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## Protective role of bortezomib in steatotic liver ischemia/reperfusion injury through abrogation of MMP activation and YKL-40 expression

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### Abstract

Steatotic liver grafts tolerate ischemia–reperfusion (I/R) injury poorly, contributing to poor survival following transplantation. However the molecular mechanisms leading to I/R injury still remain to be defined. We have previously reported that the protective effect of bortezomib towards inhibiting cold induced I/R injury in obese rat liver transplant model is through NF- $\kappa$ B down modulation. In this report using an orthotopic liver transplant (OLT) model in Zucker rats (from obese, leptin deficient donor, to lean recipient) we defined the mechanisms of steatotic liver injury, and characterized the role of bortezomib in inhibiting MMP activation and YKL-40, both of which are involved in extracellular matrix deposition and fibrosis, the key pathological features of liver allograft failure. Obese donor rats were treated with bortezomib (i.v., 0.1 mg/kg immediately prior to liver procurement) to assess the role of MMP and YKL-40 in steatotic liver I/R injury. I/R injury in steatotic livers resulted in significant increases in expression of YKL-40 (9 fold), and activation of MMP-2 (15 fold)/MMP-9 (12 fold). Bortezomib treatment reduced the expression of YKL-40 and MMP to basal levels. Bortezomib also inhibited the pro-fibrotic (VEGF, HGF, bFGF, TGF- $\beta$ ) and pro-inflammatory (IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ ) cytokines significantly in comparison to untreated animals with I/R injury. These results demonstrate that I/R injury in steatotic livers

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Conflict of interest  
None.

following transplantation are associated with MMP activation and YKL-40 upregulation resulting in pro-fibrotic and pro-inflammatory cytokine release. Administration of the proteasomal inhibitor, bortezomib, effectively attenuated the I/R injury by inhibiting MMP and YKL-40 expression and therefore support the clinical utility of this drug in donor management for preventing I/R injury and its sequelae.

## Keywords

Bortezomib; Matrix metalloproteinases; YKL-40; Liver transplantation; I/R injury

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## 1. Introduction

Liver transplantation is considered as a viable therapeutic option for end stage liver diseases. However, the shortage of available organs contributes to the waiting list mortality [1]. Hepatic steatosis remains one of the major reasons for liver organ discards with a prevalence of 13–50% [2,3]. The current prevalence of obesity and metabolic syndrome seems to be the major risk factor for the increased incidence of hepatic steatosis in potential donors [4]. Steatotic livers are more susceptible to ischemia/reperfusion (I/R) injury induced by both ischemic periods from organ procurement to engraftment, which is directly proportional to higher graft failure rate [5-7]. Severe steatosis, with greater than 60%, is generally viewed as a contraindication for liver transplantation [6,7], while livers with moderate steatosis (30–60%) are sometimes used for transplantation due to limited availability of the donor organs [2]. These grafts usually have a higher incidence of I/R injury [6,7]. However the molecular mechanisms that contribute to increased susceptibility of steatotic grafts to I/R injury remain poorly understood.

Ischemia reperfusion (I/R) injury of the liver consists of direct ischemic damage during organ procurement and the subsequent reperfusion injury during engraftment. The activation of Kupffer cells in I/R injury induces proinflammatory cytokines including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) [8,9]. These mediators facilitate neutrophil sequestration in the ischemic liver and induce subsequent neutrophil-dependent organ dysfunction through the release of reactive oxygen species and proteases from the neutrophils [10]. Matrix metalloproteinases (MMPs) are proteolytic enzymes that induce the extracellular matrix deposition and play an important role in inflammation, and fibrotic damage [11]. MMPs play an important role in I/R injury in the liver, lung, and heart [12-14]. Along with MMP activation, our previous studies have demonstrated elevated fibrotic biomarker YKL-40, a chitinase like glycoprotein, in the sera of patients with liver diseases, including hepatic fibrosis associated with hepatitis C virus [15]. Serum concentrations of YKL-40 correlated with extracellular matrix (ECM) products secreted by hepatic stellate cells (HSCs) and fibroblasts in hepatic fibrosis [16]. Therefore, YKL-40 and MMPs appear to be two important mediators of tissue remodeling in the setting of liver injury [17].

Bortezomib (PS-341), a selective inhibitor of 26S proteasome is an FDA approved drug for the treatment of multiple myeloma and mantle cell lymphoma [18]. We have previously reported that bortezomib mediated blockade of NF- $\kappa$ B activation attenuated the I/R injury in

rat steatotic liver transplant model [19]. In the current study we used the leptin deficient Zucker rat liver transplant model which exhibits uniform macrovesicular steatosis that closely resembles the clinical setting of human fatty livers employed in transplantation. Using this well established rat model of cold I/R injury, we demonstrate that the activation of MMPs and the upregulation of YKL-40 are the major downstream mechanisms responsible for steatotic liver I/R injury and this can be abrogated with the administration of the proteasome inhibitor bortezomib to donors prior to liver procurement.

## 2. Objective

The overall objective of the study is to characterize the early molecular events mediated by pro-fibrotic biomarkers, matrix metalloproteinases (MMPs) and YKL-40, that contribute to the exacerbated I/R injury using an established rat steatotic liver transplantation in the steatotic liver transplantation model and to determine the ability of chemical small molecule bortezomib (PS-341) to ameliorate I/R injury in the steatotic liver transplantation model.

## 3. Materials and methods

### 3.1. Animal model

Zucker rats represent a well-characterized model of leptin receptor deficiency induced obesity [20] and were used in this study. Animals were housed in pathogen-free conditions with 12-hour diurnal light cycle and access to standard rodent chow and water ad libitum. Surgical procedures were performed under aseptic conditions approved by the Washington University Animals Studies Committee and in accordance with the National Institutes of Health guidelines in “The Guide for the Care and Use of Laboratory Animals”. Obese Zucker rats aged 9–11 weeks and weighing 250–350 g were chosen as donors with weight-matched heterozygous (lean) rats used as recipients. Donor–recipient pairs were matched for sex and weight as controls.

Drugs: bortezomib (PS-341, i.v., 0.5 mL of 0.1 mg/kg) (Millenium Pharmaceuticals, Boston, MA) was given to obese donors immediately prior to liver procurement, while control animals received intravenous saline.

### 3.2. Orthotopic liver transplantation and cold I/R injury

Orthotopic liver transplantation was performed under isofluorane (Baxter, IL) inhalational anesthesia using Kamada's modified cuff technique [21]. Prior to organ procurement, 1 mL of saline containing 200 units of heparin was given intravenously, and the donor liver perfused via portal vein with 10 mL of cold physiological saline, followed by 10 mL of cold University of Wisconsin (UW) solution. Following cuff preparation, the graft was stored in UW solution at 4 °C for 2 h. Following storage, the graft was slowly flushed with 20 mL of cold saline prior to transplantation.

### 3.3. MMP2 and MMP9 determination

The MMP2 and MMP9 activity in the engrafted livers collected 2 h and 24 h following transplantation was determined by gelatin zymography [22-24]. The protein concentration of

the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL), and 5 µg of supernatant proteins were resolved by non-reducing 10% SDS-PAGE through Novex Tris–Glycine gels containing 0.1% gelatin (Invitrogen, Carlsbad, CA). The gels were then developed as per manufacturer's instructions (Invitrogen, Carlsbad, CA). The gelatinolytic activity of the MMPs were quantitatively analyzed by the optical density of the bands using the Kodak image analysis system (Gel Logic 100 System; Kodak, Inc., Rochester, NY).

The MMP2 and MMP9 activity is further quantitated using gelatinase substrate activity [25] (MMP-2/RPN2631, MMP-9/RPN2634, biotrack activity assay kit, GE Healthcare, Pittsburgh, PA) as per manufacturer protocols. Briefly, MMPs were captured by specific antibodies precoated microplate, the activity of which was eventually measured using chromogenic peptide substrate read at 405 nm. The concentration of active MMP is interpolated from a standard curve obtained using the manufacturer provided standard.

The MMP2 and MMP9 mRNA level expressions were analyzed using the FAM-labeled RT-PCR primers (Applied Biosystems, Foster City, CA) as per the manufacturer's recommendation. Briefly, total RNA was extracted from liver using TRIzol reagent (Sigma-Aldrich). The RNA was reverse-transcribed and Real-time PCR was performed in a final reaction volume of 20 µL using iCycler 480 Probes Master (Roche Diagnostics). Each sample was analyzed in triplicate. Cycling conditions consisted of an initial denaturation of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, followed by 61 °C for 1 min [26].

#### 3.4. YKL-40 determination

Protein levels of YKL-40 were analyzed using the Western blot. The retrieved livers were lysed using 4% SDS cell lysis buffer supplemented with protease inhibitor cocktail and EDTA. The lysates were boiled for 20 min in sample buffer (200 mmol/L Tris (pH6.8), 20% glycerol, 2% SDS, 0.1% bromophenol blue, and 10% β-mercaptoethanol) and centrifuged for 30 min and run on 4–12% gradient Bis–Tris denaturing gel (Invitrogen). The gel was transferred onto nitrocellulose membrane, probed with appropriate primary and secondary antibodies, developed and analyzed on using Bio-Rad Universal Hood II (Hercules, CA). Densitometric analysis was done using the software provided by the company [27].

#### 3.5. Growth factor and cytokine gene expression analysis

Expression profiles of intracellular inflammatory cytokine (IL-1β, IL-10, TNF-α and IFNγ), pro-fibrotic (VEGF, HGF, TGF-β and bFGF), and YKL-40 signal genes in the retrieved livers were analyzed using the FAM-labeled RT-PCR primers (Applied Biosystems, Foster City, CA) as per the manufacturer's recommendation. Briefly, total RNA was extracted from the liver using TRIzol reagent (Sigma-Aldrich). The RNA was reverse-transcribed and Real-time PCR was performed in a final reaction volume of 20 µL using iCycler 480 Probes Master (Roche Diagnostics). Each sample was analyzed in triplicate. Cycling conditions consisted of an initial denaturation of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30s, followed by 61 °C for 1 min [26].

### 3.6. Statistical analysis

All the molecular studies were performed from the tissues obtained from independent experiments performed on animals (n = 5) from each cohorts. The data were analyzed using Graphpad prism 4 software, statistical difference between groups were analyzed by ANOVA, and when significant differences were observed Bonferroni's multiple comparison was performed. Results are presented as mean  $\pm$  standard error, and considered significant when  $p < 0.05$ .

## 4. Results

### 4.1. Inhibition of MMP activation by PS341 following IR injury

The activation of matrix metalloproteinases (MMP) has been implicated in the I/R injury leading to poor steatotic graft survival [28]. We performed gelatin zymography to determine the role of bortezomib on MMP activation. As shown in Fig. 1, cold I/R injury of the steatotic liver induced the activation of MMP 9 and MMP 2 when steatotic livers were transplanted to lean recipients (2 h and 24 h following transplantation). However, following treatment with bortezomib there is significant reduction in the densitometry based zymographic activation (Fig. 1A-C) of MMP 9 (MMP9 to pro-MMP9 ratio at 2 h: without bortezomib  $9.3 \pm 3.7$  fold vs. with bortezomib  $0.8 \pm 0.3$  fold,  $p < 0.05$ ; 24 h without  $12.2 \pm 2.4$  fold and with bortezomib  $1.8 \pm 0.5$  fold,  $p < 0.05$ ) and MMP2 (MMP2 to pro-MMP2 ratio at 2 h: without bortezomib  $12.8 \pm 5.1$  fold vs. with bortezomib  $1.8 \pm 0.5$  fold,  $p < 0.05$ ; 24 h without  $17.1 \pm 3.8$  fold and with bortezomib  $0.6 \pm 0.4$  fold,  $p < 0.05$ ). Further to more quantitatively demonstrate the changes in MMP 2 and MMP9 activity, we have performed a chromogenic activity assay (Fig. 1D,E). Similar to zymographic analysis, chromogenic data demonstrate that MMP2 (MMP2 activity in blank control, untreated non-transplanted:  $5.7 \pm 2.1$  U/ $\mu$ g of total protein; at 2 h: without bortezomib  $31.4 \pm 7.9$  U/ $\mu$ g of total protein, vs. with bortezomib  $9.8 \pm 3.7$  U/ $\mu$ g of total protein,  $p < 0.05$ ; 24 h without  $39.2 \pm 8.3$  U/ $\mu$ g of total protein and with bortezomib  $11.6 \pm 4.7$  U/ $\mu$ g of total protein,  $p < 0.05$ ) and MMP9 (MMP9 activity in blank control, untreated non-transplanted:  $0.7 \pm 0.3$  U/ $\mu$ g of total protein; at 2 h: without bortezomib  $6.2 \pm 1.9$  U/ $\mu$ g of total protein, vs. with bortezomib  $1.4 \pm 0.6$  U/ $\mu$ g of total protein,  $p < 0.05$ ; 24 h without  $9.7 \pm 2.3$  U/ $\mu$ g of total protein and with bortezomib  $1.8 \pm 0.5$  U/ $\mu$ g of total protein,  $p < 0.05$ ) activity was reduced upon bortezomib treatment in our orthotopic liver transplant animal model. The MMP2 and MMP9 expression was quantitatively determined by RT-PCR (Fig. 1F). The ratio of MMP9 to MMP2 demonstrated that the expression of constitutively expressed MMP2 was significantly reduced over the inducibly expressed MMP9 [29,30] and thus increasing the numerical ratio of MMP9 to MMP2 following bortezomib treatment (MMP9 to MMP2 expression in blank control, untreated non-transplanted:  $0.17 \pm 0.05$ ; at 2 h: without bortezomib  $0.39 \pm 0.11$ , vs. with bortezomib  $0.75 \pm 0.19$ ,  $p < 0.05$ ; 24 h without  $0.37 \pm 0.14$  and with bortezomib  $0.67 \pm 0.15$ ,  $p < 0.05$ ). These results demonstrate that bortezomib has direct inhibitory effect on MMP and therefore ECM deposition in the transplanted livers.

#### 4.2. Inhibition of YKL-40 expression following PS341 treatment

Increased expression of the pro-fibrotic and pro-inflammatory signaling molecule, YKL-40, has been demonstrated in hepatic injury related disease states [31]. As shown in Fig. 2, we demonstrate that cold I/R injury of the steatotic liver increases the expression of YKL40 following obese to lean liver transplants at 2 h and 24 h following I/R injury. However, following treatment of donors with bortezomib prior to procurement and transplantation, there is decreased expression of YKL-40 at 2 h ( $6.7 \pm 1.4$  fold vs.  $1.9 \pm 0.7$  fold,  $p < 0.05$ ) and 24 h ( $8.9 \pm 1.7$  fold vs.  $1.3 \pm 0.5$  fold,  $p < 0.05$ ). These results suggest that the proteasomal inhibitor bortezomib can decrease the inflammatory and fibrotic injury by inhibition of YKL-40 protein.

#### 4.3. Inhibition of pro-inflammatory cytokines with PS341 treatment

Poor outcome of steatotic liver allografts have been shown to be associated with increased pro-inflammatory cytokines in the circulation [19]. To determine the effect of bortezomib on the pro-inflammatory cytokines in the serum in the cold I/R injury of the steatotic liver we performed LUMINEX assay on the expression of IL-1 $\beta$ , IL-10, TNF- $\alpha$  and IFN- $\gamma$  following obese to lean liver transplant As shown in Fig. 3, IL-1 $\beta$  serum concentration increased upon 2 h and 24 h warm I/R injury. However, bortezomib treatment significantly decreased pro-inflammatory cytokine, IL-1 $\beta$ . on 2 h  $983 \pm 177$  pg/mL vs.  $271 \pm 67$  pg/mL,  $p < 0.05$ ; blank control, untreated non-transplanted was  $93 \pm 26$  pg/mL and 24 h ( $1187 \pm 237$  pg/mL vs.  $139 \pm 41$  pg/mL,  $p < 0.05$ ). Similarly, other pro-inflammatory cytokines, TNF- $\alpha$  (2 h:  $594 \pm 178$  pg/mL vs.  $158 \pm 81$  pg/mL,  $p < 0.05$ ; and 24 h:  $643 \pm 91$  pg/mL vs.  $197 \pm 48$  pg/mL,  $p < 0.05$ ; blank control, untreated non-transplanted was  $87 \pm 23$  pg/mL), and IFN- $\gamma$  (2 h:  $472 \pm 66$  pg/mL vs.  $103 \pm 54$  pg/mL,  $p < 0.05$ ; and 24 h:  $513 \pm 49$  pg/mL vs.  $129 \pm 48$  pg/mL,  $p < 0.05$ ; blank control, untreated non-transplanted was  $69 \pm 18$  pg/mL) were also inhibited by bortezomib. Conversely, serum concentration of the anti-inflammatory cytokine, IL-10 increased (2 h:  $234 \pm 76$  pg/mL vs.  $567 \pm 108$  pg/mL,  $p < 0.05$ ; and 24 h:  $145 \pm 56$  vs.  $637 \pm 152$  pg/mL,  $p < 0.05$ ; blank control, untreated non-transplanted was  $887 \pm 132$  pg/mL) following bortezomib treatment These results strongly suggest that bortezomib correlates with the inhibitory effect on the induction of pro-inflammatory cytokines along with an increase in the anti-inflammatory cytokine, IL-10.

#### 4.4. Inhibition of pro-fibrotic cytokine profile with PS341 treatment in I/R injury

To determine the effect of bortezomib on the pro-inflammatory and pro-fibrotic growth factor expression in the warm IR injury of the steatotic liver we have performed quantitative RT-PCR assay on the expression of VEGF, HGF, TGF- $\beta$  and bFGF in the obese to lean liver transplant with and without bortezomib administration. As shown in Fig. 4, VEGF expression increased in 2 h and 24 h cold IR injury. However, bortezomib treatment of donors prior to procurement decreased the expression of VEGF on 2 h ( $8.1 \pm 3.3$  fold vs.  $1.6 \pm 0.8$  fold,  $p < 0.05$ ) and 24 h ( $9.4 \pm 2.9$  fold vs.  $2.6 \pm 1.7$  fold,  $p < 0.05$ ; fold increase determined as ratio expression over blank control, untreated, non-transplant) warm perfusion. Similarly, other fibrotic growth factors, HGF (2 h:  $6.2 \pm 2.1$  fold vs.  $1.2 \pm 0.7$  fold,  $p < 0.05$ ; and 24 h:  $7.2 \pm 2.8$  fold vs.  $1.8 \pm 1.1$  fold,  $p < 0.05$ ), TGF- $\beta$  (2 h:  $5.8 \pm 2.4$  fold vs.  $1.5 \pm 0.6$  fold,  $p < 0.05$ ; and 24 h:  $6.4 \pm 1.8$  fold vs.  $1.4 \pm 0.9$  fold,  $p < 0.05$ ) and



bFGF (2 h:  $8.9 \pm 2.4$  fold vs.  $2.1 \pm 0.9$  fold,  $p < 0.05$ ; and 24 h:  $11.4 \pm 3.9$  fold vs.  $3.2 \pm 1.8$  fold,  $p < 0.05$ ) were all inhibited by bortezomib. These results demonstrate that bortezomib strongly correlates with the inhibitory effect on the fibrotic growth factors.

## 5. Discussion

Obesity and metabolic syndrome, major risk factors for hepatic steatosis are one of the major hurdles in the usage of marginally steatotic livers for transplantation [4-7]. The lack of understanding of the mechanisms of injury in the steatotic liver remains a major challenge in the inability to initiate specific therapeutic targeting of the steatotic liver to improve its function. Previously, we have reported the protective effect of proteasomal inhibitor, bortezomib, towards reducing I/R injury in the setting of steatosis by p65 NF- $\kappa$ B down regulation [19]. In the present study we demonstrate that MMP activation and YKL-40 upregulation resulting in the induction of pro-inflammatory and pro-fibrotic cytokines appear to play a significant role in initiating I/R injury following transplantation of steatotic livers which is efficiently abrogated by bortezomib.

Matrix metallo-proteinases (MMPs) have been associated with increased ECM turnover and fibrosis. Using murine MMP knock out models Hamada et al. [28] have demonstrated that MMP deficiency has a protective effect on hepatic I/R injury. Further MMP activation has also been demonstrated in various vascular and inflammatory disease models [32]. Further, reports by Shirhane et al. [33] have suggested that MMP inhibitor (ONO-4817) prevented hepatic I/R injury in non-steatotic rat liver transplant models [33]. These studies have also determined that this MMP inhibitor (ONO-4817) also reduced serum pro-inflammatory cytokine including TNF- $\alpha$  and IL-1 $\beta$  [33]. Transcription factors, especially NF- $\kappa$ B, induce IL-1 $\beta$  and TNF- $\alpha$ , have been shown to promote expression of other transcriptional factors [34]. Previous studies from our lab have demonstrated that bortezomib (PS-341) prevents hepatic I/R injury by suppression of p65 NF- $\kappa$ B activity [19]. Results presented in the current report demonstrate that bortezomib also inhibits MMP activity (Fig. 1) and NF- $\kappa$ B transcription factor expression [19], which play a significant role in inhibiting key downstream pro-inflammatory cytokines (Fig. 3) such as (IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ ). It is of interest that along with reduction in pro-inflammatory cytokines noted following bortezomib administration to the donors it also up regulated the expression of cytokine IL-10 (Fig. 3) which is known to have strong anti-inflammatory properties. These results are in good agreement with reports by Yoshidome et al. [35] which demonstrate a protective effect of IL-10 cytokine administration in preventing hepatic I/R injury following liver transplantation in a murine model [35]. These cytokine data need to be further studied by functional characterization studies such as Luciferase promoter assays.

Several studies have found a correlation between serum YKL-40 levels and liver fibrosis [36,37]. Fibrous deposition is a major reason for hepatic allograft failure [38]. YKL-40 is considered to be an important fibrotic growth factor that stimulates proliferation of cells that produce ECM proteins [39]. Although liver fibrosis and cirrhosis are characterized by inflammatory infiltration, a process in which a great number of cells participate, analysis using immunochemistry have demonstrated increased YKL-40 expression in fibrotic areas in the liver tissue effected by alcohol and viral hepatitis associated injury [16]. Our results

demonstrate that YKL-40 expression increased in the liver allografts following cold I/R injury and the expression was significantly elevated at both 2 h (6.5 fold) and 24 h (9 fold) following the insult (Fig. 1), and specific RNA levels began to increase at 12 h, with a maximum peak at 24 h (Fig. 2). Along with YKL-40, we also noted increased expression of other downstream pro-fibrotic growth factors following the cold I/R injury. An important finding in our study is that the administration of bortezomib to the donor rats prior to procurement of steatotic livers for transplantation resulted in the return of expression levels of fibrotic biomarker YKL-40 (Fig. 2) to basal levels along with significant reduction in down stream pro-fibrotic signaling molecules. Although the attenuation of hepatic fibrosis by bortezomib (PS-341) has not yet described in literature, its role in inhibiting lung and skin fibrosis has been documented [40]. The results presented in Figs. 2 and 4 clearly demonstrate that bortezomib has an inhibitory role towards attenuation of fibrotic cascade in the I/R injury model. As YKL-40 induction has been correlated with NF- $\kappa$ B signaling in other hepatitis models [15] we propose that similar signaling inhibition might play a critical role in our finding of bortezomib induced inhibition of YKL-40 and other pro-fibrotic signaling molecules which needs to be further characterized.

In summary, our data demonstrate an important role for MMP and YKL-40 in the pathogenesis of I/R injury in steatotic livers. The current study demonstrates that proteasome inhibitor bortezomib, decreased MMP activation and YKL-40 expression which in collaboration with NF- $\kappa$ B inhibition downregulates the pro-inflammatory (IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ ) and pro-fibrotic (VEGF, TGF- $\beta$ , HGF, bFGF) cascade of destructive events and thus ultimately leading to the reduction in I/R associated graft injury in fatty liver. Defining the mechanisms by which MMP activation and YKL-40 signaling leads to upregulation of pro-inflammatory and pro-fibrotic cascade may provide important therapeutic targets to prevent I/R injury in following transplantation of steatotic livers in clinical settings.

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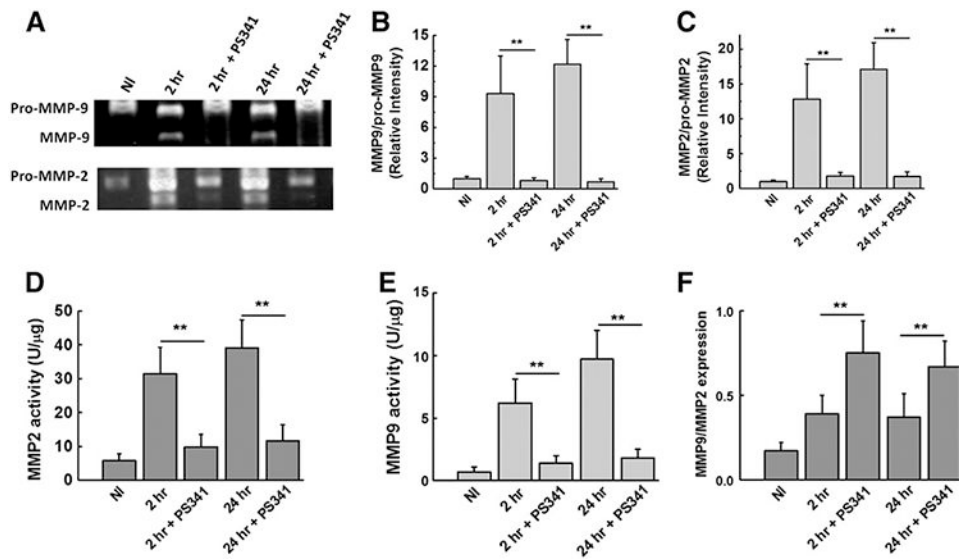
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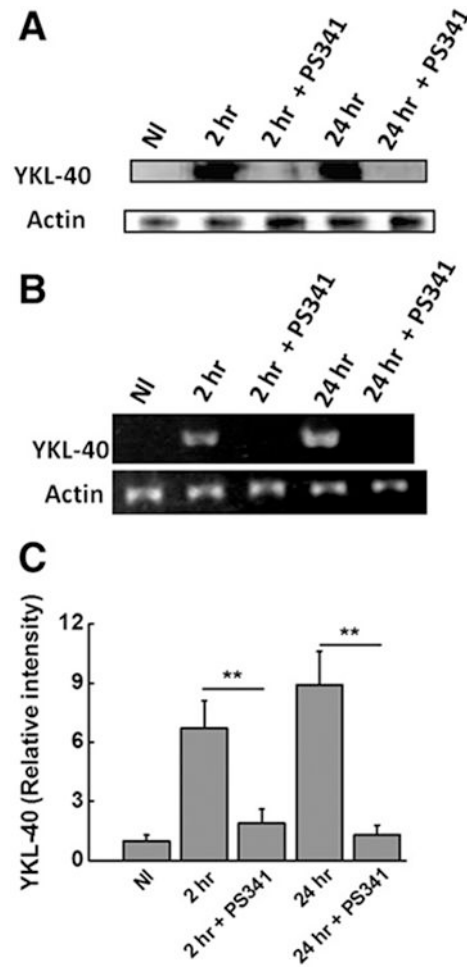


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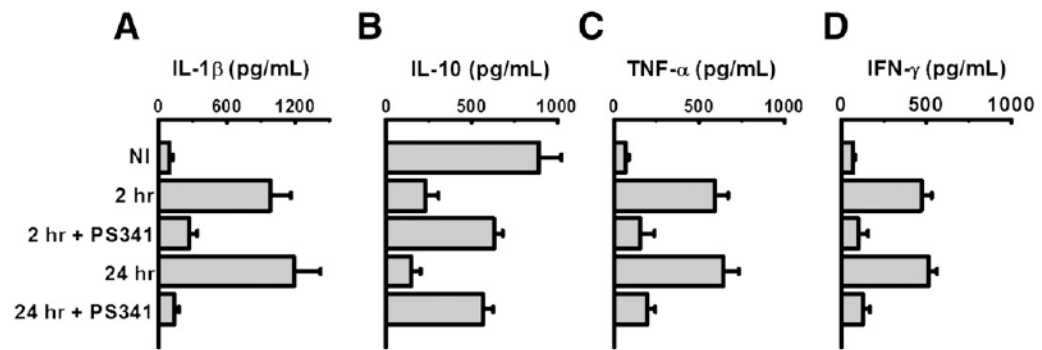
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**Fig. 1.**

Inhibition of MMP activation by bortezomib (PS341). (A) Gelatin zymography assay to determine the activity of MMP2 MMP2 (66 kDa) and MMP9 (97 kDa) at 2 h and 24 h following cold I/R injury with and without treatment of bortezomib; (B and C) Densitometric analysis of the ratio of activated over pro-form, MMP9 and 2 at 2 h and 24 h following cold I/R injury with and without treatment of bortezomib, respectively; (D, E) Gelatinase substrate activity assay of MMP2 (D), and MMP9 (E) were performed in the above mentioned cohorts; (F) Ratio of MMP9 to MMP2 expression determined by RT-PCR, performed in the above mentioned cohorts. All the studies were performed from the tissues obtained from independent experiments performed on animals (n = 5) from each cohorts. The significance, (\*\*),  $p < 0.05$ , determined by between groups was analyzed by ANOVA, and when significant differences were observed, Bonferroni's multiple comparison was performed.

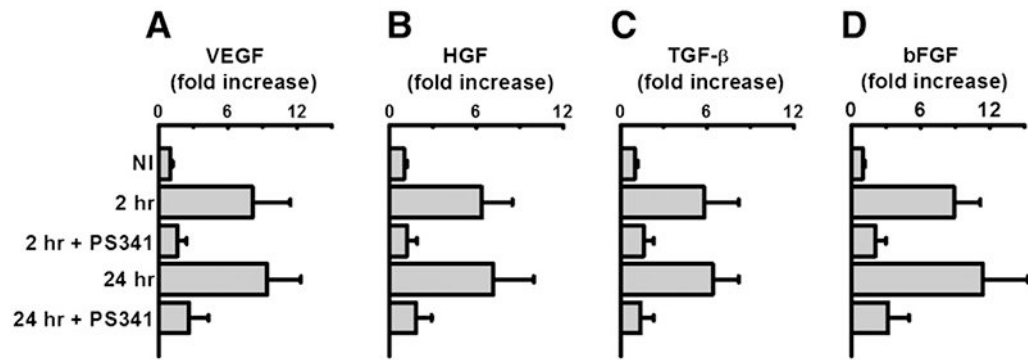


**Fig. 2.** Inhibition of YKL-40 expression by bortezomib (PS341). (A) Western-blot analysis to determine the hepatic expression of YKL-40 at 2 h and 24 h following cold IR injury with and without treatment of bortezomib; (B) mRNA expression analysis to determine the hepatic expression of YKL-40 at 2 h and 24 h following cold IR injury with and without treatment of bortezomib; (C) RT-PCR analysis to determine the hepatic expression of YKL-40 at 2 h and 24 h following cold I/R injury with and without treatment of bortezomib. All the studies were performed from the tissues obtained from independent experiments performed on animals (n = 5) from each cohorts. The significance, (\*\*),  $p < 0.05$ , determined by between groups was analyzed by ANOVA, and when significant differences were observed, Bonferroni's multiple comparison was performed.



**Fig. 3.**

Hepatic expression of inflammatory associated cytokines IL-1 $\beta$  (A), IL-10 (B), TNF- $\alpha$  (C) and IFN- $\gamma$  (D) at 2 h and 24 h following cold IR injury with and without treatment of bortezomib. All the studies were performed from the tissues obtained from independent experiments performed on animals (n = 5) from each cohorts.



**Fig. 4.** Hepatic expression of pro-fibrotic growth factors VEGF (A), HGF (B), TGF- $\beta$  (C) and bFGF (D) at 2 h and 24 h following cold IR injury with and without treatment of bortezomib. All the studies were performed from the tissues obtained from independent experiments performed on animals (n = 5) from each cohorts. The fold increase in the expression was presented as a ratio of expression of the pro-fibrotic growth factor over the blank negative control (untreated, non-transplanted animal cohort).