Andro inhibits breast tumor growth by down regulating OPN expression and PI3-kinase signaling

Natural science does not simply describe and explain nature; it is part of the interplay between nature and ourselves; it describes nature as exposed to our method of questioning.

Werner Heisenberg
RESULTS AND DISCUSSION

6. Andro inhibits breast tumor growth by down regulating OPN expression and PI3-kinase signaling

6.1 Introduction

Cancer of the breast is a major public health problem which accounts 0.45 million deaths annually across the globe (33). In spite of more awareness and early detection in the developed countries, the incidence of breast cancer is rapidly growing in India and around the world. The conventional therapeutic and surgical procedures have been shown to be insufficient in the management of breast cancer. Therefore, there is an urgent need of hunt for novel compounds/agents that can be effective and selective in the prevention and treatment of cancer. One such potential candidate is Andrographolide (Andro), a bicyclic diterpenoid lactone, isolated from medicinal plant called Andrographis paniculata. Previous studies have focused on role of Andro on its anti-inflammatory effects and regulation of tumor cell proliferation, migration and invasion. However, the effects of Andro on tumor growth and angiogenesis remain to be elucidated. A recent study has shown that Andro is capable of activating p53 function via ROS-dependent JNK activation, leading to DR4 up-regulation and sensitization to TRAIL-induced apoptosis in TRAIL-resistant cancer cells (216). Previously, it has been reported that Andro inhibits NF-κB activation in vivo in a dose-dependent manner by binding to the reduced cysteine 62 of p50, thereby inhibiting NF-κB DNA binding (217, 218). Moreover it also suppresses viral replication by inhibiting virus-infected cell proliferation and prevents binding of the virus to T cells by inhibiting proprotein convertases, which cleave the HIV envelope glycoprotein gp160 (137). OPN has shown to be upregulated in breast cancer and its overexpression is correlated with enhancer angiogenesis, tumor growth and metastasis (42, 43, and 79). Therefore, targeting OPN and/or its downstream molecules is likely to have therapeutic benefit (69, 175). In this study, the effects of Andro on OPN expression, PI3 kinase/Akt activation, the mechanism underlying this process and its role in vivo tumorigenicity have been investigated.

In this study, using multiple in vitro and in vivo approaches, we have purified and characterized Andro from Andrographis paniculata and demonstrated the inhibitory effect of Andro in breast cancer cell proliferation and motility. We further demonstrated that Andro
inhibits cell cycle at G2-M phase and induces ROS mediated apoptosis in MDA-MB-231 cells. Our experimental findings suggest that Andro inhibits HUVEC motility, \textit{in vitro} angiogenesis and tumor-endothelial cell interaction. Andro attenuates the expression of pro-angiogenic molecules such as OPN, Cox-2 and VEGF through downregulation of PI3 kinase/Akt pathway in breast cancer cells. Moreover, Andro suppresses both \textit{in vitro} and \textit{in vivo} tumorigenicity in orthotopic breast cancer model.

6.2 Results

6.2.1 Purification and characterization of Andro

Andro was purified from the DCM precipitates of organic extracts of \textit{Andrographis paniculata} as described in material and method. The crystalline compound purified was chemically characterized by HPLC, $^1$H and $^{13}$C NMR, crystallography and identified as Andro (Fig. 11, A-D). The spectral data suggest the purity of the compound greater than 99.90%.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{Extraction, purification and characterization of Andro from \textit{Andrographispaniculata}. (A) HPLC plot showing single peak confirms the purity of Andro. (B) LC-MS UV chromatograph of Andro. (C) LC-MS mass spectra of Andro. (D) The chemical structure of Andro derived on the basis of crystallography and NMR data.}
\end{figure}
6.2.2 Andro inhibits breast cancer cell proliferation

Previous studies have shown that Andro acts as anti-proliferative agent in variety of cell lines (127). Here, we have studied the effect of Andro on breast cancer cell viability. Breast cancer (MCF-7 and MDA-MB-231) cells were incubated in presence of 0-500 µM of Andro for 24 h and viability was determined using MTT assay. The effect of Andro on growth of breast cancer cells is expressed as percentage of viable cells as compared to control. As low as 10µM of Andro displayed significant anti-proliferative effects in both MDA-MB-231 (Fig. 12A) and MCF-7 (Fig. 12B) cells. To further study the effect of Andro on normal cells, NIH-3T3 cells were treated with Andro (0-500 µM) and MTT assay was performed. The result showed that Andro is relatively less toxic to normal cells than cancerous cell lines (Fig. 12C).

**Figure 12 Andro reduces cell viability and arrests cell cycle progression in breast cancer cells.** (A) MDA-MB-231, (B) MCF-7 and (C) NIH-3T3 cells were treated with Andro (0-500 µM) for 24 h and MTT assay were performed. The results are the mean ± SE of the measurement of three assays and analyzed statistically by ANOVA. (*, P< 0.001 vs. control). The results showed are the representative of three different experiments. (D) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h, stained with propidium iodide and analyzed the cell cycle arrest by flowcytometer.
6.2.3 Andro induces G2/M phase arrest and apoptosis in a dose dependent manner

The anti-proliferative effect of Andro suggested that growth inhibition of cells may be the result of cell cycle inhibition or induction of apoptosis. Previous reports suggested that Andro inhibits cell cycle progression at different stages depending upon the cell types (214, 219). To investigate the effects of Andro on cell cycle arrest, MDA-MB-231 cells were treated with Andro for 24 h, stained with propidium iodide and analyzed by flowcytometer. The results demonstrated that MDA-MB-231 cells exposed with Andro (0-150µM) for 24 h showed significant increase in percentage of G2-M phase of cell cycle in a dose dependent manner (Fig. 12D). Percentage of cells in the G0-G1 and G2-M phases were 79% and 11% respectively in control. However, when treated with Andro (150 µM) for 24 h, percentage of cells in G2-M phase was noticeably enhanced (22%) whereas percentage of cells decreased to 60% in G0-G1 phases.

**Figure 13** Andro induces apoptosis in breast cancer cells through caspase independent pathway. (A) MDA-MB-231 cells were treated with Andro (0-150 µM) for 36 h, stained with annexin V-FITC and propidium iodide and analyzed by flowcytometer. (B) MDA-MB-231 cells were treated with Andro (0-150 µM) for 36 h and PARP cleavage was analyzed by Western blot. Actin was used as loading control. (C) And (D) MDA-MB-231 cells were treated with Andro (0-150 µM) and levels of apoptosis related proteins were detected by Western blot. Actin was used as loading control.
To further determine whether cell cycle inhibition is associated with apoptosis, annexin V-FITC staining were performed. MDA-MB-231 cells were treated with Andro (0-150 µM) for 36 h, stained with annexin V-FITC and analyzed by flowcytometry. The data showed that Andro induces apoptosis in MDA-MB-231 cells in a dose dependent manner (Fig. 13A). PARP cleavage is one of the hallmarks associated with apoptotic cells (220, 221). Accordingly, MDA-MB-231 cells were treated with Andro (0-150 µM) and PARP cleavage was analyzed by Western blot. The results showed that PARP cleavage is enhanced with increasing doses of Andro (Fig. 13B). These results indicated that Andro induced prolonged G2-M phase arrest resulting in the induction of apoptosis in MDA-MB-231 cell.

6.2.4 Andro induces cellular apoptosis through caspase independent pathway

Cell death can be either necrotic or apoptotic in nature. According to the cellular morphology different stages of programmed cell death can be defined (222). Necrosis is characterized by swelling of the cell, early disruption of plasma membrane and lack of chromatin condensation, whereas apoptosis is characterized by blebbing of plasma membrane, rounding, shrinkage and detachment of cells from substratum and loose chromatin condensation before plasma membrane disrupts (222). Caspases play important role in majority of apoptotic events. To investigate the role of Andro in caspases, Bcl2, Bim, Bad and p-p27 dependent cell death, cells were treated with Andro (0-150 µM) and Western blot analysis were performed. The results showed that the expression of apoptosis related molecules (Bcl2, Bim, Bad, caspase3, caspase9 and p-p27) remained unchanged upon Andro treatment (Fig. 13C and 13D). To further investigate whether this apoptotic event is caspase dependent, MDA-MB-231 cells were treated with Andro (150 µM) along with either Pan-caspase inhibitor (Z-VAD, 0-100 µM) or caspase-3 inhibitor (Z-DEVD, 0-100 µM) and MTT assay was performed. The data showed that caspase inhibitors failed to confer any protection against Andro-induced cell death in MDA-MB-231 cells suggesting that apoptosis induced by Andro is mediated by caspase independent pathway (Fig. 14B). These data indicated that caspases are not involved in cell death induced by Andro in MDA-MB-231 cells. ROS have been shown to play an important role in cellular signaling which modulates cell proliferation and death; it is further possible that Andro may impact these apoptotic pathways via alteration of ROS levels. Therefore to analyze the level of total ROS in MDA-MB-231 cells,
cells were treated with Andro (0-150 µM), stained with DHE (dihydroethidium) and superoxide levels were measured by flowcytometer.

**Figure 14** Andro induced apoptosis is caspase independent and ROS mediated in breast cancer cells. (A) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h, stained with FITC conjugated phalloidin and analyzed by confocal microscopy. The data showed disorganization of actin filaments as well cell and nuclear morphology. Micron bar: 10 µm. The results showed are the representative of three different experiments. (B) MDA-MB-231 cells were used either alone or treated with Andro (150 µM) along with either pan caspase inhibitor, Z-VAD (0-100 µM) or caspase 3 inhibitor, Z-DEVD (0-100 µM) for 36 h and MTT assay was performed. The results are the mean ± SE of three assays, analyzed statistically by ANOVA and represented in the form of graph. (#, P> 0.05 vs. only Andro treated). All the results showed are the representative of at least three independent experiments. (C & D) MDA-MB-231 cells were treated with Andro (0-150 µM) for 36 h and stained with DHE to detect total ROS in the cells by flow cytometry and by confocal microscopy. Red fluorescence indicating the presence of superoxide was significantly increased in cells exposed with Andro as compared to control (Fig. 14C & D). The results showed the
significant increase of ROS levels upon Andro treatment highlighting the possible cause for apoptosis.

6.2.5 Breast cancer cells motility is inhibited by Andro

Considering the cytotoxic effects of Andro at higher doses, we sought to establish experimental conditions under which motility rather than viability was affected. For this, wound assay was performed using confluent monolayer of MDA-MB-231 cells with typical cobblestone morphology. Wounds with a constant diameter were made, and cells were treated with Andro (0-30 µM) for 12 h, the concentration that does not have much effect on cell viability. The wound photographs were taken and analyzed with image Pro Plus software. The data demonstrated that Andro inhibits MDA-MB-231 cell motility in a dose dependent manner (Fig. 15A & B). To further confirm the results of wound healing, Boyden chamber migration assays were performed, in which MDA-MB-231 cells were seeded on the upper chamber treated with Andro under similar condition as described in wound assay. After 12 h of incubation, cells were fixed, stained with Giemsa and images were captured and counted at three different high power field (C/HPF) under inverted microscope (Fig. 15C). Cells migrated to the lower side of membrane was analyzed statistically. The results showed that Andro inhibits MDA-MB-231 cell migration in a dose dependent manner (Fig. 15D). Thus the data further confirmed the inhibitory activity of Andro on MDA-MB-231 cell motility. Cell migration is mediated by a protrusions commonly called as lamellipodia and filopodia depending upon the types of actin polymerization. Actin polymerization is often branched in lamellipodia whereas in filopodia it often formed parallel bundle. These two forms of protrusion serve different roles: filopodia acts as mechanosensory, exploratory devices, whereas lamellipodia provide wide surfaces that help in forward movement. Recently it has been shown that actin serves as a structural element during directional movement (223). Therefore to further study the effect on actin reorganization, MDA-MB-231 cells were treated with Andro, stained with FITC conjugated phalloidin and analyzed with confocal microscopy. The data showed that Andro destabilized the actin filaments and reduces the cellular polarity in MBA-MB-231 cells in a dose dependent manner (Fig. 14A).
6.2.6 Andro blocks endothelial cell motility, tumor-endothelial interactions and *in vitro* angiogenesis.

Tumor angiogenesis is a multistep process in which branching of blood vessels and its migrations towards the distant tumors occur by breaking the basement membrane. Each phenomenon such as basement membrane disruption, cell migration, cell proliferation, and tube formation can be target for intervention to inhibit tumor growth (224). In the present study, the effect of Andro on HUVEC wound migration was studied. The wound migration
data revealed that Andro significantly inhibits HUVEC migration in a dose dependent manner (Fig. 16A & B).

The chemotactic model of co-migration and co-invasion of HUVEC using Boyden chamber is correlated with a typical tumor-endothelial interaction study (18). To study this interaction, HUVEC were seeded on transwell coated with or without matrigel for migration and invasion assays. Conditioned media collected from Andro treated MDA-MB-231 cells were used as chemotactic agent in the lower chamber. The data showed that Andro significantly inhibits endothelial cell migration and invasion towards the lower side of

Figure 16 Andro inhibits endothelial cell motility and attenuates in vitro angiogenesis. (A) HUVEC monolayer were wounded and treated with Andro (0-30 µM) and incubated for 12 h. Wound closure was photographed at 10x magnification. (B) Graphical representation of wound closure of HUVEC when treated with Andro (0-30 µM) for 12 h, mean ± SE. *, P<0.001 (C) HUVEC cells were seeded on matrigel coated plate and treated with Andro (0-30 µM). Photographs were captured for 8 h at 10x magnification. (D) Tubes formed were counted, analyzed statistically and represented graphically. Mean ± SE. *, P<0.001
Boyden chamber suggesting the inhibitory effect of Andro on tumor-endothelial cell interaction (Fig. 17A & B).

Endothelial cells proliferate into surrounding matrix to provide the necessary number of cells for making a new vessel. Subsequent to this proliferation, the new outgrowths of endothelial cells need to reorganize into a three-dimensionally tubular structure. In vitro tube formation is a reliable method for quick assessment of angiogenesis by measurement of the ability of endothelial cells to form tubular structures (225). In the present study, HUVEC were seeded into matrigel coated plates and treated with Andro (0-30 µM). After 12 h, tubes formed were photographed, quantified and analyzed statistically. The data showed that

Figure 17 Andro attenuates breast tumor-endothelial interaction in in vitro conditions. (A) HUVEC (1 x 10⁴ cells) were plated on transwell with or without precoated matrigel, conditioned media collected from control or Andro (0-30 µM) treated MDA-MB-231 cells and used in the lower chamber as chemoattractant. Migrated or invaded endothelial cells were stained with Giemsa and photographed (B) Invaded or migrated cells were quantified, analyzed statistically and represented in the form of bar graph. The error bar mean ± SE. *, P<0.001 vs. control, **, P=0.011 vs. control. The data represents three experiments exhibiting similar results.
Andro significantly inhibits the endothelial cells alignment into tubes in a dose of as low as 10 µM (Fig. 16C & D).

6.2.7 Andro inhibits OPN expression

OPN has been detected both in serum and tumors of a majority of human cancers, including breast, lung, prostate, gastric, oesophageal, ovarian and glioma (42, 43 and 69). Moreover, it has been shown that OPN has an anti-apoptotic effect in breast cancer and it is established that suppression of OPN expression or inhibition of downstream signaling pathways of OPN could be an effective strategy to inhibit tumor growth (77). Therefore we sought to examine the effect of Andro on OPN expression in MDA-MB-231 cells. Cells were treated with Andro (0-150 µM) for 24 h and levels of OPN were analyzed by RT-PCR and Western blot analysis. Interestingly, we found that Andro downregulates OPN expression in a dose dependent fashion both at RNA and protein level (Fig. 18A and B). The data suggested that Andro inhibits two isoforms of OPN (OPN-a and OPN-c) expression at RNA level. To further investigate whether Andro specifically regulates OPN expression at transcription level, reporter assay was employed to determine Andro’s attenuation of OPN expression. MCF-7 cells were cotransfected with renilla and OPN promoter reporter construct. The ratio was obtained by normalizing firefly with renilla and fold change with respect to control was calculated. The results demonstrated that Andro decreases OPN promoter activity, and the decrease in activity is statistically significant in a dose of 100 and 150 µM Andro which accounts to 48%±18% and 25%±20% inhibition respectively as compared to control (P<0.05). This result suggests that Andro inhibits OPN expression at transcription level by regulating its promoter.

6.2.8 Andro downregulates PI3-kinase/Akt pathway

Several studies have demonstrated that many aggressive tumors have up-regulated PI3-kinase/Akt and MAPK pathway and their inhibition could be potential approach for treatment of cancer (226). Recently, Lee et al. have shown that Andro inhibits PI3-kinase/Akt signaling in A549 cells (227). It is of great interest to know whether Andro led to inhibition of PI3-kinase/Akt signaling in breast cancer. Accordingly, MDA-MB-231 cells were incubated with Andro (0-150 µM) for 24 h and lysates were analyzed by Western blot to check the level of Akt activation. The data demonstrated that Andro suppresses Akt activation in a dose dependent manner (Fig. 18D).
Figure 18 Andro suppresses OPN expression and downregulates PI3-kinase/Akt signaling by inhibiting p85α expression. (A & B) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h and levels of OPN were checked at RNA (A) and at protein (B) level. (C) MCF-7 cells were cotransfected with OPN full length promoter reporter construct and renilla reporter construct (pRL) and then treated with Andro (0-150 µM) for 24 h. Cell lysates prepared were used to measure luciferase activity. The values were normalized to Renilla luciferase activity and fold changes were calculated. Columns, mean of triplicate determinations; bars, SE. *, P=0.05 vs. control and **, P<0.001 vs. control. All the experiments were repeated thrice. (D) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h and expression of signaling molecules (p-Akt, Akt, p85α and p110α) were detected by Western blot. Actin was used as loading control.

To further investigate whether Andro has any affect on components of PI3-kinase, Western blot analysis were performed to examine the levels of P85α (regulatory subunit) and P110α (catalytic subunit). Interestingly, P110α was unaffected whereas P85α was downregulated in a dose dependent fashion (Fig. 18D). Thus growth inhibition by Andro may be attributed by downregulation of PI3-kinase/Akt signaling pathway. Taken together, our results suggest that growth inhibition by Andro is associated with the down regulation of
P85α subunit of PI3 kinase and thereby suppresses the activation of Akt in breast cancer cells.

6.2.9 Andro downregulates NF-κB and AP-1 activation in breast cancer cells

Both NF-κB and AP-1 are reported to be up-regulated in various cancers (175). Particularly, in breast cancer, activated NF-κB and AP-1 regulate varieties of tumor promoting and angiogenic genes. Previously it has been shown that Andro inhibits NF-κB activation and attenuates neointimal hyperplasia in arterial restenosis (228). Thus to study the effect of Andro on expression and activation of these transcription factors, MDA-MB-231 cells were treated with Andro and the levels of c-Fos, c-Jun and phospho p65 were studied by immunofluorescence. The results showed that Andro inhibits the expression and nuclear localization of c-Jun and p-p65 but not c-Fos in a dose dependent manner (Fig. 19A). This was further confirmed by Western blot analysis (Fig. 19B). To further study whether Andro could inhibit NF-κB and/or AP-1-DNA binding, electrophoretic mobility shift assay (EMSA) was performed. The nuclear extracts of Andro treated cells were incubated with labeled oligonucleotides containing NF-κB or AP-1 consensus sequences and resolved on native polyacrylamide gel electrophoresis. Our EMSA results showed that Andro inhibits NF-κB or AP-1-DNA binding suggesting that Andro essentially regulates the activation of NF-κB or AP-1 in MDA-MB-231 cells (Fig. 19C & D).

To study further the cause-and-effect relationship between OPN expression and AP-1 activation, MDA-MB-231 cells were treated with either wortmannin, SN50 or Andro and Western blot analysis were performed. In separate experiments, MDA-MB-231 cells were transfected with either OPN siRNA or dn c-Jun and Western blot analysis was done. The result revealed that inhibiting PI3-kinase (Wortmannin) or blocking NF-κB (SN50) has no effect on OPN expression, but when transfected with dn-c-Jun, OPN expression was decreased (Fig. 20A). Silencing OPN down regulates p-Akt indicating that OPN regulates Akt phosphorylation. This study demonstrated that Andro suppresses OPN expression through down regulation of c-Jun expression in MDA-MB-231 cells.

6.2.10 Andro regulates NF-κB and AP-1-regulated Cox-2, cyclin D1, VEGF and Flk1 expression

At the molecular level, binding of VEGF to its receptor, Flk1 induces receptor dimerization, activation of intrinsic receptor kinase activity, and tyrosine
autophosphorylation that associated with its migration (229). In the present study, we have analyzed the effect of Andro on VEGF and Flk1 expression. MDA-MB-231 cells were treated with Andro (0-150 µM) and analyzed by Western blot. Interestingly, the result showed that VEGF and Flk1 expressions are downregulated in MDA-MB-231 cells exposed with Andro (Fig. 20B). Previous reports have suggested that NF-κB and AP-1 regulate Cox-2 and cyclin D1 expression (113, 230). Therefore to investigate whether Andro inhibits NF-κB and AP-1 dependent Cox-2 and cyclin D1 expression, MDA-MB-231 cells were treated with

**Figure 19** Andro suppresses NF-κB and AP-1 activation in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with Andro and the expressions as well as localization of c-Jun, c-Fos and p-p65 were detected by confocal microscopy. c-Jun and c-Fos were stained with Cy2 (green) conjugated IgG and p-p65 was stained with Cy3 (red) conjugated IgG whereas nuclei were stained with DAPI (blue). Micron bar, 10 µm. (B). MDA-MB-231 cells were treated with Andro (0-150 µM) for 2 h and levels of p-p65, p65, c-Jun and c-Fos were analyzed by Western blot analysis. Actin was used as loading control. (C & D) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h, nuclear extracts was prepared; NF-κB and AP-1-DNA binding were analyzed by EMSA.
Andro and the levels of cyclin D1 and Cox-2 were examined by Western blot. The results revealed that Andro inhibits the expression of these proteins in a dose dependent manner (Fig. 20C). Endothelial cell migration can be regulated by chemotactic factors, such as vascular endothelial growth factor (VEGF). To further study the role OPN and VEGF in Andro inhibited tumor-endothelial interaction, conditioned media collected from MDA-MB-

![Figure 20 Andro inhibited OPN expression is associated with down regulation of c-Jun expression in MDA-MB-231 cells. (A) MDA-MB-231 cells were either transfected with OPN siRNA or dn-c-Jun for 48 h. In separate experiments, MDA-MB-231 cells were either treated with Andro, wortmannin or SN 50 for 24 h and the levels of OPN, pAkt, p-p65, c-Jun and c-Fos were analyzed by Western blot. Actin was used as internal control. (B & C) MDA-MB-231 cells were treated with Andro (0-150 µM) for 24 h, protein lysates were prepared and levels of VEGF, Flk-1, COX-2 and cyclin-D1 were detected by Western blot analysis. Actin was used as loading control. (D) MDA-MB-231 cells were seeded onto matrigel coated plate and treated with Andro for 24 h, media was replaced in every alternate day. Photographs of the colony formed were taken on the 10th day of experiment. All panels are representative of three independent experiments.](image-url)
231 cells were either treated with Andro or OPN blocking antibody or VEGF blocking antibody or in combination of OPN and VEGF antibodies in absence or presence of Andro and then comigration and coinvasion experiments were performed. The results demonstrated that blocking OPN or VEGF inhibits the migration and invasion of endothelial cells (Fig. 21A-C). This suggests that Andro inhibits tumor-endothelial interaction by down-regulating OPN and VEGF expression.

**Figure 21** Andro inhibited breast tumor-endothelial interaction is mediated through OPN and VEGF expressions. HUVEC (1 x 10^4 cells) were plated on transwell with or without precoated matrigel, conditioned media collected from MDA-MB-231 cells were treated with either Andro (30 µM) or OPN blocking antibody (10 µM) or VEGF blocking antibody (5 µM) or in combinations and used in the lower chamber as chemoattractant for comigration and coinvasion assay. Migrated or invaded endothelial cells were stained with Giemsa and photographed. (A) & (B) represents migrated and invaded endothelial cells respectively. (C) Migrated and invaded cells were quantified, analyzed statistically and represented in the form of graph. The error bar mean ± SE. *, P<0.001 vs. control.
6.2.11 Andro inhibits breast tumor growth in *in vitro* and *in vivo* models

To investigate the role of Andro on *in vitro* tumorigenicity, colony formation assay was performed. MDA-MB-231 cells were seeded on matrigel coated plates and treated with Andro (0-150 µM) for 24 h. Media was replaced with fresh complete media on every alternate day and incubated at 37°C for 10 days. The data demonstrated that Andro inhibits the anchorage-independent growth of MDA-MB-231 cells in a dose-dependent manner as compared to control (Fig. 20D).

**Figure 22** Andro inhibits breast tumor growth and angiogenesis in orthotopic NOD/SCID mice model. (A): (I) photographs of bioluminescence imaging of representative tumor bearing NOD/SCID mice. (II) External appearance of excised tumors of respective mice. Six mice per group were used. (B) The line graph depicts the growth of tumor in terms of mean flux (photons/sec/cm²/sr). The graph represents mean flux ± SE. *, P=0.002 and **, P=0.008. (C) Tumor volumes were calculated and fold change with respect to control was analyzed statistically. The graph represents fold change in mean tumor volume ± SE. *, P=0.004 and **, P=0.015. (D) Tumors were excised, weighed and analyzed statistically. Bar graph represents fold change in mean tumor weight with respect to control. Error bar represent SD. *, P<0.001 and **, P=0.04.
We further investigated the effect of Andro on \textit{in vivo} breast tumorigenesis in mice xenograft model. MDA-MB-231-Luc cells were injected into the upper right mammary fat pad of NOD/SCID mice. After 7 days, tumor-bearing mice were randomly separated into three groups (six mice per group). Vehicle or two dosages of Andro (25 mg/kg and 100 mg/kg body weight) were injected intraperitoneally twice a week for five weeks. Tumor growths were monitored in a real time manner with \textit{In Vivo} Imaging System (IVIS) and tumor sizes were measured using vernier calipers every week. At the end of experiments, mice were sacrificed, tumors were dissected, photographed and weight were measured (Fig. 22A, panels I and II). Treatment with Andro resulted in significant reduction in both tumor volume (Fig. 22C) and tumor weight (Fig. 22D) as compared to vehicle-treated group. Mean fluxes were calculated from the bioluminescence study of the tumor and analyzed statistically (Fig. 22B).

To further correlate our \textit{in vitro} experimental results with \textit{in vivo} data, tumor samples were analyzed by Western blot, histopathology and immunohistochemistry. The results revealed that tumors generated in Andro treated cohort exhibit less tumor infiltration (Fig. 23B, panel a-c) in mammary fat pad of mice compared with control. Moreover, to examine whether reduced tumor growth is associated with the inhibition of tumor angiogenesis, tumor sections were analyzed by immunohistochemistry using anti-CD31 antibody. The results indicated that Andro reduced microvessel density as compared to vehicle treated cohort (Fig. 23B, panel p-r.). We next examined the status of OPN and other signaling molecules such as p-Akt, c-Jun and p-p65 in control and Andro treated tumors by immunohistochemistry. The data revealed that Andro inhibits the expression of OPN, p-Akt, c-Jun and p-p65 as compared with control which is consistent with our \textit{in vitro} observations (Fig. 23B, panel d-o). To further confirm these results, expression profiles of these signaling and angiogenesis specific molecules in tumor samples were analyzed by Western blot and similar results were obtained (Fig. 23A, panels I-II). Moreover, NF-κB and AP-1 regulated genes such as cyclinD1 and VEGF were found to be downregulated in Andro treated tumors. Taken together, these results suggest that Andro exhibit anti-tumor and anti-angiogenic activity by downregulating the expressions of OPN, p-Akt, c-Jun, p-p65, VEGF and Flk 1. Collectively our \textit{in vitro} and \textit{in vivo} results indicated that Andro suppresses breast tumor growth.
6.3 Discussion

Despite the best treatment available today, breast cancer is one of the most common types of cancer throughout the world and leading cause of death. Agents with high anti-cancer activity with least side effects are desirable. There are emerging evidences of activation of PI3 kinase/Akt signaling and OPN overexpression during development and progression of many cancers (87). Therefore targeting PI3 kinase/Akt signaling and OPN may have the novel strategy for the management of breast cancer. This inhibition may be
achieved by small-molecule inhibitors that may target any cancer specific gene either at transcriptional and/or post transcriptional level. In this study, we have developed a fast and easy method for extraction and purification of Andro from *Andrographis paniculata* and studied its anti-cancer properties in breast cancer cells. Previous reports suggested that Andro treatment induces apoptosis in many cancer cells (231, 232). In this study, we confirmed that Andro inhibits breast cancer cell proliferation. In particular, growth inhibition in MCF-7 was more than in MDA-MB-231 cells. Majority of the experiments were performed in MDA-MB-231 cells because it is triple negative, highly aggressive cell line and assuming that drug targeting triple negative and aggressive cancer cells could prove better efficiency for the treatment of cancer.

In fact, we for the first time report that Andro arrests cell cycle at G2/M phase in MDA-MB-231 cells. Interestingly, no significant apoptosis was observed upon 24 h of Andro treatment even at higher doses. However, at 36 h, apoptosis is observed in a dose dependent manner, suggesting that prolonged G2/M arrest could have triggered apoptotic pathway in these cells. The inhibition of cell cycle and apoptosis are consistent with the findings using hepatic carcinoma (HepG2) cells (214). To further investigate the detailed mechanism of Andro induced apoptosis, we have shown that Andro-induced apoptosis is mediated by caspase independent pathway. Earlier reports suggested that change in redox system of cells may trigger either apoptotic or proliferative signaling. Here we have demonstrated that ROS content in Andro treated cells was much higher than untreated cells. The molecular mechanism by which Andro regulates ROS mediated p53 independent apoptosis in MDA-MB-231 cells is the subject of intense investigation. The results of the present study provide a basis for further evaluation of Andro as a potential chemotherapeutic agent against breast cancer.

Metastasis is a migration of cancer cells to the distant sites, and the phenomenon is attributed by tumor-endothelial interaction, migration and invasive potential of a cancer cells (233). Earlier reports suggested that Andro can inhibit tumor cell motility (227). Here we provide evidence that Andro inhibits both breast cancer and endothelial cell motility. Tumor-endothelial interaction is crucial for tumor growth and angiogenesis and VEGF is shown to be one of the key players in this event. Here we have demonstrated that Andro inhibits tumor-endothelial interaction and is further correlated with the downregulation of OPN and
VEGF expression in MDA-MB-231 cells. Previous studies have indicated that in vitro endothelial tube formation is the process of vascular mimicry of in vivo angiogenesis and is an appropriate method to screen anti-angiogenic compound. Here we provide evidence that Andro inhibits the tubular alignment of endothelial cells.

The PI3 kinase/Akt signaling plays a crucial role in cell growth, survival, migration and invasion; and even act as an important protective pathway during oxidative stress. Majority of cancers are associated with aberrant activation of PI3 kinase/Akt signaling. Blocking of constitutively active PI3 kinase/Akt signaling could provide a novel strategy for cancer therapy (226). Here we provide evidence that Andro downregulates PI3 kinase (p85α subunit) leading to inhibition of Akt activation in breast cancer cells. This downregulation suggests that PI3-kinase/Akt is unable to confer protection against ROS ins response to Andro leading to apoptosis. Andro induced NF-κB and AP-1 activation played crucial role in several cellular processes, including proliferation, cell adhesion, apoptosis and migration. Moreover, it has been reported that Andro attenuates NF-κB by covalent modification of reduced cysteine 62 of p50 subunit (217). In the present study, we have found that treatment of MDA-MB-231 cells with Andro attenuates NF-κB and AP-1 activation. OPN is one of the key players in breast cancer development and growth (79). As reported earlier, OPN itself regulates various signaling pathway leading to cancer cell proliferation and tumor advancement (175). Moreover, OPN is reported to be involved in breast cancer cell migration, proliferation and apoptosis (79). Here we report that Andro downregulates PI3 kinase/Akt signaling and OPN expression in a dose dependent manner. Moreover, breast tumor growth was significantly reduced by Andro in mice orthotopic model. The anti-tumor activity shown in this report ascribes new anti-cancer property of Andro.

In conclusion, our study delineated the detailed mechanism by which Andro induces apoptosis and inhibits breast cancer and endothelial cell migration and tumor-endothelial interaction. Finally, we have shown for the first time that Andro inhibits OPN and VEGF expression and attenuates NF-κB and AP-1 activation leading to the suppression of tumor progression. Our results further warrant that the anti-tumor activity shown in the mouse model might provide a new strategy for the management of breast cancer.