Tumoricidal effects of the JAK inhibitor Ruxolitinib (INC424) on hepatocellular carcinoma in vitro

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ABSTRACT

Hepatocellular carcinoma (HCC) is an aggressive tumour with limited treatment options. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway plays a key role in promoting tumorigenesis in HCC. Recently a new JAK inhibitor Ruxolitinib (INC424) has been developed by Novartis Pharmaceuticals and it shows high affinity for JAK signalling with very low affinity for non-JAK targets. Clinical trials have demonstrated that Ruxolitinib has good therapeutic efficacy for the treatment of myelofibrosis and is currently FDA approved for the treatment of advanced stages of this disease. Our study tested the effects of Ruxolitinib on HCC tumorigenesis in vitro. Ruxolitinib effectively inhibited JAK/STAT signalling in HCC cells with a significant reduction in the expression of JAK downstream targets pSTAT1 and pSTAT3. Ruxolitinib also caused a marked reduction in the proliferation and colony formation of HCC cells. The antiproliferative effect of Ruxolitinib on HCC cells is unlikely due to off-target effects with no inhibition of key regulators of other cell proliferative pathways. To our knowledge this study is the first to report on the effect of Ruxolitinib on liver cancer cells.

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1. Introduction

Hepatocellular carcinoma (HCC) is the 5th most common cancer worldwide and the 3rd most common cause of cancer related mortality [1]. The mortality rate for HCC has increased at a greater rate compared to all other forms of cancer [2]. Despite advances in surgery and liver transplantation survival rates have only improved slightly in recent decades [3]. As a result the majority of patients who develop HCC succumb to the disease within 1 year of diagnosis [3].

JAK/STAT signalling is a key signalling pathway in a wide variety of cancers including haematological malignancies, head and neck squamous cell carcinomas, and HCC [4–7]. In HCC, the JAK/STAT signalling pathway is up-regulated in tumour tissues compared to adjacent normal tissues [6]. JAK/STAT signalling pathway can also directly mediate hepatitis B driven HCC tumorigenesis [7]. Furthermore, methylation induced silencing of SOCS1 and SOCS3, the negative regulators of JAK/STAT signalling, have been observed in the majority of HCC tumours, leading to activation of JAK/STAT signalling and increased cell growth and resistance to apoptosis [6,8,9].

There is currently a strong interest in JAK inhibitors to inhibit JAK/STAT signalling pathway in cancer. The best characterised JAK inhibitors are shown in Table 1. Published studies have shown that these JAK inhibitors affect tumorigenesis in a variety of cancers (such as ovarian cancer, glioma, leukemia, myeloma, and gastric cancer) by inhibiting cell growth and promoting apoptosis [10–17]. However, to date none of these inhibitors have been approved for the treatment of any solid tumours. Studies on the effect of JAK inhibitors on HCC tumorigenesis are limited. To our knowledge only AG490, a small molecule JAK2 inhibitor has been tested in HCC [11]. Treatment of AG490 on HCC cell lines resulted in reduced cell viability and increased apoptosis and cell cycle arrest [11]. Recently a new JAK inhibitor Ruxolitinib (INC8018424, INC424) developed by Novartis Pharmaceuticals has shown clinical benefits in patients with myelofibrosis and has thus been approved by FDA for the treatment of patients with intermediate or advanced diseases [15,18]. Ruxolitinib is currently the only JAK inhibitor that can effectively inhibit JAK1 and JAK2 with very low affinity for non-JAK targets (Table 1).
Given the clinical benefits of Ruxolitinib in the treatment of myelofibrosis and the key role JAK/STAT signalling plays in HCC tumour progression, our group tested the effect of Ruxolitinib in HCC. To our knowledge this is first study to report the effects of Ruxolitinib on solid tumours.

2. Materials and methods

2.1. Culture of hepatocellular carcinoma cell lines

Three HCC cell lines were used in this study. All cell lines were purchased from ATCC (VA, USA). SNU182 and SNU423 were cultured in DMEM + 10% FCS. HuH7 was cultured in DMEM + 10% FCS. All HCC cell lines were cultured at 37 °C in 5% CO2.

2.2. Antibodies and chemical inhibitors

Mouse monoclonal antibody against pSTAT3, mouse monoclonal antibody against pJNK, rabbit monoclonal against pSTAT1, rabbit monoclonal against pAKT and rabbit monoclonal antibody against pERK1/2 were purchased from Cell Signaling Technology (MA, USA). Anti-β-actin, anti-mouse IgG-HRP and anti-rabbit IgG were purchased from Sigma–Aldrich (MO, USA). The JAK inhibitor Ruxolitinib (INCBO18424, INC424) was provided by Novartis Pharmaceuticals (NSW, Australia) under a material transfer agreement (MTA). INC424 was dissolved in DMSO at a concentration of 10 mM, and kept at 4 °C as the stock solution. In all controls, 1% DMSO was added to the culture medium.

2.3. Protein extraction, SDS–polyacrylamide gel electrophoresis and Western blots

NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1% glycerol, 1% Triton X-100, and protease inhibitor cocktail (Roche, NSW, Australia) and homogenized using steel beads on a Tissue Lyser (Qiagen, CA, USA). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentration in the supernatant was quantified using DCA Protein Assay (BioRad, NSW, Australia) using bovine serum albumin as the standard. Equal amount of protein (30 μg) for each sample was loaded onto an 8% or 10% SDS polyacrylamide gel and transferred to PVDF membrane (Hybond-P, Amershams Biosciences Piscataway, NJ, USA). The membranes were blocked in TBST (tris buffered saline +0.1% Tween 20) + 5% skim milk, incubated overnight in a 1:1000 dilution of primary antibodies in TBST for 1 h. The membranes were then washed for five times each for 5 min in TBST, developed using the Amersham ECL Western blotting detection reagents (GE Healthcare, NSW, Australia). All membranes were stripped with stripping buffer [2% SDS, 100 mM 2-ME, and 62.5 mM Tris–HCl (pH 6.7)] at 40 °C for 30 min with gentle shaking and re-probed for β-actin as a control for equal protein loading. The Western blot data were quantitated by densitometry analysis and expressed as mean ± SE.

2.4. Apoptosis and necrosis assay

HCC cells were prepared and stained with APC Annexin V (AnV) and propidium iodine (PI) according to manufacturer’s instructions (BD, NJ, USA). Cells were then analysed on a FACS Canto-6-Color Flow Cytometer (Becton Dickinson, CA, USA) by gating viable cells (AnV−/PI−), apoptotic cells (AnV+/PI−, AnV+/PI+) and necrotic cells (AnV+/PI+).

2.5. Colony formation assay

HCC cells (500 cells/well) were seeded in 1 mL of growth media + 10% FCS in a 6-well tissue culture plate for overnight. Cells were then incubated for 2 weeks in the presence or absence of 50 μM INC424 at 37 °C in 5% CO2. Colonies were then

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**Table 1**

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Information obtained from Selleckchem (www.selleckchem.com).

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![Figure 1](https://example.com/image1.png)

Fig. 1. A dose-dependent effect of INC424 on the proliferation of HCC cells. HuH7, SNU182, and SNU423 cells were treated with a wide range of INC424 (0–100 μM) for 24 h, and the cell proliferation was examined by FACScan analysis following Annexin V/PI staining. Low dose range (0–25 μM) of INC424 did not significantly inhibit the proliferation of all three HCC cell lines (A), but INC424 at the concentration of > 50 μM significantly inhibits the proliferation of 3 HCC cell lines (A, B). Data were expressed as mean ± SE (n = 3 in each bar). *Indicates a significant difference (p < 0.05) between treatments as determined by a two sample t-test.
washed twice in PBS and stained with crystal violet (2 g/L in formalin) and counted using an inverted microscope.

2.6. Statistic analysis

Differences between treatments were determined using two sample t-tests assuming equal variances on Microsoft Excel 2010 (Microsoft, CA, USA). Differences were deemed significant if the p value is <0.05.

3. Results

3.1. Effect of INC424 on cell proliferation and apoptosis

The effect of INC424 on cell proliferation of HCC cell lines was determined. As shown in Fig. 1A, exposure of cells to 50 μM INC424 for 24 h significantly inhibited the proliferation of HCC cell lines HuH7, SNU182 and SNU423 (two sample t-test, p < 0.05). Longer treatment with 50 μM of INC424 or larger dose led to a marked reduction in cell proliferation in HuH7 cells (>82% reduction after 48 h), SNU182 cells (>64% reduction after 48 h), and SNU423 cells (>81% reduction after 48 h) (Fig. 1B). Because antibodies to pJAK1 and pJAK2 do not effectively measure the inhibition of JAK signalling by INC424, we used pSTAT1 and pSTAT3 as the major readouts of JAK inhibition. As expected, at the 50 μM level, INC424 significantly reduced the expression of pSTAT1 and pSTAT3 in all 3 cell lines (by >80–90%) (Fig. 2). This dose barely had any effect on cell viability in three HCC cell lines as determined by Annexin V assay (data not shown). The sensitivity of these cell types to INC424-induced apoptosis varies. INC424 treatment at 50 μM appears to have a slight apoptotic effect on HuH7 (Fig. 3A) with a 12% increase in apoptosis (two sample t-test, p < 0.05) (Fig. 3B).
p < 0.05). In contrast, 50 μM INC424 only marginally induced apoptosis in SNU423 cells (a 6% increase, p > 0.05) and had no pro-apoptotic effect on SNU182 cells (data not shown). By phase contrast morphology study, it was revealed that 50 μM INC424 for 24 h caused a marked reduction in the number of attached HuH7 cells (Fig. 3B).

3.2. Effect of INC424 on colony formation

To determine the potential anticancer effect of INC424 in HCC, in vitro colony formation assays were undertaken. HuH7, SNU182 and SNU423 were treated with INC424 at a concentration of 50 μM. As shown in Fig. 4, 50 μM INC424 almost completely abolished the colony-forming ability of these 3 HCC cell lines.

3.3. Off-target effect of INC424 in HCC cells

In order to test if the above observed effects on cell proliferation and colony formation were due to the potential off-target effect of INC424, HCC cells were treated with 50 μM INC424 for 24 h and the expressions of pJNK, pAKT, pERK, pAMPK, pPKM2, pP53, pP38 and c-Jun were examined by Western blot. As shown in Fig. 5, INC424 did not consistently affect the expression of these proteins across all cell lines (HuH7, SNU182 and SNU423).

3.4. Toxicity of INC424 on HCC cells

To determine drug toxicity of INC424, cell necrosis was measured in all three HCC cell lines (HuH7, SNU182 and SNU423) after treatment with 50 μM INC424 for 24 h. As shown in Fig. 6, no sig-

![Fig. 4. Effect of 50 μM INC424 on the colony-forming ability of HCC cells. HuH7, SNU182, and SNU423 cells were treated with or without 50 μM of INC424 and the number of colonies were counted 2 weeks later after cells being stained with crystal violet. Data shown are representatives of three separate experiments.](image1)

![Fig. 5. Off-target effect of INC424 on HCC cells. HuH7, SNU182, and SNU423 cells were treated with 50 μM INC424 for 24 h. The expression of pJNK, pAKT, pERK, pAMPK, pPKM2, pP53, pP38, and c-Jun was examined by Western blot. The Western blot bands were quantitated by densitometric analysis and the density normalised against that of the corresponding β-actin in each group. -: control (vehicle only); +: INC424 50 μM, 24 h.](image2)
significant increase in cellular necrosis in SNU182 and SNU423 cells were observed (two sample t-test, \( p > 0.05 \)). In fact, in HuH7 cells, a significant reduction in cell necrosis was observed after INC424 treatment (two sample t-test, \( p < 0.05 \)).

4. Discussion

Constitutive activation of JAK/STAT pathway plays a major role in promoting tumorigenesis in a variety of cancers including haematological malignancies, head and neck cancers, colon cancer, breast cancer, and pancreatic carcinomas [4,5,19–24]. Overexpression and constitutive activation of JAK/STAT pathway has also been observed in human liver cancer [6].

Constitutive activation of the JAK/STAT signalling in cancers may be due to increased levels of activating cytokines and growth factors secreted in an autocrine or paracrine manner by tumour and stromal cells, and/or deactivation of endogenous inhibitor such as SOCS1 and SOCS3 [8,9,25].

HCC is a typical example of inflammation related cancer in which increased IL-6 and TNF\(\alpha\) may be responsible for activation of JAK/STAT3 signalling [26,27]. In vitro studies have shown that activation of JAK/STAT signalling in HCC cells leads to increased cell proliferation, invasion and migration [8,9,28,29]. In clinical practice, most HCC cases are resistant to conventional chemotherapy or radiotherapy. Thus, more effective therapeutic approaches are needed. Various JAK inhibitors have been tested in animals and in vitro studies for their efficacy on various cancers including HCC [11]. Some JAK inhibitors are now in various stages of clinical trials [30].

The JAK inhibitor Ruxolitinib (INC424) has been approved by the FDA for treatment of myelofibrosis [31]. As activation of JAK/STAT pathway may play an important role in the development of HCC, we performed a preliminary study to test if INC424 has any anticancer effect against HCC cells. Our data show that INC424 has a dramatic anti-proliferative effect. At doses of \( \geq 50 \mu M \), INC424 markedly suppresses the proliferation and colony forming ability of three HCC cell lines. Importantly this dose does not appear to have any hepatotoxicity with no increase in cell necrosis after 50 \( \mu M \) INC424 treatment. The strong inhibition of colony forming capacity of HCC cells indicates that this inhibitor may possess a strong anticancer effect. Further in vivo studies are needed to confirm this aspect.

The inhibition of cell growth appears to be consistent with JAK/STAT inhibition as we have observed a significant reduction in the tyrosine phosphorylated forms of JAK downstream targets STAT1 and STAT3. To determine if the antiproliferative effect of INC424 at this dose was due to JAK/STAT inhibition and not due to inhibition of other major signalling pathways, we tested the impact of INC424 on the levels of activation of the most important cell proliferation related pathways by measuring pJNK, pAKT, pERK, pAMPK, pPKM2, pP53, pP38 and c-Jun. Our data showed that 50 \( \mu M \) INC424 did not consistently inhibit these pathways. However, as 50 \( \mu M \) INC424 exceeds concentrations reported elsewhere to be active in JAK-dependent cell lines [18], we cannot completely rule out the contribution of other non-JAK activities.

HCC is notoriously known as one of the most refractory cancers against chemotherapy or radiotherapy. In patients with myelofibrosis, the FDA approved dose of INC424 is 25–50 mg daily [15]. In our study, the antiproliferative effect was only observed at \( \geq 50 \mu M \) which equates to a dosage of \( \geq 100 \) mg. One might argue that the concentration of INC424 which reduced HCC cell viability may be too high to be used as a single agent to treat HCC in clinical setting. However, the in vitro data from two dimensional cell cultures need to be verified in perhaps more biologically relevant models and at a range of drug concentrations. For example, Looyenga et al. reported that INC424 is significantly more active in three dimensional cultures of lung cancer cells compared to two dimensional assays [32]. It is also likely that JAK/STAT signalling can promote HCC tumorigenesis by mediating tumour angiogenesis which can only be effectively tested in vivo [33–35]. Hence further in vivo testing is warranted. In the clinic, INC424 may also be useful at lower doses as an adjunct therapy for HCC, just like other JAK inhibitors that shown to enhance the sensitivity of anticancer agents such as Doxorubicin [10,17]. The dosage of INC424 can also be reduced in patients by 50% when co-administered with potent inhibitors of the major drug metabolisng enzyme CYP3A4 [36]. Finally, it may be feasible to use higher doses of INC424 to treat HCC patients given there appears to be no hepatotoxicity in vitro at 50 \( \mu M \) INC424 treatment. There are a range of new drug delivery systems being utilised in the clinical setting such as highly selective transcatheter arterial chemoembolization which may reduce toxic side effects by localising the drug to HCC tumour [37]. Again, these possibilities needs to be properly tested in vitro and in vivo conditions.

Abnormal apoptotic machinery is an important mechanism for cancer formation, and refractoriness of cancer cells to undergo...
apoptosis is responsible for treatment failure. Besides cell proliferation, activation of JAK/STAT signalling pathway can also enhance tumorigenesis via inhibition of apoptotic pathways. We thus tested if INC424 could induce apoptosis on HCC cells. Our results showed that at the doses tested, INC424 did not cause an obvious increase in apoptosis, suggesting that the antitumor effect of INC424 is likely through its anti-proliferative effect rather than induction of apoptosis.

In summary, this preliminary in vitro study is the first to report the potential of JAK inhibitor INC424 therapy for liver cancer. Our study has shown that INC424 may hold a great promise as a therapeutic drug to treat liver cancer. INC424 is able to inhibit JAK/STAT signalling in HCC, and this inhibition was associated with a marked inhibition of cell proliferation and colony formation. Importantly, the antiproliferative effect of INC424 could not be attributed to the off-target effects on the other major signalling pathways tested. Given the broad inhibition of JAK signalling and the antitumor effect in HCC, we suggest Ruxolitinib (INC424) may possess therapeutic potential in the treatment of liver cancer. Further studies particularly in vivo studies are warranted.

Conflict of interest

None.

Acknowledgements

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