Carbidopa suppresses estrogen receptor-positive breast cancer via AhR-mediated proteasomal degradation of ERα

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Summary
Estrogen receptor-α (ERα) promotes breast cancer, and ER-positive cancer accounts for ~80% of breast cancers. This subtype responds positively to hormone/endocrine therapies involving either inhibition of estrogen synthesis or blockade of estrogen action. Carbidopa, a drug used to potentiate the therapeutic efficacy of L-DOPA in Parkinson’s disease, is an agonist for aryl hydrocarbon receptor (AhR). Pharmacotherapy in Parkinson’s disease decreases the risk for cancers, including breast cancer. The effects of carbidopa on ER-positive breast cancer were evaluated in cell culture and in mouse xenografts. The assays included cell proliferation, apoptosis, cell migration/invasion, subcellular localization of AhR, proteasomal degradation, and tumor growth in xenografts. Carbidopa decreased proliferation and migration of ER-positive human breast cancer cells in vitro with no significant effect on ER-negative breast cancer cells. Treatment of ER-positive cells with carbidopa promoted nuclear localization of AhR and expression of AhR target genes; it also decreased cellular levels of ERα via proteasomal degradation in an AhR-dependent manner. In vivo, carbidopa suppressed the growth of ER-positive breast cancer cells in mouse xenografts; this was associated with increased apoptosis and decreased cell proliferation. Carbidopa has therapeutic potential for ER-positive breast cancer either as a single agent or in combination with other standard chemotherapies.

Keywords Carbidopa · Estrogen receptor-positive breast cancer · AhR · Proteasomal degradation · ERα

Introduction
Breast cancer is one of the most prevalent cancers worldwide and the leading cause of cancer death in women [1]. The estrogen receptor-positive (ER +) breast cancer
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dev elopment of resistance to antiestrogen therapy [7, 8].

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ractor therapy (selective estrogen receptor modulators and aromatase inhibitors) is a standard component of front-line therapy for ER+ breast cancer. Unfortunately, most patients who initially respond to antiestrogen therapy such as tamoxifen eventually become resistant and stop responding to the therapy [7, 8]. Studies have suggested that the antiestrogen therapy induces mutations in ERα as a means to develop resistance in some cases [9, 10]. Interestingly, even though the effectiveness of tamoxifen and other selective estrogen receptor modulators gradually decreases with continuous treatment, breast tumors generally retain ERα, indicating that alterations in other signaling pathways at least partly contribute to the development of resistance to antiestrogen therapy [7, 8].

Carbidopa, an inhibitor of aromatic amino acid decarboxylase, is used to potentiate the therapeutic efficacy of L-DOPA in Parkinson’s disease (PD) [11]. Epidemiological evidence indicates that patients with PD have decreased incidence of most cancers (e.g., pancreatic cancer and breast cancer) with a notable exception of melanoma [12–14]. This protection against cancer in PD is most likely due to the combination therapy involving carbidopa and L-DOPA. However, studies have shown that L-DOPA has no or little role in this protection [15, 16], though it might play a role in the increased incidence of melanoma based on its biological role as a precursor in melanin synthesis in melanocytes. Recently, we addressed the possible role of carbidopa in cancer prevention and found that carbidopa functions as an agonist for aryl hydrocarbon receptor (AhR) and suppresses pancreatic cancer as a single agent [17]. AhR is essential to maintain cellular homeostasis in health and disease [18–21], but its expression pattern varies among cancers. It is upregulated in some cancers (e.g., colorectal cancer, pancreatic cancer, renal cancer, esophageal cancer) but down-regulated in others (e.g., breast cancer, prostate cancer) [22]. Activation of AhR as a therapeutic approach has been demonstrated by several studies with AhR agonists such as raloxifene [23–25]. Similar to raloxifene, carbidopa is also an FDA-approved drug and therefore can be potentially repositioned for cancer therapy if its anticancer efficacy is demonstrated convincingly. The anticancer effect of carbidopa, possibly via AhR activation, has been demonstrated in pancreatic cancer, but its effects have not been studied in breast cancer. The relevance of AhR to breast cancer is particularly interesting because this receptor functions not only as a transcription factor but also as a component of the substrate-recognition machinery in the E3 ubiquitin

ligase complex which targets steroid hormone receptors for proteasomal degradation [26–29]. As most cases of breast cancer are dependent on the steroid hormone receptor ERα for their growth, we initiated the current project to evaluate the impact of carbidopa on ER + breast cancer.

Materials and methods

Cell culture

Human breast cancer cell lines MCF7, MB231, T47D and BT549 were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2-95% air. T47D cells were cultured in DMEM medium with 10% fetal bovine serum (FBS), 50 units/mL streptomycin and 100 units/mL penicillin; MCF7, MB231 and BT549 cells were cultured in RPMI medium with 10% FBS, 50 units/mL streptomycin and 100 units/mL penicillin. All cell lines were tested and found to be free of mycoplasma contamination using MycoAlert® Mycoplasma Detection Kit (Lonza, Basel, Switzerland). When used for assays, cells were seeded in multi-well culture plates, and cultured in phenol red-free DMEM/RPMI-1640 medium. After 24 h, cells were washed with phosphate-buffered saline (PBS) and then treated with carbidopa (Sigma, St. Louis, MO, USA) for 24 h. CH223191 (Sigma, St. Louis, MO, USA) was used as a selective blocker of AhR. MG132 (Sigma, St. Louis, MO, USA) was used as a proteasome inhibitor.

Animals

Female athymic BALB/c-nude mice (7-week-old) were purchased from Vital River Laboratories (Beijing, China). The animals were maintained under a specific pathogen-free housing conditions in a barrier facility. They had free access to rodent chow and water. All animal studies were carried out in accordance with the Guidelines for Animal Experimentation of Wenzhou Medical University, and the Animal Ethics Committee of the institution approved the protocol.

Cell proliferation assay

Cell proliferation was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded in 96-well plates (2 × 10^3 cells/well). After 12 h, the cells were incubated for 24 h in the medium containing 0–30 μM carbidopa. Then, the medium was replaced with fresh medium containing MTT reagent (5 mg/mL in PBS; Solarbio, Beijing, China) and incubated for additional 4 h. The reaction product was dissolved in 150 μL of dimethyl sulfoxide and the absorbance at 490 nm quantified with a plate reader.
Colony-formation assay

For colony-formation assay, the cells were seeded into 6-well plates (2 × 10^4 cells/well). After 12 h, the cells were incubated in the medium containing carbidopa. The medium was changed every 3 days. When control cells reached 60–70% confluency, control cells and carbidopa-exposed cells were fixed with ice-cold 100% methanol for 20 min. The cells were then incubated with 0.1% crystal violet (Solarbio, Beijing, China) for 2 h. The cells were then washed with water and then imaged. The colonies counted under a light microscope (Leica, Wetzlar, Germany).

Cell-migration assay

Cells were seeded in six-well plates and incubated in the medium until grown to 90% confluency. A wound was created by scraping the cells in the middle of the confluent culture with a sterile 200 μL pipette tip. Cells were cultured in the medium containing 2.5% FBS for another 24 h. Then, the medium was replaced with PBS and the wound gap was photographed with an inverted microscope (Leica DM 14000B microscope fixed with digital camera, Wetzlar, Germany) at 0 h and 24 h.

Apoptosis analysis

Apoptosis was determined by flow cytometry and TUNEL staining. For flow cytometry, the cells were stained with Annexin V/FITC-propidium iodide (PI) using Apoptosis Detection kit (Beyotime Biotechnology, Shanghai, China). Tumor tissues were fixed in 10% formalin and embedded in paraffin. Specimens were sectioned at 5-µm thickness. For TUNEL assay, the MCF7 cells and tumor tissue sections were stained with an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). The stained cells were imaged with a confocal laser scanning microscope (TCS SP8, Leica, Wetzlar, Germany). The number of cells per field was counted and the percent of TUNEL-positive cells was calculated.

RNA isolation and PCR

RNA was extracted from the cells using TRIZOL Reagent (Takara, Japan). RNA (2 μg) was reverse-transcribed into cDNA (GoScript Reverse Transcription Kit; Promega, Madison, WI, USA). RT-PCR was conducted in a Thermal Cycler (T100, Bio-Rad, Hercules, CA, USA). PCR products were electrophoresed in agarose gel and visualized with ethidium bromide (GelDoc XR +; Bio-Rad, Hercules, CA, USA). Quantitative PCR (qPCR) was conducted using FastSYBR Green qPCR Master Mix (Takara, Japan) and Viia7 real time PCR machine (Bio-Rad). Changes in expression were calculated as the ratio of expression in treated samples to expression in control samples. The expression levels for target genes were normalized to the expression of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase). PCR primers were as follows: ERα: 5’-TTCTTCAATTTCCCAAG CGT-3’ (sense) and 5’-CTAAACACAAAGGCTA-3’ (antisense); cyclin D1: 5’-CCGTCATCGGAAGATCT-3’ (sense), 5’-ATGGCCACCGGAAACG-3’ (antisense); c-Myc: 5’-GCCAGAGGGAGAAGG-3’ (sense), 5’-GCT TGGACCGACAGAT-3’ (antisense); GAPDH: 5’-GCC AGTCAACGGATTTGCTAT-3’ (sense), 5’-GC CCTCTCCATGTTGGTGAAGA-3’ (antisense); CYP1B1: 5’-GGGCTACCACATCCCA-3’ (sense), 5’-GAGGCCATC CTTGTCCA-3’ (antisense); ARNT: 5’-GTTTGGCAGCA CACTCTATG-3’ (sense), 5’-ACAGTTATCTGCGCTCC GT-3’ (antisense); CYP1A1: 5’-CAAGGGGGCGTTGT GCTTTC-3’ (sense), 5’-GTGCATAGCACCACCATCAGGG-3’ (antisense).

Western blot

Lysates from cells and tumor tissues were prepared and protein determined using the BCA assay (Beyotime Biotechnology). Protein (30 μg) from each sample was resolved on SDS-PAGE, and transferred to PVDF membrane. Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibodies overnight at 4 °C. Membranes were washed thrice, 5 min each time, and then incubated with either horseradish peroxidase (HRP)-conjugated goat anti-mouse (ab6789, Abcam, Cambridge, UK) or HRP-conjugated goat anti-rabbit (ab6721, Abcam) secondary antibodies for 2 h at room temperature. Immunoreactive bands were visualized using Pierce ECL plus Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies used were: ERα (sc-8002, Santa Cruz Biotechnology, Dallas, TX, USA), HSP90 (ab203126, Abcam), β-actin (4970S, Cell Signaling Technology, Boston, MA, USA), Bax (2774S, Cell Signaling Technology), Bel-2 (15071S, Cell Signaling Technology), ubiquitin (3936S, Cell Signaling Technology), CYP1A1 (ab124295, Abcam) and AhR (ab190797, Abcam). The protein bands were analyzed using Image-Quant 5.2 software. β-Actin was used as loading control.

Immunofluorescence staining

Immunofluorescence staining was performed for nuclear localization of AhR in MCF7 cells. Cells in chamber slides were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton-X 100 (Solabio, Beijing, China) for 10 min at 37 °C. After washing, the cells were blocked in 5% BSA for 1 h and incubated with primary rabbit antibody against AhR at 4 °C overnight. Cells were then washed.
and incubated with Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (ab150113, Abcam) for 1 h. After subsequent washing with PBS, the cell nuclei were stained with DAPI for 15 min. Photo capture was performed by the confocal laser scanning microscope (TCS SP8, Leica).

**Luciferase reporter assay**

MCF7 cells were transfected with AhR-luciferase reporter plasmid (YEASEN, Shanghai, China) and pRL (renilla luciferase)-null plasmid (internal control) using Lipofectamine 2000. After 24 h, cells were serum-starved and treated with or without carbidopa. Cell lysates were used to measure luciferase activity using the Dual Luciferase Reporter Assay System (E1910, Promega, Madison, WI, USA). Firefly luciferase and renilla luciferase were detected on a Veritas Microplate Luminometer (Promega). Normalization was done with renilla luciferase.

**Immunoprecipitation**

MCF7 cells were lysed with IGEPAL CA-630 buffer: 50 mM Tris–HCl, pH 7.4, (Sigma, T5030), 1% IGEPAL CA-630 (Sigma, 18896), 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 μM leupeptin (Sigma, L5793), 0.1 μM aprotinin (Sigma, SRE0050). Primary antibody was covalently immobilized on protein A/G agarose using the Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific, 26,147). Samples were incubated with immobilized antibody beads for at least 2 h at 4 °C. Cell lysates were also subjected to immunoprecipitation with either mouse IgG isotype control (Cell Signaling Technology, 5415) or rabbit IgG isotype control (Cell Signaling Technology, 3900) according to the immunoglobulin type of the primary antibody. After immunoprecipitation, the samples were washed with PBS for 5 times. They were then eluted with glycine–HCl (0.1 M, pH 3.5) and the immunoprecipitates were then subjected to immunoblotting using specific primary antibodies described in the Western blot part.

**Molecule docking**

As there is no information available in the literature on the structure of the ligand-binding domain of AhR, a homology model has been developed for the PAS-B domain in AhR that interacts with agonists for the purpose of molecular modeling and docking simulations [30, 31]. With this model as the template, we analyzed the interaction of carbidopa and the endogenous AhR agonist L-kyurenine with the PAS-B domain of human AhR based on AutoDock Vina [32]. The size of the grid box was set to 12 Å × 12 Å × 12 Å. The exhaustiveness value was set to 8.

**Xenograft studies in athymic BALB/c nude mice**

BALB/c nude mice were randomly divided into two groups. The investigators performing the outcome analyses were blinded to the group assignment. MCF7 cells were subcutaneously injected into the right flank of each mouse (0.1 mL, 1 × 10⁶ cells per mouse). When tumors reached a volume of 100 mm³, mice were randomized into control and treatment groups (6 mice per group); no specific criteria or method was used for randomization. The treatment group received intraperitoneal (i.p.) injection of carbidopa (7.5 mg/kg; 0.1 mL volume) and the control group the same amount of vehicle twice a day, every 12 h. The treatment continued for 14 days continuously. The body weight and tumor volume were recorded every two days throughout the procedure. Tumor volumes were determined by measuring length (L) and width (W) of the tumors periodically and then calculating the volume using the formula: V = 0.5 × L × W² [33]. After 14 days, mice were euthanized under anesthesia. Tumor specimens were harvested, weighed, and then processed for histology and protein assays.

**Immunohistochemistry**

Tumor tissues were fixed in 10% formalin and embedded in paraffin. Specimens were sectioned at 5-μm thickness. The sections were stained using routine immunohistochemical techniques. Briefly, after pre-treatment with heat-mediated antigen retrieval for 20 min in a microwave oven, the endogenous peroxidase activity was blocked by incubating the tissues in 3% H₂O₂ for 30 min. After blocking for 15 min with 10% normal goat serum, the sections were treated with primary antibodies against Ki67 (1:500 dilution; overnight exposure) (ab15580, Abcam). HRP-conjugated secondary antibodies were used for detection. Next, the sections were exposed to Diaminobenzidine (DAB) solution (P0203, Beyotime Biotechnology), and counterstained by Hematoxylin Staining Solution (C0107, Beyotime Biotechnology). Finally, tumor specimens were observed under the microscope. The substitution of PBS for primary antibody was used as negative control. For Ki-67 index, numbers of positively stained cells were expressed as a percentage of the total number of cells examined.

**Statistical analysis**

Results are expressed as means ± SD. Statistical differences were assessed with the unpaired two-tailed Student’s t-test for two groups and with one-way ANOVA for multiple groups using SPSS software. Bonferroni’s post-hoc testing was employed after ANOVA to test for significant
differences between groups. \( p < 0.05 \) was considered statistically significant. Statistical analyses were done using GraphPad Prism (GraphPad Software).

**Results**

**Carbidopa inhibits proliferation and migration, and promotes apoptosis in ER\(^+\) breast cancer cells**

We evaluated the effects of carbidopa on four human breast cancer cell lines: MCF7, T47D, MB231 and BT549. MCF7 and T47D are ER\(^+\)-positive whereas MB231 and BT549 are ER\(^-\)-negative. First, we examined cell proliferation using MTT assay and colony formation assay. Carbidopa significantly reduced cell viability selectively in MCF7 and T47D cells as determined in MTT assay; there was little effect on MB231 and B549 cells (Figs. 1A, S1A). The inhibitory effect on the ER\(^+\) cell lines by carbidopa was dose-dependent; the IC\(_{50}\) values were 15–20 \( \mu \)M. Similar results were observed with colony formation assay (Figs. 1B, C, S1B, C). The inhibition by carbidopa was again selective for ER\(^+\) cell lines. We then examined the effect of carbidopa on cell migration using the wound healing assay. The

![Fig. 1](image_url)

Fig. 1 Carbidopa selectively inhibits the proliferation and migration of the ER\(^+\) breast cancer cell line MCF7. (A) MCF7 and MB231 cells were treated with the increasing doses of carbidopa for 24 h, and the cell viability was examined by MTT assay, data represent means ±SD of 3 independent experiments. (B, C) Colony formation assay in human breast cancer cell lines MCF7 (B) and MB231 (C). Cells were cultured in the presence of increasing doses of carbidopa for 2 weeks and then stained 0.1% crystal violet. (D, E) Migration assay with MCF7 (D) and MB231 (E) cells with vehicle alone (control) and carbidopa (15 \( \mu \)M). (F) Quantitative analysis of cell migration. Data represent means ±SD of 3 independent experiments. **, \( p < 0.01 \) versus control. ns, not significant.
migration of MCF7 and T47D was inhibited by carbidopa (15 µM) (Figs. 1D-F, S1D-F). Again, there was no effect of carbidopa on the migration of the two ER-negative cell lines MB231 and BT549.

To determine the effect of carbidopa on apoptotic cell death, we used two methods: detection of Annexin V-positive cells by flow cytometry and detection of DNA fragmentation in cells by TUNEL assay. The same ER+ (MCF7 and T47D) and ER-negative cell lines (MB231 and BT549) were used. Carbidopa promoted cell death in ER+ cells (Figs. 2A, S2B) with no noticeable effect on ER-negative cells (Fig. S2A, C); the effect of carbidopa was dose-dependent. The TUNEL assay confirmed the apoptotic cell death induced by carbidopa in MCF7 cells (Fig. 2B, C).

**Fig. 2** Carbidopa induces apoptosis selectively in the ER+ breast cancer cell line MCF7. (A) Flow cytometry analysis of apoptosis in MCF7 cells following 24 h treatment with increasing doses of carbidopa and vehicle alone (control). Apoptotic cells positive for annexin V were quantified. (B) TUNEL assay in MCF7 cells following 24 h treatment with increasing doses of carbidopa and vehicle alone (control). Apoptotic cells were labelled green, and nuclei were labeled blue (DAPI). Scale bars = 100 µm. (C) Quantitative analysis of TUNEL+ cells in at least 5 separate fields. Data represent means ± SD of 3 independent experiments. *, p < 0.05, **, p < 0.01, ***, p < 0.001 versus control. (D) Cell lysates of MCF7 cells were used to monitor the protein levels for Bax and Bcl-2 by immunoblotting in control cells and in cells treated with carbidopa (20 µM) for 24 h. (E) Quantitative analysis of the immunoblots; results were normalized to control; data represent means ± SD of 3 independent experiments. *, p < 0.05, **, p < 0.01 versus control.
The carbidopa-induced apoptosis in MCF7 cells was accompanied with an increase in the pro-apoptotic protein Bax and a decrease in the anti-apoptotic protein Bcl-2 (Fig. 2D). The blots were quantified and the data are presented as Bax/Bcl-2 ratio in carbidopa-treated cells normalized to control cells (Fig. 2E). The increase in Bax/Bcl-2 ratio was clearly evident in MCF7 cells exposed to carbidopa.

Involvement of AhR in carbidopa-mediated inhibition of proliferation and migration, and induction of apoptosis in MCF7 cells

Carbidopa is an agonist for the nuclear receptor AhR [17]. To examine the involvement of this receptor in the effects of carbidopa on MCF7 cells, we used CH223191 as a blocker of the receptor. When the treatment of cells with carbidopa was done in the presence of CH223191, the inhibitory effect of carbidopa on cell proliferation and migration was almost completely blocked; similarly, the carbidopa-induced apoptosis was also blocked markedly by the AhR antagonist (Fig. 3).

Carbidopa decreases ERα protein levels in MCF7 cells via ubiquitin–proteasome pathway

The selectivity of carbidopa effects towards ER+ breast cancer cells strongly suggests ERα as a potential target for carbidopa. Therefore, we monitored the levels of ERα protein...
in control and carbidopa-treated MCF7 cells. We found the levels of ERα to be decreased in response to carbidopa; the effect was dose-dependent (Fig. 4A, B). But, there was no change in ERα mRNA (Fig. 4C). This indicates that the carbidopa-mediated decrease in ERα protein occurs at the post-transcriptional level. Several studies have reported that the steroid hormone receptors AR (androgen receptor) and ERα are subject to degradation via the ubiquitin–proteasome pathway promoted by agonist-activated AhR [28, 29]. The findings that carbidopa is an agonist for AhR, its effects on cell proliferation and migration in ER+ breast cancer cells are abrogated by an AhR blocker, and that carbidopa decreases ERα protein post-transcriptionally point to the possibility that AhR-mediated proteasomal degradation is a possible mechanism for the effects of carbidopa. To validate this, we treated MCF7 cells with carbidopa (15 μM) for 24 h in the presence or absence of MG132 (10 μM), a cell-permeable inhibitor of the proteasomal protein degradation pathway, during the final 6 h of the 24 h treatment. We found that inhibition of proteasomal degradation prevented the carbidopa-mediated decrease in ERα protein (Fig. 4D, E). We then examined the ubiquitination of ERα protein in MCF7 cells treated with or without carbidopa in the presence of MG132. This was done by immunoprecipitation of

Fig. 4 Carbidopa decreases ERα protein levels through ubiquitin–proteasome pathway. (A) MCF7 cells were treated increasing doses of carbidopa for 24 h. Immunoblot analysis of ERα. (B) Quantitative analysis of ERα immunoblot; results were normalized to control; data represent means ± SD of 3 independent experiments. *, p < 0.05, **, p < 0.01, ***, p < 0.001 versus control. (C) MCF7 cells were treated increasing doses of carbidopa for 24 h. Quantitative RT-PCR (qRT-PCR) analysis of ERα mRNA. Results were normalized to control and data are given as means ± SD of 3 independent experiments. ns, not significant. (D) Influence of the proteasomal inhibitor MG132 on carbidopa-mediated decrease in ERα protein levels. MCF7 cells were incubated in the presence or absence of carbidopa (15 μM) for 24 h in the presence or absence of MG132 (10 μM) during the final 6 h of the 24 h treatment. (E) Quantitative analysis of the immunoblots; results were normalized to control; data represent means ± SD of 3 independent experiments. *, p < 0.05 versus control. ns, not significant. (F) MCF7 cells were treated with or without carbidopa (15 μM) for 24 h in the presence of MG132 (10 μM) during the final 6 h of the 24 h treatment. ERα was immunoprecipitated with an anti-ERα antibody, and the immunoprecipitated ERα was then used for immunoblot with an anti-ubiquitin antibody.
ERα protein with an anti-ERα antibody and subjecting the immunoprecipitate to immunoblot using an anti-ubiquitin antibody. The results showed that carbidopa increased the level of ubiquitinated ERα in the presence of MG132 (Fig. 4F). This demonstrates that carbidopa-dependent activation of AhR promotes ubiquitination of ERα for subsequent degradation by proteasomes. When this degradation is blocked with MG132, ubiquitinated ERα is increased.

**Carbidopa activates AhR, promotes its localization to the nucleus, and potentiates its transcriptional activity in MCF7 cells**

AhR promotes E3 ubiquitin ligase activity on selective protein substrates; ligand-activated AhR engages steroid hormone receptors for ubiquitination and proteasomal degradation [24–27]. Given that carbidopa decreases the levels of ERα in MCF7 cells and the supporting evidence for the involvement of AhR in carbidopa-mediated ubiquitination and proteasomal degradation of ERα in the process, we sought direct evidence for AhR activation by carbidopa. We first examined the impact of carbidopa on AhR. Carbidopa increased AhR target gene CYP1A1 protein level in MCF7 cells (Fig. 5A, B). We then monitored the transcriptional activity of AhR by examining the expression of selective AhR target genes. The levels of mRNA for CYP1B1, ARNT, and CYP1A1 increased in response to carbidopa (Fig. 5C). The transcriptional activity of AhR requires its localization in the nucleus. Ligand-free AhR resides in the cytoplasm, forming a complex with heat shock proteins [34]. Upon binding with an agonist, the cytoplasmic complex dissociates, and the ligand-bound AhR gets translocated to the nucleus to function as a transcription factor [35]. Therefore, we used immunofluorescence to localize AhR in control and carbidopa-treated MCF7 cells. Carbidopa promoted AhR translocation from cytoplasm into nucleus (Fig. 5D). We

Fig. 5 Direct evidence for activation of AhR and promotion of its transcriptional activity by carbidopa in MCF7 cells. MCF7 cells were treated with carbidopa (15 μM) and vehicle alone (control) for 24 h. (A) Lysates from control and treated cells were used for immunoblot to monitor CYP1A1 protein levels. (B) Quantitative analysis of the immunoblot. Results were normalized to control; data represent means±SD of 3 independent experiments. **, p<0.01 versus control. (C) Quantitative-RT-PCR (qRT-PCR) analysis of CYP1B1, ARNT and CYP1A1 mRNA. MCF7 cells were treated with or without carbidopa (15 μM) for 24 h, and then the cells were fixed and probed with an antibody specific for AhR. DAPI was used to stain nuclei. Scale bars = 50 μm. (E) AhR luciferase reporter activity assay. MCF7 cells were transfected with AhR luciferase reporter plasmid using Lipofectamine 2000. After 24 h transfection, cells were serum-starved and treated with carbidopa (15 μM; 24 h treatment) and vehicle alone (control). Data represent means±SD of 3 independent experiments. **, p<0.01 versus control.
also examined the transcriptional activity of AhR directly in MCF7 cells with an AhR-luciferase reporter. In this method, the transcriptional expression of the reporter luciferase is controlled by activated AhR. We found in MCF7 cells that have been transfected with the reporter plasmid, treatment with carbidopa increased the reporter activity (Fig. 5E), thus providing evidence for the promotion of the transcriptional activity of AhR by carbidopa.

**Involvement of AhR in carbidopa-mediated ubiquitination and proteasomal degradation of ERα in MCF7 cells**

Studies described thus far show that carbidopa promotes ubiquitination and proteasomal degradation of ERα and that carbidopa activates AhR. We then determined if these two processes were connected. For this, we evaluated the influence of the AhR blocker CH223191 on carbidopa-mediated alterations in ERα in MCF7 cells. Carbidopa decreased the levels of ERα, and co-treatment with CH223191 blocked this effect (Fig. 6A, B). Similarly, carbidopa increased the levels of CYP1A1 protein, and CH223191 blocked this effect. We then checked the ubiquitination of ERα with and without CH223191 treatment. These experiments were carried out in the presence of MG132 to prevent proteasomal degradation of ubiquitinated ERα. Carbidopa increased ERα ubiquitination, and CH223191 prevented this effect (Fig. 6C). As ligand-activated AhR promotes ubiquitination of selective substrates by E3 ubiquitin ligase, we inferred direct interaction between ERα and AhR. ERα interacts with the chaperone protein Hsp90. We used co-immunoprecipitation to monitor protein–protein interactions among ERα, AhR and Hsp90 in MCF7 cells with and without carbidopa treatment. In control cells without carbidopa treatment, ERα interacted with Hsp90 more robustly than with AhR (Fig. 6D, E). This preference was reversed in cells treated with carbidopa where the interaction of ERα with AhR was greater than with Hsp90. These data show that activation of AhR with carbidopa promotes dissociation of ERα from Hsp90 so that ERα could now interact with the activated AhR for subsequent ubiquitination by E3 ubiquitin ligase.

**Carbidopa suppresses the growth of MCF7 cells in nude mouse xenografts in vivo**

We assessed the in vivo anticancer effect of carbidopa in mouse xenografts. MCF7 cells were implanted in BALB/c nude mice. Once the tumors grew to a volume of ~100 mm³, mice were treated with either vehicle or carbidopa (7.5 mg/kg). Carbidopa significantly suppressed the tumor growth (Fig. 7A, B, D). There was little or no effect on body weight in carbidopa-treated mice (Fig. 7C). Immunohistochemical analysis of tumor tissue sections was performed to detect the expression of Ki-67, a key marker for cellular proliferation. The results showed a profound decrease of Ki-67 expression in carbidopa-exposed tumor tissues (Fig. 7E, F). There was also strong evidence of carbidopa-induced apoptosis as assessed by TUNEL assay (Fig. 7G). Even in in vivo grown tumors, the increase in CYP1A1 protein and the decrease in ERα protein in response to carbidopa were evident (Fig. 7H, I). Similarly, the increase in the pro-apoptotic protein Bax and the decrease in the anti-apoptotic protein Bcl-2 that were observed in vitro in cell culture were also evident in vivo tumors (Fig. 7H, I).

**Expression pattern of AhR in breast cancer and molecular docking of carbidopa with the ligand-binding pocket in AhR**

The results of this study have shown that activation of AhR with carbidopa blocks the proliferation and growth of ER + breast cancer cells in vitro and in vivo, suggesting that AhR functions as a tumor suppressor in breast cancer. These observations prompted us to examine the TCGA (The Cancer Genome Atlas) database for AhR expression in breast cancer. The expression of AhR is markedly reduced in primary breast tumors (no differentiation among the major subclasses) at the mRNA level (p = 1.1 E−16) (Fig. 8A). When the major subclasses were examined individually, the decrease is evident in all three major classes of breast cancer (ER+, HER2-positive, and TNBC) (Fig. 8B). Kaplan–Meier survival analysis indicates a better survival probability in patients with higher expression of AhR (p = 0.013) (Fig. 8C). Here no information is available with regard to the relative contribution of the different subclasses of breast cancer to the survival analysis, but it is likely that most of the cases examined were ER + subtype. These findings corroborate the proposed tumor suppressive function of AhR in breast cancer.

We also performed a molecular docking modeling for the interaction of carbidopa with the ligand-binding pocket of AhR. For this, we employed a computer-based molecular docking approach using the homology model available in the literature for the structure of the ligand-binding domain (PAS-B) in human AhR [30, 31]. As a positive control, we used the endogenous agonist L-kynurenine. This docking simulation revealed that the binding modes for carbidopa and L-kynurenine at the ligand-binding pocket of human AhR are quite similar. The aromatic ring in both of these agonists insert into a hydrophobic core of the ligand-binding pocket, which is lined by Phe-287, Leu-308, Phe-324, and Leu-353 (Fig. S3). In addition, carbidopa and kynurenine form a hydrogen bond with His-337 and Thr-289, respectively.
Discussion

Carbidopa is used in combination with DOPA to treat Parkinson’s disease; it is not used by itself for any indication. It is a potent inhibitor of DOPA decarboxylase, and thus prevents the conversion of DOPA into dopamine. But the pharmacologic action of carbidopa occurs only outside the brain because the drug does not cross the blood-brain barrier. Since DOPA is metabolized outside the brain primarily in enteroendocrine cells of the intestinal tract, carbidopa blocks DOPA metabolism at this site, thus increasing the half-life of DOPA in circulation. Consequently, more
DOPA is available to the brain where it gets converted to dopamine to treat PD. Until recently, the only pharmacologic target known for carbidopa was DOPA decarboxylase [36]. Several studies have now revealed that carbidopa induces apoptosis in many types of cancer cells in vitro, and inhibits the growth of transplanted human cancer cell lines in vivo [17, 37]. Carbidopa is selectively cytotoxic to human pulmonary carcinoid and small cell lung carcinoma cells and retard prostate tumor growth [33, 38]. The effects of carbidopa on prostate cancer seem to be related to the recently discovered role of DOPA decarboxylase as a co-activator of androgen receptor AR [33].

Our study is focused on the role of carbidopa as an agonist for AhR [17]. AhR is known for its role as a ligand-activated transcription factor, but a more recent discovery about this receptor is its role as a promoter of ubiquitination of steroid hormone receptors ER and AR in a ligand-dependent manner, a function unrelated to the receptor role as a transcription factor. Our study is related to the epidemiological findings that the incidence of multiple cancers is significantly less in patients with PD [12–14]. Since PD is treated with a combination of carbidopa and DOPA, either of the two drugs could contribute to this phenomenon. But, it has already been shown that DOPA is not responsible for the...
decreased cancer risk even though it might be relevant to the increased incidence of melanoma in PD patients [39]. This led us to ask the question: could the decreased incidence of most cancers in PD be due to carbidopa? Our efforts to address this question led to our recently published study that showed the anticancer efficacy of carbidopa as a single agent in pancreatic cancer [17]. Subsequently, we turned our attention to AR-driven prostate cancer and ERα-driven breast cancer with regard to carbidopa based on the literature evidence that activation of AhR promotes proteasomal degradation of steroid hormone receptors [28, 29]. We have recently established that carbidopa does suppress prostate cancer via proteasomal degradation of AR [37]. In the present study, we asked if carbidopa would be useful to treat ER + breast cancer via depletion of ERα in a manner similar to what we found with regard to AR in prostate cancer.

The results of the present study can be summarized as follows. (i) The growth and proliferation of ER + breast cancer cells are suppressed by carbidopa; such effects are significantly less in ER-negative breast cancer cells; (ii) Carbidopa induces apoptosis in ER + cells as evident from increased levels of pro-apoptotic protein Bax and decreased levels of anti-apoptotic protein Bcl-2; (iii) Carbidopa decreases the cellular levels of ERα via increased ubiquitination and consequent proteasomal degradation; (iv) the effects of carbidopa on cell proliferation and apoptosis in ER + cells are AhR-dependent as these effects are nullified by an AhR blocker; (v) Carbidopa activates AhR and promotes its nuclear localization with concomitant increase in AhR transcriptional activity; (vi) The anticancer efficacy of carbidopa is also evident in vivo with regard to growth of ER + breast cancer cells into tumor in nude mouse xenografts.

The TCGA database shows decreased expression of AhR in breast cancer and an association between high levels of AhR expression and improved survival. Molecular docking simulation provides evidence for the binding of carbidopa to the ligand-binding pocket of AhR. All these data demonstrate that activation of AhR underlies the anticancer efficacy of carbidopa. The pathways involved in the pharmacological actions of carbidopa in terms of its potential use as an anticancer drug in ER + breast cancer are schematically represented in Fig. S4. The function of carbidopa as an AhR agonist in its anticancer efficacy is two-fold: (i) activation of AhR and the resultant changes

![Image](image_url)
in the expression of AhR target genes might underlie the tumor-suppressive effect as we have shown for pancreatic cancer [17]; (ii) promotion of ubiquitination of ERα and consequent proteasomal degradation by ligand-activated AhR is another mechanism by which carbidopa suppresses tumor growth in ER+ breast cancer. These two mechanisms are not directly related. The transcriptional activity of AhR is restricted to the nucleus whereas the modulation of the ubiquitination machinery by AhR occurs in the cytoplasm. Even though the relevance of AhR expression levels to survival of breast cancer patients is not stratified in terms of the breast subtype, it is likely that most of the patients included in the analysis are ER+ . Interestingly, when the expression of AhR is analyzed individually in different subtypes, the downregulation of the receptor is evident across the board in all subtypes irrespective of their hormone receptor status. This could be due to the tumor-suppressive function of AhR independent of its role in ER degradation. Activation of AhR triggers anoikis resistance and metastasis in TNBC cell lines [40]. In contrast, in the ER+ breast cancer cells, AhR agonists inhibit estrogen-induced proliferation and growth; these effects are related to AhR-mediated suppression of expression of ER target genes [41].

In vivo studies with mouse xenografts of ER+ breast cancer cells have shown that carbidopa inhibits tumor growth and increases apoptosis, which are consistent with the effects of carbidopa in vitro on cell lines. The maximal recommended human dose for carbidopa for PD is 200 mg/day, but the drug is safe even at a dose as high as 450 mg/day [42]. The effective dose in mice found in the present study translated to a human dose is ~200 mg/day [43, 44].

Our study demonstrates that FDA-approved drug Carbidopa suppresses ER+ breast cancer through AhR-mediated proteasomal degradation of ERα. Acting as an agonist of AhR, Carbidopa could degrade ERα in an AhR-dependent manner. These findings highlight the repurposing potential of carbidopa for ER+ breast cancer treatment.

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Declarations

Ethics approval and consent to participate The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (approval number: wydw2019-0704).

Conflict of interest The authors declare that they have no conflict of interest.

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