

# Inhibition of DDR1 and DDR2 receptors: The Potential Therapeutic Option to Treat Pancreatic Fibrosis Demonstrated in Experimental Chronic Pancreatitis Model

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## Research Article

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# Abstract

Discoidin domain receptors (DDR1 and DDR2) are the collagen receptors of the family tyrosine kinases, which play significant role in the diseases like inflammation, fibrosis and cancer. Chronic pancreatitis (CP) is a fibro-inflammatory disease in which recurrent pancreatic inflammation leads to pancreatic fibrosis.

**Methods:** In the present study for the first time, we have investigated the role of DDR1 and DDR2 in cerulein-induced CP. Pancreatic DDR1 and DDR2 expression was examined by immunoblotting and immunostaining analysis. Subsequently, the protective effects of selective DDR1 and DDR2 inhibitor, imatinib (IMT) was investigated in CP mice as a therapeutic intervention.

**Results:** Activated pancreatic stellate cells (PSCs) and CP mice showed significant upregulation of DDR1 and DDR2 expression. Pharmacological intervention with IMT effectively downregulated DDR1 and DDR2 expression. Further, we found that pancreatic injury, inflammation, extracellular matrix (ECM) deposition and PSCs activation were significantly inhibited by IMT. Further, we found a significant inhibition of TGF- $\beta$ 1/Smad signaling pathway by IMT and showed its protective effects against CP and associated fibrosis.

**Conclusions:** Taken together, these results suggest that inhibition of DDR1 and DDR2 controls pancreatic inflammation and fibrosis, which could represent an attractive and promising therapeutic strategy for the treatment of CP.

## Introduction

Chronic pancreatitis (CP) is a fibro-inflammatory disease of the pancreas, characterized by recurrent pancreatic inflammation and fibrosis leading to irreversible impairment of exocrine and endocrine functions of pancreas [1]. CP is a major risk factor for the progression of diabetes and pancreatic cancer in the developed countries [2]. Although there has been an extensive research in CP during last few years, but the exact pathogenesis and specific molecular mechanism of CP needs to be elucidated. The well-established theory behind CP development is premature activation of pancreatic digestive enzymes leading to autodigestion of pancreatic tissue and the recruitment of the excess inflammatory cells that results in pancreatic inflammation. Pancreatic fibrosis is a complex pathological event in which dysregulation of production and degradation of extracellular matrix (ECM) in the pancreas. Persistent inflammation of pancreas causes the activation of pancreatic fibroblasts, known as pancreatic stellate cells (PSCs) which promotes development of pancreatic fibrosis. Upon activation, PSCs undergo morphological changes and initiate pancreatic fibro-inflammation through the secretion of a number of inflammatory mediators and excessive synthesis of ECM proteins such as collagen I, III and fibronectin [3]. Among these, transforming growth factor (TGF)- $\beta$  is the potent profibrotic cytokine which regulates PSCs activation and pancreatic fibrogenesis through its downstream Smad signaling pathway [4]. Currently, CP treatment is only limited to the supportive care, there is no clinically available therapeutic

agent to treat CP and associated fibrosis progression. Thus, there is a pressing need to identify novel therapeutic agents for the treatment of CP on high priority, which could be helpful to improve the quality of life and reduce costs of long-term therapy.

Discoidin domain receptors (DDR1 and DDR2) are new class of tyrosine kinase receptors, which get activated in response to collagens and involved in the early embryonic development [5]. DDR1 is majorly expressed in epithelial cells and activated by both fibrillar and nonfibrillar collagens (type I to V, VIII, and XI), while DDR2 is expressed in fibroblasts and activated by fibrillar collagens (type I and III). This binding causes phosphorylation and dimerization of the tyrosine kinase receptors leads to the activation of different signaling pathways involved in inflammation and fibrosis [6]. Accumulating evidences have reported that upregulated expression of DDR1 and DDR2 are observed in the chronic injury, inflammation and fibrotic conditions [7]. Majority of the studies reported that inhibition of DDR1 reduced deposition of collagen I and IV in alcoholic liver fibrosis, lung fibrosis, cirrhotic liver and chronic nephropathies [8–10]. On the other hand, DDR2 is a key regulator of epithelial to mesenchymal transition (EMT) program, which is involved in several pathological changes such as fibrosis and tumor progression [11]. Although, the DDRs are involved in inflammatory and fibrotic diseases but their role in CP and associated fibrosis still not explored. Therefore, targeting DDR1 and DDR2 are considered as a potential therapeutic strategy for the treatment of CP.

Imatinib mesylate (IMT) is a standard Food and Drug Administration (FDA)-approved drug for the treatment of patients with chronic myelocytic leukemia. IMT is the only tyrosine kinase inhibitor which potentially inhibited both the profibrotic cytokines TGF- $\beta$  and PDGF which are involved in regulation of organ fibrosis process. Recently, several other targets of IMT have been explored. Further, emerging studies have shown that IMT has the potent tyrosine kinase DDR1 and DDR2 inhibitory activity [12, 13]. IMT is the type-2 DDRs inhibitor which inhibits tyrosine kinase domain by leveraging the ATP binding site as well as the allosteric site, which is freely accessible in the inactive conformation. However, the effect of IMT on CP has not been studied yet and the underlying molecular mechanism remains to be explored. Therefore, in the present study we investigated the role DDR1 and DDR2 in progression of CP and associated fibrosis. In addition, we investigated the protective effects of pharmacological inhibitor of DDR1 and DDR2, IMT against CP and pancreatic fibrosis. Our results showed the upregulated expression of DDR1 and DDR2 in CP mice and isolated mouse PSCs. Further, we found the activation of PSCs and TGF- $\beta$ 1/Smad signaling pathway is associated with upregulated DDR1 and DDR2 expression. However, inhibition of these receptors signaling by DDR1 and DDR2 inhibitor, IMT could effectively attenuated CP and associated fibrosis. Our data suggest the DDR1 and DDR2 could be a potential therapeutic target for treatment of CP and associated fibrosis.

## Materials And Methods

### Reagents

Imatinib mesylate (IMT) was procured from Sigma Aldrich, USA. Cerulein was obtained from Ana Spec, France. TGF- $\beta$ 1 was procured from Bio-legend, USA. Antibodies used in this study were  $\beta$ -actin (Catalogue no. sc-47778), collagen1a (Catalogue no. sc-393573), collagen3a (Catalogue no. sc-271249),  $\alpha$ -SMA (Catalogue no. sc-53142), CTGF (Catalogue no. sc-373936), TGF- $\beta$ 1 (Catalogue no. sc-146), DDR2 (Catalogue no. sc-81707), pSmad2/3 (Catalogue no. #8828), Smad2/3 (Catalogue no. #8685), pDDR1 (Catalogue no. #14531), DDR1 (Catalogue no. #5583), pNF- $\kappa$ B (Catalogue no. #3033), NF- $\kappa$ B (Catalogue no. #8242) were procured from Santa Cruz Biotechnology Ltd and Cell Signaling Technology, USA. The ELISA kits of TGF- $\beta$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (catalogue no. TGF- $\beta$ : 88-8350, IL-1 $\beta$ : 88-7013, IL-6: 88-7064, and TNF- $\alpha$ : 88-7324) were obtained from eBioscience, USA.

### **PSCs isolation, culture and treatment**

PSCs were isolated from the mice according to the methods as described with slight modifications [14]. Briefly, pancreas was isolated from adult male Swiss albino mice. Isolated pancreas was immediately kept in sterile PBS. Further, small portion of pancreas was cut, minced with scissors and single cell suspension was prepared by using 1 ml syringes in Dulbecco's Modified Eagle (DME) medium. Then, cells suspension was centrifuged at 3000 rpm for 3 min followed by two times washing with PBS. Cells pellet was mixed and cultured in DMEM medium containing 1% antibiotic solution and supplemented with 20% fetal bovine serum (FBS). PSCs were separated by using the Histodenz density gradient centrifugation [15].

### **Oil-red O staining for PSCs identification**

After 70% confluency, PSCs were fixed with 4% paraformaldehyde and 0.1% Triton X-100 reagents and stained with oil-red O working solution (1% in isopropanol) at 60°C for 30 min. Next, PSCs were counterstained using hematoxylin, mounted with DPX and observed under the microscope [16].

### **Confocal microscopy**

Freshly isolated and cultured PSCs were treated with TGF- $\beta$ 1 (10 ng/ml) and IMT (1  $\mu$ M). After 24 hr of treatment, cells were fixed with 4% paraformaldehyde and 0.1% Triton X-100 followed by blocking with 3% bovine serum albumin (BSA) for 1 hr. Then cells were labeled with primary antibodies against  $\alpha$ -SMA, collagen 1a, pSmad2/3, pDDR1, and DDR2 at 4°C for overnight. Next day, cells were probed with secondary antibody conjugated with rhodamine (1:200 dilution) or fluorescein isothiocyanate (FITC) (1:100 dilution) for 1 hr. After washing, cells were mounted with fluoroshield DAPI (Sigma-Aldrich, USA) medium and fluorescent signals were captured by confocal laser-scanning microscopy (Leica, Germany) [17].

### **Animals and experimental design**

Animals were kept in well-controlled housing facilities at temperature (25  $\pm$  2°C) and 12/12 hr light/dark cycle with free access to water as ad libitum and pellet food. The Animal experiments were designed, conducted and reported as per the ARRIVE guidelines [18]. Specifically, all the animal experiments were

performed according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), which is the approval body for animal experimentation in India. The CPCSEA certified Institutional Animal Ethical Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER)-Hyderabad, has reviewed the animal protocol and approved it (IAEC Protocol Approval No.: NIP/01/2019/RT/365).

Male Swiss albino mice (age: 6–8 weeks, body weight 25-30 g) were purchased from Palamur Biosciences Pvt. Ltd, Mahabubnagar, India. All mice were randomly divided into 6 groups (7 mice per group, n = 42): Normal control, IMT alone, Cerulein, Cerulein + IMT low dose (LD - 1 mg/kg), Cerulein + IMT mid dose (MD - 3 mg/kg) and Cerulein + IMT high dose (HD - 10 mg/kg). CP was induced by 6-hourly exposures of cerulein (50 µg/kg/intraperitoneal (i.p.)), 3 alternative days per week, for the period of 3 weeks as per our previously published protocol [19]. Cerulein + IMT group mice were administered IMT (1, 3 and 10 mg/kg) orally, every other day after cerulein exposures for 3 weeks. Normal control animals received i.p. injection of sterile normal saline same as cerulein group. IMT alone group mice were administrated with IMT (10 mg/kg) orally 4 alternative days in the week for 3 weeks (Fig. 1). Mice were sacrificed after the completion of 21 days. Doses of IMT were selected on the basis of previous literature [20]. However, cerulein needed minimum 12 hr to produce maximum inflammatory response, IMT was given every other day after cerulein exposures for the period of 3 weeks [19].

### **Blood collection and plasma analysis**

Blood samples were collected by cardiac punctured on the day of sacrifice in heparin containing tubes and plasma was separated after centrifugation. Amylase and lipase levels were assessed in plasma and measured by using Accurex kinetic enzymatic kits and values are expressed as IU/L [19].

### **Collagen estimation by Sircol assay**

Pancreatic tissues were homogenized in PBS and then supernatants were incubated with collagen binding dye picosirius red for 1 hr at 37°C. Next, samples were centrifuged and obtained pellet was resuspend in 100% ethanol followed by centrifugation at 10,000 rpm for 10 min. Then pellet was mixed with 0.5 M NaOH solution and incubated for 30 min at 37°C. The final absorbance was taken at 540 nm and values were expressed as relative collagen content per milligram of protein [21].

### **Enzyme-linked immunosorbent assay**

Cytokines concentrations in pancreas were determined by eBioscience ELISA kits of IL-6, IL-1β, TNF-α and TGF-β1 as per the previously described protocol [22]. Concentration of cytokines were expressed as pg per milligram of protein. Total protein was estimated by Bicinchoninic acid assay kit (Sigma Aldrich, USA).

### **Histopathology and immunohistochemistry analysis**

Pancreas were fixed with 10% formaldehyde solution and paraffin-embedded pancreatic sections were taken at 5  $\mu$ m. Further, pancreatic sections were stained with H & E, picrosirius red (PSR) and Masson's trichrome (MT) as per previously described protocol [23]. For immunostaining, antigen retrieval was carried out by using citrate buffer. After washing, sections were incubated with 3% hydrogen peroxide for 15 min followed by blocking with 3% BSA. The primary antibodies against  $\alpha$ -SMA, collagen1a, pDDR1 and DDR2 (1:100) were added and kept at 4°C overnight. The HRP-conjugated secondary antibodies were added and sections were stained with DAB reagent. Further, sections were counter stained with hematoxylin and images were observed using a light microscope [24]. Quantitative analysis of fibrotic and immunopositive area were analyzed by using ImageJ software (NIH, USA).

## **Immunoblotting**

To analyze the pancreas protein expression, immunoblotting technique was used as described earlier [25, 26]. Small portion of pancreatic tissues were homogenized in RIPA lysis buffer. Then equal amounts of proteins were separated using SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane. The protein blots were then detected using ECL (Bio-Rad Laboratories, USA) and densitometry analysis of respective protein band was carried out by ImageJ software (NIH, USA).

## **Statistical analysis**

All results are given as mean  $\pm$  SEM. Student's t-test was used to determine the difference between two groups. However, differences among more than two groups were analyzed using nonparametric test one-way analysis of variance by GraphPad Prism scientific software version 6.01. Value of P less than 0.05 was considered statistically significant.

# **Results**

## **Upregulation of DDR1 and DDR2 expression in pancreatic fibrosis**

We evaluated the expression of DDR1 and DDR2 via western blot and immunohistochemical analysis in CP mice. Interestingly, we found marked upregulation of phosphorylated DDR1, DDR2 and collagen1a expression in pancreatic tissue of CP mice (Fig. 2A-D). In addition, immunohistochemical (IHC) staining and quantification analysis demonstrated that expression of DDR1 and DDR2 were significantly increased in CP model mice as compared to the normal control mice (Fig. 2E). These results clearly indicated the involvement of DDR1 and DDR2 receptor signaling in the progression of pancreatic fibrosis.

## **Inhibition of DDR1 and DDR2 prevents progression of pancreatic fibrosis**

Next, to investigate whether collagen receptors DDR1 and DDR2 play key role in the development of CP and associated fibrosis or not, we evaluated the effect of selective DDR1/DDR2 inhibitor, IMT in cerulein-induced CP model. Treatment with IMT (1, 3 and 10 mg/kg) showed significant downregulation of phosphorylated DDR1 and DDR2 expression in a dose-dependent manner (Fig. 3A-C). In addition, we found that cerulein-treated mice demonstrated significantly higher collagen expression, whereas oral

administration of IMT dose-dependently reduced collagen expression as compared to cerulein-induced mice (Fig. 3D-E). On the other hand, we further validated the effect of IMT on TGF- $\beta$ 1-induced PSCs activation by using confocal microscopy using antibodies against pDDR1, DDR2 and collagen1a. Our results demonstrated that TGF- $\beta$ 1-treated PSCs showed notably upregulated pDDR1, DDR2 and collagen1a expression and these protein expressions were markedly inhibited by IMT treatment (Fig. 3F-G). Together, these results suggest that inhibition of DDR1 and DDR2 expression suppressed collagen deposition and pancreatic fibrosis.

### **IMT controls collagen deposition in the pancreas**

IHC staining and their quantification analysis showed that the expression of DDR1, DDR2 and collagen1a were strongly increased in CP mice. However, IMT treatment prevented the elevated pancreatic DDR1, DDR2, and collagen1a expression (Fig. 4A-C). Further, collagen deposition in the pancreatic tissue were examined by PSR, MT staining and Sircol assay. Pancreatic sections of cerulein-treated mice showed marked massive pancreatic fibrosis illustrated by red color in PSR staining while blue color in MT staining and which is observed around the pancreatic ducts. Interestingly, treatment with DDRs inhibitor, IMT significantly attenuated collagen deposition in the pancreas (Fig. 4D-E). In addition, Sircol assay was performed to measure total collagen content in the pancreas. Our results demonstrated that significantly increased collagen content in CP mice, and this increase collagen content was effectively attenuated by IMT treatment (Fig. 4F).

### **IMT attenuates cerulein-induced pancreatic injury and inflammation**

Next, we investigated the protective effects of DDRs inhibitor, IMT against cerulein-induced CP model. Cerulein challenged mice demonstrated significant elevation in plasma amylase and lipase levels, while IMT administration effectively reduced these levels (Fig. 5A-B). Further, to evaluate the antifibrotic and anti-inflammatory effects of IMT, histopathological examination was performed in the pancreatic tissue by H&E staining. Our results revealed that the hallmark characteristics of CP including acinar cell atrophy, vacuolization, inflammatory cells infiltration and collagen deposition in the cerulein control pancreas. On the other hand, pharmacological intervention with IMT markedly ameliorated cerulein-induced histopathological alterations as shown in Fig. 5C. These results suggest that IMT attenuated cerulein-induced pancreatic injury in CP mice. Furthermore, we examined the effect of IMT on NF- $\kappa$ B p65 expression by western blot analysis. Interestingly, we found that the expression of phosphorylated NF- $\kappa$ B p65 was significantly increased in cerulein challenged mice, while IMT mid dose and high dose mice demonstrated significant inhibition of phosphorylated NF- $\kappa$ B expression in the pancreatic tissues (Fig. 5D & E). In addition to this, we also investigated the effects of IMT on cerulein-induced pancreatic inflammation in CP model. We observed that repetitive cerulein exposure significantly increased the cytokines production in the pancreas, while IMT treatment markedly suppressed these cytokines levels, indicating downregulation of local production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Fig. 5F-H). Our results indicated that IMT shows potent anti-inflammatory effects and prevent pancreatic injury in CP mice.

## **IMT inhibits the activation of PSCs studied *in-vivo* and *in-vitro* model**

PSCs activation is a major event involved in the synthesis and accumulation of ECM proteins resulting in the development of pancreatic fibrosis. In response to chronic injury, quiescent PSCs convert to an activated PSCs synthesizing excessive ECM proteins. In this study, we evaluated the effect of IMT on the activation of PSCs by western blot analysis and immunostaining. Our *in-vivo* results showed the expression of  $\alpha$ -SMA was profoundly increased in cerulein-treated CP mice, indicating increased number of activated PSCs during CP. Further, pharmacological treatment with IMT significantly inhibited the PSCs activation via downregulation of  $\alpha$ -SMA expression in the pancreas (Fig. 6A-D). Consistent with the *in-vivo* results, we observed marked upregulation of  $\alpha$ -SMA expression in TGF- $\beta$ 1-stimulated mouse PSCs, which is the main characteristic indication of PSCs activation. However, treatment with IMT successfully decreased  $\alpha$ -SMA expression, indicating the effective inhibition of PSCs activation by IMT (Fig. 6E). These results clearly indicated that IMT successfully inhibited PSCs activation.

## **IMT inhibited TGF- $\beta$ /Smad signaling**

TGF- $\beta$ /Smad is a well-established fibrotic signaling pathway, get activated in response to the chronic injuries (Fig. 7A). Our western blot results revealed the expression of CTGF was markedly increased in cerulein-induced CP mice, which was effectively inhibited by IMT treatment (Fig. 7B). Further, we found that IMT treatment significantly downregulated the expression of phosphorylated Smad2/3 in CP mice (Fig. 7C). In addition to this, our results demonstrated that significant increase in TGF- $\beta$ 1 levels and expression in cerulein-treated mice. Interestingly, treatment with IMT significantly inhibited expression of TGF- $\beta$ 1 in a dose-dependent manner (Fig. 7D-E). On the other hand, TGF- $\beta$ 1-induced PSCs also showed the marked increase in phosphorylated Smad2/3 expression, while this expression was effectively downregulated by IMT treatment (Fig. 7F). Taken together, these results indicated that IMT significantly inhibited TGF- $\beta$ /Smad signaling in CP model and in PSCs.

## **Discussion**

CP is a fibro-inflammatory disease which is majorly associated with excess synthesis and deposition of ECM in the pancreas and leads to the development of pancreatic fibrosis [17, 19, 23]. Due to limited treatment options, there is a need to understand the exact pathogenesis and explore therapeutically effective treatment option for CP. Although, there are multiple signaling pathways involved in the pathogenesis of CP, activation of collagen-producing PSCs is the foremost event in the progression of pancreatic fibrosis. PSCs are the main cells of pancreas involved in the ECM deposition and activation of fibrotic signaling during pancreatic fibrosis. It is well-known that TGF- $\beta$ 1, a profibrotic cytokine and its downstream signaling mediators are responsible for excessive synthesis and accumulation of ECM proteins in the pancreas. TGF- $\beta$ 1 induces the activation of PSCs resulting in excess synthesis and secretion of collagen by PSCs consequently leading to pancreatic fibrosis [27]. Recently, studies have reported that collagen receptors DDR1 and DDR2 play a central role in the regulation of inflammation and fibrotic conditions [28, 29]. Apart from this, DDR1 and DDR2 receptors also play a decisive role in the

activation of myofibroblasts and TGF- $\beta$ /Smad signaling which is majorly involved in the initiation and progression of fibrosis [30, 31].

Collagen-activated receptors, DDRs gained a considerable amount of attention because of their involvement in the chronic inflammatory and organ fibrosis conditions and are classified into 2 types: DDR1 and DDR2 [32–34]. These receptors are majorly involved in both physiological as well as pathological processes [35]. DDR1 is majorly expressed in epithelial cells and activated by both fibrillar and nonfibrillar collagens including type I to V, VIII, and XI, while DDR2 is expressed in fibroblasts which gets activated by fibrillar type I and III collagen [36]. The hallmark feature of DDRs is that they directly activated upon binding to components of ECM protein. This binding leads to phosphorylation of receptor which further leads to the activation of inflammatory and fibrotic signaling pathways [37]. Studies reported that inhibition of DDR1, reduced deposition of collagens I and IV in alcoholic liver fibrosis, cirrhotic liver and in chronic nephropathies [38–40]. In addition, upregulated DDR1 expression is also observed in idiopathic pulmonary fibrosis [34, 41]. Moreover, DDR2 plays an important role in the development and progression of lung fibrosis [42]. On the other hand, selective inhibition of DDR1 provided protective effects against fibrosis and inflammation in lung fibrosis [43]. Further, researchers reported that DDR1 null mice showed reduction in the development of renal injury associated inflammation and fibrosis [44, 45]. Furthermore, DDR2 deficiency prevented lung fibrosis and deletion of DDR2 inhibited angiotensin-induced collagen I expression in cardiac fibroblasts [33]. Accordingly, the inhibition of DDR2 resulted in attenuation of fibrosis and inflammation in kidneys [46]. Notwithstanding, the exact involvement of DDR1 and DDR2 in CP and associated pancreatic fibrosis remains unclear and has not been studied yet. In CP, repetitive injury of the pancreas triggers release of variety of cytokines and chemokines, which result in the activation of collagen-producing PSCs accompanied by excessive accumulation of ECM. Based on the above literature, in the present study, we put forward a hypothesis that upregulation of DDR1 and DDR2 receptors in CP provoke the activation of pro-inflammatory and profibrotic mediators, which accelerate the conversion of quiescent PSCs into activated PSCs resulting in excessive accumulation of ECM in the pancreas (Fig. 8).

To prove our hypothesis, we investigated the role of DDR1 and DDR2 in CP model and in PSCs. The significant upregulation of DDR1 along with DDR2 was observed in the present study suggested that these receptors play a major role in the development of pancreatic fibrosis. These results were consistent with previous literature where activation of DDR1 and DDR2 were found to be involved in the progression of fibrosis in kidney and lungs [7, 41]. Moreover, Ruggeri et al. reported the overexpression of DDR1 plays a significant role during pancreatic injury, tumor development, and tumor progression [47]. On the other hand, we observed that inhibition of DDR1 and DDR2 by IMT was associated with reduced fibrosis and inflammation suggesting a link between DDRs and pancreatic fibrosis. Our results were found to be in correlation with previous studies showing role of DDRs in fibrosis. Moll et al., reported that pharmacological inhibition of DDR1 significantly inhibited fibrotic and inflammatory protein expression in the human crescentic glomerulonephritis [38]. Further, the deletion of DDR1 in mice provided a significant reduction in the deposition of collagen in the kidneys. In addition, Li et al. demonstrated that deletion of DDR2 significantly alleviated renal interstitial fibrosis induced by unilateral ureteral obstruction [48]. In

light of the results of present as well as previous studies, it was evident that DDRs play a major role in the progression of pancreatic fibrosis and the inhibition of these receptors could become a novel approach for the fibrotic disorders.

The inflammation is one of the most important factors involved in the initiation and progression of the fibrosis in different organs. In context of pancreatic injury, the inflammatory cells infiltration along with acinar cell atrophy were major players in the CP [49]. Although, the DDRs are essential for normal development and tissue homeostasis; the overexpression of these receptors is associated with tissue injury [50]. Similarly, we also observed marked pancreatic injury and histopathological alterations including atrophy of acinar cells, vacuolization, infiltration of inflammatory cells and excessive deposition of collagen in CP pancreas. However, the inhibition of DDR1 and DDR2 resulted in reduced pancreatic injury and histopathological alterations in the present study. Our findings suggested that overexpression of DDRs is associated with pancreatic injury and inflammation. Further, the reduction in pancreatic injury by inhibition DDRs provided evidences for a strong link between induced expression of DDRs and pancreatic inflammation. Our results were consistent with the earlier reports where DDR1 and DDR2 were reported to be involved in the regulation of tissue homeostasis, cell proliferation, migration and remodeling of the ECM in the injured pancreas [47]. In addition, we found that CP mice showed activated NF- $\kappa$ B signaling accompanied with elevation of inflammatory mediators namely, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the pancreas. There is a high possibility that this increased inflammatory process could be due to the elevated DDR1 and DDR2 expression as the previous reports have already shown that DDR1 and DDR2 activation was accompanied by the induction of pro-inflammatory cytokines [50, 51]. It is highly likely that DDR1 and DDR2 could specifically initiate the release of several inflammatory mediators by the activation of NF- $\kappa$ B in CP pancreas. On the other hand, the inhibition of DDR1 and DDR2 showed anti-inflammatory potential via successful inhibition of the inflammatory signaling in CP mice further supporting the association of DDRs with inflammation. [29, 52].

PSCs are the major effector cells, play a central role in the progression and development of pancreatic fibrosis in response to persistent injury [53]. PSCs activate and proliferate in response to profibrotic cytokines such as TGF- $\beta$ 1, CTGF and PDGF [54]. Activated PSCs show induced expression of  $\alpha$ -SMA and increases the accumulation of ECM proteins including collagens type I and III, and fibronectin [55]. In fact, the activation of PSCs has been observed in pancreatic injury of humans as well as animals. Haber et al., demonstrated that PSCs are activated in the experimental and human pancreatic fibrosis [56]. In the light of previous study, we observed activation of PSCs in CP mice which could be due to the upregulation of DDR1 and DDR2 proteins. Growing evidences showed that excessive deposition of collagen type I and III activates DDR1 and DDR2 signaling, which is associated with proliferation and activation of myofibroblasts [57, 58]. Interestingly, we found the pharmacological inhibition of DDR1 and DDR2 significantly blunted PSCs activation and ECM deposition. Our results were complying with the earlier literatures, where inhibition of DDR1 and DDR2 prevented the fibroblast proliferation and migration [32, 36]. These results suggest that the suppression of PSCs activation and ECM markers might be the result of the downregulation of DDR1 and DDR2 expression, which ultimately resulted in the attenuation of

pancreatic fibrosis. Our findings imply that inhibition of DDR1 and DDR2 could be an attractive molecular target for the prevention of PSCs activation and pancreatic fibrosis.

Growing amount of evidences reported that TGF- $\beta$ 1 plays a phenomenal role in all types of fibrotic diseases including pancreatic fibrosis. It is the main mediator for the activation of PSCs which subsequently increasing deposition of ECM proteins [27]. TGF- $\beta$  binds to its receptors expressed by PSCs and initiates its fibrotic responses through the activation of Smad pathway. Further, activated Smad2/3 make a complex with Smad4 and translocate to the nucleus, where it increases the transcription of targeted genes such as collagen and  $\alpha$ -SMA. In addition, our earlier studies reported that activation of TGF- $\beta$ /Smad stimulates PSCs activation and consequently results in the progression of the pancreatic fibrosis [17, 23]. In context with CP, researchers have documented that there is an activation of TGF- $\beta$ 1/Smad signaling in response to the overexpression of DDR1/DDR2 [59], but its role in the regulation of TGF- $\beta$ 1/Smad signaling and PSCs activation in CP has not been explored. In accordance with the earlier reports, we have observed the activation of TGF- $\beta$  and its downstream mediators, which might be due to the upregulation of DDR1 and DDR2 expression in CP. Our results are in accordance with the previous study showing that DDR1 interacts with TGF- $\beta$  pathway to restrict calcifying extracellular vesicle-mediated mineralization and fibrosis in vascular smooth muscle cells [60]. Further, studies have also demonstrated that genetic deletion of the expression of DDR1 is directly associated with the downregulation of TGF- $\beta$  and CTGF in renal fibrosis [51, 61]. In addition, Zhao et al., reported that upregulation of DDR2 activates the TGF- $\beta$  signaling, while DDR2 knockdown resulted in the inhibition of TGF- $\beta$  signaling in lung fibroblasts [41]. Consistent with these findings, we observed that pharmacological inhibition of DDR1 and DDR2 effectively inhibited TGF- $\beta$ 1 and subsequent Smad signaling. Here, our results suggested that DDRs are involved in the activation of TGF- $\beta$ /Smad signaling and pharmacological inhibition of DDRs attenuated pancreatic fibrosis by inhibiting TGF- $\beta$ /Smad signaling.

## Conclusion

Taken together, the data from this study strongly provides evidences that activation of DDR1 and DDR2 are predominantly implicated in the progression of CP and associated fibrosis. Further, activation of DDR1 and DDR2 is involved in the progression of CP and associated fibrosis particularly through the enhancement of pancreatic injury, PSCs activation and TGF- $\beta$ /Smad signaling. However, pharmacological inhibition of these receptors showed promising protective effects against CP and associated fibrosis. Thus, it can be concluded that inhibition of DDRs significantly ameliorated CP and associated fibrosis and could prove to be a potential target for halting the progression of CP in humans.

## Abbreviations

DDRs, discoidin domain receptors; CP, chronic pancreatitis; IMT, imatinib; PSCs, pancreatic stellate cells; TGF- $\beta$ , transforming growth factor- $\beta$ ; ECM, extracellular matrix; PDGF, platelet-derived growth factor;  $\alpha$ -SMA,  $\alpha$ -Smooth muscle actin; CTGF, connective tissue growth factor; NF- $\kappa$ B, nuclear factor kappa B.

# Declarations

## Author contributions

S.B. performed the experiments, analyzed the data and wrote the manuscript. M.A.S. performed the experiments, wrote the manuscript. C.G. designed the study and corrected the manuscript.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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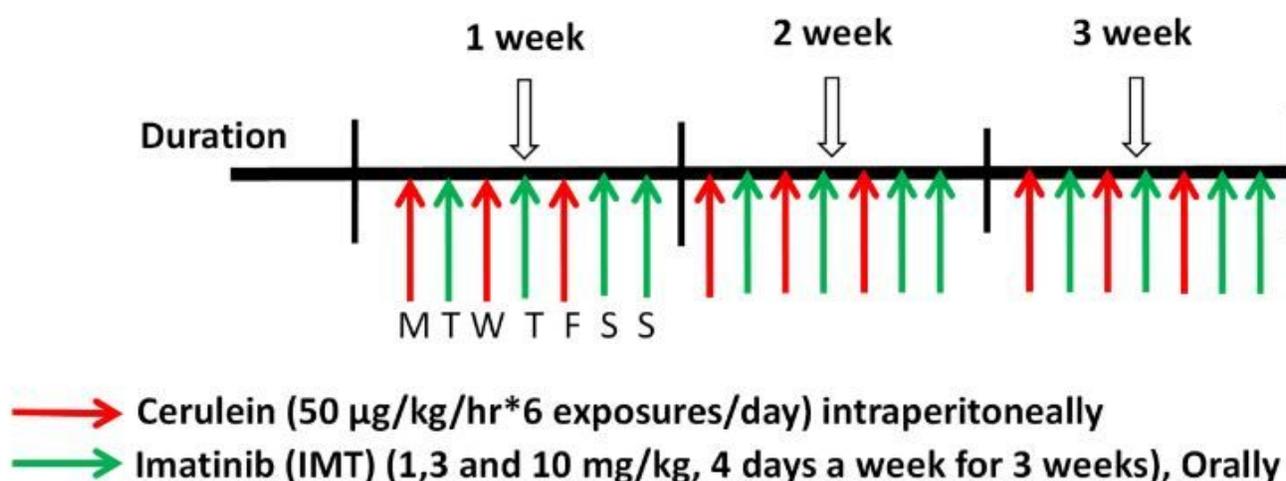
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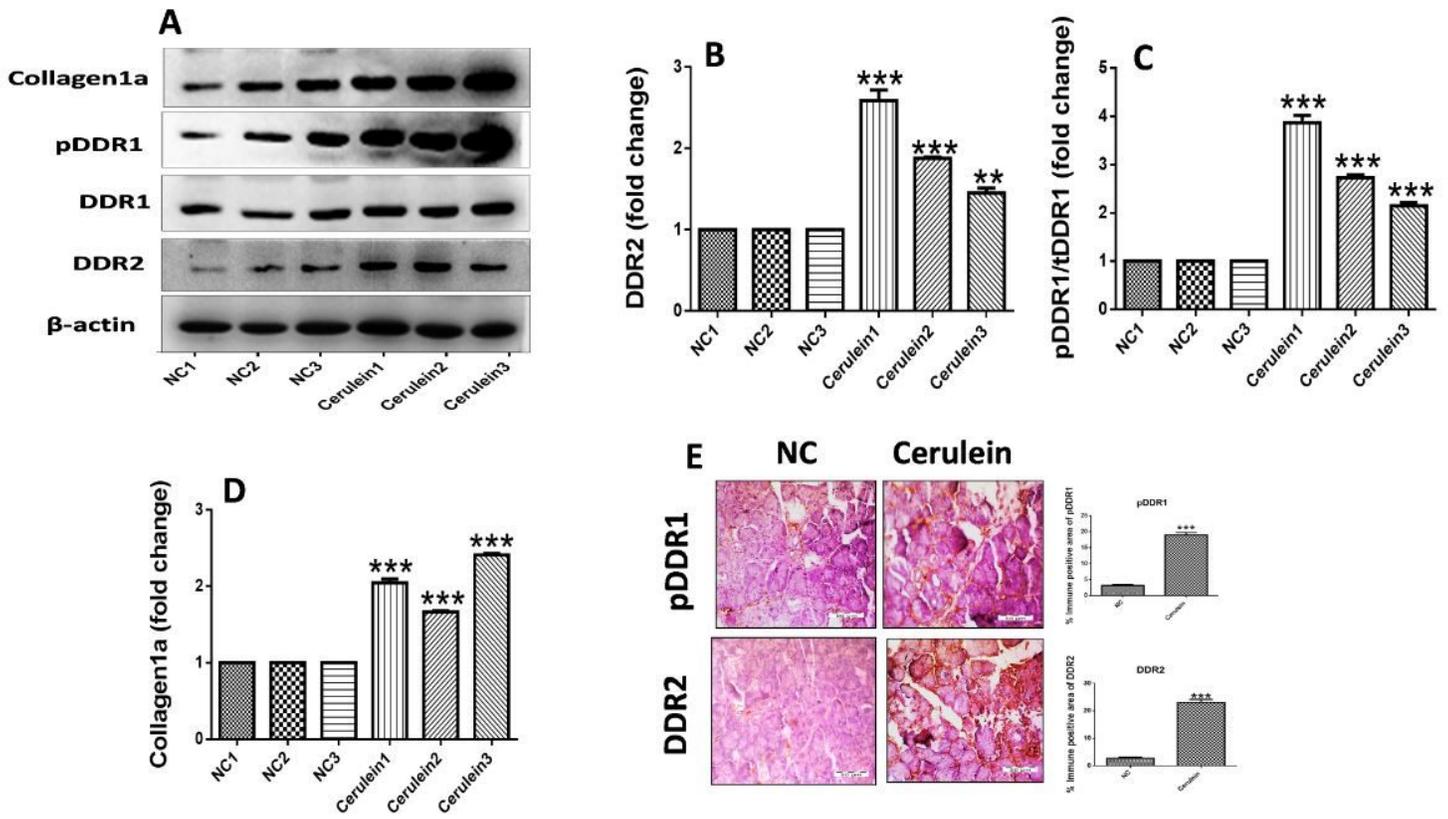
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## Figures



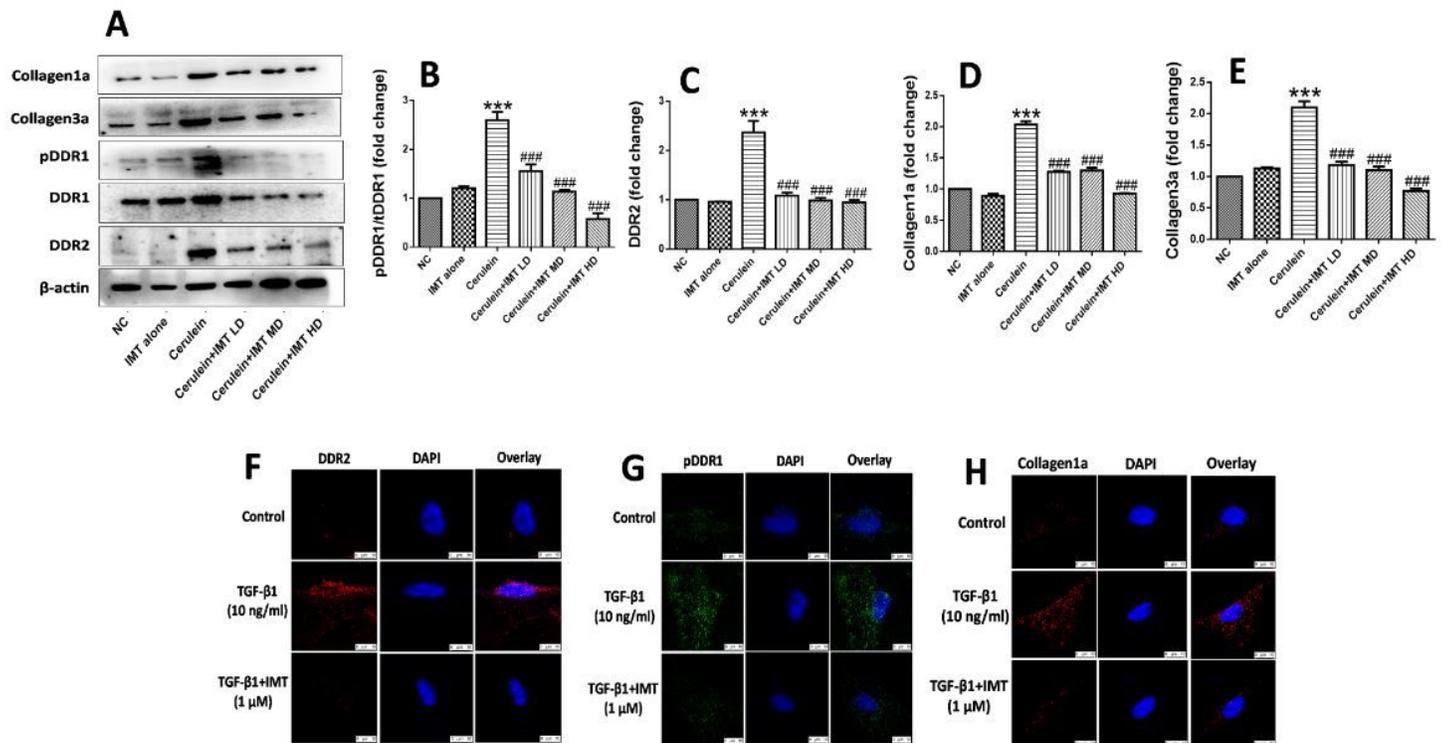
**Figure 1**

Experimental design for development and treatment of CP in mice. Male Swiss albino mice were randomly divided into 6 groups (7 mice per group): Normal control, IMT alone, Cerulein, Cerulein + IMT low dose (IMT LD - 1 mg/kg), Cerulein + IMT mid dose (IMT MD - 3 mg/kg) and Cerulein + IMT high dose (IMT HD - 10 mg/kg). CP was induced by 6-hourly intraperitoneal (i.p.) injections of cerulein (50 µg/kg), 3 alternative days per week, for the period of 3 weeks. Cerulein + IMT group mice were administered IMT (1, 3 and 10 mg/kg) orally, every other day after cerulein exposures for 3 weeks. Normal control group mice received normal saline same as cerulein group. IMT alone group mice were administered with IMT (10 mg/kg) orally 4 alternative days in the week for 3 weeks.



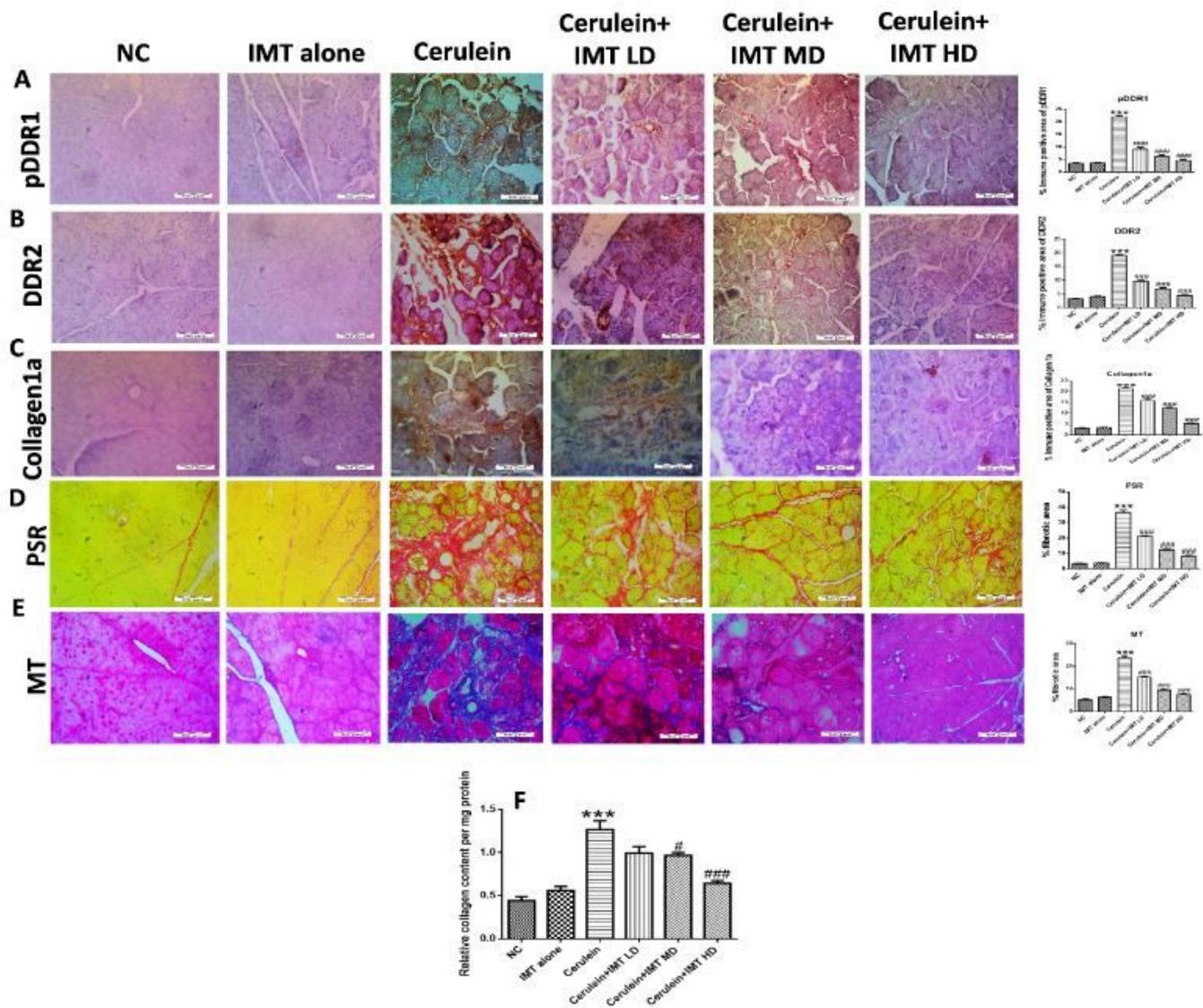
**Figure 2**

Upregulation of DDR1 and DDR2 in pancreatic fibrosis. (A-D) Western blot and corresponding densitometric analysis of protein expression of phosphorylated DDR1, total DDR1, DDR2 and collagen1a in pancreatic tissue. (E) Representative photographs of immunohistochemistry of phosphorylated DDR1 and DDR2 in the pancreatic tissue. All values are represented as mean  $\pm$  SEM (n = 3-10). \*\*P < 0.01, \*\*\*P < 0.001 Vs Normal control.



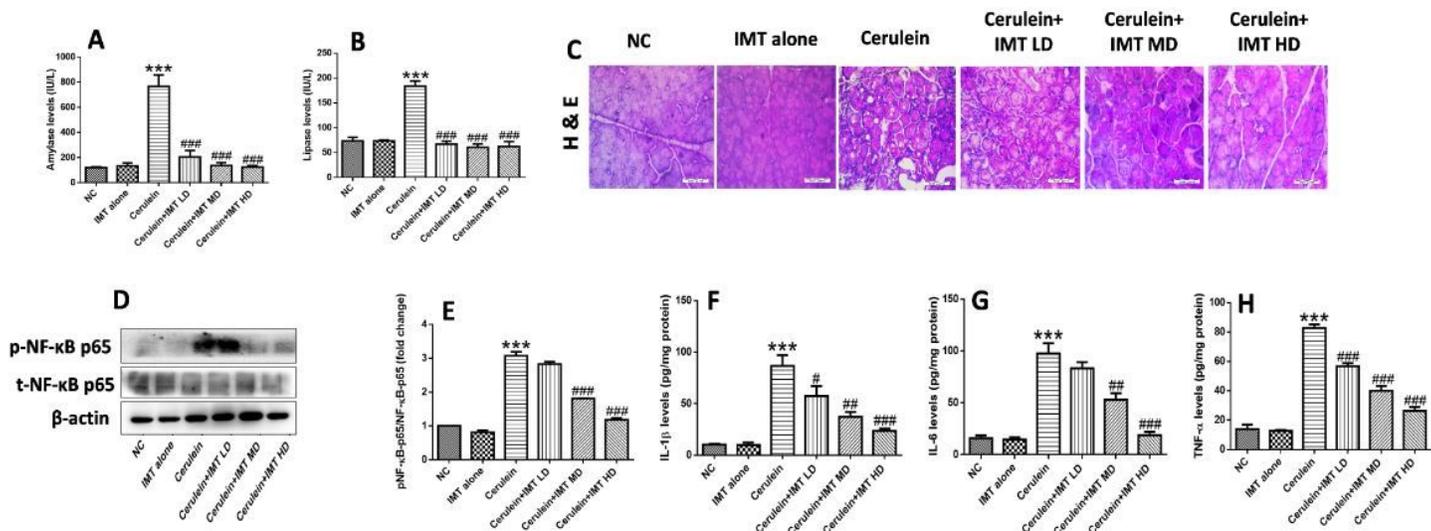
**Figure 3**

Inhibition of DDR1 and DDR2 expression prevents progression of pancreatic fibrosis. (A-E) Western blot and corresponding densitometric analysis of protein expression of phosphorylated DDR1, total DDR1, DDR2, collagen1a and collagen3a in pancreatic tissue. (F-H) Immunofluorescence (IF) staining for phosphorylated DDR1, DDR2 and collagen1a in TGF-β1-induced PSCs. All values are represented as mean ± SEM (n = 3). \*\*\*P < 0.001 Vs Normal control, ###P < 0.001 Vs Cerulein.



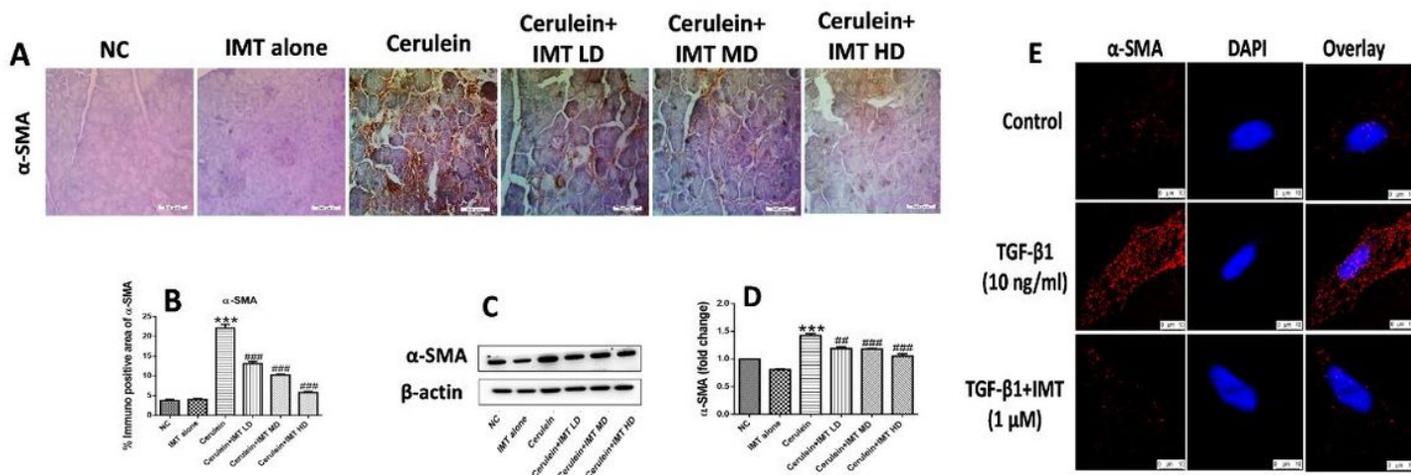
**Figure 4**

IMT controls collagen deposition in the pancreas (A-C) Representative photographs and quantitative analysis of immunohistochemistry of phosphorylated DDR1, DDR2 and collagen1a protein, (D-E) Representative photographs and quantitative analysis of PSR and MT staining in the pancreatic tissue, (F) Relative collagen content in pancreas estimated by Sircol assay. All values are represented as mean  $\pm$  SEM (n = 10). \*\*\*P < 0.001 Vs Normal control, #P < 0.05, ###P < 0.001 Vs Cerulein.



**Figure 5**

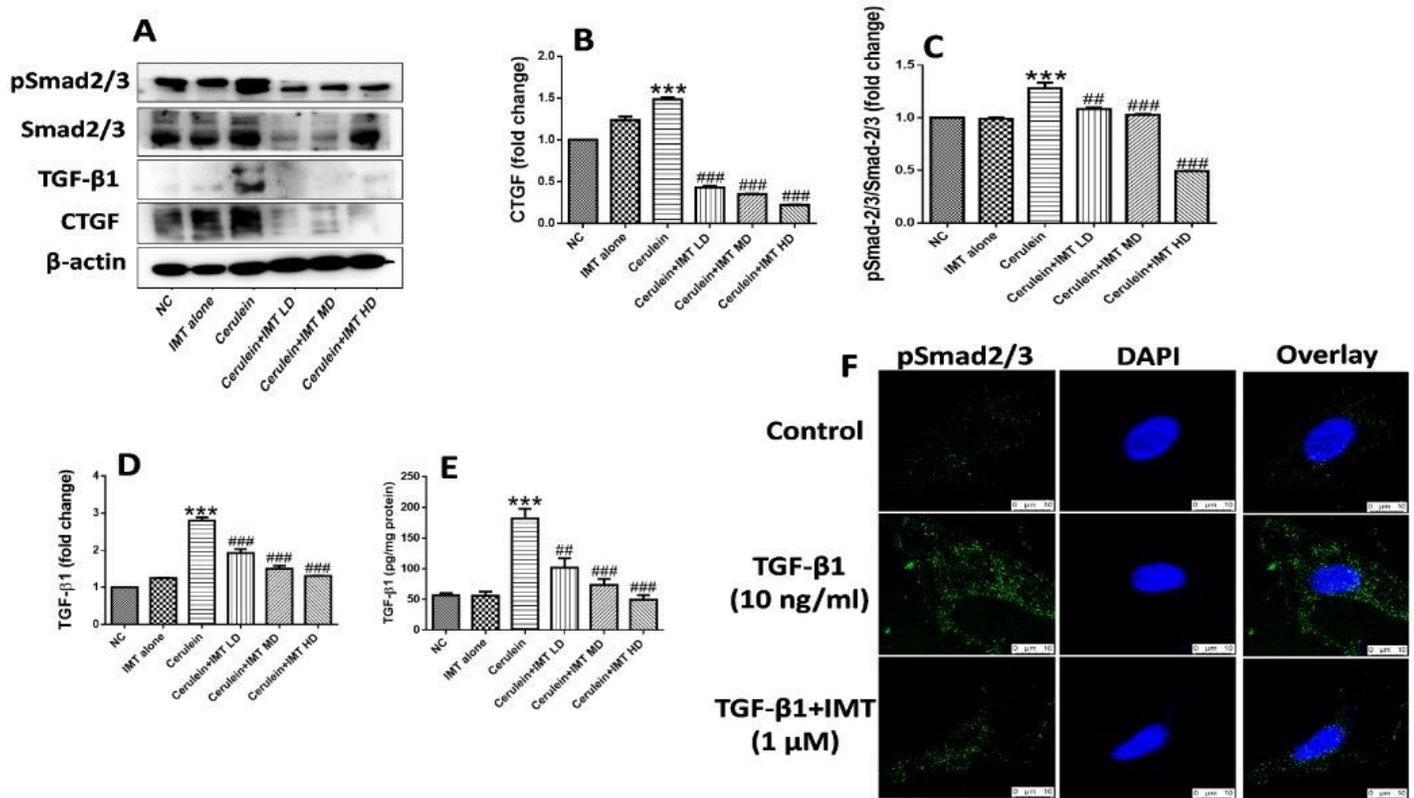
Effect of IMT on cerulein-induced pancreatic injury and inflammation. (A-B) Plasma amylase and lipase levels, (C) Representative photographs of H & E staining of pancreas at 40X magnification. (D-E) Western blot and corresponding densitometric analysis of protein expression of total and phosphorylated NF- $\kappa$ B in pancreas, (F-H) Pancreatic IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels studied by ELSA. All values are represented as mean  $\pm$  SEM (n = 7). \*\*\*P < 0.001 Vs Normal control, #P < 0.05, ##P < 0.01, ###P < 0.001 Vs Cerulein.



**Figure 6**

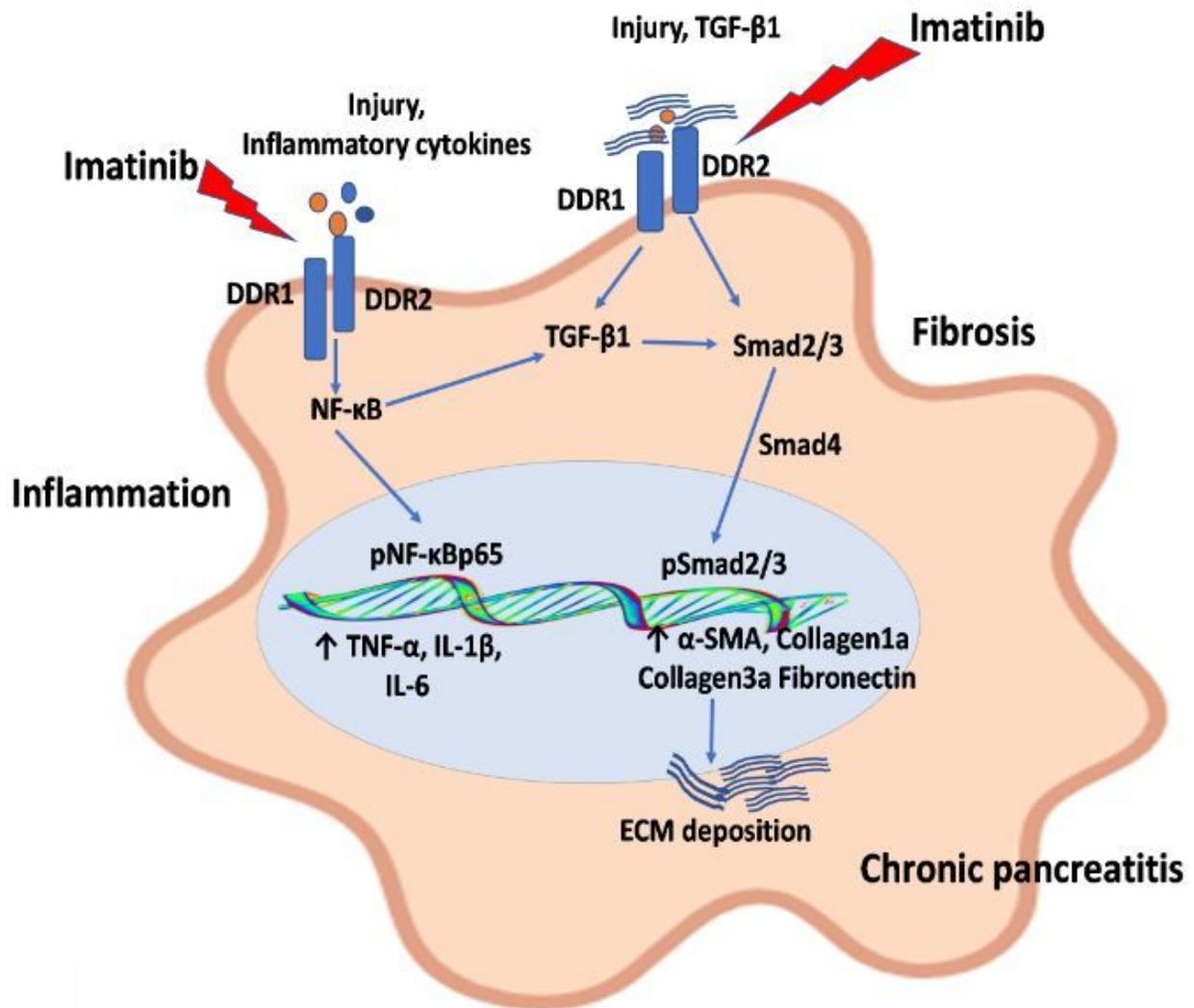
Effect of IMT on pancreatic stellate cells activation (PSCs). (A-B) Representative photographs of immunohistochemistry and quantitative analysis of  $\alpha$ -SMA protein in the pancreatic tissue, (C-D) Western

blot and corresponding densitometric analysis of protein expression of  $\alpha$ -SMA, (E) Immunofluorescence (IF) staining for  $\alpha$ -SMA in TGF- $\beta$ 1-induced PSCs. All values are represented as mean  $\pm$  SEM (n = 3-10). \*\*\*P < 0.001 Vs Normal control, ##P < 0.01, ###P < 0.001 Vs Cerulein.



**Figure 7**

Effect of IMT on TGF- $\beta$ 1/Smad signaling pathway. (A-D) Western blot and corresponding densitometric analysis of protein expression of CTGF, phosphorylated and total Smad2/3 and TGF- $\beta$ 1 in pancreatic tissue, (E) Pancreatic TGF- $\beta$ 1 levels studied by ELISA. (F) IF staining for phosphorylated Smad2/3 in TGF- $\beta$ 1-induced PSCs. All values are represented as mean  $\pm$  SEM (n = 3). \*\*\*P < 0.001 Vs Normal control, ##P < 0.01, ###P < 0.001 Vs Cerulein.



**Figure 8**

Schematic diagram of the molecular mechanisms of discoidin domain receptors (DDR1/DDR2) in the activation of pancreatic stellate cells (PSCs) and progression of pancreatic fibrosis. During pancreatic injury, release of variety of inflammatory mediators and transforming growth factor (TGF)- $\beta$  promotes the upregulation of expression of DDR1 and DDR2, which results in the activation of TGF- $\beta$ 1 downstream pathway and NF- $\kappa$ B signaling, consequently leading to excessive ECM deposition and pancreatic fibrosis. Moreover, IMT produced its protective effects by inhibiting DDR1/DDR2 signaling pathways in CP and associated fibrosis.

## Supplementary Files

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