Chapter IV

Effect of Hypoxic Cell Sensitizer on Transcription of \textit{hif-1a} and its Target Genes in Tumor Cells
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Hypoxia-selective tumor therapy has great importance since hypoxic environment makes the tumors refractory to radiation and antineoplastic agents. The compounds with hypoxic cell sensitizing property have been evaluated in association with various therapeutic strategies and some of them are found to be operational. Sanazole (a nitrotriazole compound), a well-known hypoxic radiation sensitizer, has been attested for its hypoxia-selective activity at molecular level. In the present study, we have investigated the effect of Sanazole on transcription of major genes responsible for hypoxia-associated tumor growth, such as *ashif-1α, vegf* and *egfr*, in tumor cells under *in vitro* and *in vivo* conditions. The transcriptional expressions of these genes were studied by quantitative real time PCR. The levels of nitric oxide in the tumor cells and tissues were studied by Griess test. The transcription of these genes were up-regulated in hypoxic tumor cells while it was down regulated significantly in these cells treated with Sanazole compared to the control (normoxic) cells. The same pattern of expression was observed in tumor tissues of animals treated with Sanazole. Thus, the study revealed the effect of Sanazole in hypoxia-induced tumor growth which makes this compound useful for targeting cytotoxic drugs to hypoxic solid tumor.

### 4.1 INTRODUCTION

The sensitivity to therapy is mostly dependent on the genetic-epigenetic modifications and the altered microenvironment in the tumor. The reduction in the oxygen bioavailability, hypoxia, in solid tumor due to the uneven dissemination of blood vessels, made tumor more resistant to the treatments. Most of the cells respond to hypoxia by the activation of adaptive cellular responses. These responses include the transcriptional activation of factors induced by hypoxia, which encourages the expression of a series of genes that promotes aggressive tumor growth. These hypoxia-inducible factors generate resistance to chemotherapy and radiation therapy [Rockwell et al., 2009; Cosse and Michiels, 2008; Song et al., 2006; Sullivan et al., 2008].

The hypoxia-inducible transcription factor-1 (HIF-1) is considered as an important link that coordinates the tumor progression in hypoxic condition. Structurally HIF-1 is a heterodimer of two subunits- HIF-1α and HIF-1β. In fully oxygenated cells, HIF-1α is hydroxylated at proline residues and ubiquitinated in proteasome system. Under hypoxic conditions, HIF-1α is translocated to the nucleus and it gets dimerized with HIF-1β to form active HIF-1[Semenza, 2000; Semenza, 2001]. HIF-1 regulates the altered expression of genes promoting angiogenesis, activation of enzymes responsible for cellular energy-acquiring metabolic pathways and cell proliferation [Maxwell et al., 1997]. Hypoxia in tumor therefore must be judged as a leading factor influencing tumor therapy. Hence, many approaches to evading the effects of hypoxia have been checked extensively in
preclinical and clinical studies. By exploiting tumor hypoxia, directed drug targeting developed has attained a lot of importance in tumor therapy [Thambi et al., 2014]. Brown and Wilson in 2004, and Ajduković in 2016 reviewed the importance of hypoxia in tumor therapy and provided an overview about the on-going research strategies including hypoxia-selective pro-drug therapy, specific targeting to hypoxia-inducible factor-1, and hypoxia-selective gene therapy.

Several hypoxic cell sensitizers, also known as radiosensitizers, are developed to enhance the efficacy of the treatments mainly radiation therapy [Masunaga et al., 2006] and chemotherapy [Millar, 1982; Candelaria et al., 2006] in hypoxic solid tumors. In hypoxic condition, these sensitizers could overcome the treatment-resistance and enhance the therapeutic damage by mimicking the oxygen [De Ridder et al., 2008]. Sanazole (SAN), a nitrotiazole based radiosensitizer, is effective in sensitizing hypoxic cells and solid tumors [Pasupathy et al., 2001]. SAN, also known as AK2123, has a favourable level of accumulation in solid tumors with good tumor-to-blood and -muscle ratio [Das et al., 2004]. In the present study, the influence of SAN in the transcription of key hypoxia regulatory factor hif-1α and its target genes- vegf and egfr- was studied under both in vitro and in vivo conditions to expound the mechanism of action of SAN in hyoxic tumor cells.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All chemicals and reagents were purchased from Sigma Aldrich, India. Sanazole was from Dr. Kagiya, Health Research Foundation, Kyoto, Japan. For PCR studies the reagents were from Genei, Bangalore, India.

4.2.2 Animals

Swiss albino mice (female) weighing 25-27 were purchased from Small Animal Breeding Section, Government College, Mannuthy, Thrissur, Kerala. The animals were fed with standard mouse chow and water ad libitum. The experiments using animals were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC), consistently adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India. The Dalton’s Lymphoma Ascites (DLA) cells were maintained in the peritoneal cavity of mice.
4.2.3 Studies- In vivo

To obtain animals bearing solid tumors, DLA cells (1×10^6 cells/animal) were transplanted subcutaneously in the left hind limb of female mice. When the volume of tumor reaches approximately 1cm^3, the animals were divided into 3 groups.

Group1 was kept as control (untreated- administered with sterile distilled water); Group 2 and 3 were administered i.p. with SAN (2.5μmoles/animal). The animals in group2 and 3 were sacrificed after 1hr and 2hr of SAN administration, respectively. The blood was collected by cardiac puncture and the tissues - tumor and liver - were excised for further analyses.

4.2.4 Studies- In vitro

The suspension culture of cells (2×10^6 cells/ml) was prepared in Dulbecco's modified Eagle's medium (DMEM), supplemented with 12% foetal bovine serum (FBS) and nutrients.

Experimental procedure: The cultured cells were divided into four tubes.
Tube 1: Normoxia (Control; Normoxic cells)
Tube 2: Hypoxia (Hypoxic cells)
Tube 3: Normoxia-SAN (Normoxic cells treated with SAN (1mM) prior to incubation)
Tube 4: Hypoxia-SAN (Hypoxic cells treated with SAN (1mM) prior to incubation)

[Normoxic condition was created by supplying 95% atmospheric air and 5% CO₂ at 37°C, while the hypoxic condition was created by 95% N₂ and 5% CO₂ at 37°C.]

All tubes were incubated for 3hrs and the cells were separated by centrifugation. RNA was isolated from the cells and the transcriptional level expression of genes was studied by Real Time PCR (qRT-PCR). The level of nitric oxide in these cells was analysed indirectly in the supernatant based on Griess reaction.

4.2.5 Transcriptional expression of genes

Acid guanidium thiocyanate phenol- chloroform extraction method was used to isolate RNA from cells and tumor tissues [Chomczynski and Sacchi, 1987]. The cDNA was prepared by reverse transcription with the use of random-hexamer primers as the initiating sequence. Using thecDNA qRT-PCR was performed using gene-specific primerst to amplify the genes hif-1α (X95580.1), vegf (AB086118.1), and egfr (AF275367.1). The house keeping gene β-actin (NM 007393.3) was used as internal control. The relative fold
change in the transcription level expression of genes was calculated in comparison with the untreated control [Schmittgen and Livak, 2008].

4.2.6 Nitric oxide assay
Based on the previous reports, the release of nitric oxide by SAN was measured indirectly as concentration of nitrite in the samples by Griess reaction [Grisham et al., 1996]. The samples were incubated with equal volume of Griess reagent for 10min at RT and the absorbance was measured at 543nm. Sodium nitrite (0.1-1.0nmoles) was used for the preparation of calibration curve.

4.2.7 Statistical analysis
The results are presented as mean±SD and were analyzed by GraphPad PRISM software version 5. Statistical significance of the results was determined using One-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test.

4.3 RESULTS

4.3.1 In vitro experiments

4.3.1.1 Transcriptional expression of hif-1α and its targeted genes
The transcriptional level expression of hif-1α is presented in figure 4.1. In hypoxic condition, the expression of hif-1α was up regulated significantly compared to the cells in normoxic condition (control). Under hypoxic conditions, the reduced cellular oxygen level alters the growth and metabolism in tumor. The over expression of hif-1α may counteract this by supporting angiogenesis, cell proliferation and metastasis of tumor [Xiao et al., 2015; Gilkes and Semenza, 2013]. The incubation of normoxic cells with SAN, which is a nitrocompound, increases the levels of hif-1α through enhanced stabilization [Sandau et al., 2001]; however, SAN down regulates the expression of hif-1α in hypoxic cells. These results indicate that the influence of SAN in the expression of the gene hif-1α is totally depending on the availability of oxygen. Under normoxic condition, SAN stabilizes hif-1α and increases its expression level, while under hypoxia SAN act in an entirely different way that down regulates hif-1α expression.
Figure 4.1: Relative fold change in the expression of \textit{hif-1\alpha}. Note: The values are expressed as mean± SD. Normoxia: Normoxic, untreated, Control cells; Hypoxia: Hypoxic cells; Normoxia-SAN: Normoxic cells treated with SAN; Hypoxia-SAN: Hypoxic cells treated with SAN

Figure 4.2 and 4.3 depicts the relative fold changes in the expression of \textit{vegf} and \textit{egfr} in DLA cells after various treatments. The expression of genes \textit{vegf} and \textit{egfr} was up-regulated in cells under hypoxia, similar to \textit{hif-1\alpha}. This up-regulation could be the consequence of the higher expression levels of \textit{hif-1\alpha} in these cells. The treatment with SAN in hypoxic cells down regulated the expression of \textit{hif-1\alpha} which in turn led to the down regulation of \textit{vegf} and \textit{egfr}.

Figure 4.2: Relative fold change in the expression of \textit{vegf}. Note: The values are expressed as mean±SD. Normoxia: Normoxic, untreated, Control cells; Hypoxia: Hypoxic cells; Normoxia-SAN: Normoxic cells treated with SAN; Hypoxia-SAN: Hypoxic cells treated with SAN
Figure 4.3: Relative fold change in the expression of $egfr$. Note: The values are expressed as mean±SD. Normoxia: Normoxic, untreated, Control cells; Hypoxia: Hypoxic cells; Normoxia-SAN: Normoxic cells treated with SAN; Hypoxia-SAN: Hypoxic cells treated with SAN

4.3.1.2 Level of nitric oxide

The nitrotriazole compound SAN could act as NO donor. We evaluated the concentration of NO in these cells by Griess reaction in terms of nitrates and the results are presented in Table 4.1. The cells incubated with SAN under hypoxic condition showed statistically significant ($p<0.05$) increase in the concentration of NO compared to the cells in normoxic condition. Under normoxic conditions, there was an increase in NO levels, but it was not significant.

Table 4.1: Concentration of NO in cells after various treatments under in vitro condition

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration of NO (nmoles/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia (Control)</td>
<td>29.0± 0.3</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>38.0± 0.05 ns</td>
</tr>
<tr>
<td>Normoxia-SAN</td>
<td>41.0± 1.0 ns</td>
</tr>
<tr>
<td>Hypoxia-SAN</td>
<td>45.0± 1.0 *</td>
</tr>
</tbody>
</table>

Note: The values are expressed as mean± SD. $ns$ indicates non-significance ($p>0.05$) and * indicates significance with $p<0.05$ compared to control.
4.3.2 **In vivo experiments**

4.3.2.1 **Effect of SAN in the transcription of hif-1α in tumor tissues**

The relative fold change in the transcriptional activation of *hif-1α* in tumor tissue is presented in figure 4.4. The transcription of *hif-1α* in control (tumor-bearing, untreated) animals was elevated compared to SAN treated animals (tumor-bearing), indicating the presence of hypoxic cells in the tumor. The administration of SAN decreased *hif-1α* expression significantly compared to the untreated control. This would suggest the down regulation of *hif-1α* by SAN under hypoxia, corroborating the results from the *in vitro* studies.

![Figure 4.4: Relative fold change in the expression of hif-1α in tumor tissues. Note: The values are expressed as mean±SD.](image)

4.3.2.2 **SAN down regulates the transcriptional expression of vegf and egfr**

![Figure 4.5: Relative fold change in the expression of vegf and egfr in tumor tissues. Note: The values are expressed as mean±SD.](image)
The relative fold changes in the transcriptional expression of the genes - *vegf* and *egfr* - in tumor following SAN treatment under hypoxic conditions are given in figure 4.5. The expression of *vegf* was down regulated about five fold in SAN treated tumors with respect to the control. Approximately five fold down regulation in the transcription of *egfr* was observed in SAN treated animals.

### 4.3.2.3 Level of NO following the treatment with SAN

Table 4.2 presents the data on concentration of NO in tumor, liver and serum of the animals following SAN administration. The concentration of NO was found increased significantly (p<0.01) in tumor tissues after 2hrs of SAN administration. In the liver tissues of the animals, also, there was an increase (p<0.05) in the NO concentration. However, in the serum of these animals there was no increase (p>0.05) in the concentration of NO following SAN administration compared to the control.

Table 4.2: Concentration of NO in tumor, liver and serum following various treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration of NO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor (nmoles/g)</td>
</tr>
<tr>
<td>Control</td>
<td>113.3± 4.9</td>
</tr>
<tr>
<td>SAN (1hr)</td>
<td>127.0± 2.1\textsuperscript{ns}</td>
</tr>
<tr>
<td>SAN (2hrs)</td>
<td>197.7± 2.3\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Note: The values are expressed as mean± SD. \textsuperscript{ns} indicates non-significance (p>0.05) and \textsuperscript{*} indicates significance with p<0.05 and \textsuperscript{**} indicates significance with p<0.01 compared to control.

### 4.4 DISCUSSION

Targeted delivery of drugs to tumor is an important strategy for cancer treatment for enhanced therapeutic efficiency and to avoid undesirable side effects. Since tumor hypoxia is a major contributor of drug resistance, hypoxia targeted drug delivery is of paramount importance in tumor therapy. Several hypoxic cell sensitizers have been reported and a number of them have undergone clinical trials in chemotherapy and radiotherapy. SAN has completed phase III clinical trials and presently it is used in several centres as a hypoxic cell radiosensitizer [Koizumi et al., 2005; Dobrowsky et al., 2005]. SAN gets accumulated in hypoxic solid tumors following administration to tumor-bearing animals [Murugesan et
al., 2001; Das et al., 2004]. SAN has also been shown to activate caspase-3 and induce apoptosis in tumor [Rajagopalan et al., 2003].

In the present study, the tumor cells incubated with SAN showed increased expression of hif-1α and its target genes under normoxic condition. Sandau et al (2001) demonstrated that NO donors can stabilize HIF-1α under normoxic condition, however, in hypoxic condition HIF-1 activity is inhibited by them [Sogawa et al., 1998; Berchner-Pfannschmidt et al., 2010]. SAN, being a nitric oxide donor [Kondakova et al., 2004], may inhibit prolyl hydroxylase (PHD)-dependent enzymatic hydroxylation of proline residues of hif-1α and thereby prevent its degradation under normoxic condition. However, the incubation of the cells with SAN in hypoxic condition could down regulate hif-1α expression as a result of the re-distribution of O2 by the competitive interaction of the functional group of SAN with mitochondrial cytochrome C oxidase [Kasuno et al., 2004]. The increase in the concentration of NO in hypoxic cells following the incubation with SAN suggested the role of NO in the down regulated transcription of hif-1α.

The level of expression of hif-1α was found reduced in tumor tissues of animals after SAN treatment confirming the results obtained from the in vitro study. The increased concentration of NO in the tumor tissue of SAN treated animals suggested NO-mediated down regulation of hif-1α. Hypoxia in tumor enhances tumor angiogenesis and tumor cell proliferation by hif-1α mediated activation of several growth factors [Xiao et al., 2015]. The expression of hif-1α and associated target genes - vegf and egfr - are considered as adaptive mechanisms of tumor tissues in response to hypoxia. The down regulated expression of these genes after SAN administration revealed anti-angiogenic and anti-proliferative potential of SAN in enhancing therapeutic efficiency.

4.5 CONCLUSION

The hypoxic cell radiosensitizer SAN up-regulated the expression of hypoxia-inducible factors under normoxic condition, while under hypoxic condition it down-regulated their transcription. Radiation- and chemo-sensitizing property of SAN could be ascribed to the O2-dependent differential expression of these factors. As SAN gets accumulated in hypoxic regions of tumor specifically, this compound could be used for targeting drugs for tumor therapy.