Synergistic Effects of Andrographolide on DNA Damage Repair Mechanism and Apoptosis in Breast Cancer Cells

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Abstract

Breast cancer is the second leading cause of cancer deaths in women. Several drugs including cisplatin and carboplatin have shown tremendous effectivity in reducing cancer; however development of drug resistance by breast cancer cells to overcome cytotoxic insults and recurrence of the disease is a major concern at the moment. Andrographolide is a diterpenoid with a potent anti-inflammatory and anti tumor activity and it ' s usage in combination therapy would be ideal as it is proven for it ' s apoptotic capability in varied number of cells. Antiproliferative and apoptotic activity of andrographolide in triple negative MDA-MB-231 cells was evaluated by clonogenic assay and flow cytometric analysis. Expression and phosphorylation of proteins were evaluated by immunoblotting. Our results revealed dose-dependent cytotoxic effects of andrographolide in MDA-MB-231 cells with and without carboplatin. It resulted in G2/M arrest of cells when treated alone, and further enhanced upon treatment in combination with carboplatin. Andrographolide alone and in combination with carboplatin enhanced apoptotic cells in early, mid and late stages and increased expression of DNA damage repair response proteins including FANCJ, FANCD2, RAD51, pRPA32 and p53. The present study strongly suggests that andrographolide inhibits breast cancer cell proliferation by apoptosis mediated through cell cycle arrest and up regulation of DNA damage repair response gene expression and shows synergistic effects upon usage in combination with carboplatin.

Keywords: Andrographolide; Apoptosis; Carboplatin; Cell cycle; DNA damage; Cancer

Introduction

Cancer remains the second leading cause of mortality around the globe with an incidence rate of more than 2.6 million cases per year [1]. Among the malignancies, breast cancer is the most prevalent diagnosed neoplasm and the main cause of cancer death among females, accounting for 23% of the total cancer case s and 14% of the cancer deaths [2]. In India, breast cancer is estimated to be 1.5 lakh (over 10 per cent of all cancers) new cases during 2016, and overall is the leading cancer [3]. Earlier studies reported that most cancers are triggered by dysfunction of genes encoding for growth factors, growth factor receptors, anti-apoptotic proteins, transcription factors, and tumor suppressors making them a target for the treatment of cancer by natural products [4]. Despite several breast cancer drugs such as cisplatin and carboplatin in use, a significant risk of recurrence and resistance to therapy is observed resulting in treatment of breast cancer a troublesome affair. Therefore, finding effective anticancer drugs with minimal side effects and higher efficacy has been one of the main criteria in cancer research [5]. Combinaton therapies have been tried successfully with cisplatin in various cancer cells and found to be effective in reduction of tumors.

Over the years, scientific research has been focused on identifying the naturally occurring chemopreventive agents, especially those present in dietary and medicinal plants due to their bioactive molecules [6]. Plant-derived natural products play an important role in the field of cancer chemotherapy as they had been a promising source for breakthrough discovery and development of chemotherapeutic agents [7]. Plant products such

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as vincristine, vinblastine, paclitaxel, epipodophyllotoxin, campothecin derivatives etc., are invaluable contributions of nature to modern medicine [8]. However, the quest to identify new therapeutic compounds for cancer therapy and management is a never-ending task.

Andrographis paniculata Nees, belongs to the family of Acanthaceae, commonly called "King of bitters" is a well-known plant of traditional Indian and Chinese system of medicine. Many researchers focused on andrographolide in recent times due to its amazing therapeutic and medical properties. Diterpenoid lactone, a ndrographolide (C_{20} H_{30} O_5), is the principal bioactive chemical constituent found in *A. paniculata* which is concentrated in leaves and stem, exhibits diverse biological activities, such as immunomodulatory, hepatoprotective, anti-inflammatory, antiviral, anti-HIV and anti-tumor etc., [9]. In terms of its potential anticancer properties, andrographolide has been reported to exhibit potent antiproliferative activity against variety of human cancer cell lines, viz; neuroblastoma, melanoma, hepatoma, prostate cancer, and gastric cancer [10–12]. This compound exerts anticancer activity on tumor cells by different mechanisms, such as cell-cycle arrest, cellular migration and angiogenesis and growth factor signaling modulation [13]. It also showed potent antiangiogenic and immunomodulatory activites in tumor tissues [14]. Androagrapholide has been reported to sensitize breast cancer cells to chemotherapeutic drug cisplatin and exert synergistic effects in the treatment of cancer [15]. As andrographolide is valuable of further investigation as a potent anticancer drug entity, a better understanding of its cellular mechanism of action is warranted. Therefore, the present study is aimed to evaluate the anti-cancer effects of andrographolide and understand its molecular mechanism of action in human breast cancer cell lines.

In the present study, we investigated whether andrographolide could inhibit the growth of breast cancer cells namely triple negative MDA-MB-231. We found that andrographolide reduced the proliferation of MDA-MB-231 cells and also showed enhanced anti-proliferative effects when treated in combination with carboplatin. Andrographolide was also found to enhance the levels of DNA damage repair proteins during co-treatment of cells with andrographolide and carboplatin.

Materials and methods

Cell Culture

The human breast cancer cells, MDA-MB-231 were procured from NCCS, Pune, India and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100U/ml penicillin. The cells were maintained at 37°C and 5% CO_2. Mycotest kit (In-

vitrogen) was used to detect mycoplasma contamination and cells within 8-10 passages were used in the experiments. Stock solutions of (100mM and 10mM) of andrographolide and carboplatin (Sigma, St. Louis, MO) were prepared in DMSO and stored at −20°C and subsequently, these stock solutions were further diluted to required concentration prior to treatment of cells.

Clonogenic survival assay

6-well dishes in triplicates were plated with triple negative MDA-MB-231 cells and incubated for overnight for cell attachment and treated with respective concentrations of andrographolide (2,4,6,8,10 μM) and 20 μM carboplatin and allowed for colony formation by replacement of the medium once in every three days for 7-12 days. Later colonies were fixed in methanol, stained with crystal violet and counted using Gene Tools, Syngene Imaging system [16].

Flow Cytometric Cell Cycle Analysis

MDA-MB-231 cells were seeded into 6-well dishes and pre-cultured for 24 hrs. The cells were then treated with 15 μ M Andrographolide and 20 μM Carboplatin alone and both for 12 hrs. The cells were trypsinized, washed with PBS and fixed in 70% ice-cold ethanol. The cells were further washed with PBS and re-suspended in 1 ml PBS. The cells were then incubated with RNase A (250 μg/ml) for 30 min and stained with propidium iodide (PI, 10 μg/ml) for 10 min and cell cycle analysis was performed by flow cytometry (FACS Calibur, BD Biosciences, CA). A minimum of 10,000 cells per samples were acquired and analyzed using Cell Quest Pro software [16].

Analysis of Nuclear Morphology

A nuclear morphological study was performed as described. After treatment, the cells were collected, washed with PBS, fixed with 4% formaldehyde for 10 mins and incubated in 500 □ l of PBS containing 10 □g/ml Propidium Iodide, incubated in dark for 10 mins, centrifuged and mounted in PBS with 90% glycerol, 1mg/ml anti-fade (paraphenylenediamine) and 0.5mg/ml 4, 6-diamidino-2-phenylindole (DAPI). The cells were imaged using Olympus BX51 flourescent microscope at 20X magnification.

Cell apoptosis assay

Cells undergoing apoptosis were assessed using PE-annexin-V apoptosis detection kit (Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 and PI kit) according to the manufacturer's instruction (Life Technologies Inc.) Briefly, cells were plated in 6-well plates at a density of 1×10^5 cells/well and were treated for 48 h with 15 μM andrographolide, 20 μM carboplatin

and combination of both. Post treatment, cells were recovered, washed with binding buffer (BB) and centrifuged. Annexin-V FITC (10 μl) was added to the pellet and incubated in the dark for 15 min at room temperature. Subsequently, cells were washed and re-suspended in BB, and PI solution (20 μg/mL) was added immediately prior to analysis by flow cytometry. The flow-cytometric analysis was performed using a FACS Calibur (BD Biosciences, CA).

Western Blotting

Cells (1×10^5 cells) were treated with 15 μM andrographolide, 20 μM carboplatin and combination of both for 12 hr for western blot analysis. After treatment, the cells were collected and washed with ice cold PBS for two times. Cells were then lysed in ice-cold lysis buffer (1% Triton X-100, 1 mol L^{-1} HEPES, 5 M NaCl and 0.5 M EDTA) freshly supplemented with protease and phosphatase inhibitors (Roche, Germany). The lysates were then centrifuged at 14,000 *g* at $4^{\bigcirc}$ C for 20 min. Samples were prepared in 4x SDS-PAGE sample buffer, boiled at $100°$C for 15 min. SDS PAGE was used to resolve denatured samples and transferred to nitrocellulose membranes, which were later incubated with respective antibodies followed by incubation with specific HRP-conjugated secondary antibodies. The blots were then developed by chemiluminescence detection kit. Antibodies used to the following antigens in the study include: FANC D2, FANC J and GAPDH (Santa Cruz Biotechnology, Inc.); phospho RPA, RAD51, p53, Phospho p53 and acetyl p53 (Cell Signalling Technologies) respectively.

Results

Andrographolide exhibits chemotherapeutic activity

Clonogenic potential of the cell lines was significantly influenced by andrographolide and carboplatin in a dose dependent manner. In cell lines, triple negative MDA-MB-231 andrographolide exhibited growth inhibitory properties with an IC$_{50}$ value of 4.5 μM (Figure. 1). High sensitivity of triple negative MDA-MB-231 cell line towards treatment of andrographolide alone and in combination with carboplatin confirms its sensitivity and makes an ideal model to undertake chemotherapy based studies for breast cancer.

Andrographolide treatment induces G2/M arrest of cells.

Exposure of triple negative MDA-M B-231 cells to andrographolide and combination of both attenuated cell cycle progression through G2/M phase after 12hr treatme nt compared to control or carboplatin alone treated cells. Most of the cells were found to be in S-phase and appeared to progress slowly

towards G2/M phase indicating that the cells were under replication stress (Figure. 2). The cell cycle arrest at G2/M phase indicated that andrographolide may have the ability to interfere with DNA replication directly or indirectly and induce replication associated DNA damage which in turn may further activate checkpoint responses to arrest the progression of cell cycle by blocking mitotic phase entry [17].

Andrographolide treatment and DNA damage responses

Since andrographolide treatment and combination treatment with carboplatin affected cell cycle, we hypothesized that it may increase the expression of DNA damage repair (DDR) proteins such as FANC J, FANCD2, RAD51, pRPA32, P53 and may have a role in regulation of expression and phosphorylation of DDR proteins. For this experiment, triple negative MDA-MB-231 cells were subjected to 15 μM andrographolide and 20 μM carboplatin and both together. After 12 hr of exposure, whole cell lysates were normalized with GAPDH for protein concentrations and probed for different D NA damage repair proteins. Breast cancer cells treated with andrographolide alone and in combination with carboplatin both induced ATM/ATR-mediated DDR as evidenced by enhanced expression of FANC J, FANCD2 and RAD51. Several studies recognized functional interactions among FANCD2, BRCA2 and Rad51 in stabilization of stalled replication forks and repair of collapsed forks [18]. Further, earlier studies have shown that replication protein A (RP A) is phosphorylated at several sites during different stages of cell cycle, one among them is phosphorylation of RPA-32 at ser-29 during M-phase which is dephosphorylated at completion of M-phase [19]. In our study, we found phosphorylation of RPA-32 indicating that the cells are in M-phase which is in consistent with cell cycle studies. Further, pRPA32 is known to induce the expression of p53 which is found enhanced and phosphorylated in our observations (Fig 3). Thus, the results indicate that andrographolide alone or in combination with carboplatin leads to replication stress-associated DDR, which slows cell cycle progression though S-phase with the accumulation of cells in G2/M phases in MDA-MB-231 cells.

Andrographolide induces apoptosis

Further, apoptosis was monitored in triple negative MDA-MB-231 cells treated with andrographolide, carboplatin and a combination of both by annexin V staining. Figure 4 (a and b), reveals an increase of apoptotic cells at early, mid and late stages, with annexin-V positive cells after 48 hours treatment of MDA-MB-231 cells. An increased number of cells in early and mid stages of apoptosis in andrographolide treated cells, compared to the control or treated with carboplatin alone point to in-
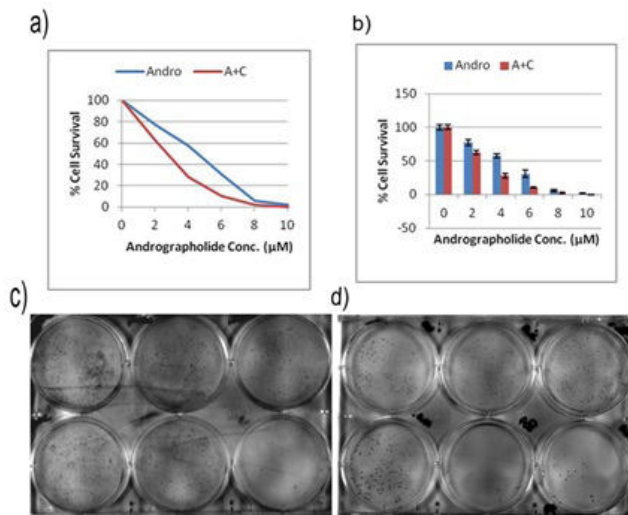
Figure 1 Clonogenic survival assay. MDA-MB-231 were treated with different concentrations of Andrographolide and 20 µM Carboplatin and allowed for colony formation by replacement of the medium once in every three days for 7-12 days. The colonies formed were fixed in methanol, stained with crystal violet and counted using Gene Tools, Syngene Imaging system. Figure 1a represents survival curve of MDA-MB-231 cells, 1b represents bar diagram of the same. Figure 1c represent colonies stained with crystal violet i) control, ii-vi) 2, 4, 6, 8, and 10 ☐M andrographolide. Figure 1d represent colonies stained with crystal violet i) 20 ☐M carboplatin, ii-vi) 2, 4, 6, 8,and 10 ☐M andrographolide in the presence of 20 ☐ M carboplatin. The blue bar represents andrographolide alone and red bar represents combination treatment of andrographolide and carboplatin. The bars represent mean $\pm$ standard error
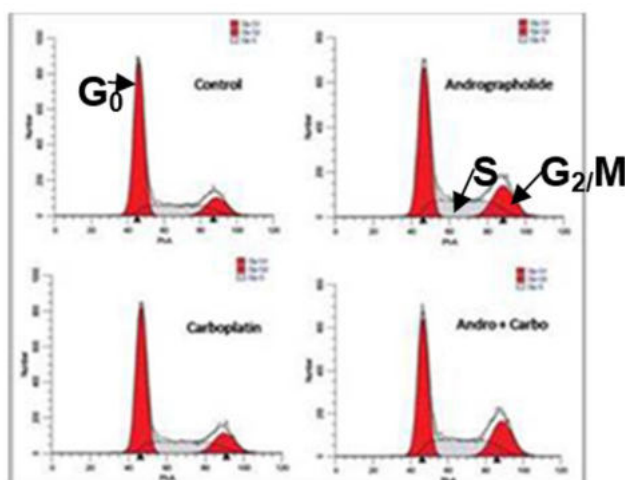

Figure 2 Cellcycle analysis and Nuclear Morphology. MDA-MB-231cells were exposed to 15 μM Andrographolideand 20 µM Carboplatin alone and in combination. Cell cycle profiles wereassessed after 12 hr treatment by flow cytometry (FACS Calibur, BD Biosciences,CA). A minimum of 10,000 cells per sample were acquired and analyzed using CellQuest Pro software. Figure is a graphical representation of PI stained cells.Red coloured areas represent cells in G_0 and G_2/M phasesrespectively; striked areas represent cells in S phase. The figure is arepresentative of independent experiments conducted thrice
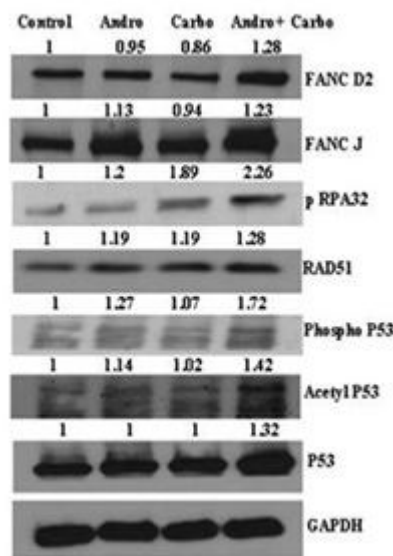
Figure 3 Expression and Phosphorylation status of FANC D2, FANC J, pRPA32, RAD51, P53, and Acetyl P53. The expression and phosphorylation status of these proteins were analyzed using specific antibodies. The figures are western blots and the various panels represent the effects of 15 ☐ M Androgarpholide alone, 20 ☐ M Carboplatin and co-treatment of both. The blots represent three independent experiments.

creased apoptosis due to andrographolide. Androgarpholide was found to further increase the cells in early and mid stages when treated in combination with carboplatin. Cells treated with andrographolide alone, when stained with Propidium Iodide and DAPI exhibited rounded and apoptotic cells whose number increased in cells treated with combination of both (Fig ure.4c). This strengthens the process of apoptosis as negative regulator of cancer and chemotherapeutic nature of andrographolide as an agent of effective synergistic treatments.

Discussion

Natural product, andrographolide and its analogs are widely used as chemotherapeutic agents for various cancers [20]. However, the mechanism of the anti-tumor activities of andrographolide and its synergistic properties are not well defined in breast cancer cells. Studies on andrographolide in many tumor cell models have demonstrated the anti-neoplastic effects which are partly due to the G2/M cell cycle arrest and apoptosis [21]. Recently, studies also suggested that andrographolide induces DNA damage and cell cycle arrest in tumor cells [22]. In our study we explored its DNA damage inducing capability in triple negative breast cancer cells i.e MDA-MB-231 cells. The DNA damage induced by replication stress results in the formation of stalled or collapsed forks, which further activates the ATM/ATR-mediated cell cycle check point responses to promote fork stability for repli cation through Rad18 and other

DNA repair mechanisms associated with Fanconi anemia [23]. Since such compounds are highly reactive in nature and influence the functionality and stability of many proteins in cells, the prediction of the specific cellular targets responsible for DNA damage becomes complex. In this regard, the present study focused on DNA damage mediated effects of andrographolide alone and in combination with carboplatin against MDA-MB-231 breast cancer cells in a concentration-dependent manner which showed a positive correlation.

Earlier studies shown that drug induced replication stress leads to activation of the FA pathway, resulting in mono-ubiquitination of FANCD2 and its nuclear foci formation [24, 25]. To study the effects of andrographolide, MDA-MB-231 cells were treated with andrographolide alone. To study its synergistic effects, we also treated cells with andrographolide in combination with another drug named carboplatin. The concentrations of carboplatin used did not induce apoptosis. Andrographolide inhibited proliferation and enhanced anti-proliferative property of carboplatin in breast cancer cells. Interestingly, the study demonstrated that andrographolide interferes with cell cycle progression by inducing replication-associated DNA damage, as evidenced by increased number of cells in S-phase and G2-M phase together with increased expression of FANCJ, FANCD2, pRPA32, RAD 51 and p53. Studies have shown that p53 is phosphorylated and acetylated in response to DNA damage and repair mediated by ATM and ATR signaling pathways. Our studies further confirmed the activation p53 by
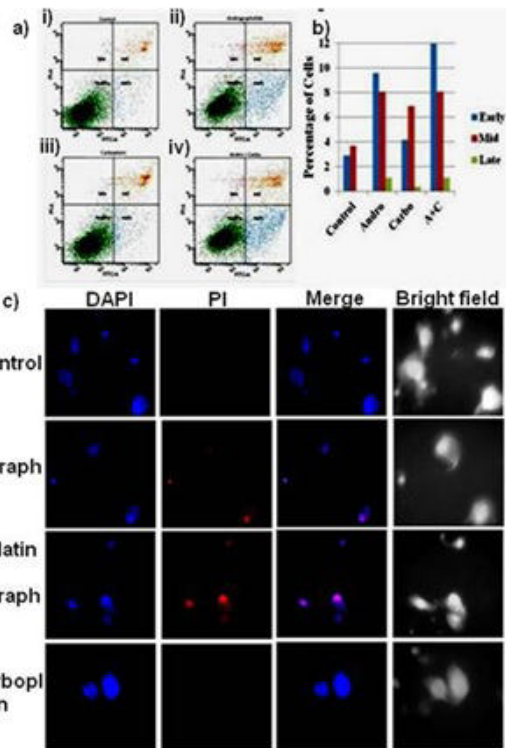
Figure 4 Analysis of apoptosis induced by andrographolide in breast cancer cells. MDA-MB-231 cells were exposed to 15 µM andrographolide, 20 µM carboplatin and combination of both for 48 hours and the cells were co-stained with Annexin V and PI antibody and analyzed by flow cytometry. Fig: 4a represents graphical representation of different stages of apoptosis. The quadrants are i) Control, ii) Andrographolide, iii) Carboplatin and iV) Androgarpholide and carboplatin respectively. 4b represents bar diagram of the same. 4C) Cells were stained with 20 □g/ml Propidium Iodide and mounted in mounatnt containing 0.5mg/ml 4, 6-diamidino-2-phenylindole (DAPI).and imaged at 40x magnification using Olympus fluorescent microscope. Scale: 10□m. The image is self explanatory

phosphorylation at ser15 and acetylation at Lys-382 in response to DNA damage. Apoptosis induction was also observed in cells treated with andrographolide confirming that DNA damaging chemotherapeutic agents result in apoptosis to limit tumor growth.

In conclusion, the data could establish an important basis for the preclinical evaluation of andrographolide with and without carboplatin in treatment of the breast cancer. Since chemotherapy remains the common standard therapy for breast cancer especially triple negative breast cancer, it is proposed that andrographolide may thus provide an important therapeutic effect, in combination with chemotherapeutic drugs to potentially eliminate locally metastasized breast cancer tumors. However, further mechanistic studies are required for the molecular mechanisms leading to apoptosis and cell death.

Conclusion

Andrograpolide induces cell cycle arrest and thereby cell death. In addition andrographolide enhances anti tumorgenic effects of carboplatin.

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Conflict of Interest

The authors do not have any conflicts of interest to disclose.

Author's Contributions:

Dr.Venkateshwari Ananthapur majorily contributed to the conceptualization of the topic, intellectual content, data acquisition, design and literature study. Dr. Pushpanjali Pendyala contributed to the data acquisition, literature study and manuscript editing.

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