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Insufficient Radiofrequency Ablation Promotes Angiogenesis of Residual Hepatocellular Carcinoma Via HIF-1α/VEGFA

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In vitro and *in vivo* effects of a cyclooxygenase-2 inhibitor nimesulide analog JCC76 in aromatase inhibitors-insensitive breast cancer cells

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Introduction

Breast Cancer is the most common cancer in women in the United States and ranks second only to lung cancer as a cause of cancer-related deaths. Among all breast cancer patients, about 65% have hormone-dependent carcinoma, which requires estrogen for tumor growth. Aromatase is one of the key enzymes responsible for the biosynthesis of estrogen. Aromatase inhibitors (Ats). which induce estrogen deprivation in postmenopausal women, have been proving to be more effective than tamoxifen for the treatment of hormone dependent breast cancer [1-3].

The 'third generation Ais' are potent and highly selective. As a consequence, they efficiently block the conversion of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone, respectively) in peripheral tissues and breast tumors. Of the three Ais currently approved by the FDA for breast cancer treatment, letrozole and anastrozole are nonsteroidal triazole compounds, whereas exemestane is a steroidal analog of androstenedione. In recent years. Ais have been used as first-line hormonal therapy for patients with advanced estrogen dependent breast cancer [1]. However, long term estrogen deprivation caused by Ais can lead to significant endocrine therapy resistance, which has been demonstrated in preclinical models by several laboratories [4-8]. As a consequence. efforts are being made to search new agents that are able to overcome AI resistance [9-11].

A growing body of experimental and epidemiological evidence suggests that the use of NSAIDs (Non-steroidal anti-inflammatory drugs) may decrease the incidence of mammary cancer, tumor burden, and tumor volume [12-16]. Celecoxib, a cyclooxygenase-2 (COX-2) selective inhibitor, has strong chemo-preventive activity against mammary carcinoma in rats in some studies [17]. In addition to COX inhibition, these small molecules also block other cellular pathways to affect cellular function. For example, celecoxib

can block the phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase (PDK)/AKT pathway to induce apoptosis in prostate cancer cells [18–20]. The COX-2 inhibitor nimesulide was found to suppress the development of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats [21,22]. Derivatives of nimesulide have been synthesized, and their pharmacological effect on breast cancer cells has been primarily studied [23,24]. These new compounds suppress aromatase in SK-BR-3 breast cancer cells through a non-direct inhibitory mechanism [23,25]. They also selectively induce apoptosis in Her2 over expressed breast cancer cells [26]. It has been well documented that growth factor pathways which include Her2 and IGFR are up regulated in AI resistant breast cancer cells [8,27,28]. We hypothesize that nimesulide analogs might potentially be able to overcome AI resistance, since they selectively inhibit Her2 over-expressing breast cancer cell growth [26].

In the present study, we examined JCC76 {Cyclohexanecarboxylic acid [3-(2,5-dimethyl-benzyloxy)-4-(methanesulfonyl-methyl-amino)-phenyl]-amide} (Fig. 1), an analog of COX-2 inhibitor nimesulide, in an AI resistance breast cancer model *in vitro* and *in vivo*. LTEDaro cell line, which was developed from MCF-7aro (MCF-7 cells stably transfected with aromatase full gene) cells by hormone deprivation over a year and was used as the AI resistance model [29]. Compound JCC76 inhibited LTEDaro cell growth via induction of cell apoptosis through the decreasing of pAKT, pBAD and BCL-2 protein levels. It also induced cell apoptosis and inhibited tumor growth in the xenograft formed by using LTEDaro cells in ovariectomized nude mice. The synthesis of JCC76 derivatives has been well established [24,30]. The information gained from this study further elucidates the biological mechanisms through which compound JCC76 over-

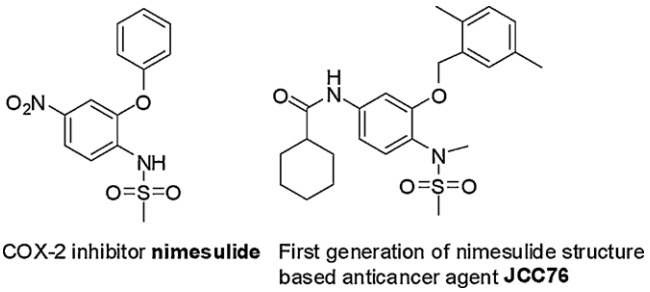

Fig. 1. Structure of nimesulide and JCC76.

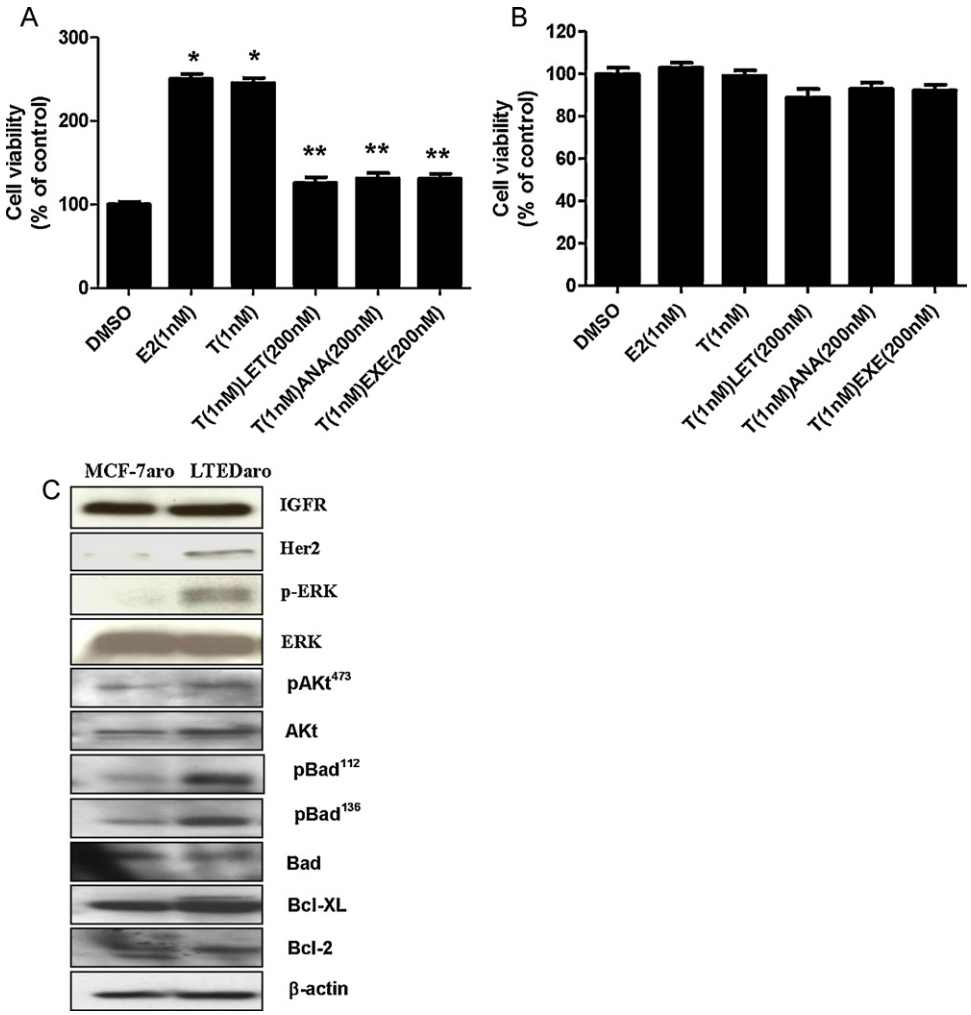

Fig. 2. Characterization of LTEDaro cells. MCF-7aro and LTEDaro cells were exposed to T or E2 and various AIs for 7days. Cell viability was measured by MTT assay as described in Section 2. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of six independent determinations ± SE. Similar results were obtained in at least three independent experiments. (A) AIs decreased MCF-7aro cell proliferation stimulated by testosterone. $^{*}P < 0.005$ *vs.* DMSO by unpaired *t* test, $^{**}p < 0.005$ *vs.* T treatment by unpaired *t* test. (B) LTEDaro cells did not respond to hormone and AIs in the proliferation assay. (C) MCF-7aro and LTEDaro cells were harvested as described in Section 2. Levels of IGFR, Her2, pAKt, AKT, pERK, ERK, pBAD, BAD, BCL-2, BCL-XL proteins were analyzed by Western blotting of cell extracts with specific antibodies.

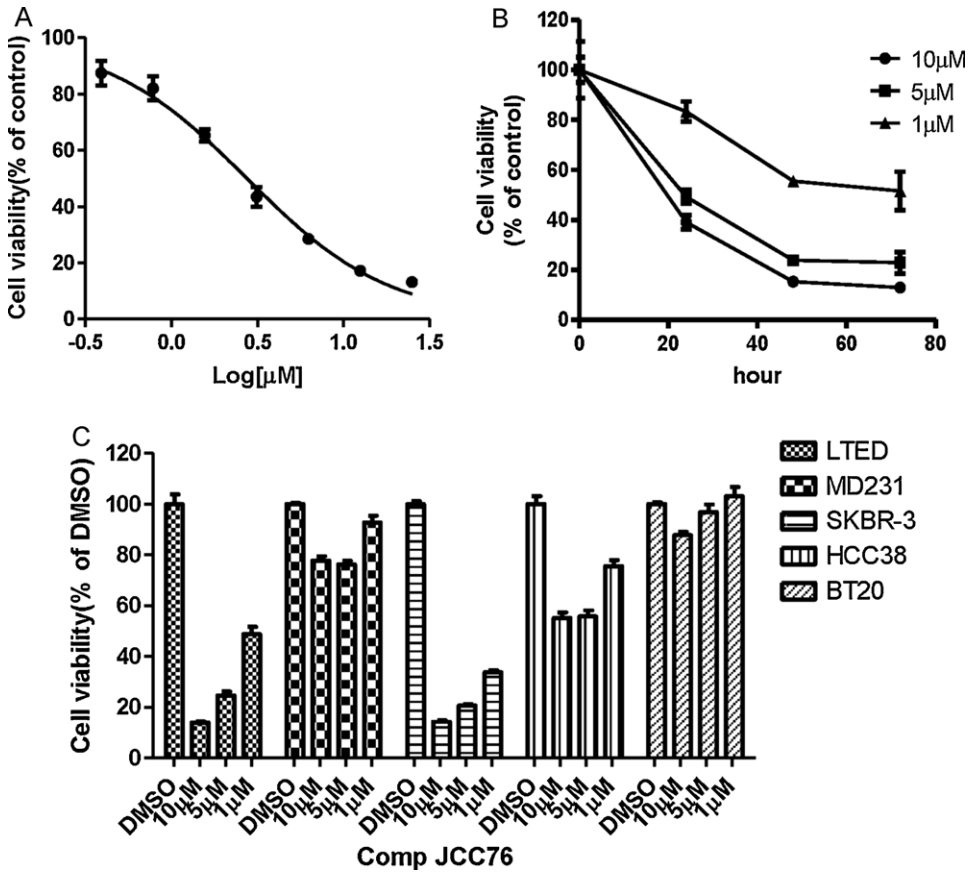

Fig. 3. Inhibition of LTEDaro cell growth by JCC76. Cells were treated with compound as indicated for 72 h. Cell viability after drug treatment was determined by the MTT assay. Each data point represents the mean results of six independent determinations ± SE. Similar results were obtained in at least three independent experiments. (A) JCC76 dose-dependently suppressed LTEDaro cell proliferation. (B) JCC76 time-dependently inhibited LTEDaro cell growth. (C) JCC76 selectively inhibited Her2 overexpressing breast cancer cell growth.

comes AI resistance, which makes the compound a promising lead for the further drug discovery.

Materials and methods

Reagents

Trypsin and all enzymes were obtained from Invitrogen (Carlsbad, CA). Testosterone and 17β-estradiol were from Sigma Chemical (St. Louis, MO). All antibodies were from Cell Signaling (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA).

Synthesis of JCC76

Compound JCC76 was synthesized according to the published procedure [24,30], and its structure and purity was confirmed by NMR and HPLC (Beckman HPLC, C18 column from Phenomenex, 10 mM ammonia acetate in 90% methanol as mobile phase, flow rate as 0.2 mL/min, UV detector setting up at 290 and 296 nM). Compound JCC76 used in the study has a purity of 98.6%.

Cell culture

The ER-positive aromatase-overexpressing MCF-7aro was prepared by stable transfection with the human placental aromatase gene and neomycin selection, as described previously [31]. The cells were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-Glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin–streptomycin and 200 mg/L G418 (Invitrogen, Carlsbad, CA). SKBR-3, MDA-MB-231, HCC38 and BT20 cells were obtained from ATCC (Rockville, MD). These cell lines were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-Glutamine, 1 mmol/L sodium pyruvate, and 100 U/mL penicillin–streptomycin. LTEDaro cells, which were developed by hormone deprivation of MCF-7aro cells for more than one year, were cultured in the same media, with charcoal stripped fetal bovine serum (CSFBS). Cell cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

Western blot

Cells were cultured in 60-mm culture dishes, incubated with DMSO or JCC76 for 24 h, and then lysed with CelLytic M (Sigma–Aldrich) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN). Cell lysates were sonicated briefly to reduce viscosity. Sixty micrograms of protein for each sample were boiled with 1× loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 min. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 100 mA for 90 min. The membrane was blocked for 2 h with 5% nonfat milk in 1x TBS-T at room temperature to reduce background and then incubated with primary antibody in 1% bovine serum albumin at 4 °C overnight. After the membrane was incubated with the primary antibody and washed four times with 1x TBS-T for 5 min each wash, it was incubated with the secondary antibody in 5% nonfat milk for 60 min at room temperature. The membrane was washed four

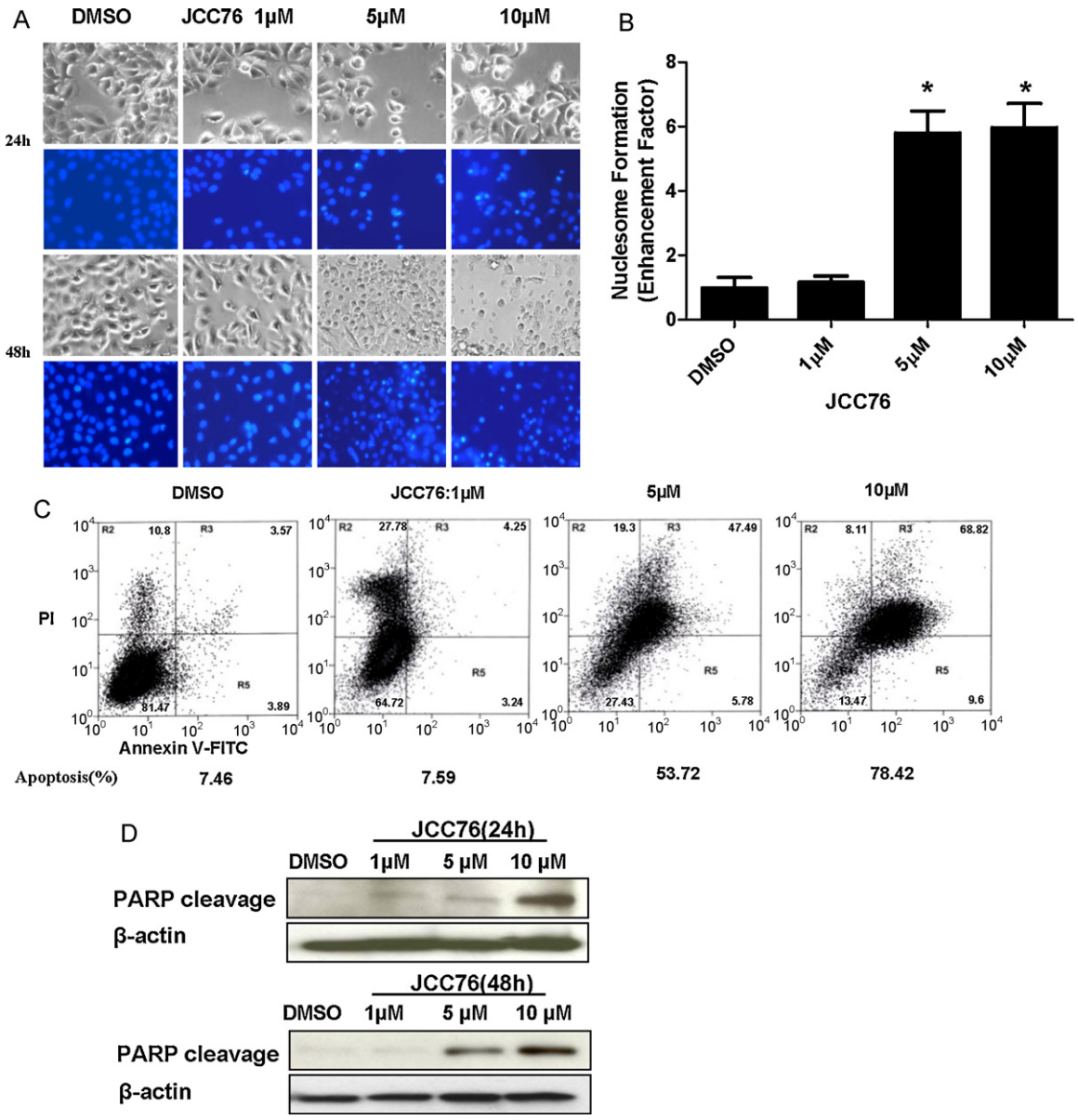

Fig. 4. JCC76 induced LTEDaro cell apoptosis. (A) JCC76 treatment led to LTEDaro cell shrinking, rounding, and detaching from the dish. In addition, staining cells with the DNA-binding fluorescent chromatin dye Hoechst 33342 revealed irregularly punctate nuclei, characteristic of cells undergoing programmed cell death. (B) Cell Death Detection ELISA assay exhibited cytoplasmic histone-associated DNA fragments after the treatment. Columns, mean of three independent experiments; bars, ±SE. $^{*}P < 0.005$ *vs.* DMSO by unpaired *t* test (C) Annexin V analysis of phosphatidylserine externalization revealed that JCC76 at 5 and 10 μmol/L induced 54% and 78% apoptotic death in LTEDaro cells after 48 h treatment, respectively. Cells were treated as indicated for 48 h and assessed for phosphatidylserine externalization by flow cytometry after staining with fluorescence-labeled Annexin V and propidium iodide. Representative quadrantal plots of data from dual-color flow cytometry of treated LTEDaro cells. (D) Apoptosis was also characterized by western blot analysis of PARP cleavage to the apoptosis-specific 85-kDa fragment examined by immunoblotting as described in Section 2.

times again for 5 min each time with 1x TBS-T and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce), according to the protocol of the manufacturer. The membrane blot was exposed to Basic Autorad Film (ISC Bioexpress, Kaysville, UT) and developed using a Konica SRX-101A (Konica, Tokyo, Japan). The blot was reprobed with anti-actin (Santa Cruz Biotechnology) at 1:2000 dilution as a loading control. Anti-pAKt, AKt, p-ERK, ERK, Her2, pBAD112, pBAD136, BAD, BCL-2, BCL-X were diluted (1:1000).

Cell proliferation

The effect of compound JCC76 on breast cancer cell proliferation was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT) in six replicates. Cells were grown in culture medium in 96-well, flat-bottomed plates for 24 h, and exposed to various concentrations of compound I dissolved in DMSO (final concentration ≤0.1%) in medium for different time intervals. For androgen and estrogen stimulated cell growth, cells were hormone starved for three days before the assay, and the cells were allowed to growth for 7 days in the assay. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in fresh medium, and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

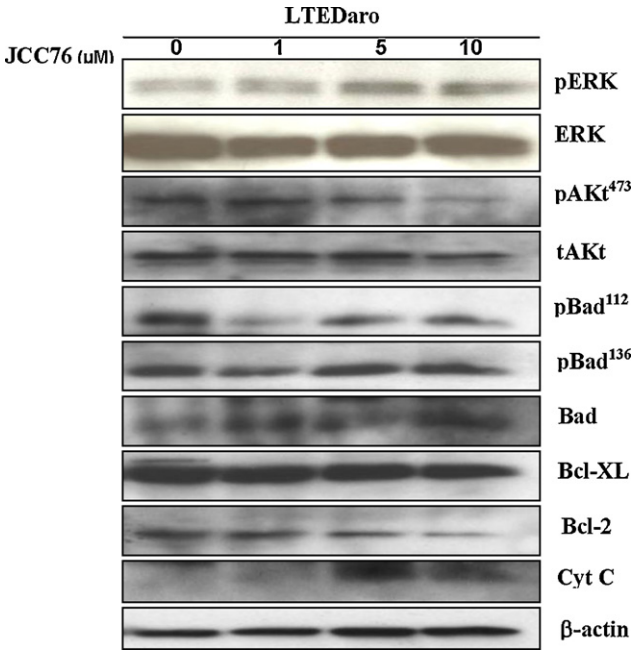

Fig. 5. Analysis of the pathways involved in JCC76-induced apoptosis. LTEDaro cells were treated with JCC76 with various concentrations for 48 h. Levels of pAKT, AKT, pERK, ERK, pBAD, BAD, BCL-2, BCL-XL and cytochrome *c* proteins were analyzed by Western blotting of cell extracts with specific antibodies as described in Section 2.

Morphological examination of apoptotic changes

Cells were treated with the indicated compound at various concentrations and times, and then stained with Hoechst 33342 (5 μg/mL) at 37 °C for 30 min. The stained cells were examined by fluorescence microscopy using a microscope (Nikon, Tokyo) equipped with an epi-illuminator and appropriate filters.

Apoptosis detection by enzyme-linked immunosorbent assay (ELISA)

Drug-induced apoptotic cell death was assessed using the Cell Death Detection ELISA kit (Roche Diagnostics), which quantitates cytoplasmic histone-associated DNA fragments in the form of mononucleosomes or oligonucleosomes. Cells were seeded and incubated at 10,000 cells per well in 12-well flat-bottomed plates in 10% CDFBS-supplemented MEM medium. After 24 h, cells were treated with JCC76 for 48 h at the indicated concentrations. Both floating and adherent cells were collected and the assay was done according to the manufacturer's instructions.

Flow cytometry analysis

For all of the assays, cells were treated for 48 h. To analyze cell cycle profile, treated LTEDaro cells were fixed overnight with 70% EtOH at −20 °C and stained with propidium iodide buffer [38 mM sodium citrate (pH 7.5), 69 μM propidium iodide and 120 μg/mL RNase A]. For assessment of apoptosis, treated cells were labeled with 5 μL Annexin V-FITC (Invitrogen) and 0.1 μg propidium iodide (Sigma–Aldrich) in 100 μL binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$ (pH 7.4)] containing 5×10^5 cells. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Immediately before analysis by flow cytometry, 400 μL binding buffer was added to each sample. Two-color analysis of apoptosis was done using a BD FACSCalibur System (BD Biosciences). Fluorescence compensation on the flow cytometer was adjusted to minimize overlap of the FITC and propidium iodide signals. A total of 1.2×10^4 cells were acquired for each sample and a maximum of 1×10^4 cells within the gated region were analyzed.

Cytochrome c release

DMSO or compound JCC76 treated LTEDaro cells were collected and triturated with 100 μL of chilled hypotonic lysis solution [220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol/L $MgCl_2$ and 1 mmol/L DTT in 50 mmol/L PIPES-KOH (pH 7.4)] for 45 min. The solution was centrifuged at $600 \times g$ for 10 min to collect the supernatant. The supernatant was further centrifuged at 14,000 rpm for 30 min, and equal amounts of proteins (50 μg) from the supernatant were resolved in 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome ***c*** antibodies [32].

Animal experiment

Five- to six-week-old female BALB/c *nu*/*nu*, athymic, ovariectomized mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). LTEDaro cells were routinely maintained in MEM, supplemented with 10% charcoal-stripped fetal bovine serum (FBS), 2 mmol/L L-Glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin–streptomycin and 200 mg/L G418 (Invitrogen, Carlsbad, CA). Subconfluent cells were harvested from monolayer culture and resuspended in an equal volume of Matrigel (BD Biosciences, San Jose, CA) to a final concentration of 1×10^7/0.2 mL. At 10 weeks of age, each animal received s.c. inoculations in one site per flank with 200 μL of LTEDaro cell suspension. Six animals were randomly grouped into two. Tumors were measured twice weekly with calipers, and tumor volume was calculated by the following formula $4/3\pi r_1^2 \times r_2$, where r_1 is the smaller radius and r_2 is the larger radius. Treatments began when the tumors reached a measurable size ($\sim$100 mm^3). One group ($n = 3$) was injected s.c. 100 mg/kg/48 h with compound JCC76. The second group ($n = 3$) received the vehicle (DMSO) and served as control group. Body weights were monitored weekly as an indicator of the animals' overall health. After 18 days of treatment, the mice were euthanized and the tumors were removed, weighed, and sent for hematoxilin and eosin (H & E) histological staining through the Cleveland Clinic Core Facility. Tumor specimens were also stained using cleaved-caspase-3 antibody (Cell Signaling Technology, Danvers, MA) for apoptosis and Ki-67 antibody (Dakocytomation, Carpinteria, CA) staining for cell proliferation. Data are expressed as mean ± SEM ($n \geq 5$).

Statistical analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Determination of IC_{50} values was performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student's *t*-test and *P* values reported at 95% confidence intervals.

Results

Characterization of LTEDaro cells

MCF-7aro cells express high level of aromatase which converts androgen to estrogen in the cells. Estrogen then stimulates the cell proliferation by ER pathways. This stimulation was inhibited by

AIs (Fig. 2A). LTEDaro was developed from MCF-7aro by hormone deprivation over a year, which mimicked the estrogen deprivation condition for the ER positive breast cancer patients in clinic. The cells were no longer sensitive to androgen and estrogen after this process. Subsequently, they were not responding to AI treatment anymore in the cell proliferation assay (Fig. 2B). Several laboratories developed similar models to study endocrine resistance mechanisms [6–8,33]. It was demonstrated that IGFR (Insulin-like Growth Factor) and/or Her2 growth factor pathways were up-regulated in these cells, and that downstream pathways of these growth factors were activated [6,33]. Corresponding signaling transduction inhibitors restored sensitivity to AIs in these cells [9–11,34]. In our LTEDaro model, we found similar, but slightly different phenomenon from other studies (Fig. 2C). Her2 protein expression was significantly increased, but IGFR remained the same. The level of activity of the Her2 downstream kinase, pERK, was significantly increased where pAKT activity remained the same. These results suggest that the MAPK (mitogen activated protein kinase) pathway was up regulated in our LTEDaro cells. In addition, the expression of the antiapoptotic protein BCL-2 was increased in LTEDaro cells, compared with that in MCF-7aro cells. The level of pBAD expression was increased where BCL-XL was only slightly increased. This could prevent BAD and BCL-2 family dimerization and protect the cells from apoptosis. These results suggest that the Her2 overexpression and anti-apoptotic protein up-regulation are responsible for AI resistance in LTEDaro cells. Her2 inhibitor AG825 exhibited clar toxic effect to LTEDaro cells at 10 μM (data not shown), which matches with several reports in which Her2 mono-antibody Trastuzumab, PI3K inhibitor wortmannin could inhibit LTEDaro cell growth [10,11].

Compound JCC76 significantly inhibited LTEDaro cell growth

COX-2 inhibitor nimesulide inhibited cancer cell growth at 200–500 μM concentrations and has been reported in many studies [35–38]. This agent did not show any selectivity of the inhibition among these cancer cells. It is more like a general cytotoxic agent to the cancer cells. Interestingly, its analogs selectively inhibited Her2 over-expressing breast cancer cell growth [26,39]. Since Her2 is unregulated in AI resistant breast cancer cells, it is very likely that nimesulide analogs are able to overcome AI resistance. Compound JCC76 (Fig. 1) is a lead compound from the nimesulide derivative pool [25,39]. It selectively inhibited the proliferation of SK-BR-3 and BT474 breast cancer cells which all express Her2 protein in previous studies [39]. We hypothesize that the compound might be able to inhibit LTEDaro breast cancer cell growth since the cells depend on the elevated Her2 signal for proliferation. To test our hypothesis, experiments were performed to determine whether compound JCC76 could do so. The results exhibited that compound JCC76 significantly inhibited LTEDaro cell growth with an IC_{50} of 2.75 ± 0.31 μM (Fig. 3A). Further study reveals that the inhibition was time-dependent (Fig. 3B). However, it is unclear whether this is just a general cytotoxic effect or JCC76 selectively interfere with the Her2 pathway to achieve this biological effect. To answer this question, we examined whether the compound inhibits the proliferation of other breast cancer cells. The Her2 over-expressing cell lines LTEDaro, SKBR-3, and the Her2 negative cells (MDA-MB-231, HCC38 and BT20) were used to study the selectivity of compound JCC76. The results revealed that JCC76 selectively inhibited LTEDaro and SKBR-3 breast cancer cell growth (Fig. 3C). More specifically, it inhibited SKBR-3 cell growth with IC_{50} of 1.80 ± 0.31 μM, MDA-MB-231 cell growth with IC_{50} of 22.35 ± 2.16 μM, HCC38 cell growth with IC_{50} of 20.03 ± 1.23 μM and BT20 cell growth with IC_{50} of 29.77 ± 4.13 μM. These results showed the selectivity of the compound on Her2 positive breast cancer cells verses Her2 negative breast cancer cells, which matches with our previous study [26].

Table 1
Altered cell cycle distribution of LTEDaro cells in response to JCC76 treatment. LTEDaro cells were treated with the compound for 48 h. Cells were processed for FACS using propidium iodide staining as described in Section 2. Percent distribution of cells in each cell cycle phase was displayed.

JCC76	Sub-G1 %	G1 %	S %	G2/M %	Dead cells
DMSO	4.82	64.71	21.76	7.18	1.53
1.0 (μM)	6.42	67.66	18.65	5.32	1.95
5.0 (μM)	20.61	38.43	22.32	9.07	9.57
10.0 (μM)	22.94	18.12	24.1	14.2	20.64

Compound JCC76 did not cause cell arrest, but significantly induced LTEDaro cell apoptosis

JCC76 may inhibit LTEDaro cell growth by causing cell cycle arrest, producing necrosis, or inducing apoptosis. Several experiments were performed to assess the possible mechanisms of cell growth inhibition of compound JCC76. Firstly, in the cell cycle study, a significant amount of cells were halted in the Sub-G1 phase (Table 1), which indicated cell apoptosis. The G1/S ratio did not change significantly at 1 μM. Only after 5 and 10 μM treatment, G1/S was dramatically changed, but mainly due to cell death (Data not shown). This suggests that the compound did not cause cell cycle arrest. Secondly, LTEDaro cells treated with JCC76 shrank, rounded, and detached from the dish, as shown in Fig. 4A by phase-contrast microscopy. Morphological evidence of apoptosis was also provided by nuclear fragmentation detected by staining cells with the DNA-binding fluorescent chromatin dye Hoechst 33342. A large portion of the cells treated with JCC76 at 5 and 10 μM for 48 h had apoptotic bodies and/or irregularly punctate nuclei, characteristic of cells undergoing programmed cell death (Fig. 4A). Thirdly, apoptosis was assessed by using a Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany). This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death (Fig. 4B). Significant DNA fragmentation was shown after the treatment of JCC76 at 5 and 10 μM for 48 h. Fourthly, apoptosis was detected by the analysis of phosphatidylserine externalization using Annexin V, as shown in Fig. 4C. After normalization to the control cells treated with DMSO, JCC76 at 5 and 10 μM induced 54% and 78% apoptotic death in LTEDaro cells after a 48 h treatment, respectively. Finally, apoptosis was characterized by western blot analysis of the 85-kDa apoptosis-specific cleavage fragment of PARP (Fig. 4D). After 24 h, the sample treated with 10 μM JCC76 showed a clear 85-kDa fragment; after 48 h, both 5 and 10 μM JCC76 treatments resulted in the cleavage of PARP. All of these results demonstrated that the compound induced apoptosis in LTEDaro cells, suggesting that it could overcome AI resistance in breast cancer cells through the induction of cell apoptosis.

Compound JCC76 decreased the levels of pAKt, BCL-2 and pBAD112 in LTEDaro cells.

To further explore the mechanisms of apoptosis in LTEDaro cells induced by JCC76, Her2 downstream PI3K/AKt and MAPK pathways, apoptosis related BCL-2 family proteins which were up regulated in LTEDaro cells, were examined after the treatment of the compound. It was found that protein levels of pAKt, pBAD112 and BCL-2 decreased, as shown in Fig. 5. The results suggest that JCC76 was able to suppress the growth factor-mediated signals which are up-regulated after long-term estrogen deprivation of MCF-7aro cells. In addition, cytochrome ***c*** release was observed after the treat-

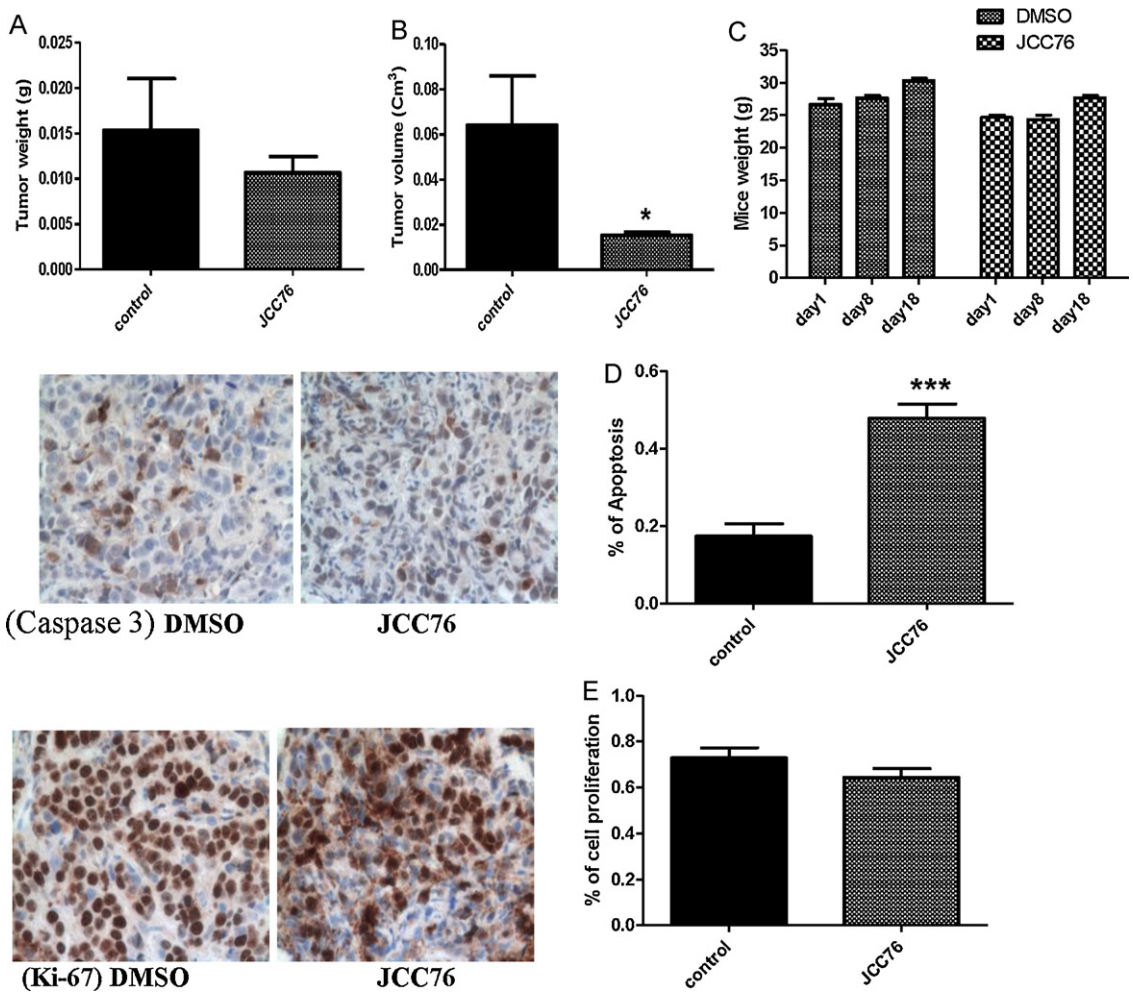

Fig. 6. *In vivo* effect of JCC76 on LTEDaro breast cancer tumor growth. Five- to six-week-old female BALB/c *nu*/*nu*, athymic, ovariectomized mice were given injections of LTEDaro cells in Matrigel (BD Biosciences, San Jose, CA). After tumor reach measurable size, the animals were treated with either JCC76 (in DMSO) s.c. or DMSO control every other day for 18 days. Mice were then euthanized and A: tumor weights ($n = 3$ for each group); B: tumor size ($n = 3$ for each group); C: mice weights ($n = 3$ for each group); D: cleaved-caspase-3 antibody staining for apoptosis; and E:Ki-67 antibody staining for cell proliferation were evaluated. Five identical sizes of areas with the staining slides were randomly picked for counting, and the counting numbers were plotted. Data represent mean in each group ($n \geq 5 \pm$ standard error of the mean in Figure D and E). * indicates statistical significance from the control group ($P \leq 0.05$);*** indicates statistical significance from control group ($P \leq 0.005$).

ment of JCC76, which indicated that apoptosis was mediated by the mitochondria.

Induction of apoptosis in vivo

To examine the overcoming AI resistance effects of JCC76 *in vivo*, we investigated the ability of the compound to induce LTEDaro-derived tumor apoptosis in female, ovariectomized nude mice. The mice bearing LTEDaro xenograft were treated for only 18 days, because the tumor size in the treatment group was significant decreased, which may limit the tumor tissue quantity for the histological examination if the experiment was continued. The results of the *in vivo* experiment showed that the treatment of JCC76 slightly decreased LTEDaro tumor weight and significantly decreased tumor size compared to control mice (Fig. 6A and B). The compound showed low toxicity to the mice since the mouse weights of the treatment group were not decreased (Fig. 6C). Histological examination of LTEDaro tumors revealed that cell proliferation marker Ki-67 was not changed in the JCC76 group compared to the control, whereas the level of apoptosis marker cleaved caspase-3 in the treated tumors was increased significantly (Fig. 6D and E). The data illustrated the ability of JCC76 to induce apoptosis of AI-insensitive breast cancer cells *in vivo*.

Discussion

AIs have been proven to be more effective than tamoxifen for hormone dependent breast cancer [2]. However, resistance to AIs eventually develops after long-term usage, which has been reported in preclinical animal models by several laboratories as well as in clinical studies [5,7,9,33,34]. Therefore, it is important to find additional strategies to control tumor growth after patients fail AI treatment. Due to the unique character of LTEDaro cell, it is generally used as a model to search new agents to overcome AI resistance. Her2 and its downstream molecule pERK, and the anti-apoptotic protein BCL-2 were all elevated in this endocrine resistant cell line, which could promote cell survival. These results also agree with the reports that Her-2 transfected MCF-7 cells showed higher BCL-2 protein compared with parental cells [40–42]. Furthermore, pBAD protein levels were also increased which could prevent BAD and BCL-2 family protein dimerization and protect the cells from apoptosis. Such findings indicate that protecting cells from apoptosis could be one of the mechanisms for resistance to AIs.

To search for new agents that can overcome AI resistance has been a new task in ER positive breast cancer research field. Many small molecules that interfere with Her2 downstream had been tested. Some of the compounds worked well in the *in vitro* model,

but show marginal effects in the clinic [34]. There is no clear explanation for this phenomenon yet. We focus our research on screening other agents to overcome AI resistance. The effects of a novel COX-2 inhibit nimesulide analog JCC76 was examined in the AI resistance model LTEDaro cells. The rationale for the study is based on the observation that nimesulide analogs selectively inhibited Her2 over-expressing breast cancer cell growth, and Her2 protein is significantly unregulated in AI resistant breast cancer. Our results demonstrated that JCC76 dramatically inhibited LTEDaro cell growth with a sub-micromole IC_{50}. Several assays were performed to examine if JCC76 can cause cell cycle arrest or apoptosis in LTEDaro cells. Results from these experiments revealed that the agent did not cause cell cycle arrest, and it mainly inhibited cell growth via induction of LTEDaro cell apoptosis. Furthermore, the results proved that JCC76 was able to decrease pAKt, pBAD112 and BCL-2 protein levels. These three proteins are involved in the regulation of cell apoptosis. Our findings suggest that JCC76 inhibited LTEDaro cell growth via promoting cell apoptosis, probably through the mitochondria pathway since cytochrome-c released from mitochondria was increased after the compound treatment. In addition, JCC76 was found to selectively inhibit Her2 over-expressing breast cancer cell growth among a panel of breast cancer cell lines. This agrees with the results showing that compound JCC76 interfered some molecules related to the Her2 pathway. In addition, our *in vivo* studies illustrated that treatment of JCC76 suppressed the growth of LTEDaro tumors in mice without affecting the weight of the animals. This result showed that the compound was active *in vivo* and low toxic to the animals. Further, the suppression of growth was due to induction of apoptosis as measured by cleaved caspase-3 antibody staining. This result is in agreement with the *in vitro* results.

In conclusion, we investigated the mechanisms of JCC76 for selectively inhibiting LTEDaro breast cancer cell growth. The results demonstrated that the compound was able to overcome AI resistance via induction of cell apoptosis. Our results have revealed several proteins that play roles in JCC76-mediated apoptosis. These anticancer properties of JCC76 make it a potential new therapeutic agent for overcoming AI-resistance. Further optimization of this compound to generate more potent derivatives is currently underway.

Acknowledgment

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