AZD1480, a JAK inhibitor, inhibits cell growth and survival of colorectal cancer via modulating the JAK2/STAT3 signaling pathway

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Abstract. Interleukin (IL)-6 and the downstream Janus kinase (JAK)/signal activator of transcription (STAT) pathway have been found to be important in the development of colorectal cancer (CRC). To develop novel therapies for CRC, we have explored the effects of a novel small-molecule JAK inhibitor (AZD1480) on IL-6/JAK/STAT3 pathway and its potential anti-tumor activity on the human CRC cell lines (HCT116, HT29 and SW480). The results showed that, AZD1480 effectively prevents constitutive and IL-6-induced JAK2 and STAT-3 phosphorylation and exerted antitumor functional effects by a decrease in proliferation and an increase in apoptosis in CRC cells. The inhibition of tumorigenesis was consistent with the decreased phosphorylated JAK2 and phosphorylated STAT3, and the decreased expression of STAT3-targeted genes c-Myc, cyclin D2 and IL-6. Thus, AZD1480 is a potential new clinical therapeutic agent for patients with CRC.

Introduction

Colorectal cancer (CRC) is a common malignancy and it remains the third leading cause of mortality among all human malignancies (1-3). The risk of CRC development is determined by genetic predisposition combined with environmental influences, such as bacterial infections that disrupt the mucosal barrier of the gastrointestinal tract leading to aberrant inflammation (4). Tumor-associated inflammation contributes to tumor growth and progression through multiple mechanisms including increased cell proliferation and anti-apoptotic signaling, promotion of angiogenesis, tumor immune evasion and metastasis (5,6). Many inflammatory signals promote tumorigenesis by activating nuclear transcription factor κB (NF-κB) and the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathways, both in tumor and stroma cells. Constitutively activated STAT3 and STAT5 are expressed in a wide variety of human malignancies including colorectal carcinomas, and often correlate with a poor prognosis and resistance to therapies.

The JAK/STAT pathway is involved in inflammation, proliferation and invasion/migration (7). Constitutive activation of STAT3, resulting in an unregulated increase in cell proliferation and reduction in cell apoptosis, is strongly correlated with the development of numerous types of cancer including CRC (8). Therefore, inhibiting cell proliferation and/or promoting apoptosis by the suppression of STAT3 activation has been a major focus in the development of anticancer drugs. Patients with CRC often exhibited elevated levels of interleukin (IL)-6 in the serum (9) and constitutively activated STAT3, which is expressed in the majority of colorectal tumors is associated with a significantly higher mortality (10). Recently, it has been demonstrated that the constitutive activation of JAK/STAT signaling is involved in the development of CRC in cell growth, survival, invasion and migration (11), thereby shedding light on new therapeutic strategies for CRC treatment by influencing the IL-6/JAK/STAT3 pathway.

AZD1480, an ATP competitive inhibitor of JAK1 and JAK2, was recently shown to inhibit the growth of tumors including human glioblastoma, myeloma, multiple sclerosis and lung cancer (12-15). AZD1480 inhibited constitutive and IL-6-induced STAT3 activation and subsequent nuclear translocation. The ability of AZD1480 to effectively limit tumor volume was attributed to the inhibition of STAT3. Stuart et al conducted a study with AZD1480 to confer the therapeutic benefits in two murine models of inflammation-associated
gastrointestinal cancer. Their results provide the first evidence that the pharmacologic targeting of AZD1480 affords the therapeutic suppression of inflammation-associated gastrointestinal cancer progression in vivo (16). In the present study, we performed an in vitro study to examine the efficacy and potential antitumor effects of AZD1480 in CRC, which demonstrated a therapeutic benefit for targeting JAK/STAT signaling in CRC.

Materials and methods

Drugs. AZD1480 was provided by Selleckchem (Houston, TX, USA). For the in vitro experiments, AZD1480 was dissolved in 100% DMSO to prepare a 10 mM stock and stored at -20°C. Recombinant human IL-6 and tumor necrosis factor (TNF)-α (purchased from PeproTech) were reconstituted in sterile 1X phosphate-buffered saline (PBS) containing 0.1% BSA to prepare a 10^3 ng/ml stock and stored at -20°C.

Cell lines and cell culture conditions. Human colon carcinoma HCT116, SW480 and HT29 cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and preserved in our institute. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 50 U/ml penicillin and streptomycin at 37°C in an atmosphere of 5% CO2, and passed twice a week.

Western blot analysis. Tissues were homogenized and cells were lysed. Total proteins were extracted by RIPA lysis buffer (Beyotime, Shanghai, China) and equal amounts of protein were electrophoresed on a 12% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skim milk in PBS containing 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated with the following primary antibodies at 4°C overnight: rabbit anti-phospho-JAK2 (Y1007/1008), STAT3, JAK2, PARP, anti-phospho-NF-κB p65 and GAPDH were purchased from Cell Signalling Technology (BSSN, USA). The membranes were then washed three times with Tris-buffered saline Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) at room temperature for 2 h. After three TBST washes, the membranes were disposed, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Rabbit IgG (A0562; Beyotime) was used as a secondary antibody to visualize phospho-STAT3. Nuclei were stained with DAPI.

Immuno fluorescence assay. HT29 cells were cultured with AZD1480 at different doses. Forty-eight hours after being disposed, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Rabbit anti-phospho-STAT3 was used as a primary antibody, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (A0562; Beyotime) was used as a secondary antibody to visualize phospho-STAT3. Nuclei were stained with DAPI.

Results

AZD1480 prevents constitutive STAT3 and JAK2 activation in CRC cells. The inhibitory effect of AZD1480 on JAK/STAT3 signaling in three human CRC cell lines (HCT116, HT29 and SW480) was examined. Treatment of CRC cells with AZD1480 at different doses (0, 0.5, 1, 2, 5 and 10 µM) prevented constitutive STAT3 and JAK2 phosphorylation in the three CRC cell lines for 2 h. Western blot analysis showed that phosphorylated JAK2 and STAT3 were markedly decreased from 1 µM (HCT116) (n=6, P<0.05), 2 µM (HT29) (n=6, P<0.05), and 5 µM (SW480) (n=6, P<0.05) respectively, and lasting for 10 µM (Fig. 1A-C). The results showed that AZD1480 inhibited the constitutive activation of JAK2 and
STAT3 in CRC cell lines. We also determined the effect of AZD1480 on the signaling by immunofluorescence.

Results of recent studies have shown that phosphorylated STAT3 translocates to the nucleus by the treatment of IL-6 (17), thus we explored whether AZD1480 prevented this process. HT29 cells were treated with the indicated doses of AZD1480 for 2 h prior to the 2 h stimulation with IL-6. The cells were fixed and stained by the anti-phosphorylated STAT3 primary antibody and the FITC-conjugated secondary antibody. The nucleus was stained with DAPI. Fig. 1D shows that phosphorylated STAT3 and non-activated STAT3 are almost located in the cytoplasm instead of the nucleus. Thus, STAT3 translocates to the nucleus when activated.

AZD1480 treatment inhibits proliferation and induces apoptosis of human CRC cell lines. Since inhibition of JAK/STAT signaling can decrease proliferation and induce apoptosis of CRC cells (18), we initially detected the effects of treatment with AZD1480 on the proliferation of HCT116, HT29 and SW480 cells. Results of the CCK-8 assays suggested that AZD1480 markedly inhibits the growth of HCT116, HT29 and SW480 cells in a time- and dose-dependent manner (Fig. 2A and B). The concentration for inhibition of proliferation at 48 h was from ~0.5 µM (n=5, P<0.05) and from 1 µM (n=5, P<0.05), respectively. SW480 cells required higher concentrations of AZD1480 at 72 h from ~2 µM (n=5, P<0.05). However, these concentrations of AZD1480 did not significantly alter the proliferation of HCT116, HT29 and SW480 cells at 24 h (data not shown).

Flow cytometry was applied to analyze the apoptotic effect of AZD1480 in the HCT116, HT29 and SW480 cell lines. The data suggested that AZD1480 induces the apoptosis of HCT116, SW480 and HT29 cells in a time- and dose-dependent manner (Fig. 2C-J). The percentage of apoptosis was markedly elevated from 1 µM at 48 h (n=5, P<0.05) and 0.5 µM at 72 h (n=5, P<0.05) of HCT116 cells. In addition, the apoptotic ratio at 48 h of HT29 and SW480 cells increased 23.93% at 2 µM (n=5, P<0.05) and 25.29% at 5 µM (n=5, P<0.05), respectively, while at 72 h the data increased 42% (n=5, P=0.05) and 45.89% (n=5, P<0.05) for HT29 and SW480 cells, respectively, compared with the untreated cells. Western blot analysis was used to assess the effect of AZD1480 inducing human CRC cell death by testing the presence of cleaved PARP. After treatment with AZD1480 of CRC cells for 24 h, the cleavage of PARP was significantly increased from 2 µM (HCT116) (n=6, P<0.05), 5 µM (HT29) (n=6, P<0.05), and 10 µM (SW480) (n=6, P<0.05) respectively, indicating induction of cell death (Fig. 3A-C).
Our results also showed that treatment with AZD1480 was more effective in inhibiting proliferation and inducing apoptosis in vitro.

AZD1480 inhibits IL-6-inducible JAK2/STAT3 signaling pathways. IL-6 is known as an important tumor-promoting cytokine that enhances proliferation and anti-apoptotic effects in tumor cells (11). Moreover, IL-6/JAK/STAT signaling has a critical role in various aspects including initiation, development and formation in CRC (19). Thus, we detected whether AZD1480 attenuated IL-6-induced JAK/STAT signaling by immunoblotting, and subsequently induced antitumor effects in CRC cells. Western blot analysis showed that treatment with AZD1480 decreased the IL-6-induced activation of JAK/STAT in a dose-dependent manner in the three CRC cell lines. However, the protein level of JAK2 and STAT3 did not change after treatment with AZD1480. We also observed that phosphorylated JAK2 and STAT3 were
markedly increased following IL-6 stimulation in HCT116 (n=6, P<0.05), HT29 (n=6, P<0.05), and SW480 cells (n=6, P<0.05) (Fig. 4A-C).

The CCK-8 assay (Fig. 5A and B) showed that AZD1480 inhibited IL-6-induced cell proliferation in HCT116, HT29, and SW480 cells at 48 and 72 h. The concentration significantly changed from ~1, 2 and 5 µM for HCT116 (n=5, P<0.05), HT29 (n=5, P<0.05) and SW480 cells (n=5, P<0.05), respectively, at 48 h, and in the same cell lines the concentration at 72 h was changed from ~0.5 µM (n=5, P<0.05), 1 µM (n=5, P<0.05) and 2 µM (n=5, P<0.05), respectively. CRC cells stimulated with IL-6 showed marked cell proliferation enhancement compared with the untreated cells.

AZD1480 inhibited the survival of the three CRC cell lines in the presence of IL-6, inducing apoptosis in a time- and dose-dependent manner (Fig. 5C and D). The apoptosis ratio at 48 h of HCT116, HT29 and SW480 cells increased 24.03% at 1 µM (n=5, P<0.05), 23.11% at 2 µM (n=5, P<0.05) and 25.01% at 5 µM (n=5, P<0.05), respectively. However, at 72 h the results increased 39.49% at 0.5 µM (n=5, P<0.05), 41.24%...
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at 1 µM (n=5, P<0.05) and 44.22 % at 2 µM (n=5, P<0.05) for HCT116, HT29 and SW480 cells, respectively, compared with the untreated cells stimulated by IL-6.

Furthermore, we investigated the effect of AZD1480 on STAT3 targets in CRC cells by qPCR to confirm whether the inhibition of STAT3 phosphorylation associated with inhibition of downstream gene expression. The data showed that the gene expression of c-Myc, cyclin D2 and IL-6 was markedly decreased from ~1 µM for HCT116 cells (n=6, P<0.05), 2 µM for HT29 cells (n=6, P<0.05) and 5 µM for HT29 cells (n=6, P<0.05). Following IL-6 stimulation, AZD1480 also significantly blocked the IL-6-induced expression of c-Myc (n=6, P<0.05), cyclin D2 (n=6, P<0.05) and IL-6 mRNA (n=6, P<0.05) (Fig. 6A-I). These results correlated with the changes of phosphorylated JAK2 and STAT3.

Effects of AZD1480 on NF-κB pathway. NF-κB pathway plays a crucial role in many steps of CRC initiation and progression (20). Notably, the NF-κB pathway cooperates with other signaling pathways such as the JAK/STAT3 pathway (21). In the present study, we found that AZD1480, as a JAK1/2 inhibitor, suppressed the JAK/STAT pathway, allowing the close association with the NF-κB and JAK/STAT pathways. We also determined the effects of AZD1480 in the NF-κB pathway in HCT116 cells. HCT116 cells were treated with AZD1480 (1 µM) for 2 h followed by treatment with TNF-α.

Figure 7. Effects of AZD1480 on NF-κB pathway. (A) The protein level of phosphorylated NF-κB p65 in HCT116 cell lines was determined by western blot analysis after treatment with the indicated concentrations of AZD1480 for 2 h, followed by stimulation with TNF-α for 30 min. Protein expressions used GAPDH as the loading control. (B) HCT116 cell lines were treated with the indicated concentrations of AZD1480 for 2 h followed by stimulation with TNF-α for 1 h, and quantitative PCR was used (n=6/group). Data show means ± SD.
Our results showed that AZD1480 does not inhibit the TNF-α-induced NF-κB p65 phosphorylation or expression of IL-8 (Fig. 7A and B), an NF-κB driven gene, supporting the absence of pleiotropic effects of AZD1480 on signaling pathways in CRC cells.

**Discussion**

In the present study, we investigated the biologic mechanism of the novel small molecule JAK1/2 kinase inhibitor AZD1480 on human CRC cells. AZD1480 inhibited constitutive JAK/STAT signaling in three established CRC cell lines (HCT116, SW480 and HT29). AZD1480 reduced the expression of several downstream gene targets of STAT3 (c-Myc, cyclin D2 and IL-6). Additionally, AZD1480 exerted antitumor functional effects in CRC cells by a decrease in proliferation and an increase in apoptosis. Antitumor activity of AZD1480 was also observed in CRC cell growth stimulated by IL-6. We found that AZD1480 prevented the IL-6-induced activation of JAK2 and phosphorylation of STAT3. In the three CRC cell lines, the inhibition of tumorigenesis was associated with decreased phosphorylated JAK2 and STAT3, and the decreased expression of the targeted genes c-Myc, cyclin D2 and IL-6. Allowing for the efficacy and potential antitumor effects of AZD1480 in CRC, we may draw the conclusion that AZD1480 demonstrates a promising therapeutic benefit for targeting JAK/STAT signaling in CRC.

The tumor microenvironment possesses a rich source of inflammatory cytokines, among which the IL-6 family, particularly IL-6 and IL-11, are markedly upregulated in many types of cancer and regarded as one of the most important cytokine families during tumorigenesis and progression (22). Furthermore, IL-6 drives many of the cancer ‘hallmarks’ through the downstream activation of the JAK/STAT signaling pathway, which is involved in a poor prognosis in many solid cancers including CRC (16). Cytokine IL-11 also shows a strong correlation with elevated STAT3 activation in many solid cancers including CRC (16). Cytokine IL-11 also shows a strong correlation with elevated STAT3 activation in many solid cancers including CRC (16).

Abnormalities on the level of IL-6-driven JAK/STAT pathways are important in the processes of hyperproliferative and invasive phenotype of CRC cells (24). The JAK/STAT signaling pathway is associated with many types of tumors, and AZD1480, a JAK1/2 kinase inhibitor, has been verified to suppress tumorigenesis, for instance, in metastatic prostate cancer (25), gastrointestinal malignancy (16), hematological malignancies (26), myeloma (13), small cell lung cancer (27), pediatric sarcomas (28) and glioblastoma (12). Stuart et al. found that AZD1480 confers therapeutic benefits in two murine models of inflammation-associated gastrointestinal cancer strictly dependent on excessive STAT3 activation (16), which is consistent with our findings in CRC.

The JAK/STAT signaling pathway intervenes in many aspects of CRC development, such as cell growth, survival, invasion and migration (29,30). Suppression of this pathway is therefore a valuable regulative strategy for CRC. A number of natural products such as resveratrol, flavopiridol and piceatannol were utilized in preclinical trials indicating the ability of inhibiting pathways involved in inflammation, whose mechanisms include the reduction of STAT3 phosphorylation, inhibition of the cytokine production and direct inhibition of the JAK (31). Additionally, the role of JAK inhibition in solid tumors was tested preclinically. The JAK1/2 inhibitor AZD1480 was reported inhibiting tumor development in models of IL-6-driven breast, ovarian and prostate cancers (32). Thus, AZD1480 may be considered potential material for the treatment of cancer including CRC. The findings of the present study are in concordance with a previous study, which suggested a key role of AZD1480 in inhibiting JAK activity to suppress the progression of CRC in vivo (16). The finding of key clinical importance in the present study is that pharmacological targeting of IL-6/JAK/STAT signaling by the JAK1/2 inhibitor AZD1480 effectively suppressed IL-6-induced CRC cell. This finding is also important as JAK1/2 inhibitors are currently under active clinical development for hematopoietic proliferative disorders and malignancies (33,34). Developing therapeutic strategies for selective STAT3 inhibition is challenging. Therefore, targeting of upstream components reveals a pharmacoologically viable alternative; for instance, monoclonal antibodies directed towards IL-6 or its α-chain receptor subunit or small molecule inhibitors for the JAK family. Our results showed marked efficacy of AZD1480 in inhibiting JAK1 and JAK2 to confer a cytostatic effect. AZD1480 inhibited constitutive JAK/STAT signaling in three established CRC cell lines (HCT116, SW480 and HT29). Moreover, AZD1480 suppressed the expression of c-Myc, cyclin D2 and IL-6 which are downstream gene targets of STAT3. Additionally, AZD1480 exerted antitumor functional effects in CRC cells with a decrease in proliferation and an increase in apoptosis. Notably, the antitumor activity of AZD1480 was observed in CRC cells growth stimulated by IL-6.

Genetic alterations lead to numerous aberrant signal transduction pathways, which are closely related to oncogenesis. The JAK/STAT signaling pathway is a major contributor to CRC transformation, and other pathways such as the NF-κB pathway play critical roles in the physiological and pathological processes of CRC. Crosstalk between the JAK/STAT and NF-κB pathways has been verified at multiple levels, including activation of STAT3 which was induced by IL-6 and COX-2, which are NF-κB-induced factors, and STAT3, which accelerates NF-κB processing, leading to pro-apoptotic responses (35) and STAT3 promoting the nuclear translocation of NF-κB (36). Furthermore, in the context of colitis-associated cancer, it has been demonstrated that as an NF-κB regulated cytokine, IL-6 is a critical tumor promoter during early colitis-associated cancer tumorigenesis, and that the proliferative and survival effects of IL-6 are modulated by STAT3, which also plays a vital role in JAK/STAT signaling pathway (37). IL-6 is currently known as an NF-κB pathway-targeted gene (38) particularly in response to TNF-α. The elevated levels of IL-6 detected in many types of cancer have been thought to result from activation of the NF-κB pathway. NF-κB and STAT3 activate IL-6, as well as other genes that promote cell survival, growth, angiogenesis, invasiveness and motility. The complex crosstalk between the NF-κB and JAK/STAT pathways is beginning to be elucidated, and data have shown that the JAK/STAT/NF-κB axis is critical for tumor progression. Given the inter-dependency of the two pathways, inhibitors such as AZD1480 may attenuate NF-κB Activation in vitro in the tumor microenvironment, as well as suppress the JAK/STAT pathway. In the present study, we found AZD1480 does not
inhibit the TNF-α-induced NF-κB p65 phosphorylation or expression of IL-8. These results indicate that AZD1480 shows the absence of pleiotropic effects of AZD1480 on signaling pathways in CRC cells. Thus, AZD1480 specifically affects the JAK/STAT pathway, which is consistent with the findings of McFarland et al (12).

In summary, to the best of our knowledge, the present study provides the first evidence on treatment with AZD1480 through IL-6/JAK/STAT pathway in CRC cells, which confers antitumor effects by inhibiting cancer cell proliferation, differentiation, invasion, inflammation and immune function. Together with the previous findings that AZD1480 inhibits progression of gastrointestinal tumors in vivo, the present findings reveal the underlying mechanisms by which AZD1480 inhibits growth and survival of human CRC cells, suggesting that AZD1480 has a practical clinical use for treating CRC.

Acknowledgements

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References