



The histone deacetylase (HDAC) inhibitor PCI-24781 decreases pro-inflammatory cytokine secretion *in vitro* and *in vivo*, and protects against endotoxemia in a sepsis model

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ABSTRACT

Inhibitors of histone deacetylases (HDACs) are currently in clinical testing for treating various cancers, and two have been recently approved by the US FDA for treating cutaneous T-cell lymphoma. Here we describe novel anti-inflammatory properties of the HDAC inhibitor PCI-24781 which is in clinical trials for multiple indications including lymphoma¹.

Cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have been shown to be involved in human inflammatory disorders, and an anti-IL-6 treatment was recently approved for rheumatoid arthritis (RA). Therefore, the effect of PCI-24781 on cytokine production by lipopolysaccharide (LPS)-stimulated human peripheral mononuclear blood cells (PBMC) as well as isolated monocytes was studied at the RNA expression level by microarrays and Taqman, and at the protein level by ELISA. PCI-24781 potently inhibits the production and secretion of several pro-inflammatory cytokines, including IL-6, TNF-α and interleukin-1beta (IL-1β), at both RNA and protein levels. In murine RAW macrophages as well, PCI-24781 inhibited IL-6 secretion at 20nM. PCI-24781 was most effective when given with or before LPS, but was still effective when given an hour after LPS.

Similarly, PCI-24781 greatly attenuated *in vivo* pro-inflammatory cytokine production in LPS-treated Balb/c mice; the IC50 for IL-6 inhibition was < 5 mg/kg. Both the *in vitro* and *in vivo* IC50s for IL-6 inhibition are considerably less than the concentrations required to inhibit growth and induce apoptosis in tumor cells (0.2-0.5μM) and in xenograft models (60-80 mg/kg). The mechanism by which these cytokines are controlled involves attenuation of the LPS receptor TLR4 signaling at multiple levels, including acetylation of targets such as MKP-1 and NF-κB subunit p65 in the MAPK and NF-κB pathways; other factors include reduced expression of proteasome, IKK and other NF-κB subunits. Interestingly, we observed a large reduction in levels of NOS2, which causes hypotension during sepsis by producing the inflammatory mediator nitric oxide (NO).

Therefore the activity of PCI-24781 was tested in a model of sepsis where mice were treated with a lethal dose of 100 mg/kg LPS, an endotoxin known to be a major mediator of sepsis in humans. Control mice died within 2 days, but pretreatment with PCI-24781 led to dose-dependent increase in survival with 60% of the mice surviving past 6 days with 2 doses of 50mg/kg PCI-24781. These data show that the HDAC inhibitor PCI-24781 protects mice from lethal endotoxemia.

Thus, taken together, our data suggest that PCI-24781 has potent anti-inflammatory activities and may be useful to treat inflammatory disorders including RA and sepsis in humans.

INTRODUCTION

Cytokines, inflammation and human disease

- Chronic inflammation is a hallmark of several diseases in which aberrant immune activity is required for maintenance of the diseased state, for example, rheumatoid arthritis, systemic onset juvenile idiopathic arthritis, lupus erythematosus, inflammatory bowel disease, and psoriasis.
- The critical roles of the pro-inflammatory cytokines TNFα and interleukin-1β (IL-1β) in inflammatory disease has been demonstrated by the efficacy of three TNF blockers, infliximab, etanercept, and adalimumab, and the IL-1β receptor antagonist IL-1RA in treating these diseases.
- Similarly IL-6 has been shown to be a critical mediator in acute phase inflammatory conditions including sepsis.
- Current cytokine targeted agents such TNF blockers, IL-1RA and anti-IL-6R Ab are all administered by infusion or injection, and these also carry a significant risk of opportunistic infections and tuberculosis.
- Therefore the development of new cytokine targeted therapies is highly desired.

HDAC inhibitors have anti-inflammatory properties

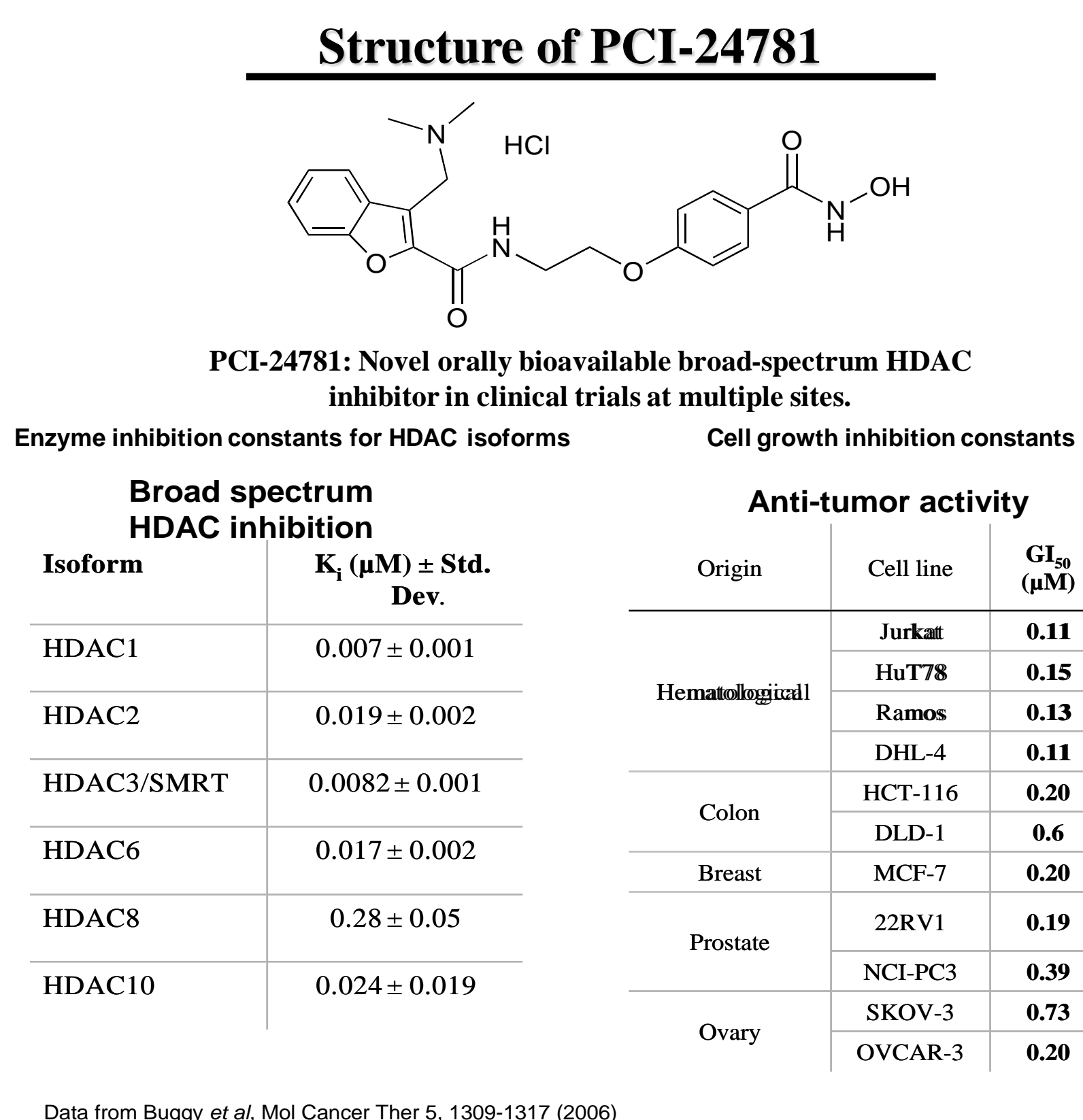
- Two HDAC inhibitors have received US FDA approval for treating cutaneous T-cell lymphoma.
- HDAC inhibitors have been shown to cause reduction of TNFα, IFNγ, IL-6, IL-1β and ameliorate injury in animal models (PNAS 2002 99:2995; Shock 2009 32:517).

PCI-24781 is a safe, well-tolerated, oral pan-HDAC inhibitor

- PCI-24781 is a potent, orally dosed pan-HDAC inhibitor with significant anti-tumor activity in lymphoma and other cancers in clinical trials. Here, we show that it also has potent anti-inflammatory properties in cells and animal models.

RESULTS

Figure 1. PCI-24781 is a potent inhibitor of all Class I and IIb HDAC isoforms, and inhibits proliferation of tumor cells



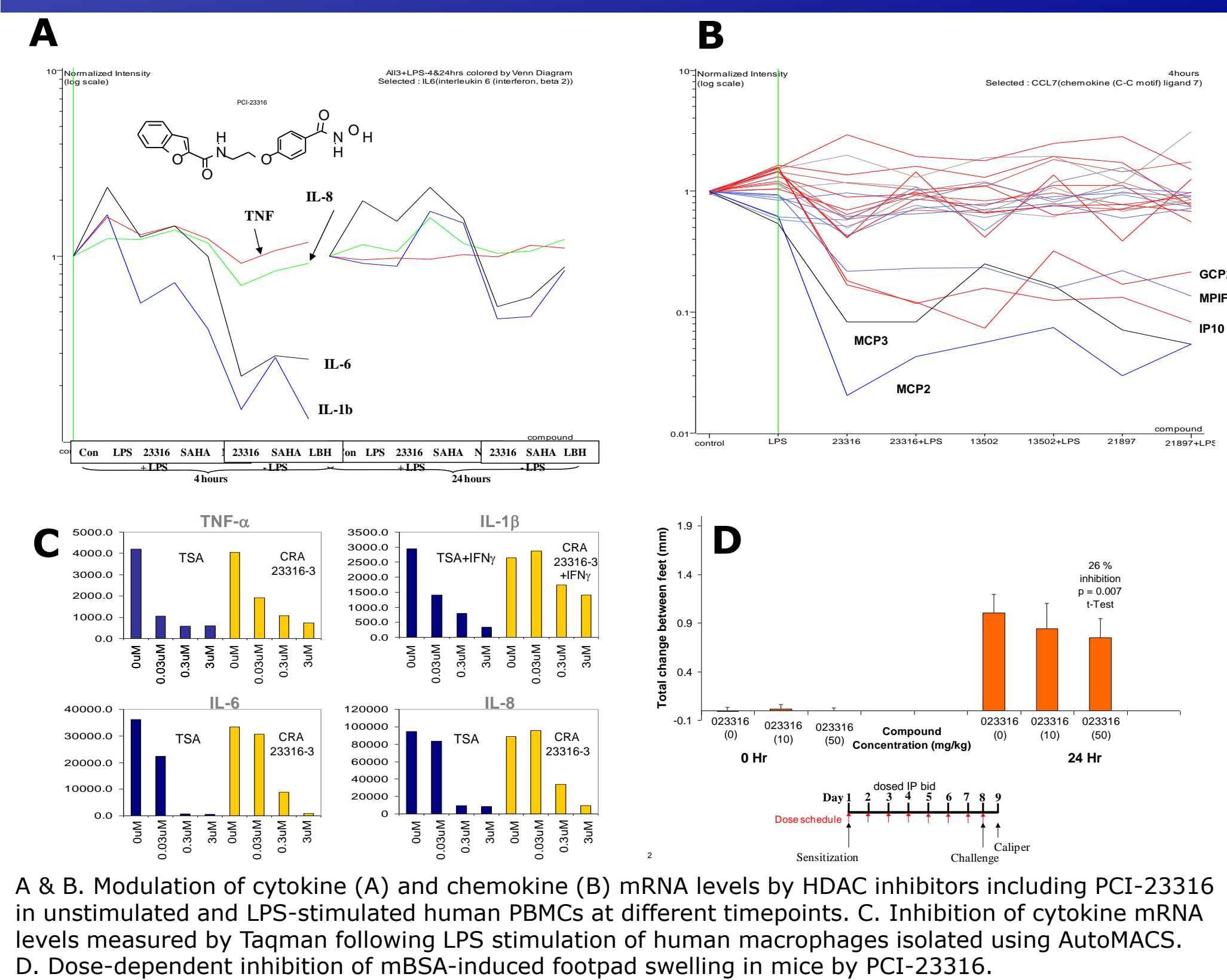
PCI-24781 potently inhibits all Class I and IIb HDACs (table, left column)². It inhibits proliferation and induces apoptosis in all tumor lines tested, most with GI50 <500nM (table, right column). It does not induce apoptosis in normal PBMC or fibroblasts at up to 1μM (data not shown)

Table 1. PCI-24781 has significant clinical activity in treating several subtypes of B- and T-cell NHL. Data from the Phase I arm of a Phase I/II lymphoma monotherapy trial¹

Results from 20 evaluable patients

Histology	n	Response	DLT	Mean # days on study
DLBCL	7	1 PR	2	36
Follicular	4	1 CR 2 PR 1 SD	1	197
Hodgkins	2	1 SD		42
CLL/SLL	2	2 SD		88
AILT	2	1 SD	2	29
MCL	1	1 PR		63
CTCL	1	1 SD		102
MALT	1	1 SD		44

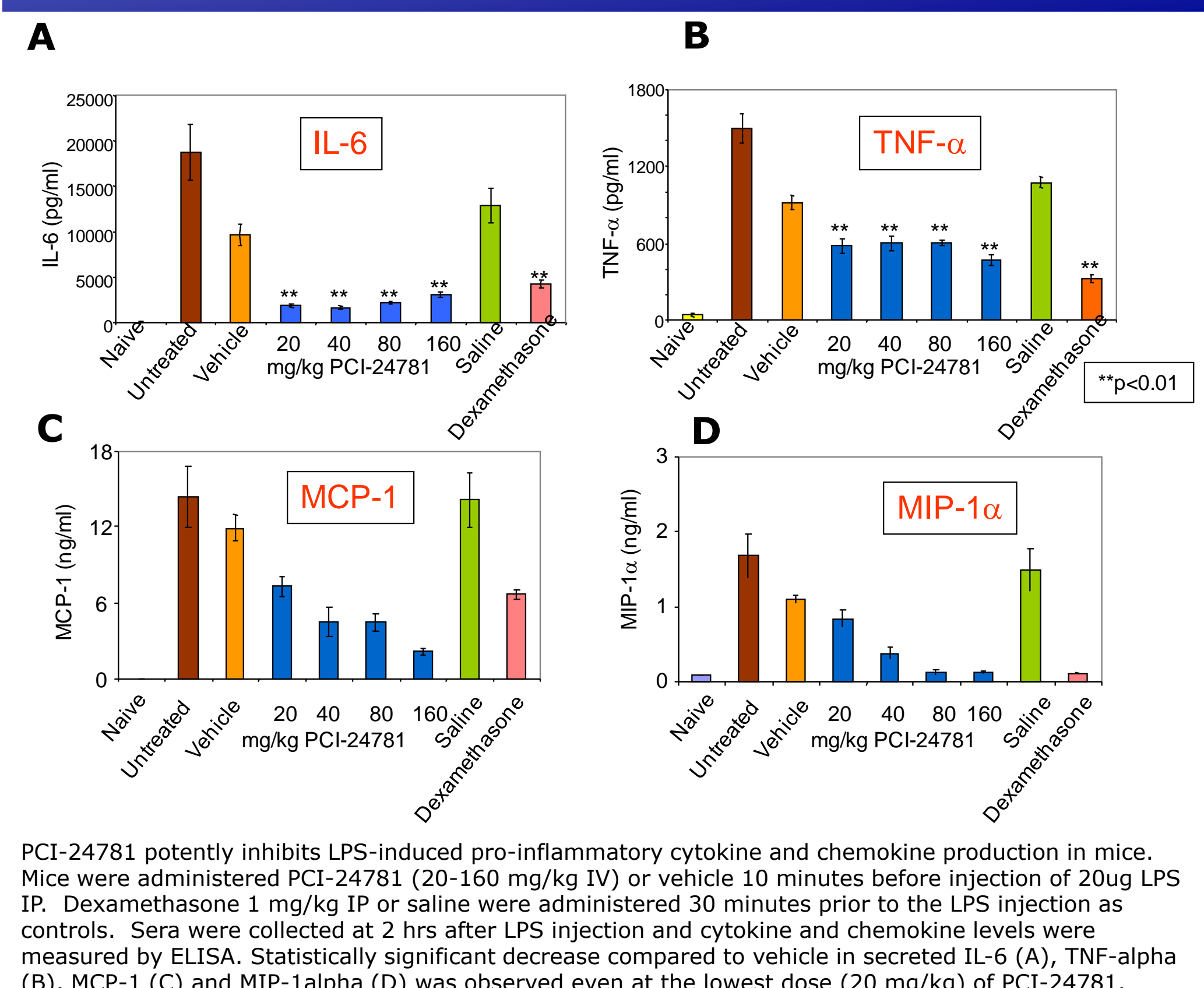
Figure 2. PCI-23316, a closely-related analog of PCI-24781, inhibits pro-inflammatory cytokine production in LPS-stimulated PBMCs as well as isolated macrophages, and inhibits footpad swelling in a mouse model of inflammation



A & B. Modulation of cytokine (A) and chemokine (B) mRNA levels by HDAC inhibitors including PCI-23316 in unstimulated and LPS-stimulated human PBMCs at different timepoints. C. Inhibition of cytokine mRNA levels measured by Taqman following LPS stimulation of human macrophages isolated using AutoMACS. D. Dose-dependent inhibition of mBSA-induced footpad swelling in mice by PCI-23316.

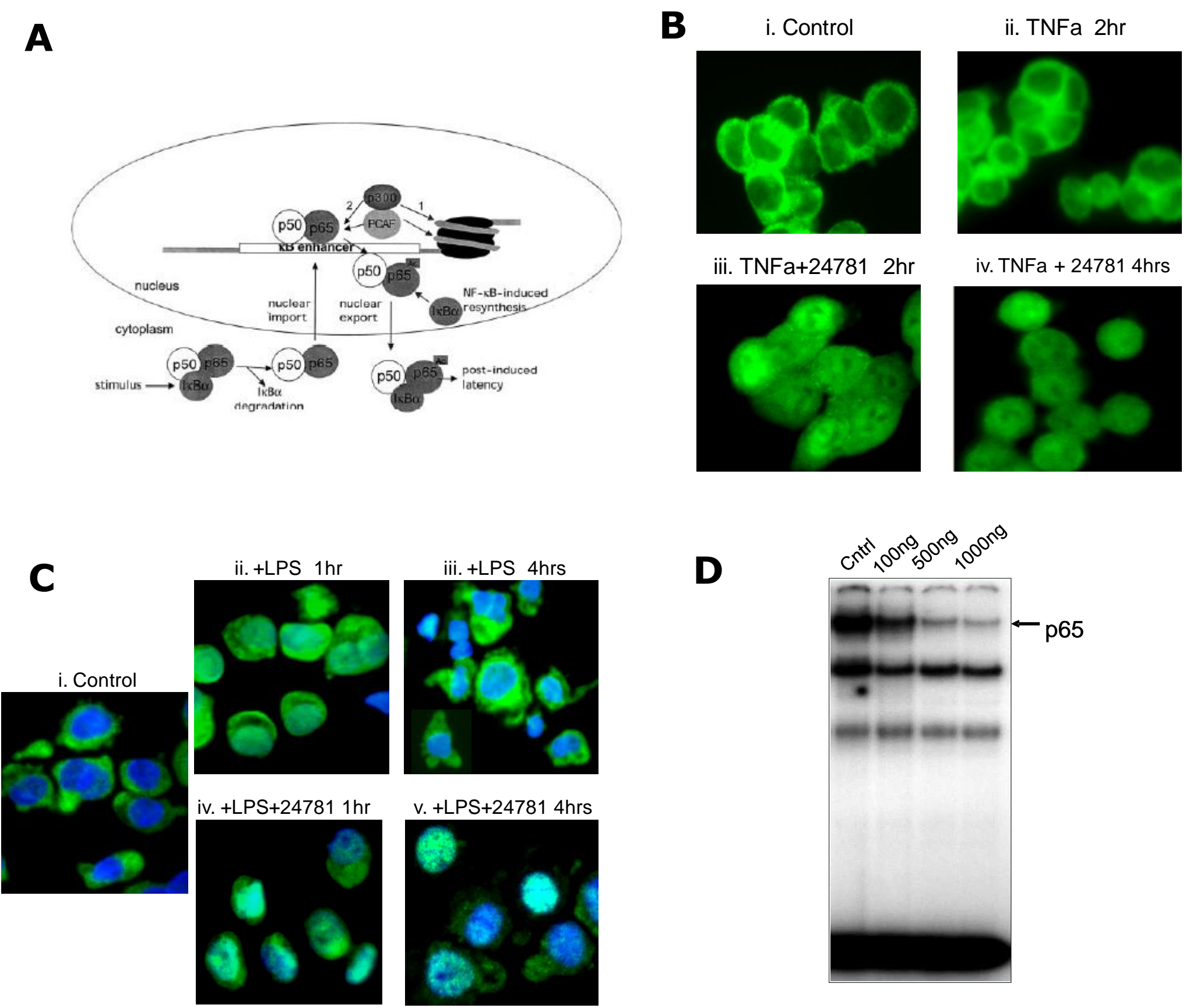
RESULTS

Figure 3. PCI-24781 inhibits LPS-induced pro-inflammatory cytokine and chemokine secretion *in vivo*



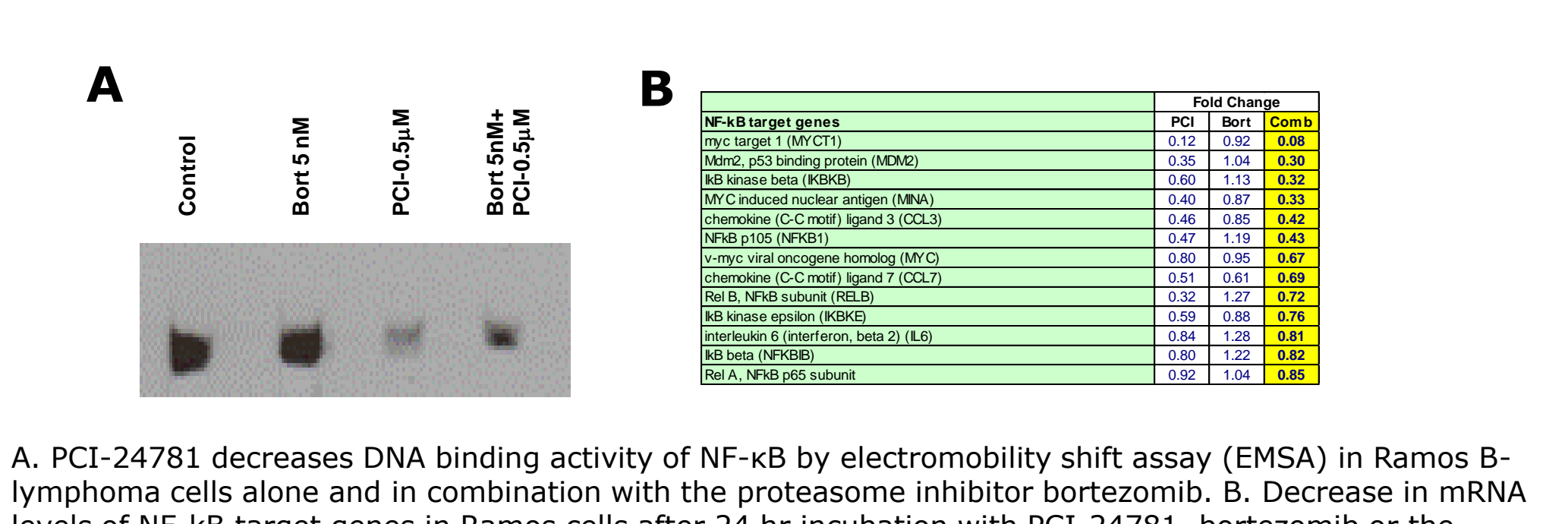
PCI-24781 potently inhibits LPS-induced pro-inflammatory cytokine and chemokine production in mice. Mice were administered PCI-24781 (20-160 mg/kg IV) or vehicle 10 minutes before injection of 20ug LPS IP. Dexamethasone 1 mg/kg IP or saline were administered 30 minutes prior to the LPS injection as controls. Sera were collected 2 hrs after LPS injection and cytokine and chemokine levels were measured by ELISA. Statistically significant decrease compared to vehicle in secreted IL-6 (A), TNF-α (B), MCP-1 (C) and MIP-1α (D) was observed even at the lowest dose (20 mg/kg) of PCI-24781.

Figure 4. PCI-24781 affects the intracellular localization and reduces DNA binding of NF-κB subunit p65 in many cell types



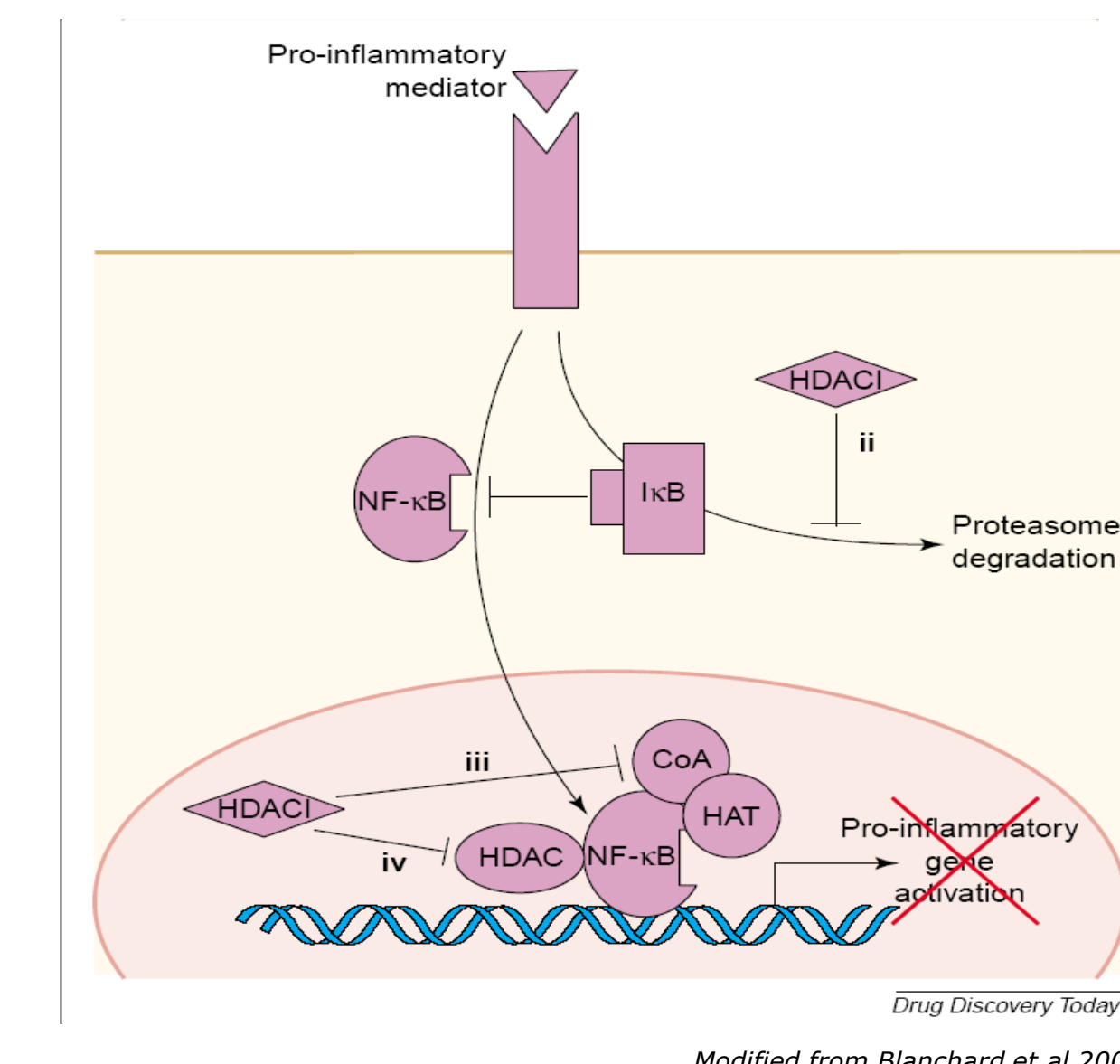
A. HDAC-induced acetylation of p65 and inhibition of NF-κB transcription can modulate normal function of the NF-κB pathway (adapted from Ref 5). B & C. NF-κB p65 localization by immunofluorescence in B) HCT-116 cells treated with TNFα or C) THP-1 cells stimulated with LPS. Upon stimulation, p65 moves into the nucleus, normally reverting to the cytoplasm after 1-2 hours. But in PCI-24781-treated cells, p65 is trapped in the nucleus for >4 hrs. D. After treatment with PCI-24781 in Jurkat cells, electrophoretic mobility shift analysis (EMSA)⁴ shows that p65 binding to DNA is decreased greatly.

Figure 5. PCI-24781 inhibits NF-κB p65 binding to DNA and NF-κB-induced transcription, alone and in combination with the proteasome inhibitor bortezomib



A. PCI-24781 decreases DNA binding activity of NF-κB by electromobility shift assay (EMSA) in Ramos B-lymphoma cells alone and in combination with the proteasome inhibitor bortezomib. B. Decrease in mRNA levels of NF-κB target genes in Ramos cells after 24 hr incubation with PCI-24781, bortezomib or the combination shown by microarray gene expression analysis⁵.

CONCLUSIONS



• PCI-24781 is a potent broad-spectrum inhibitor of Class I and IIb HDACs with promising clinical activity in Non-Hodgkin's lymphoma patients

• PCI-24781 (and its close analog PCI-23316) inhibits pro-inflammatory cytokine production in many different cell types and in response to a variety of stimuli:

- In monocyte/macrophage lineage cells, PCI-24781 greatly attenuates LPS-induced transcription and secretion of NF-κB target cytokines and chemokines (TNFα, IL-6, MCP-1, MIP-1α).
- In vivo, PCI-24781 decreases secretion of NF-κB regulated cytokines and chemokines following LPS administration to mice.

• Mechanistically, this effect is likely due to acetylation of NF-κB subunit p65, which prevents it from binding to DNA and activating transcription. Paradoxically, inhibition of NF-κB target genes including IκB prevents the re-export of p65, trapping it in an inactive acetylated state within the nucleus.

• PCI-24781 decreases expression of iNOS, a pro-inflammatory mediator known to be involved in sepsis.

- In a mouse model of sepsis, PCI-24781 confers a dose-dependent protection against endotoxemic shock and decreases LPS-induced lethality >70% at a dose of 50mg/kg.

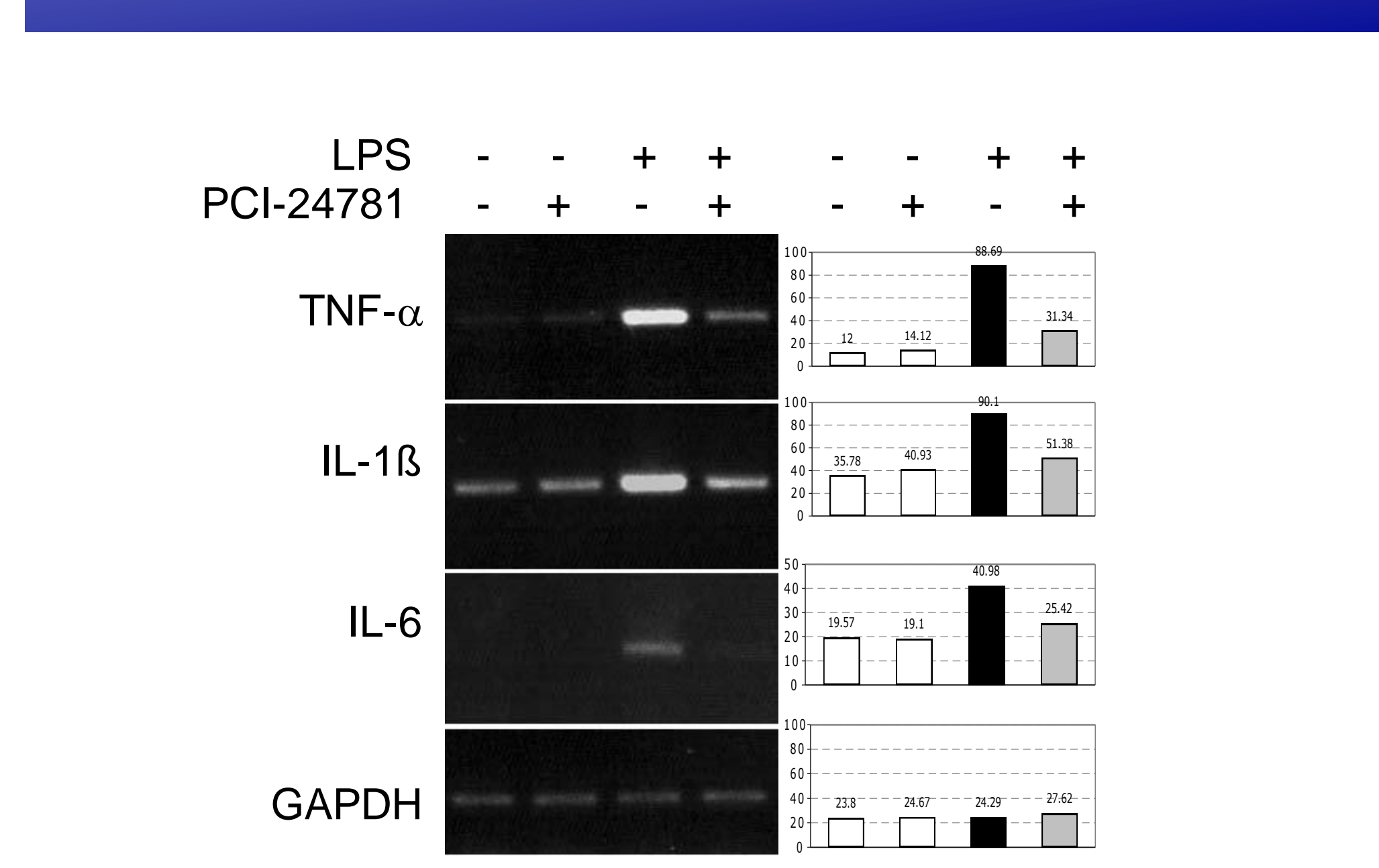
These studies suggest HDAC inhibition by PCI-24781 could be an useful therapy in inflammatory disorders and warrants further clinical investigation.

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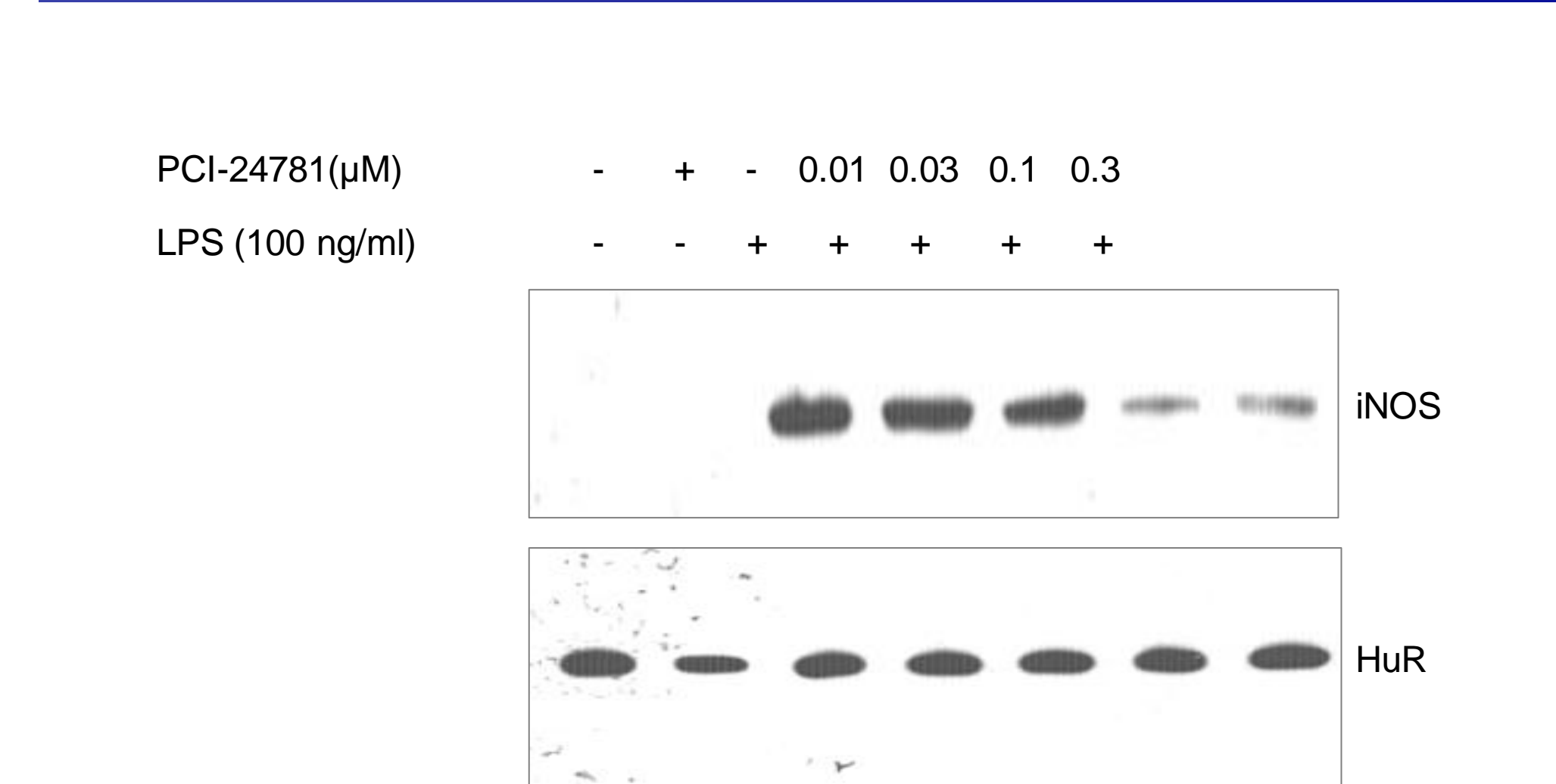
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Figure 6. PCI-24781 decreases LPS-induced pro-inflammatory gene transcription in murine RAW macrophages



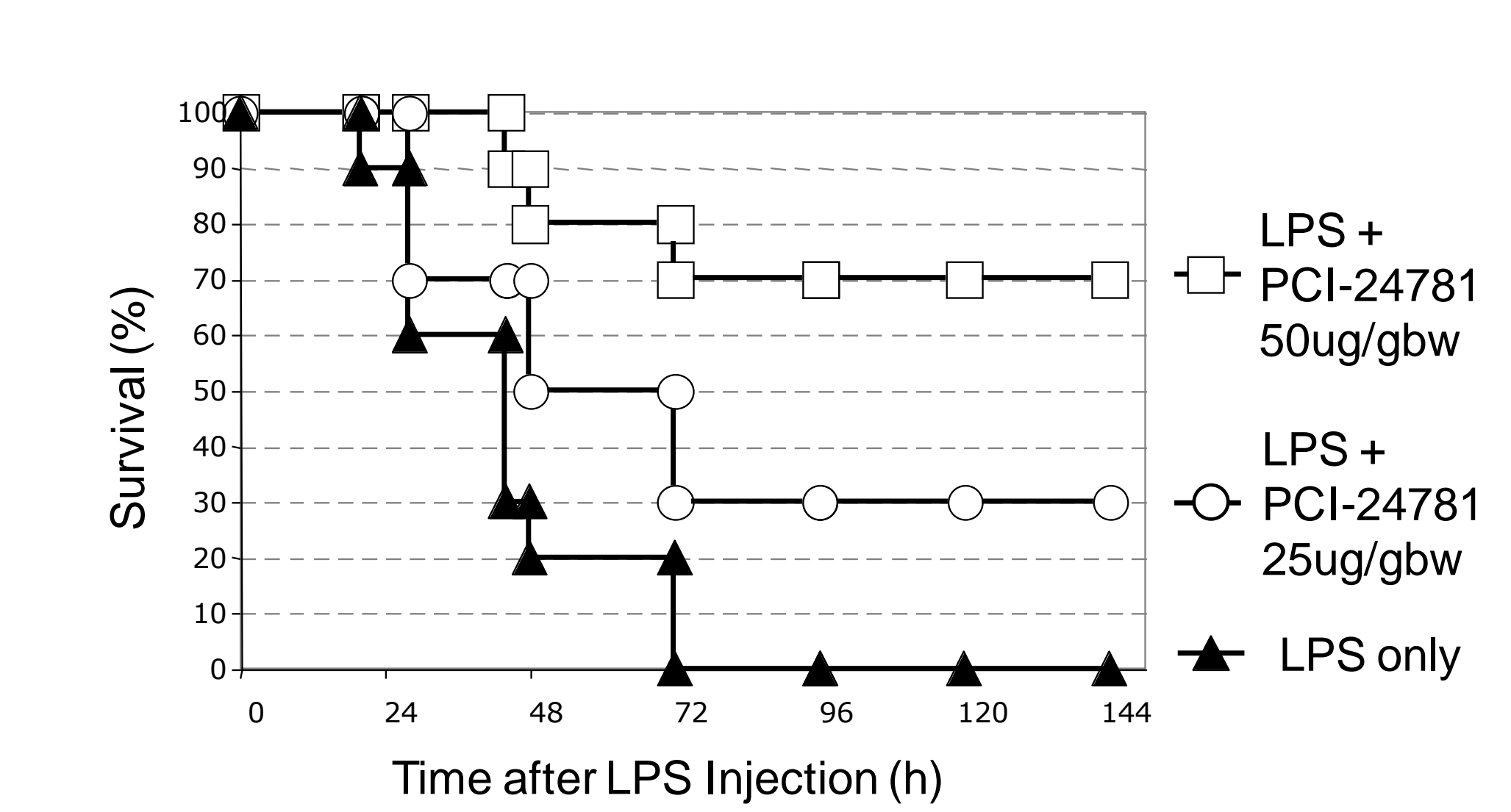
PCI-24781 decreases expression of cytokine mRNA. RAW cells were treated with 0.1 μM of PCI-24781 for 30 min, then 100 ng/ml LPS was added for 6 hours. RT-PCR was performed (30 cycles), and PCR products were analyzed on a 1% agarose gel (left). PCI-24781 decreases RNA levels (right) of TNF, IL-1β, and IL-6, but not GAPDH.

Fig. 7. PCI-24781 dose-dependently decreases LPS-induced iNOS expression in murine RAW macrophages



PCI-24781 decreases iNOS expression. RAW cells were treated with various amounts of PCI-24781 for 30 min, and LPS 100 ng/ml was added to the cells for 16 hours. Cell lysates were analyzed by Western blot with antibodies to the inducible nitric oxide synthase (iNOS) (above) and HuR (below) as a negative control. PCI-24781 decreases iNOS expression.

Fig. 8. PCI-24781 greatly decreases mortality from LPS-induced endotoxemia in a mouse model of sepsis



PCI-24781 decreases mortality from LPS. Mice were treated with saline, PCI-24781 25 μg/gbw IP BID, or PCI-24781 50 μg/gbw IP BID on days -2, -1, 0, 1, and 2. On d 0, a lethal dose of LPS 50 μg/gbw was injected IP, and survival was observed (n = 10 per group). PCI-24781 increases survival from endotoxemia by 70%.