Palmatine Modulates Triple Negative Mammary Carcinoma by Regulating the Endogenous Function of p53, p21 and Mdm2

Ativui Selase¹*, Danquah A. Cynthia¹, Osafo Newman¹ and Ka-Chungu Sammy²

¹Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, PMB, Kumasi, Ghana.
²Department of Pathology, Komfo Anokye Teaching Hospital, P.O. Box 1932, Kumasi, Ghana.
*Corresponding author E-mail: Selase Ativui; montecelacy@gmail.com

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Natural products and their bioactive constituents have been investigated for centuries and recognized as a source of valuable therapeutic candidates in the development of contemporary anticancer drugs. Triple negative breast cancers are a subtype of malignant cells formed in the breast tissue caused by uncontrolled and abnormal division. Palmatine, a naturally occurring alkaloid extracted from several medicinal plants in West Africa, has not been extensively investigated for its anti-breast cancer properties especially in triple-negative mammary carcinoma. The 4T1 triple-negative breast cancer cells were transplanted orthotopically into the mammary fat pad of the female balb/c mice. Tumor volume, tumor weight, histology and immunohistochemical analysis were carried out. After 28 days, palmatine (1, 5 and 10 mg/kg) in a dose-dependent manner decreased tumor volume (190.80 ± 19.14, 25.40 ± 2.82, 14.20 ± 1.85), reduced tumor weight (1.035 ± 0.04, 0.8027 ± 0.01, 0.5090 ± 0.04), inhibited tumor growth (31%, 46%, 66%) and protected against morphological dysplasia induced by the carcinoma (3.50 ± 0.29, 2.25 ± 0.25, 1.75 ± 0.25) respectively. Also, palmatine increased the activity of the tumor protein p53, cyclin-dependent kinase inhibitor 1 (p21) and mouse double minute 2 (Mdm2) compared to the untreated carcinoma bearing mice. Overall, palmatine protected against triple negative mammary carcinoma and can be a valuable anticancer compound to treat breast diseases.

Keywords: anti-breast cancer, natural compounds, palmatine, triple negative.

Breast cancer disease is characterized by the malignant growth of cells in the mammary gland. The triple negative subtypes account for 10–20% of breast cancers diagnosed worldwide. This subtype has a negative expression for the estrogen receptor, progesterone receptor and human epidermal growth factor 2.¹² Triple negative breast cancers (TNBC) present a great challenge for successful therapy. They are especially difficult to treat due to their aggressive phenotype, disease relapse and metastasis.¹³

Currently, Anthracycline (doxorubicin) chemotherapy is the main treatment strategy for triple negative breast cancers since they do not respond to hormonal therapies. Doxorubicin, however, is associated with poor tumor selectivity.
and severe side effects in healthy tissues such as cardiotoxicity \(^5\), \(^6\). Research to investigate lead compounds to aid the treatment of this disease is needed urgently. Consequently, natural compounds have been proposed as a valuable source for discovering new drugs for treating and preventing breast cancer \(^7\).

Palmatine, an isoquinoline alkaloid has been extracted from many medicinal plants used in Africa. This compound exhibits various pharmacological properties such as antiviral, antimalarial, antioxidant, anti-inflammatory and anticancer as reported by several studies \(^8\)–\(^11\). The antiproliferative properties of palmatine investigated on several cancerous cells \textit{in vitro} shows much promise and deserve great attention \(^12\), \(^13\). Nevertheless, to the best of our knowledge, there is little report on the \textit{in vivo} activity of palmatine in triple negative breast cancer.

In this study, 4T1 triple-negative breast cancer cells derived from the epithelial mammary gland of the female balb/c mice \(^14\) were used to investigate the anti-breast cancer properties of palmatine. Orthotopic transplantation of the highly tumorigenic/aggressive 4T1 triple negative breast cancer cells into the mammary fat pad of Balb/c mice represents the most relevant preclinical model of human triple-negative breast cancer \(^15\)–\(^17\). This study further investigated the activity of palmatine in preventing triple negative mammary carcinoma through the activation of endogenous tumor suppressor genes. Inactivation of tumor suppressor genes such as Tumor protein p53 greatly contributes to breast cancer formation and progression. Several clinical studies have reported deletion/mutation of the p53 gene in 80\% of TNBC patients. This mutation prevents the recessive function of p53 and impedes its ability to activate other transcriptional genes to promote disease regression \(^18\)–\(^20\). Restoring the endogenous function of p53 is critical in tumor regression and treating triple negative breast cancers.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Palmatine (ChemShuttle, Trust Way, USA), Doxorubicin, Trypan blue, Phosphate buffered saline Carmine stain, Aluminum potassium sulfate (Sigma Aldrich, St. Louis, USA), Roswell Park Memorial Institute (RPMI) 1640 medium, Trypsin/EDTA (Pan Biotech, Aidenbach, Germany), Fetal bovine serum (FBS), Penicillin-Streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), L-glutamine (Pan Biotech, Aidenbach, Germany) Antibodies for p53, p21 and Mdm2 (Sigma Aldrich, St. Louis, USA).

**Cell culture**

The mouse 4T1 triple-negative breast cancer cell line (ATCC CRL-2539) was purchased from AddexBio (San Diego, CA 92117, USA). The 4T1 breast cancer cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10\% of fetal bovine serum (FBS), penicillin 100 U/mL, streptomycin 100 µg/mL, and L-glutamine 2 mM. The cells were maintained at 37\°C in a CO2 5\% humidified atmosphere. Before experimenting, the trypan blue assay was performed to determine the viability of the cells.

**Animals**

Female Balb/c mice 20-30g, 6-8 weeks were obtained from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, kept in the Animal House of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The mice were housed in stainless steel cages with delicate wood shavings as bedding, fed with pellet diet (GAFCO, Tema) with water given \textit{ad libitum}. The room was kept at a temperature of 24 - 28 °C, relative humidity 60 - 70\%, and 12 h light-dark cycle. All protocols used in this study were done in compliance with Animal Welfare Regulations and established guidelines on the use and care of Laboratory animals \(^21\). The animals were acclimatized for a week before the experiment.

**Orthotopic transplantation of 4T1 breast cancer cells**

Female Balb/c mice were anesthetized, 4T1 breast cancer cells (4× 10\(^5\)) were suspended in 100 iL of serum-free media and injected into the 4th inguinal mammary fat pad to induce carcinogenesis. The mice were divided into the following groups;

- **Group1-** Normal saline (1 mL/kg) i.p.
- **Group 2-** 4T1 breast cancer cells (4× 10\(^5\)) only.
- **Group 3-5-** Palmatine (1, 5 and 10 mg/kg) i.p.
- **Group 6-** Doxorubicin (5 mg/kg) i.p. per week. Treatment of animals began 48 hours after
induction of carcinogenesis and continued for 28 days\textsuperscript{22}. At the end of the experiment, the mice were euthanized by cervical dislocation and analytical procedures were performed.

**Tumor data**

The mice were palpated for the presence of tumors and tumor size was measured at weekly intervals. The tumor length i.e., largest diameter (a) and the width i.e., shortest dimension (b) perpendicular to (a) of the tumor was measured on days 7, 14, 21 and 28. The tumor volume was calculated using the formula:

\[
\text{Tumor volume (mm}^3\text{)} = \frac{a \times b^2}{2}
\]

At the end of the experiment, the following data were analyzed:

(A) Tumor volume: the total tumor volume calculated at each palpation over time (B) Tumor growth rate: The change in palpable tumor volume from palpation until sacrifice divided by time. (C) Tumor weight: The tumor weight was recorded at necropsy. (D) Tumor Growth Inhibition (\%) = (Tumor weight of 4T1 group - Tumor weight of Treated group)/ (Tumor weight of 4T1 group) \times 100

**Histopathology**

The mammary glands were excised, fixed in 10\% neutral-buffered formalin, and embedded
Fig. 1. Effect of palmatine and doxorubicin on 4T1 mammary carcinoma. A) tumor volume, B) tumor growth rate, C) tumor weight, D) tumor growth inhibition. Data represent mean ± SEM. n=10. One-way analysis of variance followed by Dunnett’s Multiple Comparison Test. ++p< 0.0001 vs. control and +++p< 0.0001 vs. 4T1 group.

Fig. 2. Representative photomicrographs of the mammary gland H&E sections. A) control B) 4T1 C-E) palmatine 1-10 mg/kg F) doxorubicin 5 mg/kg; AT-adipose tissue; C-carcinoma; D-duct; I-inflammatory cells; ME- mammary epithelia; MY- myoepithelial. Scale bar 20 mm (A-E).
in paraffin. Then, 4-µm-thick sections were cut and transferred onto slides. Deparaffinized tissue sections were stained with hematoxylin and eosin (H&E) and examined at 10× magnification. Histology sections from the mammary gland were evaluated for neoplastic morphological changes. A quantitative score of 0-4 representing; 0 - Normal morphology, 1-3 - Mild/moderate/severe dysplasia and 4 - Abnormal morphology with invasive carcinoma.

**Immunohistochemical detection of p53, p21, and Mdm2 Expression**

The immunoreactions of p53, p21 and Mdm2 were determined in the mammary tissues using their respective mouse polyclonal antibodies. Briefly, 5 µm sized paraffin-embedded tissue sections were de-paraffinized with xylene and endogenous peroxidase activity was quenched with 3% H2O2 in methanol for 30 minutes in the dark. Tissue sections were dehydrated through graded alcohols and subjected to heat-induced epitope antigen retrieval using 10mM sodium citrate. Sections were washed with TBST (Tris Borate Saline Tween-20) and then blocked with 5% BSA (Bovine Serum Albumin) for one hour. Slides were incubated with the respective mouse monoclonal primary antibody diluted with TBS. Slides were then washed for 5 minutes in TBST and incubated for 1 hour with the respective HRP (Horse Radish Peroxidase) conjugated anti-mouse secondary antibody diluted with TBS in a ratio of 16:200. After washing, slides were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride) and immediately washed under tap water after color development. Slides were then counterstained with hematoxylin. Slides were mounted with dibutyl phthalate xylene and images under a 10× light microscope. The manifestation of a dark, brown-colored, intracytoplasmic precipitate is indicative of the presence of p53, p21, and Mdm2. The antibody stain intensity of the images obtained was analyzed with an IHC Profiler plugin in Image J. The Immunohistochemistry optical density score (IODS) was calculated.

\[
\text{IHC optical density score} = \frac{(\text{Percentage contribution of high positive } \times 4 + \text{Percentage contribution of positive } \times 3 + \text{Percentage contribution of low positive } \times 2 + \text{Percentage contribution of negative } \times 1)}{100}
\]

**Statistical Analysis**

Experimental data were expressed as Mean ± Standard error of the mean. Analysis of data was done by Graph Prism software version 8.0.1. using One-way analysis of variance (ANOVA) followed by Dunnett’s post hoc comparisons. The criteria for significant difference between groups was set at p ≤ 0.05.

**RESULTS**

**Effect of palmatine on mammary carcinoma**

Growth-dependent changes on the mammary gland of carcinoma bearing mice were assessed in the different groups. Tumor volume and weight recorded in the 4T1 group, 28 days post-injection with the 4T1 breast cancer cells was 547.20 ± 18.11 and 1.49 ± 0.02 respectively (Figure 1 A, C). The growth rate of the tumor from the untreated 4T1 group on day 28 was 5.0 ± 2.24
compared to the control 0.0 ± 0.0 (Figure 1 B). After 28 days, administering palmatine 1, 5 and 10 mg/kg significantly (p < 0.001) in a dose-dependent manner decreased mammary tumor volume 190.80 ± 19.14, 25.40 ± 2.82, 14.20 ± 1.85, tumor weight 1.035 ± 0.04, 0.8027 ± 0.01, 0.5090 ± 0.04 and effectively inhibited tumor growth 31%, 46% and 66% respectively compared to carcinoma bearing mice (Figure 1 A-D).

**Histopathology**

The morphology of the mammary gland from the control group presents a vacuolated appearance of normal acini arranged radially, surrounded by myoepithelial structures with a dysplasia score of 0.0 ± 0.0 (Figure 4 A, 5). Histological analysis established the presence of mammary carcinoma in the hematoxylin and eosin (H&E) stained sections from the 4T1 group. The 4T1 group showed severe mammary carcinoma characterized by solid aggregation of the 4T1 breast cancer cells defined by dilated ducts and lobules filled with cancerous cells with a deterioration of the conjunctive tissue with numerous degrees of cellular and nuclear pleomorphism consistent with malignant epithelial proliferation; a dysplasia score of 3.75 ± 0.25 was recorded (Figure 4 B, 5). On the other hand, the histoarchitecture of mammary glands from carcinoma bearing mice treated with palmatine 1, 5, 10 mg/kg in a dose dependent manner presented a high regression of tumor growth, wide regions of necrosis, little sign of neoplasia, well-separated acini and various areas of tumor cell remnants; dysplasia scores of 3.50 ± 0.29, 2.25 ± 0.25, 1.75 ± 0.25 was recorded respectively (Figure 4C-D, 5).

![Representative photomicrographs of p53 expression in IHC sections of the mammary gland: A) control B) 4T1 C-E) palmatine 1-10 mg/kg F) doxorubicin 5 mg/kg. Data were obtained from five fields per slide with seven slides analyzed from each group. Red arrows show immunoreactive areas. Scale bar 20 mm (A-E)](image_url)
Immunohistochemical Detection of p53 Expression

The expression of p53 indicated by a brown stain was evaluated by a semi-quantitative method using the Immunohistochemistry Optical density score (IODS). Tissue sections from mice treated with palmatine at doses 5 mg/kg and 10 mg/kg showed positive immunoreactivity in the cytoplasm for the p53 tumor protein; IODS $1.77 \pm 0.12$, $2.15 \pm 0.12$ respectively (Figure 6D–E, 7) compared to the low expression in the untreated 4T1 group; IODS $1.15 \pm 0.03$ (Figure 6B, 7).

Immunohistochemical Detection of p21 Expression

Tissue sections from animals treated with palmatine at doses 1 mg/kg, 5 mg/kg and 10 mg/kg showed positive immunoreactivity in the cytoplasm for the p21 protein (brown stain); IODS $1.72 \pm 0.06$, $1.86 \pm 0.04$ and $2.0 \pm 0.07$ respectively (Figure 8C–E, 9) compared to the low expression in the untreated 4T1 group; IODS $1.19 \pm 0.03$ (Figure 8B, 9).

Fig. 5. Effect of palmatine and doxorubicin on p53 IHC Optical density score in 4T1 mammary carcinoma. Data represent mean ± SEM. n=10, One-way analysis of variance followed by Dunnett’s Multiple Comparison Test, ns (not significant) vs. control and “p< 0.002, ****p< 0.0001 vs. 4T1 group

Fig. 6. Representative photomicrographs of p21 expression in IHC sections of the mammary gland; A) control B) 4T1 C-E) palmatine 1-10 mg/kg F) doxorubicin 5 mg/kg. Data were obtained from five fields per slide with seven slides analyzed from each group. Red arrows show immunoreactive areas. Scale bar 20 mm (A-E)
Immunohistochemical Detection of Mdm2 Expression

Tissue sections from mice treated with palmatine at doses 1 mg/kg, 5 mg/kg and 10 mg/kg showed positive immunoreactivity in the cytoplasm for the Mdm2 protein (brown stain); IODS 1.63 ± 0.06, 1.69 ± 0.06 and 1.91 ± 0.03 respectively (Figure 10C-E, 11) compared to the low expression in the untreated 4T1 group; IODS 1.07 ± 0.03 (Figure 10B, 11).

DISCUSSION

The mouse 4T1 triple negative mammary carcinoma model has several characteristics that make it an effective model for the preclinical evaluation of new anti-cancer therapies. The 4T1 breast cancer cells mimic human triple negative breast cancer in the lack of the expression of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2). The 4T1 breast cancer cells are easily transplanted into the mammary fat pad allowing for easy quantification of tumor growth and progression.

The present study was carried out to investigate the protective activity of palmatine in triple-negative mammary carcinoma. The intraperitoneally administered dose of palmatine used in this study was obtained from effective pharmaceutical concentrations from published reports.

Consistently, there were no symptoms of intoxication, weight loss and anorexia with the doses of palmatine administered.

Following transplantation of the 4T1 breast cancer cells, carcinoma was formed. This involves alterations in the micro and macro-environment of the breast cancer cells imparting a selective advantage to the cancerous cells which promotes increased cell proliferation and tumor growth. Such changes often inactivate components of several signal transaction pathways. This is supported by the evidence that a majority of human cancers display persistent inactivation of one or more pathways. Palmatine administration effectively demonstrated a dose dependent reduction in tumor burden in carcinoma bearing mice suggesting a dose-related beneficial and protective inhibition of mammary carcinoma.

Elevated levels of tumor suppressor genes have been associated with the prevention of malignant growth of cancerous cells. Tumor protein p53 through several mechanisms plays a critical role in cell cycle regulation, DNA repair, inhibition of angiogenesis and apoptosis. The p53 gene has been described as “the guardian of the genome” referring to its function in preserving stability by preventing genome mutation. Anticancer drugs that modulate the p53 tumor suppressor pathway are important mediators for better cancer prognosis.

The endogenous tumor protein p53 when synthesized in cells has a short half-life. Nonetheless, the tumor protein p53 when activated by anticancer agents in response to cellular stress signals such as DNA damage, hypoxia, nitric oxide signaling, and oncogene activation result in an increased half-life and transcriptional activity. There was a statistically significant increase in the levels of the p53 protein when carcinoma
bearing mice were treated with palmatine. This confirms published reports which state that the 4T1 mammary carcinoma is deficient in the p53 protein and harbor genes that inactivate the p53 protein. Thus, changes in the level of p53 may be a result of administering a therapeutically effective compound.

Several proteins mediate cell cycle regulation by p53, however, p21 is a major therapeutic target for the transcriptional activity of p53. The progression of abnormal cell division into the S phase requires the Cyclin-dependent kinase 2 enzymes, which is inhibited by p21. Also, progression into the M phase requires Cell division control 2 which is also inhibited by p21. The tumor protein p53 increased the expression of p21, an inhibitory protein to induce growth arrest as such, a significant increase in the expression of the p21 protein was observed with palmatine in a dose-dependent manner.

The Mdm2 is the product of a p53-activated gene. An increase in p53 activity leads to the upregulation of Mdm2. The Mdm2 is also a p53 inducible gene whose protein product binds to p53 and acts as an E-3 ubiquitin ligase that adds ubiquitin to p53 which results in its degradation. This produces an autoregulatory loop where a synthesis of p53 results in the synthesis of Mdm2, which in turn degrades p53. Upregulation of Mdm2 observed for palmatine in a dose-dependent manner is a reliable indication of the endogenous activation of the tumor protein p53 by palmatine.

**CONCLUSION**

Palmatine administration effectively inhibits the growth of mammary carcinoma in
female balb/c mice evident from tumor growth inhibition and the observation of a quasi-normal mammary morphology. These effects of palmatine were associated with the endogenous upregulation of the tumor p53 protein, the translational gene p21 and its negative regulatory gene Mdm2.

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Conflict Of Interest

The authors declare no competing interest.

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Author contributions

Selase Ativui – Project administration, manuscript writing, data analysis and interpretation. Cynthia Amaning Danquah – Project conceptualization, protocol optimization and data interpretation. Osafo Newman - Manuscript writing and review. Ka-Chungu Sammy – Performed the histopathology and immunohistochemistry assays.

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