Research Paper:
Blockade of Hypoxia: The Impact on Tumor Growth in an Experimental Tumor Model

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A B S T R A C T

Background: Tumor microenvironment is an active factor participating in immunoregulation, thereby preventing immunosurveillance and limiting the efficacy of anticancer therapies. Hypoxia as a major characteristic of solid tumors causes the expression of Hypoxia-Inducible Factor-1α (HIF-1α). This is a transcription factor that mediates hypoxic responses of tumor cells and involves in the expression of tumor immunosuppression-related genes.

Materials and Methods: In this study, we used a mouse 4T1 breast cancer model.

Results: Our obtained data revealed that in vivo administration of PX-478, an inhibitor of oxygen sensitive HIF-1α, reduced the expression of Forkhead box P3 (Foxp3) transcript, a molecule that is directly controlled by HIF-1. The level of vascular endothelial growth factor, another gene controlled by HIF-1, remained unchanged. The observed results were in correlation with delayed tumor growth in tumor-bearing mice.

Conclusion: Our findings indicate that the reduction in Foxp3 expression through HIF-1α inhibition using PX-478 may contribute to tumor regression.

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Introduction

Hypoxia, a common characteristic of all solid tumors, provokes mechanisms involved in suppressing antitumor immunity and protects cancerous tissues [1-5]. The immuno-regulatory effect of hypoxia is elicited by Hypoxia-Inducible Factor (HIF). HIF mediates adaptive transcriptional responses to hypoxia, involved in different cell biology aspects, including cell survival, glucose metabolism, angiogenesis, and invasion [6, 7]. HIF is a heterodimer molecule consisting of constitutively expressed beta (HIF-1β) subunit and one of the three oxygen-regulated alpha subunits (HIF-1α, HIF-2α, or HIF-3α). In the absence of oxygen, alpha subunits are post-translationally stabilized and heterodimerized with HIF-1β, then be translocated into the nucleus and transactivate its target genes [8].

Hypoxia plays a critical role in immune regulation and resistance to therapy. Thus, targeting HIF-1 activity could be a potential immunotherapeutic approach for cancer therapy. Suppressing HIF-1 in animal models decreases tumorigenesis, and increases survival rate [9]. A growing number of medications have been introduced as anticancer agents that are HIF-1inhibitors [8, 10]. PX-478, [S-2-amino-3-(4V-N,N,-bis[2-chloroethyl]amino)-phenyl propionic acid N-oxide dihydrochloride], is a small molecule that suppresses hypoxic and normoxic translations of HIF-1α, as well as HIF-1α transcriptional activity and Vascular Endothelial Growth Factor (VEGF) expression under hypoxic conditions, in various cancer cell lines [11, 12]. Moreover, hypoxia promotes Foxp3 expression and regulatory T-cell function through direct transcriptional activation of Foxp3 mRNA by HIF-1α [13].

Given the impact of hypoxic stress on angiogenesis, tumor progression and immune tolerance, hypoxia attracted particular attention in tumor immune biology. The present study investigated the effect of HIF-1α inhibition using PX-478 on tumor growth in the mouse model of cancer, as well as its potential effect on VEGF and Foxp3 expression.

Materials and Methods

Mice and cell lines

Six- to 8-week-old BALB/c and C57BL/6 female mice were obtained from the Laboratory Animal Center, Pasteur Institute of Iran. Animal protocols were approved by the Institutional Animal Care and Use Committee of Tehran University of Medical Sciences. The 4T1 carcinoma and F10 melanoma cell lines which are of BALB/c and C57BL/6 origin, respectively, were cultured in complete media RPMI-1640 (Biosera, UK) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L L-glutamine (Biosera, UK), as well as 10% heat-inactivated FBS (Gibco, Grand Island, USA), in a 5% CO_2 humidified incubator at 37° C. Tumors were created by subcutaneously injecting 7×105 4T1 or 5×105 F10 tumor cells into the right flank of syngeneic mice, with tumor size (in mm^2), assessed every 2 days thereafter.

PX-478 treatment

HIF-1α inhibitor, PX-478, was purchased from MedKoo Biosciences. For in vivo use, tumor-bearing mice were Intraperitoneally (IP) administered 20, 40 or 60 mg/kg PX-478 or normal saline in a total volume of 200 μL, 3 times a week for one week, beginning after tumors were palpable with an approximate size of ~25 mm^2 in the area.

RNA isolation and real-time quantitative PCR

RNA was extracted from frozen tissues with Hybrid-R RNA purification kit (GeneAll Biotechnology, Korea). One microgram of RNA was reverse transcribed into complementary DNA (cDNA), using a QuantiTect Reverse Transcription kit (Qiagen). cDNAs were quantified by real-time PCR using an SYBR Green Real-time PCR master mix (Primer design, UK), on an ABI 7500 detection system (Applied Biosystems, United States). The relative mRNA levels were determined using the ΔCt method. The values were expressed relative to endogenous β-actin. The following PCR primers were used: HIF-1α forward, 5′-AGCTTCTGTTATGAGGCTCACC-3′; HIF-1α reverse, 5′- TGACTTGATGTTCATCGTCCTC-3′ [14]; VEGF forward, 5′- GCGGAGAAAGCATTTGTTTG-3′; VEGF reverse, 5′-TCTTTCCGGTGAGAGGTCTG-3′; Foxp3 forward, 5′-GCAGGGCAGCTAGGTATCTGTAG-3′; Foxp3 reverse, 5′-TCGGAGATCCCCTTTGTCTTATC-3′; β-actin forward, 5′-GGTCATCACTATTGGCAACG-3′; and β-actin reverse, 5′-ACGGATGTCAACGTCACACT-3′.

Statistical analysis

Between-group comparisons were performed using 1-way Analysis of Variance (ANOVA), and Tukey's test. GraphPad Prism was used for graphs and statistical analysis. P values less than 0.05 were considered as significance.

Results

PX-478 administration interferes with HIF-1α and Foxp3 expression

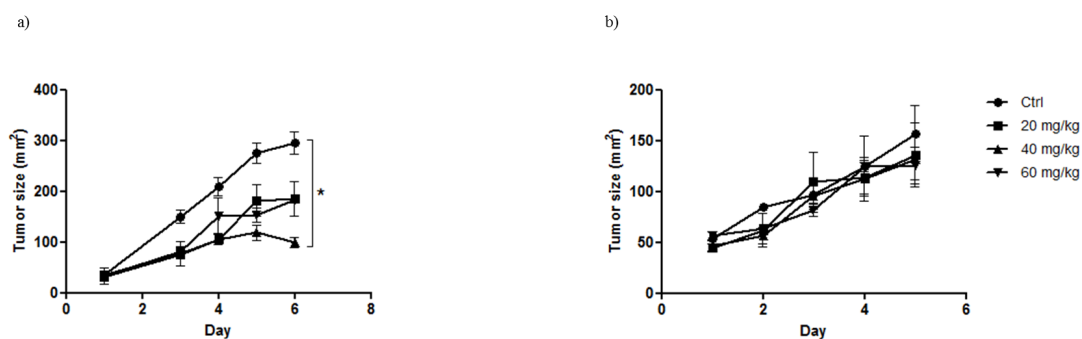PX-478 is a HIF-1α inhibitor currently being evaluated in phase I/II clinical trials. Previous studies indicated that

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a)

b)

Figure 1. Effect of PX-478 treatment on the expression of HIF-1α and its downstream genes   **ImmunoRegulation**

Tumor-bearing mice were left untreated or they were given PX-478 (IP at 20, 40, or 60 mg/kg every other day for a week). Tumors were excised one day after the final injection. Then, HIF-1α mRNA in both models, as well as VEGF and Foxp3 mRNA in breast carcinoma model was measured in tumors by real-time RT-PCR, calculated relative to housekeeping gene β-actin. Data were expressed as the mean±SEM for the three mice per group. *$P<0.05$ by 1-way ANOVA.

PX-478 administration inhibits HIF-1α expression and its downstream target gene, VEGF. The direct effect of HIF-1α on the induction of Foxp3 has also been indicated. To determine whether PX-478 involves VEGF and Foxp3 expression, PX-478 was IP administered in palpable tumors, at dosages of 20, 40, or 60 mg/kg every other day for a week. Treated animals were sacrificed 1 day after receiving the last dose of the drug, and tumors were excised. Then, the transcript levels of HIF-1α, FOXP3, and VEGF were measured via real-time RT-PCR.

As shown in Figure 1, HIF-1α transcription significantly decreased in tumors obtained from 4T1 tumor-bearing mice that received 40 mg/kg PX-478. However, in the case of F10 tumors, HIF-1α expression, while not at a significant level, indicated a decreased expression at the dosages of 40 and 60 mg/kg. These studies revealed that HIF-1α blockade diminished the tumoral expression of Foxp3 when the inhibitor was administrated at 40 mg/kg in breast cancer models. However, VEGF expression evaluated at mRNA level suggested no remarkable change in tumors.
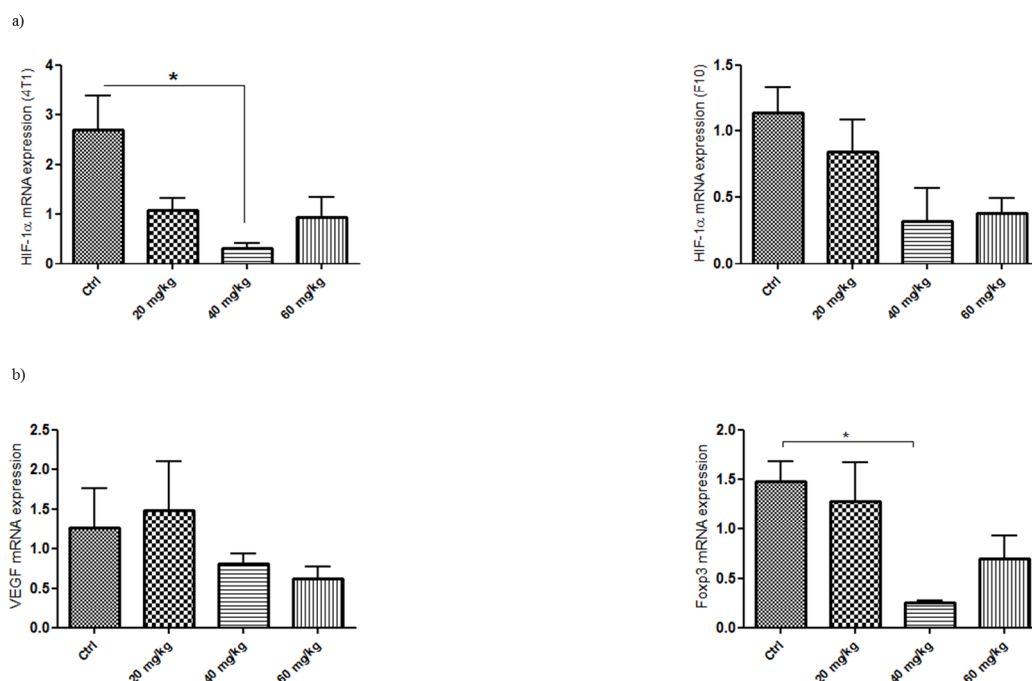
a)

b)

Figure 2. Delay of tumor growth in mice treated with PX-478   **ImmunoRegulation**

Tumor size (mean±SEM) of mice in each treatment modality reported in mm² followed till the end of the experiment (a. 4T1; and b. F10 model). *$P<0.05$ (ANOVA, n=3).

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PX-478 administration at 40 mg/kg promotes tumor regression in BALB/c mice

The effect of different dosages of PX-478 on tumor progression was investigated using subcutaneously implanted 4T1 and F10 tumor cell grafts in syngeneic animals. As shown in Figure 2, untreated tumors in 4T1 tumor bearing-mice displayed rapidly progressive growth, whereas tumors in animals treated with 20 and 60 mg/kg PX-478 grew more slowly. The growth of tumors in mice treated with 40 mg/kg PX-478 significantly decreased, compared with the untreated group. PX-478 treatment in mice bearing F10 tumors indicated no significant effects on tumor growth.

Discussion

The hypoxia of solid tumors as a tumor microenvironment hallmark is strongly associated with malignant phenotypes and therapy resistance [15]. HIF-1, the master regulator of the cellular response to hypoxia, is targeted for cancer therapy. Due to the critical role of HIF-1 in cancer progression, there is interest in the discovery of medications that target this molecule [6, 16, 17]. PX-478 is a specific agent that suppresses HIF-1α levels under normoxic and hypoxic conditions in various cancer cell lines [11, 12].

During hypoxia, multiple anti-inflammatory mechanisms might be elicited via HIF-dependent transactivation, like VEGF induction that plays an important role in tumor angiogenesis and immune escape [18, 19]. Another example is the induction of extracellular adenosine pathway that contributes to Regulatory T cells (Treg) differentiation and function [20-22]. Another mechanism is the enhanced suppressing effect of tumor Myeloid-Derived Suppressor Cells (MDSCs), that involves HIF-1α-mediated induction of PDL-1 on these cells [23]. Moreover, upon TCR activation, CD4+ T cells upregulate Foxp3 expression in a HIF-1α and Transforming Growth Factor (TGF)-β-dependent manner; thereby differentiate to Tregs. The differentiated Tregs demonstrate defects in their inhibitory functions in the absence of HIF-1α [13].

The present study explored the effects of HIF-1α blockade by PX-478 on tumor growth, as well as the tumor expression of VEGF and Foxp3 genes. When used at 40 mg/kg, PX-478 was particularly effective in the breast cancer model. This is because it led to a marked reduction in tumor growth in correlation with a significant decrease in HIF-1α mRNA. However, with no significant effect on HIF-1α expression, the drug was not effective on the F10 tumor model. This finding indicates that tumors induced by F10 cells might be resistant to HIF-1 inhibition, or the selected range of drug concentration is not effective on the tumors induced by this

cell line. Prior research also reported that the effectiveness of PX-478 is positively correlated with HIF-1 expression in tumors [12]. The microenvironment of the melanoma or the administered drug dose might influence the effectiveness of PX-478 on HIF-1α inhibition. Moreover, original studies were performed using higher dosages of this drug.

We also investigated the expression of VEGF and Foxp3 genes that are directly controlled by HIF-1α. The treatment of BALB/c mice with 40 mg/kg PX-478 significantly reduced Foxp3 expression. However, an unexpected observation was that PX-478 indicated no significant effect on VEGF expression in tumor tissues obtained from the treated mice. Various studies also emphasized the role of HIF-1α in tumor progression and angiogenesis by macrophages. Doedens et al. indicated that VEGF is a HIF-1α regulated gene [24].

The targeted deletion of HIF-1α in myeloid cells failed to change VEGF-A levels or tumor vasculature. The tumors, however, exhibited reduced progression attributed to their escape from immune suppression. The reduced expression of tumoral Foxp3 observed in the present study might be an indication of impairments in the number of Treg cells or their function. However, another study reported that HIF-1α promotes Foxp3 degradation in the proteasome [25], emphasizing the determinant role of different microenvironments in the effects of HIF-1 on Foxp3, and Treg function. The precise effect of hypoxia on Tregs in the tumor microenvironment remains largely unexplored.

Our study revealed that HIF-1α inhibition using PX-478 is highly effective on the mouse models of breast cancer. PX-478 treatment significantly reduced Foxp3 expression and decreased tumor growth in a dose-dependent manner in the breast carcinoma model, when given as monotherapy for just 3 days.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; Moreover, They were allowed to leave the study whenever they wish, and if desired, the results of the research would be available to them.

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Authors contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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