INCB018424 induces apoptotic cell death through the suppression of pJAK1 in human colon cancer cells

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Janus kinase (JAK) is one of the main upstream activators of signal transducers and activators of transcription (STAT) that are constitutively activated in various malignancies and are associated with cell growth, survival, and carcinogenesis. Here, we investigated the role of JAKs in colorectal cancer in order to develop effective therapeutic targets for INCB018424, which is the first JAK1/2 inhibitor to be approved by FDA. After examining the basal expression levels of phospho-JAK1 and phospho-JAK2, we measured the effects of INCB018424 on the phosphorylation of JAK1/2 using western blot analysis. Cell viability was determined using the trypan blue exclusion assay. The cell death mechanism was identified by the activation of caspase 3 using western blot and annexin V staining. The basal levels of phospho-JAK1 and phospho-JAK2 were cancer cell type dependent. Colorectal cancer cell lines that phosphorylate both JAK1 and JAK2 include DLD-1 and RKO. INCB018424 inactivates both JAK1 and JAK2 in DLD-1 cells but inactivates only JAK1 in RKO cells. Cell death was proportional to the inactivation of JAK1 but not JAK2. INCB018424 causes caspase-dependent cell death, which is prevented by treatment with z-VAD. The inhibition of JAK1 phosphorylation seemed sufficient to allow INCB018424-mediated apoptosis. JAK1 is a key molecule that is involved in colon cancer cell survival and the inhibition of JAK1 by INCB01424 results in caspase-dependent apoptosis in colorectal cancer cells. The use of selective JAK1 inhibitors could be an attractive therapy against colorectal cancer, but further clinical investigations are needed to test this possibility.

Key words: INCB018424, JAK, apoptosis, colon cancer

Colorectal cancer is the third most common cancer and the fourth most common cause of death worldwide with approximately 950,000 newly diagnosed patients each year [1]. The incidence of colorectal cancer is rising steeply as diet, lifestyle, and demographics change. The general therapy for colorectal cancer is surgery, which can be used along with chemotherapy or radiation to treat advanced cancer-stage. These therapeutic protocols however frequently result in the development of resistance in the cancer cells to the treatment. Resistance could be acquired by the results of drug treatment, with the primary risk factors including complex genomic alterations. Furthermore, it has also been reported that signaling interactions between cancer cells and mesenchymal stromal cells induce drug resistance by protecting cancer cells from the effects of therapy [2,3]. One of many different signaling mechanisms associated with this kind of resistance is the activation of signal transducers and activators of transcription (STAT). It has been reported that STAT family proteins are usually implicated in chemotherapy-related resistance [4]. In addition, STAT, especially STAT3, has been identified as a target of EGFR (Epidermal Growth Factor Receptor) inhibitors in large-scale synthetic screens for novel cancer drugs [5]. Several studies have also confirmed that STAT3 is required for the complete cellular transformations that are induced by various oncogenes such as Ras, Src, and EGFR [6-10], which means that the STATs are important for tumorigenesis and cancer progression [11]. For STAT3 activation, tyrosine (Y705) phosphorylation is important and is mediated by the Janus kinase (JAK) protein families [12]. JAKs were first identified as unique tyrosine kinases and play important roles in the proliferation, differentiation, and apoptosis of both normal and cancer cells [13]. JAKs transfer
signals between cytokine and growth factor receptors, sequentially activating various downstream signaling proteins such as STATs [14]. The JAK/STAT signaling pathway is involved in tumorigenesis and cancer progression as much as it is involved in normal physiological processes such as cell growth, survival, development, and immunological processes [15]. This pathway is constitutively activated in myeloproliferative diseases and in various solid tumors, including head and neck, lung, prostate, breast, colorectal, and hepatocellular carcinomas. Although the mechanism of dysregulated JAK/STAT signaling in colorectal cancers has not been elucidated, it has been implied that the JAK/STAT signaling pathway might be an attractive therapeutic target for the treatment of colorectal cancers and other human malignancies [16,17]. In practice, small-molecule inhibitors of the JAK protein received approved for therapeutic uses from the US FDA in 2012 [18].

INCB018424 (Jakari, Ruxolitinib) is a potent JAK1/2 inhibitor, and its activity is exerted through the competitive inhibition of the ATP-binding sites of the JAK kinase. It is approved for the treatment of myelofibrosis based on a phase II clinical trial that demonstrated that INCB018424 decreases circulating inflammatory cytokines and improves clinical manifestations [19]. Clinical studies on solid tumors, such as breast and pancreatic cancer, have been processed but INCB018424 is generally applied to limited types of cancer such as leukemia and myelofibrosis.

In our present study, we report for the first time the effects of INCB018424 when targeting JAK1/2 in colorectal cancers. Our data show that INCB018424 demonstrates different inhibitory activities against JAK1 and JAK2 depending on the colorectal cancer cell type. In addition, INCB018424-mediated JAK1 inhibition is sufficient to cause apoptotic cell death in colorectal cancer cells.

Materials and methods

Cell lines, reagents and antibodies. Human colorectal cancer cell lines DLD-1, HCT116, HT29, and RKO were purchased from the American Type Culture Collection (Manassas, VA, USA). Human colonic epithelial cells were provided from SciencCell Research Laboratories (Carlsbad, CA, USA). HCT116, HT29, and RKO cells were grown in Dulbecco’s modified Eagle’s medium (Gibco BRL;Grand Island, NY, USA), and DLD-1 cells were maintained in RPMI 1640 medium (Gibco-Invitrogen, Carlisle, CA, USA) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2 mM glutamine. Human colonic epithelial cells were maintained with human colonic epithelial cell medium (Sci- enCell Research Laboratories). All media were supplemented with 10% fetal bovine serum. The cells were grown at 37°C in a 5% CO₂ atmosphere. INCB018424, a specific JAK1/2 inhibitor, was purchased from SelleckChem (Houston, TX, USA), dissolved and diluted in dimethyl sulfoxide (DMSO;Sigma, St Louis, MO, USA), and stored at -20°C. z-V AD-fmk, a pan-caspase inhibitor, was obtained from R&D Systems (Minneapolis, MN, USA). Primary antibodies against the following proteins were used in the western blot analyses: anti-phospho-JAK1 (Y1003/1004), anti-phospho-JAK2 (Y1007/1008), anti-JAK1, anti-JAK2, anti-caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blot analysis. After treatment with INCB018424 for 48 hours at the indicated doses, protein expression was measured using western blot analysis. Briefly, whole cell lysates (20 µg) were subjected to SDS-PAGE and transferred to PVDF membranes (New England Nuclear, Boston, MA, USA). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and probed with the primary antibodies. After washing with the TBS-T buffer, the cell lysates were incubated with a horseradish peroxidase-coupled secondary antibody. Protein expression was visualized using an enhanced chemiluminescence system (Amer sham, Buckinghamshire, UK).

Cell viability assay. Cells in 60 mm plates were treated with INCB018424 at the indicated doses. After treatment with INCB018424 for 48 hours, cell viability was determined using the trypan blue exclusion method and MTS assay (Promega Inc, Madison, WI, USA). IC₅₀ of INCB018424 was calculated with GraphPad prism ver 5.01 software.

Annexin-V staining. Cells were pretreated with z-V-AD for one hour and then treated with 25 µM INCB018424 for 48 hours. Cells were fixed in 3.7% paraformaldehyde for 10 minutes at room temperature and then washed twice with PBS. Cells were stained with annexin-V-FITC (fluorescein isothiocyanate) at room temperature for two hours and counterstained with 4’,6-diamidino-2-phenylindole (DAPI). The stained cells were observed using an EVOS inverted fluorescence microscope (AMG, Bothell, WA).

Statistical analysis. All values are shown as mean ± SD. Two side t-test was used to calculate the P value and P<0.05 was considered significant.

Results

INCB018424 exerts its effects by suppressing JAK1 phosphorylation (rather than JAK2) in colorectal cancer cell lines. It has been reported that INCB018424 is generally effective against both JAK1 and JAK2 for the treatment of myeloma [20]. To study the inhibitory effects of INCB018424 on JAK activity in colorectal cancer in our present analyses, we first examined the endogenous phosphorylation status of JAK1 and JAK2 in various colorectal cancer cell lines by western blot analysis. The colorectal cancer cell lines we examined were found to phosphorylate JAK1 and JAK2 differently, in addition to different JAK1 and JAK2 expression levels (Fig. 1). Very low or zero JAK1 and JAK2 phosphorylation in HCT116 cells and JAK1 phosphorylation in HT29 cells was observed. The phosphorylation of both JAK1 and JAK2 was detected only in DLD-1 and RKO cells. Based on this information, we selected these two cell lines to deter-
To further clarify the inhibitory effects of INCB018424 on the JAK1/2 signaling pathways of colorectal cancers, we treated DLD-1 and RKO cells with different concentrations of INCB018424 (Fig. 2). The phosphorylation of JAK1 was inhibited in a dose-dependent manner in both colorectal cancer cell lines when treated with INCB018424. The phosphorylation of JAK1 in DLD-1 and RKO cells was almost inhibited by 25 µM INCB018424, however INCB018424 differently inhibited the phosphorylation of JAK2 in these two cell lines. Treatment with INCB018424 blocked the phosphorylation of JAK2 in DLD-1 cells; likewise, JAK1 was inhibited but not in RKO cells. Even though we treated these cells with 25 µM INCB018424, JAK2 phosphorylation in RKO cells did not decrease following the addition of INCB018424. Based on our data, the inhibitory effects of INCB018424 on JAKs differ depending on the colorectal cancer cell type.

**INCB018424 induces cell death through caspase-dependent apoptosis in colorectal cancer cells.** Our data show that INCB018424 may inhibit the phosphorylation of JAK1 and/or JAK2 depending on the colorectal cancer cell line. We then examined how different inhibitory patterns of INCB018424 can be observed in colorectal cancer cells and how this influences cell death. INCB018424 inhibits cell viability on both cells in a dose dependent manner (Supplemental Fig. 1). To do this, we treated DLD-1 and RKO cells with 10 µM or 25 µM INCB018424, concentrations that block JAK1 and/or JAK2 phosphorylation, respectively. Caspase 3, the representative caspase, was activated and significantly decreased cell viability following the treatment of DLD-1 cells with 25 µM INCB018424 (Fig. 3a). For RKO cells, in which JAK1 phosphorylation is inhibited by INCB018424, caspase 3 was activated and cell viability was decreased, similar to DLD-1 cells (Fig. 3b). Interestingly, there was no distinct difference found between DLD-1 and RKO cells in terms of caspase 3 activation and cell viability, even though only JAK1 phosphorylation was inhibited in RKO cells by INCB018424. This suggests that INCB018424 suppresses JAK1 phosphorylation and may be sufficient to induce apoptosis in colorectal cancer cell lines via the activation of caspase 3, regardless of JAK2 phosphorylation.

To determine if INCB018424-induced cell death is caspase-dependent, cells were treated with a pan-caspase inhibitor, z-VAD, and caspase 3 activation and cell viability were examined. As observed in Figures 3A and B, cleaved caspase 3 were increased by INCB018424 in both DLD-1 and RKO cells. Treatment with z-VAD rescued DLD-1 and RKO from...
INCB018424-mediated cell death and completely inhibited the cleavage of caspase 3 (Figs. 3c and 3d). In this case, the inactivation of JAK1 by INCB018424 alone, which was observed in RKO cells, resulted in apoptosis similar to that observed in DLD-1 cells. We thus suggest that INCB018424 induces caspase-dependent cell death through the inhibition of JAK1.

Another question to be answered was whether or not the INCB018424-mediated cleavage of caspase3 could be practically used to induce apoptosis. To investigate this, we treated DLD-1 and RKO cells with INCB018424, Z-V AD, or Z-V AD/INCB018424 and then immunostained the cells using annexin-V after fixation and permeabilization (Fig. 4). Under inverted fluorescence microscopy, annexin-V staining was observed at very low levels in the majority of the both cells. On the other hand, INCB018424 profoundly increased annexin-V staining in both cells, though co-treatment with z-V AD effectively reduced the apoptotic potential. As shown by the results in Figure 3, INCB018424 activates caspase 3 to cause cell death, and this type of caspase-dependent cell death might be considered apoptosis. Furthermore, it seems that the inhibition of JAK2 is actually not needed to induce INCB018424 cytotoxicity in colorectal cancer.

**Discussion**

The JAK/STAT signaling pathway is constitutively activated in most human colorectal carcinoma tissues, is associated with nodal metastasis, and generally carries a poor clinical prognosis [21]. It has been also reported that the JAK/STAT pathway is significantly involved in tumor survival via interactions with the tumor stroma. Furthermore, tumor-associated inflammatory cells, which are influenced by the JAK/STAT signaling pathway, are critical to tumorigenesis in colorectal cancers [22, 23]. However, the possible use of JAKs as therapeutic targets and their inhibitory mechanisms as pharmacologic agents for the treatment of colorectal cancers has been poorly understood until recently. Even though the importance of JAKs as therapeutic targets for colorectal cancers is beginning to be explored, JAK1 is still underestimated in terms of its role in carcinogenesis and cancer progression in comparison with JAK2.

In our present study, we investigated the use of JAK1/2 as an effective therapeutic target of INCB018424, which is the first JAK1/2 inhibitor to be approved by the US FDA. Our data show that the inhibition of JAK1/2 phosphorylation by
INCB018424 can differ depending on the cell line. In other words, INCB018424 can inactivate JAK1 and JAK2 in some colorectal cancer cell lines (e.g., DLD-1), but only inhibit JAK1 phosphorylation in others (e.g., RKO). However, the INCB018424-mediated inhibition of JAK1 phosphorylation is sufficient to induce caspase-dependent apoptosis in colorectal cancers. This is interesting because INCB018424 generally inhibits JAK2 more profoundly than JAK1 in order to exert its effects in leukemia, myelofibrosis, and other solid tumors such as NSCLC [26]. However, it has been shown that EGFR signaling is highly activated in colorectal cancers and activates JAK1 preferentially to JAK2 [27]. In addition, another activating signaling pathway in colorectal cancer is interferon-γ [28]. These studies suggest that there are no reported somatic mutations in JAK1, but activating the signaling pathway may make colorectal cancer cells addictive to JAK1, so consequently INCB018424 more effectively inactivates JAK1 which is enough to cause cell death.

Preclinical studies have reported that JAK1 knockdown by siRNA or JAK inhibitors like AG490 or pyridine can result in growth arrest or apoptosis [29]. On the other hand, there is
no available information at present regarding the functions of JAK1, and the effects of JAK1 on colorectal cancer are poorly understood. According to our current findings, JAK1 may be a potential target when treating colorectal cancer, but more studies are needed to determine the cellular context for constitutive JAK1 activation in colorectal cancer. Further studies should also be performed to determine if JAK2 can be influenced by INCB018424 and, if so, how different this mechanism is from the mechanism of JAK1. Small-interfering RNA against JAK2 or JAK2-specific inhibitors could help identify the exact JAK mechanisms that are involved in colorectal cancer.

In conclusion, the results of our present study show that INCB018424, a JAK1/2 inhibitor, can cause caspase-dependent apoptosis by suppressing JAK1 in colorectal cancer cell lines. Among the subsets of colorectal cancer with constitutive JAK activities, the results of this study also suggest another possible targeted molecular therapy for use against the JAK/STAT signaling pathway.

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References


