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***In Vitro* and *In Vivo* Evaluation of Novel Anticancer Agents in Triple Negative Breast Cancer Models**

KiTani Parker Johnson, Duane E. Johnson, Diana Stoute, Matthew E. Burow, Lyndsay V. Rhodes, Marian Gray, Patrick Carriere, Syreeta L. Tilghman, John A. McLachlan, and Josiah Ochieng

Xavier University of Louisiana (XULA), College of Pharmacy, Division of Basic Pharmaceutical Sciences, New Orleans [KPJ, DEJ, DS, PC, SLT]; XULA, Department of Biology [DS, MG]; Tulane University School of Medicine (TUSOM), Department of Medicine, New Orleans [MEB, LVR]; TUSOM, Department of Pharmacology [MEB, JAM]; Meharry Medical College, Department of Cancer Biology and Biochemistry, Nashville [JO]

Abstract

Triple negative breast cancer (TNBC) is subtype of breast disease devoid of the estrogen, progesterone, and Her2/neu receptors which are targets for pharmacological intervention. There is a need for novel anti-breast cancer agents that target TNBC. Therefore, novel isochalcone DJ52 was evaluated using the alamar blue dye exclusion assay, the luciferase colony assay, and xenograft models to determine its efficacy and potency. DJ52 significantly decreased proliferation of cells measured by using the alamar blue dye method and produced IC50 values of DJ52, DJ56, and DJ82 at 10-6M, 10-5M, and 10-5M, respectively. *In vivo* studies were conducted by injecting MDA-MB-231 cells into SCID mice to determine tumor regression was measured over 20 days. DJ52 at 50mg/kg caused significant decrease in tumor volume (p value <.05) by nearly 50% compared with the control with vehicle alone. These data suggest that DJ52 has merit for further evaluation as a novel anticancer agent.

Keywords

Triple negative breast cancer; isochalcone; chalcone

Breast cancer is the leading cause of cancer death in the United States and the most common form of malignant cancer diagnosed in women.¹ At least five subtypes (luminal A, luminal B, basal-like, ERBB2, and normal-like) have been identified on the basis of the pattern of expression.² The luminal A and luminal B subtypes express ER+ tumours, while the basal-like, ERBB2 and normal-like subclasses express ER– tumours. Interestingly, the luminal subtype A exhibits a more favorable prognosis, while the luminal B tumours present a worse prognosis. The basal-like and ERBB2 subsets show the worst clinical outcome.^{3,4} The triple negative breast cancer (TNBC) is defined as breast epithelial cancer cells which lack the HER-2/neu (HER2) receptor over-expression, the estrogen receptor (ER) and the progesterone receptor (PR). These patients do not respond to either the typical hormonal treatments tailored to the ER and PR or the inhibition of the EGFR.⁵ TNBC comprises between 7%–20% of all breast cancers and patients typically have a very poor prognosis. Chemotherapy benefits some patients, but the relapse rate in these patients is very high and

the survival rate is extremely low. Patients that present with these tumor types have a higher mortality rate than any other ethnic group.¹ According to reports from the Americana Cancer Society, Although breast cancer is found to occur in all races, the rate of TNBC is higher in incidence within African American women than in other races, creating a disparity among this population.⁶ Therefore there is a need for novel pharmacological intervention and novel targets for this subclass of breast disease.

Chalcones are a group of drugs that are known as phytochemicals and have demonstrated anticancer activity, such as blueberry extracts, soy, garcinol, and curcumin.⁶ There have been several recent studies that various chalcones possess antiproliferative activity against various types of human breast cancers.^{6,7} Therefore, a series of novel chalcones, chemicals derived from natural products, were developed. These agents were screened in TNBC cell lines MDA-MB-231 and MDA-MB-468 for antiproliferative activity. *Therefore, this study was to determine if these novel isochalcones have antiproliferative effects in triple negative breast cancer cells by* to evaluating DJ compounds for growth inhibition in breast cancer cell lines MDA-MB231 and MDA-MB-468; and 2) measuring the effect of novel isochalcones in decreasing the tumor volume of MDA-MB-231 cells injected into SCID xenograft mouse models.

Methods

Cell lines and cell culture

Breast cancer cell lines MDA-MB-231 and MDA-MB-468 were purchased from American Type Culture Collection. All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 250 mg/ml Amphotericin B and 10,000 I.U./ml Penicillin/Streptomycin. Cells were incubated at 37° C, 5% CO₂ in a humidified incubator Cells were passaged with 0.05% Trypsin-EDTA every 2–3 days.

Colon assay

MDA-MB 231 and MDA-MB 468 cells were placed in phenol red containing DMEM supplemented with 10% FBS. 1×10³ cells/well were plated in 6-well plates and treated with 10⁻¹² M, 10⁻⁹ M and 10⁻⁶ M DJ52 or vehicle (DMSO) stimulation. Cells were incubated at 37° C, 5% CO₂ in a humidified incubator for 10 to 14 days. Ten to 14 days later, cells were fixed with formaldehyde for 30 min and then stained with crystal violet 0.1% in methanol 20% for 30 min. The number of colonies were counted using ImageJ software. The data were reported as percentage of control ± standard error of the mean (SEM).

Dose response assay

Pharmacological treatments were performed in 96-well plates in assay medium containing each of the respective drugs. The alamar blue assay was done to treated cell lines to determine the IC₅₀. The cancer cell lines MBA MB 468, MBA MB 231, and MCF-7 were harvested with trypsin and plated on to separate 96 well plates with 5000 cells per well. The cells were allowed 24 hours to recover from plating. After 24 hours, the cells were treated with novel agents in concentrations ranging from 10⁻³ M to 10⁻⁹ M and the alamar blue dye was added to each well at 10% its total volume and incubated for 48hrs. Biotek Synergy 4 Plate Reader was used to measure absorbance and background wavelengths at 550nm and 630nm to determine proliferation and calculated as a percent of the controls as follows:

$$\text{Antiproliferative activity} = \frac{[\text{viable cells (control)}] - [\text{viable cells (treated)}]}{\text{Viable cells (control)}}$$

Data are the mean \pm SEM of triplicate determinations.

In vivo studies

Twenty female SCID mice 3–4 weeks old were purchased from Jackson Laboratories and housed five animals per cage with food and water *ad libetum* with 12 hour light/dark cycles. The density of MDA-MB-231 cells injected into the mammary fat pad was 5×10^5 cells and the tumors were allowed to form over 10–14 days. The tumor volumes were determined by caliper measurements and calculated as a percent of the untreated control tumors. Measurements were taken every day for 24 days.

EGF-stimulated inhibition of A431 cells

A431 cells were grown in RPMI with 10% FBS or in media containing 10% BSA. The cells were plated at a density of 5000 cells/well and were exposed to DJ56, the parent compound, genestein, and tyrophosphitin for 48 hours in concentrations of $1-10^4$ nM. Alamar blue was added to each well and measured as described above. Data are the mean \pm SEM of triplicate determinations.

Results

DJ52, DJ53, and DJ56 demonstrated an IC_{50} at 10^{-6} M in MDA-MB-468 cells. DJ82 demonstrated an IC_{50} at 10^{-3} M. Neither DJ64 nor control drug tamoxifen demonstrated to inhibit the growth of these cells by at least 50%. See Figure 1.

This graph (Figure 2) indicated that DJ52 caused an IC_{50} at 10^{-6} M. DJ56, DJ64, and DJ82 all demonstrate an IC_{50} at approximately the millimolar concentration. 5-fluorouracil demonstrated a drastic decrease in proliferation at the 10^{-3} M concentration.

Colony formation assays were performed in both MDA-MB231 and MDA-MB468 triple negative breast cancer cells to further examine the mechanism of action of the isochalcone DJ52. (See Figure 3.) Results demonstrate that DJ52 caused a dose dependent decrease in the cellular viability of MDA-MB 231. At the 10^{-6} M concentration, cellular viability was 32% of the control value. Interestingly, the 10^{-6} M dose of DJ52 caused a modest decrease in the viability of MDA-MB 468 cells, which was approximately 67% of the control value. As the MDA-MB 231 cells were more responsive to the antiproliferative properties of DJ52, this may suggest that DJ52 has enhanced selectivity for MDA-MB 231 cells over MDA-MB 468 cells.

Tumor volumes of MDA-MB-231 cells in the SCID mice treated with DJ52 demonstrated 50% tumor regression with the average tumor volume at 60 mm^3 after 21 days of treatment (Figure 4). The tumor volume in the untreated control mice doubled over the same period when compared with the experimental group.

Parent novel compound DJ56 demonstrated significant decrease of tyrosine kinase activity in A431 cells with an IC_{50} at 10^{-3} nM concentration (Figure 5). Genestein demonstrated an IC_{50} at the same concentration. Tyrophostin demonstrated an IC_{50} at 10^{-4} nM. Data are the mean \pm SEM of triplicate determinations.

Discussion

Triple negative breast cancer is an aggressive subtype of breast disease that demonstrates poor prognosis in patients. Chalcones are derivatives from soy and have been reported to have both antiproliferative and chemopreventative properties.^{7,8} Isochalcones are precursors to the flavonoids that are synthesized by plants. Chalcones have been reported to inhibit

proliferation in MCF-7 and MDA-MB231 cells by blocking cell cycle progression.⁹ Chalcones licochalcone-A, isoliquiritigenin and flavokawain A are found to induce apoptosis and cell cycle arrest in cancer cell lines.^{10,11} Some flavonoids have been reported to induce apoptosis or decrease COX-2 expression.^{12,13,14,15}

DJ52 was reported to decrease both proliferation and COX-2 expression in androgen dependent prostate cancer cell line LNCaP, which suggest that the DJ compounds work through a mechanism other than as antiestrogens or anti-androgens because proliferation is decreased in cell line models from both hormone sensitive and hormone refractory disease.¹⁶ In Figure 1, DJ52 significantly decreased the proliferation of MDA-MB-468 cells when compared with DJ56, DJ82, DJ64, DJ53, and tamoxifen ($p < .05$). It is demonstrated in Figure 2 that indicates that DJ56 demonstrated an IC_{50} value at the millimolar concentration, as did 5-fluorouracil (5FU). DJ52, however, indicated an IC_{50} value at the micromolar concentration. The colony assay in Figure 3 confirms the antiproliferative effects of DJ52 in both MDA-MB-231 and MDA-MB-468 cells. However, DJ52 was more selective for the MDA-MB-231 cells by causing a more significant antiproliferative effect in this model ($p < .05$). Figure 4 demonstrates that by evaluating MDA-MB-231 tumors that developed when these cells were injected into the mammary fat pad of SCID mice, the tumor volumes decreased significantly over 21 days compared with the untreated control. DJ52 has demonstrated to both slow the growth of TNBC cells, but also to cause death in MDA-MB-231 cells as indicated in Figure 3, which is significant. Chalcones have been reported to cause cell death in MCF-7 cells, which are estrogen positive.^{17,18,19} There are some reports that indicated that chalcones may have a role in increasing ROS levels.^{20,21,22} Figure 5 demonstrated that when parental compound DJ56 was compared with potent inhibitors of tyrosine kinase stimulation, genestein and tyrophostin, DJ56 significantly decreased the binding of the EGF receptor to its ligand. These data suggest that receptor tyrosine kinase inhibition may be involved with the mechanism of action of these novel agents. However, the mechanism of action for these DJ compounds is not fully understood. The receptor tyrosine kinase pathway is a potential signaling pathway to evaluate for pharmacological intervention in patients with triple negative breast cancer. Although African American women, many of whom may be poor and have limited access to medical care, have a lower breast cancer incidence, their mortality rate from breast cancer is significantly higher than that of their White counterparts. Therefore, these data suggest that DJ52 has merit for further investigations for its antiproliferative effects in triple negative breast cancer and to evaluate DJ52 for its mechanism of action to identify potential biomarkers for pharmacological intervention to relieve the burden of disease in this population.

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Notes

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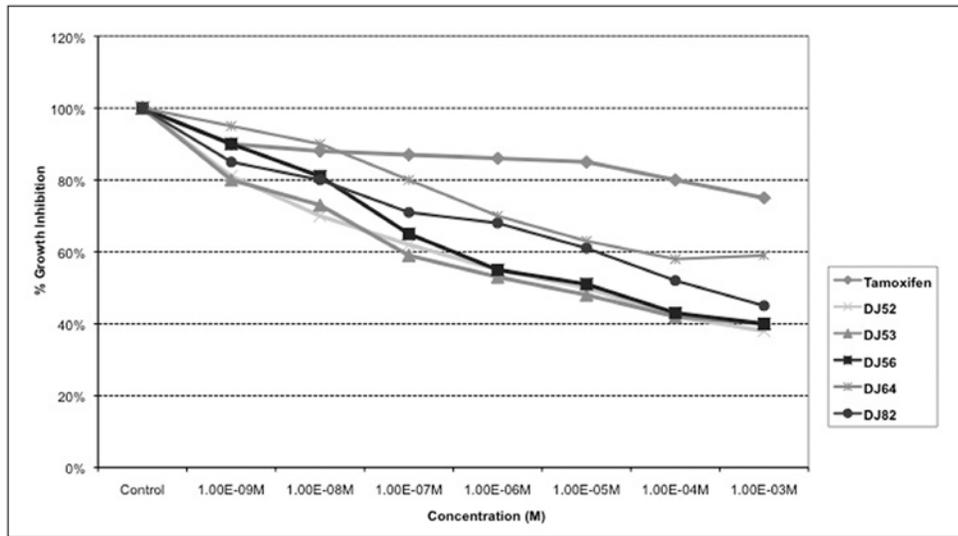


Figure 1. Dose response curve of metastatic human breast cancer MDA-MB-468 cells.

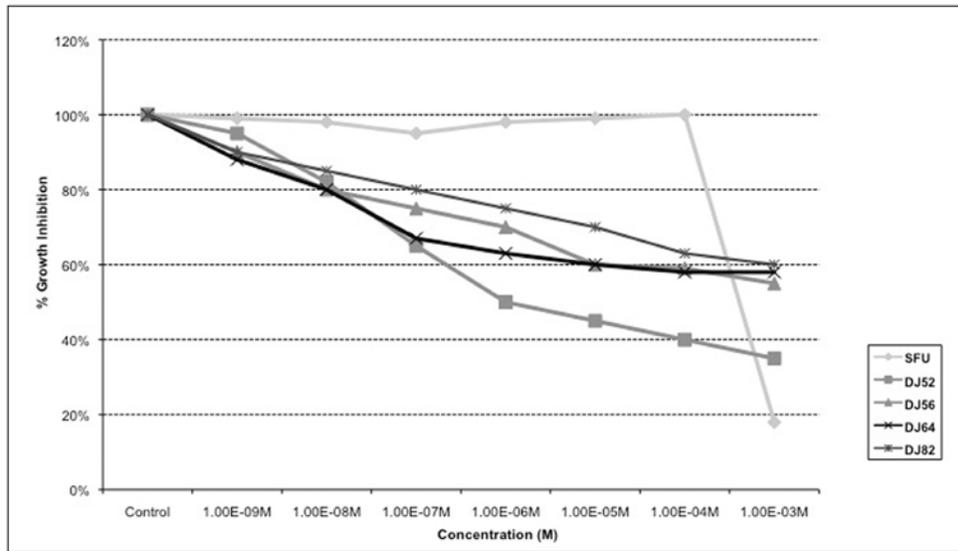


Figure 2.
Dose response curve of human breast cancer MDA-MB-231 cells.

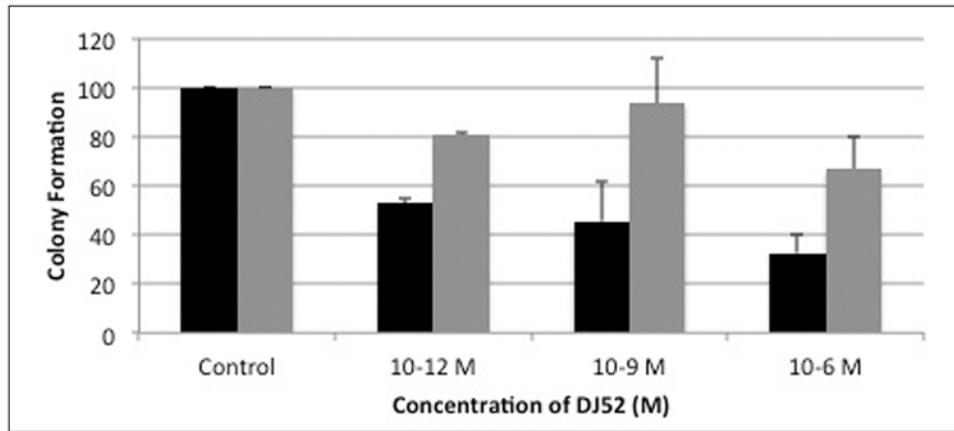


Figure 3.
DJ 52 causes dose-dependent decrease in growth measure by colony assay.

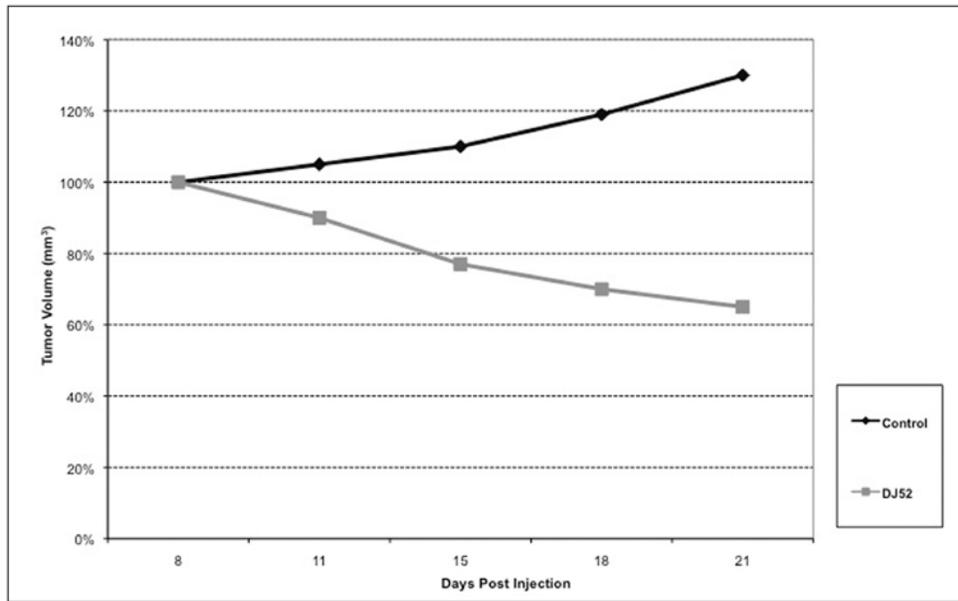


Figure 4. Effects of DJ52 on MDA-MB-231 tumors in SCID mice.

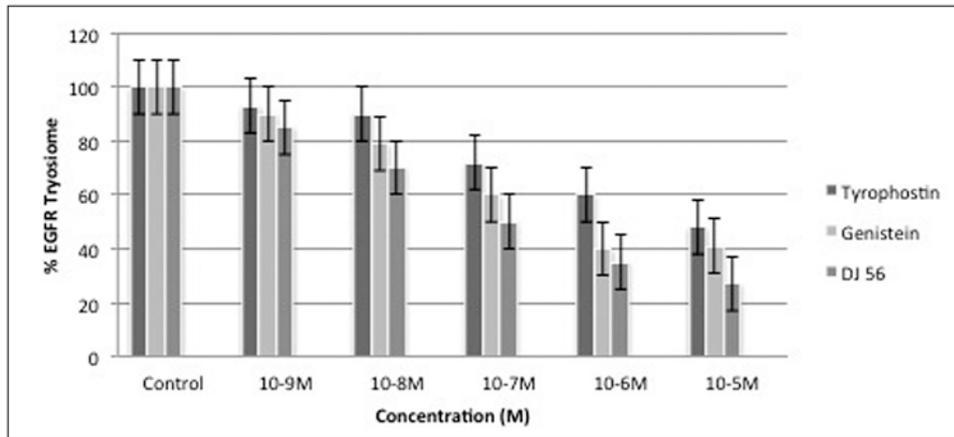


Figure 5.
%EGF-receptor tyrosine kinase phosphorylation of serum-stimulated A431 cells.