Atovaquone: An Antiprotozoal Drug Suppresses Primary and Resistant Breast Tumor Growth by Inhibiting HER2/β-Catenin Signaling

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Abstract

Breast cancer is the second leading cause of cancer-related mortality in women. In the current study, we evaluated the antitumor effects of an antiprotozoal drug, atovaquone, against several breast cancer cell lines. Our results showed that atovaquone treatment induced apoptosis and inhibited the growth of all the breast cancer cell lines tested, including several patient-derived cells. In addition, atovaquone treatment significantly reduced the expression of HER2, β-catenin, and its downstream molecules such as pGSK-3β, TCF-4, cyclin D1, and c-Myc in vitro. Efficacy of atovaquone was further evaluated in an in vivo tumor model by orthotopic implantation of two highly aggressive 4T1 and Cl66 breast cancer cells in the mammary fat pad of female mice. Our results demonstrated that oral administration of atovaquone suppressed the growth of Cl66 and 4T1 tumors by 70% and 60%, respectively. Paclitaxel is the first-line chemotherapeutic agent for metastatic breast cancer. We demonstrate that atovaquone administration suppressed the growth of 4T1 paclitaxel-resistant tumors by 40%. Tumors from atovaquone-treated mice exhibited reduced HER2, β-catenin, and c-Myc levels alongside an increase in apoptosis. Taken together, our results indicate that atovaquone effectively reduces the growth of primary and paclitaxel-resistant breast tumors. Atovaquone is already in the clinics with high safety and tolerability profile. Therefore, the findings from our studies will potentially prompt further clinical investigation into repurposing atovaquone for the treatment of patients with advanced breast cancer.

Introduction

HER2 is a membrane tyrosine kinase that belongs to the family of EGFRs (1). It plays a crucial role in the complex signaling network that controls normal cell growth and differentiation. However, when activated, it is responsible for rapid proliferation of cells (2, 3). HER2 is known to be overexpressed in several neoplasms such as breast, ovarian, gastric, colorectal, and pancreatic cancers (4–6). About 30% of patients with breast cancer show overexpression of HER2, which contributes to tumor development and progression (7, 8). Few studies including a recent study from our laboratory found that HER2 overexpression is responsible for chemotherapeutic resistance (9, 10). Another study suggested that under certain conditions, cleavage of HER2 leads to the activation of intrinsic apoptotic pathway (11). Therefore, targeting HER2 signaling proves to be an effective therapeutic strategy in the clinic. Trastuzumab (Herceptin) is the first humanized antibody that targets HER2. However, acquired resistance to trastuzumab occurs after initial treatment phase, providing a therapeutic roadblock in improving patient’s overall survival (12–14). Hence, there is an unmet medical need of novel therapeutic agents that can target HER2 and suppress resistant and metastatic breast cancer.

Wnt/β-catenin signaling regulate crucial aspects of cell fate such as homeostasis, cell migration, and immune control (15). Wnt signaling is a complex pathway in which β-catenin is typically viewed as a central mediator. β-catenin signaling has an important role in controlling cellular proliferation and death, and has been shown to be associated with maintenance of cancer stem cells and metastasis (16, 17). Perturbation of β-catenin or downstream signaling molecules lead to the development of several human cancers (18), including breast cancer (19); making it a potential therapeutic target (20, 21).

Paclitaxel and other taxanes are approved for the treatment of metastatic breast cancer (22). The optimal dosage for paclitaxel as a single agent is 175 mg/m² as 3-hour infusion (23). However, it is well established that paclitaxel administration is associated with hematologic and neural adverse effects that constitute: neutropenia, peripheral neuropathy, and bone marrow suppression (24). Moreover, resistance toward paclitaxel occurs after several mechanisms has hindered its therapeutic use. To overcome the adverse effect and resistance to paclitaxel, it is important to explore novel therapeutic approaches that could be effective in treating paclitaxel-resistant breast tumors. In a recent study, we observed that overexpression of HER2/β-catenin signaling.
β-catenin signaling is one of the mechanisms that imparts resistance to paclitaxel (10).

Few studies have indicated the use of antiprotozoal and antimalarial drugs for the treatment of a variety of cancers such as hepatocellular carcinoma, acute myeloid leukemia, pancreatic, and colorectal cancer (25–28). Atovaquone is an antiprotozoal and an antimalarial drug that was approved by the FDA in 1999 for Pneumocystis carinii pneumonia and was eventually approved for the treatment of malaria in combination with proguanil. Previous studies have shown the anticancer potency of atovaquone in various cancer models (29–31). Interestingly, clinical studies on atovaquone for lung cancer and acute myeloid leukemia are currently underway (NCT03568994, NCT02628080). Therefore, these facts strengthen the necessity for more preclinical studies to establish the mechanism of the anticancer effects of atovaquone. This study provides an account of the preclinical investigation into the mechanism of atovaquone action and establishes the anticancer property of atovaquone against breast cancer. We demonstrated that oral administration of atovaquone not only suppressed the primary breast tumor growth but also reduced paclitaxel-resistant breast tumor growth by inhibiting HER2/β-catenin signaling.

Materials and Methods

Cell culture

Human breast carcinoma cell lines MCF-7, HCC1806, and T47D and murine breast cancer cells 4T1 were obtained from ATCC and were maintained in DMEM supplemented with 10% FBS, 1% PSN (penicillin, streptomycin, and neomycin). The MCF-7–overexpressing HER2 cells (MCF-7HH) were kindly provided by Dr. Huang Fei (Bristol-Myers Squibb) and CI66 cells were provided by Dr. Rakesh K. Singh (UNMC, Omaha, NE). Both of these cell lines were cultured in DMEM supplemented with 10% FBS and 1% PSN. SKBR3 cells were kindly provided by Dr. Marc Antonyak (Cornell University, Ithaca, NY) and were maintained in RPMI supplemented with 10% FBS and 1% PSN. 4T1 paclitaxel-resistant cells (4T1PR) were developed in our laboratory by gradually exposing the 4T1 cells to paclitaxel for several months. Patient-derived cells (TX-BR-237 and TX-BR-290) were obtained from Children’s Oncology Group (Texas Tech University Health Sciences Center, Lubbock, TX). These cells were maintained in IMDM supplemented with 20% FBS, 1% PSN, and 1× ITS (5 μg/mL insulin, 5 μg/mL transferrin, 5 μg/mL selenous acid). All the cell lines were periodical authenticated by short tandem repeats analysis.

Reagents and chemicals

HER2 shRNA was obtained from Genecopoeia. The chemicals, atovaquone, sulforhodamine B, and antibody against actin were obtained from Sigma-Aldrich. Atovaquone (Mepron) was purchased from PRASCO laboratories and was used for in vitro study. Xfect Transfection Reagent Kit was obtained from Clontech. All the antibodies and siHER2 were purchased from Cell Signaling Technology. TUNEL Assay Kit was purchased from Calbiochem.

Cytotoxicity studies

Breast cancer cells were plated at the density of 4,000 to 5,000 cells/well in 96-well plates and allowed to attach overnight. Next day, cells were treated with different concentrations of atovaquone for 24, 48, and 72 hours. At desired time points, cells were fixed with ice cold 10% trichloroacetic acid, washed, and stained with sulforhodamine B dye, and the optical density was measured in 10 mmol/L Tris base solution using plate reader, after washing the dye with 1% acetic acid solution as described by us previously (32).

 Colony formation assay

Approximately 400 to 600 4T1 and MCF-7 cells/well were seeded in a 6-well plate. Next day, cells were treated with 10, 15, and 20 μmol/L of atovaquone. After 72 hours of atovaquone treatment, media were replaced with fresh medium and 4T1 and MCF-7 cells were cultured for another 5 and 10 days, respectively. At day 9 or 14, cells were fixed and stained with 0.5% crystal violet solution after washing with PBS. Finally, the colonies with >50 cells were counted under an image J-software.

Annexin V-FITC apoptosis assay

Apoptosis assay was performed using a kit (BD Biosciences) according to the manufacturer’s instructions and as described by us previously (33). Approximately, 0.2 × 10⁶ cells were plated in 6-well plate and left overnight for attachment and further treated with 10, 20, and 30 μmol/L atovaquone. After 72 hours of treatment, cells were harvested by trypsinization, suspended in PBS to have cell density of 1 × 10⁶/mL. Furthermore, cells were suspended in 100 μL of binding buffer and 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added to the cell suspension and incubated for additional 20 minutes at room temperature in dark. Additional binding buffer was further added to make the final sample volume up to 500 μL. Samples were kept on ice in dark and analyzed by flow cytometer (Accuri C6).

Western blot analysis

Various breast cancer cells were treated with varying concentrations of atovaquone (10, 20, and 30 μmol/L) for 72 hours. Whole-cell lysates were prepared using 4% (w/v) CHAPS buffer, whereas tumor lysates were prepared by homogenizing the tumors in PBS and lysed using RIPA buffer. About 40 to 60 μg of protein was subjected to SDS-PAGE and the segregated protein was transferred on PVDF membrane. The membrane was developed as described by us previously (34, 35).

HER2 knockdown

Approximately 0.2 × 10⁶ SKBR3 and MCF-7HH cells were plated in a 6-well plate and left overnight for attachment. Next day, cells were transfected with HER2 siRNA (Cell Signaling Technology) as per the manufacturer’s instructions using siPORT transfection reagent. After 8 hours of transfection, cells were treated with 20 μmol/L atovaquone for 72 hours and the cell lysate was used for Western blotting. In another experiment, HER2 shRNA and scrambled shRNA (Genecopoeia) were transfected in SKBR3 cells using Xfect Transfection reagent (Clontech) as per the manufacturer’s instructions. After 8 hours of transfection, cells were treated with 20 μmol/L atovaquone. After 72 hours of atovaquone treatment, cell lysate was analyzed by Western blotting.

Immunoprecipitation assay

Immunoprecipitation assay was performed to examine the effect of atovaquone on the interaction of HER2 with β-catenin. Briefly, MCF-7 and MCF-7HH cells were treated with 20 μmol/L atovaquone for 72 hours. The cells from control...
and atovaquone-treated group were collected, lysed using RIPA buffer, and immunoprecipitated with β-catenin antibody using Dynabeads magnetic beads as per the manufacturer’s instructions. The samples were then resolved by SDS-polyacrylamide gel and transferred to PVDF membrane as described by us earlier (36). The membrane was immuno-blotted using HER2 antibody. Input is the cell lysate from control and atovaquone (20 μmol/L) treated MCF-7/HH cells. Negative control is the sample with the beads not immunoprecipitated with β-catenin.

Tumor therapy model
Female Balb/c mice (4–6 weeks old) were obtained from Envigo. Exponentially growing 4T1 and CI66 cells were harvested, washed twice with PBS, and resuspended in 1:1 PBS/Matrigel at a density of 0.7 × 10^6 and 1 × 10^6 cells/mL, respectively. A suspension of 0.1 mL containing 0.07 × 10^6 4T1 cells or 0.1 × 10^6 CI66 cells were injected orthotopically in the right and left mammary fat pad of female Balb/c mice, respectively. Tumor volumes were measured three times a week and animal weights were taken once a week as described by us previously (37). When the tumors reached a size of about 70 mm³, mice were randomly segregated into two groups with 7 mice in each group. Treated groups of mice received 30 mg/kg atovaquone by oral gavage according to approved protocol by IACUC. The tumors were removed aseptically from each mouse and snap frozen in liquid nitrogen for Western blot analysis.

Immunostaining of tumor sections
Female Balb/c mice (4–6 weeks old) were obtained from Envigo. Exponentially growing 4T1 and CI66 cells were harvested, washed twice with PBS, and resuspended in 1:1 PBS/Matrigel at a density of 0.7 × 10^6 and 1 × 10^6 cells/mL, respectively. A suspension of 0.1 mL containing 0.07 × 10^6 4T1 cells or 0.1 × 10^6 CI66 cells were injected orthotopically in the right and left mammary fat pad of female Balb/c mice, respectively. Tumor volumes were measured three times a week and animal weights were taken once a week as described by us previously (37). When the tumors reached a size of about 70 mm³, mice were randomly segregated into two groups with 7 mice in each group. Treated group of mice received 30 mg/kg atovaquone by oral gavage every day till day 25, whereas control mice received the vehicle. Mice were sacrificed on day 25 by CO₂ overdose and death was confirmed by cervical dislocation in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. The tumors were removed aseptically from each mouse and were snap frozen in liquid nitrogen for Western blot analysis. A part of tumor from control and treated group were kept in formalin for immunofluorescence analysis. All the mice experiments were conducted in accordance with the ethical standards and according to approved protocol by IACUC.

Resistance to paclitaxel was developed in 4T1 cells by gradually exposing these cells to paclitaxel for several months. In another in vivo study, approximately 0.07 × 10^6 4T1 cells were injected orthotopically in the left and right third mammary fat pad of mice. Once mice had stable tumor (~ size: 70 mm³), mice were divided into two groups (n = 10) and the treatment group was administered with 25 mg/kg atovaquone every day by oral gavage. Mice were sacrificed at day 19 and tumors were collected for further analysis as described earlier.

Immunostaining of tumor sections
The IHC was performed as described previously by us (38). Briefly, paraffin-embedded tissues were sectioned into 5- to 7-μm thick sections using microtome (Leica Microsystems Inc.). After deparaffinization and rehydration, antigens were retrieved by boiling the sections in 10 mM/L sodium citrate buffer (pH 6.0). The slides were washed with distilled water and incubated in 3% hydrogen peroxide methanol solution. The sections were then washed, blocked in 200 μL of blocking solution (5% goat serum diluted), and incubated with anti–β-catenin (1:100; Cell Signaling Technology) overnight at 4°C. Next day, primary antibody was removed and the sections were washed with wash buffer followed by 30 minute incubation with Ultravision ONE HRP polymer (Thermo Fisher Scientific) as per the manufacturer’s instructions. Subsequently, sections were washed with wash buffer and incubated with DAB Plus chromogen for 15 to 20 minutes. The sections were counterstained with hematoxylin and dehydrated. The slides were mounted using Permount (Thermo Fisher Scientific) and analyzed under a bright field Olympus microscope (Olympus America Inc). TUNEL assay was carried out according to the manufacturer’s protocol (Calbiochem).

Statistical analysis
Statistical calculations and analysis were performed using Prism 7.0 (GraphPad software Inc.). Results represent means ± SD or SEM of at least three independent experiments. Data were analyzed by Student t test or Fisher (F) test. Differences were considered statistically significant at P < 0.05.

Results
Atovaquone inhibits the proliferation of breast cancer cells
Initially, we determined the optimum concentration at which atovaquone could inhibit the proliferation of breast cancer cells. We performed the cytotoxicity assay in a panel of various breast cancer cell lines (MCF-7, SKBR3, HCC1806, 4T1, CI66, and T47D) with atovaquone. Our results showed that increasing concentrations of atovaquone significantly suppressed the growth of all the breast cancer cells in a concentration- and time-dependent manner. The IC₅₀ of atovaquone after 72 hours treatment ranged from 11 to 18 μmol/L. In all the cell lines tested (Fig. 1A–F). We further evaluated the growth-suppressive effects of atovaquone in two adult patient-derived cells, TX-BR-237 and TX-BR-290. The IC₅₀ of atovaquone was found to be in the range of 18 to 60 μmol/L in patient-derived cells after 72-hour treatment (Fig. 1G–H). These results suggest the potential cytotoxic effects of atovaquone in all the breast cancer cells in a concentration- and time-dependent manner. We also performed colony formation assay to investigate whether atovaquone could inhibit the proliferation of breast cancer cells. Results from this assay clearly showed that atovaquone treatment significantly decreased the size and number of colonies in a concentration-dependent manner (Fig. 1I–L). We did not observe much toxic effects of atovaquone in HEK293 cells, which are normal human embryonic kidney cells (Supplementary Fig. S1).

Atovaquone causes induction of apoptosis
To gain further insight into the mechanism of the cell growth–inhibitory effects of atovaquone, SKBR3, HCC1806, 4T1, and MCF-7 cells were treated with various concentrations of atovaquone for 72 hours and analyzed for apoptosis using Annexin V/ PI assay. Our results showed that 20 to 30 μmol/L atovaquone caused significant apoptosis in all the breast cancer cell lines after 72 hours of treatment. A concentration-dependent increase in apoptosis with atovaquone treatment was observed. For instance, treatment of HCC1806 and MCF-7 cells with 20 and 30 μmol/L atovaquone resulted in approximately 25% to 35% and 20% to 30% apoptosis, respectively, compared with controls (Fig. 2B and D). On the other hand, SKBR3 and 4T1 cells were more sensitive to atovaquone-induced apoptosis. Percentage of apoptotic cells in SKBR3 and 4T1 at 10, 20, and 30 μmol/L atovaquone concentrations were 15% to 70% and 40% to 95%, respectively (Fig. 2A and C). Approximately 30% to 90% apoptosis was observed in all the cell lines after 72-hour treatment with 30 μmol/L atovaquone as shown in Fig. 2A–D.
Atovaquone exhibits potent antitumor activity in breast cancer cell lines. HCC1806 (A), SKBR3 (B), MCF-7 (C), 4T1 (D), T47D (E), and CI66 (F) cells were treated with increasing concentrations of atovaquone for 24, 48, and 72 hours. Cell survival was measured with sulforhodamine B (SRB) assay to estimate the IC50 values. Patient-derived cell lines TX-BR-237 (G) and TX-BR-290 (H) were also evaluated for cytotoxic effect of atovaquone. The experiments were repeated at least three times with 8 replicates in each experiment. Colony formation assay was performed by seeding 400 to 600 cells/well. 4T1 cells were fixed and stained using crystal violet (0.5%) after 9 days and MCF-7 cells after 14 days. Number of colonies formed in control and atovaquone-treated wells was quantitated using ImageJ software. Representative images of colonies and their quantification in 4T1 cells (I and J) and MCF-7 cells (K and L). Data shown as mean ± SD; (n = 3). Student t test for unpaired samples was used to perform statistical comparisons.
Atovaquone treatment depletes HER2 levels and inhibits β-catenin signaling

To further elucidate the mechanism of cell death induced by atovaquone, we performed Western blot analysis using lysates from SKBR3, 4T1, HCC1806, MCF-7, CI66, and T47D cells treated with 10, 20, and 30 μmol/L atovaquone for 72 hours. First, we compared the basal level of HER2 and β-catenin in all the cells (Fig. 2E). Consistent with previous observations, MCF-7, 4T1, and HCC1806 cells showed very low constitutive levels of HER2. Our results showed that atovaquone treatment significantly reduced HER2 expression in a concentration-dependent manner in all the cell lines tested (Fig. 3; Supplementary Fig. S2). Interestingly, complete abrogation of HER2 expression was observed in 4T1, MCF-7, and CI66 breast cancer cells after treatment with 30 μmol/L of atovaquone (Fig. 3). Moreover, atovaquone treatment also suppressed the phosphorylation of HER2. β-Catenin was also found to be reduced significantly
Atovaquone inhibits HER2/β-catenin to suppress breast cancer

Figure 3. Atovaquone downregulates HER2/β-catenin signaling in breast cancer cells. SKBR3 (A), 4T1 (B), MCF-7 (C), and HCC1806 (D) cells were treated with varying concentrations of atovaquone for 72 hours. Representative blots showing the concentration dependent effect of atovaquone on p-HER2, HER2, β-catenin, p-GSK3β, Gsk-3β, c-Myc, TCF-4, TCF-1, cyclin D1, MMP-7, cleaved caspase-3, and cleaved PARP. Actin was used as loading control. Each experiment was repeated three times independently.

by atovaquone treatment in all the breast cancer cells (Fig. 3; Supplementary Fig. S2). β-Catenin signaling plays a critical role in the regulation of cell proliferative pathways and is known to be activated in breast tumors. c-Myc, a proto-oncogene regulated by β-catenin, was also significantly down-regulated by atovaquone. In addition, atovaquone treatment substantially reduced the expression of other downstream markers of β-catenin signaling, such as p-GSK3β, TCF-4, TCF-1, cyclin D1, and MMP-7. Accumulating evidences have established GSK3β as a viable therapeutic target in multiple tumor types including breast cancer (44–46). Our results showed reduction in GSK3β expression with atovaquone treatment in SKBR3 and MCF-7 cells, whereas its expression remains unchanged in 4T1 and HCC1806 cells (Fig. 3A–D). As shown in Fig. 3, we also observed significant increase in the cleaved fragments of caspase-3 and PARP after treatment with 20 and 30 μmol/L atovaquone, indicating apoptosis. These results suggest that atovaquone induces apoptosis in breast cancer cells by inhibiting HER2/β-catenin signaling (Fig. 3).
Effect of atovaquone treatment in cells stably overexpressing HER2

Next, we sought to determine whether HER2 overexpression could affect atovaquone induced apoptosis. For this, we determined the effect of atovaquone on MCF-7/HH cells. First, we compared the cytotoxic effects of atovaquone in MCF-7/HH with MCF-7 cells. We observed that atovaquone significantly suppressed the growth of MCF-7/HH cells similar to MCF-7 cells (Fig. 1C) and the IC_{50} of atovaquone was comparable in both the cell lines (Fig. 4A). To determine the effect of atovaquone on HER2 and other oncogenic proteins, MCF-7 and MCF-7/HH cells were treated with varying concentrations of atovaquone for 72 hours and evaluated by Western blotting. Although the expression of HER2 was drastically reduced by atovaquone treatment in both the cell lines, the extent of HER2 downregulation was diminished in MCF-7/HH cells when compared with MCF-7 cells. As shown in Fig 4B, MCF-7/HH cells showed diminished down-regulation of β-catenin, c-Myc, and other downstream markers as compared with the MCF-7 cell line in response to atovaquone treatment. Furthermore, slightly reduced apoptosis was seen by atovaquone treatment as indicated by less cleavage of PARP and caspase-3 in MCF-7/HH cells when compared with MCF-7 cells. Interestingly, we observed that not only HER2 expression was greater in MCF-7/HH cells but also the expression of β-catenin and its downstream markers such as p-GSK3β, c-Myc, TCF-4, and MMP-7 was constitutively higher. Taken together, these results clearly indicate that atovaquone was almost equally ineffective in suppressing the growth of MCF-7/HH cells by inhibiting HER2/β-catenin signaling.

HER2 silencing enhances atovaquone-induced apoptosis

Furthermore, to confirm the role of HER2 in atovaquone-induced apoptosis in breast cancer cells, HER2 was knocked down using HER2 siRNA in SKBR3 cells, which has high constitutive level of HER2 expression. It is important to note that knocking down HER2 resulted in the reduction of β-catenin and c-Myc levels (Fig. 4C). Atovaquone treatment further reduced the expression of these proteins and enhanced the cleavage of caspase-3 and PARP in HER2 knocked down SKBR3 cells as compared with SKBR3 cells with constitutively high HER2 expression (Fig. 4C). These results were also confirmed in MCF-7 cells stably overexpressing HER2 (MCF-7/HH), siRNA as well as shRNA was used to knock down HER2 in MCF-7/HH cells, which were further treated with 20 μmol/L atovaquone. As shown in Fig. 4D–E, knocking down HER2 resulted in significant cleavage of caspase 3 and PARP in MCF-7/HH cells. Interestingly, we observed that silencing HER2 also lead to reduced expression of β-catenin, c-Myc, and cyclin D1, which was further reduced by atovaquone treatment. These results not only suggest the regulation of β-catenin and c-Myc by HER2 but also the role of HER2/β-catenin in atovaquone-induced apoptosis (Fig. 4C–E).

Degradation of HER2 and β-catenin by atovaquone is proteasome dependent

We performed experiments using proteasome inhibitor MG-132 to understand the mechanism of HER2 and β-catenin degradation in MCF-7/HH cells by atovaquone. MCF-7 cells were treated with either MG-132 or 20 μmol/L atovaquone or a combination of both. We observed a humongous increase in the expression of HER2 and β-catenin in the cells treated with MG-132 and then with atovaquone, indicating that these proteins are degraded by proteasome pathway (Fig. 4F). We also observed that the effect of atovaquone was blocked when used in combination with MG-132. These results further corroborate our hypothesis that atovaquone promotes degradation of HER2 and β-catenin in a proteasome-dependent manner as evidenced by the rescue of proteins by MG-132 (Fig. 4F).

Atovaquone treatment disrupts HER2/β-catenin interaction

To further analyze the cross-talk of HER2 with β-catenin and to evaluate the effect of atovaquone on the interaction of HER2 and β-catenin, immunoprecipitation studies were performed. MCF-7 cells were treated with or without 20 μmol/L atovaquone for 72 hours; β-catenin was immunoprecipitated with β-catenin antibody and immunoblotted with HER2. We observed a clear immunoblot of HER2 in cells immunoprecipitated with β-catenin, suggesting a direct interaction of β-catenin with HER2. Our results also showed that the cells treated with atovaquone and immunoprecipitated with β-catenin antibody exhibited low expression of HER2, indicating reduced association of HER2 with β-catenin after atovaquone treatment (Fig. 4G). To confirm these results, we also performed a similar experiment in HER2-over-expressing MCF-7/HH cells. We observed similar results suggesting interaction between HER2 and β-catenin was completely blocked after atovaquone treatment (Fig. 4H). Taken together, these results demonstrate that HER2/β-catenin is a possible target of atovaquone in breast cancer cells.

Tumor therapy model

Furthermore, to evaluate the efficacy of atovaquone treatment in vivo and to establish the mechanism of tumor growth inhibition in breast cancer, highly aggressive 4T1 and CI66 cell lines were used. About 0.07 × 10^6 4T1 and 0.1 × 10^6 CI66 were orthotopically implanted in the mammary fat pad of female Balb/c mice. After each mouse had a tumor growth of about 70 mm^3, mice received 30 mg/kg atovaquone by oral gavage every day. Because of the high tumor burden in control mice, all animals were sacrificed at day 25. Our results showed that atovaquone treatment significantly suppressed the growth of breast tumors (Fig. 5A and E). At day 25, tumor volume of 4T1 tumors in atovaquone-treated group was reduced by 60% as compared with control group (865.53 vs. 342.99 mm^3; Fig. 5A). Similarly, atovaquone treatment significantly suppressed the growth of CI66 tumors by 70% (1,778.55 mm^3 vs. 538.57 mm^3; Fig. 5E). In addition, average weight of CI66 tumors dissected from atovaquone-treated mice was approximately 55% less than the weight of the tumors from control mice (Fig. 5F). The tumors of control- and atovaquone-treated mice were further analyzed by Western blotting and IHC. Consistent with our in vitro results, Western blots from 4T1 tumor lysate showed that atovaquone treatment substantially downregulated HER2 and β-catenin expression (Fig. 5B). We also observed a decrease in c-Myc expression in the tumors of atovaquone-treated mice. It is
Figure 4.
Change in HER2 expression modulates the effect of atovaquone. A, Percentage cell survival of MCF-7HH cells when treated with atovaquone at indicated concentrations and time points. B, MCF-7 and MCF-7HH cells were treated with 10, 20, and 30 μmol/L atovaquone for 72 hours, and whole lysate was analyzed by Western blotting for HER2, β-catenin, p-GSK3β, c-Myc, TCF-4, cyclin D1, MMP-7, c-caspase-3, and c-PARP. C, SKBR3 cells transfected with either scrambled or HER2 siRNA and treated with or without 20 μmol/L atovaquone for 72 hours. Whole-cell lysate was evaluated for HER2, β-catenin, c-Myc, cyclin D1, c-caspase-3, and c-PARP. MCF-7HH cells were treated with atovaquone (20 μmol/L) for 72 hours after transfecting the cells with HER2 siRNA or scrambled siRNA (D) and HER2 shRNA or scrambled shRNA (E). Levels of HER2, β-catenin, c-Myc, cyclin D1, c-caspase-3, and c-PARP were evaluated by Western blotting. Blots were quantitated and normalized with actin. Each experiment was repeated three times independently. F, MCF-7HH cells treated with 10 μmol/L MG-132 and 20 μmol/L atovaquone alone and in combination for 72 hours. β-Catenin was immunoprecipitated from control and 20 μmol/L atovaquone-treated cells and probed for HER2 in MCF-7 (G) and MCF-7HH (H) cells. IgG was used as loading control.
noteworthy that substantial cleavage of caspase-3 and PARP was observed in the tumors from atovaquone-treated group as compared with control, indicating apoptosis (Fig. 5B). Similar results were observed in the tumor lysates from CI66 tumors (Fig. 5G). These observations were confirmed by IHC in the tumor sections from control- and atovaquone-treated mice. Figure 5C shows reduced staining for HER2 and β-catenin in 4T1 tumors from atovaquone-treated mice. TUNEL staining showed increased...
staining in the tumor sections from atovaquone-treated mice as compared with control mice, indicating apoptosis (Fig. 5D). No change in the body weight of atovaquone-treated mice was seen as compared with control mice (Fig. 5H).

**Atovaquone suppresses the growth of paclitaxel-resistant tumors**

Paclitaxel is a chemotherapeutic agent used for breast cancer treatment. However, frequent development of resistance to paclitaxel dampens the clinical response. We developed >100-fold paclitaxel-resistant 4T1 breast cancer cell line (4T1PR). Interestingly, we observed enhanced expression of HER2, β-catenin, and other downstream markers in 4T1PR cells when compared with 4T1 cells (10). Furthermore, we wanted to determine whether atovaquone would be effective against paclitaxel-resistant breast cancer cells. For this purpose, we first evaluated the cytotoxicity of atovaquone in 4T1PR cells. We observed that the IC50 of atovaquone was in the range of 13 to 40 μmol/L at 24 to 72 hours of treatment (Fig. 6A). Next, we determined the percentage apoptosis with atovaquone treatment using Annexin V-FITC apoptosis assay in 4T1PR cells. We observed a concentration-dependent increase in apoptosis with atovaquone treatment (Fig. 6B). Furthermore, we implanted paclitaxel-resistant 4T1 (4T1PR) cells in the left and right mammary fat pad of female mice. Once each mouse had palpable tumors, mice were treated with 25 mg/kg atovaquone every day by oral gavage for 19 days. Our results showed that atovaquone significantly suppressed the growth of 4T1PR tumors by 42% (Fig. 6C). In addition, we noted a decrease in the weight of tumors isolated from atovaquone-treated mice when compared with control mice (Fig. 6D). At the end of the experiment, tumors were collected and analyzed by Western blotting. Our results showed that atovaquone treatment further suppressed the expression of HER2 and β-catenin in 4T1PR tumor lysates, while cleavage of caspase-3 and PARP increased (Fig. 6E). Furthermore, similar to Western blot results, IHC staining showed reduction in the expression of HER2 and β-catenin in the tumor sections of atovaquone-treated mice as compared with controls. In addition, increased expression of c-caspase-3 was observed in the tumor sections from atovaquone-treated mice (Fig. 6G). This suggests atovaquone inhibited 4T1PR tumor growth by inhibiting HER2/β-catenin signaling. We also observed increased apoptosis in tumor samples obtained from atovaquone-treated mice as analyzed by TUNEL assay (Fig. 6H). Taken together, our results indicate that paclitaxel-resistant tumor growth suppression by atovaquone treatment was associated with downregulation of HER2/β-catenin signaling and increased apoptosis.

**Discussion**

HER2 and β-catenin signaling pathways are upregulated in breast cancer and lead to tumor development and progression. Moreover, these pathways are also accountable to impart chemotherapeutic resistance (47, 48). To develop effective therapies for breast cancer, one salient approach is to target HER2 and β-catenin signaling. In the current study, we demonstrate that the FDA-approved drug atovaquone is an attractive candidate for breast cancer treatment. Atovaquone is an antiprotozoal agent that belongs to the class of naphthoquinones. It is used for the prevention and treatment of malaria, toxoplasmosis, and pneumocystis pneumonia (PCP; refs. 49, 50). Atovaquone primarily works by inhibiting the mitochondrial electron transport chain of malarial parasite and thus causes cell death (51). Herein, we report the novel anticancer mechanism of atovaquone by inhibiting HER2/β-catenin signaling in breast cancer.

HER2, an EGFR-related oncogene is found to be amplified in breast cancer and its overexpression is associated with an aggressive progression of the disease with poor prognosis (52). Wnt/β-catenin signaling has recently gained attention as an important target for therapy in various cancer types (53). Recently, a phase II clinical trial (NCT02413853) has been undertaken for metastatic colorectal cancer using PRI-724—a β-catenin antagonist. The outcome of this study indicated that PRI-724 blocks the expression of many proteins by inhibiting β-catenin and thereby suppresses cancer growth. Similarly, several other clinical trials also have been initiated by the NCI (NCT01764477, NCT01398462, and NCT01302405) to evaluate the efficacy of β-catenin antagonists or inhibitors in various cancer types such as pancreatic adenocarcinoma, acute myeloid leukemia, and advanced solid tumors. Hence, for the rational design of new therapies, it is crucial to establish the mechanism of interaction of HER2 with β-catenin, paving a way to better understand the underlying biology for drug targeting. Herein, our results showed significant suppression of HER2/β-catenin signaling by atovaquone treatment. Interestingly, we observed that silencing HER2 also reduced the expression of β-catenin and c-Myc in SKBR3 and MCF-HH cells, indicating a cross-talk between HER2 and β-catenin. In a previous study, we evaluated the interaction of HER2 with β-catenin and found that HER2 regulates β-catenin (10). In agreement, results from current study also show an association of HER2 and β-catenin with its further reduced by atovaquone treatment as evaluated by immunoprecipitation studies.

Our results demonstrate that atovaquone treatment significantly suppresses the viability of several breast cancer cells, including MCF-7, SKBR3, HCC1806, 4T1, Cl66, and T47D with IC50 ranging from 11 to 18 μmol/L. In addition, atovaquone also reduces the proliferation of patient-derived breast cancer cells, TX-BR-247 and TX-BR-290. It is noteworthy that the average steady-state plasma concentration of atovaquone in humans is 57 μmol/L (54, 55), concentration that is approximately 3- to 4-fold higher than the IC50 concentration in our cell model. This suggests that the effective therapeutic concentration of atovaquone is clinically achievable. The reduced viability of breast cancer cells by atovaquone was also associated with HER2/β-catenin downregulation. In addition, atovaquone treatment reduced the expression of downstream markers of β-catenin signaling such as p-GSK3β, TCF-4, and TCF-1 as well as its transcriptionally regulated oncogenes such as c-Myc, cyclin D1, and MMP-7 in most of the breast cancer cell lines tested. Knocking down HER2 using siHER2 or shRNA enhanced the cytotoxic potential of atovaquone in these cells as demonstrated by an increase in the cleavage of caspase-3 and PARP. On the other hand, stable overexpression of HER2 in MCF-7 cells made the cells less sensitive toward atovaquone cytotoxicity. To determine the efficacy of atovaquone in vivo, we tested atovaquone in three different breast tumor models in mice. We used a highly aggressive cell lines, CI66 and 4T1 representing stage IV breast cancer, which is considered to be untreatable. Atovaquone suppressed the growth of both 4T1 and Cl66 breast tumors. Tumor growth suppression by atovaquone was associated with reduced HER2 and β-catenin signaling markers as demonstrated by Western blotting, IHC, and TUNEL staining of the excised tumors. In addition, oral administration of atovaquone suppressed the growth of paclitaxel-resistant breast
tumors. Paclitaxel is a clinically employed first-line treatment option for the patients with metastatic breast cancer. However, inherited or acquired resistance to paclitaxel is a major limiting factor for successful therapy. Our recent studies demonstrate the significance of overexpression of HER2, β-catenin, and associated proteins in paclitaxel-resistant breast cancer cells (10). As atovaquone downregulates HER2/β-catenin signaling markers, it can be successfully used as a potential agent to treat...
Atovaquone Inhibits HER2/β-Catenin to Suppress Breast Cancer

To the best of our knowledge, ours is the first study to demonstrate the use of atovaquone for breast cancer by inhibiting HER2/β-catenin signaling.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Gupta, S.K. Srivastava
Development of methodology: N. Gupta, S.K. Srivastava
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Gupta
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Gupta, S.K. Srivastava
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Srivastava
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