

Research papers

Reversal of pancreatitis-induced pain by an orally available, small molecule interleukin-6 receptor antagonist

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ABSTRACT

Pancreatic pain resulting from chronic inflammation of the pancreas is often intractable and clinically difficult to manage with available analgesics reflecting the need for more effective therapies. The mechanisms underlying pancreatitis pain are not well understood. Here, the possibility that interleukin-6 (IL-6) may promote pancreatitis pain was investigated with TB-2-081 (3-O-formyl-20R,21-epoxy-resibufogenin, EBRF), a small molecule IL-6 receptor antagonist that was semi-synthetically derived from natural sources. The potential activity and mechanism of TB-2-081 were investigated following the induction of persistent pancreatitis using dibutyltin dichloride (DBTC) in rats. TB-2-081 displaces the binding of IL-6 to the human recombinant soluble IL-6 receptor with apparent high affinity and inhibits IL-6 mediated cell growth. Systemic or oral, but not intrathecal, administration of TB-2-081 reversed DBTC-induced abdominal hypersensitivity in a dose- and time-dependent manner. IL-6 levels were significantly up-regulated in the dorsal root ganglia (DRG) of rats with pancreatitis on day 6 after DBTC injection. IL-6-enhanced capsaicin-evoked release of calcitonin gene-related peptide from cultured DRG neurons was blocked by TB-2-081. Our data demonstrate that TB-2-081 acts as a systemically available and orally active small molecule IL-6 receptor antagonist. TB-2-081 effectively reduces pancreatitis-induced pain through peripheral mechanisms that are likely due to (a) increased expression of IL-6 in the DRG and (b) IL-6-mediated sensitization of nociceptive neurons. The activity of TB-2-081 implicates an important role for IL-6 in sustaining pancreatitis pain. Strategies targeting IL-6 actions through small molecule antagonists may offer novel approaches to improve the therapy of chronic pancreatitis and other chronic pain states.

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1. Introduction

Chronic pancreatitis is a continuous inflammatory disease of the pancreas characterized by irreversible morphological changes that typically cause pain and permanent loss of function. Pain is the most challenging and debilitating symptom associated with this condition. Initial treatment is generally conservative and includes non-opioid analgesics, followed by opioids [9]. However, a sub-

group of patients ultimately develop severe, incapacitating epigastric pain that markedly reduces their quality of life [34]. Conservative pain control efforts in these patients can lead to a vicious cycle of increasing need for narcotic analgesics and significant unwanted side-effects including somnolence, mental confusion and gastrointestinal problems [3] as well as potential for addiction. Therefore, there is a high and unmet need for novel therapies for the treatment of pancreatitis-induced pain.

The pleiotropic cytokine, interleukin-6 (IL-6) is markedly up-regulated during various pathological conditions and is generally associated with increased pain and hyperalgesia. Most experimental studies report pro-inflammatory and pro-nociceptive roles for IL-6. Intramuscular, intradermal, intracerebroventricular, or intrathecal injection of IL-6 induces allodynia or hyperalgesia in rats [6,30]. Several experimental models have shown that IL-6 is a

Abbreviations: CGRP, calcitonin gene-related peptide; DBTC, dibutyltin dichloride; DRG, dorsal root ganglia; IL-6R, interleukin-6 receptor; TB-2-081, 3-O-formyl-20R,21-epoxyresibufogenin.

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mediator of hyperalgesia in the inflamed tissues. Moreover, overproduction of IL-6 contributes to the pathogenesis of various autoimmune and inflammatory diseases, including rheumatoid arthritis (RA), systemic-onset juvenile chronic arthritis (JCA), osteoporosis, psoriasis, insulin-dependent diabetes mellitus, and inflammatory bowel disease [14,25]. IL-6 has been suggested to play a role in acute pancreatitis and pancreatic cancer [13,27,32].

IL-6 is produced by lymphoid and non-lymphoid cells such as T cells, B cells, monocytes, pancreatic acinar cells, fibroblasts, keratinocytes, endothelial cells, mesangial cells and several tumor cells, including myeloma and hypernephroma cells [18]. When IL-6 reaches an IL-6-responsive cell, it binds to its specific receptor, IL-6R, that exists in both transmembrane and soluble forms [33]. Binding of IL-6 triggers an association of the IL-6R with the transducer protein, gp130 [12], initiating an intracellular cascade of phosphorylation of several signal proteins including Janus kinases, STAT factors [11] as well as the MAPKs [16,41]. Based on the known role of IL-6 in inflammation and pain-promoting properties, we hypothesized that IL-6 might be an important mediator of pancreatitis-induced pain. Inhibition of IL-6 signaling, therefore, may represent a potential non-narcotic therapeutic target for the treatment of this disorder.

TB-2-081 (3-O-formyl-20R,21-epoxyresibufogenin) [17] (Fig. 1A; structure confirmed by X-ray crystallographic analysis, $M_w = 428.5$) has been identified in the extracts of Chinese toad skin [15] and has been reported to function as an IL-6R antagonist [7,17]. However, its potential activity in experimental models of pain is not known. Our results indicate that TB-2-081 is an orally active small molecule antagonist of the IL-6R, and is effective for alleviation of chronic pancreatitis pain in rats. Our data suggest that IL-6 may enhance nociceptive sensitivity in conditions of persistent pancreatitis.

2. Materials and methods

2.1. Competition of IL-6 binding to human recombinant soluble IL-6R by TB-2-081

Ninety-six well-plates (Nunc) were coated with 500 ng/mL of human recombinant soluble IL-6 receptor (R&D Systems) overnight at 4 °C. Blank wells were used as a control. The plates were washed three times with 300 μ L of phosphate buffered saline (PBS) and pre-blocked with 1% BSA (Sigma) in PBS at room temperature (RT) for 1 h and washed three times with PBS. Twelve concentrations (50 pM–100 nM) of human recombinant IL-6 (R&D Systems) were added to the wells, in triplicates, and incubated at RT with shaking for 2 h. After washing, to remove unbound IL-6, the wells were incubated with a biotinylated polyclonal anti-human IL-6 antibody (200 ng/mL, R&D Systems) at room temperature for 2 h, washed and incubated with streptavidin–horseradish peroxidase (R&D Systems) for 20 min. Sample wells were washed and developed with tetramethylbenzidine (100 μ L/well; 5–10 min at RT and stopped with 1 M HCl). Optical density in each well was determined using a microplate reader (Multiskan Ascent, Thermo) set to 450 nm with a correction wavelength of 570 nm. Non-specific binding of IL-6 is defined by that in the absence of sIL-6R. Data were analyzed by non-linear regression analysis using GraphPad Prism4 (Graph Pad, San Diego, CA). Based on the titration analysis, competition of IL-6 binding to sIL-6R by TB-2-081 was carried out as follows. Ninety-six well-plates were prepared as described above. After pre-blocking with the BSA-containing buffer, the wells were incubated with 1.2 nM of recombinant human IL-6, or 1.2 nM IL-6 plus one of six concentrations of TB-2-081 (10^{-12} – 10^{-7} M), in triplicates. All incubation conditions and colorimetric development were as described above. Maximum binding of IL-6 (100% bound) is defined as the

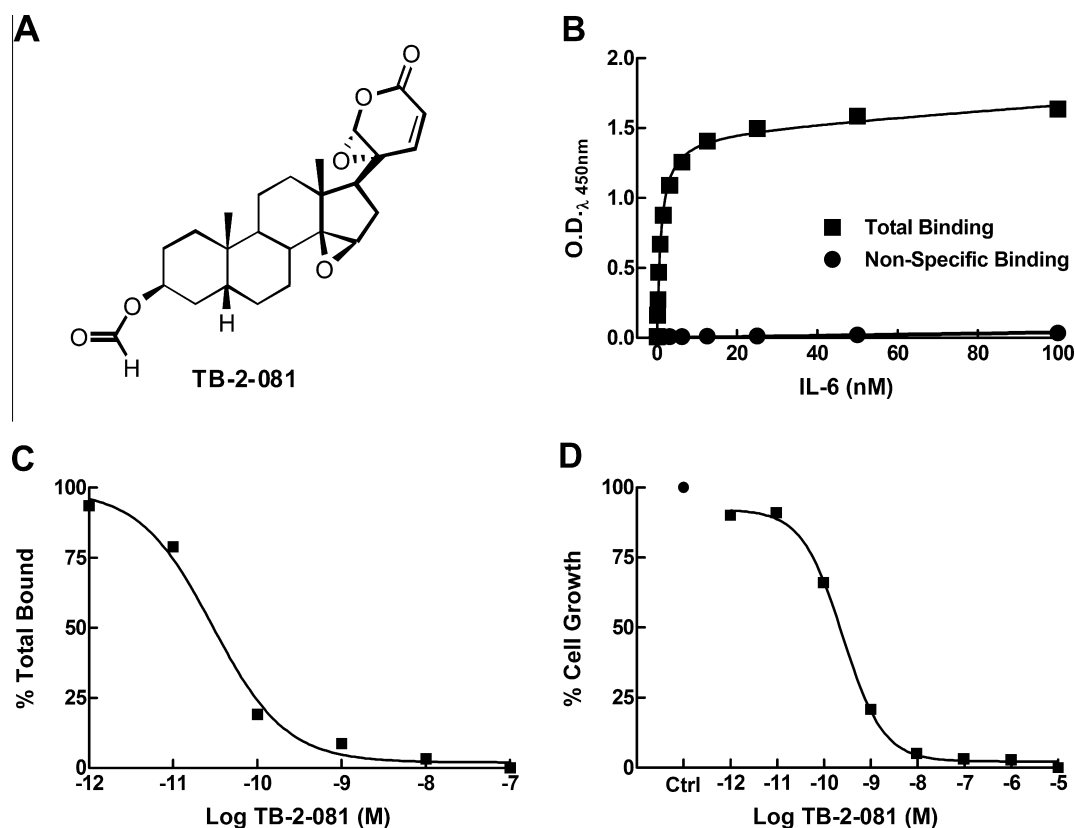


Fig. 1. (A) Structure of TB-2-081. (B) Saturation of IL-6 at human recombinant sIL-6R. Half saturation concentration is 960 pM. (C) Competitive binding of TB-2-081 to IL-6R ($IC_{50} = 29.4$ pM). (D) TB-2-081 inhibits the growth of IL-6-dependent cell line TF-1 ($IC_{50} = 250$ pM).

absorbance in the absence of TB-2-081. The absorbance in the presence of various concentrations of TB-2-081 is calculated as percent of maximum binding of IL-6. Data were analyzed by non-linear regression analysis to determine the IC₅₀ value of TB-2-081 using GraphPad Prism4 (Graph Pad, San Diego, CA).

2.2. Functional assay for IL-6

TF-1 is a factor-dependent human erythroleukemic cell line that proliferates in response to many cytokines including human IL-6 [5]. The cells were grown and maintained in RPMI 1640/10% fetal bovine serum/2 mM L-glutamine/100 U/mL penicillin/100 µg/mL streptomycin/2 ng/ml rhGM-CSF (R&D Systems). Cells were seeded at 50,000 cells/mL or higher in the medium and were passaged every 3–4 days with fresh medium. For the bioassay, the cells were collected and washed with PBS and suspended at 200,000 cells/mL in the above-mentioned medium but omitting rhGM-CSF (assay medium). Human recombinant IL-6 (2 ng/mL) or various doses of the drug with or without IL-6 were prepared in assay medium in a final volume of 50 µL in 96-well-plates (BD Biosciences), and incubation was initiated by adding 50 µL of the cell suspension to each well. The plates were incubated for 72 h at 37 °C in humidified 95% air/5% CO₂. Cell proliferation was measured by CellTiter[®] 96 AQueous One Solution Cell Proliferation Assay (Promega). Data were analyzed by non-linear regression analysis using GraphPad Prism4 (Graph Pad, San Diego, CA).

2.3. Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN), weighing 150–200 g at the time of testing, were maintained in a climate-controlled room on a 12 h light–dark cycle (lights on at 07:00 h) and food and water were available *ad libitum*. All testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain (IASP) and National Institute of Health (NIH) and received approval from the Institutional Animal Care and Use Committee of the University of Arizona.

2.4. Induction of pancreatitis

Chronic pancreatic inflammation was induced by tail vein injection of dibutyltin dichloride (DBTC, Aldrich Milwaukee, WI) dissolved in 100% ethanol vehicle at a dose of 8 mg/kg under isoflurane anesthesia (2–3 l/min of 4% isoflurane delivered in a mixture of 95% O₂ and 5% CO₂ to induce anesthesia and reduced to 2.5% isoflurane to maintain anesthesia) throughout the procedure [39,40]. Control rats received the same volume of ethanol vehicle solution only. This is a well established model of experimental pancreatitis that produces elevations of pancreatic enzymes and histological evidence of pancreatic inflammation without evidence of histological damage to other organs including the liver, heart, kidney or lungs [39,40]. Elevation in serum levels of amylase and lipase caused by DBTC injection has also been previously reported [38,39], consistent with what is observed in humans [10].

2.5. Intrathecal catheter implantation

Implantation of intrathecal catheters was performed as described by Yaksh and Rudy [44]. The rats were anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (12 mg/kg, i.p.) and a 5.5 cm length of PE-10 tubing was inserted through an incision made in the atlanto-occipital membrane, to the level of the lower thoracic levels (T₈–T₁₂). The catheter was then secured to the musculature at the site of incision, which was then closed.

Drugs were dissolved in saline and administered in a volume of 5 µL through a length of PE20 tubing connecting the catheter with the injection syringe. The catheter was cleared by flushing with 9 µL saline after drug. The animals were allowed to recover for 6 days after surgery before pharmacological manipulations were made.

2.6. Behavioral testing: assessment of pancreatitis-induced pain

Pancreatitis pain was assessed by referred abdominal hypersensitivity to probing the upper left abdominal quadrant of rats with calibrated von Frey filaments (4 g). The rats were allowed to acclimate in a suspended wire-mesh cage for 30 min before testing. A response was indicated by the sharp withdrawal of the abdomen, licking of abdominal area, or whole body withdrawal. A single trial consisted of 10 applications of a von Frey filament applied once every 10 s (intervals to allow the animal to return to a relatively inactive position). The mean occurrence of withdrawal events in each trial is expressed as the number of responses to 10 applications [39,40]. A significant increase in the number of abdominal withdrawals is interpreted as “pancreatitis-induced pain” [36,37].

2.7. Acute thermal nociception

The 52 °C hot-plate test was employed to assess the potential antinociceptive activity of TB-2-081. Rats were placed on a metallic hot-plate maintained at 52 ± 0.5 °C and latency to withdrawal, stamping or licking of the hindpaw was determined. A significant (*P* < 0.05) elevation in hot-plate latency from baseline values indicated antinociception.

2.8. ELISA assay for IL-6

Pancreas and the lower thoracic DRG (T₈–T₁₂) innervating the pancreas were collected and frozen in –80 °C before use. The tissue was ultrasonicated in PBS (pH 7.4) in the presence of protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche) after which the samples were centrifuged and the supernatant was collected. IL-6-like immunoreactivity was determined by ELISA (IL-6 ELISA kit, R&D systems) according to the manufacturer's instructions.

2.9. CGRP release from adult rat DRG culture

Male Sprague–Dawley rats (200–250 g) were used. Dorsal root ganglia (DRG) from all levels (~45/animal) were excised. After the ganglia were freed of capsular connective tissue and the nerve trunk, DRG were dissociated enzymatically with collagenase A (1 mg/mL, 25 min) and collagenase D (1 mg/mL) with papain (30 U/mL) for 20 min at 37 °C. Dissociated cells were seeded in 24 well titer plates and incubated at 37 °C in a humidified 95% air/5% CO₂ incubator. On day 4, the cells were incubated in a media containing IL-6 only (10 ng/mL), TB-2-081 only (5 µM), or both drugs, for 30 min, followed by incubation with a media containing the same drug(s) and capsaicin (30 nM) for an additional 15 min. The Media were collected from the sample wells and measured for CGRP-like immunoreactivity by ELISA according to the manufacturer's instructions (CGRP (rat) ELISA, Cayman Chemicals).

2.10. Data analysis

The results of withdrawal events are presented as mean ± SEM. Statistical analysis was performed in FlashCalc (www.u.arizona.edu/~michaelo). One-way ANOVA was used to detect significant differences in behavioral outcomes within each experimental group over time. Two-factor ANOVA was employed

to detect changes between treatment groups over time. In order to generate dose–response curves, data were converted to % inhibition by the formula: $100 \times (\text{maximum} - \text{response}) / \text{maximum}$, where maximum = 10 withdrawals. Linear regression analysis of the log dose–response curves was used to determine the A_{50} (95% confidence limits), representing the dose producing a 50% response. The area under the curve (AUC) for each of the time–effect curves at each dose was calculated from % inhibition vs. time by using the trapezoid rule. For all statistical comparisons, significance was established at $P = 0.05$.

3. Results

3.1. TB-2-081 competes with the binding of IL-6 to sIL-6R

IL-6 binds to the sIL-6R in a saturable, concentration-dependent manner. Under the assay conditions, the half saturation concentration of IL-6 was 960 pM (Fig. 1B). TB-2-081 displaced the binding of IL-6 (1.2 nM) to sIL-6R with an IC_{50} value of 29.4 pM and a Hill slope of -1.0 suggesting a single binding site for TB-2-081 (Fig. 1C).

3.2. TB-2-081 antagonizes the biological activity of IL-6

TB-2-081 inhibited IL-6-mediated growth of IL-6-dependent TF-1 cells in a dose-dependent manner with an IC_{50} of 250 pM (Fig. 1D). Treatment with TB-2-081 alone had no effect on the growth of TF-1 cells, suggesting that TB-2-081 acts as an antagonist of IL-6R.

3.3. Treatment with TB-2-081 blocks pancreatitis-induced referred abdominal hypersensitivity

Pancreatitis-induced pain was evaluated by probing the abdomen with von Frey filaments on day 6 after injection of DBTC. The mean number of responses to 10 applications of von Frey filaments was 8.33 ± 0.18 in the DBTC-treated rats compared with 0 ± 0.0 in ethanol vehicle-treated control rats, indicating significant abdominal hypersensitivity when compared to pre-injection baselines. To investigate the potential role of IL-6 in pancreatitis-induced referred abdominal hypersensitivity, rats were given TB-2-081 (0.001, 0.01, 0.1 or 1 mg/kg) by s.c. injection on day 6 after induction of chronic pancreatitis. TB-2-081 produced a dose- and time-dependent reversal of referred abdominal hypersensitivity while injection of the vehicle (i.e.; 50% PBS and 50% ethanol) was without effect (Fig. 2A). The peak inhibitory effect of TB-2-081 occurred rapidly, 15 min after subcutaneous administration and lasted for about 60 min. Injection of the ethanol vehicle (i.e.; DBTC vehicle) plus PBS/ethanol (TB-2-081 vehicle) did not produce increases in the numbers of withdrawal (Fig. 2A). The area under the time–effect curve analysis of the TB-2-081 effect suggests that the compound abolishes referred abdominal hypersensitivity in a dose- and time-dependent manner, consistent with a specific receptor mechanism (Fig. 2B and C). The A_{50} value for s.c. TB-2-081 was 0.006 mg/kg (0.0001–0.26 mg/kg; 95% confidence limits).

Based on the potent activity of systemically administered TB-2-081, we explored the possibility that this molecule might also have oral activity. TB-2-081 was given by oral gavage at doses of 0.1 mg/kg or 1 mg/kg on day 6 after DBTC induction of pancreatitis. Oral TB-2-081 produced dose- and time-dependent reversal of referred abdominal hypersensitivity (Fig. 3A and B). Analysis of the area under the time–effect curve for orally administered TB-2-081 shows that the compound reverses referred abdominal hypersensitivity induced by pancreatitis in a time- and dose-dependent manner (Fig. 3B). The potency of orally administered TB-2-081 was approximately one-fifth that of s.c. administration.

In contrast, the oral administration of up to 3 mg/kg of TB-2-081, representing three times the effective dose against pancreatitis-induced abdominal hypersensitivity, did not reverse acute nociception in the 52 °C rat hot-plate test (data not shown).

In order to determine if TB-2-081 acted through central sites, a dose of 5 µg was given i.th. to rats with referred abdominal hypersensitivity on day 6 after DBTC administration. Intrathecal TB-2-081 did not reverse abdominal hypersensitivity in rats with DBTC-induced pancreatitis (data not shown).

3.4. IL-6 levels in the pancreas and DRG after the induction of pancreatitis

The results of the behavioral analysis suggested that endogenous IL-6 may drive pancreatitis pain. To determine if this may be due to increased synthesis of IL-6, the levels of IL-6 were quantified in pancreatic tissue and in thoracic DRGs from naïve rats and from treated rats 6 days following ethanol vehicle or DBTC injection.

A significant ($P < 0.05$) upregulation of IL-6 was seen in the pancreas in both ethanol vehicle and DBTC-treated groups when compared with that from naïve rats (Fig. 4A). Additional analyses over a period of 1–14 days after ethanol vehicle or DBTC treatment showed that increased levels of IL-6 were already evident 1 day after the administration of either ethanol vehicle or DBTC and these levels remained elevated on day 6 post-injection. By day 14 post-injection, pancreatic levels of IL-6 returned to baseline levels for the ethanol vehicle group but remained elevated in the DBTC group (Fig. 4A). There were no significant ($P > 0.05$) changes in IL-6 levels in liver, thoracic spinal cord or brain tissue on day 6 after DBTC (data not shown). This is consistent with our previous reports that DBTC did not affect morphological changes in the liver, intestines, lungs, kidneys or heart [39,40].

Levels of IL-6 were also measured in the T8–T12 thoracic DRG on day 6 after DBTC or ethanol vehicle injection (Fig. 4B). IL-6 was significantly elevated in the DRG of animals pretreated with DBTC ($P < 0.05$) compared with levels detected in naïve or ethanol vehicle pretreated rats. There was no significant difference in the IL-6 levels between ethanol vehicle control and naïve rats.

3.5. IL-6 potentiates capsaicin-evoked CGRP release from DRG neurons

As elevated levels of IL-6 in thoracic DRG might correlate with abdominal hypersensitivity after DBTC pretreatment, the possible effect of IL-6 on these cells was examined using cultured DRG neurons from adult rats by measuring the effect of IL-6 on capsaicin-evoked CGRP release. Stimulation of DRG cultures with capsaicin (30 nM) induced an increase in CGRP release that was further potentiated by IL-6 (20 ng/mL). The potentiating effect of IL-6 was blocked by TB-2-081 (5 µM), which had no effect on capsaicin-evoked CGRP release (Fig. 5A). IL-6 alone, TB-2-081 alone, or their combination did not activate CGRP release in the absence of capsaicin (Fig. 5A).

As previous studies have proposed that exogenous soluble IL-6R (sIL-6R) administration is required to induce IL-6 competency [8,26], we tested whether sIL-6R administration would further augment IL-6 effects on capsaicin-evoked CGRP release. Cultured DRG cells were incubated with IL-6 (20 ng/mL), sIL-6R (25 ng/mL), or both, for 30 min followed by 15 min incubation with capsaicin (30 nM). Neither IL-6 nor sIL-6R or the combination of both stimulated CGRP release (Fig. 5B). IL-6, but not sIL-6R, potentiated capsaicin-evoked CGRP release (Fig. 5B). Co-administration of IL-6 and sIL-6R did not further augment evoked CGRP release compared with IL-6 alone (Fig. 5B). These experiments suggest that exogenous soluble IL-6R is not required for IL-6 augmentation of capsaicin-evoked CGRP release under these conditions.

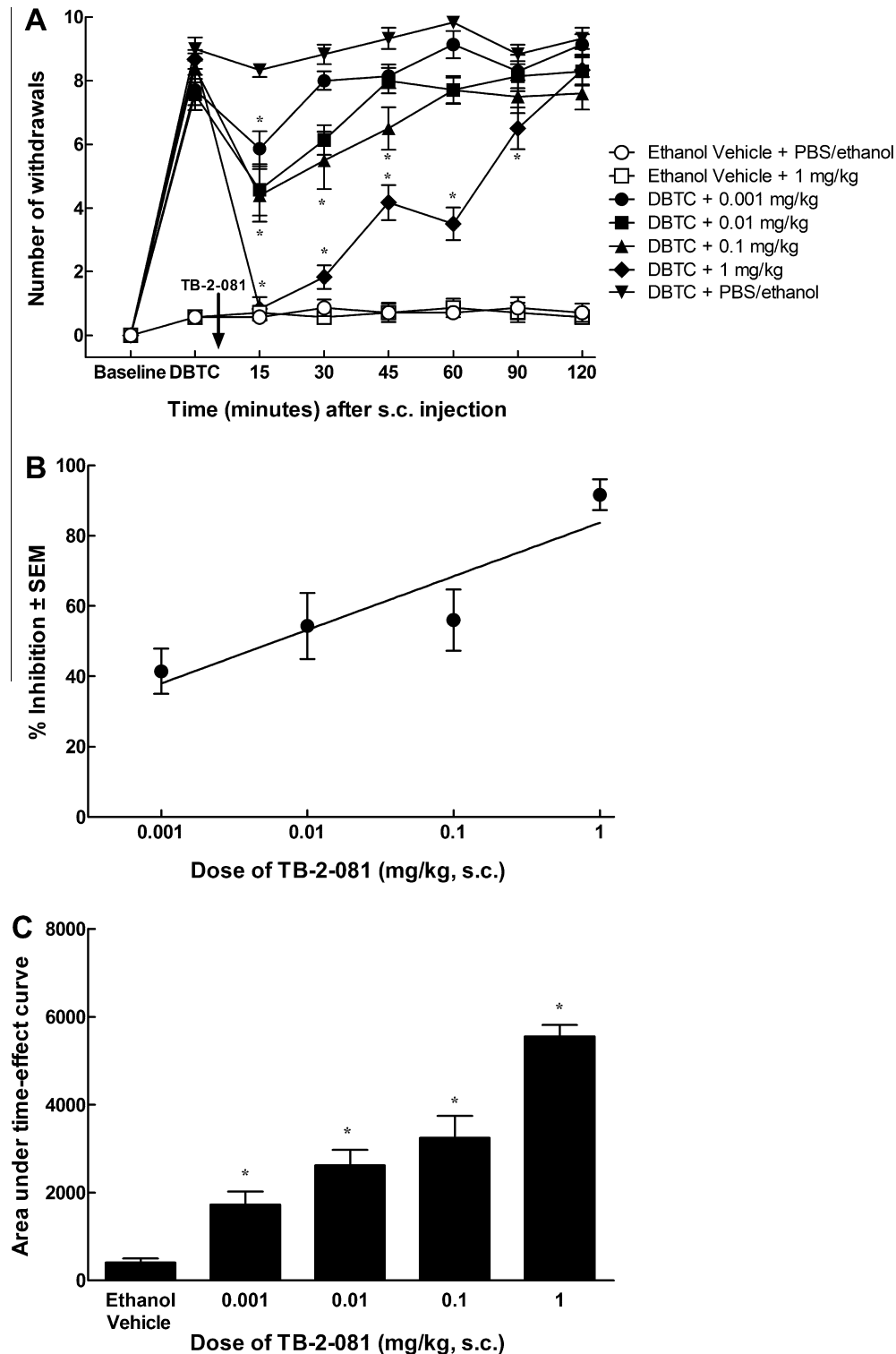


Fig. 2. Effects of systemic TB-2-081 in the DBTC pancreatitis model. (A) Time-course of the effects of TB-2-081 in rats with pancreatitis (DBTC) or without pancreatitis (ethanol vehicle). The doses of TB-2-081 are represented as mg/kg. PBS/ethanol indicates the vehicle for TB-2-081. $N = 6-10$ animals in all groups ($*P < 0.05$). (B) Dose-response curve for TB-2-081 induced reversal of abdominal hypersensitivity 15 min after subcutaneous (s.c.) administration in animals with experimental pancreatitis. (C) Area under the time-effect curve for subcutaneously administered TB-2-081 against referred abdominal hypersensitivity in rats injected with DBTC. $N = 6-10$ animals in all groups. *Indicates significant difference from the control group (ethanol vehicle) ($*P < 0.01$).

4. Discussion

In the present study, we used a novel, small molecule IL-6R antagonist TB-2-081 [8,15] to assess the role of IL-6 in pancreatitis-induced pain. TB-2-081 suppressed IL-6-induced mRNA

expression of the hepatic acute phase protein $\alpha 1$ -antichymotrypin (AACT) in human hepatoma HepG2 [17] cells and inhibited IL-6-induced phosphorylation of STAT3, thereby antagonizing IL-6 signaling via its canonical signaling pathway [17,26]. Our data show that TB-2-081 displaces the binding of IL-6 from sIL-6R with apparent

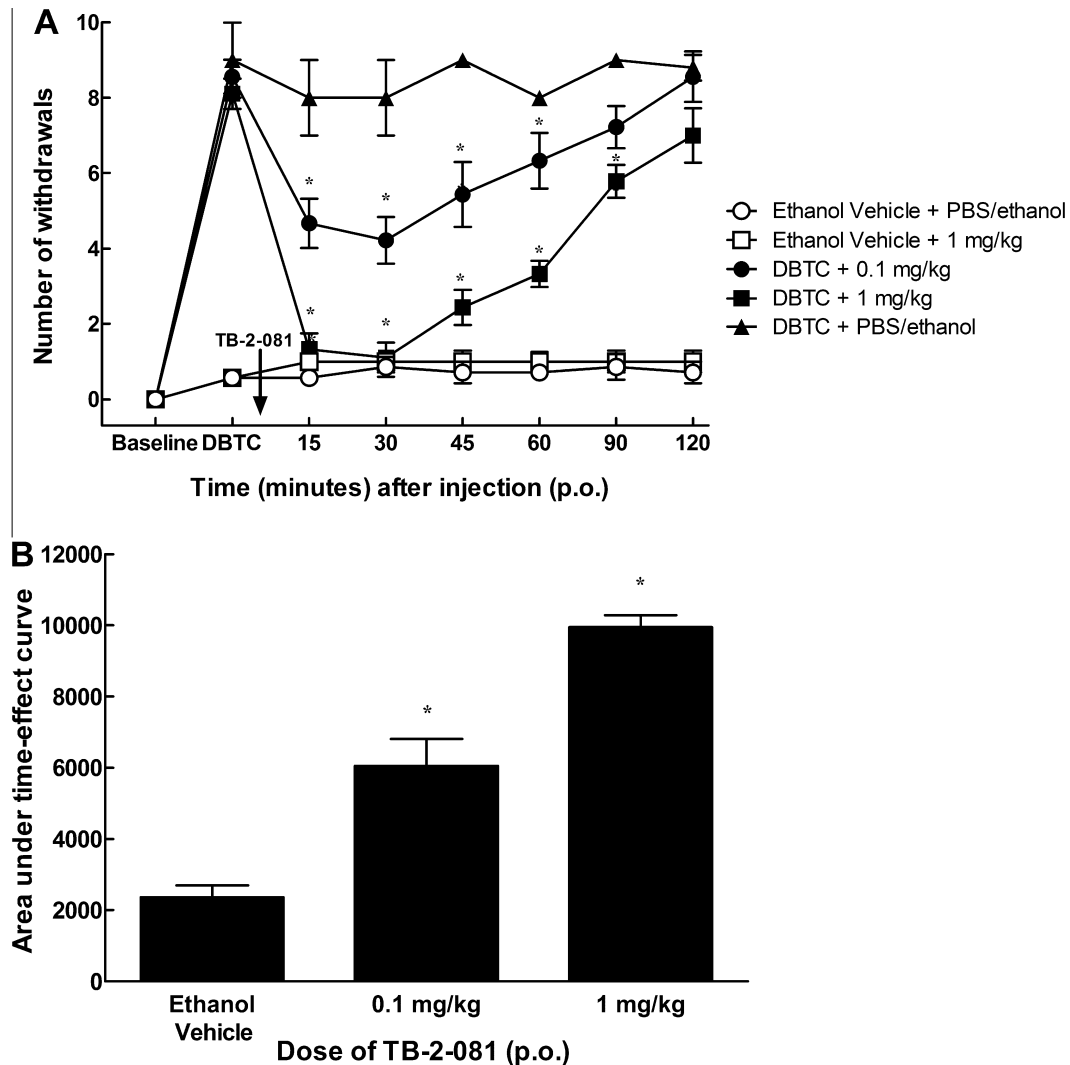


Fig. 3. Effects of orally administered TB-2-081 in the DBTC pancreatitis model. (A) Time-course of the effects of TB-2-081 in rats with pancreatitis (DBTC) or without pancreatitis (ethanol vehicle). The doses of TB-2-081 are represented as mg/kg. PBS/ethanol indicates the vehicle for TB-2-081. $N = 5-9$ animals per group. (B) Area under the time-effect curve for orally administered TB-2-081 against referred abdominal hypersensitivity in rats injected with DBTC. *Indicates significant difference from the control group (ethanol vehicle) ($*P < 0.01$).

high affinity and inhibits IL-6-induced growth of TF-1 cells, which further support TB-2-081 as a natural product that exhibits characteristics of antagonist for IL-6R. The major finding of this study is that TB-2-081 effectively reverses pancreatitis-induced referred abdominal hypersensitivity; its action as an IL-6R antagonist further implies that IL-6R blockade, or IL-6 sequestration, may be therapeutic avenues for the treatment of pancreatitis pain in humans. Furthermore, TB-2-081 was shown to be orally active.

We found that systemic or oral, but not intrathecal administration of TB-2-081 reversed pancreatitis-induced referred abdominal hypersensitivity, suggesting that this compound may have a peripheral mechanism of action. These data also suggest that peripheral IL-6R is likely to be critical in maintaining the pancreatitis-induced hypersensitivity. This observation may be important in the development of novel therapeutics for pancreatitis pain. Furthermore, the rapid onset of reversal of referred abdominal hypersensitivity by TB-2-081 is also consistent with a direct interaction with IL-6R and disruption of a signaling pathway that modulates sensory input. Previous evidence suggests that the IL-6-IL-6R-gp130 complex stimulates MAPK activation and several MAPKs such as p38 and ERK. Activation of these pathways promotes the

excitability of primary afferent neurons [16]; the relevance of these signaling pathways by IL-6R in pancreatitis-induced pain states warrants further investigations.

Our findings on the alteration of IL-6 expression in the pancreas and DRG in the experimental model of pancreatitis also points to a possible role of IL-6 in the maintenance of the chronic inflammatory pain states. Both DBTC and ethanol vehicle caused a significant increase in IL-6 levels in the pancreas, compared to a very low expression of IL-6 in pancreata of naïve animals. However, as ethanol vehicle-treated animals do not develop abdominal hypersensitivity or pancreatitis, it seems unlikely that pancreas-derived IL-6 alone is sufficient to promote the chronic pain state. One hypothesis that may explain the effects of alcohol on the pancreas is that the alcohol sensitizes the pancreas to the pathobiological processes including inflammation, necrosis and fibrosis. Ethanol feeding alone, even at high doses, has minimal and inconsistent effects on morphological findings in the pancreas of animals [20,38]. A 6-week, ethanol-containing diet increases the sensitivity of rats to acute pancreatitis induced by cholecystikinin (CCK)-8 [31]. An ethanol diet and CCK-8 infusion increase pancreatic NF κ B activation compared with injection of CCK-8 alone. Failure to produce

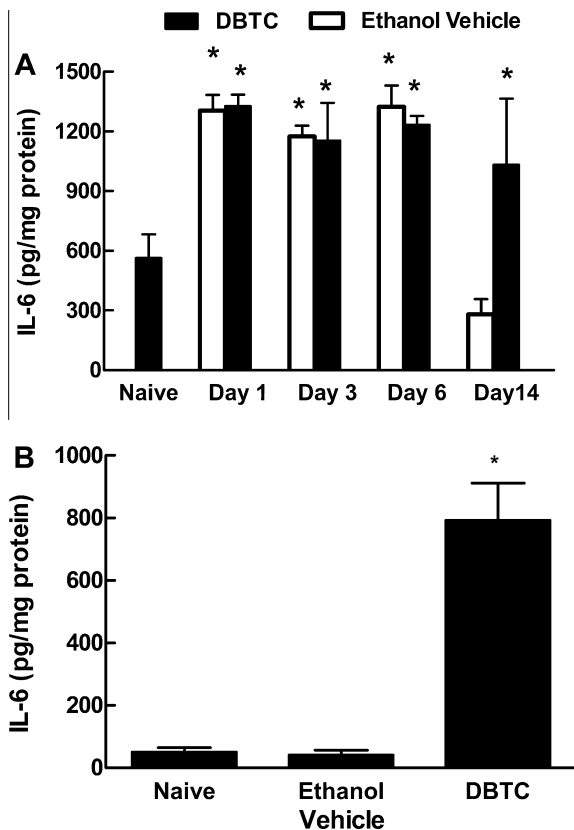


Fig. 4. Pancreatitis-induced changes in IL-6 expression in the pancreas and DRG. (A) Time-course of changes in levels of IL-6 in pancreata of naïve, ethanol vehicle or DBTC-treated animals (*indicates significant difference from naïve group, $P < 0.01$). (B) The levels of IL-6 in the T_8-T_{12} DRG obtained from naïve animals or animals treated with ethanol vehicle or with DBTC-induced visceral inflammation at 6 day after injection are shown (* $P < 0.01$, $N = 6$ rats per group in all panels).

experimental alcoholic pancreatitis and the fact that alcohol abuse causes pancreatic pathology in only a minority of patients suggest that ethanol may only increase predisposition to pancreatitis. Therefore, elevated levels of IL-6 in vehicle-treated group might be a necessary prerequisite for pancreatic injury induced by DBTC.

In contrast, we observed a large increase in IL-6 in the thoracic DRG after DBTC treatment but not in the ethanol vehicle-treated controls. While the mechanisms underlying this increase in IL-6 expression in the DRG are presently unclear, IL-6 has been shown to be up-regulated in injured nerves [1,2] and studies from IL-6 knockout mice indicate that the effects of IL-6 are critical for full expression of neuropathic pain in these models [24]. Thus, the upregulation of IL-6 in the thoracic DRG may be an autocrine or paracrine mechanism in the sensitization of primary afferents through activation of IL-6R in the peripheral terminals. Therefore, our data indicate that a possible mechanism of action for TB-2-081 in the DBTC model is an abrogation of IL-6 effects within the DRG of animals with chronic pancreatitis leading to a decrement in abdominal hypersensitivity. To determine if IL-6 promotes the excitability of primary afferents, particularly of nociceptors, the release of the excitatory neuropeptide, CGRP, from isolated DRG was used as a functional measure of the activation of sensory neurons in the absence or presence of capsaicin, a ligand for the TRPV1 channel [4,37]. Previous evidence suggests that IL-6 enhances the electrophysiological properties of TRPV1 [28]. IL-6 does not directly activate CGRP release, at least at the dose tested here, but significantly enhanced capsaicin-evoked CGRP release; the enhancing effect of IL-6 was blocked by TB-2-081. These data are

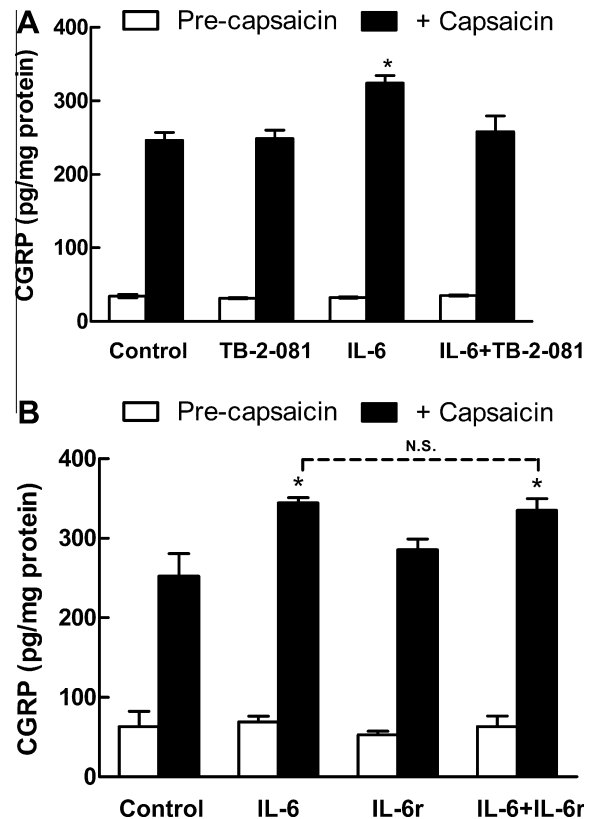


Fig. 5. Effects of IL-6 on capsaicin-evoked CGRP release from adult DRG neurons in culture. (A) Adult cultured DRGs were treated with capsaicin (30 nM) alone (i.e.; Control), TB-2-081 (5 M) followed by capsaicin (TB-2-081), IL-6 (20 ng/ml) followed by capsaicin (IL-6), or IL-6 with TB-2-081 followed by capsaicin (IL-6 + TB-2-081). Treatment with IL-6 increased the levels of capsaicin-evoked CGRP release by 30% (* $P = 0.01$). (B) Application of IL-6, soluble IL-6R (20 ng/ml) or a combination of both on the basal release of CGRP (* $P = 0.02$, $n = 6$ per group in all panels).

consistent with a pro-nociceptive role of IL-6, and furthermore, suggest that the effect of IL-6 may be mediated by IL-6R present on the primary afferent terminals, where the signaling process through IL-6R activation modulates the function of TRPV1. Previous behavioral and electrophysiological studies suggested that exogenously applied soluble IL-6R was required for IL-6-mediated sensitization of TRPV1 currents or TRPV1-dependent thermal hyperalgesia [29,42]. However, we found that the enhanced release of CGRP by IL-6 does not require sIL-6R under our experimental conditions.

Thus, in the DBTC model of pancreatitis, the upregulation of IL-6 and the blockade of IL-6R by TB-2-081 implicate a critical role for IL-6 in inflammatory hyperalgesia. The mechanisms by which IL-6R activation potentiates the probable excitability of nociceptors remain to be elucidated. Previous work has suggested that TRPV1 plays a critical role in the etiology of acute and chronic pancreatitis [21,43]. It would be of interest to ascertain if potentiation of TRPV1 constitutes a critical mechanism for the pro-nociceptive effect of IL-6/IL-6R.

It is possible that TB-2-081 may block the effects of other members of IL-6 family of cytokines, including IL-11 and oncostatin M [17]. However, these members of the IL-6 family of cytokines have been suggested to be involved in hematogenesis and oncogenesis and their role in nociception is not clearly established. Additionally, while oncostatin M also plays a role in the development of small TRPV1⁺ neurons, this is not likely to be relevant to our study [23]. Further studies are necessary to determine the antagonistic effects of TB-2-081 on IL-6 type cytokines *in vivo*.

A variety of studies have demonstrated that overproduction of IL-6 contributes to the pathogenesis of various autoimmune and inflammatory diseases, including rheumatoid arthritis (RA), systemic-onset juvenile chronic arthritis (JCA), degenerative lumbar spinal disorder. A large number of diseases and pathological conditions in which IL-6 plays a key role, and existing clinical validation of mechanism, make the development of molecules which block the actions of IL-6 desirable targets for drug discovery and development.

Therapeutics that target IL-6R are currently limited to monoclonal antibodies. Blocking IL-6 actions by use of a humanized antibody, tocilizumab, has been shown to be therapeutically effective for rheumatoid arthritis [22,35,36], systemic juvenile idiopathic arthritis and Crohn's disease [25]. While these are exciting developments, there remain some contraindications regarding the use of monoclonal antibodies in risks of infections and immunogenicity [19]. The promise of a small molecule, orally available IL-6R antagonist such as TB-2-081 and/or its derivatives, offers a viable alternative strategy to the development of therapeutics targeting the pathological actions of IL-6.

Conflict of interest

All authors declare that they have no conflicts of interest.

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The authors have nothing to disclose.

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