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Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis

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Abstract

Effective and well-tolerated anti-fibrotic drugs are currently lacking. Therefore, this study was carried out to investigate the potential anti-fibrotic effects of imatinib, nilotinib and silymarin on established hepatic fibrosis in the carbon tetrachloride (CCl₄) rat model. Male Wistar rats received intraperitoneal injections of CCl₄ twice weekly for 8 weeks, as well as daily intraperitoneal treatments of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) during the last 4 weeks of CCl₄-intoxication. At the end of the study, hepatic damage was evaluated by analysis of liver function tests and hepatic oxidative stress parameters. Hepatic fibrosis was evaluated by histopathology and morphometry, as well as collagen and 4-hydroxyproline contents. Nilotinib (20 mg/kg) was the most effective treatment to counteract CCl₄-induced hepatic injury as indicated by liver function tests and histopathology. Nilotinib (10 mg/kg), nilotinib (20 mg/kg) and silymarin (100 mg/kg) treatments reduced the mean score of hepatic fibrosis by 31%, 68% and 47%, respectively, and hepatic collagen content by 47%, 49% and 18%, respectively in CCl₄-treated rats. Hepatic morphometric evaluation and 4-hydroxyproline content revealed that CCl₄-induced fibrosis was ameliorated significantly by nilotinib (20 mg/kg) and imatinib (20 mg/kg). Unlike nilotinib, imatinib (20 mg/kg) showed some sort of hepatic injury evidenced by elevation of serum aminotransferases and total bilirubin levels, and hepatic total nitrate/nitrite content, as well as characteristic anisonucleosis visualized with the hematoxylineosin staining. In conclusion, this study provides the evidence that nilotinib exerts anti-fibrotic activity and suggests that it may be valuable in the treatment of hepatic fibrosis in humans.

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Conflict of interest

The authors have no conflict of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2011.02.004.

Keywords

Imatinib; Nilotinib; Silymarin; Carbon tetrachloride; Hepatic fibrosis

Introduction

Hepatic fibrosis represents a major worldwide medical problem with significant morbidity and mortality. Fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins in the liver as a consequence of chronic hepatic injury of any etiology (Friedman, 2008a). Hepatic stellate cells (HSCs), the main hepatic cells implicated in the accumulation of ECM proteins, are activated by several cytokines, such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), tumor necrosis factor- α (TNF- α), insulin-like growth factor (IGF-I) and endothelin-1 (ET-1), as well as reactive oxygen species (ROS) (Gabele et al., 2003). Among these, PDGF and TGF- β are well known to be the most potent stimuli for proliferation and fibrogenesis of HSCs, respectively (Friedman, 2008b).

Tyrosine kinases are involved in diverse cellular activities, including metabolism, growth, differentiation and apoptosis (Levitzki and Gazit, 1995). The common mode of action of tyrosine kinases is phosphorylation of tyrosine residues in target proteins, using ATP (Robinson et al., 2000). The two general classes of tyrosine kinases are receptor tyrosine kinases, such as PDGF receptors (PDGFRs), and nonreceptor tyrosine kinases, such as c-Src and Abelson kinase (c-Abl) (Robinson et al., 2000). Beside the physiological roles of tyrosine kinases, recent advances have implicated the role of their activation in the pathophysiology of carcinogenesis, vascular remodeling, rheumatoid arthritis and fibrogenesis (Krause and Van Etten, 2005; Distler and Distler, 2008; D'Aura Swanson et al., 2009; Hassoun et al., 2009). Thus, inhibitors that block the activity of tyrosine kinases and their signaling pathways may prove useful for the treatment of these diseases.

Tyrosine kinase inhibitors are a relatively new class of therapeutic agents that have been used clinically to treat chronic myeloid leukemia, gastrointestinal stromal tumors and a number of other malignancies (Cohen et al., 2002; Johnson et al., 2003). Imatinib was the first tyrosine kinase inhibitor to be approved by FDA on May 10, 2001, whereas nilotinib, a new rationally designed tyrosine kinase inhibitor, was approved on October 29, 2007 (Manley et al., 2010). There has been a recent interest in the potential efficacy of imatinib in the treatment of the most common and progressive forms of human fibrosis. These include treatment of pulmonary fibrosis (Daniels et al., 2004; Aono et al., 2005; Vittal et al., 2007), renal fibrosis (Wang et al., 2005; Chandran et al., 2009), dermal fibrosis (Distler et al., 2007) and hepatic fibrosis (Yoshiji et al., 2005, 2006; Neef et al., 2006). Unlike imatinib, the therapeutic efficacy of nilotinib has not been tested against *in vivo* fibrosis models except for bleomycin-induced dermal fibrosis (Akhmetshina et al., 2008).

Despite efforts to develop anti-fibrotic agents, no drug has been approved as an anti-fibrotic agent in humans to date. In the current study, we marshal the first investigation to look at the anti-fibrotic activity of nilotinib using the carbon tetrachloride (CCl₄) rat model. This model is considered the “gold standard” model of hepatic fibrosis (Laleman et al., 2006) that has the advantage of mimicking that of human caused by different etiologies (Mitsuhashi et al., 2004; Natarajan et al., 2006). Herein, we report that nilotinib possesses anti-fibrotic activity greater than its predecessor, imatinib, and the standard hepatoprotective treatment, silymarin. In addition to the anti-fibrotic activity, nilotinib did not show any signs of hepatotoxicity.

Materials and methods

Drugs and chemicals

Imatinib mesylate (formerly STI571; Gleevec®) and nilotinib hydrochloride monohydrate (formerly AMN107; Tasigna®) were generously supplied by Novartis (Basel, Switzerland). Silymarin (milk thistle powder containing 80% silymarin) was purchased from Bulk Nutrition (Northborough, MA, USA). 1-Methyl-2-phenylindole, 1,1,3,3-tetramethoxypropane, 5,5'-dithiobis(2-nitrobenzoic acid), vanadium III chloride (VCl₃), 4-hydroxy-L-proline and sirius red F3B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanesulfonic acid was purchased from Merck (Darmstadt, Germany). 3,3',5,5'-Tetramethylbenzidine and hexadecyltrimethylammonium bromide were purchased from MP Biomedicals (Irvine, CA, USA). L-Glutathione reduced (GSH) and pyrogallol were purchased from Fluka (Buchs SG, Switzerland). 4-Hydroxynonenal was purchased from Cayman (Ann Arbor, MI, USA).

Animals

Adult male Wistar rats (225–250 g) were purchased from Nile Pharmaceuticals (Cairo, Egypt). The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Induction of hepatic fibrosis

Hepatic fibrosis was induced by intraperitoneal (i.p.) injections of CCl₄ (1 ml/kg) twice a week for 8 weeks as previously described (Shaker et al., 2009), but with using a concentration of 40% (v/v) of CCl₄ in olive oil, rather than 50% (v/v). All inhibitor treatments were administered during the last 4 weeks of CCl₄-intoxication to simulate the clinical application of anti-fibrotic drugs.

Experimental design

The rats were divided into seven groups as follows: (1) CTRL: biweekly olive oil (1 ml/kg, i.p.) in addition to saline (1 ml/kg/day, i.p.); (2) CCl₄: biweekly CCl₄ as described in addition to saline (1 ml/kg/day, i.p.); (3) CCl₄ + imatinib (10 mg/kg): received CCl₄ and imatinib (10 mg/kg/day, 1% w/v in saline; 1 ml/kg, i.p.); (4) CCl₄ + imatinib (20 mg/kg): received CCl₄ and imatinib (20 mg/kg/day, 2% w/v in saline; 1 ml/kg, i.p.); (5) CCl₄ + nilotinib (10 mg/kg): received CCl₄ and nilotinib (10 mg/kg/day, 1% w/v in saline; 1 ml/kg, i.p.); (6) CCl₄ + nilotinib (20 mg/kg): received CCl₄ and nilotinib (20 mg/kg/day, 2% w/v in saline; 1 ml/kg, i.p.); (7) CCl₄ + silymarin (100 mg/kg): received CCl₄ and silymarin (100 mg/kg/day, 12.5% w/v in saline; 1 ml/kg, i.p.).

Twenty four hours after the last injection of CCl₄, blood samples were withdrawn from thiopental-anesthetized animals *via* cardiac puncture and centrifuged at 2000 g for 10 min at 4 °C for serum preparation. Liver tissues were homogenized (10% w/v) in 20 mM Tris-HCl (containing 1 mM EDTA, pH 7.4) and centrifuged at 3000 g for 20 min at 4 °C. Serum samples and supernatants from liver homogenates were assayed on the same day of collection. Protein contents of supernatants from liver homogenates were determined as previously described (Beyer, 1983).

Evaluation of liver functions

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (GGT), total

bilirubin and albumin levels were determined using commercial kits from Spectrum Diagnostics (Cairo, Egypt).

Hepatic histopathological evaluation

Portions of livers were fixed in 10% neutral buffered formalin solutions for 24 h. Standard histopathological techniques were followed for processing the tissue and preparation of paraffin blocks. Fibrosis and necroinflammation changes were evaluated in sections (5 μ m thick) stained with Masson's trichrome and hematoxylin-eosin, respectively, using the Ishak-modified HAI system (Ishak et al., 1995). The following scores were applied to quantify fibrosis: 0, no fibrosis; 1, fibrous expansion of some portal areas with or without short fibrous septa; 2, fibrous expansion of most portal areas with or without short fibrous septa; 3, fibrous expansion of most portal areas with occasional portal-to-portal (P-P) bridging; 4, fibrous expansion of portal areas with marked portal bridging (P-P as well as portal-to-central (P-C)); 5, marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis); and 6, cirrhosis, probable or definite. The necroinflammation scores were calculated as the sum of the 4 categories of necroinflammation: periportal or periseptal interface hepatitis (0–4); confluent necrosis (0–6); focal (spotty) lytic necrosis, apoptosis and focal inflammation (0–4); and portal inflammation (0–4). The final numerical score is the result of multiplying the number of rats per grade by the grade, adding these products and dividing by the total number of rats per group.

Hepatic morphometric evaluation of fibrosis

The morphometric evaluation of hepatic fibrosis was performed as previously described (Abdalla et al., 2009) using the fully automated Leica image processor with automated stage and Leica Quin software 2004. The liver biopsy slide, stained with sirius red, was placed on the x-y motorized stage of Leica microscope. At a magnification of 10 $\times$, automated sequential digitalized images were taken and stored. To evaluate the relative fibrosis area, the measured collagen area was divided by the net field area and then multiplied by 100. Artifacts created during slide preparation were eliminated by both automatic and interactive procedures. The area of the liver parenchyma was considered the reference area, and the fractional surface occupied by fibrosis was measured within the above-defined area.

Measurement of hepatic collagen content

Hepatic collagen content was assayed using the previously described method (Gascon-Barre et al., 1989). Briefly, liver sections (15 μ m thick) were taken from liver blocks embedded in paraffin and were deparaffinised resulting in tissue areas of approximately 50–100 mm², then incubated in 0.04% (w/v) solution of fast green FCF in saturated picric acid for 15 min. The sections were then washed with distilled water, incubated in the presence of fast green FCF 0.1% (w/v) and sirius red F3B 0.04% (w/v) for 30 min and washed again with distilled water until the eluted fluid is completely free of color. Then 1 ml of 0.05 N NaOH in 50% (v/v) aqueous methanol was added, and each tube was gently mixed. The eluted color was quantified using a spectrophotometer at 530 and 605 nm (corresponding to the maximal absorbance of sirius red and fast green, respectively).

Measurement of hepatic 4-hydroxyproline content

Hepatic 4-hydroxyproline content was quantitated colorimetrically using the chloramine T method as previously described (Lee et al., 2005) with minor modifications. In brief, liver specimens (50 mg) were weighed in Eppendorf tubes and hydrolyzed in 0.5 ml of 6 N HCl at 100 °C for 24 h. The hydrolysate was then cooled, neutralized with 0.5 ml of 6 N NaOH and centrifuged at 8000 g for 10 min. Two hundred microliters of the supernatant was added to 0.125 ml of chloramine T solution [1 part of 7% chloramine T and 4 parts of citrate/

acetate buffer (pH 6.0, 57 g sodium acetate, 37.5 g trisodium citrate, 5.5 g citric acid, 385 ml of isopropanol, and dissolved in H₂O to a final volume of 1 L)] and was incubated at room temperature for 10 min. Thereafter, 0.75 ml of Ehrlich's solution was added. The Ehrlich's solution was prepared by dissolving 2 g of P-dimethylamino-benzaldehyde in 3 ml of 60% HClO₄ and then mixed with 9 ml of isopropanol. The final mixture was incubated at 60 °C for 35 min and then at room temperature for 10 min, and the absorbance was determined at 560 nm. Standard solutions containing 0, 20, 40, 60, 80 and 100 µg/ml of authentic 4-hydroxy-L-proline were treated likewise. The standard curve was linear in this range ($r=0.988$). The value of hepatic 4-hydroxyproline content was expressed as µg/g wet tissue.

Hepatic oxidative stress parameters

Measurement of hepatic malondialdehyde and 4-hydroxynonenal contents

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations were determined as previously described (Gerard-Monnier et al., 1998). Briefly, 0.65 ml of 10.3 mM 1-methyl-2-phenyl-indole in acetonitrile diluted with methanol containing 32 µM FeCl₃ (3:1) was added to 0.2 ml of liver homogenate sample with vortexing. After adding 0.15 ml of either 37% (v/v) HCl (for MDA alone) or 99% (v/v) methanesulfonic acid (for MDA+4-HNE), samples were mixed well, closed with a tight stopper and incubated at 45 °C (60 min for MDA alone and 40 min for MDA+4-HNE). The samples were then cooled on ice, centrifuged at 4000 g for 10 min, and the absorbance was measured spectrophotometrically at 586 nm. Two standard curves comprised of 1,1,3,3-tetramethoxypropane and 4-HNE (0–20 nmol/ml) were also run for quantitation. The amount of 4-HNE was obtained by the subtraction of the MDA values from the total MDA+4-HNE values.

Measurement of hepatic nitric oxide content

Total nitrate/nitrite (NO_x) products were estimated as an index of nitric oxide (NO•) synthesis as previously described (Miranda et al., 2001). Liver homogenate (0.5 ml) was added to 0.25 ml of 0.3 N NaOH. After incubation for 5 min at room temperature, 0.25 ml of 5% (w/v) ZnSO₄ was added for deproteinization. This mixture was then centrifuged at 3000 g for 20 min at 4 °C, and 0.3 ml of the resultant supernatant was added to 0.3 ml VCl₃ (8 mg/ml) in 1 N HCl and 0.3 ml Griess reagent [0.15 ml of 2% (w/v) sulfanilamide in 5% (v/v) HCl and 0.15 ml of 0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water]. After incubation for 45 min at 37 °C, samples were measured spectrophotometrically at 540 nm. Concentration of NO_x in samples was determined from a standard curve of NaNO₃ (0–100 nmol/ml).

Measurement of hepatic myeloperoxidase activity

The extent of neutrophils accumulation in the liver was measured by assaying myeloperoxidase (MPO) activity as previously described (Schierwagen et al., 1990) with a slight modification. After processing the supernatant of liver homogenate, a part of the corresponding pellet (100 mg) was weighed, homogenized in 2 ml of the buffer (100 mM NaCl; 20 mM NaH₂PO₄; 15 mM EDTA, pH 4.7) and centrifuged at 6000 g for 20 min at 4 °C. The pellets were then resuspended in 1 ml of 50 mM 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. The reaction was started by mixing 0.2 ml of 1.6 mM 3,3',5,5'-tetramethylbenzidine in dimethylsulfoxide, 0.8 ml of 50 mM sodium phosphate buffer (pH 5.4) containing 0.006% (v/v) H₂O₂ and 0.2 ml of the supernatant from the tissue sample. MPO activity in the supernatant was assayed by measuring the change in optical density (OD) for 5 min at 450 nm. Results were expressed as change in OD per g of wet tissue.

Measurement of hepatic non-protein sulfhydryl content

Hepatic content of non-protein sulfhydryl (NPSH), an indicator of hepatic GSH, was measured as previously described (Moron et al., 1979) with a slight modification. Briefly, the protein in 0.45 ml of liver homogenate was precipitated with 0.05 ml of 50% (w/v) trichloroacetic acid and then centrifuged at 1000 g for 5 min. The reaction mixture containing 0.25 ml of supernatant, 1 ml of 200 mM Tris-HCl (containing 1 mM EDTA, pH 8.9) and 0.05 ml of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in absolute methanol was kept at room temperature for 5 min, and the yellow color developed was measured spectrophotometrically at 412 nm. Results were calculated from a standard GSH curve (0–500 nmol/ml) and expressed as nmol/mg protein.

Measurement of hepatic superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured as previously described (Marklund and Marklund, 1974) with a slight modification. This method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. Briefly, 0.1 ml of liver homogenate was mixed with 1.5 ml of 20 mM Tris-HCl (containing 1 mM EDTA, pH 8.2), then 0.1 ml of 15 mM pyrogallol was added. Thereafter, the change in OD per min was determined for the samples by monitoring the increase in OD at 420 nm for 3 min. The percentage of inhibition for the samples was calculated by the aid of running a control with no sample under the same conditions. SOD enzyme activity was expressed as U/mg protein, where one unit was defined as the amount of the enzyme that inhibited the rate of pyrogallol autoxidation by 50%.

Statistical analysis

Data are expressed as means±S.E. in each experimental group. Statistical evaluations of the results, except those of histopathology scoring, were carried out by means of one way analysis of variance, followed by the Tukey–Kramer multiple comparison test. The results of histopathology scoring were analyzed by the non-parametric Mann–Whitney *U* test (two-tailed). Statistical tests were performed with GraphPad InStat V 3.05 (GraphPad Software Inc, San Diego, CA, USA).

Results

Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on liver function tests

The administration of CCl₄ for 8 weeks produced severe liver damage characterized by significant elevation in serum ALT, AST and ALP activities ($P<0.001$), as well as LDH activity and total bilirubin level ($P<0.01$), compared with the control group (Fig. 1). A significant reduction in serum ALT and AST activities was observed in the groups treated with imatinib (10 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg), but not imatinib (20 mg/kg), compared with the CCl₄ group. However, the elevation in serum ALP and LDH activities was attenuated in the groups treated with imatinib (10 and 20 mg/kg), nilotinib (20 mg/kg) and silymarin (100 mg/kg), but not nilotinib (10 mg/kg). An increase in total bilirubin level was observed in rats treated with imatinib (20 mg/kg) and silymarin (100 mg/kg) rather than a decrease (Table 1). The activity of GGT in serum was higher in rats treated with CCl₄+imatinib (10 and 20 mg/kg) than those treated with CCl₄ alone, but was lower in rats given nilotinib, especially 20 mg/kg. The serum albumin level in the CCl₄ group was relatively lower than that in the control group and none of the used treatments succeeded to restore it to the normal control value.

Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on hepatic histopathology, morphometric evaluation of fibrosis, and contents of collagen and 4-hydroxyproline

CCl₄-administration for 8 weeks produced marked inflammation, necrosis and pronounced macrovesicular/microvesicular steatosis. According to the observations from Masson's trichrome stained sections, fibrosis score was markedly increased in rats treated with CCl₄, but this increase was significantly reduced in rats treated with imatinib (10 mg/kg), nilotinib (10 mg/kg), nilotinib (20 mg/kg) and silymarin (100 mg/kg) by 36%, 31%, 68% and 47%, respectively (Table 2, Fig. 2A). Hematoxylin-eosin stained sections revealed that rats treated with CCl₄+nilotinib (20 mg/kg) was the only group to show a significant decrease in necroinflammation score ($P<0.05$), compared with the CCl₄ group (Table 2, Fig. 2B). However, rats treated with CCl₄+ imatinib (20 mg/kg) showed characteristic anisonucleosis.

Interestingly, hepatic morphometric evaluation (Fig. 3) revealed that CCl₄-induced fibrosis was counteracted pronouncedly by nilotinib (20 mg/kg) and to less extent imatinib (20 mg/kg). Additionally, intoxication with CCl₄ twice weekly for 8 weeks was associated with an increase in hepatic collagen (almost 2-fold) and 4-hydroxyproline contents (almost 5-fold) (Fig. 4). The 2-fold increase in hepatic collagen content was reduced in rats treated with nilotinib (10 mg/kg), nilotinib (20 mg/kg) and silymarin (100 mg/kg) by 47%, 49% and 18%, respectively. The 5-fold increase in 4-hydroxyproline content was reduced in rats treated with imatinib (10 mg/kg), imatinib (20 mg/kg), nilotinib (10 mg/kg), nilotinib (20 mg/kg) and silymarin (100 mg/kg) by 10%, 55%, 22%, 74% and 28%, respectively.

Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on hepatic oxidative stress parameters

The administration of CCl₄ caused an increase of approximately 32% and 20% in hepatic MDA and 4-HNE contents, respectively, compared with the control group (Fig. 5A). Nilotinib (20 mg/kg) treatment slightly decreased the elevated hepatic MDA (not significant) and 4-HNE ($P<0.01$) contents, compared with the CCl₄ group. However, significant decreases in both hepatic MDA ($P<0.001$) and 4-HNE ($P<0.01$) contents were observed with the silymarin (100 mg/kg) treatment, compared with the CCl₄ group. Hepatic NO_x content was found to be higher in rats treated with CCl₄ and CCl₄+imatinib (20 mg/kg) than in the control group by 42% and 89%, respectively. Interestingly, nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments counteracted this elevation effectively (Fig. 5B). Hepatic MPO activity was markedly increased in the CCl₄ group (4-fold), but this increase was significantly ($P<0.001$) reduced in rats treated only with imatinib (20 mg/kg) and nilotinib (10 mg/kg and 20 mg/kg) (Fig. 5C). Hepatic NPSH content and SOD activity were significantly reduced in CCl₄-treated rats by 39% and 52%, respectively, compared with the control group (Fig. 5D and E). Imatinib (20 mg/kg) was the only treatment to exhibit a highly significant ($P<0.001$) elevation in hepatic NPSH content and SOD activity, compared with the CCl₄ group.

Discussion

The present data show an increase in oxidative stress due to CCl₄-administration for 8 weeks as indicated by the elevation of hepatic MDA, 4-HNE, NO_x and MPO levels, and the reduction of hepatic NPSH and SOD levels. The severity of changes was associated with the degree of hepatic damage as confirmed by the elevation of liver function tests, and by the changes of liver morphology and histopathology. The mechanism of CCl₄-hepatotoxicity involves oxidative stress initiated by CCl₄-derived reactive free radical metabolites, including trichloromethyl radical ($\bullet\text{CCl}_3$), which rapidly reacts with molecular oxygen to form the more electrophilic trichloromethyl peroxy radical ($\bullet\text{OCCl}_3$) (Ruch et al., 1986).

•CCl₃ is responsible for the covalent binding to cell components, such as GSH, resulting in inhibition of lipoprotein secretion and thus steatosis. On the other hand, •OCCl₃ initiates the lipid peroxidation process with the consequent formation of by-products such as, MDA and 4-HNE, resulting in loss of membrane integrity and cell death (Boll et al., 2001; Weber et al., 2003). The elevation of hepatic MPO activity in CCl₄-treated rats originates from neutrophils that are activated and recruited in response to Kupffer cell-derived TNF-α (Edwards et al., 1993).

In the present study, nilotinib (20 mg/kg) treatment was found to prevent the progression of hepatic injury in rats intoxicated with CCl₄ for 8 weeks as confirmed by decreasing the elevated levels of serum ALT, AST, ALP, LDH and total bilirubin, as well as the necroinflammation score. In addition, the protective effects exerted by nilotinib (20 mg/kg) against CCl₄-induced hepatic injury was further confirmed by reducing the elevated contents of MDA, 4-HNE and NOx, as well as MPO activity in the liver. Previous *in vitro* studies have shown that the cytoplasmic form of c-Abl is activated in the cellular response to ROS (Sun et al., 2000; Kumar et al., 2001). Activation of c-Abl is associated with targeting of c-Abl to the mitochondria, loss of mitochondrial transmembrane potential, release of cytochrome c and cell death (Kumar et al., 2003). Based on these findings, the protective effects exerted by nilotinib against CCl₄-induced hepatic injury and oxidative stress may be attributed to the inhibition of c-Abl activity.

Scoring of hepatic fibrosis and morphometric evaluation, as well as hepatic collagen and 4-hydroxyproline contents revealed anti-fibrotic activity for nilotinib at 20 mg/kg and, to a lesser extent, 10 mg/kg. Nilotinib has been reported to suppress the tyrosine kinase activities of the PDGFRs and c-Abl (Akhmetshina et al., 2008). As a consequence, both PDGF-induced proliferation of fibroblasts and TGF-β-induced fibrogenesis, mediated *via* c-Abl through a non-Smad pathway, are inhibited. More recently, nilotinib has been suggested to inhibit the collagen receptors, discoidin domain receptors (Day et al., 2008). Thus, nilotinib interferes simultaneously with three major pathways involved in fibrogenesis.

At the dose level of 20 mg/kg, but not 10 mg/kg, imatinib however showed some hepatic injury, rather than protection, evidenced by the elevation of serum ALT, AST and total bilirubin levels, as well as the hepatic NOx content. It is worth noting that our biochemical findings correlated with the characteristic anisonucleosis visualized with the hematoxylin-eosin staining. The last observation is an indication of hepatotoxicity due to high dose and is in agreement with previous studies (Kong et al., 2007; Mindikoglu et al., 2007), but the pathogenic mechanisms behind remain unknown. Nevertheless, imatinib (20 mg/kg) effectively increased the NPSH content and SOD activity, as well as decreased the MPO activity in the liver to almost near the normal control values. Previous studies have reported that imatinib-induced inhibition of c-Abl decreases glutathione peroxidase activity, resulting in prevention of GSH oxidation to its disulfide form (Cao et al., 2003; Saad et al., 2006). Imatinib has been also reported to inhibit c-Abl-induced stimulation of NADPH oxidase 5 that leads to production of superoxide anion radical (O₂•⁻), the main substrate for SOD (El Jamali et al., 2008). Additionally, imatinib has been reported to modulate TNF-α production by Kupffer cells, and thereby inhibition of neutrophil activation and dependent MPO elevation (Wolf et al., 2005).

In dramatic contrast, hepatic NPSH content in CCl₄-intoxicated rats was strongly reduced, rather than elevated, by nilotinib (20 mg/kg) treatment. This effect might be unique to CCl₄-intoxication, because our previous study has revealed that nilotinib and to a lesser extent imatinib treatments ameliorated TAA-induced depletion of hepatic GSH in rats as indicated by NPSH (Shaker et al., 2011). Therefore, we believe that nilotinib at 20 mg/kg may compete with oxygen molecules involved in the transformation of •CCl₃ to •OCCl₃

(Weber et al., 2003), resulting in $\bullet\text{CCl}_3$ accumulation and hepatic GSH depletion. This believe was further supported by the suppression of $\bullet\text{OCCl}_3$ -mediated increase in total lipid peroxidation products (MDA+4-HNE) due to nilotinib (20 mg/kg) treatment, as well as the fact that nilotinib is metabolized in the liver *via* oxidation and hydroxylation pathways (Deremer et al., 2008). On the other hand, imatinib is metabolized in the liver mainly by N-demethylation (Peng et al., 2004).

Imatinib (20 mg/kg) treatment relatively failed to alter the fibrogenic responses as it showed patterns of bridging fibrosis observed by Masson's trichrome staining for collagen, and this was further supported upon standardization of the values of collagen to those of non-collagenous protein. However, these patterns of bridging fibrosis were found to be significantly thinner upon standardization of the values to area and weight as assessed by hepatic morphometry and 4-hydroxyproline content, respectively. Like nilotinib, imatinib has been postulated to interfere with the same three major pathways involved in fibrogenesis, but to a lesser extent (Distler and Distler, 2008; Manley et al., 2010). Although imatinib when used at 20 mg/kg exhibited anti-fibrotic activity, it was overwhelmed by toxicity for hepatocytes as indicated by the elevation of serum aminotransferases and total bilirubin levels. Accordingly, our data suggest that imatinib has a limited effect on CCl_4 pre-induced hepatic fibrosis, which were also consistent with previous studies concerned with bile duct-induced hepatic fibrosis (Neef et al., 2006) and bleomycin-induced pulmonary fibrosis (Vittal et al., 2007).

In conclusion, nilotinib was more effective than imatinib and silymarin as an anti-fibrotic therapy. Furthermore, this study suggests that nilotinib may be valuable in the treatment of hepatic fibrosis in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (A), and alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) (B) in carbon tetrachloride (CCl₄)-induced fibrosis. Bars are means $\pm$ SE (n=7–8). Statistically significant differences are indicated as: ** P <0.01 and *** P <0.001 vs. CTRL; # P <0.05, ## P <0.01 and ### P <0.001 vs. CCl₄; + P <0.05 and +++ P <0.001 vs. CCl₄+Imatinib (10 mg/kg).

Fig. 2. Representative hepatic histopathology showing effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on fibrosis (A, Masson's trichrome staining, 40×) and necroinflammation (B, hematoxylin-eosin staining, 100× unless otherwise stated) in carbon tetrachloride (CCl₄)-intoxicated rats.

Fig. 3.

Representative captured pictures of hepatic morphometry (A, sirius red staining, 10×) showing effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on fibrosis in carbon tetrachloride (CCl₄)-intoxicated rats, as well as the corresponding fibrosis area (%) as assessed by the image analyzer (B). Bars are means $\pm$ SE (n=7–8). Statistically significant differences are indicated as: ** P <0.01 and *** P <0.001 vs. CTRL; # P <0.05, ## P <0.01 and ### P <0.001 vs. CCl₄; + P <0.05 and ++ P <0.001 vs. CCl₄+imatinib (10 mg/kg).

Fig. 4. Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on hepatic contents of collagen (A) and 4-hydroxyproline (B) in carbon tetrachloride (CCl₄)-induced fibrosis. Bars are means ± SE (n = 7-8). Statistically significant differences are indicated as: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs. CTRL; ##*P* < 0.01 and ###*P* < 0.001 vs. CCl₄; +*P* < 0.05 and +++*P* < 0.001 vs. CCl₄+imatinib (10 mg/kg).

Fig. 5. Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on hepatic levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (A), total nitrate/nitrite (NOx) (B), and myeloperoxidase (MPO) (C), reduced glutathione (GSH) (D) and superoxide dismutase (SOD) (E) in carbon tetrachloride (CCl₄)-induced fibrosis. Bars are means ± SE (n = 7–8). Statistically significant differences are indicated as: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs. CTRL; #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001 vs. CCl₄; ++*P* < 0.01 and +++*P* < 0.001 vs. CCl₄ + imatinib (10 mg/kg).

Table 1

Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20 mg/kg) and silymarin (100 mg/kg) treatments on serum γ -glutamyl transpeptidase (GGT), total bilirubin and albumin levels in carbon tetrachloride (CCl₄)-induced fibrosis.

Groups	n	GGT (U/L)	Total bilirubin (mg/L)	Albumin (g/dL)
CTRL	7	2.01 $\pm$ 0.39	4.15 $\pm$ 0.96	3.37 $\pm$ 0.08
CCl ₄	8	3.33 $\pm$ 0.34	12.64 $\pm$ 1.83 **	2.86 $\pm$ 0.18
CCl ₄ + imatinib (10 mg/kg)	7	5.02 $\pm$ 0.63 **	7 $\pm$ 1.08	2.91 $\pm$ 0.13
CCl ₄ + imatinib (20 mg/kg)	7	4.69 $\pm$ 0.62 *	19.4 $\pm$ 2.17 ***#+++	2.59 $\pm$ 0.07 ***
CCl ₄ + nilotinib (10 mg/kg)	7	2.77 $\pm$ 0.21 +	2.8 $\pm$ 0.61 ##	1.93 $\pm$ 0.11 ***###+++
CCl ₄ + nilotinib (20 mg/kg)	8	2.26 $\pm$ 0.39 ++	7.99 $\pm$ 0.82	2.37 $\pm$ 0.1 ***,+
CCl ₄ + silymarin (100 mg/kg)	7	3.28 $\pm$ 0.57	16.84 $\pm$ 1.42 ***+++	2.42 $\pm$ 0.11 ***,+

Values are means $\pm$ SE (n = 7–8).

* Statistically significant at $P < 0.05$ vs. CTRL.

** Statistically significant at $P < 0.01$ vs. CTRL.

*** Statistically significant at $P < 0.001$ vs. CTRL.

Statistically significant at $P < 0.05$ vs. CCl₄.

Statistically significant at $P < 0.01$ vs. CCl₄.

Statistically significant at $P < 0.001$ vs. CCl₄.

+ Statistically significant at $P < 0.05$ vs. CCl₄ + imatinib (10 mg/kg).

++ Statistically significant at $P < 0.01$ vs. CCl₄ + imatinib (10 mg/kg).

+++ Statistically significant at $P < 0.001$ vs. CCl₄ + imatinib (10 mg/kg).

Table 2

Effects of imatinib (10 and 20 mg/kg), nilotinib (10 and 20mg/kg) and silymarin (100 mg/kg) treatments on fibrosis and necroinflammation scores in carbon tetrachloride (CCl₄)-induced fibrosis.

Groups	n	Fibrosis	Necroinflammation
CTRL	7	0	0
CCl ₄	8	3.5 ± 0.19	5.38 ± 0.42
CCl ₄ + imatinib (10 mg/kg)	7	2.29 ± 0.29 [#]	5.57 ± 0.37
CCl ₄ + imatinib (20 mg/kg)	7	2.57 ± 0.48	4.71 ± 0.78
CCl ₄ + nilotinib (10 mg/kg)	7	2.43 ± 0.2 [#]	5.43 ± 0.57
CCl ₄ + nilotinib (20 mg/kg)	8	1.13 ± 0.3 ^{###}	3.5 ± 0.5 [#]
CCl ₄ + silymarin (100 mg/kg)	7	1.86 ± 0.34 ^{##}	5.29 ± 0.52

Values are means ± SE (n = 7–8).

[#] *P* < 0.05 compared with the CCl₄ group.

^{##} *P* < 0.01 compared with the CCl₄ group.

^{###} *P* < 0.001 compared with the CCl₄ group.