marker for endothelial cells) and then stained for the GRP-78 (red fluorescence), an ER chaperone. In untreated
control, CD144 stained endothelium appears as a thin line around the vessel so did the GRP-78 (Fig. 7C and D). As the tumors were treated with Tunicamycin, a high level expression of GRP-78 was observed in microvascular endothelial cells of the tumor (Fig. 7D). CD144 also stained the same area (Fig. 7C). Both green and red fluorescence of CD144 and GRP-78 co-localized in tumor microvasculature supporting unfolded protein response mediated by ER stress in tumor microvasculature. GRP-78 staining in tumor cells following Tunicamycin treatment appears to be increased (Fig. 7E). This may be an indirect effect because of nutritional deprivation due to reduced blood flow in the tumor and not necessarily due to an induction of upr.

**DISCUSSION**

Deaths from breast cancer have dropped more than 2% since 1990, but it still is the second largest killer of women after heart disease. Approximately 15% of diagnosed breast cancers are also triple negative, have no treatment, and obviously, no cure. Tumors can stay dormant for many years due to a balance between cell proliferation and apoptosis in the absence of angiogenesis. In such condition, there is also a balance between pro-angiogenic and anti-angiogenic factors (31, 32). Dormant tumors secrete inhibitory factors that prevent the tumor from switching to the angiogenic phenotype and arrest the growth of tumors. The quantification of tumor angiogenesis, i.e., microvascular count (MVC) is a measure of tumor staging for growth, invasive, and metastatic potential as well as for diagnosis (28, 33). Many preclinical studies have suggested that anti-angiogenic strategy could be a promising approach for cancer control because it is less likely to acquire drug resistance (34).

Anti-angiogenic agents conceptually categorized as: endothelial toxin (35), growth factor receptor antagonists (36), protease inhibitors (37, 38), and normal inhibitors (39). In addition, several chemotherapeutic agents routinely employed in breast cancer treatment have true anti-angiogenic activity (40). Unfortunately, none of these and others targeting different areas of vasculature could cure breast cancer, all have serious side effects, and are detrimental to patients’ quality of life.

The study we have presented here with Tunicamycin as a model offers a possibility for developing a new drug regimen for treating breast cancer including a triple negative. The outcome is from a central hypothesis that Tunicamycin inhibits angiogenesis (both in vitro and in vivo) and built on the confidence that inhibition of capillary endothelial cell proliferation was by arresting cells in G1 (22).

To establish and evaluate the survivability of Tunicamycin in tumor microenvironment, the effect of Tunicamycin in the presence of VEGF165 was studied. Tumor-derived VEGF is a most important angiogenic factor associated closely with the induction and maintenance of the neo-vasculature in human tumors (41). The results in Fig. 1A demonstrate that VEGF165 tries to protect the cells from Tunicamycin initially and the reduction of cellular proliferation during the first 24 hours was modest when Tunicamycin was present together with VEGF than alone. The inhibition of cellular proliferation, however, was comparable from 48 h to 144 h whether Tunicamycin was present alone or in combination with VEGF165. The cells in control continued to respond to VEGF165 stimulation and exhibited higher rate of proliferation till 120 h and before reaching to a plateau at 144 h. This may be due to (i) the exhaustion of VEGF165, and/or (ii) desensitization of the VEGF receptors. Nevertheless, it clearly demonstrates that VEGF165 is unable to override the anti-angiogenic action of Tunicamycin. Mechanistic details indicate that phosphorylation of VEGFR1 and VEGFR2 started down regulating as early as 3 h of Tunicamycin treatment but became more at 32 h (Figure 1D). The behavior of phosphoVEGFR2 in cells treated with Tunicamycin for 12 h is not sustainable and may indicate the cells are fighting back to stay alive under the experimental condition. This could be addressed in the future while studying the cellular survival. Since, VEGF signals through tyrosine phosphorylation, the tyrosine kinase activity was measured in cells treated with Tunicamycin and stimulated with VEGF165. The VEGF-stimulated phosphotyrosine kinase activity in Tunicamycin treated cells was reduced significantly (i.e., ~46.6%, p<0.001; Fig 1B) which is analogous to the reduction of phosphorylated VEGFR1 and VEGFR2 levels (i.e., ~30.5% and ~44.4%), respectively. There is a 1.6-2.4 fold reduction of VEGFR1 and
VEGFR2 phosphorylation in cells treated with Tunicamycin for 32 h over 3 h. However, a difference of 2.7-2.9 fold has been observed in the ratio of phosphorylated VEGFR1 and VEGFR2 to total VEGFR1 and VEGFR2 between cells treated with Tunicamycin for 3 h and 32 h (Table 1). It would have been ideal if the cells would have maintained a steady level of total VEGFR1 and VEGFR2 receptors throughout. The apparent changes are believed to physiological and did not deter our conclusion. In fact, irrespective of the levels of total VEGFR1 and VEGFR2, the levels of phosphorylated VEGFR1 and VEGFR2 are reduced when the cells were treated with Tunicamycin for either 3 h or 32 h compared to the untreated control. Thus, strongly supporting that down regulation of phosphorylated VEGFR1 and VEGFR2 in Tunicamycin treated cells is primarily due to a reduced phosphotyroine kinase activity in these cells. We anticipate that phosphorylated VEGFR1 and VEGFR2 would be much higher in VEGF-stimulated cells. Our primary reason not to use the VEGF-stimulated cells is that we have observed uprmediate apoptosis in Tunicamycin-treated cells in the absence of VEGF. The kinase activity was VEGF-specific because no additive or synergistic reduction was observed in the presence of the VEGF-receptor antagonist, CBO-II. Higher basal level of phospho-tyrosine kinase activity may be due to the presence of VEGF-like molecule in the serum since the cells were cultured in the presence of fetal bovine serum or due to an endogenous VEGF acting in a paracrine fashion (unpublished observation). Any unparallel changes in VEGFR1 and VEGFR2 observed here may be due to the fact that activation and signaling of VEGFR2 receptor may be positively or negatively influenced by the co-expression and activation of VEGFR1 receptor as proposed earlier (42). But, it has not been verified independently in this study.

When tested, Tunicamycin reduced microvessel density by ~75% (Fig. 2B) as well as the expression of CD34 and CD144 in Matrigel™ plugs in athymic mice (Fig. 2C and D). TSP-1 is a known natural inhibitor of angiogenesis and its down-regulation plays a critical role in the angiogenic switch in several tumor types (30). In this study high expression of Tsp-1 gene in Matrigel™ plugs correlated with the TSP-1 protein and gene expression in Tunicamycin treated capillary endothelial cells (Fig. 3). The anti-angiogenic effect of TSP-1 has been proposed to mediate in part by the CD36 (TSP-1) receptor, which triggers a signaling cascade that leads to apoptosis in activated endothelial cells via the CD95 (Fas ligand) death receptor. There is no Fas ligand receptors detected in our capillary endothelial cell model (unpublished data). Up-regulation of TSP-1 expression in Tunicamycin treated capillary endothelial cells as well as in Matrigel™ implants supports inhibition of angiogenesis but the mechanism through which TSP-1 is exerting its anti-angiogenic effect in our system is currently unknown and warrants future studies to delineate the molecular details. Based on the above in vitro and in vivo studies, Tunicamycin was used as a therapeutic for treating a double negative (ER/PR/EGFR+) and a triple negative (ER/PR/EGFR-) breast tumor in nude mice. These results are shown in Figs. 4 and 5. The tumor volume was reduced ~55%-65% in one to three weeks irrespective of the tumor type or the mode of administration of the therapeutic. Taxol was used as an internal control, and to see an effect similar to that of Tunicamycin (i.e., 1.0 mg/Kg) 15 times more taxol was needed. The reduction of tumor growth correlated well with reduced Ki-67 and VEGF expression, microvascular density, and decreased mitotic index of the tumor cells in paraffin sections (Fig. 4). Most importantly, the animals showed no sign of abnormal behavior. There was no weight loss and the animals exhibited normal intake of food and water.

The origin of MDA-MB-435 cell line has been questioned by comparative genomic hybridization studies and claimed that both MDA-MB-435 and M14 melanoma are identical lines (43). However, as reviewed (44) both cell lines are of breast cancer origin rather than of melanoma origin due to the following rationale. The MDA-MB-435 cell line was isolated from a pleural effusion of a female patient with breast cancer and still has two X chromosomes; express milk proteins and lipids; and when transfected with the mm23 metastasis suppressor gene, MDA-MB-435 cells show the morphologic feature of normal breast epithelial cells, including acinus formation in three-dimensional culture.
Apart from the loss in cellular proliferation and induction of cell death due to apoptosis, Tunicamycin blocked cellular invasion through Matrigel™ as well as their chemotactic activity (Figs. 6B and C). Cumulatively, these processes are required for active angiogenesis during tumor progression, invasion and metastasis. We have taken an approach here that develops ER stress in the tumor microvasculature mediated by unfolded protein response. It has been a challenge to image such a process since the tumor is highly heterogeneous. Identifying the microvascular endothelial cells by staining for CD144 followed by imaging the expression of GRP-78 turned out to be an excellent tool. Using the dual imaging technique our study has convincingly and unequivocally supported that Tunicamycin reduces the breast tumor growth by triggering the unfolded protein response in the tumor microvasculature (Fig. 7C and D). The microvessels from the treated groups are thin and smaller. In addition, quantification of WGA fluorescence intensity by IMAGE J program indicates that the expression of WGA-positive glycans per vessel was reduced as the dose of Tunicamycin was increased (Fig. 7A and Fig. 8). Tumor epithelial cells also react to WGA staining giving a distinct structural appearance in untreated control but both the staining intensity and the structural integrity seemed to be collapsing with morphological changes as the Tunicamycin dose was increased (Fig. 7B). Increased GRP-78 staining of tumor cells in Tunicamycin treated tissue (Fig. 7E) may indicate the presence of an ER stress developed indirectly as a result of nutritional deprivation due to reduced blood flow and not necessarily due to the induction of upr. This is also supported by an earlier report claiming that Tunicamycin does not induce ER stress or develop upr in tumor cells (45). It is obviously impossible to settle this question in paraffin sections of breast tumor tissue and needs to be addressed with breast cancer cells in culture in the future.

In view of these results we conclude that (i) anti-angiogenic action of Tunicamycin survives in tumor microenvironment; (ii) tumor associated high-density neo-vascularization responsible for the development of tumor growth is blocked by Tunicamycin through a distinct mechanism of unfolded protein response mediated by ER stress; and (iii) Tunicamycin itself could develop into a new generation anti-angiogenic therapeutic treating breast cancer in the clinic. Down-regulation of protein tyrosine kinase activity as well as reduced phosphorylated VEGFR1 and VEGFR2 receptors level as early as 3 h of Tunicamycin treatment supports the initiation of the apoptotic process. Furthermore, strengthening of apoptosis through a 1.6-2.4 fold down-regulation of phosphoVEGFR1 and VEGFR2 receptors in 32 h over that of 3 h, and/or a 2.7-2.9 fold difference (i.e. down regulation) in the ratio of phosphorylated VEGFR1 and VEGFR2 to that of the total receptors between 3 h and 32 h of Tunicamycin treatment may indicate recruiting other down-stream pathways remain a strong possibility.

Acknowledgements- The work was supported in part by grants NIH U54-CA096297, Susan G. Komen for the Cure BCTR0600582 (DKB), and NIH/NCRR/RCMI grant G12-RR03035 (KB). The authors greatly acknowledge the pathological consultation of María Marcos, MD., María Almira, MD, the editorial assistance of Laura M Bretana and the technical help from Meriandrys Miller Burgos, Frances Candelas and Weiya Xia.

REFERENCES


FOOTNOTES

Abbreviations: TSP-1, thrombospondin-1; LLO, lipid-linked oligosaccharide; PKA, cAMP-dependent protein kinase; ER, endoplasmic reticulum; UPR, unfolded protein response; VEGFR, vascular endothelial growth factor receptor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin; GRP-78, glucose responsive protein; HRP, Horseradish peroxidase; qRT-PCR, quantitative real time PCR; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ANOVA, analysis of variance.

FIGURE LEGENDS

Fig. 1. Time course of Tunicamycin inhibition of VEGF-stimulated capillary endothelial cell proliferation. A, Synchronized culture of capillary endothelial cells was treated with VEGF165 (10 ng) in the presence or absence of Tunicamycin (1 µg/ml) and the cell numbers were counted microscopically. VEGF165 was added prior to the addition of Tunicamycin. B, Phosphotyrosine-kinase activity in capillary endothelial cells. The experiment was performed as per instructions from the manufacturer and the measurement was made in an automated microplate reader/EIA plate reader (Model 2550, BioRad Laboratories; Hercules, CA) at 450nm. The synchronized cells were treated with Tunicamycin (1 µg/ml) for 3 h and then treated with VEGF165 for 10 min. CBO-II was added 30 min prior to the addition of VEGF165. C, Status of VEGFR1 and VEGFR2 receptors. Synchronized cells were incubated with Tunicamycin for 3 h-32 h, and the levels of total VEGFR1, phospho VEGFR1, total VEGFR2 and phospho VEGFR2 receptors were analyzed by western blot using anti-VEGFR1 total (1:2,000; v/v), anti-phospho VEGFR1 (1:1,000; v/v), anti-VEGFR2 total (1:2,000; v/v) and anti-phospho VEGFR2 (1:1,000; v/v) antibodies. Actin (1:5,000; v/v) was used as a loading control. D, Quantification of VEGFR1 and VEGFR2 receptors: Densitometer scanning (arbitrary unit) of the western blots was plotted against the time of treatment. The results are an average from three representative immunoblots of each experiment.

Fig. 2. Tunicamycin inhibition of VEGF-induced angiogenesis and CD34 and CD144 expression in Matrigel™ implants. A, Representative images of Matrigel™ plugs containing heparin (Left), heparin + VEGF165 (middle) and heparin + VEGF165 + Tunicamycin (5 µg) (right). B, H & E staining of Matrigel™ implants (100x). Microvessels are identified with arrows. Magnified views (200x) are in Insets. Histograms represent microvessels per fields [mean ± SE (n=5); p<0.001]. C, Immunohistochemistry of CD34 of Matrigel™ sections (100x). The histograms are for CD34 positive cells (mean ± SE) per field. D, Photomicrographs of immunostained CD144 from Matrigel™ sections (100x). The histograms are for CD144 positive cells (mean ± SE). The numbers in each case are an average from five different slides and five regions per slide; p<0.001.

Fig. 3. Thrombospondin-1 expression in Matrigel™ implants and in capillary endothelial cells. A, Quantification of TSP-1 mRNA expression in Matrigel™. B, Quantification of TSP-1 mRNA expression in capillary endothelial cells after 3 h and 32 h of Tunicamycin treatment. C left, TSP-1 protein expression in capillary endothelial cell was examined by immunoblotting (40 µg total protein) from control and Tunicamycin treated cells (3 h and 32 h). The blot was developed with an anti-TSP-1 antibody (1:1,000; v/v) and anti-Actin. C right, Histogram representing quantification of the TSP-1
protein expression as measured by Chem Doc Densitometry (Bio-Rad Laboratories). The results are an average from three blots done independently.

**Fig. 4.** Tunicamycin slows down the growth of a double negative breast adenocarcinoma. The breast tumor was developed orthotopically in athymic nude mice after injecting MDA-MB-435 (ER/PR-/EGFR+) human breast cancer cells and monitored by caliper measurements. The mice were treated intravenously once a week with Tunicamycin (0-1.0 mg/Kg). A left, tumor growth as a function of time and Tunicamycin treatment. A right, weight of the excised tumor after 23 days. B, H & E stained tumor sections identifying the microvascular density in control and following Tunicamycin treatment. Arrows indicate microvessels. C, H & E staining identifies the mitotic index (arrow heads) in control and Tunicamycin treated breast tumor section. Histogram on the right represents the quantitative results of mitotic index averaged from ten representative areas of each tumor. The results are expressed as mean ± SE; p<0.001. D, Immunohistochemistry of Ki-67 and VEGF expression in breast tumor tissue sections (400x).

**Fig. 5.** Tunicamycin inhibits the growth of a triple negative breast tumor. The xenografts were developed in athymic nude mice after injecting MDA-MB-231 (ER/PR/EGFR) human breast cancer cells and treated with Tunicamycin (0.25 mg/Kg) orally twice a week. A, Tumor size in a control xenograft (untreated) and after Tunicamycin treatment (TM-treated); B, Tumor volume (mm³) as a function of time. ♦---♦, Control; ■---■, Tunicamycin.

**Fig. 6.** Inhibition of Matrigel™ invasion and chemotaxis of capillary endothelial cells after Tunicamycin treatment. A, Synchronized culture of capillary endothelial cells either alone or after pre-treating with Tunicamycin (1 µg/ml) for 32 h were seeded in control and growth factor reduced Matrigel™ coated transwell plates. B, Cells were cultured in EMEM containing 2% fetal bovine serum in the upper chamber. Conditioned media from human breast cancer cells MCF-7 cultured in 10% fetal bovine serum along with VEGF (10 ng/ml) was used in the lower chamber as a chemo-attractant. After incubation for 24 h at 37°C in a CO₂ incubator the transwells were removed, cells passed through the membrane were fixed and stained with H & E. The invaded cells were quantified by counting in an optical microscope at 200x magnification, and averaged after counting five fields per membrane. Histogram at right is the quantification of cell invasion through Matrigel™ (p<0.001). C, The image of chemotaxis of endothelial cells. Endothelial cells grown to confluence in a regular media containing 10% fetal bovine serum and switched to a media containing 0.2% fetal bovine serum for 6 h. The monolayers were scratched with a 10 µl pipette tip and cultured with or without VEGF (10 ng/ml), or Tunicamycin (1 µg/ml), or Tunicamycin (1 µg/ml) + VEGF₁₆₅ (10 ng/ml) for an additional 6 h. The migrated cells were quantified microscopically and the histogram at right is the quantification of cell migration (averaging the position of the migrating cells at the wounding edges; p<0.001); broken line indicated by →.

**Fig. 7.** Expression of cell surface glycans and induction of unfolded protein response-mediated ER stress in breast tumor microvasculature. A, WGA staining for N-glycans in microvessels from control and Tunicamycin (0-1.0 mg/Kg) treated breast tumor tissue sections. Images were captured under a fluorescence microscope. B, WGA staining of tumor cells. Detection of upr-mediated ER stress in tumor microvasculature. Tumors were fixed, sectioned and stained dually with anti-CD144 (endothelial cell marker) antibody (1:50; v/v) followed by AlexaFluor 488-conjugated secondary antibody (1:100; v/v), and anti-GRP-78 antibody (1:40; v/v) followed by Rhodamin-conjugated secondary antibody (1:100; v/v). C, Microvessel (arrows) from peripheral region of breast tissue section immunostained with anti-CD144 antibody (green) and D, microvessel immunostained with anti-GRP-78 antibody (red) were monitored under a fluorescence microscope. E, GRP-78 (red) staining of tumor tissue. Histology scale: 20 µm.
Fig. 8. Quantification of WGA staining of breast tumor microvasculature. Tumor tissue was stained with Texas-red conjugated WGA as described in Experimental Procedures. The fluorescence intensity of microvessles was quantified with ImageJ program. The results are an average of vessels from five different areas in each group.

Table 1: Ratio of phosphoVEGFRs to Total VEGFRs. The ratios are calculated from the values in Figure 1D. The results are expressed as mean ± SE

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con-3h</td>
</tr>
<tr>
<td>Phospho-VEGFR1/Total VEGFR1</td>
<td>0.96 ± 0.09</td>
</tr>
<tr>
<td>Phospho-VEGFR2/Total VEGFR2</td>
<td>0.49 ±0.02</td>
</tr>
</tbody>
</table>
Figure 1

A

![Graph showing the number of cells over time with different conditions.]

B

![Bar graph showing absorbance at 450 nm with different conditions.]

C

![Western blot images showing Phospho VEGFR1, Total VEGFR1, Phospho VEGFR2, Total VEGFR2, and Actin with molecular weights indicated.]

D

![Bar graphs showing activity units for different conditions for Phospho VEGFR1, Total VEGFR1, Phospho VEGFR2, and Total VEGFR2 with significance levels indicated.]

Downloaded from http://www.jbc.org/ by guest on October 1, 2017
**Figure 3**

**A**

![Bar graph showing relative expression of TSP-1](image)

- Con
- VEGF
- TM + VEGF

Significance levels:
- P < 0.001
- P < 0.001

**B**

![Bar graph showing relative expression of TSP-1](image)

- Con
- 3h
- 32h

Significance levels:
- P < 0.01
- P < 0.01

**C**

![Western blot images of TSP-1 and Actin](image)

- TSP-1 at 170 kDa
- Actin at 42 kDa

Significance levels:
- P < 0.01
- P < 0.001
Figure 5

A

Untreated

TM-tREATED

B

Tumor Volume (mm$^3$)

1st week 2nd week 3rd week 4th week

19
Figure 8
Unfolded protein response is required in nu/nu mice microvasculature for treating breast tumor with tunicamycin

Aditi Banerjee, Jing Yu Lang, Mien-Chie Hung, Krishanu Sengupta, Sushanta K. Banerjee, Krishna Baksi and Dipak K. Banerjee

J. Biol. Chem. published online June 15, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.169771

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2011/06/15/jbc.M110.169771.full.html#ref-list-1