Determination of Inhibitory Effects of Newly Synthesized Potential HIF Inhibitors on Non-Small Cell Lung Cancer Under Hypoxic Conditions

Yeni Sentezlenen Potansiyel HIF İnhibitörlerinin Hipoksik Koşullarda Küçük Hücre Dışı Akciğer Kanseri Üzerindeki İnhibitör Etkilerinin Belirlenmesi

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Öz

Küresel tahminlere göre her yıl akciğer kanseri nedeniyle 2,3 milyon yeni vaka ve 1,8 milyon ölüm yaşanmaktadır. Tanı ve tedavideki son gelişmelere rağmen devam eden zorluklar, akciğer kanseriyle etkili bir şekilde mücadele etmek için yeni tedavi yöntemlerine ve yenilikçi yaklaşımlara olan acil ihtiyacı vurgulamaktadır. Dolayısıyla, bu çalışmada HIF-1α'nın potansiyel inhibitörleri olan 7a ve 7b bileşiklerinin antikanser özelliklerinin araştırılması amaçlanmıştır. Çalışmada HTB-54 ve BEAS-2B hücre hatları kullanılmıştır. Yeni sentezlenen HIF inhibitörleri 7a ve 7b'nin normoksik ve hipoksik koşullar altında hücre canlılığı üzerindeki etkisini belirlemek için MTT hücre canlılığı deneyleri yapılmıştır. HIF1A'nın kantitatif ekspresyon seviyeleri, Real-Time PCR yöntemi ile belirlenmiştir. HTB-54 hücrelerinde 7a bileşiğinin yarı maksimum inhibitör konsantrasyonu (IC_{50}) normoksik koşullar altında 10,37 µM iken hipoksik koşullar altında 10.63 µM olarak bulunmuştur. HTB-54 hücrelerindeki diğer bir HIF inhibitörü 7b'nin IC_{50} değeri normoksik koşullar altında 8,80 µM, hipoksik koşullar altında ise 9.54 µM olarak bulunmuştur. Hipoksik koşullarda, 7a ve 7b bileşikleri ile maruz bırakılan hücrelerde HIF1A ekspresyon düzeyinin kontrol grubuna göre daha düşük olduğu gösterilmiştir. Normoksi koşullarda ise HIF1A ekspresyon düzeyi 7a bileşiği ile maruz bırakılan hücrelerde kontrol grubuna göre 6,5 kat ($p<0.0001$), 7b'ye maruz kaldığında ise yaklaşık 9 kat ($p<0.0001$) arttığı bulunmuştur. Sonuç olarak hem 7a hem de 7b bileşikleri akciğer kanserine yönelik gelecekteki terapötik girişimler için büyük umut vaat etmektedir.

Anahtar Kelimeler: Akciğer Kanseri, HIF inhibitörleri, Hipoksi

Abstract

According to global estimates, there are 2.3 million new cases and 1.8 million fatalities due to lung cancer each year. Despite recent progress in diagnosis and treatment, persistent challenges highlight the urgent need for novel therapeutics and innovative approaches to combat lung cancer effectively. Accordingly, in the present study, we aimed to investigate the anticancer properties of potential inhibitors of HIF-1α, compound 7a and 7b. In the study, HTB-54 and BEAS-2B cell lines were used. MTT cell viability experiments were performed to determine the effect of newly synthesized HIF inhibitors 7a and 7b on cell viability under normoxic and hypoxic conditions. Quantitative expression levels of HIF1A were determined by real-time PCR approach. While the half maximum inhibitory concentration (IC_{50}) of compound 7a in HTB-54 cells was 10.37 µM under normoxic conditions, it was found to be 10.63 µM under hypoxic conditions. The IC_{50} value of another HIF inhibitor 7b in HTB-54 cells was found to be 8.80 µM under normoxic conditions and 9.54 µM under hypoxic conditions. The expression level of HIF1A was found to be lower in cells exposed to compounds 7a and 7b under hypoxia compared to the control group. Conversely, in normoxia, HIF1A expression level in cells exposed to compound 7a increased 6.5-fold ($p<0.0001$) compared to the control group, while it was found to increase approximately 9-fold ($p<0.0001$) when exposed to 7b. Consequently, both compound 7a and 7b holds great promise for future therapeutic interventions to lung cancer.

Keywords: Lung Cancer, HIF Inhibitors, Hypoxia

Introduction

The worldwide incidence of lung cancer is estimated to be approximately 1.86 million new cases, and the associated death rate is around 1.6 million annually (1-2). The majority of cases of lung cancer (85%) are non-small cell lung cancer (NSCLC). More than 50% of patients are diagnosed at an advanced stage and have a poor prognosis (2-4).

Current treatment strategies in NSCLC include surgical resection, chemotherapy, radiotherapy, targeted therapy, or combinations of these treatments (5). Many cancer cases are diagnosed at a late stage, which unfortunately reduces the chance for surgical treatment. As a result, chemotherapy and radiotherapy are the primary treatment options for managing non-small cell lung cancer (NSCLC). Currently, cisplatin and carboplatin are commonly used as the first-line chemotherapy agents for treating various solid tumors, including lung cancer. Nevertheless, the development of resistance to cisplatin over time remains a significant challenge,

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impacting the prognosis and survival of patients with the disease (3,5,6). Hence, the development of novel drugs that target specific oncogenic mechanisms is crucial for effectively treating patients with NSCLC. Instead of focusing exclusively on the malignant cells themselves, the tumor microenvironment (TME) contributes significantly to the emergence of drug resistance and the dysfunction of immune system cells in the course of cancer treatment. (7). Studies have shown that TMD in solid tumors supports the progression and metastasis of cancer through various mechanisms including drug resistance. Studies have also highlighted the critical role of the tumor microenvironment (TME) in driving the progression of non-small cell lung cancer (NSCLC) (2). The rapid and uncontrolled growth of tumors leads to the expansion of tumor tissue. However, within the tumor, certain regions experience chronically reduced oxygen levels due to inadequate blood vessel development. This persistent and uninterrupted hypoxic state is a common characteristic of the microenvironment in almost all solid tumors (2,7,8). The increased level of HIF protein in hypoxic TME activates the transcription of a number of genes, resulting in an acidic tumor environment. Decreased extracellular pH inhibits cellular and humoral immune functions, leading to drug resistance by multiple mechanisms, including reduced apoptotic potential, genetic alterations, and high activity of the multidrug transporter P-glycoprotein (P-gp) (7-9).

New selective HIF inhibitors were synthesized for lung cancer in our project, which was previously funded by TÜBITAK (114Z960). These 34 compounds are new chiral molecules designed by combining benzoxadiazole and sulfonamide on the same molecule. All compounds were examined on the A549 alveolar epithelial lung cancer cell line for their in vitro cytotoxic activities, apoptotic effects, and effects on the HIF gene. As a result, several compounds have been designed which inhibit the HIF gene and induce apoptosis in cancer cells. The compounds 7a and 7b, whose HIF potential was determined in the previous study, are enantiomers of one another and were selected for this study.

For the study, a hypoxic environment was artificially induced using cobalt chloride ($CoCl_2$) and increased HIF-1α gene levels were validated using the RT-PCR method. The potential cytotoxic effects of compounds 7a and 7b on NSCLC cell HTB-54 under hypoxia/normoxia conditions were determined by cell viability assays.

Material and Method

Cells culture conditions

Commercially, ATCC provided HTB-54 non-small cell lung epidermoid carcinoma and BEAS-2B bronchial epithelial cells. The medium was Roswell Park Memorial Institute 1640 (RPMI-1640; Hyclone, USA), which contained 1% penicillin-streptomycin-amphotericin B solution (Sigma Aldrich, USA), 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), and 2 Mm L-glutamine (Sigma Aldrich, USA). Cells were kept at 37°C in an incubator with 95% humidified CO_2 (5%) (10).

$CoCl_2$-induced chemical hypoxia mimetic model

HTB-54 and BEAS-2B cell lines were seeded in 12-well culture dishes at $2x10^5$ cells/ml and incubated overnight. For hypoxic conditions, cells were exposed to SF, 50,100, 200 and 400 µM doses of $CoCl_2$ prepared in serum-free RPMI-1640 medium for 3, 6, 12 and 24 hours as previously described (11-12). Cells were not exposed to $CoCl_2$ under normoxic conditions. At each time interval, cells were collected using Trypsin-EDTA solution for RNA isolations.

Cell viability assay

MTT (3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability test was performed to evaluate cell viability. Briefly, human HTB-54 and BEAS-2B cells were seeded into 96-well plates at approximately $6x10^5$ cells/ml. Under hypoxic and normoxic conditions, cells were exposed to compound 7a and 7b at varying concentrations (6.25, 12.5, 25, 50 and 100 µM) for 24 hours. Following incubation, cells were rinsed with PBS and treated with 1mg/ml MTT for 60 minutes. Then, MTT solution was discarded and formed formazan crystals were solubilized using 100 µl DMSO. Plates were read at 550 nm with the help of Multiskan GO spectrophotometer (Thermo Scientific). The obtained optical density (OD) values were used to calculate half maximum inhibitory concentration (IC_{50}) of hypoxia inhibitors 7a and 7b.

RNA isolations, cDNA synthesis and qPCR

For the RNA isolations, Qiagen RNeasy mini kit (Qiagen, Germany) was used. Briefly, cells were lysed using buffer RLT supplemented with β-mercaptoethanol. Lysate was further mixed with ethanol and transferred to spin columns. Columns were then rinsed with wash buffers and RNA eluted using ddH2O. RNA quality and quantity was determined using NanoDrop ND1000 spectrophotometer. Complementary DNA (cDNA) synthesis was achieved using RT^2 HT First Strand (Qiagen, Germany) according to the recommendations of the manufacturer. RNA concentrations were adjusted to 100 ng/□L for cDNA synthesis. For the qPCR analysis, QuantiTect SYBR green PCR kit was used. Gene-specific primer pairs for HIF-1α (Forward: 5'-GATCACCCTCTTCGTCGCTT -3', Reverse: 5'-CCTCCATGGTGAATCGGTCC- 3') and GAPDH (Forward: 5'- GATCATCAGCAATGCCTCCT -3', Reverse: 5'- TGTGGTCATGAGTCCTTCCA - 3') were designed using Primer Blast. For the following

PCR reaction was prepared; 10 µL SYBR Green Master Mix, 1 µL forward primer, 1 µL reverse Primer, 5 µL RNase-free water and 3 µL cDNA. Reactions were held in Rotor-Gene Q instrument using following cycling conditions; 15 minutes at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C. After the reaction, Ct (cycling threshold) values were taken at an appropriate threshold. The $2^{-\Delta Ct}$ formula was used to determine gene expressions ($\Delta Ct= (Ct_{HIF1A} – Ct_{GAPDH})$.

Statistical analysis

At least three times each of the experiments were repeated. All data were statistically analyzed with GraphPad Prism version 8.0 (GraphPad, San Diego, CA, USA). Shapiro-Wilk test and Kolmogorov-Smirnov test were used for normalization of the data. For multiple group comparisons, ANOVA or Kruskal-Wallis test was used according to normality of distribution, And t-test was used for pairwise group comparisons. The mean ± SD (standard deviation) and median (25%, 75%) values of the results were calculated. Differences between groups were considered statistically significant when the p value was <0.05.

Results

$CoCl_2$ was used to establish the mimetic hypoxia model. Cells were exposed to different concentrations of $CoCl_2$ for different time periods and HIF1A expression level was determined. Significant increase in expression level of HIF1A gene was detected in both cells after exposure with 400 µM $CoCl_2$ for 3 h and hypoxia conditions were determined (p<0.0001) (Figure 1).

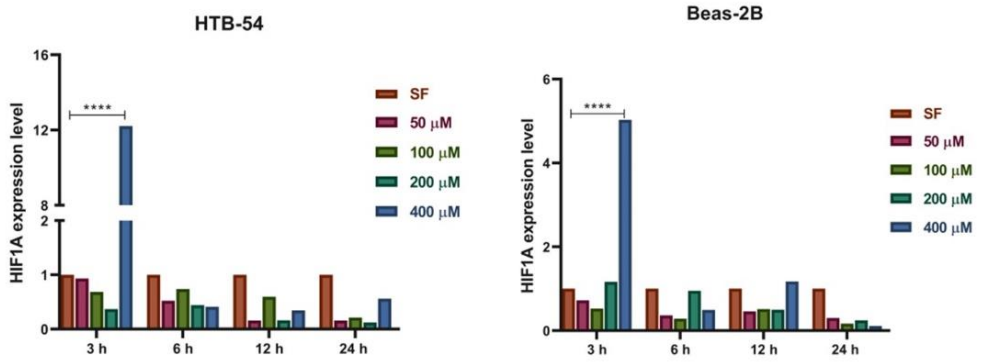

Figure 1. The expression level of HIF1α was demonstrated in HTB-54 and Beas-2B cells by exposure to $CoCl_2$ at different periods and different concentrations. **** p<0.001. One Way ANOVA test was used to analyze the HIF1A expression level in each time period.

MTT experiments were performed to determine the effect of newly synthesized HIF inhibitors 7a and 7b on cell viability under normoxic and hypoxic conditions. In HTB-54 cells, the IC_{50} value of compound 7a was found to be 10.37 µM (95% CI: 5.73-17.96; p=0.0047) under normoxic conditions, while it was 10.63 µM (95% CI: 5.24-18.93; p=0.0052) under hypoxic conditions (Figure 2A). In addition, the IC_{50} value of compound 7a in BEAS-2B cells was 24.89 µM (95% CI: 15.80-34.01; p=0.0011) under normoxic conditions, while it was 33.32 µM (95% CI: 17.12-61.29; p=0.0037) under hypoxic conditions. (Figure 2B). The IC_{50} value of another HIF inhibitor 7b in HTB-54 cells was 8.80 µM (95% CI: 5.78-13.12; p=0.0053) under normoxic conditions and 9.54 µM (95% CI: 5.73-15.05; p=0.0067) under hypoxic conditions. found (Figure 3A). In BEAS-2B cells, it was found to be 22.01 µM (95% CI: 15.21-29.35; p=0.0013) under normoxic conditions and 31.27 µM (95% CI: 17.89-50.69; p=0.0044) under hypoxia (Figure 3B).

qPCR experiments were performed to determine the effect of the effective dose of HIF inhibitors 7a and 7b on the expression level of the HIF1A gene in HTB-54 lung cancer cells under normoxic and hypoxic conditions. The expression level of HIF1A was found to be lower in cells exposed to compounds 7a and 7b in hypoxia compared to the control group. Conversely, in the normoxia condition, the expression level of HIF1A in cells exposed to compound 7a increased 6.5-fold compared to the control group (p<0.0001), while it was found to increase approximately 9-fold when exposed to 7b (p<0.0001) (Figure 4).

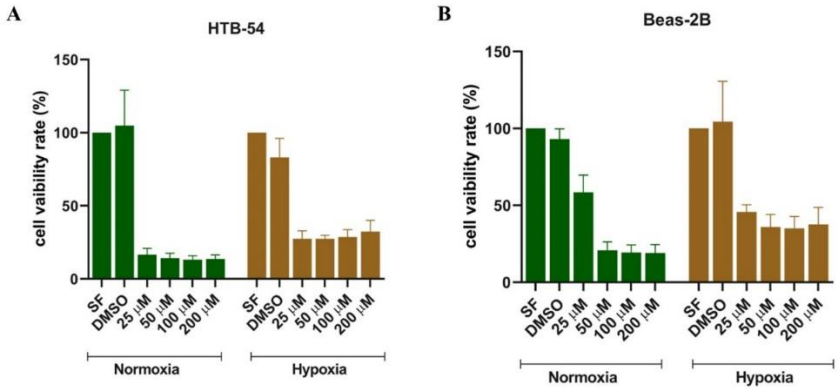

Figure 2. The effect of compound 7a on cell viability was demonstrated under normoxic and hypoxic conditions in HTB-54 (A) and Beas-2B (B) cells. Kruskal-Wallis test was used for the statistical evaluations.

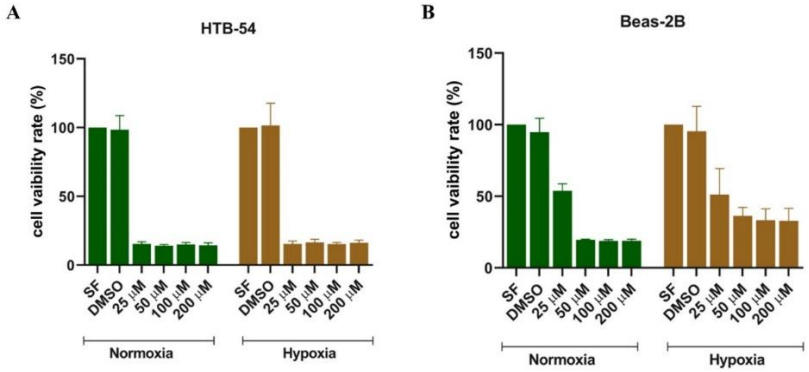

Figure 3. The effect of compound 7b on cell viability was demonstrated under normoxic and hypoxic conditions in HTB-54 (A) and Beas-2B (B) cells. Kruskal-Wallis test was used for the statistical evaluations.

Discussion

In recent years, with the clearer understanding of the HIF-1 pathway in the pharmaceutical industry, researchers have turned to genetic manipulations to increase or decrease the cellular HIF-1 gene level. The HIF gene has become an attractive target, especially since it can lead to non-invasive treatments for diseases such as ischemia and cancer (13). Hypoxic TME plays an active role in the proliferation of cancer cells by reprogramming the cellular energy metabolism through regulating the transition to anaerobic metabolism within tumor cells (7). Increased HIF expression is a sign of a poor prognosis in many cancers (14). Therefore, it is thought that the progression of cancer can be suppressed by inhibiting the HIF1 gene under hypoxic conditions. Therefore, it is of great interest to develop novel chemical inhibitors of HIF1 expression (15). Cobalt or nickel are frequently used to provide a hypoxic environment in vitro hypoxic cellular study. Since cobalt (II) and Nickel (II) ions in cells remove iron from the active sites of 2OG hydroxylases, it is thought to increase HIF-1 activity and provide in vitro hypoxic TME (16).

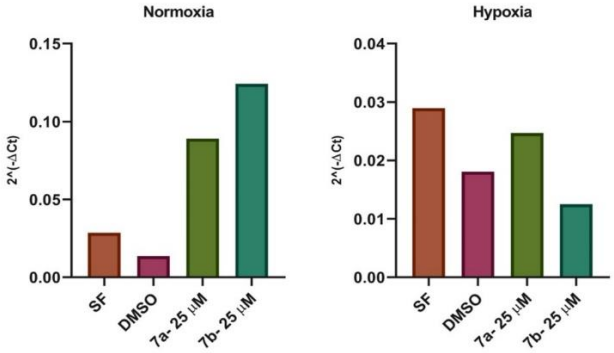

Figure 4. The changes in HIF1α expression level after exposure of HTB-54 cells with 25 µM 7a and 25 µM 7b under normoxia and hypoxia conditions are shown. t-test was used to analyze the HIF1A expression level.

Clinical and preclinical research on HIF inhibition is still in progress, and drug development studies focusing on the HIF signaling pathway have shown that these medicines have very high anticancer potential. Recently, arylsulfonamide has been identified as a new group of pharmacophores with high HIF-1α inhibitory activity in a cell-based study (17). Another study discovered that benzoxadiazole-containing drugs rapidly and specifically linked to HIF-2a (18). The sulfonamide structure, which was found to be an inhibitor of HIF-la, and the benzoxadiazole core, which is an inhibitor of HIF-2a, were combined to create new HIF inhibitors as 7a and 7b compounds.

In our study, compounds 7a and 7b, which are novel potential HIF inhibitory molecules (19), showed cytotoxic effects on the lung cancer cell HTB-54 at low doses (7a: 10.37 and 7b: 10.63 μM). The fact that they do not affect healthy cells at the determined IC_{50} doses makes them promising candidate anticancer agents for lung cancer chemotherapy. In hypoxic conditions, the decrease in oxygen level together with the increase of HIF gene expression much above normal accelerates the development of tumor cells from the vessels (20). Therefore, targeted small molecule synthesis increases in chemotherapy. Our medical study, it is tried to suppress the increased HIF gene expression in cancer with newly synthesized 7a and 7b compounds. The most crucial aspect of this, however, is that there won't be a mechanism by which these inhibitors may control or affect healthy cells because healthy cells, unlike cancer, won't have an excessive increase in the HIF gene. As a result, we believe that cancer cells will have less of an impact on healthy cells. Notably, both compounds were found to increase HIF gene expression under normoxia conditions and suppressed the increased cellular HIF level under hypoxia conditions compared to control. This suggests that both compounds have the potential to regulate hypoxic TME by suppressing increased HIF expression in lung cancer therapy.

Conclusion

Consequently, herein we showed that two candidate HIF1A inhibitors, 7a and 7b, have strong inhibitory activity against HIF1A under hypoxic conditions. Both compounds were shown to be selectively activated under hypoxic conditions but not under normoxia in lung cancer cells. To better understand the mechanistical details, further comprehensive studies are needed. Particularly, gain of function and loss of function studies of HIF1A will be more valuable. Moreover, further studies utilizing tumor xenograft models and/or genetically or chemically modified mouse models of lung cancer are necessary to gain a comprehensive understanding of the anticancer potential of HIF1A inhibitors 7a and 7b.

Conflict of interest statement

There is no conflict of interest to declare.

Ethics Committee Approval: This study did not involve the use of any human or animal tissues.

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