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Noscapine chemosensitization enhances docetaxel anticancer activity and nanocarrier uptake in triple negative breast cancer

Ravi Doddapaneni¹, Ketan Patel¹, Nusrat Chowdhury, and Mandip Singh*

College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA

Abstract

Chemosensitization and enhanced delivery to solid tumor are widely explored strategies to augment the anticancer efficacy of existing chemotherapeutics agents. The aim of current research was to investigate the role of low dose Noscapine (Nos) in potentiating docetaxel cytotoxicity and enhancing tumor penetration of nanocarriers. The objectives are; (1) To evaluate the chemosensitizing effect of Nos in combination with docetaxel (DTX), and to elucidate the possible mechanism (2) To investigate the effect of low dose Nos on tumor stroma and enhancing nanocarrier uptake in triple negative breast cancer (TNBC) bearing nude mice. Cytotoxicity and flow cytometry analysis of DTX in Nos (4 μ M) pre-treated MDA-MB-231 cells showed 3.0-fold increase in cell killing and 30% increase in number of late apoptotic cells, respectively. Stress transducer p38 phosphorylation was significantly upregulated with Nos exposure. DTX showed remarkable downregulation in expression of bcl-2, survivin and pAKT in Nos pre-treated MDA-MB-231 cells. Nos pre-sensitization significantly ($p < 0.02$) enhanced the anti-migration effect of DTX. *In vivo* studies in orthotopic TNBC tumor bearing mice showed marked reduction in tumor collagen-I levels and significantly ($p < 0.03$) higher intra-tumoral uptake of coumarin-6 loaded PEGylated liposomes (7-fold) in Nos treated group. Chemo-sensitization and anti-fibrotic effect of Nos could be a promising approach to increase anticancer efficacy of DTX which can be used for other nanomedicinal products.

Keywords

Triple negative breast cancer; Chemosensitization; Noscapine; Docetaxel; Tumor stroma

1. Introduction

Triple negative breast cancer (TNBC) is a diverse subgroup of breast cancers that lack ER, PR, and EGFR/Her2 receptors making them unresponsive to current hormone targeted chemotherapies [1]. TNBC is highly aggressive with a high histological grade and it also relapses quickly in response to chemotherapy [2,3]. Common treatment for TNBC includes docetaxel (DTX), paclitaxel (PTX), cisplatin and doxorubicin etc. However, there is a huge

*Corresponding author. mandip.sachdeva@fam.u.edu (M. Singh).

¹Both the authors have equally contributed to paper.

Conflict of interest

The authors declare that they have no competing interests.

lacuna conventional chemotherapy due to dose dependent side effects and development of chemo-resistance which limit the success of chemotherapy in TNBC [4,5]. Despite recent advances, clinical outcome among TNBC patients has not significantly improved. Combination therapies involving use of taxanes with synergistic elements is greatly needed in order to overcome limitations with existing chemotherapeutic regimens.

Natural compounds have been explored to act as potent chemo-sensitizers in combination with conventional chemotherapeutic drugs and are pharmacologically safe over several synthetic chemicals due to low systemic toxicity [6,7]. Noscapine (Nos) is a commonly used cough suppressant, and has been extensively investigated as a single agent anticancer therapy against melanoma, lung, prostate, ovarian and breast cancers and works by acting on microtubules like taxanes, inducing apoptosis and inhibiting angiogenesis [8–11].

Multiple investigations have shown that Nos acts through various mechanisms including its ability to inhibit microtubule assembly [12], suppress expression of hypoxia-inducible factor-1 α [13], induce p21 and p53 [14], induce apoptosis-inducing factor (AIF) [15], activate c-Jun-N-terminal kinase (JNK) [16] and repress bcl-2 [10]. It has been reported that Nos can efficiently inhibit the growth of both paclitaxel-sensitive and paclitaxel-resistant ovarian cancer cells [16], suggesting that Nos may mediate anticancer effects through other mechanisms. In addition, it has been suggested that various anti-microtubule agents might trigger apoptosis through a variety of phospho-regulatory pathways [16]. Our laboratory and other researchers have provided evidence that enhanced tumor growth inhibition of various tumors was achieved by combining Nos with chemotherapeutic drugs [10,11,17–20]. Further, our group also demonstrated previously that Nos enhances the anticancer activity of doxorubicin in a synergistic manner via inactivation of NF- κ B and anti-angiogenic pathways while stimulating apoptosis against TNBC tumors [19].

However, low dose oral Nos has never been investigated as chemosensitizing agent for taxanes against triple negative breast cancer. Thus, even though Nos cannot be used as a standalone agent in TNBC treatment, its chemo-sensitizing effect can be critically important for enhancing the tumor specific toxicity of DTX and will help in reducing the dose of DTX and its dose dependent side effects.

Poor availability of anticancer drug and nanocarrier in solid tumor is one of the major limitations in their therapeutic outcome [21,22]. Tumor blood vessels are abnormal and characterized by increased permeability and retention. Poor vascular perfusion decreases delivery of anticancer drugs and, as a result, it impairs the efficacy of antitumor agents given by IV route. In addition, the collagen fibers-rich dense tumor interstitial matrix further hinders drug transport to cancer cells—especially nanoparticles [21,23]. In such scenario, stromal disruption could be important for harnessing the potential of anticancer therapy. Various strategies like use of hyaluronidase, relaxin, collagenase and selective TGF- β inhibitors have been investigated to disrupt the tumor stroma for facilitating the uptake of nanoparticles. However, use of orally bioavailable small molecule inhibitors are better choice over intravenous proteolytic enzyme based approaches for reducing tumor stroma. In our previous report we have demonstrated respiratory delivery of an antihypertensive drug—telmisartan to reduce collagen in lung tumors and enhance the uptake of fluorescent

nanoparticles [24]. Also, an increase of collagen deposition in the extracellular matrix (ECM) indicated a high mammographic density that is correlated with an increased risk of breast cancer. Antifibrotic effect of telmisartan is contributed by its TGF- β inhibitory effect. Very recently, the anti-fibrotic activity of Nos has been demonstrated in pulmonary fibrosis and the mechanism proposed was based on protein kinase A activation via EP2 prostaglandin receptors [25]. Liu et al. (2012) have reported that TGF- β blockade decreased the tumor interstitial matrix density by reducing collagen I content and, consequently, increased tumor tissue penetration of the nanoliposomal doxorubicin (Doxil) in TNBC solid tumor [21]. Based on that, we have investigated the effect of Nos on tumor fibrosis in orthotopic TNBC solid tumor bearing mice and exploiting its antifibrotic effect as means to improve the intratumoral delivery of nanocarrier. The rationale of using oral NOS in TNBC chemotherapy are based on; 1. NOS will enhance anticancer efficacy of DTX and 2. NOS mediated disruption of tumor ECM will increase the tumor bioavailability of nanocarrier loaded with anticancer drugs. Thus, oral NOS treatment will result in significant enhancement in clinical outcome of existing chemotherapy with minimum dose and minimal side effects.

In the present study, we are investigating low dose oral Nos as a chemosensitizer and solid tumor anti-fibrosis agent. The objectives of this study were (a) to examine the DTX chemosensitizing effects of Nos in TNBC cells and (b) evaluate the effect of Nos treatment on intratumoral uptake of fluorescent PEGylated liposomes in TNBC orthotopic xenografts tumor bearing mice.

2. Materials and methods

Noscapine, docetaxel and Coumarin-6 were purchased from Sigma Chemicals, St. Louis, MO, USA and AK Scientific Chemicals, CA, USA. The TNBC cell lines MDA-MB-231 and MDA-MB-468 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM:F12K medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum. The cell culture media contained antibiotic antimycotic solution of penicillin (5000 U/ml), streptomycin (0.1 mg/ml), and neomycin (0.2 mg/ml) purchased from life technologies, USA. The cells were maintained at 37 °C in the presence of 5% CO₂. All other chemicals were either reagent or tissue culture grade.

2.1. Analysis of cytotoxicity of DTX after Nos chemosensitization

To determine the chemo-sensitizing effect of Nos on DTX in TNBC cells, crystal violet cytotoxic assay was performed. The MDA-MB-231 and MDA-MB-468 TNBC cells were plated in 96-well micro titer plates, at a density of 1×10^4 cells/well and allowed to incubate overnight and were treated with various dilutions of Nos made in cell growth medium (10–160 μ M) from Nos stock solution in DMSO. To study the interaction between Nos and DTX, the treatment strategy included as cells treated with (i) control (ii) only Nos (4 μ M) for 24 h (iii) only DTX (0.4 μ M) (iv) Nos 4 μ M for 24 h followed by DTX 0.4 μ M for 48. In group (ii) and (iv), Nos was discarded after 24 h and replaced with fresh media and DTX, respectively. After 48 h of DTX exposure, cells were fixed with 0.5% v/v glutaraldehyde and

viability was assessed by crystal violet assay [26]. The absorbance was measured by a microtiter plate reader (Spectramax 190, Molecular devices, USA) at 540 nm.

2.2. Flow cytometry

MDA-MB-231 cells were seeded in 24-well plate at a density of 1×10^5 cells/well and incubated in 1 ml of complete growth medium for 24 h to allow cell adherence. Cells were treated with 4 μ M Nos. After 24 h, media and Nos were completely discarded and cells were washed with PBS. Cells were exposed to 0.4 μ M DTX in control and Nos pretreated cells. At different time points, the medium was removed and washed cells with PBS twice and incubated with annexinV/FITC. The intracellular fluorescence of apoptotic cells was determined using fluorescence-activated cells sorter (FACS) Calibur flow cytometer (BD, Franklin Lakes, NJ, USA).

2.3. Immunoblotting

The protein extraction, sample preparation, SDS page and transfer of proteins to nitrocellulose membrane was performed according to our previous methods [27]. Membranes were probed with antibodies against JNK, phospho-JNK, p38, phospho p38, bcl-2, α -tubulin, Akt, pAkt, survivin, and β -actin at the dilution of 1:1000. Rest of the procedures such as secondary antibody incubation, protein band visualization, densitometric analysis of bands was performed as per our previous methods using Chemi-Doc XRS+ Imaging system (Bio-Rad).

2.4. Immunofluorescence assay

MDA-MB-231 cells were plated and allowed to adhere for 24 h. Cells were exposed to Nos (4 μ M) for 24 h. DTX (0.4 μ M) treatment was given after replacing media and Nos with fresh media. After 48 h, cells were fixed in 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton X-100 in PBS according to previously published method [25]. Cells were further incubated in 2% BSA in PBS, followed by incubation with mouse α -tubulin antibody at 4 °C overnight and washed with PBST for 10 min. Coverslips were rinsed with PBST for 10 min and incubated with DAPI nuclear stain (SantaCruz, CA, USA). Finally, coverslips were mounted using mounting medium and immunofluorescence images were taken using the fluorescent microscope (Olympus Inc., USA).

2.5. In vitro migration assay

In vitro migration (scratch) assay was carried out in MDA-MB-231 cells as per the protocol described earlier with slight modification to improve precision of the result [28]. MDA-MB-231 cells (10,000 cells per well) were plated 96-well plate in complete media, and cultured overnight. A uniform scratch was made in the center of well with a cell-scraper. Cells were treated in a similar manner as described in apoptosis assay. After 48 h of DTX exposure, cells were fixed with 0.5% glutaraldehyde and stained with crystal violet for 15 min. The images of scratch area were taken using Olympus microscope to calculate the area using ImageJ software. Areas of gap (scratch) before and after treatment were analyzed for calculating the percent bridging of migration area.

2.6. Preparation of Coumarin-6 PEGylated liposomes

Coumarin-6 PEGylated liposomes (C6PL) were prepared using thin film hydration method. Coumarin-6 (1 mg), DPPC (40 mg), DSPE-PEG 2000 (5 mg) and cholesterol (10 mg) were dissolved in chloroform and evaporated under vacuum in round bottom flask. Resultant thin film was hydrated with 4 ml of HPLC grade water at 50 °C for 1 h. The particle sizes of the liposomes were reduced by ultrasonication (Branson Probe Sonicator, USA) for 2 min and then allowed to cool to room temperature. The hydrodynamic diameter and zeta potential of the C6PL was determined using Nicomp 380 ZLS (Particle Sizing Systems, Port Richey, FL) which measures particle size on the basis of dynamic light scattering and zeta potential on the basis of electrophoretic mobility.

2.7. Liposome uptake in TNBC solid tumor in mice

Orthotopic xenograft TNBC solid tumor was developed by injecting MDA-MB-231 cells (2×10^6 cells/animal) into mammary fat pad of female BALB/c mice. The protocol for *in vivo* experiments with nude mice was approved by the Animal Care and Use Committee, Florida A&M University, Tallahassee, FL. The mice were randomized into vehicle control and treatment groups (n = 8) when average tumor volume reached to $\sim 100 \text{ mm}^3$. The mice were treated with (i) Control, PBS only; (ii) Nos (100 mg/kg/day) every alternate day for 2 weeks. On 15th day, 4 animals from each group were sacrificed and tumor tissues were collected immediately. Tumor samples were sectioned, fixed in paraffin and used for picro-sirius red staining for collagen content. Paraffin fixed tumor sections were dewaxed, rehydrated and stained with Weigert's haematoxylin for 10 min before washing in running tap water. Sections were then stained with picro-sirius red solution (0.5 g sirius red in 500 ml saturated aqueous picric acid solution) for 1 h. Sections were washed in two changes of acidified water solution (5 ml acetic acid in 1 L water). Finally, sections were dehydrated in three changes of 100% ethanol, cleared in xylene and mounted in a resinous medium. Sections were viewed under a light microscope and imaged by an inverted microscope (Olympus Inc., USA).

For analyzing the qualitative uptake of C6 PEGylated liposomes in solid tumor, on 15th day, C6 PEGylated liposomes were intravenously injected in remaining 4 animals of each group. Animals were sacrificed 1 h after injection and tumors tissues were collected immediately. Tumor tissues were cryosectioned (40 μM) and analyzed using fluorescence microscope (Olympus Inc., USA).

2.8. Statistics

One-way ANOVA followed by Tukey's Multiple Comparison Test was performed to determine the significance of differences among groups using GraphPad PRISM version 3.0 software (San diego, CA, USA). Differences were considered significant in all experiments at $p < 0.05$ (significantly different from untreated controls).

3. Results

3.1. Effect of Noscapine pre-sensitization on DTX cytotoxicity and apoptosis against triple negative breast cancer cells

Effect of Nos pretreatment on cytotoxicity of DTX in MDA-MB-231 and MDA-MB-468 TNBC cell lines were evaluated using crystal violet assay (Fig. 1A). Nos showed negligible cell killing at 4 μ M ($p < 0.001$) and showed behavior similar to control group. However, cell killing of DTX was significantly higher in Nos pretreated cells compared to DTX alone treated cells (Fig. 1A). IC₅₀ of DTX was 3-fold lower in Nos pre-sensitized cells compared to non-sensitized MDA-MB-231 cells. Due to significant cytotoxic results found with MDA-MB-231 cells ($p < 0.01$) compared to MDA-MB-468 cells ($p < 0.05$), further studies were carried out using MDA-MB-231 cells. Nos was reported to induce apoptosis in various cancer cell lines at higher concentration. Based on encouraging cytotoxicity data, flow cytometry analysis was carried out to understand the effect of low dose Nos exposure in DTX induced apoptosis in MDA-MB-231 cells. The results of cytotoxicity studies were in complete agreement with flow cytometry data. As shown in Fig. 1B, a significant increase in the percentage of late apoptotic cells in Nos sensitized DTX treated cells (69.03%) compared to DTX alone (38.03%). There was 4.79-fold higher number of early apoptotic cell population was observed with DTX treatment in Nos sensitized cells. These findings provided us with strong evidence that Nos sensitization followed by DTX treatment is a promising therapeutic approach to kill TNBC cells.

3.2. Noscapine chemosensitization induces P38 and JNK activation

To elucidate the underlying mechanism of action of Nos chemo-sensitization and combination treatment, we have evaluated the expression of stress proteins using western blot. We observed that Nos chemo-sensitization induces stress signals initially and subsequent exposure to DTX results in higher levels of apoptosis in part by lowering pAkt, bcl-2 and survivin (Fig. 2). In addition, in the current study we observed that there was no significant change in expression level of early stress markers like P38 and JNK at different Nos concentrations. Interestingly, we found that pre-sensitization of breast cancer cells with Nos at different time intervals (6 h, 12 h and 24 h), the expression of stress transducer phospho p38 stress activated protein kinase was upregulated in a dose and time dependent manner (Fig. 2A). Phospho p38 expression was increased significantly ($p < 0.01$) in MDA-MB-231 cells at 12 h, 24 h, 4 μ M and 20 μ M Nos treatment (Fig. 2A and B). The phospho JNK, also known as stress-activated protein kinases, (group of mitogen-activated protein kinases) expression was also increased in Nos sensitized DTX group in time and dose dependent fashion and found to be increased significantly ($p < 0.05$) at 24 h, 20 μ M concentration (Fig. 2B). Our studies demonstrate for the first time that treatment of cells with Nos at low concentrations leads to phospho p38 and phospho JNK activation in breast cancer cells and promotes apoptotic activity of DTX *in vitro*. To confirm this, further, the expression of various molecules playing a role in cell survival and death was analyzed.

3.3. Noscapine chemosensitization augmented DTX induced apoptosis of breast cancer cells

Having identified the chemo-sensitizing effect of Nos in TNBC cell line, we next examined the levels of various apoptotic proteins as shown in (Fig. 2C and D). Consistent with the results from cytotoxic and apoptotic study, western blot analysis of anti-apoptotic protein bcl-2 was significantly ($p < 0.001$) down regulated in Nos pre-sensitized DTX treated cells when compared to control, Nos and DTX only treated cells. Further, the densitometric analysis of western blot bands revealed that β -actin relative bcl-2 expressions were found to be reduced by 0.7 and 1.6-fold, in DTX alone and DTX in Nos pretreated cells, respectively in comparison untreated control cells. The expression of Akt was not changed in any of the samples. Furthermore, Nos pre-sensitization attenuated phosphorylation of Akt significantly ($p < 0.01$) by 1.3 and 1.9-fold in Nos pre-sensitized DTX treated cells compared to Nos and control cells, respectively. We observed the relative expression of survivin decreased (0.5 and 1.0-fold) significantly ($p < 0.01$) in DTX alone and Nos pre-sensitized DTX treated cells, respectively. On the other hand, the relative expression of α -tubulin was found to be reduced 0.4 and 1.2-fold significantly ($p < 0.05$) in DTX alone and Nos pre-sensitized DTX treated cells, respectively compared to Nos and untreated control samples.

3.4. Effect of Noscapine and DTX on α -tubulin binding by immunofluorescence assay

In vitro studies of α -tubulin by other researchers suggest that Nos does not promote or inhibit the gross microtubule polymerization, but rather effects the steady-state dynamics of microtubule assembly in cancer cells [29]. Immunocytochemical analysis showed that immunofluorescence staining of α -tubulin of MDA-MB-231 cells treated with Nos and DTX (Fig. 3). When the microtubule cytoskeleton was visualized using immunofluorescence staining of α -tubulin, there were no gross morphological changes observed at low concentration of Nos (4 μ M), which was almost similar to untreated control cells. However, we found that Nos chemosensitization followed by DTX treatment showed significant decrease of immunofluorescence staining of free α -tubulin needed for microtubule formation, leads to further preventing cancer cell progeny.

3.5. Migration assay

The two dimensional migration gap/scratch closure assays is a simple method to study cell migration *in vitro* (Fig. 4). Results of migration assay further confirmed the enhancement of DTX activity in the Nos pretreated cells. As shown in Fig. 4A, control group showed $> 80\%$ bridging of scratch within 48 h, whereas DTX alone and Nos sensitization DTX treated group showed significant inhibition of proliferation and migration of cells within the scratched area. Percentage bridging of migration area in Nos (4 μ M) well was similar to control which means Nos did not showed any effect on migration of MDA-MB-231 cells. However, as anticipated, Nos pre-sensitization enhanced the antimigration effect of DTX. DTX alone at 0.4 μ M concentration showed 53.8% bridging of scratch area while Nos pretreated DTX group showed just 24.5% bridging of scratch area (Fig. 4B). The results clearly indicated that even at non-cytotoxic concentration, Nos promoted the DTX anticancer activity. Calculation of area of scratch or gap is more accurate than calculating

length of scratch because gap was found to be uneven and showed very high standard deviation.

3.6. Picrosirius red staining of TNBC xenograft tumors

Histological assessment of breast tissue sections were evaluated by stained with picro-sirius red staining and hematoxylin & eosin (H&E) (Fig. 5). Picro-sirius red stain was used to measure collagen content in tissue specimens to assess the degree of fibrosis in tissue samples and stained tumor sections are shown in Fig. 5A. In MDA-MB-231 cell derived orthotopic TNBC tumor model, multiple oral doses of Nos resulted in significant reduction in tumor collagen-I level. Bright red collagen deposits were observed in control as compared to Nos treated group in which the red colored fibers were considerably decreased, indicating significant reduction in tumor collagen-I levels. In addition, breast tissue sections stained with H&E also show attenuation of fibrosis was observed in Nos treated group compared to control (Fig. 5B).

3.7. Intratumor uptake of C6PL in TNBC xenograft tumors

As shown in Fig. 6A, intensity of green fluorescence in control breast cancer tissue is nearly negligible but it was significantly higher Nos treated tumor tissue (Fig. 6A). Fluorescent intensity is proportional to C6 concentration in tumor. Therefore, this study clearly indicated the higher uptake of C6PL in Nos treated animals compared to untreated control. Quantitative estimation of intensity revealed that tumoral uptake of C6PL was 7-fold higher in Nos treated tumors compared to control which has confirmed the tumor stromal disrupting effect of Nos (Fig. 6B).

4. Discussion

Triple negative breast cancer (TNBC) has more aggressive disease progression with limited treatment options due to the lack of standard chemotherapy [30,31]. DTX has shown significant anticancer activity against TNBC [2,32], however its clinical utility has been limited due to dose dependent toxicities and associated adverse side effects. Besides toxicity issue, the development of drug resistance has led to a search of new drugs or combination regimens which can enhance therapeutic efficacies with minimal toxicity. Nos has been studied extensively in cancer research due to its effect on microtubule dynamics and its antitumor activity has been demonstrated in various *in vivo* studies [14,18,20]. In the present study, we demonstrated for the first time that chemo-sensitizing and tumor stromal disrupting effect of low dose Nos in augmenting DTX cytotoxicity *in vitro* and compromising barriers for nanoparticle uptake *in vivo*.

Essentially chemosensitization would increase drug efficacy at lower-dose levels thus, reducing high dosage toxicity and drug resistance. Vital goal is to increase efficacy and tumoral uptake of nanocarrier while reducing side effects and toxicity; we sought to investigate drug combinations of DTX with agents that have superior anticancer effects. To our knowledge, this is the first study demonstrating Nos as a chemo-sensitizer in combination with DTX against TNBC. In this study, we have shown that pre-sensitization of TNBC cells with Nos increase cytotoxicity and anti-migration effect of DTX significantly (p

< 0.01). We determined that when MDA-MB-231 and MDA-MB-468 TNBC cells were exposed to low non-cytotoxic dose of Nos (4 μ M), the cytotoxicity of DTX was increased by 3.0 and 2.3-fold respectively. Our results indicated significant cell killing of MDA-MB-231 cells ($p < 0.01$) compared to MDA-MB-468 cells ($p < 0.05$), hence further studies were carried out using MDA-MB-231 cells. It is our hypothesis that MDA-MB-231 cells are more prone to taxols because of their lack of DNA repairing capability compared to MDA-MB-468 cells. This observation was supported by a study demonstrating synergistic interaction of DTX (0.001–0.1 μ M) and Nos analog EM011 (0.1– 10 μ M) on prostate cancer cells [33]. These workers also suggested that both the drugs bind at different sites of tubulin by *in silico* model techniques. Novelty of our study was that at very low subtherapeutic doses, Nos acts as a chemo-sensitizer of breast cancer cells to enhance DTX cytotoxic activity.

Although, both drugs share a common cellular target (microtubules), they may occupy different regions of the target protein and pre-sensitization with Nos seems to trigger changes at cellular level, suggesting the therapeutic advantage for decreasing the dose of DTX. Our results highlight the dire need to explore the underlying mechanisms of increasing DTX anticancer effect after Nos chemo-sensitization. Several studies have suggested that Nos induces multiple proapoptotic responses that induce apoptosis against variety of cancer cells [9,14,15,18]. Previously, our lab also reported the synergistic activity between Nos with cisplatin and gemcitabine in A549 and H460 lung cancer cells [9,19]. The role of Nos as a sensitizer has also been shown by Sung et al. (2010) who demonstrated that Nos (50 μ M) significantly potentiated the cytotoxic and apoptotic effects of TNF, thalidomide, paclitaxel, and bortezomib in human leukemia KBM-5 cell lines by activating NF- κ B [20]. A synergistic interaction between bromo-Nos and paclitaxel was also observed due to different binding sites on microtubules for these actions in prostate cancer cells [34,35]. We have observed that the after Nos chemo-sensitization, DTX treated breast cancer cells downregulated the expression of bcl-2 and survivin most effectively which was in agreement with the previous findings in KBM-5 leukemic and U266 multiple myeloma cells [20].

It was well known that a number of constitutively activated signaling pathways play critical roles in proliferation and survival of cancer cells [36]. Significant downregulation of phosphorylated Akt and survivin levels in Nos sensitized DTX treated cells further supported the apoptotic effects of these drugs. Interestingly, at this dose level of Nos (4 μ M) alone, we did not find any significant changes in the expression of bcl-2, survivin, pAkt and α -tubulin. We observed that early stress markers like P38 and JNK are activated at lower Nos concentrations in a time dependent fashion but this level was not sufficient to induce apoptosis. Nos activates the JNK pathway at a very low dose (20 μ M), which might not be sufficient to induce cell death. Our findings are in agreement with the Zhou et al. (2002) reported that apoptosis induced by taxanes like DTX and paclitaxel was a JNK-dependent but AP-1-independent process [16]. Nos pretreatment of ovarian cancer cells activated JNK and increased p53 levels, this was associated with increased sensitivity of these cells to apoptotic stimuli [16]. Shi et al. (2006) further demonstrated that enhancement of the JNK pathway down-regulates P-glycoprotein expression and reverses P-glycoprotein efflux mediated multidrug resistance in cancer cells. Down regulation of P-glycoprotein level led to

significant enhancement in intracellular drug accumulation and cytotoxicity in multidrug resistant cancer cells [37]. Thus, the role of Noscapine to overcome the resistance in MDA-MB-231 cells has a strong co-relation with JNK levels and is being explored further in resistant tumor models in our laboratory.

In the current study, we have evidently showed that Nos treatment lead to the activation of early stress markers such as phospho p38 and Phospho JNK (family of MAP kinases) in a time and dose dependent manner, thus may sensitize TNBC cells to DTX to induce apoptosis as shown in graphical abstract. Hence, we construe from this study that lower concentrations of Nos act as a chemo-sensitizer and treatment with DTX may produce superior anticancer effect that warrants further investigation for its potential clinical applications.

Effect of oral Nos treatment on tumor collagen-I level and nanoparticle uptake was investigated on TNBC solid tumor bearing nude mice. Picro-sirius red stain was used to measure collagen content in tissue specimens to assess the degree of fibrosis in tissue samples. In this study, we have shown tumor anti-fibrotic effect of by its collagen-I reducing effect in TNBC solid tumor. Recently, the antifibrotic activity of Nos in pulmonary fibrosis has been demonstrated and described the mechanism of Nos action though a rapid activation of cAMP/protein kinase A via EP2 prostaglandin receptors [25]. However, the investigators have used 100 mg/kg dose of Nos via intraperitoneal route, which was a significantly higher dose compared to 100 mg/kg dose administered by oral route used in our study. It has been demonstrated that Nos in dose ranges of 150–500 mg/kg have been used for melanoma, breast, ovarian, lung and pancreatic cancer treatment *in vivo* [18,38–40]. Furthermore combinations of Nos (300 mg/kg) with cisplatin and gemcitabine have been studied in lung and breast cancer [9,18].

Anti-tumor fibrosis effect of Nos has a potential clinical relevance for anticancer nano-medicinal products. Increase in collagen-1 results in highly fibrous tissue network in tumor which restricts the deeper permeation of anticancer drug. Dense collagen network of extracellular matrix (ECM) restrict the diffusion and availability of drug to deeper tumor layers [41]. The same is responsible for high tumor interstitial fluid pressure which may obstruct adequate penetration of macromolecules and nanocarriers in tumor tissue [42–44]. Higher uptake of coumarin-6 PEGylated liposomes can be correlated with the disruption of collagen network and tumor stromal barriers. Interestingly, at these dose levels of Nos, there was no impact on tumor progression or growth. Moreover, oral route of administration was preferred over intravenous route due to high patient compliance [45]. Low dose oral treatment of Nos has successfully enhanced uptake of liposome suggested that it may have the potential to amplify efficacy of clinically approved nanocarriers e.g. Abraxane®, Doxil® etc. Treatment with chemo-sensitizing plus tumor stroma disrupting agent to enhance the cytotoxicity and preferential distribution of drug loaded nanocarrier to deeper tumor tissue could be a very promising strategy to leverage the maximum clinical outcome with minimal dose of drug.

5. Conclusions

Low dose noscapine exposure sensitizes the TNBC cells to docetaxel which results in enhanced cytotoxicity of DTX at similar concentration. Tumor anti-fibrotic effect of low dose oral noscapine could be of great importance for clinical applications of anticancer nanomedicines. Even though, Nos cannot be used as a standalone agent in TNBC treatment; its chemo-sensitizing effect can be critically important for enhancing the tumor specific toxicity of DTX and will help in reducing the dose of DTX and its dose dependent side effects.

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Fig. 1.

Noscapine chemosensitization increases cytotoxicity of MDA-MB-231 and MDA-MB-468 cells followed by docetaxel treatment. (A) Breast cancer cells were chemosensitized with noscapine for 24 h followed by DTX treatment for 48 h and percentage cell killing was measured by the crystal violet assay. (B) Representative annexin V-FITC staining profiles of MDA-MB-231 cells treated with noscapine, docetaxel and noscapine pre-sensitization followed by docetaxel treatment. The quadrants demarcate annexin V-FITC negative and positive staining populations analyzed by flow cytometry (i) Control, (ii) Noscapine, (iii) Docetaxel, (iv) Noscapine+ Docetaxel. Each value represents the average of the independent experiments with triplicate determinations. One-way ANOVA followed by post Tukey test was used for statistical analysis (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, statistically significant). Data presented are means $\pm$ SD ($n = 3$).

Fig. 2.

Noscapine increases the expression of phospho p38 and phospho JNK levels in a dose- and time-dependent manner and decreases anti-apoptotic and survival proteins in MDA-MB-231 cells. (A) Western blot analysis of p38, phospho p38, JNK, phospho JNK and β -actin levels in cells treated with different dose- and time-dependent noscapine concentrations of representative images. (B) The intensity of indicated proteins was quantified by densitometric analysis of the western blot bands and β -actin was used as a housekeeping protein. (C) Representative images of bcl-2, α -tubulin, Akt, pAkt and survivin expression in TNBC tumor lysates of equal amounts, and (D) The intensity of indicated proteins were quantified by densitometric analysis. Data are calculated from triplicate experiments and presented as mean, and error bars refer to SD, * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$ compared with control.

Fig. 3.

Noscapine has no effect on gross microtubule polymerization state but noscapine pre-sensitization followed by docetaxel treatment stabilizes microtubules, hence inhibits cell proliferation. TNBC cells were treated with noscapine (4 μM) or docetaxel (0.4 μM) for 24 h, followed by immunostaining with α -tubulin antibody (green) and counter staining for nuclei with DAPI (blue). Shown are the representative images at 400 $\times$ magnification (micron bar = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Noscapine pre-sensitization followed by docetaxel treatment inhibits migration of MDA-MB-231 cells. (A) Representative images of cells which were treated with Noscapine (4 μ M) and docetaxel (0.4 μ M) in 96-well plate for 48 h by scratch assay after crystal violet staining ($n = 6$), and (B) Quantitative analysis of inhibition of bridging migration area. ANOVA–Bonferroni test was used to calculate the p values and $p < 0.05$ considered as significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Pre-sensitization of animals with noscapine inhibits fibrosis in TNBC xenograft breast tumors. (A) Representative images of picro-sirius red staining of breast tumor sections showing reduced bright red collagen fibers in noscapine treated group, and (B) Hematoxylin and Eosin stained sections of triple negative breast cancer xenograft tissues. Each data point was represented as mean $\pm$ sem (n = 3). Original magnification 400 $\times$ (micron bar = 200 μ m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Noscapine pre-sensitization increases intratumoral distribution of Coumarin-6 liposomes. (A) Representative images of noscapine pre-treatment of xenograft triple negative breast cancer tissues at 100 mg/kg. (B) Noscapine pre-treatment increased the coumarin-6 liposomes fluorescence by 7-fold. ANOVA–Bonferroni test was used to calculate the p values and $p < 0.05$ considered as significant. Original magnification 400 $\times$ (micron bar = 200 μ m).