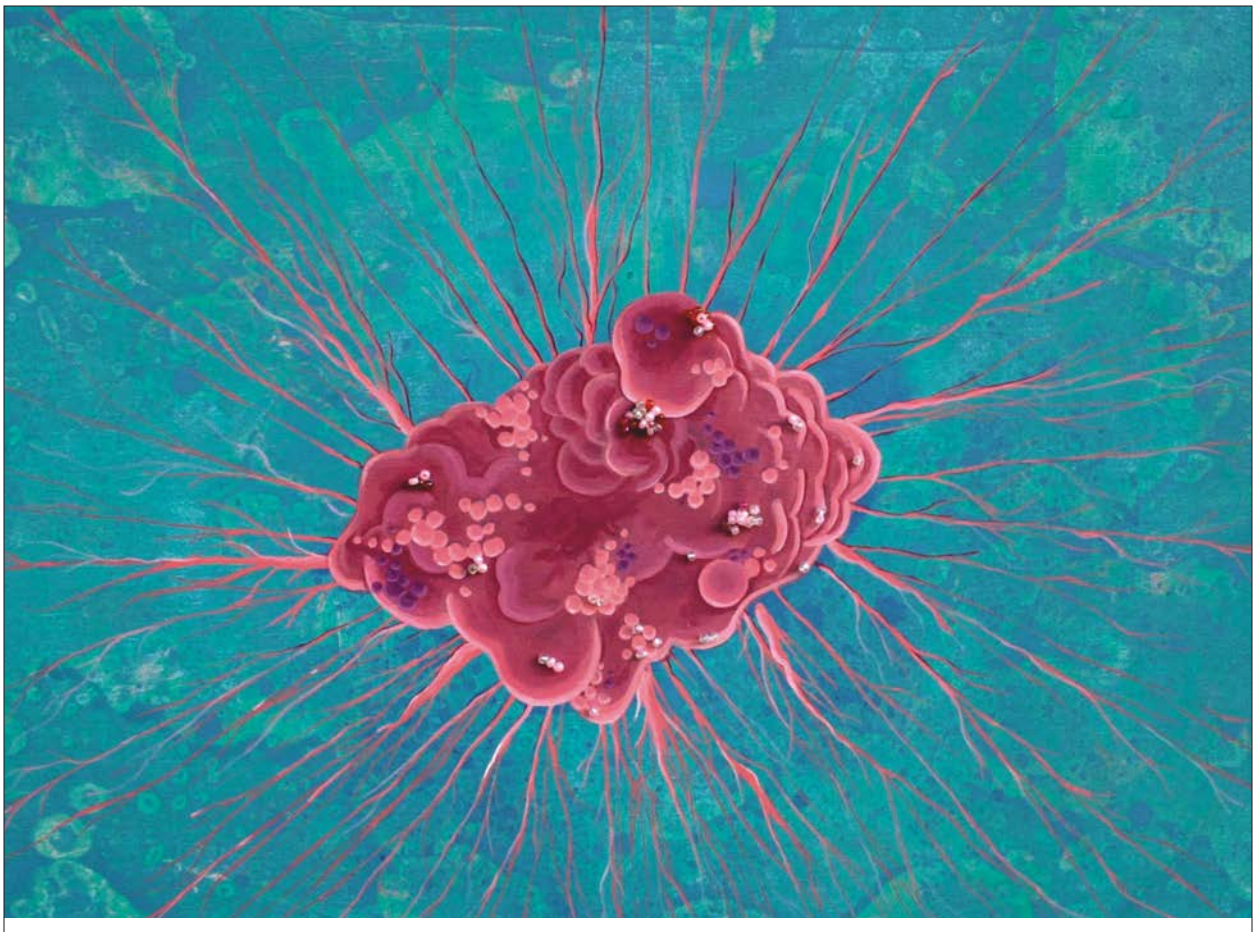

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Research Article
Effect of Hypoxic Cell Sensitizer on Transcription of *hif-1α* and its Target Genes in Tumor Cells

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Abstract

Background: Hypoxia-selective tumor therapy has great importance since hypoxic environment makes the tumor 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 nitro-imidazole compound), a well-known hypoxic radiation sensitizer, has been attested for its hypoxia-selective activity at molecular level. In the present study, the effect of Sanazole on the transcription of major genes responsible for hypoxia-associated tumor growth such as *hif-1α*, *vegf* and *egfr* in tumor cells under *in vitro* and *in vivo* conditions was investigated. **Methodology:** 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. **Results:** The transcription of these genes were up-regulated in hypoxic tumor cells while it was down regulated significantly in the 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. **Conclusion:** 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.

Key words: Sanazole, tumor hypoxia, hypoxia-inducible factor, vascular endothelial growth factor, endothelial growth factor receptor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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 and hypoxia in solid tumor due to the uneven dissemination of blood vessels, made tumor more resistant to 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[1-4].

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[5,6]. The HIF-1 regulates the altered expression of genes-promoting angiogenesis, activation of enzymes responsible for cellular energy-acquiring metabolic pathways and cell proliferation[7]. 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[8]. Brown and Wilson[9] and Ajdukovic[10] 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[11]and chemotherapy[12-13] in hypoxic solid tumors. In hypoxic condition, these sensitizers could overcome the treatment-resistance and enhance the therapeutic damage by mimicking the oxygen[14]. Sanazole, (SAN), a nitroimidazole based radiosensitizer, is effective in sensitizing hypoxic cells and solid tumors[15]. SAN, also known as AK2123, has a favourable level of accumulation in solid tumors with good tumor-to-blood and muscle ratio[16]. 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.

MATERIALS AND METHODS

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

Animals: Swiss Albino mice weighing 25-27 were purchased from Small Animal Breeding Section, Government Veterinary College, Mannuthy, Thrissur, Kerala. The animals were fed with normal 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.

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 1 cm^3 and the animals were divided into three groups.

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

Transcriptional expression of genes: The RNA was isolated from cells and tumor tissues by acid guanidium thiocyanate phenol-chloroform extraction method[17]. The cDNA was prepared by reverse transcription with the use of random-hexamer primers as the initiating sequence. Using the cDNA, qRT-PCR was performed, using gene-specific primers 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 untreated control[18].

Nitric oxide assay: Based on the previous study, the release of nitric oxide by SAN was measured indirectly as the concentration of nitrites in the samples by Griess reaction[19]. The samples were incubated with equal volume of Griess reagent for 10 min at room temperature and absorbance was measured at 543 nm. Sodium nitrite (0.1-1.0 nmoles) was used for the preparation of calibration curve.

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.

The cultured cells were divided into four tubes as follow:

Tube 1: Normoxia (Control and normoxic cells)
Tube 2: Hypoxia (Hypoxic cells)
Tube 3: Normoxia-SAN (Normoxic cells treated with 1 mM SAN prior to incubation)
Group 4: Hypoxia-SAN (Hypoxic cells treated with 1 mM SAN prior to incubation)

Normoxic condition was created by supplying 95% atmospheric air and 5% CO_2 at 37°C while, the hypoxic condition was created by 95% N_2 and 5% CO_2 at 37°C.

All tubes were incubated for 3 h and the cells were separated by centrifugation. The 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.

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

RESULTS

In vitro experiments

Transcriptional expression of *hif-1α* and its targeted genes: The transcriptional level expression of *hif-1α* is presented in Fig. 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 levels alter the growth and metabolism in tumor. The over expression of *hif-1α* may counteract this by supporting angiogenesis, cell proliferation and metastasis of tumor[20-21]. The incubation of normoxic cells with SAN, which is a nitro compound, increases the levels of *hif-1α* through enhanced stabilization[22] 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 can stabilize *hif-1α* and increase the levels while, under hypoxia SAN act in an entirely different way that can down regulate *hif-1α* expression.

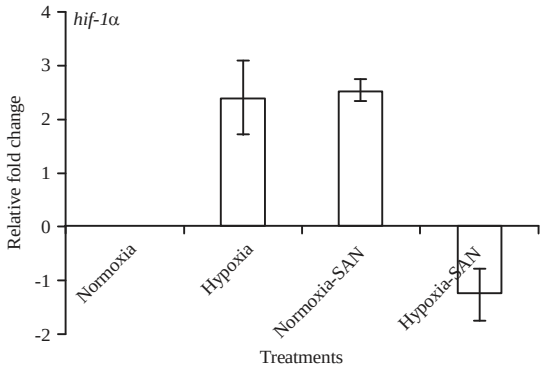

Fig. 1: Relative fold change in the expression of *hif-1α*, values are expressed as Mean±SD, Normoxia: Normoxic and control cells, Hypoxia: Hypoxic cells, Normoxia-SAN: Normoxic cells treated with SAN and Hypoxia-SAN: Hypoxic cells treated with SAN

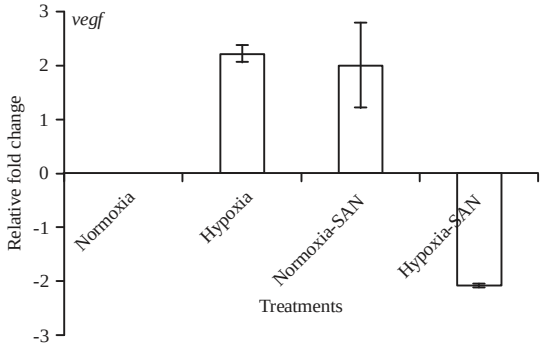

Fig. 2: Relative fold change in the expression of *vegf*, values are expressed as Mean±SD, Normoxia: Normoxic and control cells, Hypoxia: Hypoxic cells, Normoxia-SAN: Normoxic cells treated with SAN and Hypoxia-SAN: Hypoxic cells treated with SAN

Figure 2 and 3 depicts the relative fold changes in the expression of *vegf* and *egfr* in DLA cells after various

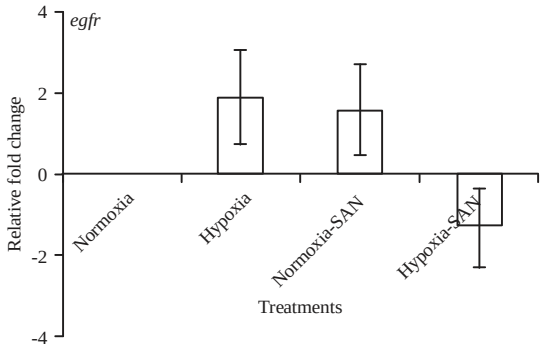

Fig. 3: Relative fold change in the expression of *egfr*, values are expressed as Mean±SD, Normoxia: Normoxic and control cells, Hypoxia: Hypoxic cells, Normoxia-SAN: Normoxic cells treated with SAN and Hypoxia-SAN: Hypoxic cells treated with SAN

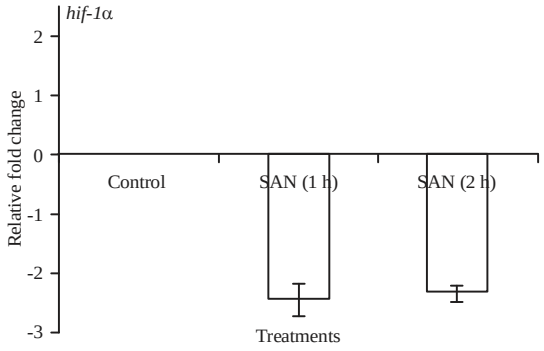

Fig. 4: Relative fold change in the expression of *hif-1α* in tumor tissues and the values are expressed as Mean±SD

treatments. The expression of genes *vegf* and *egfr* was up-regulated in cells under hypoxia similar to *hif-1α*. This up-regulation could be the consequence of the higher expression levels of *hif-1α* in these cells. The treatment with SAN in hypoxic cells down regulated the expression of *hif-1α* which in turn led to the down regulation of *vegf* and *egfr* as can be evidenced from Fig. 2 and 3.

Level of nitric oxide: The nitrotriazole compound SAN could act as NO donor. The concentration of NO in these cells was evaluated by Griess reaction in terms of nitrite and the results are presented in Table 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 nomoxic condition. Under normoxic conditions, there was an increase in NO levels but it was not significant.

Table 1: NO-concentration in cells after various treatments under *in vitro* condition

Treatments	Concentration of nitric oxide (nmoles g^{-1} tissue)
Normoxia (Control)	29.0±0.3
Hypoxia	38.0±0.05^{ns}
Normoxia-SAN	41.0±1.0^{ns}
Hypoxia-SAN	45.0±1.0*

Values are expressed as Mean±SD, ^{ns}indicates non-significance ($p>0.05$) and *indicates significance with $p<0.05$ compared to control

Table 2: Concentration of NO in tumor, liver and serum following various treatments

	Concentration of NO		
Treatments	Tumor (nmoles g^{-1})	Liver (nmoles g^{-1})	Serum (nmoles mL^{-1})
Control	113.3±4.9	8.3±0.4	50.8±0.2
SAN (1 h)	127.0±2.1^{ns}	12.6±0.2^{ns}	14.0±1.4^{ns}
SAN (2 h)	197.7±2.3**	15.7±0.1*	48.0±0.5^{ns}

Values are expressed as Mean±SD, ^{ns}indicates non-significance ($p>0.05$), *indicates significance with $p<0.05$ and **indicates significance with $p<0.01$ compared to control

In vivo experiments

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 Fig. 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.

SAN down regulates the transcriptional expression of *vegf* and *egfr*: 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 Fig. 5. The expression of *vegf* was down regulated about five fold in SAN treated tumor with respect to the control. Approximately, five fold down regulation in the transcription of *egfr* was observed in SAN treated animals.

Level of NO following the treatment with SAN: Table 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 2 h 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 ($p>0.05$) of the NO concentration following SAN administration compared to the control.

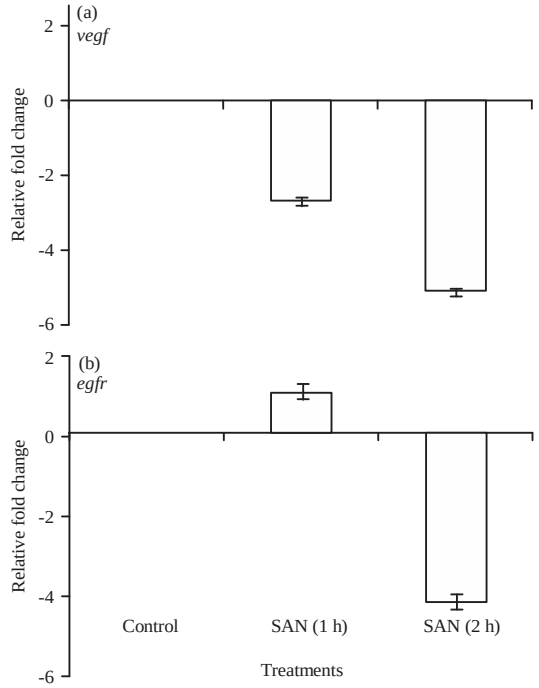

Fig. 5(a-b): Relative fold change in the expression of (a) *vegf*, (b) *egfr* in tumor tissues and the values are expressed as Mean±SD

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. The SAN has completed phase III clinical trials and presently it is used in several centres as a hypoxic cell radiosensitizer[23,24]. SAN gets accumulated in hypoxic solid tumors following administration to tumor-bearing animals[16,25]. SAN has been shown to activate *caspase-3* and induce apoptosis in tumor[26]. The chemoprevention-potential of synthetic organo-selenium compounds has been shown to be mediated through controlling the gene expression at the transcriptional level[27-29].

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.*[22] demonstrated that NO donors can stabilize HIF-1α under normoxic condition however, in hypoxic condition HIF-1 activity is inhibited by them[30,31]. SAN being a nitric oxide donor[32], 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 O_2 by the competitive interaction of the functional group of SAN with mitochondrial cytochrome C oxidase[33]. 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[20]. 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.

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 O_2-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.

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