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The novel Janus kinase inhibitor ruxolitinib confers protection against carbon tetrachloride-induced hepatotoxicity via multiple mechanisms

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ABSTRACT

Therapeutic targeting of the JAK/STAT pathway, the principal signaling mechanism for numerous cytokines, might be an effective approach for limiting inflammation in different organs, including the liver. Therefore, we investigated whether targeting this pathway by the novel JAK inhibitor ruxolitinib could mitigate hepatic damage provoked by carbon tetrachloride (CCl_4). Male mice received ruxolitinib treatments (75 and 150 mg/kg, oral) 2 h prior to intoxication with CCl_4 (10 ml/kg of 0.3% v/v CCl_4 solution in olive oil, intraperitoneal) for 24 h. Our results showed that ruxolitinib treatments dose-dependently alleviated CCl_4-induced hepatic injury and necroinflammation, as indicated by biochemical markers of injury and histopathology. We unraveled also the mechanisms involved in these hepatoprotective effects. These comprise (i) reducing infiltration of neutrophils and macrophages, as demonstrated by reducing myeloperoxidase activity and F4/80 positive macrophages; (ii) abating apoptosis of hepatocytes, as denoted by decreasing hepatocytes positive for Bax protein; (iii) inhibiting elevation of TNF-α, IL-1β and IL-10; (iv) inhibiting NF-κB activation and translocation to the nucleus, as visualized immunohistochemically; (v) attenuating activation of the IL-23/IL-17 pathway via targeting IL-17, but not IL-23; (vi) antagonizing hepatic oxidative stress by increasing the antioxidant levels (reduced glutathione, glutathione-S-transferase and superoxide dismutase) and decreasing products of lipid peroxidation (malondialdehyde and 4-hydroxynonenal) and total nitrate/nitrite; and (vii) more interestingly, modulating hepatocyte regeneration according to the extent of damage, as quantified by PCNA-immunohistochemistry. In conclusion, our study sheds light on the therapeutic usefulness and the potential underlying mechanisms of the novel JAK inhibitor ruxolitinib in hepatic inflammatory disorders.

1. Introduction

Hepatic inflammation is a key process in the pathogenesis of several disorders, such as drug-induced liver injury, ischemia/reperfusion, nonalcoholic and alcoholic steatohepatitis, as well as viral and bacterial infections [1,2]. Moreover, hepatic inflammation is a major determinant for the progression of hepatic fibrosis to cirrhosis and carcinogenesis [3,4]. Among the various pathways implicated in hepatic inflammation, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway appears to be the most intimately related to signal transduction of proinflammatory cytokines.

JAKs are a family of intracellular non-receptor tyrosine kinases that transmit signals arising from the interaction of various cytokines with extracellular receptor to the nucleus via phosphorylating STAT3 [5]. Generally, JAK/STAT signaling regulates many cellular processes including development, cell proliferation, differentiation and apoptosis [6]. In the liver, JAKs are activated by several proinflammatory cytokines and growth factors, such as interferon-γ, interleukin (IL)-4, IL-6, IL-12, IL-13 and growth hormones [7], as well as hepatitis viral proteins [8]. Recently, activation of the JAK/STAT pathway has been shown to regulate fibrogenic cytokines like transforming growth factor-β1 and connective tissue growth factor [9], as well as enhancement of liver fibrosis and cancer [10,11]. Hence, drugs that target this pathway will indeed dampen the signaling of these proinflammatory and fibrogenic cytokines involved in hepatic inflammation and fibrosis in humans.

JAK inhibitors are a novel class of medications that inhibit the activity of one or more of JAK enzymes, including JAK1, JAK2, JAK3, TYK2 [12]. Of these inhibitors, ruxolitinib is considered the most important predecessor. Ruxolitinib, a novel oral JAK1/JAK2 inhibitor, is the first approved therapy for the treatment of myelofibrosis by FDA on November 16, 2011 [13]. Mechanistically,

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ruxolitinib functions through competitive inhibition at the ATP binding site of JAK1 and JAK2, and thereby interferes with cytokine-induced STAT3 phosphorylation and translocation to the nucleus [14]. Clinically, ruxolitinib elicits a meaningful reduction in spleen size and symptom burden in the majority of myelofibrosis patients with maintaining an acceptable toxicity profile and a low treatment-discontinuation rate [15].

Recent studies have shown the potential efficacy of ruxolitinib in the treatment of life-threatening inflammatory and allergic disorders, such as rheumatoid arthritis [16] and food allergy [17], respectively. Besides, ruxolitinib exhibited an antiproliferative effect on liver cancer cells *in vitro* [18]. Nevertheless, no *in vivo* data exist about the potential efficacy of ruxolitinib in hepatic inflammatory disorders. On this background, we investigated whether the novel JAK inhibitor ruxolitinib could mitigate hepatic damage provoked by carbon tetrachloride (CCl_4), and, if so, what would be the potential mechanisms.

2. Materials and methods

2.1. Drugs and chemicals

Ruxolitinib phosphate (formerly known as INCB018424; Jakafi®) was generously supplied as pure powder by Novartis (Basel, Switzerland). 1-Methyl-2-phenylindole, 1,1,3,3-tetramethoxypropane, 5,5′-dithiobis(2-nitrobenzoic acid), L-glutathione reduced and vanadium III chloride (VCl_3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CCl_4 and methanesulfonic acid were purchased from Merck (Darmstadt, Germany). 3,3′,5,5′-Tetramethylbenzidine and hexadecyltrimethylammonium bromide were purchased from MP Biomedicals (Irvine, CA, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) and AEBSF were purchased from Acros Organics (Morris Plains, NJ, USA). Pyrogallol was purchased from Fluka (Buchs SG, Switzerland). All other chemicals used were of the highest grade available.

2.2. Animals

Adult male BALB/c mice (33–37 g) were allowed free access to food and tap water throughout the acclimatization and experimental periods. All animals received humane care in compliance with the National Institutes of Health and the Research Ethics Committee Criteria for Care of Laboratory Animals at Mansoura University.

2.3. Induction of CCl_4-induced acute liver injury and experimental design

Ruxolitinib powder was suspended in its vehicle (0.5% w/v carboxymethyl cellulose in saline) and orally administered to overnight-fasted mice 2 h prior to intraperitoneal (i.p.) intoxication with CCl_4 (10 ml/kg of 0.3% v/v CCl_4 solution in olive oil, 0.35 ml/35 g) for 24 h as previously described [19].

The mice were divided into four groups as follows: (1) control: received the vehicle (0.35 ml/35 g, oral), followed by olive oil (0.35 ml/35 g, i.p.) 2 h afterwards; (2) CCl_4: received the vehicle (0.35 ml/35 g, oral), followed by CCl_4 as described; (3) CCl_4 + Ruxolitinib (75 mg/kg): received 75 mg/kg of ruxolitinib (0.35 ml/35 g, 0.75% w/v in the vehicle, oral), followed by CCl_4 as described; (4) CCl_4 + Ruxolitinib (150 mg/kg): received 150 mg/kg of ruxolitinib (0.35 ml/35 g, 1.5% w/v in the vehicle, oral), followed by CCl_4 as described. The used doses of ruxolitinib (75 and 150 mg/kg) were based on our preliminary experiments and guidance from Novartis.

After 24 h from CCl_4 challenge, blood samples were withdrawn from thiopental-anesthetized animals via cardiac puncture and allowed to clot for 10 min at room temperature, followed by centrifugation at 2000*g* for 10 min at 4 °C for separation of serum. Serum samples were collected and stored at −80 °C till used. Portions of liver tissues were stored at −80 °C for the enzyme-linked immunosorbent assay (ELISA) and oxidative stress/antioxidant assays. Another portion of liver tissue was also fixed in 10% (v/v) neutral-buffered formalin solutions for 24 h for histopathological and immunohistochemical evaluations.

2.4. Biochemical parameters of hepatic injury

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities were determined using commercial kits from Spectrum Diagnostics (Cairo, Egypt).

2.5. Hepatic histopathological and immunohistochemical evaluations

Standard histopathological techniques were followed for processing portions of liver, preparation of paraffin blocks and staining of the slides (5 μm thick) with hematoxylin–eosin for evaluating CCl_4-induced necroinflammation. The following scores were applied for assessing the necroinflammation injury: 0, absent; 1, spotty necrosis; one or few necrotic hepatocytes; 2, confluent necrosis; 3, bridging necrosis [20,21]. For immunohistochemical analysis, primary antibodies for F4/80, Bax, nuclear factor-kappa B (NF-κB) p65 and proliferating cell nuclear antigen (PCNA) (BioLegend, San Diego, CA, USA) were used to detect their targeted proteins using standard immunohistochemical methods.

2.6. Enzyme-linked immunosorbent assay (ELISA) for cytokines

Portions of livers were homogenized (10% w/v) in ice-cold lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM AEBSF). The lysates were centrifuged at 8000*g* for 10 min at 4 °C, and the supernatants were transferred for ELISA. Mouse IL-1β, IL-17A, IL-23(p19/40), IL-10 and tumor necrosis factor-α (TNF-α) concentrations were determined using the ELISA MAX™ Deluxe set (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. The protein content in lysate was assayed by the Bradford method [22]. Some of the cytokines mentioned were also determined in serum.

2.7. Measurement of hepatic myeloperoxidase (MPO) activity

The extent of neutrophil accumulation in the liver was measured by assaying myeloperoxidase (MPO) activity as previously described [23] with a slight modification. After processing the supernatant of liver homogenate, a part of the corresponding pellet (50 mg) was weighed, homogenized in 1 ml of the buffer (0.1 M NaCl, 0.02 M NaH_2PO_4, 0.015 M EDTA, pH 4.7) and centrifuged at 6000*g* for 20 min at 4 °C. The pellets were then resuspended in 0.5 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% (w/v) hexadecyltrimethylammonium bromide. The suspensions were freeze–thawed three times, heated for 2 h at 60 °C to increase myeloperoxidase recovery, and finally centrifuged at 6000*g* for 20 min at 4 °C to separate the supernatants for MPO assay. The reaction was started by mixing 0.2 ml of 1.6 mM 3,3′,5,5′-tetramethylbenzidine in dimethylsulfoxide with 0.8 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.006% (v/v) H_2O_2 and 0.2 ml of the 6000*g* supernatant from the liver tissue sample. MPO activity was assayed by measuring the change in optical density (OD) for 5 min at 650 nm. Results were expressed as change in OD per g of wet tissue.