Original Article

Naringenin and metformin enhance the antitumor effect of doxorubicin against experimental models of breast carcinoma

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Abstract:

Breast cancer is the most common malignancy in women worldwide and is curable in patients at an early stage. The present work is aimed to evaluate the potential of naringenin and metformin concomitant addition with doxorubicin chemotherapy against experimental breast carcinoma models. The antitumor potential of drugs under the study was evaluated *in vivo* against methylnitrosourea (MNU)- induced breast cancer in rats and 4T1- induced orthotropic mouse model. Tumor-bearing animals were randomly divided into various groups to assess the effect of each single drug and concomitant drug treatments. Parameters like tumor growth, body weight, survival rate, blood glucose, hematology and histology study were determined. There was significant reduction in tumor weight and an observed decrease in tumor multiplicity in naringenin and metformin concomitant addition with doxorubicin treatment as compared to doxorubicin alone against MNU-induced breast carcinoma. Likewise, significant reduction of tumor volume and tumor weight was also observed in 4T1 mouse model suggesting combination treatment enhanced antitumor activity *in vivo*. Further, histology of tumor biopsies presented enhanced antitumor activity of doxorubicin through increasing tumor necrosis. Hematological parameters, body weight and survival data presented better safety of combination treatment without compromising efficacy using lower dose of doxorubicin as compared to large dose of doxorubicin alone. These results demonstrate that naringenin and metformin enhanced the antitumor effect of doxorubicin in animal models of breast carcinoma and useful as an adjunct to increase the effectiveness of doxorubicin at lower dose.

**Keywords:** Naringenin, Metformin, Doxorubicin, Breast carcinoma
Introduction:

Breast cancer is the most common malignancy in women around the world. Early stage and locally advanced disease is curable in 70–80% of patients (Harbeck et al. 2019). Unless some urgent action is taken, the number of females diagnosed with breast cancer in the world would almost double to 3.2 million a year by 2030 (Noori et al. 2020). Advanced metastatic breast cancer is considered incurable using currently available anticancer therapies. Strategies using cancer chemotherapy commonly require the combination of agents. However, development of resistance to chemotherapeutic agents and toxicity to normal cells are the major problems. Therefore, it is an unmet need to evaluate the currently available medicines and some plant based active component for their potential role for the treatment of breast carcinoma.

Doxorubicin is one of the most frequently used anticancer agents in the treatment of human malignancies. However, the therapeutic use of doxorubicin is limited by its severe cumulative dose-related cardiotoxicity and resistance. Naringenin, a naturally occurring flavonoid has shown anti-inflammatory, anti-atherogenic, anti-mutagenic, hepatoprotective, antidiabetic, cardioprotective and anticancer potential in many non-clinical studies (Salehi et al. 2019). The cytotoxic mechanism of naringenin does not depend on p53, a tumor suppressor gene. Therefore naringenin is a promising co-chemotherapeutic agent for cancer which has inactivated p53 (Kanno et al. 2005). Metformin has shown antitumor activity (Viollet et al. 2012; Aljofan and Riethmacher 2019) and is associated with a lower incidence of cancer as well as reported to improve overall survival of several cancers (Kasznicki et al. 2014; Lee et al. 2020; Ugwueze et al. 2020).
Despite available literature for naringenin, metformin and doxorubicin for their potential use against cancers, no reports are available for *in vivo* efficacy of these drugs together for the treatment of breast cancer. In the present study, we evaluated the efficacy and safety of combining naringenin and metformin with doxorubicin chemotherapy using *in vivo* experimental breast carcinoma models to explore the scope of treatment option for breast cancer.

**Materials and Methods:**

**Culture condition and reagents**

The mouse breast cancer cell line 4T1 (CRL-2539™) was purchased from the American Type Culture Collection (ATCC; Bangalore, India). Cells were cultured with RPMI-1640 medium (Sigma-Aldrich, Merck) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cultures were incubated at 37°C in humidified atmosphere of 95% air and 5% CO₂. Methylnitrosourea (MNU) and naringenin were purchased from Sigma-Aldrich (Bangalore, India). Metformin was procured from Parth Medicine (Vadodara, India). Liposomal doxorubicin was obtained from Sun Pharmaceutical Industries Limited (Vadodara, India).

**Animals**

Female Sprague Dawley rats (80-120 gm) and female Balb/c mice (18-22 gm) were used for the studies. Animals were housed in group (2 to 3 animals/cage) and cages were maintained under temperature (18-26°C), humidity (30%- 70%) and 12 h light and 12 h dark condition. Animals received water and rodent diet ad libitum. Animal experiments were conducted according to the
guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA). The project proposal was approved by the Institutional Animal Ethics Committee of Sardar Patel College of Pharmacy, Gujarat Technological University, Gujarat, India (IAEC No. SPCP/IAEC/RP-03/2017).

**Chemical-induced breast carcinoma (MNU-induced rat model)**

Breast tumor was induced in SD rats by injecting 50 mg/kg MNU intraperitoneally as described earlier (Thompson and Adlakha 1991). After the development of tumor i.e., 90 days from the injection, the animals were screened and selected animals were divided in to different treatment groups on the basis of tumor occurrence and body weight as shown in study design (Table 1). Naringenin (50 mg/kg) or/and metformin (100 mg/kg) was administered orally to its respective treatment groups of animals for 28 days. Intravenous injections of liposomal doxorubicin (2 mg/kg or 4 mg/kg), or saline were administered on days 0, 7, 14 and 21 to the respective group of animals as shown in Table 1. Tumor development and body weights were recorded weekly up to day 28. Blood glucose was measured on day 21 for each animal to check any hypoglycemic effect of treatments under the study. The animals were checked daily throughout the study for any mortality. On 28th day, tumor was isolated and weighed. Antitumor activities of a single drug or combination of drugs were assessed based on tumor number and incidence, tumor multiplicity (total number of tumor/total number of animal) and tumor weight (Table 2).

**Orthotropic breast carcinoma (4T1 mouse model)**

Breast tumors were induced using orthotropic injection of 1 x 10⁶ 4T1 breast carcinoma cells at mammary fat pad of Balb/c mice as described earlier (Paschall 2016; Zhang et al. 2018).
Animals were divided into different treatment groups on the basis of tumor volume and body weight (tumor volume 50 to 100 mm$^3$ and had a body weight 18-24 gm at the time of treatment initiation) as shown in study design (Table 1). Naringenin (50 mg/kg) or/and metformin (100 mg/kg) was administered orally to its respective treatment groups of animals for 28 days. Intravenous injections of liposomal doxorubicin (3 mg/kg or 6 mg/kg), or saline were administered on days 0, 7, 14 and 21 to its respective group of animals as shown in Table 1. Tumor diameter using digital vernier caliper and body weights were recorded twice weekly up to day 28. For humane reasons, animals were euthanized when tumor volume reached >4000 mm$^3$. The animals were checked daily throughout the study for mortality. On day 28th, blood was withdrawn for the hematology estimation. The tumor was isolated and weighed. Tumor volume (V) was calculated using the formula of a sphere as follows:

$$V (\text{mm}^3) = \left[\frac{(D1 + D2)}{2}\right]^3 \times 0.5236$$

where D1 and D2 were the largest and smallest diameters of tumor respectively.

Percentage test/control (%T/C) was calculated as follows:

$$\%T/C = \frac{\text{Mean tumor volume of drug treated group on day } X}{\text{Mean tumor volume of control group on day } X} \times 100$$

where X was the day of observation.

The optimal %T/C value for each group was the minimal %T/C ratio, thus reflecting the maximal tumor growth inhibition. According to NCI standard criteria, %T/C ≤ 42% indicates acceptable antitumor activity; %T/C ≤ 20% indicates moderate antitumor activity; %T/C ≤ 10%
indicates high antitumor activity (Bissery and Gueritte-Voegelein 1991; Kruczynski and Hill 2002; Burade et al. 2017).

Animal body weight changes were calculated as follows:

\[
\text{Body weight change (\%) = } \frac{\text{Mouse weight on Day X} - \text{mouse weight on Day 0}}{\text{Mouse weight on Day 0}} \times 100
\]

where X was the day of observation.

A dose producing a mean weight loss ≥15% of initial body weight was considered toxic (Kruczynski and Hill 2002; Burade et al. 2017).

Hematology estimation

The blood was collected from the mice at the end of the study (on day 28) and processed for hematology parameters analysis using with the ADVIA 120 hematology system.

Histology

Histology was performed using hematoxylin and eosin (H&E) staining for the tumor sections of MNU- induced rat model as well as orthotropic mouse model. Tissue samples were fixed in 10% formalin and embedded in paraffin, cut in 4 mm sections, stained with H&E and then observed for tumor necrosis based on morphology under a bright field microscope (Alyahya et al. 2015; Liu et al. 2017).

Statistical analyses
Tumor volume data were analysed using two-way ANOVA followed by Bonferroni’s test. Body weight, hematology and blood glucose parameters were analysed using one-way ANOVA followed by Dunnett’s test. The Kaplan-Meier method was used to estimate survival, and differences were analysed by log-rank test. Statistical analyses were carried out using Graph Pad Prism and p values <0.05 were considered significant.

Results:

Effect of naringenin, metformin and doxorubicin on MNU-induced breast carcinoma

In MNU-induced breast carcinoma model of rats, significant reduction (p<0.05) in tumor weight was seen in metformin + 2 mg/kg lipo-doxt, naringenin + 2 mg/kg lipo-doxt and combinations of naringenin + metformin + 2 mg/kg lipo-doxt treated groups when compared with the disease control group (p<0.05). Higher dose of lipo-doxt (4 mg/kg) showed significant reduction in tumor weight as compared to disease control group (p<0.01). Moreover, combination of treatment (lipo-doxt 2 mg/kg + naringenin + metformin) showed marked reduction in tumor weight compared to lipo-doxt 2 mg/kg alone treatment (Table 2). Combination of lipo-doxt 2 mg/kg with naringenin and metformin showed reduction in tumor incidence, total number of tumors as well as tumor multiplicity compared to lipo-doxt 2 mg/kg alone and showing effects closer to higher dose of lipo-doxt (4 mg/kg). Reduction in mean tumor weight was not found significant in naringenin or metformin or lipo-doxt 2 mg/kg individual drug treatment compared to disease control group. There was also no remarkable body weight loss observed at any dose under the study treatment groups, when compared with their respective body weight on day 0 (Fig. 1A). However, the maximum decrease in body weight was observed in the lipo-doxt at 4 mg/kg (12%); whereas all other tested treatment groups showed bodyweight loss less than 5% in rat model of MNU-induced breast carcinoma. Further, no change in blood glucose levels noticed in any
treatment group (p > 0.05; Fig. 1B) that may be due to euglycemic effect of metformin on non-diabetic animals. In addition to above, no mortality was observed in any treatment group throughout the study (p > 0.05; Fig. 1C).

Effect of naringenin, metformin and doxorubicin on orthotropic 4T1 mouse model

Significant reduction in tumor volume was observed in 3 & 6 mg/kg lipo-dox treated groups, metformin alone group and combinations of naringenin and/ or metformin with 3 mg/kg lipo-dox treated group as compared to the disease control group. Moreover, combination treatment (lipo-dox 3 mg/kg + naringenin + metformin) showed significant reduction in tumor volume compared to lipo-dox 3 mg/kg alone treatment establishing the synergistic effect (p < 0.05; Fig. 2A). Tumor weight at the end of the study (day 28) showed similar pattern with tumor volume data and thereby conforming the synergistic effect of concomitant treatments (Fig. 2B & C). There was no significant change in tumor volume observed in naringenin alone treatment throughout the study. Antitumor activities were compared based on %T/C (Table 3). Acceptable antitumor activity was observed in lipo-dox 3 mg/kg (%T/C ≤ 42, based on NCI criteria) in 4T1-bearing mice. However, combination of lipo-dox 3 mg/kg either with naringenin or with metformin showed moderate antitumor activity (optimal %T/C values on day 28 were 16.2 and 12.6 respectively). Further, combination of naringenin and metformin with lipo-dox 3 mg/kg showed highly significant antitumor activity (optimal %T/C values on day 28 were 7.2) indicating the synergistic effect of combination treatments. In addition, the efficacy of combination of naringenin and metformin with lipo-dox 3 mg/kg was comparable with highly significant antitumor activity of 6 mg/kg lipo-dox (optimal %T/C values on day 28 was 2.8) (Table 3).
In 4T1-bearing mice, bodyweight loss from the baseline value was not found significant in any of the treatment groups, except higher dose of lipo-dox (i.e. 6 mg/kg) (Fig. 3A). Looking at the survival data, mortality was observed in each treatment group including disease treatment group indicating the highly aggressive nature of 4T1 cells on mouse survival. However, the maximum mice survival (83%) was found in control group, lipo-dox 3 mg/kg + naringenin + metformin combination group, lipo-dox 3 mg/kg + naringenin combination group, and metformin alone treatment group at the end of study. 67% survival was observed in naringenin, lipo-dox 3 mg/kg, and lipo-dox 3 mg/kg + metformin combination group. At a higher dose of lipo-dox 6 mg/kg, only 50% survival was noticed. (Fig. 3B).

**Effect of naringenin, metformin and doxorubicin on hematology parameters**

The hematological parameters in whole blood of mice showed significant reduction (**p<0.001**) in total WBC and neutrophils counts in lipo-dox alone groups and combination treatment groups as compared to the control group (Table 4). Also, the higher dose of lipo-dox receiving animals showed a high reduction of WBC and neutrophils counts. This indicates the use of a lower dose of lipo-dox along with the naringenin and metformin is helpful to minimize toxicity without compromising efficacy. Although, there was no significant difference in platelets, hemoglobin, RBC and lymphocyte counts following various treatment groups under the study (Table 4).

**Effect of naringenin, metformin and doxorubicin on histopathological examination**
Histopathological examination of tumor sections showed that naringenin or metformin alone treatment was not able to affect tumor necrosis in MNU-induced rat model and 4T1-induced mouse breast carcinoma model. However, concomitant treatment of naringenin, metformin and lipo-dox showed remarkable increase in the tumor necrotic area as compared with the vehicle control group in both the models (Fig. 4A & B). These findings have further proved the efficacy of combination treatment in breast carcinoma.

**Discussion:**

The therapeutic dose of doxorubicin is frequently insufficient in cancer treatment, and the use of its higher dose produces dose-related toxicity (Hanušová et al. 2011). Moreover, long-term repeated use of doxorubicin chemotherapy develops its resistance and toxicity which can be attenuated by increasing its effectiveness. In the present study, we focused on the *in vivo* efficacy and safety of concomitant use of naringenin and metformin with doxorubicin by reducing its dose and subsequently dose-related toxicities of doxorubicin against experimental breast cancer models.

NMU is a most reliable carcinogen, mutagen and teratogen to induce breast carcinogenesis in experimental rats. 4T1 mouse model is frequently used for the evaluation of breast carcinoma. We used both models in the present study. Wang and coworkers observed that naringenin has a chemoprotective effect in breast cancer cells via inhibition of caspase-3 and caspase-9 activities (Wang et al. 2019). Recently, Noori et al observed that naringenin improves the anti-cancer effect of cyclophosphamide in breast carcinoma whereas other reports have shown the
synergistic effect of 5-Fluorouracil combined with naringin in MDA-MB-231 human breast cancer cells (Muthusamy et al. 2020; Noori et al. 2020). In addition to anticancer effects, naringenin has shown cardioprotective effects. Shabanah et al observed protective effects of naringenin against doxorubicin induced cardiac, hepatic and renal toxicities in rats (Shabanah et al. 2019). Other side, it has been reported that metformin synergistically inhibits tumor growth and reverses resistance with doxorubicin in both MCF7/ADR cells and xenograft study (Li et al. 2018). Further, metformin prevents doxorubicin resistance in MCF-7 and MDA-MB-231 through oxidative stress and modulation of cell adaptation genes (Marinello et al. 2019). Hirsch and coworkers have reported that metformin selectively targets cancer stem cells and helps chemotherapy to block tumor growth and delay remission (Hirsch et al. 2009). In addition to above, Kelleni et al have assessed the effect of metformin and sitagliptin on doxorubicin-induced cardiotoxicity in rats and the protective effect observed via oxidative stress, inflammation, and apoptosis pathways (Sheta et al. 2016).

The present study of MNU-induced breast carcinoma in rats wherein, combination of naringenin + metformin + lip-dox 2 mg/kg showed a marked reduction in tumor weight compared to lipodox 2 mg/kg alone treatment and the effects were closer to higher dose of lipo-dox 4 mg/kg alone. Also, histology using H&E revealed higher necrosis in those concomitant treatment groups. Other findings such as blood glucose levels, boy weight and survival observations indicated no significant toxicity at single drug treatment and combination treatments. Our earlier in vitro data demonstrated that the combination of naringenin and metformin enhanced the sensitivities of breast cancer cells (MDA-MB-231 and 4T1) to doxorubicin (Pateliya et al. 2020). Also, in vivo study using MDA-MB-231 xenograft showed improved efficacy and safety of
doxorubicin combination treatment (Pateliya et al. 2020). Parallel to above, the present study of in vivo 4T1 orthotropic mouse model showed statistically significant reduction in tumor volume as well as tumor weight in the three-drug concomitant treatment group compared to lipo-dox alone at 3 mg/kg treatment group. The concomitant use of naringenin and metformin with lipo-dox 3 mg/kg exhibited a comparable antitumor effect to that of the group receiving a higher dose of lipo-dox (6 mg/kg). Finally, based on MNU-induced breast carcinoma and 4T1 orthotropic mouse model, we observed that the combination of naringenin and metformin with doxorubicin increased antitumor activity compared to doxorubicin alone. Moreover, in view of hematology, body weight loss and survival of mice treated with combination treatment showed fewer signs of systemic toxicity than that treated with a large dose of doxorubicin alone.

In conclusion, our results demonstrated that use of naringenin and metformin together with doxorubicin chemotherapy has good potential as candidates for co-chemotherapy agents for the treatment of breast cancer. It may thus provide an opportunity and the scope for the use of naringenin and metformin with doxorubicin chemotherapy for reducing its dose and subsequently toxicity in patients. However, further in-depth studies including clinical research are needed to fully evaluate the value of naringenin and metformin in combination with chemotherapeutic agents for the treatment of human cancers.

**Declarations**

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**Ethics approval and consent to participate:** This article does not contain any studies with human participants performed by any of the authors. All animal studies were carried out with prior approval from the Institutional Animal Ethics Committee (IAEC) and care of animals complied according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

**Consent for publication:** Not applicable.

**Conflict of interest:** All the authors declare that they have no conflict of interest.

**Availability of data and material:** Data available with the paper in the form of tables, figures and its supplementary file.

**Code availability:** Not applicable.

**Author contributions:** The authors declare that all data were generated in-house and that no paper mill was used. Bharat Pateliya (BP): Designed and performed the experiments, contributed to the data analysis and writing original draft, Vinod Burade (VB): Reviewed and performed supervision. Sunita Goswami (SG): Reviewed, results interpretation and work supervision. All the authors read and approved the final manuscript.
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Figure captions

Fig. 1: Effect of single or combination treatment of naringenin, metformin and liposomal doxorubicin on (A) body weight (B) glucose levels and (C) survival in MNU- induced rat model

Fig. 2: Effect of single or combination treatment of naringenin, metformin and liposomal doxorubicin in 4T1 mouse model (A) tumor volume (B) tumor weight (C) representative images tumor bearing mice

Data are expressed as mean ± SD. n=6. Tumor volume data was analyzed using Two way ANOVA followed by Bonferroni posttests. *p<0.05 compared to Lipo-dox 3 alone. Tumor weight data was analyzed using One way ANOVA followed by dunnet’s posttests.*p < 0.05, **p < 0.01, ***p < 0.001 compared to control group. $$p < 0.01 compared to Lipo-dox 3 alone group.

Groups (a) Nar 50, (b) Met 100, (c) Lipo-dox 6, (d) Lipo-dox 3, (e) Lipo-dox 3 + Nar, (f) Lipo-dox 3 + Met and (g) Lipo-dox 3 + Nar + Met

Nar- Naringenin, Met- Metformin, Lipo-dox- Liposomal Doxorubicin hydrochloride, q7d*4- total 4 dose at weekly interval.qd*28- daily for total 28 days.

Fig. 3: Effect of single or combination treatment of naringenin, metformin and liposomal doxorubicin on (A) body weight and (B) survival in 4T1 mouse model

Body weight data are expressed as % change in body weight from initial body weight. The data was analyzed using One way ANOVA followed by dunnet’s posttests. *p < 0.05, compared to initial body weight of same group. A dose producing a weight loss ≥15% of initial body weight was considered toxic. No significant change in body weight was observed in any treatment group except Lipo-dox 6.
Survival was estimated using the kaplan-meier method, and differences were analyzed by log-rank test. No statically significant difference was observed in survival data.

Nar- Naringenin, Met- Metformin, Lipo-dox- Liposomal Doxorubicin hydrochloride, q7d*4- total 4 dose at weekly interval.qd*28- daily for total 28 days.

Fig. 4: Representative images of tumor sections using histology (H&E) (A) MNU- induced rat model and (B) 4T1 mouse model:

Groups (a) Nar 50, (b) Met 100, (c) Lipo-dox 6, (d) Lipo-dox 3, (e) Lipo-dox 3 + Nar, (f) Lipo-dox 3 + Met and (g) Lipo-dox 3 + Nar + Met, (100x magnification, scale bars = 50µm)