PNAS
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Supplementary Information for

Glucocorticoid Receptor Modulators CpdX and CpdX-D3 exhibit the same *in vivo* anti-inflammatory activities as synthetic glucocorticoids

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www.pnas.org/cgi/doi/10.1073/pnas.1908258116

Supplementary Information Text

SI Materials and Methods

CpdX and its enantiomers

A racemic mixture of CpdX {(R/S)-5-[4-(5-fluoro-2-methoxyphenyl)-2-hydroxy-4-methyl-2-(trifluoromethyl)pentylamino]isobenzofuran-1(3H)-one} was run through a preparative supercritical fluid chromatography (SFC) AD column (250 mm * 30 mm * 5 µm; mobile phase: Neu-MeOH; B%: 20%-20%, 2.3 minutes). The collected fractions corresponding to the first elution peak were then concentrated under reduced pressure at 30°C, lyophilized and further purified through a Phenomenex Synergi C18 column chromatography [150 mm * 25mm * 10 µm; mobile phase: water (0.1 % TFA)-ACN; B%: 50%-80%, 10 minutes]. The collected fractions were concentrated under reduced pressure at 30°C and lyophilized as a white solid, the identity of which was confirmed by LCMS (MS+1=442.1) and SFC (retention time (RT) = 1.084 mins), and named as the "CpdX(eA)" enantiomer (97.6 % purity). The collected fractions corresponding to the second elution peak were similarly concentrated under reduced pressure at 30°C, lyophilized and further purified by Phenomenex Synergi C18 column chromatography [150 mm * 25 mm * 10 µm); mobile phase: water (0.1 % TFA)-ACN; B%: 51%-81%, 12 minutes]. The collected fractions were concentrated under reduced pressure at 30°C and lyophilized as a white solid, the identity of which was confirmed by LCMS (MS+1=442.1) and SFC (RT = 1.147 minutes), and named as the "CpdX(eB)" enantiomer with a 98% purity.

CpdX-D3 and its enantiomers

A racemic mixture of the deuterated compound CpdX-D3, {(R/S)-5-{4-[2-(methoxy-D3)-5-fluorophenyl]-2-hydroxy-4-methyl-2-(trifluoromethyl)pentylamino}isobenzofuran-1(3H)-one} was run through a preparative supercritical fluid chromatography (SFC) DAICEL CHIRALPAK AD-H column (250 mm * 30 mm * 5 µm; mobile phase: 0.1% NH_3H_2O-MEOH; B%: 20%-20%, 2.3 minutes). The collected fractions corresponding to the first elution peak were concentrated under reduced pressure at 30°C and lyophilized as

a white solid, the identity of which was confirmed by LCMS (MS+1=445) and SFC (RT = 1.082 minutes), and named as the enantiomer "CpdX-D3(eA)" with a 98.7 % purity. The collected fractions corresponding to the second elution peak were concentrated under reduced pressure at 30°C and lyophilized as a white solid, the identity of which was confirmed by LCMS (MS+1=445) and SFC (RT = 1.149 minutes), and named as the "CpdX-D3(eB)" enantiomer with a 99.1% purity.

Materials

Dexamethasone (Dex), 12-O-Tetradecanoylphorbol-13-acetate (TPA), Calcipotriol (MC903), Lipopolysaccharide (LPS), Ovalbumin (chicken) and Dextran Sodium Sulfate (DSS) were from Sigma Aldrich. Aldara® Cream was provided from MEDA AB (Sweden). The neutral cream is composed of vaseline, liquid paraffin, Emulgade® 1000 NI (BASF), propyl gallate, sodium edetate, sorbic acid and purified water. Immunization Grade Chick Type II Collagen (7009) was from Chrondrex. House dust mite extract from *dermatopha goides pteronyssinus* was from Greer source material. TOBRADEX was from Novartis Pharma S.A.S. Biotinylated goat polyclonal anti-TSLP was from R&D Systems. Anti-Neutrophil antibody [NIMP-R14] (ab2557) was from Abcam. Anti-Eosinophil antibody (MBP) was a gift kindly provided by Dr. Mei LI (IGBMC, Illkirch, France). Anti-CD31/PECAM-1 antibody was from BD Pharmingen™- BD Biosciences (550274).

Topical treatments

For all topical administration, compounds were dissolved in 100% ethanol and topically applied on both sides of the ears or on shaved dorsal skin of mice.

Histology and Immunohistochemistry staining

Mouse samples were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5µm) were stained with hematoxylin and eosin. For immunohistochemistry (IHC) staining of the major basic protein (MBP) in eosinophils and NIMP-R14 in neutrophils, ears, lungs or eyelids paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block the endogenous peroxidase activity before antigen retrieval with pepsin (Invitrogen) incubated for 10 min at 37°C. Slides were then blocked with 1.5% of

normal rabbit serum (Vector Laboratories, Burlingame, Calif) and incubated with monoclonal anti-mouse MBP primary antibody (dilution 1:2000) (for IHC of MBP), or with rat monoclonal anti-NIMP-R14 primary antibody (anti-neutrophil antibody, Abcam, AB2557, dilution 1:500) (for IHC of R14),overnight at 4°C, followed by incubation with biotinylated rabbit anti-rat IgG (Vector Laboratories) for 1h at room temperature and treatment with AB complex (Vector Laboratories) for 30 min at room temperature. Staining was then visualized with AEC+ high-sensitivity substrate chromogen solution (Dako, Glostrup, Denmark).

For immunohistochemistry (IHC) staining of TSLP (13), paraffin sections were treated with citric buffer (10mmol/L citric acid, PH6) and boiled in the microwave (700W, for 2x5 min) for antigen retrieval. Slides were blocked with Streptavidin and then with a Biotin solution (Vector Laboratories) and incubated with goat polyclonal anti-TSLP antibody (Bio-TSLP, dilution 1/50), overnight at 4°C. Slides were then washed and incubated with biotinylated secondary antibody (Strep-CY3, dilution 1/400) for 1 hour at room temperature. For nucleus staining, a Dapi-Mounting medium is added (Dapi 1/100) to the slides. Staining was then visualized under a UV microscope.

Whole-Mount Immunostaining of cornea

Freshly excised corneas were fixed in PFA 4% at 4°C overnight. Cornea whole-mounts were washed in PBS, blocked in 2% bovine serum albumin, immunostained with FITC-conjugated rat anti–mouse CD31/PECAM-1 antibody (1:200; BD Pharmingen™- BD Biosciences- 550274) at 4°C overnight. Specimens were then stained at room temperature for 1 hour with Cy3 goat anti-rat IgG (H+L) secondary antibody (1:200; Interchim SA laboratory), and mounted using 50µl of mounting medium with DAPI. Staining was visualized under a fluorescent microscope.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from either mouse ears, lungs, paws or colon with Trizol reagent (Invitrogen) according to a standard protocol. RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics) and the SYBR Green kit (Roche), according to

manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analysis of each set of gene expression, an arbitrary value of 1 was given to the samples exhibiting the highest level, and the remaining samples were plotted relative to this value. PCR primers are available upon request.

Analysis of BAL cells.

Mouse lungs were lavaged with saline containing 1 mg/mL EDTA. The lavage fluid was centrifuged, and BAL cells were counted with a hemocytometer (Neubauer's chamber; VWR). Differential cell counts were determined using cytospin preparations stained with Microscopy Hemacolor (Merck).

Determination of airway responsiveness

Airway responsiveness was invasively determined using a computer-controlled small animal ventilator (FlexVent® system, SCIREQ Technologies). Mice were anesthetized with xylazine (15 mg/kg, i.p.), followed 15 minutes later by an i.p. injection of pentobarbital sodium (54 mg/kg). An 18-gauge metal needle was then inserted into the trachea and each mouse was connected to the FlexVent® ventilator, and quasi-sinusoidally ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H_2O in order to achieve a mean lung volume close to spontaneous breathing. After baseline measurement, mice were challenged for 10 seconds with a saline aerosol and, at 4.5-minute intervals, with 50 mg/mL methacholine. Airway resistance and elastance were expressed as $cmH_20.s/mL$ and cmH_2O/mL respectively.

Table S1: Primers used for Q-RT PCR analyses

mHPRT F	5’ GTTGGATACAGGCCAGACTTTGTTG
mHPRT R	5’ GATTCAACTTGCGCTCATCTTAGGC
mCCL4 F	5’ GCCCTCTCTCTCCTCTTGCT
mCCL4 R	5’ GTCTGCCTCTTTTGGTCAGG
mMMP13 F	5’ GTGTGGAGTTATGATGATGT
mMMP13 R	5’ TGCGATTACTCCAGATACTG
mCox2 F	5’ TTCAAAAGAAGTGCTGGAAAAGGT
mCox2 R	5’ GATCATCTCTACCTGAGTGTCTTT
mIL1β F	5’ GCCCATCCTCTGTGACTCAT
mIL1β R	5’ AGGCCACAGGTATTTTGTCG
mIL6 F	5’ GAGGATACCACTCCCAACAGACC
mIL6 R	5’ AAGTGCATCATCGTTGTTCATACA
mIL4 F	5’ GGCATTTTGAACGAGGTCAC
mIL4 R	5’ AAATATGCGAAGCACCTTGG
mIL5 F	5’ AGCACAGTGGTGAAAGAGACCTT
mIL5 R	5’ TCCAATGCATAGCTGGTGATTT
mIL10 F	5’ TGCTATGCTGCCTGCTCTTA
mIL10 R	5’ TCATTTCCGATAAGGCTTGG
mIL13 F	5’ GGAGCTGAGCAACATCACACA
mIL13 R	5’ GGTCCTGTAGATGGCATTGCA
mTSLP F	5’ AGCTTGTCTCCTGAAAATCGAG
mTSLP R	5’ AGGTTTGATTCAGGCAGATGTT
mTNFα F	5’ CATCTTCTCAAAATTCGAGTGACAA
mTNFα R	5’ TGGGAGTAGACAAGGTACAACCC
mIL17a F	5’ CCAGGGAGAGCTTCATCTGT
mIL17a R	5’ ACGTGGAACGGTTGAGGTAG
mIL17c F	5’ CACAGGAGACAGCATGAAGG
mIL17c R	5’ GCATCCACGACACAAGCA
mIL17f F	5’ AAGAAGCAGCCATTGGAGAA
mIL17f R	5’ ACAGAAATGCCCTGGTTTTG
mIL22 F	5’ CCGAGGAGTCAGTGCTAAGG
mIL22 R	5’ GCTGATGTGACAGGAGCTGA
mEotaxin F	5’ ATGCACCCTGAAAGCCATAG
mEotaxin R	5’ ATTCCCTCAGAGCACGTCTT
mWNT2 F	5’ CCTGATGAACCTTCACAACAAC
mWNT2 R	5’ TCTTGTTTCAAGAAGCGCTTTAC
mWNT2B F	5’ GGGCCCTCATGAACTTACAC
mWNT2B R	5’ CCACTCACACCGTGACACTT
mWNT4 F	5’ ACTGGACTCCCTCCCTGTCT
mWNT4 R	5’ TGCCCTTGTCACTGCAAA
mREDD1 F	5’ TAGTGCCCACCTTTCAGTTG

mREDD1 R	5’ GTCAGGGACTGGCTGTAACC
mK5 F	5’ ACCTCCTCACCCCTCTGAAC
mK5 R	5’ CACTTGGTGTCCAGGACCTT
mTAT F	5’ TTGGGGACCCTACTGTGTTT
mTAT R	5’ GGAGCCTCAGGACAGTGGTA
mTNFRSF19 F	5’ CACACACATTTTTGGGAAGG
mTNFRSF19 R	5’ AAGAGAATGGCAGCGAAGAG

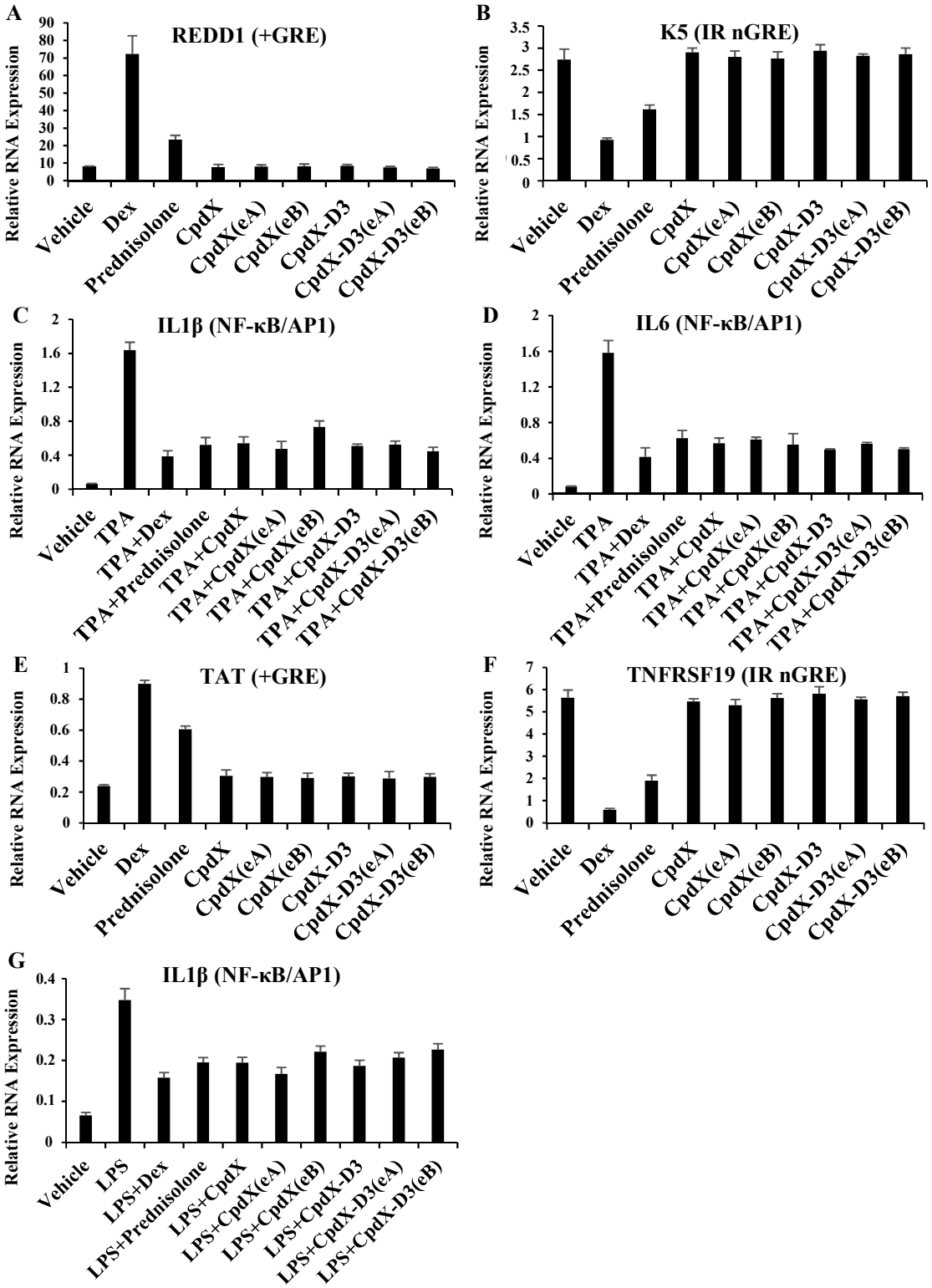

Fig. S1. CpdX, CpdX-D3, and their respective enantiomers selectively activate the indirect transrepression function of GR. Q-RT-PCR for transcripts of the (+)GRE-containing gene REDD1 (A), the IR nGRE-containing gene K5 (B), the NFκB/AP1-containing genes IL-1β (C) and IL-6 (D) in mouse ears treated as indicated (1 nmole/cm^2); the (+)GRE-containing gene TAT (E), the IR nGRE-containing gene TNFRSF19 (F), the NFκB/AP1-containing genes IL-1β (G) in livers from mice intraperitoneally injected with either Dex, prednisolone or CpdX compounds (1mg/kg of body weight) without (E and F) or with Lipopolysaccharide (LPS) (10 μg) (G) for 4 hours. All data are represented as mean ± SEM of at least three independent experiments with at least three mice per treatment.

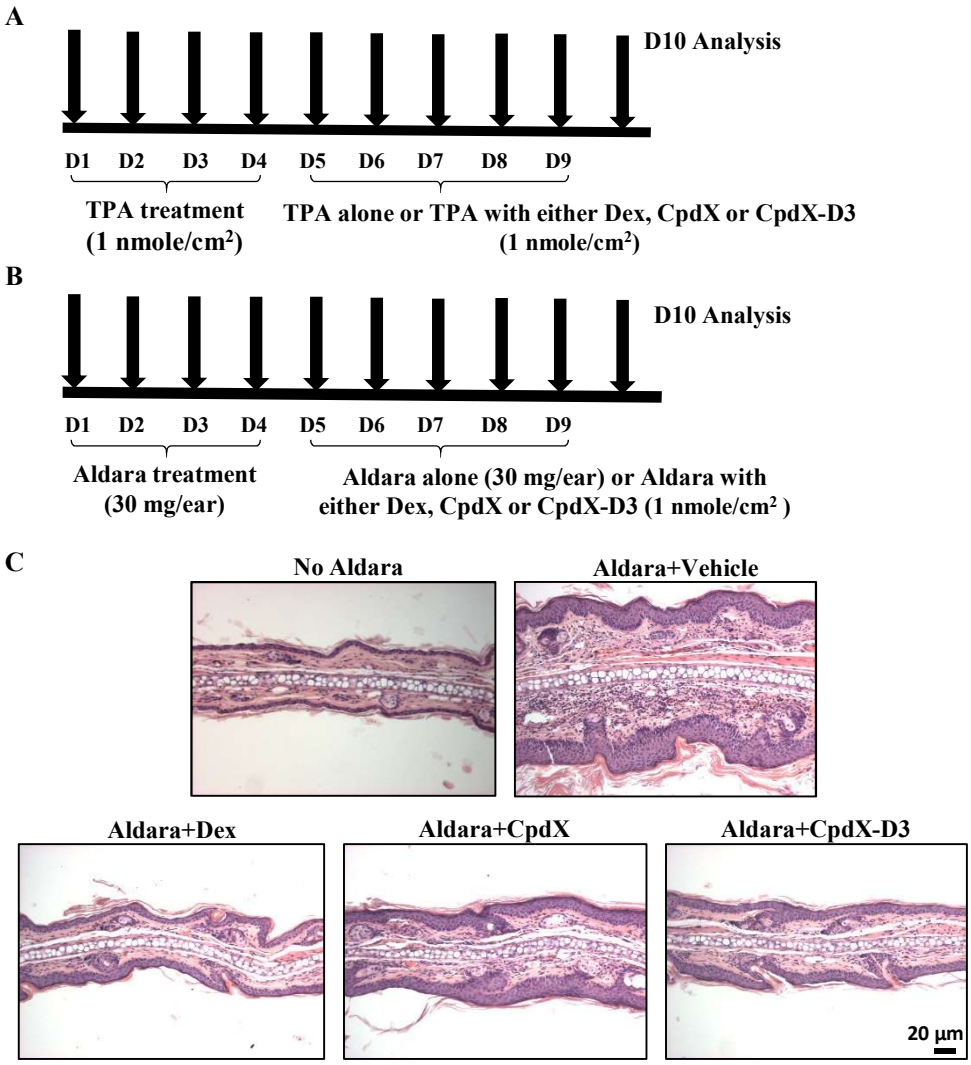

Fig. S2. CpdX and CpdX-D3 are as efficient as Dexamethasone (Dex) at decreasing skin inflammations. (A) Experimental protocol for a TPA-induced irritant contact dermatitis-like inflammation. (B) Experimental protocol for an Aldara-induced psoriasis-like inflammation. (C) Hematoxylin and eosin staining of an ear section from an Aldara-induced dermatitis. Scale bar represents 20 µm.

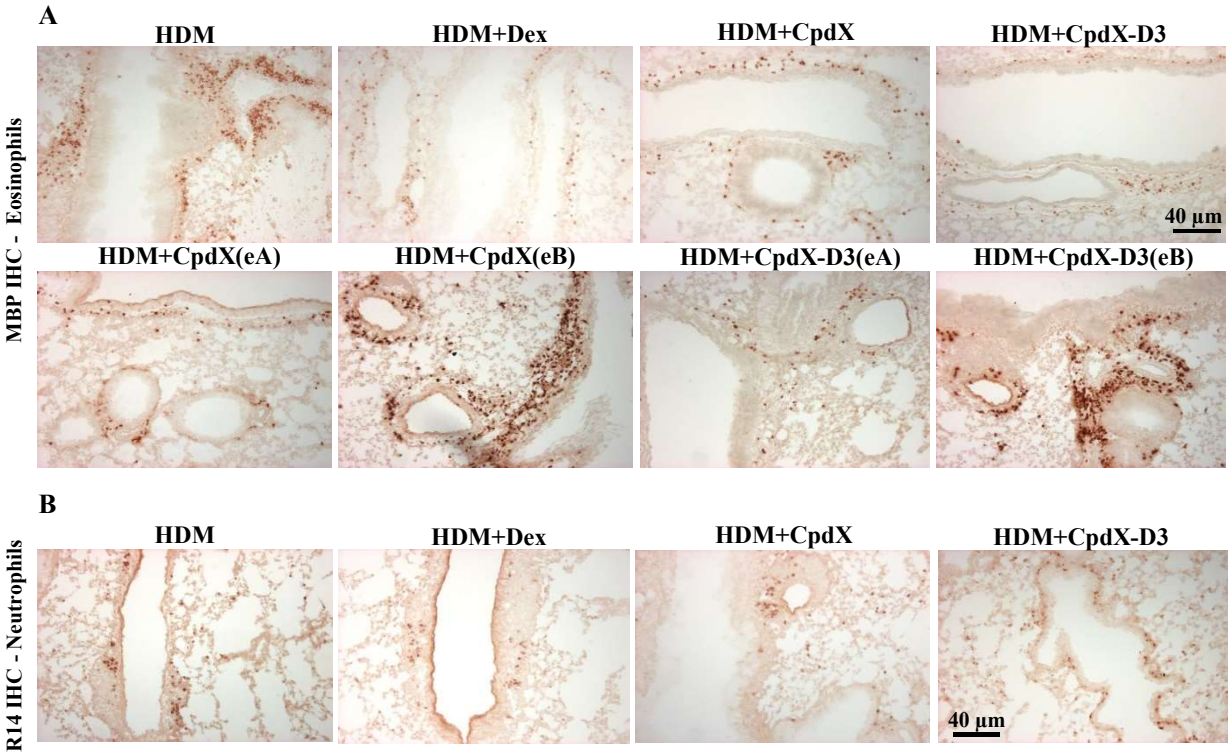

Fig. S3. CpdX, CpdX(eA), CpdX-D3 and CpdX-D3(eA), but not CpdX(eB) nor CpdX-D3(eB), are as efficient as Dexamethasone (Dex) at decreasing an HDM-induced asthma-like lung allergic inflammation. (A) Immunohistochemistry analyses of lung sections using the eosinophils specific antibody MBP. Scale bar represents 40 µm. (B) As in (A) but using the neutrophil specific antibody [NIMP-R14] (Abcam- ab2557).

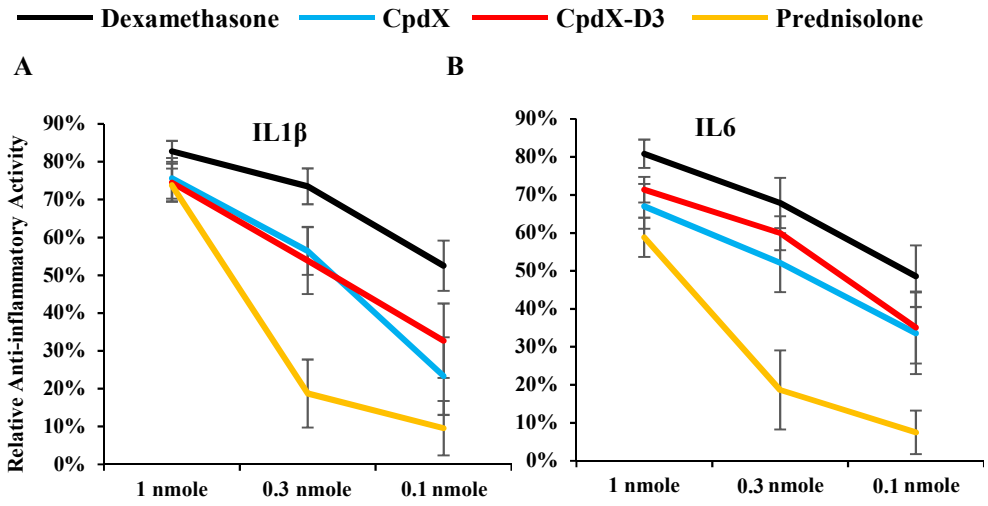

Fig. S4. Relative Anti-inflammatory Potency of Dexamethasone (Dex), CpdX, CpdX-D3 or Prednisolone. BALB/c mouse ears were topically treated overnight for 18 hours with 0.1 nmole/cm^2, 0.3 nmole/cm^2 or 1 nmole/cm^2 of either Dex, CpdX, CpdX-D3 or Prednisolone. The values correspond to the tethered repression of IL-1β (A) or IL-6 (B) induced by either Dex, CpdX, CpdX-D3 or Prednisolone. All data are represented as mean ± SEM of at least three independent experiments with at least three mice per treatment.