The beta adrenergic receptor antagonist propranolol alters mitogenic and apoptotic signaling in late stage breast cancer

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Abstract

Background: Substantial evidence supports the use of inexpensive β-AR antagonists (beta blockers) against a variety of cancers, and the β-AR antagonist propranolol was recently approved by the European Medicines Agency for the treatment of soft tissue sarcomas. Prospective and retrospective data published by our group and others suggest that non-selective β-AR antagonists are effective at reducing proliferative rates in breast cancers, however the mechanism by which this occurs is largely unknown.

Methods: In this study, we measured changes in tumor proliferation and apoptosis in a late stage breast cancer patient treated with neoadjuvant propranolol. We expounded upon these clinical findings by employing an in vitro breast cancer model, where we used cell-based assays to evaluate propranolol-mediated molecular alterations related to cell proliferation and apoptosis.

Results: Neoadjuvant propranolol decreased expression of the pro-proliferative Ki-67 and pro-survival Bcl-2 markers, and increased pro-apoptotic p53 expression in a patient with stage III breast cancer. Molecular analysis revealed that β-AR antagonism disrupted cell cycle progression and steady state levels of cyclins. Furthermore, propranolol treatment of breast cancer cells increased p53 levels, enhanced caspase cleavage, and induced apoptosis.

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Conclusion: Collectively, these data provide support for the incorporation of β-AR antagonists into the clinical management of breast cancer, and elucidate a partial molecular mechanism explaining the efficacy of β-AR antagonists against this disease.

### Materials and methods

**Neoadjuvant treatment of a late stage breast cancer patient with propranolol**

Clinical studies presented in this report were performed following approval of the Texas Tech University Health Sciences Center Institutional Review Board. A female patient, age 44, presented at the Texas Tech Breast Care Center with a stage IIIA, 6.5 cm left breast invasive lobular carcinoma (T3N1M). Tumor tissue from the diagnostic biopsy was collected for analysis. The patient was immediately prescribed 1.5 mg/kg/day propranolol for 18 days and then a tapering dose over the subsequent 7 days. After the treatment period, the tumor was removed through a radical mastectomy and tumor tissue was collected for analysis.

### Immunohistochemistry (IHC)

Tumor tissue was collected and processed from both the patient’s diagnostic biopsy (pre-treatment) and the radical...
mastectomy (post-treatment). IHC for Ki-67 was performed at the University Medical Center’s Pathology facilities according to their in-house procedures. The Ki-67 index was blindly quantified in both the pre- and post-treatment tissues by counting 10 fields under a microscope, with each field composed of 250 + nuclei. The Ki-67 index was calculated as the (number of Ki-67 positive nuclei/total number of nuclei) \times 100. For IHC staining of total p53 and Bcl-2 proteins, slides were deparaffinized, rehydrated, and stained using the rabbit specific HRP/DAB (ABC) Detection IHC Kit (ab#64261) or the mouse specific HRP/DAB (ABC) Detection IHC Kit (ab#64259) according to the manufacturer’s protocols. Antibodies included: anti-p53 (total) (Cell Signaling #2527) and anti-Bcl-2 (Abcam #ab196495).

Cell culture and treatment

MDA-MB-231 breast cancer cells (ATCC #HTB-26) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were maintained in a water-jacketed CO₂ incubator at 37 °C. DL-propranolol hydrochloride (Acros Organics) and doxorubicin hydrochloride (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and were administered as indicated for each experiment. As a solvent control, DMSO was tested at the same concentration (1% v/v) as contained in the experimental samples. Also, untreated cells were used to establish the background of dead cells provoked by factors inherent to the cell cultures and cell manipulation.

Flow cytometry

MDA-MB-231 cells were collected via trypsinization and resuspended in PBS. Nuclear isolation medium (NIM)-DAPI solution (Beckman Coulter) was added to the cell suspensions and analyzed by a Gallios flow cytometer (Beckman Coulter) according to previously described methods [23]. Determination of the main phases of the cell cycle subpopulations (G0/G1, S, and G2/M), as well as the occurrence of cells experiencing DNA fragmentation (sub-G0/G1; apoptotic subpopulation), was performed as previously described [23]. The percentage of each cell cycle phase distributions was determined by using Kaluza flow cytometry software (Beckman Coulter). Consistently, twenty thousand cells were measured in each condition.

Immunoblotting

Lysates were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride membranes using the Trans-Blot Turbo

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**Fig. 1** Neoadjuvant treatment of a breast cancer patient with propranolol. (A) Immunohistochemical staining of breast cancer tissue from the patient discussed in this study for β1-AR, β2-AR, and β3-AR proteins. Purple/brown staining indicated positivity for the antigen tested. (B) Flow diagram showing the treatment and tumor tissue collection schemes for the stage III breast cancer patient.
Transfer System (Bio-Rad). Membranes were blocked in Tris-buffered saline plus 3% bovine serum albumin and 0.05% Tween-20, and incubated with the following antibodies as indicated for each experiment: anti-cyclin A (Cell Signaling #4656), anti-cyclin B1 (Cell Signaling #4138), anti-cyclin D1 (Cell Signaling #2978), anti-cyclin D2 (Cell Signaling #3741), anti-cyclin E1 (Cell Signaling #4129), anti-cyclin E2 (Cell Signaling #4132), anti-p53 (total) (Cell Signaling #2527), anti-phospho-p53 (S9) (Cell Signaling #9286), anti-phospho-p53 (S15) (Cell Signaling #9286), anti-phospho-p53 (S20) (Cell Signaling #9287), anti-phospho-p53 (S37) (Cell Signaling #9287), anti-phospho-p53 (S46) (Cell Signaling #8641), anti-phospho-p53 (S392) (Cell Signaling #2928), cleaved caspase 3 (Cell Signaling #9664), cleaved caspase 6 (Cell Signaling #9761), cleaved caspase 9 (Cell Signaling #7237), and anti-beta actin (Santa Cruz Biotech #sc8432). Each primary antibody was detected with an appropriate 1:1000 HRP-conjugated secondary antibody, subjected to Supersignal West Dura Extended Duration Substrate (ThermoFisher Scientific), and digitally captured using a GE Image Quant Las4000 imaging system.

**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and incubated with the anti-p53 antibody (Cell Signaling #2527), rhodamine-conjugated phalloidin (Cyto-skeleton Inc), or Hoechst 33342 (Thermo Fisher Scientific). Appropriate fluorescent-conjugated secondary antibodies were used for primary antibody detection. Single plane high-resolution digital fluorescent images were captured using an LSM 700 confocal microscope (Zeiss) equipped with a 40x immersion oil objective and assisted with Zen 2009 software (Zeiss); for each channel, 1-airy unit (AU) pinhole setting was utilized as previously described [24].

**Live/dead staining**

Cells were stained with Apopxin Green Indicator, 7-AAD, and CytoCalcein Violet 450 using the Apoptosis/Necrosis Detection Kit (Abcam #ab176749) according to the manufacturer's instructions.

**Statistical analysis**

The Mann-Whitney rank sum test was performed in GraphPad Prism version 7 for [Figs. 2B, G, and 3B, F]. One-way ANOVA was performed in GraphPad Prism version 7 for [Figs. 2D, E, and 4C]. Differences were considered statistically significant if the p < 0.01.

**Results**

**Neoadjuvant treatment of a late stage breast cancer patient with propranolol**

We have previously reported that administration of propranolol to an early stage breast cancer patient resulted in a decreased tumor proliferative index [3]. To prospectively evaluate if beta blockade could reduce the proliferation rate of a late stage breast tumor in a neoadjuvant setting, propranolol was administered to a patient diagnosed with a 6.5 cm invasive lobular carcinoma that was estrogen and progesterone receptor positive, HER2-neu negative, and expressed β1-AR, β2-AR, and β3-AR. The treatment regime is outlined in [Fig. 1].

**Propranolol alters breast cancer cell cycle progression**

IHC for Ki-67 was performed on the diagnostic biopsy (before treatment with propranolol) and on the tumor tissue from the radical mastectomy (after 25 days of propranolol treatment). The Ki-67 proliferative indices at the time of diagnostic biopsy and surgical removal were 68 ± 3% (mean ± SEM) and 25 ± 2% (mean ± SEM), respectively [Fig. 2A and B]. This equated to an approximately 2.7-fold reduction in tumor proliferative index after propranolol treatment (p < 0.0001). Substantial concordance has been reported for Ki-67 indices between diagnostic biopsy and surgical resection [25], therefore an alteration in tumor proliferation, as observed in this patient, can be attributed to propranolol administration with reasonable confidence. We corroborated this finding using the MDA-MB-231 breast cancer cell line, which was originally isolated from a metastatic pleural effusion. Cell cycle analysis was performed using flow cytometry in control and propranolol (40 μM) treated breast cancer cells (24 h treatment, as substantial changes in cell viability were visually observed at this time). In our previously published study examining the effects of beta blockade on MDA-MB-231 cells, we demonstrated that the EC50 for propranolol in this cell line was approximately 78 μM [3]. In the current study, we treated MDA-MB-231 cells with a lower dose of propranolol for multiple reasons: 1) the EC50 of propranolol established in our previous publication is not a physiologically attainable plasma concentration in patients; 2) dosing of patients with chemotherapeutic compounds generally involves low dose/longer treatment regimens that are not well recapitulated in lethality testing associated with in vitro cell culture systems; and 3) we sought to examine the acute molecular effect of sub-lethal doses of propranolol on the breast cancer cells. The anthracryline chemotherapy drug, doxorubicin (3 μM), was used as a positive experimental control for this and all subsequent in vitro experiments. Addition of propranolol reduced the percentage of the cell population residing in the G2/M phase of the cell cycle [Fig. 2C and D], and increased the sub-G1 cell population, representing dead or dying cells [Fig. 2C and E]. Moreover, the expression of multiple cyclins was reduced following 24 h propranolol treatment [Fig. 2F and G], further demonstrating the impact of β-AR antagonism on breast cancer cell proliferation.

**Propranolol enhances p53 activation in breast cancer cells**

IHC for total p53 was performed on the diagnostic biopsy and tumor tissue from the radical mastectomy in the late stage breast cancer patient. The p53 indices at the time of diagnostic biopsy and surgical removal were 8 ± 2% (mean ± SEM), and 20 ± 2% (mean ± SEM), respectively [Fig. 3A and B]. This equated to an approximately 2.5-fold increase in p53 protein expression after propranolol treatment (p < 0.0001). These data were corroborated by immunofluorescent staining of MDA-MB-231 cells for total p53, revealing a marked increase in
Fig. 2 Propranolol decreases the mitogenic potential of breast cancer cells. (A & B) IHC and graphical representation for Ki-67 performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates Ki-67 antigenicity. Ten random vision fields were counted per condition. (C & D) DAPI staining of MDA-MB-231 breast cancer cells was analyzed via flow cytometry after treatment with vehicle control or with 40 μM propranolol for 24 h. Three μM doxorubicin was added as a positive experimental control. (E) Quantification of the sub-G1/G0 cell sub-population from the flow cytometry analysis. Cell cycle populations (sub-G1, G1, S, G2/M) are marked in red in the control flow cytometry graph. (F & G) Immunoblotting and quantification of cyclin protein levels in MDA-MB-231 cells treated with vehicle control or 40 μM propranolol. Three μM doxorubicin was added as a positive experimental control. For quantification, cyclins were normalized relative levels of actin. AU indicates arbitrary units.
Fig. 3 Propranolol modulates p53 steady state protein levels and post-translational modifications. (A & B) IHC and graphical representation for p53 protein performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates p53 antigenicity. Ten random vision fields were counted per condition. (C) Immunofluorescent staining for p53 (green), Hoechst 33342 nuclear counterstain (blue), and rhodamine-conjugated phalloidin as an actin counterstain (red) in MDA-MB-231 cells treated with vehicle control or 40 μM propranolol. Three μM doxorubicin was added as a positive experimental control. (D) Immunoblotting for p53 protein levels in MDA-MB-231 cells treated with 40 μM propranolol over a 6 h time course. Three μM doxorubicin was added as a positive experimental control. (E & F) Immunoblotting of p53 phosphorylation events in MDA-MB-231 cells treated with vehicle control or 40 μM propranolol for 6 h. Three μM doxorubicin was added as a positive experimental control. For quantification, phospho-p53 was normalized relative levels of both total p53 and actin. AU indicates arbitrary units.
Fig. 4 Propranolol induces apoptosis in breast cancer cells. (A) IHC for Bcl-2 protein performed on tissues from the diagnostic biopsy (pre-treatment) and surgical resection (post-treatment). Brown staining indicates Bcl-2 antigenicity. (B & C) Immunoblotting of cleavage caspase products in MDA-MB-231 cells treated 40 μM propranolol over a 6 h time course. For quantification, cleaved caspase products were normalized relative levels of beta-actin. AU indicates arbitrary units. (D) MDA-MD-231 cells were treated with vehicle control, 40 μM propranolol, or 3 μM doxorubicin as a positive control for 6 h. Cells were stained with the apoptotic marker Apopxin Green Indicator (green), the necrotic marker 7-AAD (red), and CytoCalcein Violet (blue) as a nuclear counterstain. Images were collected using confocal microscopy.
p53 protein levels by 6 h post-treatment with propranolol [Fig. 3C]. Immunoblotting of the breast cancer cells showed similar propranolol-mediated increases in total p53 levels between two to 6 h post-treatment [Fig. 3D]. Using immunoblotting, we evaluated specific post-translational modifications that occur on the p53 protein following 6 h propranolol treatment of MDA-MB-231 cell lysates. Phosphorylation events were normalized based on levels of actin and total p53, revealing that propranolol increased the phosphorylation of p53 on residues S9, S37, and S46 [Fig. 3E and F].

Propranolol induces apoptosis in breast cancer cells

IHC for the pro-survival protein, Bcl-2, was performed on the diagnostic biopsy and tumor tissue from the radical mastectomy in the late stage breast cancer patient. Decreased Bcl-2 staining intensity was observed after propranolol administration. We then treated MDA-MB-231 cells with a time course of propranolol up to 6 h and evaluated changes in cleaved caspase levels using immunoblotting. Our analysis revealed that propranolol markedly increased the levels of caspase 3, 6, and 9 cleavage products as early as 2 h post treatment [Fig. 4B and C]. MDA-MB-231 cells treated for 6 h with control or propranolol were stained with the apoptotic and necrotic markers, Apopxin Green Indicator and 7-AAD, respectively, revealing substantial apoptotic increases in propranolol treated cells [Fig. 4D]. At the time point evaluated, we observed no visible increases in necrosis for the propranolol treated cells [Fig. 4D].

Discussion

In a retrospective analysis of over 400 patients, we previously reported that use of non-selective β-AR antagonists was associated with decreased proliferation of early stage breast cancer [3]. While we did not observe an effect on late stage breast cancer patients in this study, these observations may have been confounded due to an inability to control β-AR antagonist dosing or length of administration in a retrospective setting. Therefore, we sought to evaluate, in a controlled prospective manner, whether propranolol could inhibit tumor growth in a late stage breast cancer patient. Our findings revealed that neoadjuvant propranolol reduced the proliferative index of a stage III breast tumor by 2.7-fold. We then corroborated these findings at the molecular level, showing that propranolol disrupted the cell cycle distribution of breast cancer populations and altered the protein expression of the cell cycle regulatory proteins cyclin A, D1, E1, and E2. Decreased proliferation in response to β-AR antagonists has been reported across multiple tumor cell types, and is accompanied by disruption of mitogenic signal transduction cascades including AKT, MAPK, and cAMP [3,26–29]. These β-AR antagonist-mediated changes in tumor proliferation rates and mitogenic signaling cascades may contribute to decreased breast cancer metastasis and recurrence, as well as improved overall patient survival demonstrated in a number of clinical studies [12,14,16,30–32].

In addition to decreased Ki-67 antigenicity, we observed an increase in pro-apoptotic p53 protein expression and a decrease in pro-survival Bcl-2 protein expression in late stage breast cancer tissue following neoadjuvant propranolol administration. Moreover, β-AR antagonism increased the sub-G1 cell cycle population of breast cancer cells, suggesting that blocking β-AR signaling leads to breast cancer cell death. Indeed, our live/dead assay indicated that propranolol enhanced apoptosis as indicated by higher Apopxin staining in propranolol treated cells relative to the control. We corroborated these findings at the molecular level by showing that propranolol stabilized p53 protein levels in breast cancer cells, and enhanced the phosphorylation of p53 regulatory sites including S9, S37, and S46. Stabilization of p53 by DNA damage or cell stress has been extensively shown to trigger multiple anti-proliferative and pro-apoptotic signaling cascades [33]. DNA damage induces phosphorylation of p53 at S9 and S37, while phosphorylation at the S46 residue regulates the ability of p53 to induce apoptosis [34,35]. Moreover, propranolol led to increased levels of cleaved initiator caspase 9 and execution caspases 3 and 6 in breast cancer cells, further indicating that propranolol treatment leads to programmed cell death. Similar effects on cell survival and apoptotic signaling have been observed in previous studies across other tumor types, including propranolol-induced caspase activation, increases in p53 steady state protein levels, and enhanced cell death [28,36–40]. We have previously reported propranolol selectivity against breast cancer cells, while this treatment relatively spares non-diseased mammary epithelial cells [3]. Our understanding of the mechanism behind selectivity of propranolol is currently very limited, whereas mechanism(s) have been addressed to some degree in infantile hemangiomas. While it has been reported that propranolol inhibits cell proliferation of both infantile hemangioma and normal endothelial cells in culture [27], selectivity has been observed in infantile hemangioma cells. β-AR antagonism induces apoptosis in hemangioma endothelial cells, yet spares hemangioma-derived stem cells in part through a differential regulation of Akt and other survival regulators [41], as well as a propensity for propranolol to selectively promote adipogenesis in hemangioma-derived stem cells [42]. Further studies are necessary to understand the mechanisms behind the selectivity of β-AR antagonism against diseased cells.

While the current study focused exclusively on the effects of propranolol on mitogenic and apoptotic signaling regulators in breast cancer, studies from other tumor types suggest that β-AR antagonism may disrupt a variety of processes. For instance, propranolol interferes with several steps during angiogenesis including cell proliferation and formation of capillary tubules [43,44]. β-AR antagonism also modulates the expression and activation of angiogenic signaling pathways including angiopoietin/TIE2, vascular endothelial growth factor (VEGF), and hypoxia inducible factor (HIF) [27,28,39,45–47]. Propranolol exhibits a biphasic effect on vascular resistance, with lower (10–20 mg/kg/day) and higher (20 mg/kg/day) doses inducing vasoconstriction and vasodilation, respectively [48]. This same study showed that propranolol affects tumor angiogenesis, but not capillary density in melanoma tumor models [48]. In both breast and neuroblastoma models, propranolol has been shown to increase the treatment efficacy of chemotherapy by potentiating anti-angiogenic effects [5], suggesting that combination treatments with propranolol may be more effective at decreasing vascular networks than either treatment alone. An emerging area of research where β-AR
antagonism may substantially impact the oncogenic process is regulation of tumor immunity. Catecholamine activation of β-ARs has long been known to suppress innate and cellular immunity by decreasing interleukin (IL)-12 production and increasing IL-10 secretion, thus shifting the immune reaction away from a beneficial T helper cell 1 (TH1) response [49,50]. A recent study in melanoma has demonstrated that propranolol is capable of reducing infiltration of immunosuppressive myeloid cells into the tumor and increasing infiltration of cytotoxic lymphocytes [51]. As the ability of tumors to foster an immunosuppressive microenvironment is a major impediment to current immunotherapy strategies, the addition of propranolol with this class of anti-cancer therapeutics may help shift the balance toward a more desirable immunoreactive state within the tumor.

Conclusions

Collectively, our data suggest that incorporation of β-AR antagonists may decrease tumor proliferation not only in localized breast tumors, as we previously demonstrated [3], but also in advanced stages of disease. β-ARs are expressed across many different cancer types [52] and preclinical and clinical efficacy has been reported following inhibition of these receptors across a large number of diverse cancers [5,9,12,15,16,18,28,29,31,48,51,53–70]. The propranolol-mediated, anti-cancer mechanisms reported in the current study using breast cancer models may translate into a much broader group of cancers, paving the way for an inexpensive and non-toxic drug to be incorporated as an adjuvant to current treatment regimens.

Conflicts of interest

The authors declare no conflicts of interest.

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