Chapter 7

Punarnavine: Evaluation of the possible therapeutic application in conventional radiotherapy
7.1. Introduction

Cells capable of remerging and establishing a metastatic tumour condition from its quiescent state after tolerating an episode of cancer therapeutic approaches is often called cancer stem cell or cancer initiating cell. Hypoxic condition with insufficient oxygenation and the presence of other favourable mediators like hypoxia inducible factor, vascular endothelial growth factor and cytokines will accelerate this type of cell selection within the tumour mass (Collet et al., 2015). Exposure with ionizing radiation will positively modulate existing conditions in favour of the survival and expansion of cancer stem cells in a hypoxic microenvironment. The phenomenon of radio resistance is well exhibited by the hypoxic cells than the non hypoxic ones. Major repair pathways are also negatively regulated by hypoxia (Liu et al., 2015). In solid tumours abnormal blood vessel formation and perfusion, increase in cancer cell proliferation with high oxygen consumption all these can contribute to low oxygen concentrations and the resultant hypoxic progression (Justus et al., 2015). The median value of partial oxygen pressure (pO$_2$) is 10mm Hg in breast cancers compared to the value of 65mm Hg in normal breast tissue.

Radiotherapy is one of the prime treatment modality for breast cancer. Heterogenous hypoxic areas present in locally advanced breast cancers contribute to therapeutic resistance, metastases and poor patient survival. Meagre response to radiotherapy in about 50% of locally advanced breast cancers is due to the presence of these hypoxic regimes and the inability of ionizing radiation to produce DNA damage in the absence of oxygen (Aravindan et al., 2013). Hypoxic tumour cells are capable of active proliferation, invasion, metastasis and neovascularisation (Ruan et al., 2009). Specific targeting of hypoxic process will be the future arena of novel cancer therapies. The plant derived compounds are a tremendous source of active therapeutic agents with fewer side effects that can be used alone or in combination with other therapeutic methods. In the present study we investigated the effect of punarnavine during X-irradiation in mouse 4T1 breast tumour model.
7.2. Materials and Methods

**Plant material:** - Punarnavine an alkaloid from *Boerhaavia diffusa*

**Animals:** - Female BALB/c mice (8 weeks old)

**Dosage:** - Punarnavine - 40mg/kg body weight

   Administration - Intraperitoneal (i.p) for ten consecutive days.

**Cell line:** - 4T1 mouse breast carcinoma cell line

   Site of injection subcutaneous to the abdominal mammary fat pad

7.2.1. Monitoring *in vivo* tumour growth in X-irradiated mice

Female BALB/c mice were divided into eight groups (8 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing $4 \times 10^5$ 4T1 cells into the fat pad of the 4th mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight intraperitoneally for 10 days starting from 24 hour after day 14 of tumour induction. Groups III, IV, and V animals were exposed to a single dose of 6, 4, and 2Gy radiation respectively on day 14, and kept as a radiation control. Groups VI, VII, and VIII animals were exposed to 6, 4, and 2Gy radiations respectively on day 14 and all animals were treated with punarnavine 40mg/kg body weight intraperitoneally for 10 days starting from 24 hour after X-irradiation. The tumour volume was measured on day 15, day 21 and on day 28 after radiation exposure. Solid tumour development was measured with vernier calipers and calculated using the formula $V = LW^2/2$ (V, tumour volume; L, Length; W, Width).

7.2.1.1. Irradiation procedure

Experimental animals were exposed to a single dose of X-radiation (6, 4 and 2Gy) using 6MV Linear accelerator (LINAC) (Varian Medical systems, USA). The animals were kept immobilized in a specially designed, well-ventilated cage.
without any anaesthesia and exposed to whole-body irradiation at a rate of 1Gy/min. The radiation field size was 25 × 25 cm$^2$, at a distance 100cm from the source.

### 7.2.2. Determination of serum vascular endothelial growth factor (VEGF) level

Blood was collected from the above groups of animals by caudal vein bleeding on day 21 and on day 28 after radiation exposure. Serum was separated and used for the estimation of VEGF level using enzyme linked immunosorbent assay kit (ELISA) according to the manufacturer’s instructions.

### 7.2.3. Immunohistochemical analysis

Immunohistochemical staining of the excised tissues were performed by means of a double antibody labelling method. Briefly resected primary tumours on day 28$^{th}$ were fixed in neutral buffer containing 3.7% formalin for 24 hours, processed, and embedded in paraffin. The paraffin tissue blocks were cut into 5 micrometer thick sections, placed on glass slides, and deparaffinized in sequential baths of xylene. Rehydration was done by graded alcohol series and distilled water. The endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide for 10 min followed by incubation with anti-CD-31 (diluted at 1:100) antibody overnight at 4°C. After incubation, the unbound primary antibody was washed off with PBS-T (Phosphate Buffered Saline with 0.1% Tween-20). The sections were then covered with HRP conjugated rabbit anti–mouse IgG (diluted at 1:200) secondary antibody, incubated for 45-60 min at room temperature, and washed with PBS-T. Visualisation was done using diaminobenzidine chromogen that yields a brown coloured deposit. These sections were then counterstained with Methyl green and photographed.
7.2.4. Gene expression of VEGF and HIF-1α

4T1 primary tumour tissue was excised and total RNA was isolated, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Amplification was performed using specific primers of VEGF and HIF-1α.

7.2.5. Proliferation assay

4T1 cells (5×10^3 cells/well) were cultured on several 96-well culture plates for 24 hours and the plates were then exposed to different doses of X-radiation (0.5, 1, 2, 3, 4, and 5Gy) in the presence or absence of punarnavine (5μg/ml). After 48 hours of incubation ³H thymidine was added to each well (1 μCi/well) and further incubated for an additional 18 hours. After incubation, DNA was precipitated using 10% ice-cold perchloric acid, and pellets were dissolved in NaOH and transferred to 5ml scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter.

7.2.6. Determination of in vitro VEGF level

4T1 cells were grown in complete DMEM on 24-well culture plates, 24 hour after incubation culture medium was replaced with serum-free DMEM. One group of cells were incubated with or without punarnavine (5μg/ml) and another group of cells were irradiated with 1Gy of radiation and further incubated with or without punarnavine (5μg/ml). Both groups were incubated for 72 hours. Conditioned medium was then collected on 24, 48, and 72 hours after irradiation, centrifuged, and stored for conducting invasion assays, gelatin zymographic analysis and gene expression studies that to be done after an estimation of VEGF content. VEGF was measured using ELISA kit according to the manufacturer’s instructions.

7.2.7. Invasion assay

The invasion assay was carried out in modified Boyden chambers. The lower compartment of the chamber was filled with conditioned medium containing
maximum VEGF content from the above experiment (section 7.2.6.) and a polycarbonate filter coated with 25μg of type I collagen was placed on top. 4T1 cells were irradiated with 1Gy of X-radiation and then seeded (10⁵ cells/150 μl DMEM) onto the upper chamber in the presence or absence of punarnavine (5μg/ml) and incubated at 37°C in 5% CO₂ atmosphere for 10 hours. After incubation, the filters were removed, fixed with methanol, and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate filters were counted under a microscope. The results are expressed as the percentage inhibition of invasion.

% inhibition of invasion = 100 - (mean number of migratory cells in test/ mean number of migratory cells in control) x 100

7.2.8. Gelatin zymography

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed with 0.1% gelatin incorporated in the separating gel (Billings et al., 1991). Conditioned medium with maximum VEGF level from the above mentioned experiment (section 7.2.6.) was used for zymographic analysis. Samples equivalent to 100μg protein was activated with 5μl trypsin solution (75 μg/ml) in the presence and absence of punarnavine (5μg/ml) in 0.1 M Tris–HCl, 10mM CaCl₂ buffer (pH8) and incubated for 1 hour at room temperature. Sample loading and electrophoresis was carried out in a same manner as mentioned in section 5.2.9.

7.2.9. Expression of VEGF, HIF-1α, and Flk-1 genes under hypoxic condition

4T1 cells and HUVECs (2×10⁴ cells) were seeded in 96 well plates containing conditioned medium (with maximum VEGF) from the above experiment (section 7.2.6.) and incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. These cells were then exposed to hypoxia (1% O₂, 5% CO₂ and 94% N₂) in a modular incubator chamber in the presence or absence of punarnavine (5μg/ml) for 4 hours at 37°C. cDNA was synthesized using Moloney murine leukemia
virus reverse transcriptase. Amplification was performed using specific primers of VEGF and HIF-1α for 4T1 cells and Flk-1 for HUVECs.

7.3. Results

7.3.1. Effect of punarnavine on in vivo tumour growth in X-irradiated mice

The effect of punarnavine on solid tumour reduction is presented in figure 7.1. Administration of punarnavine reduced the tumour volume in all groups compared to control (radiation alone) groups. Mice carrying 4T1 cells alone (tumour controls) had a tumour volume of $1003\pm144\text{mm}^3$ on day 28; it was reduced by the administration of punarnavine on the same day ($500\pm74\text{mm}^3$). Radiation treatment significantly decreased the tumour volume in the treated groups of animals. When tumour-bearing mice were exposed to 6Gy radiation the tumour volume was reduced to $293\pm74\text{mm}^3$ on day 28. There was a non significant reduction in tumour volume by the administration of punarnavine in 6Gy group $219\pm43\text{mm}^3$. 4Gy radiation treated group of animals showed a reduction in tumour volume of $410\pm53\text{mm}^3$, when punarnavine was administered this reduction was further lowered significantly ($p<0.001$) and had a decreased tumour volume of $275\pm70\text{mm}^3$ on day 28. The 2Gy radiation reduced tumour volume to about $701\pm147\text{mm}^3$ at the same time on 28th day. There was a significant ($p<0.001$) reduction in tumour volume in punarnavine along with 2Gy treated group $341\pm74\text{mm}^3$. This reduction in tumour volume in 2Gy radiation along with punarnavine is more effective than 4Gy alone treated control group.

7.3.2. Effect of punarnavine on serum VEGF level

The serum VEGF level during tumour progression in radiation alone and radiation along with punarnavine treated groups of animals were shown in figure 7.2. Serum VEGF level was elevated during tumour progression. Administration of punarnavine significantly ($p<0.001$) reduced serum VEGF compared with untreated tumour controls. Radiation exposure drastically enhanced the serum VEGF level. Tumour bearing animals exposed to 6Gy radiation showed a
Figure 7.1. Effect of punarnavine on *in vivo* tumour growth in X-irradiated mice

![Graph showing tumour volume (mm³) over different treatments for tumour alone, tumour + Pan, tumour + 6 Gy Pan, tumour + 4 Gy Pan, two-way ANOVA with Bonferroni post hoc test.](image1)

\[\text{a} \ p < 0.001, \ \text{b} \ p < 0.01\]

Figure 7.2. Effect of punarnavine on *in vivo* VEGF level

![Graph showing VEGF level (pg/ml) over different treatments for tumour alone, tumour + Pan, tumour + 6 Gy Pan, tumour + 4 Gy Pan, two-way ANOVA with Bonferroni post hoc test.](image2)

\[\text{a} \ p < 0.001\]
maximum VEGF level on day 28. The 4Gy and 2Gy radiation treated animals also showed a significant increase in VEGF level. This increase in all the three doses of radiation treated groups were significantly (p<0.001) reduced by radiation along with punarnavine administration.

7.3.3. Immunohistochemical analysis

Figure 7.3 shows the immunohistochemistry of tumour tissue for CD31 to analyze the vascular mass. Immunohistochemistry studies revealed an increase in the CD31 endothelial cell marker, suggesting a hypoxia mediated activation of the neovascularizing pathways. By Punarnavine treatment this vascular mass was markedly reduced in different doses of radiation treated groups showing its effect on neovascularizing process.

7.3.4. Gene expression of VEGF and HIF-1α

Gene expression of VEGF and HIF-1α is given in figure 7.4. We observed upregulated expression of these genes in the untreated tumour-bearing mice exposed to different doses of radiation. Punarnavine administration down regulated the expression of VEGF and HIF-1α in the tumour bearing mice exposed to different doses of radiation.

7.3.5. Effect of punarnavine on cell proliferation and in vitro VEGF level

Figure 7.5 shows the effect of radiation and punarnavine on the inhibition of 4T1 cell proliferation. Cell proliferation was analyzed by the $^3$H-thymidine incorporation assay. Cell proliferation was markedly decreased by the increase of radiation dose. Proliferation was further decreased when the cells were treated with punarnavine. Figure 7.6 shows the effect of radiation on in vitro VEGF level. Our data indicates that radiation elevated the VEGF level. After 48 hours by radiation treatment, 4T1 cells showed a maximum VEGF level (1415±66pg/ml) that was significantly (p<0.001) decreased by treatment with punarnavine (861±21 pg/ml).
Figure 7.3. Immunohistochemical analysis

A. Tumour alone
B. Tumour + punarnavine
C. Tumour + radiation (6 Gy)
D. Tumour + radiation (6 Gy) + punarnavine
E. Tumour + radiation (4 Gy)
F. Tumour + radiation (4 Gy) + punarnavine
G. Tumour + radiation (2 Gy)
H. Tumour + radiation (2 Gy) + punarnavine
Figure 7.5. Proliferation assay

![Graph showing proliferation count per minute (CPM) vs. radiation dose (Gy).](image)

*p < 0.001

Figure 7.6. Effect of punarnavine on in vitro VEGF level

![Graph showing VEGF level (pg/ml) vs. time periods.](image)

*p < 0.001
7.3.6 Collagen Matrix Invasion Assay

The effect of punarnavine on the invasion of irradiated 4T1 cells is shown in figure 7.7. Irradiated control cells showed the maximum invasion through the polycarbonate filter membrane. Punarnavine treatment significantly inhibited cell invasion by 86%.

7.3.7 Gelatin zymography

Punarnavine inhibited the activation of MMPs produced by irradiated 4T1 cells, as shown in figure 7.8. Conditioned medium after trypsin activation showed digested clear areas at 92 and 72 kD, which was identical to MMP-9 and MMP-2 activity (Gel no.2). Gels loaded with conditioned medium without trypsin activation did not show any clear areas, indicating the inactive form of the enzyme (Gel no.1). After incubation with 10mM EDTA, trypsin activated conditioned medium loaded gels did not show clear areas, which indicate that the enzyme responsible for degradation is metalloproteinase (Gel no.3). When conditioned medium was treated with punarnavine during trypsin activation, no clear bands were observed (Gel no.4), indicating that punarnavine inhibited the activation of proenzyme to active enzyme.

7.3.8 Expression of VEGF, HIF-1α, and Flk-1 genes

VEGF, HIF-1α, and Flk-1 genes were clearly expressed in hypoxia-exposed untreated cells (figure 7.9). The expression of all of these genes was down-regulated or suppressed by treatment with punarnavine showing its inhibitory effect on these marker genes involved in hypoxic tumour progression.

7.4. Discussion

Many studies have demonstrated that the growth of solid tumours and their metastases are dependent on angiogenesis, which is regarded as a critical event in tumour development (Bergers et al., 2003). HIF-1α contributes to tumour aggressiveness, invasiveness, and resistance to radiotherapy and chemotherapy.
Figure 7.4. Gene expression of VEGF and HIF-1α

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>T</th>
<th></th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>T+R (6 Gy)</td>
<td>grey</td>
<td>black</td>
<td>T+R (4 Gy)</td>
<td>grey</td>
<td>black</td>
</tr>
<tr>
<td>T+R (2 Gy)</td>
<td>grey</td>
<td>black</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C-Tumour control; T-punarnavine treated

Figure 7.7. Collagen matrix invasion assay

Untreated irradiated control

Irradiated punarnavine treated
Figure 7.8. Gelatin zymography

1 2 3 4

1. Conditioned medium from untreated, irradiated 4T1 cells without trypsin activation
2. Conditioned medium from untreated, irradiated 4T1 cells after trypsin activation
3. Conditioned medium from untreated, irradiated 4T1 cells after trypsin activation + EDTA
4. Conditioned medium from irradiated 4T1 cells (5 microgram/ml punarnavine) after trypsin activation

Figure 7.9. Gene expression of VEGF, HIF-1α, and Flk-1

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VEGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HIF-1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flk-1</td>
<td></td>
</tr>
</tbody>
</table>

C - Tumour control; T - punarnavine treated
Over expression of HIF-1α is correlated with vascular density in tumours, indicating that HIF-1α is a key initiator of angiogenic activity (Giatromanolaki et al., 2001; Sivridis et al., 2002). Punarnavine efficiently down regulated the expression of HIF-1α and potentially inhibited vascular endothelial growth factor levels. Although punarnavine is known to inhibit VEGF expression via multiple pathways its effect on the VEGF expression in a hypoxic condition is not well understood. In this study we have demonstrated that VEGF, the hypoxia induced potent vascularisation factor and HIF-1α the transcription factor required for its activation can be regulated by punarnavine in 4T1 mouse breast cancer cells. Punarnavine may play a direct role in degradation of HIF-1α and succeeding VEGF signalling in 4T1 cells leading to inhibition of hypoxia induced tumour angiogenesis which is consistent with previous studies that punarnavine process antiangiogenic and antimitastatic activity.

Resistance to radiation therapy is often associated with an increased cellular invasion and metastatic potential (Postovit et al., 2004). Hypoxic cells are 2 to 3 fold more resistant to radiation than well-oxygenated cells because the biological effect of radiation is greatly influenced by the presence or absence of molecular oxygen at the time of irradiation (Hall, 1994; Gray et al.,1953). In the present study, the administration of punarnavine along with radiation significantly reduced tumour volumes compared with untreated radiation controls in 4T1 tumour-bearing mice. The treatment along with the radiation also decreased the cell proliferation and survival fraction of 4T1 cells. VEGF is produced by tumour cells, and its binding with the VEGF receptor Flk-1 (which is expressed on vascular endothelial cells) leads to the proliferation and migration of endothelial cells (Ferrara, 2004; Liu et al., 2005). In our study, the serum VEGF level was elevated during tumour progression, and punarnavine significantly reduced this level in tumour-bearing animals. Ionizing radiation also drastically enhanced the serum VEGF level, which was found to be decreased by the administration of punarnavine. Gene expressions of VEGF, HIF-1α, and Flk-1 were found to be down-regulated by treatment with Punarnavine.
CD31 is a transmembrane glycoprotein that is highly expressed in endothelium. Its localization at the endothelial cell junctions suggests an important role in transendothelial cellular migration (Zocchi et al., 1996). Immunohistochemical analysis showed comparatively less tumour vasculature in punarnavine treated tumour bearing animals. MMPs are zinc-dependent proteolytic endo-peptidases (Matrisian, 1992) involved in cancer progression. They stimulate cancer cell growth, migration, invasion, and metastasis. Our study clearly demonstrated that punarnavine treatment significantly inhibited the activation of MMP-2 and MMP-9 in 4T1 cells, and decreased the invasion of 4T1 cells through a collagen coated polycarbonate filter membrane, which supports the above observations. In nutshell the results indicate that punarnavine administration significantly reduced tumour volumes in irradiated experimental mice, inhibited tumour cell invasion, MMP activation, VEGF level, and gene expressions of HIF-1α and VEGF in a condition of ionizing radiation exposure. All these strongly suggest that punarnavine inhibited the invasion of irradiated tumour cells by regulating HIF-1α, MMP-2, MMP-9, and VEGF.