Selective inhibition of BET proteins reduces pancreatic damage and systemic inflammation in bile acid- and fatty acid ethyl ester- but not caerulein-induced acute pancreatitis

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Running title: BET proteins in experimental acute pancreatitis

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Abbreviations: AP, acute pancreatitis; AUC, area under the curve; BET proteins, bromodomain and extra-terminal proteins; CCL2, chemokine (C-C motif) ligand 2; CER-AP, caerulein-induced AP; Cmax, peak serum concentration; CXCL1, chemokine (C-X-C motif) ligand 1; DMSO, dimethyl sulfoxide; FAEE-AP, fatty acid ethyl ester-induced AP; HETAB, hexadecyltrimethyl ammonium bromide; IL, interleukin; i.p., intraperitoneal; MOPS, 3-(N-morpholino)propanesulfonic acid; MPO, myeloperoxidase; POA, palmitoleic acid; TLCS-AP, tauroliothocholic acid 3-sulphate disodium salt-induced AP; Tmax, time of Cmax; TMB, 3,3′,5,5′-tetramethylbenzidine.


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ABSTRACT

Objectives: To evaluate the therapeutic potential of I-BET-762, an inhibitor of the bromodomain and extra-terminal (BET) protein family, in experimental acute pancreatitis (AP).

Methods: AP was induced by retrograde infusion of taurolithocholic acid sulphate into the biliopancreatic duct (TLCS-AP) or two intraperitoneal (i.p.) injections of ethanol and palmitoleic acid 1 h apart (FAEE-AP) or 12 hourly i.p. injections of caerulein (CER-AP). In all treatment groups, I-BET-762 (30 mg/kg, i.p.) was administered at the time of disease induction and again 12 h later. AP severity was assessed at 24 h by serum biochemistry, multiple cytokines and histopathology.

Results: TLCS-AP, FAEE-AP and CER-AP resulted in characteristic elevations in serum amylase and cytokine levels, increased pancreatic trypsin and myeloperoxidase activity, typical pancreatic histopathological changes and lung injury. Treatment with I-BET-762 significantly reduced biochemical, cytokine and histopathological responses in TLCS-AP and FAEE-AP, but not CER-AP.

Conclusions: These results suggest that in different forms of AP there are significant differences in the epigenetic control of gene transcription contributing to the severity of disease responses. There is therapeutic potential in targeting bromodomains for the treatment of gallstone- and alcohol-related pancreatitis.

Key words: acute pancreatitis, epigenetics, BET inhibition, drug discovery
INTRODUCTION

AP is an inflammatory condition of the pancreas with an incidence of 30-50 cases per 100,000 population per year and an overall mortality of circa 5% in the Western world\(^1\), for which there is no licensed treatment. Gallstones and alcohol excess account for about 80% of AP aetiology\(^2\). 15-20% of all patients suffer a severe illness in which two broad phases can be identified. Within the first 7 to 10 days after onset of a severe attack there is a pro-inflammatory phase featuring pancreatic injury and systemic inflammatory response syndrome that may result in organ failure, during which time pancreatic necrosis frequently develops. Subsequently there is relative immune anergy, which may result from earlier over-activation or disruption of the immune system. During this later phase peri-pancreatic necrotic or systemic infections are more likely to develop and contribute to a worse outcome\(^3,4\). Strategies that inhibit the early phase immune response in AP that also sustain subsequent immune function hold significant promise in drug discovery for AP.

Histones surround DNA in nucleosomes and contribute to the regulation of gene expression through epigenetic post-translational histone modifications specific to individual cells, including through acetylation, methylation, citrullination and phosphorylation. These post-translational modifications, termed epigenetic marks, facilitate the formation of macromolecular protein complexes that relax condensed nuclear DNA, increasing transcription\(^5\). Bromodomain and extra-terminal (BET) proteins (BRD2, BRD3, BRD4 and BRDT) form these complexes through binding to epigenetic marks, notably acetylated lysine residues\(^6\), reducing interaction between histones and DNA, thus increasing transcription. I-BET-762 (also known as GSK525762A) is a potent inhibitor of the BET family of proteins that has been shown to be protective in a lethal model of infection \textit{in vivo}\(^6\) and in CD4\(^+\) T-cell-mediated neuroinflammation\(^7\), and is now in early phase trials for oncological indications\(^8\). Since the immune response is a critical contributor to pancreatic and systemic injury in AP, and I-BET-762 might be a potential treatment for AP, we sought to determine the local and systemic effects of I-BET-762 in multiple experimental models of AP.
MATERIALS AND METHODS

Experimental animals

Male CD1 mice (30-35 g), purchased from Charles River UK Ltd (Margate, UK), were housed at 23 ± 2°C under a 12 h light/dark cycle with ad libitum access to standard laboratory chow and water. Twelve hours before induction of AP, the animals were deprived of food but were allowed access to water; 2 h after initiation of experimental procedures (which took less than 1 h), food was returned to animals. Studies were conducted in compliance with UK Home Office regulations (PPL 40/3320, renewed as 70/8109), together with the Institutional Ethical Review processes of the University of Liverpool.

Induction of AP and administration of I-BET-762

AP was induced using 3 different methods: (i) Retrograde infusion of 3 mM tauroliothocholic acid 3-sulphate disodium salt (TLCS) was injected at a speed of 5 µl/min for 10 min into the biliopancreatic duct (with a clamp across the upper end) by minipump (Harvard Apparatus, Kent, UK) to induce TLCS-AP\(^9,10\); control mice received the same surgical procedure but with infusion of normal saline, not TLCS. (ii) Two intraperitoneal (i.p.) injections of ethanol (1.35 g/kg) and palmitoleic acid (POA, 150 mg/kg) were administered 1 h apart to cause fatty acid ethyl ester-induced AP (FAEE-AP)\(^11-14\); control mice received 2 i.p. injections of either saline or ethanol. To avoid local damage by ethanol to the peritoneal organs at the injection site, 150 µL of saline was injected i.p. shortly before the ethanol/POA injections. (iii) Hyperstimulation by 12 hourly i.p. injections of cerulein (50 µg/kg, CER-AP)\(^14,15\), a cholecystokinin analog\(^16\), while control mice received 12 saline injections (i.p.). At the time of disease induction, analgesia was administered by subcutaneous administration of buprenorphine hydrochloride (0.1 mg/kg).

Mice received 2 i.p. injections of either I-BET-762 (30 mg/kg) or vehicle (2% dimethyl sulfoxide (DMSO)/98% kleptose (10%) solution): the first was administered at the time of disease induction and the second 12 h later. Mice were humanely killed at 24 h and blood, pancreas and lung tissue immediately sampled to assess disease severity\(^14\).

Serum amylase and circulating cytokines

Blood samples were allowed to clot naturally (serum) or were preserved in ethylenediaminetetraacetic acid (EDTA) tubes (plasma) for 30 min, followed by centrifugation at 1,500 g × 10 min. Serum amylase was measured kinetically using a Roche automated clinical chemistry analyser (GMI, Leeds, UK).
Serum or plasma multicytokine tests for interleukin-1β (IL-1β), IL-10, chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1) were carried out according to the protocols provided by R&D Systems (Abingdon, UK) using a Bio-Rad luminex machine (Hemel Hempstead, UK). IL-6 was measured using Quantikine ELISA according to the instructions provided by R&D Systems.

**Pancreatic trypsin activity**

Pancreata were homogenised in tissue buffer (pH 6.5), containing 3-(N-morpholino)propanesulfonic acid (MOPS, 5 mM), sucrose (250 mM) and magnesium sulfate (1 mM) using a motorised homogeniser on ice. The homogenates were centrifuged at 1,500 g for 5 min, and 100 µL of each supernatant were added to a cuvette containing the peptide substrate Boc-Gln-Ala-Arg-MCA (Peptide, Osaka, Japan) dissolved in 1900 µL assay buffer (pH 8.0) containing Tris (50 mM), NaCl (150 mM), CaCl₂ (1 mM) and 0.1 mg/mL bovine serum albumin. Trypsin activity was measured fluorimetrically using a Shimadzu RF-5000 spectrophotometer (Milton Keynes, UK; excitation 380 nm, emission 440 nm¹⁷). Standard curves were generated using purified human trypsin. Pancreatic protein concentration was measured by a BCA protein assay (Thermo, Rockford, USA) using a BMG FLUOstar Omega Microplate Reader (Imgen Technologies, New York, USA). Trypsin activity was expressed as fmol/mg protein.

**Pancreatic and lung myeloperoxidase (MPO) activity**

Pancreatic and lung MPO activity were tested by a modified method from Dawra et al¹⁸. Pancreatic tissue was homogenized, resuspended in 100 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethyl ammonium bromide (HETAB), 10 mM EDTA and protease inhibitors, freeze-thawed three times, sonicated for 30 sec and centrifuged for 15 min at 16,000 g. MPO activity was measured using substrate 3,3',5,5'-tetramethylbenzidine (TMB). Briefly, 20 µL of the supernatant were added to the assay mix, which consisted of 200 µL of phosphate buffer (100 mM, pH 5.4) with 0.5% HETAB and 20 µl TMB (20 mM in DMSO). This mixture was incubated at 37°C for 3 min, followed by addition of 50 µL H₂O₂ (0.01%), then incubated for another 3 min. The difference in absorbance between 0 min and 3 min at 655 nm was calculated from a human MPO standard curve using a plate reader. MPO activity was expressed as mU/mg protein.

**Histopathology**

After H&E staining of the pancreas, 10 random fields per slide (5 µm) from all groups were graded by two independent blinded observers according to the severity and extent of oedema, inflammatory cell infiltration and...
acinar necrosis (magnification \( \times 200 \)) as described by Wildi et al\(^9\). Similarly for the lung sections, 10 random fields per slide were scored according to the following thickening of alveolar septae: normal = 0, thickening <1/3 field = 1, thickening = 1/3 - 2/3 field = 2 and thickening >2/3 field = 3 (magnification \( \times 200 \)).

**Pharmacokinetic analysis**

Terminal blood (25 µL) was collected from satellite mice with TLCS-AP and CER-AP at 0.25, 1, 2, 4, 12.25 and 24 h after the first injection of I-BET-762 (n = 1 per time point for each model). An equal volume of water (25 µL) was added to the blood, mixed and frozen prior to measurement of blood levels of I-BET-762 by liquid chromatography-tandem mass spectrometry at GSK Stevenage (UK).

**Drugs and chemicals**

I-BET-762 (synthesised at GSK Stevenage UK) was dissolved in 2% DMSO/98% kleptose (10%) solution and dosed as the free base at 30 mg/kg in a dose volume of 10 mL/kg. TLCS, ethanol, POA, caerulein, human trypsin, human MPO, TMB and other chemicals if not otherwise stated were purchased from Sigma (Gillingham, UK).

**Data analysis**

Results were presented as mean ± SE obtained from three or more independent experiments. In all the figures, vertical bars denote SE values. A Student’s t-test was used for statistical evaluation of data with a normal distribution, while an ANOVA test was carried out for data with a skewed distribution. \( P \) values of < 0.05 were considered to indicate significant differences.
RESULTS

Pharmacokinetics analysis of I-BET-762 in TLCS-AP and CER-AP

Analysis of the blood samples following the first 30 mg/kg (i.p.) injection of I-BET-762 in the TLCS-AP and CER-AP models gave peak serum concentrations (Cmax) of 26.68 and 10.61 µg/mL at 0.25 h (time of Cmax, Tmax) with resulting exposures (area under the curve, AUC0-inf) of 42.03 and 19.91 µg.h/mL respectively (Table 1). Blood concentrations in both models were within the expected range following single administration over the first 12 h.

Effects of I-BET-762 in TLCS-AP

Infusion of 3 mM (50 µL) TLCS into the pancreatic duct caused marked increases in serum amylase and pancreatic MPO (Figures 1A, B). TLCS infusion also resulted in significant increases in serum cytokines (IL-6, IL-10, CCL2 and CXCL1; Table 2) and lung injury that was evident in an elevated lung MPO (Figure 1C) and thickened alveolar septae (TLCS-AP 1.16 ± 0.06 versus sham 0.22 ± 0.05, P < 0.05). There were pronounced histopathological findings in the head of pancreas at 24 h demonstrated by oedema, vacuolisation, inflammatory cell infiltration and scattered necrosis (Figure 2A). Consistent with the known features of this model10, 12-14, 20, the body and tail of the pancreas were less affected (data not shown). The severity of pancreatic histopathology was reflected in a significant increase in the overall histopathological score and the individual components compared to the sham group (Figure 2B). I-BET-762 administration significantly reduced serum amylase and showed a trend to lower pancreatic MPO (Figures 1A, B). Moreover, I-BET-762 significantly lowered serum cytokines (IL-6, IL-10, CCL2 and CXCL1; Table 2). I-BET-762 significantly reduced the overall pancreatic histopathological score and oedema score, with a trend to curtail the inflammation and necrosis scores (Figures 2A, B). Furthermore, lung MPO (Figure 1C) and alveolar septal thickening (TLCS-AP with I-BET-762 0.55 ± 0.11 versus TLCS-AP without I-BET-762 1.16 ± 0.06, P < 0.05) were significantly diminished with I-BET-762 treatment.

Effects of I-BET-762 in FAEE-AP

Concomitant i.p. injections of ethanol and POA induced a pronounced rise in serum amylase, pancreatic trypsin and MPO levels (Figures 3A, B and C). Moreover, plasma cytokines (IL-6, IL-10, CCL2 and CXCL1; Table 3), lung MPO (Figure 3D) and alveolar septal thickening (FAEE-AP 2.05 ± 0.19 versus saline 0.41 ± 0.1, P < 0.01) were significantly increased in FAEE-AP. FAEE-AP caused changes in the pancreas as in TLCS-AP, but
in contrast to TLCS-AP, the changes were not restricted to the head of the pancreas (Figure 4A) and were pronounced (Figure 4B). Treatment of FAEE-AP with I-BET-762 significantly reduced serum amylase, pancreatic trypsin and myeloperoxidase (Figures 3A, B, C). Moreover, I-BET-762 diminished serum cytokines (IL-6, IL-10, CCL2 and CXCL1; Table 3) but had no significant effect on lung injury. The overall pancreatic histopathological score was also greatly ameliorated by I-BET-762, showing significant reductions in oedema and inflammation scores with a trend to decrease the necrosis score (Figure 4B).

**Effects of I-BET-762 in CER-AP**

Hyperstimulation of the pancreas with 12 hourly i.p. injections of supramaximal caerulein caused typical features of AP at 24 h as shown by raised serum amylase, pancreatic trypsin and MPO (Figure 5A-C), serum cytokines (IL-6, CCL2 and CXCL1; Table 4) and lung injury with raised lung MPO (Figures 5D) and alveolar septal thickening (caerulein 1.75 ± 0.19 versus saline 0.38 ± 0.13, P <0.01), as well as overall pancreatic histopathology score (Figure 6A, B). The histopathological changes in pancreatic oedema, inflammation and necrosis appeared more pronounced and more homogenously distributed compared to TLCS-AP and FAEE-AP (Figure 6A).

Treatment with I-BET-762 did not have protective effects on serum amylase, pancreatic trypsin, pancreatic MPO, pancreatic histopathology or lung injury, but rather caused a modest increase of all parameters measured. Interestingly, I-BET-762 significantly increased serum cytokines (IL-6, IL-10, CCL2 and CXCL1). As these results were inconsistent with the effects of I-BET-762 in TLCS-AP and FAEE-AP, the experiments were repeated by an independent investigator (L.W.). These further experiments were conducted with CER-AP induced by either 7 or 12 hourly i.p. injections of caerulein (50 µg/kg) in separate groups; identical results were obtained with modest increases in all parameters as a result of I-BET762 (data not shown), as before.
DISCUSSION

Here we have shown that I-BET-762 significantly reduces pancreatic damage and systemic injury in 2 experimental models representing the commonest causes of clinical AP, namely gallstones and alcohol excess, although not in hyperstimulation AP, a rare cause. These findings broaden the potential applications of BET protein inhibition across inflammatory diseases, while ongoing clinical trials assess applications in oncology, cardiovascular disease, diabetes mellitus and dyslipidaemia. I-BET-762 has been shown to inhibit transcription of multiple inflammatory response genes induced in macrophages by lipopolysaccharide, conferring protection against death from lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis from caecal puncture in murine models. The inhibitory action of I-BET-762 is highly specific, not affecting genes already primed for or actively involved in transcription as primary immune response genes, but greatly reducing transcription of secondary immune response genes. Thus I-BET-762 has not previously been found to inhibit transcription of tumour necrosis factor but inhibits transcription of IL-6. The resulting inhibition of the biochemical, immunological and histopathological features of TLCS-AP and FAEE-AP confirm that the severity of these models is determined at least in part by immune responses depending directly on transcription. Transcription of other genes that contribute to the severity of AP may have been inhibited, and not necessarily the same genes in the 2 models, although the overall impact of I-BET-762 was similar in both. Thus BET inhibition could have clinical application since AP, like severe sepsis, is characterised by organ failure induced by profound systemic inflammatory responses; inhibition of these responses may reduce organ dysfunction while maintaining later phase immune competence.

The absence of any protective effect from I-BET-762 on the severity of CER-AP indicates that the mechanisms by which transcription contributes to severity in CER-AP have important differences from those in TLCS-AP and FAEE-AP. This is despite achieving pharmacologically relevant blood concentrations within the range required to see in vivo efficacy, albeit lower in CER-AP than TLCS-AP. Were the mechanisms to be the same, at least a modest beneficial effect from I-BET-762 in CER-AP would be expected. Several differences could be present, as suggested by the far lower levels of several cytokines in CER-AP than in TLCS-AP and FAEE-AP, despite the severity of CER-AP induced with 12 caerulein injections, and the increase in these levels in CER-AP following I-BET-762 administration. Administration of I-BET-762 in CER-AP enhanced expression of IL-6, IL-10, CCL2 and CXCL1, possibly from enhanced transcription of other genes through compensatory mechanisms in the macromolecular regulation of transcription following BET protein inhibition, including
histone acetylation-independent mechanisms. Histone phosphorylation contributes to the severity of CER-AP\textsuperscript{22}, likely more so than in TLCS-AP and FAEE-AP\textsuperscript{23}, and may render I-BET-762 ineffective in preventing BET protein binding to histones, as I-BET-762 is an acetylated histone mimic. The contribution of immune responses to CER-AP may depend more on the direct effects of primary immune response genes, such as those induced by tumour necrosis factor receptor type 1 ligation, which triggers receptor interacting protein kinase activation and subsequent necroptosis\textsuperscript{24}. The marked pancreatic histopathological changes in CER-AP with far lower levels of IL-6, IL-10, CCL2 and CXCL1 than in TLCS-AP and FAEE-AP suggest that secondary immune response genes have a relatively reduced role in CER-AP. Alternatively I-BET762 when administered with caerulein might have an unknown off-target effect that exacerbates AP. Further exploration of these differences would benefit from detailed comparative transcriptomic analyses at different time points following induction of AP in each model, with and without I-BET-762. Nevertheless the markedly contrasting results of I-BET-762 administration in these three models do illustrate the complexity of epigenetic control of gene transcription in AP. This is despite common mechanisms of pancreatic injury that include intracellular calcium overload\textsuperscript{11-14, 25, 26}, reactive oxygen species production\textsuperscript{27}, mitochondrial injury\textsuperscript{11, 13, 28}, trypsinogen activation\textsuperscript{29, 30}, nuclear factor-κB activation\textsuperscript{31} and induction of the inflammasomes\textsuperscript{32}.

In both TLCS-AP and FAEE-AP the most marked effect of I-BET-762 was to ablate markedly raised levels of IL-6, IL-10, CCL2 and CXCL1. These results are consistent with previous data showing that inhibition of pancreatic histone acetyltransferases by pentoxifylline ameliorates the severity of taurocholate-induced AP in rats\textsuperscript{33}, although pentoxifylline has other effects that may be protective in AP, including inhibition of tumour necrosis factor alpha production\textsuperscript{34}. The inhibition of IL-10 production by I-BET-762 in TLSC-AP and FAEE-AP removes an immunoregulator, but that occurred alongside the reduction of major pro-inflammatory cytokines including IL6, which is associated with lung injury and lethality\textsuperscript{35}. The effects of I-BET-762 on cytokine responses in these 2 models implicates post-translational modification of histones in the disease course of AP and confirms the validity of a strategy to prevent the consequences of pancreatic and leukocyte histone acetylation in the search for new, effective treatments in clinical AP.
REFERENCES


**Table 1.** Blood concentrations of I-BET-762 in male CD1 mice with TLCS-AP or CER-AP following intraperitoneal administration every 12 h at a target dose of 30 mg/kg. The first injection was administered at the time of disease induction, and a composite profile was achieved with \( n = 1 \) mouse per time-point. (A) Blood concentration at each time-point. (B) Pharmacokinetic parameters.

### A.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>I-BET-762 concentration (µg/mL)</th>
<th>CER-AP</th>
<th>TLCS-AP</th>
</tr>
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<tbody>
<tr>
<td>0.25</td>
<td>10.610</td>
<td>26.676</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>8.542</td>
<td>11.302</td>
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</tr>
<tr>
<td>2.00</td>
<td>3.417</td>
<td>8.629</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>0.821</td>
<td>2.248</td>
<td></td>
</tr>
<tr>
<td>12.25</td>
<td>38.411</td>
<td>8.518</td>
<td></td>
</tr>
<tr>
<td>24.00</td>
<td>0.039</td>
<td>0.021</td>
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### B.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg/mL)</td>
<td>CER-AP</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.25</td>
</tr>
<tr>
<td>T1/2 (h)</td>
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</tr>
<tr>
<td>AUC(0-inf) (µg.h/mL)</td>
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</tr>
<tr>
<td>AUC(0-inf)/D (µg.h/mL)</td>
<td>39.82</td>
</tr>
<tr>
<td>% Extrapolation</td>
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</table>
Table 2. Effects of I-BET-762A on serum cytokines at 24 h in TLCS-AP. TLCS-AP was induced by retrograde infusion of 3 mM TLCS into the biliopancreatic duct, and I-BET-762 (30 mg/kg, i.p.) was given at the time of disease induction with a second dose 12 h later.

<table>
<thead>
<tr>
<th>Cytokines (pg/mL)</th>
<th>Sham (n = 4)</th>
<th>TLCS (n = 8)</th>
<th>TLCS + I-BET-762A (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>2.8 ± 0.6</td>
<td>2.4 ± 0.3</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>62.5 ± 6.3</td>
<td>517.2 ± 142.1*</td>
<td>146.3 ± 71.0#</td>
</tr>
<tr>
<td>IL-10</td>
<td>10.6 ± 0.2</td>
<td>78.7 ± 32.0*</td>
<td>11.6 ± 1.5#</td>
</tr>
<tr>
<td>CCL2</td>
<td>34.3 ± 17.2</td>
<td>169.4 ± 129.7*</td>
<td>22.7 ± 15.6#</td>
</tr>
<tr>
<td>CXCL1</td>
<td>130.9 ± 44.2</td>
<td>1623.8 ± 613.0*</td>
<td>192.6 ± 62.6#</td>
</tr>
</tbody>
</table>

*P <0.01 sham versus TLCS-AP; #P <0.01 TLCS-AP versus TLCS-AP plus I-BET-762; TLCS, taurolithocholic acid 3-sulphate disodium salt; Values are means ± SE of 4-8 mice.
Table 3. Effects of I-BET-762A on serum cytokines at 24 h in FAEE-AP. FAEE-AP was induced by 2 intraperitoneal (i.p.) injection of ethanol (1.35 g/kg) and palmitoleic acid (POA, 150 mg/kg) at 1 h apart and I-BET-762 (30 mg/kg, i.p.) was given at the time of disease induction with a second dose 12 h later.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Saline (n = 4)</th>
<th>Ethanol (n = 6)</th>
<th>FAEE-AP (n = 6)</th>
<th>FAEE-AP + I-BET-762A (n = 6)</th>
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<tr>
<td>IL-1(\beta)</td>
<td>3.0 ± 0</td>
<td>2.3 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>IL-6</td>
<td>21.5 ± 2.1</td>
<td>24.8 ± 2.0</td>
<td>3807.6 ± 1913.3*</td>
<td>24.9 ± 4.2#</td>
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<tr>
<td>IL-10</td>
<td>12.4 ± 0.9</td>
<td>10.8 ± 0.3</td>
<td>82.8 ± 32.4*</td>
<td>10.0 ± 1.0#</td>
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<tr>
<td>CCL2</td>
<td>9.1 ± 0.8</td>
<td>9.1 ± 0.8</td>
<td>121.2 ± 73.6*</td>
<td>12.0 ± 2.9#</td>
</tr>
<tr>
<td>CXCL1</td>
<td>62.6 ± 11.7</td>
<td>139.4 ± 45.6*</td>
<td>1477.0 ± 444.2*</td>
<td>172.6 ± 40.6#</td>
</tr>
</tbody>
</table>

*P < 0.01 saline versus ethanol or ethanol/POA group; #P < 0.01 ethanol/POA versus ethanol/POA plus I-BET-762 group; POA, palmitoleic acid; values are means ± SE of 4-6 mice.
Table 4. Effects of I-BET-762A on serum cytokines in CER-AP at 24 h. CER-AP was induced 12 hourly intraperitoneal (i.p.) injections of carerulein (50 µg/kg), and I-BET-762 (30 mg/kg, i.p.), given at the time of disease induction and the second dose 12 h later.

<table>
<thead>
<tr>
<th>Cytokines (pg/mL)</th>
<th>Saline (n = 4)</th>
<th>Caerulein (n = 8)</th>
<th>Caerulein + I-BET-762A (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>3.1 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>21.0 ± 4.7</td>
<td>33.0 ± 3.0*</td>
<td>66.0 ± 11.4ª</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.5 ± 1.3</td>
<td>11.5 ± 0.7</td>
<td>16.3 ± 2.1#</td>
</tr>
<tr>
<td>CCL2</td>
<td>8.0 ± 0.7</td>
<td>10.4 ± 0.5*</td>
<td>17.0 ± 5.0#</td>
</tr>
<tr>
<td>CXCL1</td>
<td>127.4 ± 74.1</td>
<td>290.1 ± 55.6*</td>
<td>442.6 ± 77.7ª</td>
</tr>
</tbody>
</table>

*P <0.05 saline versus caerulein; ªP <0.05 caerulein versus caerulein plus I-BET-762 group; Values are means ± SE of 4-8 mice.
FIGURE LEGENDS

Figure 1. Protective effects of I-BET-762 on biochemical markers in TLCS-AP. Retrograde infusion of 3 mM TLCS into the biliopancreatic duct resulted in a significant elevation of (A) serum amylase, (B) pancreatic and (C) lung myeloperoxidase (MPO) activity. Control mice received sham operation with ductal saline infusion. Mice were sacrificed 24 h after ductal infusion. Intraperitoneal administration of 30 mg/kg I-BET-762 significantly reduced serum amylase and lung MPO activity, with a trend to decrease pancreatic MPO activity. TLCS, taurolithocholic acid 3-sulphate disodium salt. *P < 0.05 sham versus TLCS group, #P < 0.05 TLCS versus TLCS plus I-BET-762 group. Values are mean ± SE of 6-11 mice.

Figure 2. Protective effects of I-BET-762 on histopathological parameters in TLCS-AP. (A) Representative H&E images from pancreatic histopathology slides from sham, TLCS and TLCS plus I-BET-762 groups. (B) Histopathological scores: (i) overall histopathological score and its breakdown components: (ii) oedema, (iii) inflammation and (iii) necrosis. I-BET-762 significantly reduced overall and edema scores, with a trend to decrease inflammation and necrosis scores. *P < 0.05 sham versus TLCS group, #P < 0.05 TLCS versus TLCS plus I-BET-762 group. Values are mean ± SE of 6-12 mice. Magnification × 200.

Figure 3. Protective effects of I-BET-762 on biochemical markers in FAEE-AP. Two intraperitoneal (i.p.) injection of ethanol (1.35 g/kg) and palmitoleic acid (POA, 150 mg/kg) at 1 h apart caused marked elevation of (A) serum amylase, (B) pancreatic trypsin activity, (C) pancreatic and (D) lung myeloperoxidase (MPO) activity. Control mice received the same regimen of normal saline or ethanol. Mice were sacrificed 24 h after the first injection of ethanol/POA. Intraperitoneal administration of 30 mg/kg I-BET-762 significantly reduced serum amylase, pancreatic trypsin and MPO activity. *P < 0.05 saline versus ethanol or ethanol/POA group, #P < 0.05 ethanol/POA versus ethanol/POA plus I-BET-762 group. Values are mean ± SE of 6-10 mice.

Figure 4. Protective effects of I-BET-762 on histopathological parameters in FAEE-AP. (A) Representative H&E images from pancreatic histopathology slides from saline, ethanol, ethanol/palmitoleic acid (POA), and ethanol/POA plus I-BET-762 groups. (B) Histopathological scores: (i) overall histopathological score and its breakdown components: (ii) oedema, (iii) inflammation and (iii) necrosis. I-BET-762 significantly reduced overall, oedema and inflammation scores, with a trend to decrease necrosis. *P < 0.05 saline versus ethanol or ethanol/POA group, #P < 0.05 ethanol/POA versus ethanol/POA plus I-BET-762 group. Values are mean ± SE of 6 mice. Magnification × 200.
Figure 5. Effects of I-BET-762 on biochemical markers in CER-AP. Twelve hourly intraperitoneal injections of carerulein (50 µg/kg) resulted in significant elevation of (A) serum amylase, (B) pancreatic trypsin activity, (C) pancreatic and (D) lung myeloperoxidase (MPO) activity. Control mice received the same regimen of normal saline. Mice were sacrificed 24 h after the first injection of caerulein. Intraperitoneal administration of 30 mg/kg I-BET-762 had no protective effects, with a trend to increase biochemical parameters. *P < 0.05 control versus caerulein group. Values are mean ± SE of 6-12 mice.

Figure 6. Effects of I-BET-762 on histopathological parameters in CER-AP. (A) Representative H&E images from pancreatic histopathology slides from saline, caerulein and caerulein plus I-BET-762 groups. (B) Histopathological scores: (i) overall histopathological score and its breakdown components: (ii) oedema, (iii) inflammation and (iii) necrosis. I-BET-762 had no protective effects on pancreatic damage with a trend to increase the scores. *P < 0.05 control versus caerulein group. Values are mean ± SE of 6-9 mice. Magnification × 200.
Figure 2

A

Sham TLCS TLCS/I-BET-762

B

(i)

Histology score

(ii)

Oedema

(iii)

Inflammation

(iv)

Necrosis
Figure 3

A

Serum amylase (U/L)

Ethanol  POA  I-BET-762

-  -  -  +  +  +

B

Trypsin activity (fmol/mg protein)

Ethanol  POA  I-BET-762

-  -  -  +  +  +

C

Pancreatic MPO (mU/mg protein)

Ethanol  POA  I-BET-762

-  -  -  +  +  +

D

Lung MPO (mU/mg protein)

Ethanol  POA  I-BET-762

-  -  -  +  +  +
Figure 4

A

Saline  EtOH  EtOH/POA  EtOH/POA/I-BET-762

B

(i) Histogram score

(ii) Oedema

(iii) Inflammation

(iv) Necrosis
Figure 6

A

Saline  Caerulein  Caerulein/I-BET-762

B

(i)

Histology score

(ii)

Oedema

(iii)

Inflammation

(iv)

Necrosis

Caerulein  I-BET-762  Caerulein  I-BET-762  Caerulein  I-BET-762  Caerulein  I-BET-762