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Original Articles

Selective targeting of FAK–Pyk2 axis by alpha-naphthoflavone abrogates doxorubicin resistance in breast cancer cells

Amrita Datta [a,b], Nobel Bhasin [b], Hogyoung Kim [b], Manish Ranjan [b], Barbara Rider [a], Zakaria Y. Abd Elmageed [b], Debasis Mondal [a], Krishna C. Agrawal [a], Asim B. Abdel-Mageed [a,b,c,*]

[a] Department of Pharmacology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112, USA
[b] Department of Urology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112, USA
[c] Tulane Cancer Center, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112, USA

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ABSTRACT

Despite an initial positive response, breast cancer cells inevitably acquire resistance to doxorubicin (Dox). Alpha-naphthoflavone (ANF) is a well-known chemopreventive agent; however, its anti-cancer properties have not been established. We examined the therapeutic efficacy of ANF in doxorubicin-resistant MCF-7 (MCF-7/Dox) breast cancer cells and investigated its underlying molecular mechanisms of action. MCF-7/Dox cells expressed constitutively active forms of the tyrosine kinases: focal adhesion kinase (FAK-Y397) and protein tyrosine kinase 2 beta (Pyk2- Y579/580) compared with parental MCF-7 cells. ANF significantly enhanced the sensitivity of MCF-7/Dox cells to Dox cytotoxicity *in vitro* and when co-administered *in vivo*. This ANF-mediated chemosensitization has dual mechanisms of action: (a) intracellular Dox retention via suppression of P-glycoprotein pump activity, and (b) inhibition of clonogenic cell survival via de-phosphorylation of FAK, Pyk2, and EGF-induced Akt in MCF-7/Dox cells and tumor xenografts. Because of its strong chemosensitization action, broad safety profile, and health benefits, ANF is an attractive anti-cancer drug with therapeutic implications to circumvent drug resistance in breast cancer patients.

Introduction

Doxorubicin (Dox) is an FDA approved chemotherapeutic drug of the cell cycle non-specific class of drugs: the anthracycline antitumor antibiotics. Doxorubicin is commonly used to treat many types of cancer including leukemia, lymphoma, neuroblastoma, sarcoma, and Wilms' tumor as well as cancers of the lung, breast, stomach, ovary, thyroid, and bladder. In the treatment of breast cancer (BCa), Dox is highly effectively in early-stage as well as advanced-stage tumors, administered as single-drug or combination therapy [1–5]. However, over time, the cancer cells develop resistance to Dox which impairs treatment [6,7]. Although the mechanisms leading to this resistance are not fully established, increased drug efflux via overexpression and increased activity of multidrug resistance pumps, such as P-glycoprotein (P-gp), are well known [8–12]. Unfortunately, drug efflux pump inhibitors like cyclosporin A, ketoconazole, and verapamil add to the toxic side effects associated with Dox treatment, thus decreasing the quality of life of cancer patients [13]. Therefore, Dox must be co-administered with a chemotherapeutic agent that abrogates Dox resistance and has no overlapping benefits or side effects.

Flavonoids are a group of polyphenolic phytochemical compounds that occur ubiquitously in foods of plant origin [14]. They are capable of modulating enzyme activity, inhibiting transporters and receptors, and regulating several signaling molecules in many different cell systems [15,16]. Flavonoids have been classified as effective anti-microbial, anti-inflammatory, and anti-viral agents and in the past three decades have been effective against a wide range of diseases such as gastric ulcers, rheumatoid arthritis, diabetes mellitus, and cardiovascular diseases among others [17]. They have also been implicated in the inhibition of ABC transporters including breast cancer resistance protein (BCRP), P-gp, and multidrug resistance-associated protein 1 (MRP-1) [18,19]. Flavonoids also exhibit protective effects against anthracycline-induced cardiotoxicity (very common in patients on a Dox regimen), have potent anxiolytic activity, and improve general health [20–22]. Alpha-naphthoflavone (ANF), a synthetic flavone, is also known as 7,8-benzoflavone. Historically, the role of ANF as a chemopreventive agent has been emphasized [23,24]. ANF is also one of the most potent flavonoids

Abbreviations: ANF, alpha-naphthoflavone; BCa, breast cancer; CI, combination index; Cy, cyanine; DMSO, dimethyl sulfoxide; Dox, doxorubicin; FAK, focal adhesion kinase; P-gp, P-glycoprotein; Pyk2, protein tyrosine kinase 2 beta.

* Corresponding author. Tel.: +504 988 3634 (Office); fax: +504 988 5059.
E-mail address: amageed@tulane.edu (A.B. Abdel-Mageed).

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for inhibiting BCRP mediated mitoxantrone efflux [18,19]. Previous studies have outlined the role of ANF as a potent aromatase inhibitor and modulator of both aryl hydrocarbon receptor and cytochrome P450 (CYP450) enzymes [25–30].

We have tested whether ANF can abrogate Dox resistance and enhance breast cancer cell response to treatment with Dox. We also investigated, for the first time, the molecular mechanisms and targets of ANF's action in breast cancer cells. As a flavone, we also expect that ANF may confer health benefits, protect against Dox-induced cardiotoxicity, and abate anxiety in patients undergoing chemotherapy; our study shows that ANF protects mice from cancer and Dox related weight loss. We have also shown that MCF-7/Dox cells overexpress P-gp and have endogenously high levels of phosphorylated FAK and Pyk-2 (tyrosine kinases implicated in inhibition of apoptosis and prevalence of metastatic tumors) and that ANF inhibits P-gp pump activity and selectively targets the FAK-Pyk-2 axis leading to abrogation of Dox resistance both *in vitro* and *in vivo*. Our results suggest that the combined use of ANF with a chemotherapy agent such as Dox may enhance the effectiveness of chemotherapy and confer health benefits in breast cancer patients.

Materials and methods

Cell culture and drugs

The Doxorubicin-resistant MCF-7/Dox cells were a kind gift from Dr. Kapil Mehta (MD Anderson Cancer Center, TX). MCF-7/Dox cells and their parental MCF-7 cells (American Type Culture Collection, ATCC, Manassas, VA) were maintained in MEM (Gibco-BRL, Grand Island, NY) supplemented with 10% FBS, 0.01 mg/mL insulin, and 1% penicillin/streptomycin. Dox resistance was maintained via selective pressure by culturing MCF-7/Dox cells in a medium supplemented with 50 ng/mL of Dox (Sigma-Aldrich, St. Louis, MO) once a week. Drug resistance was verified every three months against the parental cells by MTT assay (Sigma-Aldrich, St. Louis, MO). ANF (N5757) was purchased from Sigma, St. Louis, MO.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay

Using the MTT dye, colorimetric cell viability assays were conducted according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). The mitochondrial dehydrogenases in viable cells reduced the tetrazolium ring of MTT, converting the yellow colored MTT to purple formazon crystals. For each well, 2500 cells per well were seeded in a 96-well plate, allowed to attach overnight, and then treated with ANF (25 μM) or Dox (0.5 μM) or their combination for 24, 48, and 72 h. Cell death was quantified at 540 nm using the BioTek plate reader (BioTek, Winooski, VT). The EC_{50} values of drugs alone and in combination were calculated using GraphPad Prism and used for combination index calculations. Data were normalized to the vehicle control, DMSO, and collected from three independent experiments.

Clonogenic assay

The long term effects of ANF and DOX alone, or in combination in the MCF-7/Dox cells relative to their parental MCF-7 cells, were assessed using a modified protocol for clonogenic/colony formation assay. Briefly, 100 cells were plated overnight in triplicate in 10-mm dishes. After 24 h of drug treatment: vehicle control–DMSO, ANF (25 μM), Dox (0.5 μM), ANF, and Dox combination (ANF/Dox) cells were allowed to grow in drug-free media for 12 d. Plates were fixed with 100% ethanol and stained with crystal violet to assess colony formation. Colonies (a group of ≥50 cells) were counted. Data were collected in triplicate from two independent experiments.

DNA fragmentation assay

To examine apoptotic cell death in response to therapeutic regimens, MCF-7/Dox cells were cultured at a density of 500,000 cells in 10-cm round dishes, and treated with vehicle control–DMSO, ANF (25 μM), Dox (0.5 μM), and ANF/Dox combination for 24, 48, and 96 h. DNA was extracted according to standard protocol as described by Matassov et al. [31] and analyzed by gel electrophoresis. The experiment was performed at least three times.

Assessment of P-gp pump activity and drug retention by calcein-AM assay

The inhibition of P-gp activity by ANF and its ability to increase cellular retention of Dox was examined by calcein-AM and fluorescence assays. MCF-7 and MCF-7/Dox cells were plated overnight at a density of 40,000 cells per well in 24-well black plates cultured in media supplemented with increasing concentrations of ANF over three time points (15 min, 30 min, or 3 h) and in another set of experiments with ANF (50 μM), Dox (1 μM), or their combination for 30 min. The fluorescent values were normalized to a (1) no dye control and (2) dye-control treated with calcein-AM only. Verapamil (20 μM) was used as a positive control. The data were collected from three independent experiments. Additionally, the calcein-AM assay was performed using PSC833 (10 μM) and PGP4008 (1 μM), well known inhibitors of P-gp and MK571, an MRP-1 inhibitor (25 μM), to compare the effectiveness of ANF as a P-gp inhibitor. Using the natural red fluorescence of Dox, its cellular sequestration in the presence or absence of ANF was also assessed. MCF-7/Dox cells were plated overnight at a concentration of 50,000 cells per well in chamber slides and then treated with Dox or ANF/Dox for 1 hour. The cells were then stained with mounting medium with DAPI (Vectashield, Burlingame, CA) and the images were acquired by Leica DMI3000B microscope (Leica Microsystems Inc., Buffalo Grove, Illinois).

Phosphoproteomics microarray analysis

The panorama antibody microarray cell signaling kit (CSAA1), comprising 224 human antibodies, was used for phosphoproteomics analysis of drug treated MCF-7/Dox cells, according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Whole protein extracts of cells cultured for 18 h in the presence of Dox (0.5 μM) alone or in combination with ANF (25 μM) were prepared and labeled with Cyanine-3 (Cy3) or Cyanine-5 (Cy5) dye, respectively. After incubation, slides were scanned by GeneTAC™ UC-4 microarray scanner and images were acquired and normalized using Genomics Solution software (Genomic Solutions Inc., Ann Arbor, MI, USA). Data analysis was performed using Genepix pro software (Molecular Devices LLC, Sunnyvale, CA). The foreground spot intensities formed the primary data and were corrected by subtracting the background intensities. Protein expression or activation was normalized to internal housekeeping controls (e.g. actin and α-tubulin) and fold change was expressed as Cy5 versus Cy3 (or ANF/Dox treated cells relative to Dox treated cells).

Protein extraction and western blot analysis

Following treatment with vehicle control–DMSO, appropriate growth factor and/or drugs, the MCF-7 and MCF-7/Dox cells were harvested, washed with PBS (P3813, Sigma), and protein was extracted using cell lysis buffer (9803S, Cell Signaling Technology, Danvers, MA) supplemented with 10 μL/mL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), 10 μL/mL phosphatase inhibitor cocktail 1, 2, and 4 μM PMSF (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Western blot was performed using standard protocol and antibodies against FAK, p-FAK (Y397) (Cell Signaling Technology, Danvers, MA), Pyk2, p-Pyk2 (Y579/580) (Abcam, Cambridge, MA), Akt, pAkt (Ser473) (Cell Signaling Technology, Danvers, MA), and P-gp (Santa Cruz Biotechnology, Dallas, TX) were used. Protein expression and phosphorylation were detected using the LumiGLO peroxidase chemiluminescent substrate kit (KPL, Gaithersburg, MD).

Tumor xenograft mouse studies

Five to six week old athymic nu/nu female mice were obtained from Harlan Laboratories (Frederick, MD). The animals were allowed a period of adaptation in a sterile and pathogen-free environment with food and water provided ad libitum. MCF-7/Dox cells (at a density of 1×10^6) suspended in 100 μL Matrigel reduced factors (BD Biosciences, Bedford, MA) were transplanted subcutaneously into flanks of mice. Drug treatments were started when tumors reached a volume of approximately 50 mm^3 (~2 weeks after injection). The treatment of the xenograft with intraperitoneal (IP) injections of Dox at 2 mg/kg, twice a week, with a cumulative dose of 4 mg was selected as it allowed us to be significantly below the Maximal Tolerated Dose or MTD of Dox in athymic rats (7 mg/kg IP). Using this dose in combination with ANF, we were able to show that ANF abrogated Dox resistance by improving its efficacy of tumor inhibition even at a low cumulative dose of 4 mg. However, since no pharmacological data were available regarding both dose toxicity and optimal dose for ANF in mice for chemotherapeutic use, a pilot study for dose optimization purposes was carried out. Mice with MCF-7/Dox tumor xenografts were treated via IP injections with vehicle (oil and saline, $n = 2$), Dox (2 mg/kg. $n = 2$), or ANF [100, 200, and 400 mg/kg, ($n = 2$ for each dose)] in combination with Dox for four weeks. Based on dose optimization studies, 200 mg/kg was found to be the optimal ANF concentration. For the final study, tumor bearing mice were treated with vehicle (oil and saline, $n = 6$), Dox (2 mg/kg, $n = 5$), ANF (200 mg/kg, $n = 7$) or the combination of ANF and Dox (200 mg/kg and 2 mg/kg, respectively, $n = 7$) for four weeks, where n is the number of mice/group. The frequency of Dox and ANF (in both single and combination treatments) were twice a week and five days a week, respectively. Animals' weight and tumor volumes were measured weekly. Tumor size was measured once a week with Vernier calipers. Tumor volume, V, was determined using the following formula:

$$V = longer\ side \times (shorter\ side)^2 \times 0.4$$

At the end of 4 weeks the experiment was terminated as the control mice showed one or more signs for the indication of euthanasia: hunched abnormal posture, impaired mobility and signs of lethargy. Tumors were excised at the end of the treatment