

RESEARCH PAPER

Inhibition of choriodecidual
cytokine production and
inflammatory gene
expression by selective I- κ B
kinase (IKK) inhibitorsD De Silva¹, MD Mitchell^{1,2} and JA Keelan^{1,3}¹Liggins Institute, University of Auckland, Auckland, New Zealand, ²National Research Centre for Growth and Development, University of Auckland, Auckland, New Zealand, and ³School of Women's and Infants' Health, University of Western Australia, WA, Australia

Correspondence

Jeffrey A Keelan, School of Women's and Infants' Health, University of Western Australia, King Edward Memorial Hospital, 374 Bagot Road, Subiaco, Perth WA 6008, Australia. E-mail: jeff.keelan@uwa.edu.au

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BACKGROUND AND PURPOSE

Inflammation of the extraplacental membranes plays a key role in the pathogenesis of preterm labour. The aim of this study was to screen a number of commercially available small molecule nuclear factor-kappa B inhibitors to identify candidates suitable for clinical evaluation as anti-inflammatory agents for the prevention of preterm birth.

EXPERIMENTAL APPROACH

Nine inhibitors were evaluated across a range of concentrations for their ability to inhibit lipopolysaccharide (LPS)-stimulated cytokine production in primary term choriodecidual cells in culture without affecting cell viability. Expression of 112 inflammation- and apoptosis-related genes was evaluated using boutique oligonucleotide arrays.

KEY RESULTS

Two IKK β inhibitors were found to be highly effective and non-toxic inhibitors of choriodecidual cytokine production: parthenolide and [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1). Both compounds also inhibited LPS-stimulated nuclear translocation of p65/RelA. Expression of 38 genes on the arrays (34%) was significantly ($P < 0.05$) inhibited by TPCA-1 or parthenolide. Of the 14 genes significantly stimulated by LPS, all were inhibited by TPCA-1 and 12 were inhibited by parthenolide. Overall, gene expression was more robustly inhibited by TPCA-1 than parthenolide; however, expression of two genes was only inhibited by parthenolide. Neither compound significantly altered the expression profile of anti-apoptosis genes on the arrays.

CONCLUSIONS AND IMPLICATIONS

These studies provide evidence that pharmacological inhibition of IKK β activity holds promise as a potential strategy for the prevention and/or treatment of inflammation-driven preterm birth. TPCA-1 appeared the most promising compound among those tested in this study. Different inhibitors may have subtly different effect profiles despite having similar modes of action.

Abbreviations

ABIN, A20-binding inhibitor of NF- κ B; AKT, ATP-dependent tyrosine kinase; BCA, bicinchoninic acid; BCL, B-cell CLL/lymphoma; BIRC, baculoviral IAP repeat-containing; CAPE, caffeic acid phenylmethyl ester; CARD, caspase recruitment domain; CARMA, caspase-recruitment domain (CARD) membrane-associated guanylate kinase protein 1; CBP, CREB-binding protein; CCL, chemokine (C-C motif) ligand; CSF, colony stimulating factor; CYLD, cylindromatosis; DAPI, 4',6-Diamidino-2-phenylindole; FADD, Fas-associated death domain; FCS, fetal calf serum; GAPDH, glyceraldehyde phosphate dehydrogenase; I- κ B, inhibitor of kappa B; IKK, inhibitor of kappa B kinase; IL, interleukin; IRAK, insulin receptor-activated kinase; IRS1, insulin receptor substrate 1; LPS, lipopolysaccharide; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; MAPK, mitogen-activated kinase; MDC, macrophage-derived chemokines; MEKK3, mitogen-activated protein kinase kinase kinase 3; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; NAC, N-acetylcysteine; NALP, NACHT, LRR and PYD containing protein; NEMO, NF- κ B essential modulator;

NF- κ B, nuclear factor-kappa B; PDGH, prostaglandin dehydrogenase; PDLIM, PDZ and Lim domain; PGLYRP, peptidoglycan recognition protein; PLA₂, phospholipase A₂; PTGS, prostaglandin endoperoxide synthase; PVDF, polyvinylidene fluoride; PYCARD, PYD and CARD domain containing; RBCK1, RanBP-type and C3HC4-type zinc finger containing 1; RIG, regulated in glioma; RIP, receptor interacting protein; SARM1, sterile alpha and TIR motif containing 1; SP-A, surfactant protein-A; SRC-3, steroid receptor co-activator-3; TAB, TAK1-binding protein; TAK, TGF- β -activated kinase; TBS-T, Tris-buffered saline/0.1% Tween 20; TICAM1, toll-like receptor adaptor molecule 1; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR, toll-like receptor; TNF, tumour necrosis factor; TNFR, TNF receptor; TPCA-1, [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide; TRAF, TNF receptor-associated factor; TRAM, TRIF-related adapter molecule; TSC1, tuberous sclerosis 1; VISA, virus-induced signalling adaptor

Introduction

Intrauterine inflammation plays an important role in the mechanism of parturition, both at term and preterm (Romero *et al.*, 2007a). Term labour is associated with increased infiltration of leukocytes into the cervix, uterus and fetal membranes and increased expression of inflammation-associated genes in these tissues is necessary for cervical ripening and uterine activation (Romero *et al.*, 2006). With spontaneous preterm labour, however, the inflammatory process is often exaggerated, and is manifested as chorioamnionitis and funisitis, accompanied by elevated concentrations of cytokines, chemokines, prostanoids and metalloproteases in amniotic and cervicovaginal fluids with preterm rupture of membranes (Keelan *et al.*, 2003; Romero *et al.*, 2007a). There is abundant evidence that pathological intrauterine inflammation resulting in preterm birth can be a consequence of intrauterine infection, particularly in early preterm births less than 32 weeks gestation (Romero *et al.*, 2007a; DiGiulio *et al.*, 2008). Fetal inflammatory response syndrome and a range of fetal morbidities are thought to be the results of fetal exposure to hyperinflammation *in utero* (Gotsch *et al.*, 2007; Murthy and Kennea, 2007). However, it is also apparent that in a sizeable proportion of preterm births complicated by intrauterine inflammation there is no clinical evidence of pathological intrauterine infection (Moutsopoulos and Madianos, 2006; Romero *et al.*, 2007a; Lee *et al.*, 2008). It has been hypothesized that activation of the innate immune system in the decidua and fetal membranes by bacterial products or other non-bacterial agonists might be the triggering agents in such cases (Elovitz, 2006; Lee *et al.*, 2008). In support of this, intra-amniotic administration of lipopolysaccharide (LPS), an agonist of the Toll-like receptor (TLR)4 receptor, or pro-inflammatory cytokines alone can trigger preterm labour and birth in primate models (Sadowsky *et al.*, 2006; Adams Waldorf *et al.*, 2008).

The nuclear factor-kappa B (NF- κ B) transcription factor is at the nexus of cellular inflammatory response (Lu *et al.*, 2008). In the absence of inflam-

matory stimuli, NF- κ B p50/p65 subunits are retained in an inactive complex in the cytoplasm by the chaperone-like protein I- κ B α . With exposure to pro-inflammatory stimuli, such as a TLR4 agonist or pro-inflammatory cytokines, phosphorylation of I- κ B occurs, leading to its degradation and subsequent release of p50/p65 homo- and hetero-dimers to translocate to the nucleus, driving inflammatory gene expression (Kawai and Akira, 2007). Phosphorylation of I- κ B is performed by the I- κ B kinase (IKK) complex, which consists of two catalytic subunits, IKK α , IKK β , and a regulatory subunit IKK γ (NEMO) (Schmid and Birbach, 2008). IKK β is 20-fold more active than IKK α in terms of I- κ B phosphorylation and is the primary kinase responsible for inflammatory NF- κ B activation *in vivo* (Kawai and Akira, 2007; Schmid and Birbach, 2008). For this reason small-molecular IKK β inhibitors are the focus of active investigation as potential anti-inflammatory therapeutics in a number of clinical settings (Calzado *et al.*, 2007; Strnad and Burke, 2007). However, NF- κ B activation also regulates expression of several pro-survival genes; hence, IKK inhibition can also provoke apoptosis, particularly in neoplastic tissue (Calzado *et al.*, 2007).

Several anti-inflammatory compounds have been evaluated in a variety of models for their capacity to block intrauterine inflammation. Using human choriodecidual membranes, *in vitro* studies have demonstrated efficacy of the non-selective anti-inflammatory/anti-oxidant N-acetylcysteine (NAC) (Lappas *et al.*, 2003; 2004). This drug has also been shown to be effective in rodent models (Beloosesky *et al.*, 2006; Paintlia *et al.*, 2008), and very recently it has been trialled in women at high risk of preterm birth with encouraging results (Shahin *et al.*, 2009). However, its unpalatability and lack of potency and selectivity are potential drawbacks to its administration in pregnancy. The salicylate drug sulphasalazine is another potential anti-inflammatory candidate that inhibits NF- κ B activation in intrauterine tissues (Lappas *et al.*, 2004, 2005); it has been administered to pregnant women for decades as a treatment for inflammatory bowel disease, and although there is evidence that it is safe in preg-

nancy (Norgard *et al.*, 2007; Rahimi *et al.*, 2008), a recent cohort study suggests a need for caution (Norgard *et al.*, 2007). We have recently evaluated sulphasalazine in an *ex vivo* membrane model and found that while it is an effective inhibitor of inflammation at high concentrations, it also induces apoptosis in the chorion (Keelan *et al.*, 2009), a potential concern for drugs acting via inhibition of NF- κ B signalling (Karin, 2008).

A large number of potent and selective small molecule NF- κ B inhibitors have been developed in recent years and tested in a variety of models (Pande and Ramos, 2005; Strnad and Burke, 2007). Although many are available commercially, few if any have been evaluated in placental tissues. We hypothesized that more selective inhibitors of NF- κ B activation may provide advantages for the prevention of inflammation-driven preterm birth, over historic so-called anti-inflammatory drugs, in terms of more favourable pharmacodynamics and fewer side effects. Such compounds, assuming they crossed the placenta, might also offer some protection against the deleterious effects of inflammation in the fetus and neonate (Gotsch *et al.*, 2007; Murthy and Kennea, 2007; Romero *et al.*, 2007b), and therefore could improve fetal outcomes independent of any effects on rates of preterm birth. Here we report the results of a screening study of a number of agents, shown to be effective inhibitors of NF- κ B activation in various animal and *in vitro* models, for their ability to inhibit choriodecidual inflammatory activation. Their effects on both inflammation and apoptosis in primary cultures of human choriodecidual cells were investigated with the intention of identifying potential agents suitable for subsequent evaluation in pre-clinical and clinical trials. We identified two IKK inhibitors that exhibited effective and non-toxic inhibition of inflammatory activation in choriodecidual cells, and concluded that IKK β is a promising therapeutic target for prevention of inflammation-driven preterm birth.

Methods

Cell culture and treatments

Primary choriodecidual (CD) cell cultures were prepared from placentas obtained by Caesarean section at term prior to the onset of labour according to previously published methods (Keelan and Mitchell, 1998). Tissues were collected with informed maternal consent in accordance with the approval of the local Human Ethics committee. In brief, the choriodecidia was manually separated from the reflected amnion, washed in phosphate buffered

saline (PBS), dissected and digested for 1 h at 37°C in 0.012% collagenase/0.25% dispase. DNase I (4 mg·L⁻¹) was added for the final 15 min of incubation. The liberated cells were isolated and fractionated on a 5–60% discontinuous Percoll gradient. Cells lying between the 60–20% layers were recovered by aspiration, washed and plated at 2.5×10^5 cells per cm² in M199 medium supplemented with 10% FCS, glutamax and antibiotic/antimycotics. Cells were cultured at 37°C in humidified 5% CO₂/95% air for 2–3 days prior to addition of treatments (Keelan and Mitchell, 1998). Cell composition was determined by staining with anti-vimentin and anti-cytokeratin 7 antibodies to determine the relative proportions of decidual and chorionic cells respectively (Figure S1); $20 \pm 10\%$ of the cells stained positive for the trophoblast marker cytokeratin 7.

Cytokine assays

Concentrations of IL-6 and TNF- α in conditioned media were determined by ELISA using commercially available capture and detection antibodies according to the manufacturer's instructions (PeproTech, NJ, USA). Plate reading and curve fitting was performed on a SpectraMax plate reader using SoftMax ProV software (Molecular Dynamics/GE Healthcare, Sunnyvale, CA, USA), or a Synergy 2 Multi Mode Microplate reader using Gene 5 software (BioTek Instruments Inc., VT, USA). Cytokine production rates were normalized to total cellular protein, as determined by the BCA method calibrated against BSA (Redinbaugh and Turley, 1986).

Assessment of cell viability

The viability of cells plated at 5×10^4 cells per well of a 96-well plate was assessed by MTT assay (Denizot and Lang, 1986). After 24 h treatment with LPS or inhibitors, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to a final concentration of 0.5 mg·mL⁻¹ and the cells incubated for 2–4 h. The formazan dye generated was dissolved in acidified isopropanol and absorbance at 570 nm was measured using a Spectramax plate spectrophotometer (Molecular Dynamics/GE Healthcare).

Cell lysis and nuclear extraction

Cells were homogenized in lysis buffer (10 mM HEPES pH 7.4, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 1% NP-40 and protease inhibitors). Lysates were centrifuged for 1 min at 3800× g at 4°C and the supernatant containing cytoplasmic proteins removed. The pellet was resuspended in 200 μ L high-salt resuspension buffer (10 mM HEPES pH 7.4, 400 mM NaCl, 10 mM KCL,

0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 1% NP-40, protease inhibitors), incubated on ice for 15 min, then centrifuged for 5 min at 13 200 rpm at 4°C. Protein concentrations of the resulting supernatants containing nuclear proteins were then determined by a RC-DC protein assay, calibrated against BSA.

Immunoblot analysis

Nuclear and cytoplasmic protein extracts were subjected to SDS polyacrylamide gel electrophoresis on a 4–15% gradient gel under reducing conditions (NuPage System, Invitrogen/Life Technologies, Carlsbad, CA, USA) and transferred onto PVDF membranes. Membranes were blocked for 1 h in 1–5% non-fat dried milk in TBS-T (10 mM Tris, 150 mM NaCl and 0.1% Tween-20) and incubated overnight at 4°C with either anti-p65 (1:1000), anti-p50 (1:1000), anti-pyruvate kinase (1:1000), anti-histone H1 (1:5000) or anti- β -actin (1:20 000) antibodies. After washing, the membranes were incubated for 1–2 h with HRP-conjugated secondary antibody (1:2500), washed, and immunoreactive bands visualized by chemiluminescence using Supersignal West Dura ECL reagent followed by exposure to X-ray film (Agfa-Gevaert NV, Mortsels, Belgium).

Immunofluorescence staining

Choriodecidual cells were cultured in four-well chamber slides and stimulated with LPS with or without inhibitors for 2 h. Cells were then fixed for 20 min with 4% paraformaldehyde in 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 10 mM PIPES pH 6.8, then 1 min exposure to acetone at –20°C followed by PBS washes. Cells were next pre-incubated with 4% BSA in PBS for 1 h, then incubated with anti-p65 antibody (1:100) for 1 h, washed and incubated with FITC-labelled anti-rabbit IgG (1:500) for 1 h. Cells were then washed again and mounted using DAPI-containing aqueous mounting medium. Visualization was carried out using a TH4-200 microscope equipped with a DP70 digital camera and DPManger software (Olympus Corp., Tokyo, Japan).

RNA extraction and quantitative RT-PCR

Pilot experiments showed that TNF- α mRNA expression peaked at 4 h post-LPS. Therefore, total RNA was extracted from cells ($n = 6$ placentas) after 4 h of treatment with either vehicle control, LPS (0.1 $\mu g \cdot mL^{-1}$), LPS plus [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1) (7 μM) or LPS plus parthenolide (40 μM) using PureLink™ Micro-to-Midi Total RNA Purification kits (Invitrogen/Life Technologies). RNA yield and quality was deter-

mined by measuring 260:280 nm ratios using a Nanodrop 1000 (Nanodrop Technologies, DE, USA). First-strand cDNA synthesis was performed using a Superscript III reverse transcription kit with random hexamer priming, followed by treatment with DNase-I to remove genomic DNA contamination. All RNA samples were initially screened for expression of two cytokine markers, IL-6 and TNF- α , by qRT-PCR using an ABI 7700 Sequence Detector (Applied Biosystems/Life Technologies, CA, USA) using SybrGreen detection. Relative expression was computed based on the $2[-\Delta\Delta Ct]$ method, relative to the housekeeping gene GAPDH. Following array analysis, another six genes were selected for qRT-PCR confirmation of changes in expression (PTGS1/COX-2, NOD2, CCL4/MIP-1 β , MMP-2, BIRC5/survivin). PCR primers (Table 1) were designed using OligoPerfect™ Designer (Invitrogen/Life Technologies).

Oligonucleotide array processing and analysis

Custom boutique oligonucleotide arrays ('superarrays') were fabricated by SABiosciences Corp. (Frederick, MD, USA). Each membrane contained 112 genes (4 spots per gene) related either to apoptosis, NF- κB signalling or inflammation. Five housekeeping genes, including GAPDH, β -actin and β_2 -macroglobulin, were included on the arrays. RPS27A (ribosomal protein 27a) and BAS2C (biotinylated artificial sequence 2 complementary) served as positive detection controls; negative controls and blanks were also included. The full list of genes is detailed in Table S1.

From $n = 6$ independent experiments, three representative sets of RNA samples were selected for array analysis based on (i) RNA quality; (ii) RNA yield; and (iii) representative response to LPS, as determined by increased expression of IL-6 and TNF- α . Synthesis of cDNA, cRNA and labelling of probes from 0.5 μg sample RNA was carried out using a TrueLabeling-AMP™ 2.0 kit. Amplification, hybridization and signal development were performed according to the manufacturer's instructions; 4 μg of cRNA was used for each hybridization. Signal from the arrays was determined by chemiluminescence, visualized by exposure to X-ray film using a range of exposure times (1–15 min) to obtain optimal images.

X-ray films were scanned and analysed by densitometry using ImageQuant software (GE Healthcare, Uppsala, Sweden), by employing the array analysis option and 'spot surface minimum' background subtraction. Residual blanks were then subtracted and spot intensities were normalized to GAPDH. Prior to statistical analysis of pooled data, to correct for experiment-to-experiment variations in overall

Table 1

PCR primer sequences

Locus ID gene bank	Gene name	Primer name	Sequence (5' to 3')	Amplicon size (bp)
NM_003998	NF- κ B1/p50	Forward	CTG GAA GCA CGA ATG ACA GA	172
		Reverse	TGA GGT CCA TCT CCT TGG TC	
NM_002984	CCL4/MIP-1 β	Forward	GTA GCT GCC TTC TGC TCT CC	196
		Reverse	TCA CTG GGA TCA GCA CAG AC	
NM_022162	NOD2	Forward	TCT GTC CAG ACC CTG CTC IT	148
		Reverse	CCT GTT CAG AGA AGC CCT TG	
NM_000962	PTGS1/COX-2	Forward	TGA GCA TCT ACG GTT TGC TG	158
		Reverse	TGC TTG TCT GGA ACAACT GC	
NM_004530	MMP2	Forward	AGG GCA CAT CCT ATG ACA GC	186
		Reverse	ATT TGT TGC CCA GGA AAG TG	
NM_001168	BIRC5/Survivin	Forward	AAA TGA CTT GGC TCG ATG CT	219
		Reverse	CAC CCT GCA GCT CTA TGA CA	
NM_000600	IL-6	Forward	ATG ACT CCT TCT CCA CAA GCG	287
		Reverse	AGC CAT CTT TGG AAG GTT CAG G	
NM_000594	TNF- α	Forward	TGC TTG TTC CTC AGC CTC TTC T	276
		Reverse	TAT CTC TCA GCT CCA CGC CAT T	
NM_002046	GAPDH	Forward	GAG TCA ACG GAT TTG GTC GT	185
		Reverse	GAC AAG CTT CCC GTT CTC AG	

intensity, the overall mean signals of each set of arrays was adjusted so that the overall signal of the three control arrays were equivalent. Mean and standard deviation for each gene from the $n = 3$ experiments was then calculated and these data used for subsequent analysis.

Materials and reagents

Nuclear factor-kappa B inhibitors were purchased from Calbiochem/Merck KGaA, Darmstadt, Germany. Lipopolysaccharide (*Escherichia coli* LPS, serotype 055:B5), bichinchonic acid (BCA) reagent and anti- β -actin antibody were from Sigma-Aldrich, St Louis, MO, USA. IL-6 and TNF- α ELISA development reagents were obtained from PeproTech, NJ, USA. M199 medium, fetal calf serum (FCS), glutamax, DNase 1, antibiotic/antimycotic solution, MTT reagent, PCR primers, PureLink™ Micro-to-Midi RNA purification kits, Superscript III kits, SybrGreen Mastermix and NuPage™ precast polyacrylamide gels were supplied by Invitrogen/Life Technologies (Carlsbad, CA, USA). Custom OligoGE Array™ membranes and TrueLabelling-AMP™ 2.0 kits were purchased from SABioscience Corporation, Frederick, MD, USA. Anti-p65/RelA antibody was from Santa Cruz Biotechnology Inc. (CA, USA); anti-histone H1, anti-pyruvate kinase and were purchased from Abcam, Cambridge, MA, USA.

SuperSignal West Dura extended Duration Substrate and goat anti-rabbit IgG-peroxidase conjugate were purchased from Pierce/Thermo Scientific, Rockford IL, USA. Complete protease inhibitor tablets, biotin-16-dUTP and dispase II were purchased from Roche Diagnostics, Mannheim, Germany. Percoll and PVDF transfer membranes were supplied by GE Healthcare Bio-Sciences AB, Uppsala, Sweden. RC-DC protein assay reagents were obtained from Bio-Rad Laboratories Pty Ltd, NSW, Australia. Vectashield™ mounting medium was supplied by Vector Laboratories, Burlingame, CA, USA.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 or InStat 3.0 (GraphPad Software, La Jolla, CA, USA). Significant differences between group means were determined by one-way ANOVA with *post-hoc* testing. $P \leq 0.05$ was considered significant. Data are represented as mean \pm SD.

Results

Screening of NF- κ B inhibitors

Primary choriodecidual cells were initially stimulated over an 18 h incubation period with 100 ng·mL⁻¹ LPS together with one of nine different

NF- κ B inhibitors or vehicle control. The inhibitors tested were selected based on their published anti-inflammatory efficacy from *in vitro* and *in vivo* models, relative specificity, and mode of action. Concentrations for testing were estimated from published IC_{50} data and were selected to achieve approximately 75% maximal effect: SN50, an inhibitor of NF- κ B translocation (36 μ M); SC514 (5-(thien-3-yl)-3-aminothiophene-2-carboxamide), a selective and competitive IKK β inhibitor (5 μ M); BMS 345541 (4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline), a selective allosteric IKK β inhibitor (5 μ M); evodiamine (21-methyl-3,13,21-triazapentacyclohenicosa-2(10),4,6,8,15,17,19-heptaen-14-one), an IKK inhibitor (1 μ M); wedelolactone (7-methoxy-5,11,12-trihydroxycoumestan), a non-selective IKK inhibitor (50 μ M); butein (3,4,2',4'-tetrahydroxychalcone), a partially selective IKK β inhibitor (50 μ M); CAPE (caffeic acid phenylmethyl ester), an inhibitor of NF- κ B translocation (35 μ M); parthenolide, an inhibitor of the IKK complex (40 μ M), and TPCA-1, a selective IKK β inhibitor (5–7 μ M).

Lipopolysaccharide induced a significant increase in IL-6 and TNF- α production with no decrease in cell viability (Figure 1). Out of nine inhibitors tested, only evodiamine and SN-50 failed to exert any inhibitory effects on LPS-stimulated cytokine production at the concentrations tested. SC514, wedelolactone, BMS 345541, CAPE and butein exerted modest inhibitory effects on the two cytokines measured (Figure 1A and B). TPCA-1 and parthenolide were the most effective, significantly inhibiting ($P < 0.01$) both IL-6 and TNF- α production by greater than 50%. None of the compounds tested showed evidence of toxicity as judged by MTT assay (Figure 1C).

TPCA-1 and parthenolide both inhibited LPS-induced IL-6 and TNF- α production in CD cells in a concentration-dependent manner (Figure 2). IC_{50} values for inhibition of IL-6 production by TPCA-1 and parthenolide were 7.05 μ M and 3.82 μ M respectively. However, TPCA-1 was a more potent inhibitor of TNF- α production, with an IC_{50} value 2.35 μ M compared with 4.16 μ M for parthenolide. No effects on cell viability were detected at any concentration.

Time course studies (4–24 h) of the effect of TPCA-1 (7 μ M) and parthenolide (40 μ M) on LPS-induced IL-6 production revealed that the inhibitory effects of both compounds increased with duration of exposure (Figure 3); no significant effects were observed at 4 h, but by 16 h inhibition had reached maximal levels. We also investigated the effects of time of addition of inhibitors (–1 h, 0 h, +1 h relative to LPS addition) but this did not

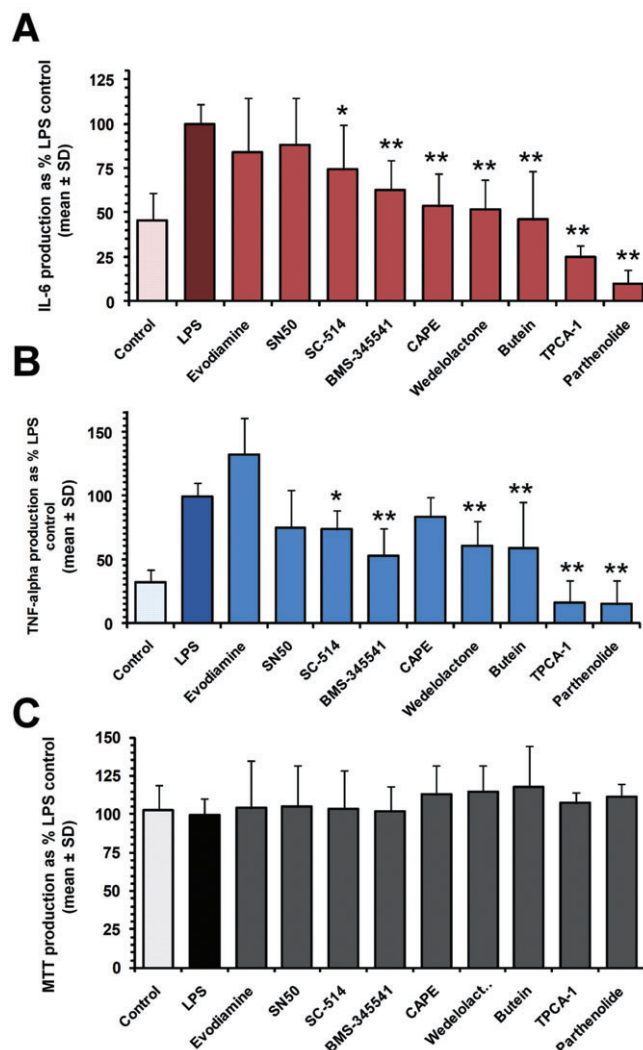


Figure 1

Initial evaluation of anti-inflammatory properties of nine commercially available NF- κ B inhibitors in primary human choriodecidual cells stimulated with LPS (0.1 μ g·mL⁻¹) for 18 h. Candidate inhibitors were tested for their ability to inhibit production of IL-6 (A) and TNF- α (B) as assessed by ELISA. Cytokine data were normalized to cellular protein and expressed relative to LPS control. Cell viability was also determined by MTT assay (C), normalized to vehicle control. Inhibitor concentrations were as follows: SN50 (36 μ M); SC514 (5 μ M); BMS 345541 (5 μ M); evodiamine (1 μ M); wedelolactone (50 μ M); butein (50 μ M); caffeic acid phenylmethyl ester [CAPE] (35 μ M); TPCA-1 (7 μ M); parthenolide (40 μ M). Data represent mean \pm SD of $n = 3$ –4 experiments performed in triplicate. * $P < 0.05$, ** < 0.01 versus LPS alone by ANOVA with Dunnett's test *post hoc*.

significantly influence the efficacy of either inhibitor (data not shown).

Effects of TPCA-1 and parthenolide on NF- κ B activation by LPS

To confirm the mode of action of the two NF- κ B inhibitors, we first investigated by immunofluores-

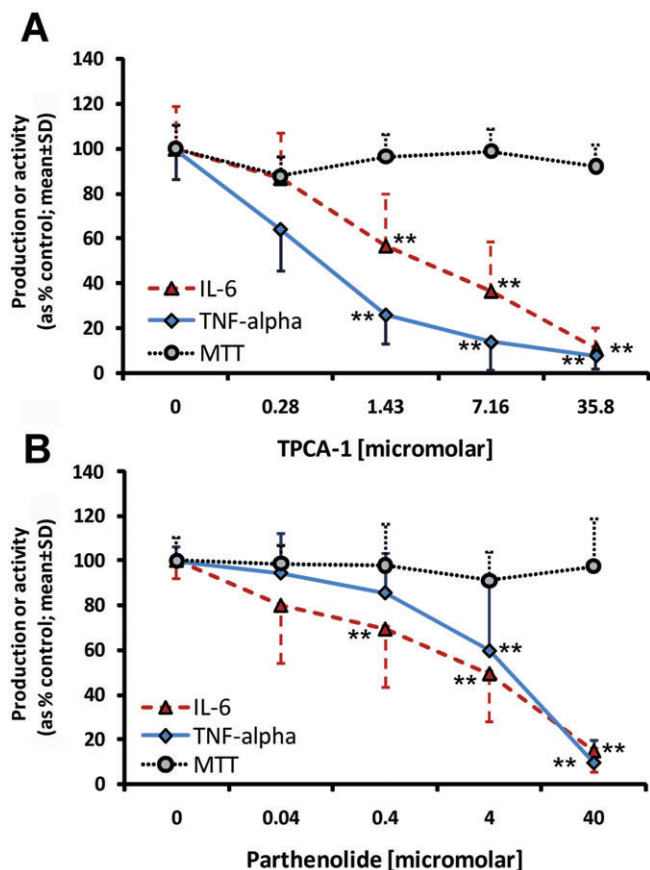


Figure 2

Concentration-dependent effects of TPCA-1 and parthenolide on LPS-stimulated cytokine production by choriodecidual cells. Cells were treated with LPS ($0.1 \text{ mg} \cdot \text{mL}^{-1}$) for 18 h together with inhibitor or vehicle for 18 h; production of IL-6 and TNF- α was normalized to cellular protein and expressed as percentage of vehicle control. MTT activity was measured to monitor effects on cell viability. Data represent mean \pm SD of $n = 3$ –4 experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$ versus vehicle control by ANOVA with Dunnett's test *post hoc*.

cence changes in the cellular localization of p65/RelA subunit in response to LPS (Figure 4). Absence of cross-contamination of the cytosolic and nuclear fractions was confirmed using immunoblotting for pyruvate kinase (cytosolic marker) and histone H1 (nuclear marker). In control cells, p65 was mainly localized in the cytoplasm. At 2 h, addition of LPS triggered translocation of p65 into the nucleus. In the presence of inhibitors, nuclear staining was partially blocked by parthenolide and completely abrogated by TPCA-1. The effects of the inhibitors on nuclear translocation of p65 were also investigated by immunoblotting of nuclear protein extracts prepared six after treatment (Figure 5). A single band that migrated at 65 kDa was detected in the nuclear protein fraction of untreated cells and its intensity

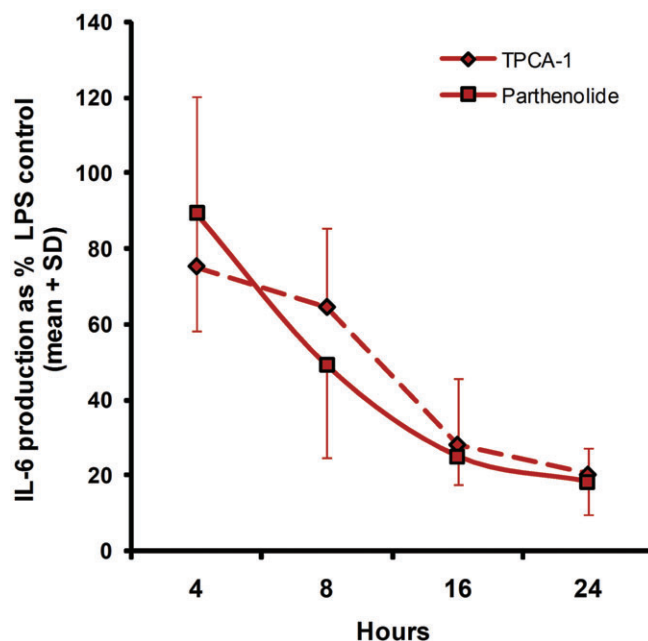


Figure 3

Time-course study of the effects of TPCA-1 ($7 \text{ } \mu\text{M}$) and parthenolide ($40 \text{ } \mu\text{M}$) on LPS-stimulated IL-6 production. The data are the mean \pm SD of $n = 3$ experiments performed in triplicate.

was increased markedly with LPS treatment. In cells co-treated with TPCA-1 and parthenolide the intensity of the p65 band was diminished to below control levels.

Array analysis of the effects of TPCA-1 and parthenolide on NF- κ B-associated gene expression

In order to more fully assess the effects of TPCA-1 and parthenolide on LPS-stimulated choriodecidual gene expression profiles, boutique pathway-specific arrays were designed and probed with biotin-dUTP-labelled RNA from three representative sets of experiments. A 4 h time-point was chosen, based on preliminary studies showing that the TNF- α mRNA response to LPS was maximal at this time. An example of a set of images is shown in Figure S2. All positive controls and housekeeping genes gave strong signals, while blanks and negative controls were devoid of spots. Analysis of the background-corrected and normalized mean signal from the arrays showed that 38 genes (34%) were consistently stimulated by LPS in all three experiments (mean stimulation approximately threefold), of which 14 were statistically significant ($P < 0.05$, ANOVA) (Figure 6). The hybridization signals for housekeeping genes and positive controls were not significantly altered by LPS treatment. Genes induced by

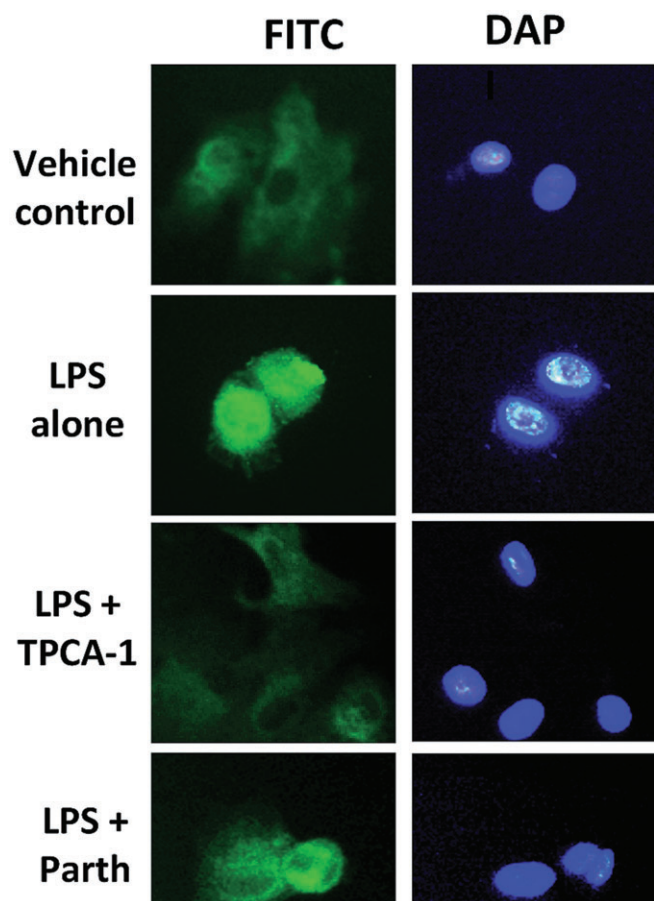


Figure 4

Immunofluorescence localization of p65/RelA cellular localization using FITC-labelled detection antibody in choriodecidual cells stimulated with LPS ($0.1 \mu\text{g}\cdot\text{mL}^{-1}$) for 2 h in the presence or absence of TPCA-1 ($7 \mu\text{M}$) and parthenolide ($40 \mu\text{M}$). Nuclei were visualized by DAPI staining (right-hand panel). LPS induced marked nuclear p65 staining, which was inhibited by both IKK inhibitors. The results are representative of $n = 3$ experiments.

LPS included inflammatory signalling proteins (A20, p65/RelA, I- κ B α , IRAK2/4, NOD1/2, TAB1-3, TAK1, TLR4, TRAF2), cytokines/chemokines (CSF2, IL-1 β , IL-6, TNF- α , MIP-1 α , MIP-1 β), apoptosis-related genes (bcl2, BIRC5/survivin, PYCARD, caspase-8) and parturition-related inflammatory genes (SP-A1/SFTPA1, PTGES, PLA₂ IV4B, PLA₂VI, MMP-2, MMP-13). On the other hand, a number of genes that were expected to be stimulated by LPS were unresponsive. These included PTGS-2/COX-2, CCL2/MCP-1, CCL22/MDC and IL-8, although it is worth noting that robust stimulation of these genes was observed on two of the three arrays. No genes on the arrays were significantly inhibited by LPS, although RIP-2 and TRADD were modestly inhibited in all three array experiments.

The two IKK inhibitors exhibited effective and widespread inhibition of LPS-responsive and non-

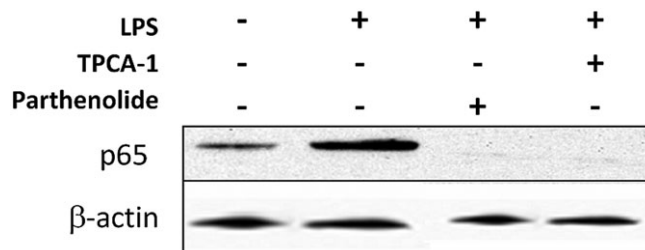


Figure 5

Immunoblotting analysis of nuclear translocation of p65/RelA in LPS-stimulated choriodecidual cells treated with IKK inhibitors. Cells were treated with LPS and either TPCA-1 ($7 \mu\text{M}$) or parthenolide ($40 \mu\text{M}$) for 6 h, then lysed and nuclear proteins extracted and immunoblotted; p65 was detected by chemiluminescence, visualized after exposure to X-ray film. Equal amounts of protein were loaded on each lane. A representative experiment of $n = 4$ is shown.

responsive genes. 38 genes were significantly down-regulated by one or more of the inhibitors (Table 2). Of these, 36 were inhibited by TPCA-1 (mean inhibition: $60 \pm 17.5\%$), while parthenolide inhibited 20 (mean inhibition: $45.1 \pm 20.1\%$). Of the 14 genes that were significantly stimulated by LPS (displayed in Figure 6), all were significantly down-regulated by either or both IKK inhibitors. Both TPCA-1 and parthenolide significantly reduced the expression of A20, BIRC5/survivin, I- κ B α , MAPK3, MIP-1 α , MIP-1 β , MMP-2, MMP-13, p50, PLA₂G6, TAB2, TAB3, TIRAP/Mal, TNF- α and TRAF2 (Table 2). In addition, a number of genes that were not significantly up-regulated by LPS were also inhibited by TPCA-1 or parthenolide: AKT1, AP1, CARD11, COX-2, I- κ B β , MAPK3, MCP-1, MIP-1 δ , MYD88, p50, RelB, SARM1, TICAM1, TIRAP/Mal, TNFR1 and TRAM1. While TPCA-1 inhibited the greatest number of genes, two genes were significantly inhibited by parthenolide alone: TAK1 and TRAM1. Notable genes that were not inhibited by either inhibitor included bcl2, bcl10, BIRC4/XIAP, caspase-8, FADD, IL-10, IKK α , IKK β , IKK γ , MALT1, PYCARD, RIP1, TAB1 and TLR4.

To validate the array findings, we used qRT-PCR to examine expression of a selection of high- and low-expression genes with a range of responsiveness to LPS: IL-6, TNF- α , MMP2, p50, BIRC5/survivin, PTGS2/COX-2 and NOD2 (Figure 7). In general, the qRT-PCR results corroborated the array results in terms of response to LPS and IKK inhibition. The effects of parthenolide, however, were much less evident in the qRT-PCR data. Unexpectedly, MMP2 expression showed no response to LPS by RT-PCR, in contrast to the twofold increase demonstrated in the array data.

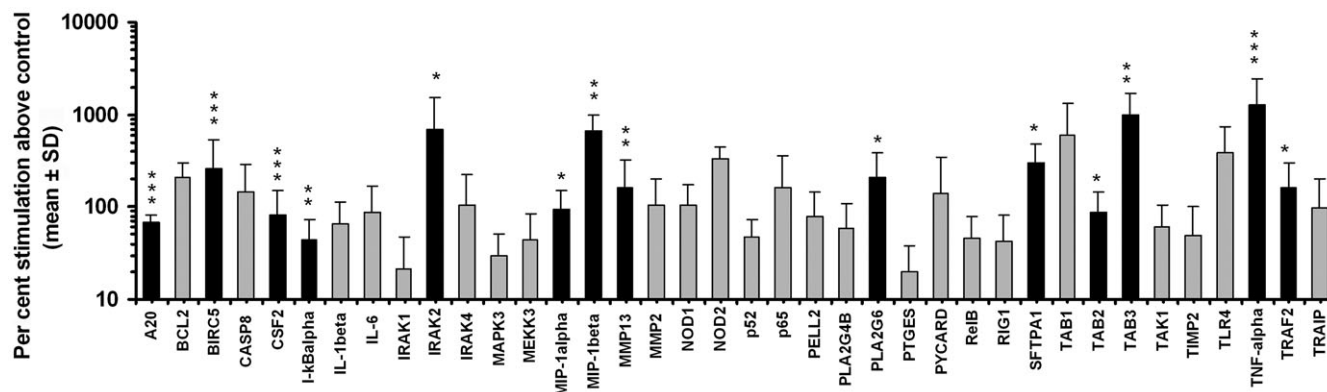


Figure 6

Identification of LPS-responsive genes in choriodecidual cells stimulated for 4 h with LPS ($0.1 \mu\text{g}\cdot\text{mL}^{-1}$) using custom oligonucleotide arrays. Expression is expressed as percentage of vehicle control after normalization to GAPDH following background subtraction. Data shown (mean \pm SD) represent those genes consistently stimulated in all three experiments; the solid black columns are significantly different from LPS control. Significance was determined by ANOVA with Tukey's test *post hoc*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control. Note log y-axis.

Discussion

The primary objective of this study was to screen a range of NF- κ B inhibitors for their anti-inflammatory effects in human primary choriodecidual cells, in order to identify candidate agents suitable for further evaluation for the prevention and treatment of inflammation-driven preterm birth. Additional objectives were to identify favourable molecular pathways or targets amenable to pharmacological manipulation, and assess the differences in the effects of different inhibitors on expression of inflammatory/apoptosis-related genes. From a total of nine potential candidate compounds tested, we identified two IKK inhibitors that effectively inhibited the production of inflammatory cytokines and the expression of genes associated with inflammatory signalling and activation without reducing cell viability: TPCA-1 and parthenolide. We conclude that IKK β inhibition holds promise as an effective pharmacological approach for the treatment of intra-uterine inflammation-driven preterm birth.

It is now well established that IKK activation is a convergence point in inflammatory signalling via the NF- κ B pathway (Schmid and Birbach, 2008) and, as such, has been of interest for over two decades because of its attraction as a therapeutic target for the treatment of chronic inflammatory disorders, such as inflammatory bowel disease, ulcerative colitis and arthritis (Strnad and Burke, 2007; Schmid and Birbach, 2008). The IKK complex contains two related kinases, IKK α and IKK β , which, despite their homology, are independently regulated and functionally distinct (Schmid and Birbach, 2008). IKK α plays an important role in the non-canonical (p52/

RelB) NF- κ B pathway which is central to keratinocyte differentiation (Descargues *et al.*, 2008), receptor-induced apoptosis and the negative regulation of NF- κ B activation (Strnad and Burke, 2007). It can translocate to the cell nucleus and phosphorylate nuclear proteins including histone H3, SRC-3 and CBP (Chariot, 2009). On the other hand, IKK β activation is key to the canonical (inflammatory) pathway and regulates the expression of many inflammation-associated genes. IKK β is approximately 20-fold more efficient than IKK α at phosphorylating Ik-B α , and knockout studies in mice have shown that IKK β , not IKK α , is essential for the activation of NF- κ B by TNF- α , IL-1 β or LPS (Li *et al.*, 1999). IKK β also phosphorylates other targets in addition to Ik-B α ; these include NF- κ B signalling proteins such as p65, p105, Bcl10, CARMA1, CYLD and NEMO, as well as other important cell regulators such as β -catenin, IRS-1, FOXO3a and TSC1 (Chariot, 2009). Therefore, selective inhibition of IKK activity could theoretically influence a wide variety of cellular processes in addition to those mediated through NF- κ B activation.

We identified two IKK inhibitors, TPCA-1 and parthenolide, as effective and non-toxic anti-inflammatory agents. This was not unexpected, although it is interesting that several other IKK inhibitors were tested [SC514 (Kishore *et al.*, 2003), BMS 345541 (Burke *et al.*, 2003), evodiamine (Takada *et al.*, 2005), wedelolactone (Kobori *et al.*, 2004), butein (Pandey *et al.*, 2007)] but none of these exerted more than a modest inhibition of IL-6 and TNF- α production. The widely utilized cell permeable peptide SN-50, which acts via blockade of NF- κ B translocation (Lin *et al.*, 1995), was also without effect. While we evaluated these com-

Table 2

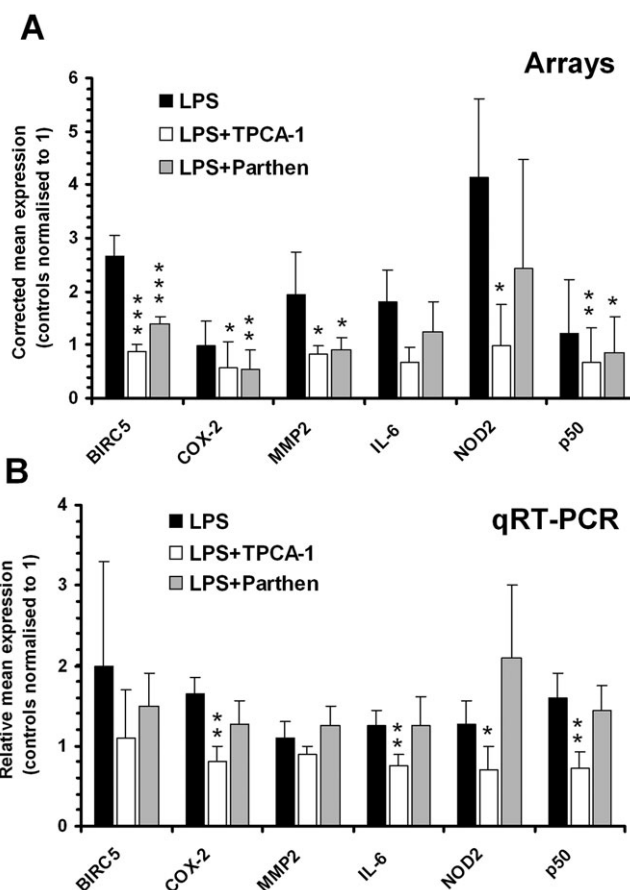
Inhibition of choriodecidual gene expression by TPCA-1 or parthenolide

Gene name	TPCA-1 (7 μ M) % change	Parthenolide (40 μ M) % change
A20	-85.30 \pm 9.0***	-26.3 \pm 6.9**
AKT1	-43.40 \pm 32.8*	-37.0 \pm 35.9 (NS)
AP-1	-55.7 \pm 37.6*	-19.5 \pm 35.9 (NS)
BCL3	-64.6 \pm 2.2*	18.2 \pm 35.9 (NS)
BIRC5/survivin	-66.1 \pm 8.0***	-46.7 \pm 6.9***
CARD11	-62.3 \pm 32.4*	-24.9 \pm 39.0 (NS)
COX-2/PTGS2	-45.9 \pm 28.9*	-40.9 \pm 36.7**
CSF2	-52.6 \pm 36.9*	-33.6 \pm 7.3 (NS)
I- κ B α	-89.6 \pm 2.25***	-54.7 \pm 10.6***
I- κ B β	-53.5 \pm 7.1**	-43.5 \pm 9.2 (NS)
MAPK3	-42.5 \pm 17.0*	-42.3 \pm 10.8*
MCP-1	-72.8 \pm 15.4**	-4.9 \pm 28.2 (NS)
MDC	-41.5 \pm 13.0*	-12.4 \pm 35.1 (NS)
MIP-1 α	-50.3 \pm 30.1**	-61.1 \pm 15.5**
MIP-1 β	-69.6 \pm 32.5**	-80.2 \pm 10.7**
MIP-1 δ	-67.6 \pm 33.6*	-62.3 \pm 24.0 (NS)
MMP13	-76.8 \pm 5.9***	-59.2 \pm 10.5**
MMP2	-50.4 \pm 25.1*	-46.6 \pm 26.0*
MYD88	-22.8 \pm 3.7*	-12.5 \pm 14.4 (NS)
NOD1	-61.8 \pm 17.1*	-43.7 \pm 46.5 (NS)
NOD2	-72.6 \pm 20.2*	-26.3 \pm 63.8 (NS)
p50	-52.3 \pm 13.4**	-33.0 \pm 24.1**
p65	-75.0 \pm 23.5*	-49.9 \pm 48.1 (NS)
PLA ₂ G6	-57.7 \pm 28.2*	-69.8 \pm 10.9**
PTGES	-36.6 \pm 30.1*	-19.4 \pm 32.7 (NS)
RELB	-56.2 \pm 37.5*	-40.9 \pm 26.4 (NS)
RIP2	-26.9 \pm 16.0*	15.2 \pm 13.0 (NS)
SARM1	-83.4 \pm 12.8*	-16.5 \pm 45.2 (NS)
SP-A1	-75.8 \pm 10.0*	-37.0 \pm 46.1 (NS)
TAB2	-75.0 \pm 4.5***	-44.0 \pm 19.5*
TAB3	-60.6 \pm 13.7*	-51.3 \pm 33.2*
TAK1	-47.1 \pm 22.9 (NS)	-61.4 \pm 23.7*
TICAM1	-87.2 \pm 12.2**	-35.6 \pm 48.8 (NS)
TIRAP/Mal	-48.5 \pm 13.8*	-48.5 \pm 18.2*
TNF- α	-89.5 \pm 10.6***	-63.7 \pm 28.0**
TNFR1	-31.8 \pm 17.7**	-17.8 \pm 13.2 (NS)
TRAF2	-58.8 \pm 25.0*	-56.7 \pm 39.7*
TRAM1	0.3 \pm 51.1 (NS)	-68.7 \pm 20.4**

The data are presented as the mean \pm SD percentage change in expression compared with LPS-stimulated controls ($n = 3$). Significance was determined by ANOVA with Tukey's test *post hoc*.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus LPS alone.

LPS, lipopolysaccharide; NS, not significant; TPCA-1, [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide.

**Figure 7**

Comparison of gene expression data using oligonucleotide arrays (A) and quantitative real-time PCR analysis (B). A selection of low and high abundance genes were chosen for analysis. Array data (A) are taken from the complete set of genes shown in Figure 6 ($n = 3$ experiments). For real-time PCR, relative expression was computed based on the $2^{-\Delta\Delta C_t}$ method relative to the housekeeping gene GAPDH. PCR data are presented as mean \pm SD ($n = 4$ experiments). * $P < 0.05$; ** $P < 0.01$ versus LPS control by ANOVA.

pounds at concentrations reported to be effective in other tissues, it is possible that higher concentrations might have had more effect. However, in previous pilot studies we tested some other NF- κ B inhibitors using choriodecidual explants and, surprisingly, these were also without effect, although strong inhibition was seen in other cell types (J.A. Keelan *et al.*, unpublished observations). On the other hand, we and others have reported inhibition of NF- κ B activity in choriodecidual tissues by salicylates (which are low potency IKK β inhibitors) and the free radical scavenger/NF- κ B inhibitor NAC (Lappas *et al.*, 2002, 2003; Beloesky *et al.*, 2006; Keelan *et al.*, 2009). These findings suggest that the NF- κ B pathway has some unusual characteristics in choriodecidual tissue that make it refractory to pharmacological inhibition. Studies are currently

underway to explore this possibility further. Regardless of the underlying mechanisms, the present results indicate that the efficacy of NF- κ B inhibitors in these tissue requires careful experimental determination and cannot be accurately predicted from studies in other models.

TPCA-1 is a thiophene carboxamide shown to inhibit LPS-stimulated TNF- α , IL-6 and IL-8 production by macrophages (IC_{50} – 0.3 μ M). In murine models it has been shown to block LPS-induced airway inflammation (Birrell *et al.*, 2005), and reduce the severity of collagen-induced arthritis with efficacy similar to that of etanercept (Kondo *et al.*, 2008). It is a potent inhibitor of IKK β with 22-fold selectivity over IKK α (Yamamoto and Gaynor, 2001). Parthenolide is a sesquiterpene lactone extract of the medicinal plant feverfew, and an inhibitor of the IKK complex (Hehner *et al.*, 1999); it does not, however, exert direct effects on either IKK α or β kinase activity (Hehner *et al.*, 1999). It has also been shown to inhibit tyrosine phosphorylation and the activity of other kinases including MAPK (Hwang *et al.*, 1996). Parthenolide inhibits inflammatory gene expression *in vitro* (Hwang *et al.*, 1996; Smolinski and Pestka, 2003), although *in vivo* it elicits a much weaker response, with only IL-6 expression being reported to be mildly suppressed by parthenolide in murine spleen and liver (Smolinski and Pestka, 2005). Hence, our findings of more widespread and robust inhibition of inflammatory gene expression by TPCA-1 are consistent with published data. The differences in specificity, potency and mode of action probably explain the observed differences in genes inhibited by the two compounds. We speculate that the two genes responsive only to parthenolide inhibition are likely to be driven by other kinases (e.g. MAPK) that are responsive to the effects of parthenolide (Hwang *et al.*, 1996).

Expression of a variety of pro-inflammatory cytokines and chemokines was markedly inhibited by TPCA-1, many of which were also responsive to LPS stimulation. TPCA-1 also blocked the expression of a number of proteins involved in the canonical signalling pathway, such as p65, MyD88, TAB2, TAB3, TICAM1/TRIF and TIRAP/Mal, plus other inflammation-associated proteins AKT1, CARD11/CARMA1 and the intracellular peptidoglycan sensors NOD1 and NOD2 (Verstrepen *et al.*, 2008). We would expect that the overall effects of these changes would be effective and sustained inhibition of the inflammatory response. TPCA-1 also inhibited the expression of a number of key signalling proteins involved in the activation of NF- κ B by TNF- α : RIP2, TRAF2 and TAK1, as well as the type I TNF receptor and TNF- α itself. TNF- α is a key pro-

inflammatory cytokine in intra-uterine tissues and mediates much of the effects of LPS in choriodecidual tissues (Sato *et al.*, 2003). Amniotic administration of a TNF antagonist has recently been shown to prevent LPS-induced preterm labour in a rodent model (Holmgren *et al.*, 2008). Blockade of TNF production and actions by TPCA-1 is likely to further enhance its anti-inflammatory efficacy. On the other hand, TPCA-1 also inhibited expression of several genes involved in the negative regulation of NF- κ B activation: A20, Bcl3, I κ B α / β / ζ (Sun and Ley, 2008). This may have implications for its efficacy long term, as inflammatory gene expression changes with time and typically undergoes a resolution phase via induction of negative feedback pathways. Inhibition of these negative regulators by TPCA-1 might result in excessive or dysregulated inflammation rather than the opposite. Extended duration time-course studies would be required to address this possibility.

Nuclear factor-kappa B is also an important driver of anti-apoptotic protein expression, particularly in transformed cells, and as such its inhibition has been investigated for the treatment of various cancers (Karin, 2008). Previous studies with sulphasalazine in full thickness fetal membranes (Keelan *et al.*, 2009) highlighted the possibility that chorionic trophoblasts might be susceptible to apoptosis following NF- κ B inhibition. However, in the present studies none of the inhibitors tested affected cell viability as determined by MTT assay over 24 h exposure. Finally, a number of genes known to be important mediators of parturition were inhibited by TPCA-1 and parthenolide, specifically genes involved in prostanoid biosynthesis (prostaglandin H and E synthases, phospholipases A₂) and collagenolysis/membrane rupture (MMP2, 9, 13). Inhibition of prostaglandin biosynthesis and metalloprotease activity would further enhance the anti-parturogenic effects of the IKK inhibitors.

From a clinical perspective, our findings suggest that IKK β inhibition has potential as a therapeutic target for prevention and treatment of inflammation-driven preterm labour and birth. Several studies in animal models have supported the use of anti-inflammatory modalities to prevent LPS-driven preterm birth, although caution should be exercised when comparing these data to human preterm labour due to the nature of the stimuli (Buhimschi *et al.*, 2003; Xu *et al.*, 2005; Holmgren *et al.*, 2008). The LPS model commonly employed revolves around activation of inflammatory responses via TLR4, which elicits a specific signal transduction pathway resulting in characteristic gene expression profile. Our data would be appli-

cable to these models. Intra-uterine inflammation, however, could be triggered by different agents (a range of different microbes, pathogen-activated molecular patterns or non-microbial TLR agonists), resulting in activation of different signalling pathways; hence, selective pharmacological inhibitors may or may not be effective in such cases. Studies using inhibitors to treat tissues delivered following spontaneous preterm delivery are currently planned to address this issue. The ultimate goal of any treatment modality is improved fetal outcomes, not just prevention of prematurity. In this regard, the use of anti-inflammatory IKK β inhibitors may hold a significant advantage over other pharmacological approaches, such as prostaglandin synthase inhibitors or progesterone analogues. Fetal inflammation is believed to play a central role in the pathophysiology of a variety of neonatal morbidities associated with preterm birth, including cerebral palsy, necrotizing enterocolitis and periventricular leukomalacia (Romero *et al.*, 2007b). In theory, treatment of the fetus via maternal administration of an IKK inhibitor should prevent damage caused by fetal inflammation and improve neonatal and perinatal outcomes, independent of any effect on timing of delivery. In a recently published trial by Shahin *et al.* (2009) the effects of administration of NAC and 17-hydroxyprogesterone caproate (17-OHPC) in mid pregnancy were compared with 17-OHPC alone in a group of 280 high risk women with bacterial vaginosis and prior PTB. Remarkably, NAC administration resulted in significantly fewer preterm deliveries (35%), an increase in birth weight (~400 g) and a reduction in neonatal morbidity/mortality compared with 17-OHPC alone (Shahin *et al.*, 2009). NAC also appeared to interfere with normal labour, increasing the requirement for induced labour more than fourfold. (Shahin *et al.*, 2009). These findings are encouraging in terms of the efficacy and benefits of an anti-inflammatory agent on both prematurity rates and neonatal outcomes. However, NAC is unpleasant to take and there was a high drop-out rate in the treatment arm of the study. A more palatable and targeted drug would be expected to have better compliance and more positive outcomes.

While our findings suggest that IKK β is an attractive target for inhibition, this approach could also suffer from potential drawbacks and unwanted side effects. The foremost concern would be that blocking NF- κ B activation might prevent proper activation of the host innate and adaptive immune system and increase maternal susceptibility to infections. This would be of particular concern in pregnant women with an already suppressed immune response (Luppi, 2003). However, this same possibil-

ity applies to glucocorticoids, salicylates and TNF biologics, and appears to be more of a theoretical concern rather than a clinical reality. Nevertheless, the period of administration would need to be kept to a minimum; a 3 month treatment window for asymptomatic high-risk women between 20 and 32 weeks gestation might be a reasonable starting point. On the other hand, somewhat counter-intuitively, chronic IKK β inhibition has been reported to induce hyper-stimulation, augmenting IL-1 β secretion by macrophages in response to LPS, raising concerns about the risk of septic shock with long-term IKK β inhibitor administration (Greten *et al.*, 2007). This effect does not appear to be mediated through inhibition of expression of negative regulators of NF- κ B activation such as A20 and I κ B proteins, but through inhibition of NF- κ B-mediated negative regulation of pro-IL-1 β processing (Greten *et al.*, 2007). The effects of maternal administration of IKK β inhibitors on the fetus would also be of concern, depending on whether the agent crosses the placenta and significant concentrations were achieved in fetal circulation. There are no published studies we are aware of describing the teratogenic effects of IKK β inhibitors, although in mice, IKK β gene deletion is embryonically lethal as a consequence of massive hepatic apoptosis (Li *et al.*, 1999), while conditional disruption of the IKK β locus in endothelial cells prevents embryonic hepatic vascular development (Hou *et al.*, 2008). However, IKK β ^{-/+} heterozygotes are born fully developed, healthy and with normal livers, despite ~50% reduced IKK β activity (Li *et al.*, 1999; Strnad and Burke, 2007). It is also reassuring to note that currently employed anti-inflammatory drugs prescribed in pregnancy such as amino-salicylates and infliximab, all of which effectively cross the placenta, do not appear to have any significant foetotoxicity or teratogenicity (Brar and Einarson, 2008; Rahimi *et al.*, 2008). The trial of NAC administration in pregnancy did not report any increase in fetal abnormalities (Shahin *et al.*, 2009), although numbers were too small to detect an increase in a rare occurrence.

In conclusion, we have tested a range of NF- κ B inhibitors for their ability to prevent LPS-induced inflammation in human choriodecidual cells and identified two IKK inhibitors as effective and non-toxic agents with widespread effects on inflammation-associated gene expression. Our studies suggest that IKK β inhibition is a potential therapeutic target for preventing inflammation in fetal membranes and as such merits investigation for the treatment prevention of preterm labour and birth. Studies are needed to determine the longer-term effects of IKK β inhibitors and their ability to cross the placenta and act upon fetal organs. The

potential risks and benefits of delivering anti-inflammatory agents to the fetus is a complex issue that will require careful investigation.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Immunocytochemical staining of choriodecidual cells. Cells were grown in 96-well tissue culture plates for 3 days then fixed for 10 min in ice-cold acetone : methanol (1:1). After washing in PBS/0.5% Tween (PBS-T), cells were incubated with anti-cytokeratin-7 monoclonal antibody (1:100; clone OV-TL 12/30, DakoCytomation, Denmark A/S) in immunobuffer (PBS-T / 1% BSA / 5% normal calf serum) for 1 h at room temperature with gentle shaking, then washed 3 \times in PBS-T. Immunostaining was developed with Cy3-labelled goat anti-mouse IgG (10 μ g/mL, BioLegend, San Diego, CA, USA). Cells were then washed and incubated with anti-vimentin monoclonal antibody (1:200; clone V9, DakoCytomation), detected with DyLight488-labelled goat anti-mouse IgG (10 μ g/mL BioLegend). Finally, nuclei were stained with Hoechst 33342 (4 μ g/mL in PBS) for 10 min before washing and storing in PBS-T. Cells were imaged in Nikon Eclipse TiR inverted fluorescent microscope and photographed at 1:200 magnification using a Nikon CCD camera. Decidual cells (vimentin positive) are stained green; chorionic trophoblasts (cytokeratin-positive) are stained red-orange; nuclei are stained blue. Ratio of decidual : chorion cells was ~4:1.

Figure S2 Custom oligonucleotide array images. Representative images of 8 \times 16 custom oligonucleotide arrays (SABiosciences Corp., Frederick, MD, USA) probed with biotin-labelled cRNA from chorionic decidual cells stimulated (4 h) with LPS \pm TPCA-1 or parthenolide. Arrays were visualized by chemiluminescence and exposed to X-ray film for 1 min. Films were then imaged by densitometry and saved as TIF files for image analysis using ImageQuant software (GE Healthcare). Spots on the left and bottom right corners are positive controls; housekeeping genes are the first six spots on the bottom row.

Table S1 Genes present on custom oligonucleotide arrays.

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