ACHP Targets the STAT3

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STAT3 is an oncogenic transcription factor that regulates the expression of genes which are involved in malignant transformation. Aberrant activation of STAT3 has been observed in a wide range of human malignancies and its role in negative prognosis is well-documented. In this report, we performed high-throughput virtual screening in search of STAT3 signaling inhibitors using a cheminformatics platform and identified 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile (ACHP) as the inhibitor of the STAT3 signaling pathway. The predicted hit was evaluated in non-small cell lung cancer (NSCLC) cell lines for its STAT3 inhibitory activity. In vitro experiments suggested that ACHP decreased the cell viability and inhibited the phosphorylation of STAT3 on Tyr705 of NSCLC cells. In addition, ACHP imparted inhibitory activity on the constitutive activation of upstream protein tyrosine kinases, including JAK1, JAK2, and Src. ACHP decreased the nuclear translocation of STAT3 and downregulated its DNA binding ability. Apoptosis was evidenced by cleavage of caspase-3 and PARP with the subsequent decline in antiapoptotic proteins, including Bcl-2, Bcl-xl, and survivin. Overall, we report that ACHP can act as a potent STAT3 signaling inhibitor in NSCLC cell lines.

Keywords: ACHP ; STAT3 signaling inhibitor ; NSCLC ; cytotoxicity

1. Introduction

Lung cancer is the second most common type of cancer in both sexes and a leading cause of cancer-related deaths [1][2][3][4]. Non-small cell lung cancer (NSCLC) and small cell lung carcinoma are the two major subtypes, which account for about 80–85% and 10–15% of all lung cancer, respectively [5][6][7][8][9][10]. The development and progression of NSCLC are tightly associated with smoking, exposure to asbestos and radon, drinking of arsenic-contaminated water, family history, and inhalation of carcinogens, such as beryllium, mustard gas, cadmium, nickel, etc. [11]. Surgical approaches, such as segmentectomy, sleeve resection, lobectomy, pneumonectomy, and non-surgical approaches, including radiation therapy, chemotherapy, and immunotherapy, have been implemented as the treatment strategies in NSCLC [12][13]. The early diagnosis and treatment of NSCLC can contribute to better survival rates and prognosis.

Signal transducer and activator of transcription (STAT) is a family of cytoplasmic transcription factors comprising of seven variants (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). STAT3 is a latent oncogenic protein transiently activated in various types of normal cells [14][15][16][17][18]. The stimulation of transmembrane receptors by cytokines (IL-6 family members) or growth factors (EGF and HGF) lead to the activation of a non-receptor tyrosine kinase, such as Src and Janus kinase (JAK). The activated upstream kinases phosphorylate STAT3 at Tyr705 to undergo dimerization, and translocation into nucleus to transcribe the genes that are involved in proliferation (cyclin D1/E1), inflammation (COX2, IL-1/6 and M-CSF), antiapoptosis (survivin and Bcl-xL), angiogenesis (VEGF, bFGF, and HIF1α), metastasis (MMP2/9), and tumor evasion (IP-10 and RANTES) [19][20][21][22]. Overactivation of STAT3 has been associated with chronic inflammation, which drives the transformation of healthy to cancerous cells [23][24][25][26][27][28][29][30]. Of note, persistent activation of STAT3 has been observed in various types of solid (lung, liver, prostate, breast, head and neck, and gastric) and hematological (leukemia, lymphoma, multiple myeloma) malignancies [31][32][33][34][35]. Given the relevance of STAT3 signaling in oncogenesis, abrogation of the STAT3 signaling cascade may be useful to counteract diverse malignancies.

In an attempt to identify new STAT3 signaling inhibitors, we performed high-throughput virtual screening (HTVS) of a library of small molecules using a cheminformatics platform and identified 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile (ACHP) as the lead inhibitor of STAT3. We further tested a predicted lead compound against lung cancer cells for possible STAT3 signaling inhibitory activity and it was found to have pronounced inhibition of the signaling cascade.

2. Discussion

The therapeutic efficacy of the blockade of the STAT3 signaling pathway in cancers has been extensively studied, and a number of STAT3 inhibitors have been developed [36][37][38][39]. Hereby we determined the cytotoxic effect of ACHP on the panel of NSCLC cells and found that ACHP possesses a good cytotoxic effect on the tested cancer cell lines. ACHP was found to mediate its cytotoxicity by abrogating the STAT3 signaling pathway. ACHP is a piperidinyl nicotinonitrile derivative, which was initially identified as a selective inhibitor of IKK-β with good aqueous solubility, cell permeability, and oral bioavailability profile in mice and rats [40][41]. In addition, ACHP has also been demonstrated to show inhibitory action towards other kinases, such as IKK-α (IC_{50}: 250 nM), IKK3, Syk, and MKK4 (IC_{50} > 20 μM) [42]. Previous studies also suggest that ACHP exhibited cytotoxicity in adult T-cell leukemia and multiple myeloma cells by interfering with NF-κB signaling [41][43]. The activation of NF-κB, in addition to controlling tumorigenesis [44][45][46][47][48], plays a key role in the induction of fibrosis and ACHP displayed strong antifibrotic effects by suppressing the TGFβ1-induced differentiation of fibroblasts into myofibroblasts and collagen synthesis [49].

Recently, ACHP has been reported to block NF-κB signaling in mouse and human keratinocytes and inhibit multiple sources of cutaneous inflammation in mouse skin [50]. Besides, persistent activity of NF-κB and STAT3 has been linked with oncogenesis [51][52], and abrogation of either of these pathways may not lead to significant cytotoxicity [53]. In addition, a small molecule inhibitor (JSI-124 or cucurbitacin I) of STAT3 signaling was reported to activate the NF-κB pathway [54]. Therefore, it may be an effective strategy to have a dual inhibitor of STAT3 and NF-κB pathways to induce potent cytotoxicity [55]. Similarly, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1) is a synthetic small molecule that has been reported as an ATP-competitive selective inhibitor of IKK2 [56], and subsequent discoveries presented TPCA-1 as a direct dual inhibitor of STAT3 and NF-κB that effectively regresses mutant EGFR-associated human NSCLC [53]. Herein, we performed HTVS of small molecules bearing various scaffolds against STAT3 inhibition and identified ACHP as the lead hit. The predicted target was experimentally validated in NSCLC cellular models.

Overexpression of STAT3 has been reported to potentiate growth, survival, and chemo- and radio-resistance of NSCLC [57][58] and human squamous cell carcinoma cells [59][60]. A significant correlation was found between STAT3 protein expression and tumor differentiation, clinical stage, and lymph node metastasis of NSCLC patients [61]. Notably, the five-year overall survival rate of patients with low STAT3 expression was significantly higher than that of patients with high STAT3 expression indicating the role of STAT3 as a prognostic marker [61]. In addition, several studies have demonstrated the persistent phosphorylation of STAT3 in 22–65% of NSCLC [57], and the deregulation of STAT3 has been associated with malignant transformation [62][63]. The phosphorylation of Tyr705 is a critical event in regulating the transcriptional activity of STAT3, and mitigation of phosphorylation can result in a decline in the STAT3 nuclear pool [64]. Therefore, blocking of nuclear translocation of STAT3 by inhibiting its phosphorylation could be a therapeutic approach. In addition, earlier studies have also demonstrated that STAT3 is present in phosphorylated, as well as in unphosphorylated form in the endosomes. This also suggested that, in addition to the signal transducer role, the membrane-associated cytoplasmic STAT3 may also have a role in STAT3 metabolism [65].

In our study, ACHP was found to significantly inhibit the phosphorylation of STAT3 at Tyr705, which was evidently demonstrated using multiple approaches, and the molecular mechanism by which the ACHP inhibits STAT3 signaling in NSCLC cells has been studied. In addition to the downregulation of phosphorylation, we also noticed the significant deprivation of nuclear STAT3 levels and reduction in DNA binding activity, which is evidence for the decline in the transcription of STAT3 driven genes. Furthermore, ACHP was found to reduce the cell viability of the tested cell lines and we speculated the cytotoxic effect is due to inhibition of STAT3 signaling. To verify this, we knocked down STAT3 using siRNA and tested the effect of ACHP on cell viability of A549 cells. We observed minimal effect on the viability of STAT3-depleted A549 cells, thus indicating the absence of off-target effects.

STAT3 protein can be positively modulated by phosphorylated upstream protein tyrosine kinases, such as Src (Tyr416) and JAK (JAK1: Tyr1022/1023; and JAK2: Tyr1007/1008) [66]. We observed a substantial decrease in the phosphorylation of Src and JAKs. H1299 cells lack the constitutive activity of STAT3 signaling, and we observed the phosphorylation of Src, JAK1, and JAK2 on treatment with IL-6. It is noteworthy that ACHP has been previously demonstrated to exhibit inhibitory activity towards serine/threonine kinases (IKK-α/β), as well as tyrosine kinase (Syk) [42]. In the present effort, we have explored another cellular target kinase of ACHP. Furthermore, ACHP treatment suppressed the IL-6 induced activation of these cascades of proteins in H1299 cells. Activation of executioner caspase (caspase 3/7) is the major biochemical event associated with the cells committed to apoptosis [67], and the activated caspase-3 cleaves PARP to induce apoptosis [68][69].

We noticed that ACHP induced the activation of caspase-3 and cleavage of PARP. Evidently, we also observed the negative modulation in the expression of apoptosis modulators such as Bcl-2, Bcl-xl, and cyclin D1. In addition to its role in the activation of oncogenic gene expression, STAT3 has also been demonstrated to repress the expression of tumor suppressor genes to encourage the survival of cancer cells [70][71][72]. In contrast, some of the studies have highlighted STAT3 as a tumor suppressor protein. In one of the early studies, simultaneous shRNA-mediated knockdown of PTEN and deletion of STAT3 showed substantial increase in in vitro proliferation cells and tumor formation in SCID (Severe combined immunodeficient) mice in astrocytes. In parallel, knockdown of PTEN alone with normal STAT3 expression displayed significantly reduced tumorigenic potential, indicating that STAT3 serves as a tumor suppressor in the absence of PTEN [73]. In similar studies, the tumor suppressor functions of STAT3 were found to have relevance with Ras and $p19^{ARF}$ protein expression [74].

In 2018, Caetano et al. developed a lung epithelial-specific K-ras mutant/STAT3 conditional knockout mouse model, and deletion of epithelial STAT3 resulted in sex-associated discrepancies in which K-ras mutant tumors were decreased in female K-ras mutant/STAT3 conditional knockout, whereas tumor burdens were increased in males [75]. These reports spread light on the multifaceted role of STAT3 in oncogenic and tumor suppressor effects. However, our study highlights the constitutive activation of STAT3 in lung cancer cells and ACHP induced cell death via blocking oncogenic STAT3 signaling. We have previously reported several natural compounds that induce their inhibitory activity towards upstream kinases (JAK/Src) in cell-based assays and displayed interaction with the SH2 domain of STAT3 in computational studies. We obtained similar results in the present study and the exact mechanisms through which ACHP can interrupt STAT3 signaling either through interaction with its SH2 domain or attenuation of phosphorylation of upstream kinases requires further investigations. With these shreds of evidence, we have conclusively reported that STAT3 is the additional signaling cascade impeded by ACHP. In summary, our study shows the feasibility of inhibiting a constitutively active STAT3 signaling pathway in NSCLC cells.

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