

RESEARCH PAPER

New use for an old drug: COX-independent anti-inflammatory effects of sulindac in models of cystic fibrosis

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BACKGROUND AND PURPOSE

Pulmonary disease is the main cause of morbidity and mortality in cystic fibrosis (CF) patients due to exacerbated inflammation. To date, the only anti-inflammatory drug available to CF patients is high-dose ibuprofen, which can slow pulmonary disease progression, but whose cyclooxygenase-dependent digestive adverse effects limit its clinical use. Here we have tested sulindac, another non-steroidal anti-inflammatory drug with an undefined anti-inflammatory effect in CF airway epithelial cells.

EXPERIMENTAL APPROACH

Using *in vitro* and *in vivo* models, we NF- κ B activity and IL-8 secretion. In HeLa-F508del cells, we performed luciferase reporter gene assays in order to measure i) IL-8 promoter activity, and ii) the activity of synthetic promoter containing NF- κ B responsive elements. We quantified IL-8 secretion in airway epithelial CFBE cells cultured at an air-liquid interface and in a mouse model of CF.

KEY RESULTS

Sulindac inhibited the transcriptional activity of NF- κ B and decreased IL-8 transcription and secretion in TNF- α stimulated CF cells *via* a cyclooxygenase-independent mechanism. This effect was confirmed *in vivo* in a mouse model of CF induced by intra-tracheal instillation of LPS, with a significant decrease of the induction of mRNA for MIP-2, following treatment with sulindac.

CONCLUSION AND IMPLICATIONS

Overall, sulindac decrease lung inflammation by a mechanism independent of cyclooxygenase. This drug could be beneficially employed in CF.

Abbreviations

CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; κ B, inhibitor of NF- κ B; KC, keratinocyte-derived chemokine; BALF, bronchoalveolar lavage fluid; COX, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug

Tables of Links

TARGETS
Ion channels^a
CFTR
Enzymes^b
COX, cyclooxygenase

LIGANDS	
CXCL2	NS-398
Ibuprofen	Sulindac
IL-8 (CXCL8)	TNF- α
Inh172, CFTR (inh)-172	VX-809, lumacaftor
KC, CXCL1	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^{a,b}Alexander *et al.*, 2015a,b).

Introduction

Cystic fibrosis (CF) is caused by mutations within the CF transmembrane conductance regulator (*CFTR*) gene leading to defective epithelial chloride transport in many organs. In the airway, CF is characterized by excessive mucus production, chronic bacterial infection, exaggerated inflammatory response, and progressive tissue damage.

The identification of novel drugs to reduce lung inflammation is therefore considered a key therapeutic approach to prevent progressive lung tissue deterioration in CF (Koehler *et al.*, 2004; Lands and Stanojevic, 2007; Taylor-Cousar *et al.*, 2010). Currently, the only therapy recommended by the CF Foundation to specifically alleviate the excessive airway inflammatory response is high-dose ibuprofen, a non-steroidal anti-inflammatory drug (NSAID). Two independent clinical trials showed that high-dose ibuprofen can slow disease progression (Konstan *et al.*, 1991; Konstan *et al.*, 2007; Lands and Stanojevic, 2007), but the risk of adverse effects such as gastrointestinal complications have limited its clinical use. Mechanisms independent of cyclooxygenase (COX) have been proposed to explain ibuprofen benefits in CF such as the inhibition of NF- κ B activity (Dauletbaev *et al.*, 2010). However, this inhibition was not accompanied by decreased IL-8 production (Dauletbaev *et al.*, 2010). IL-8 is the major PMN chemoattractant secreted by airway epithelial cells, whose secretion is reported in numerous studies to be increased in CF airway fluids (Jacquot *et al.*, 2008). Here, we have assessed compounds with anti-inflammatory activity (measured as decreased IL-8 production) and with fewer COX-related side-effects.

Sulindac sulfoxide (sulindac hereafter) (Z-5-fluoro-2-methyl-1-[4-(methylsulfinyl) benzylidene]-1H-indene-3-acetic acid) is an NSAID that decreases PG production by inhibition of COX and is currently used in the treatment of chronic inflammatory diseases such as arthritis. It is a prodrug that can either be reversibly reduced to a pharmacologically active sulfide derivative, that inhibits COX, or be irreversibly oxidized to a sulfone derivative, inactive on COX. The reversible biotransformation of sulindac in equilibrium with sulfide provides a long half-life for the active drug and a favorable gastrointestinal tolerance (Duggan *et al.*, 1980; Duggan, 1981). Moreover, sulindac can act in the lung, as it decreased induced pulmonary

fibrosis in rats (Verma *et al.*, 2013). Interestingly, sulindac possesses a chemopreventive activity shown to involve COX-independent mechanisms (Piazza *et al.*, 1997; Han *et al.*, 1998; Yamamoto *et al.*, 1999; Babbar *et al.*, 2003). The known targets modulated by sulindac to explain these properties and that are relevant to CF pathogenesis include ceramide (Chan *et al.*, 1998), the PPARs (Babbar *et al.*, 2003; Jarvis *et al.*, 2005), PDEs acting on cGMP (Soh *et al.*, 2000) and NF- κ B pathways (Yamamoto *et al.*, 1999; Loveridge *et al.*, 2008).

In this study, we investigated the action of sulindac on lung inflammation in the context of CF therapy. We found that, like ibuprofen, sulindac inhibited NF- κ B transcriptional activity. However, in contrast to ibuprofen, sulindac also decreased IL-8 transcription and secretion.

Methods

Cells

Stably transfected HeLa cells expressing wild-type CFTR (HeLa-WT), F508del-CFTR (HeLa-F508del) or pTracer (HeLa) were grown in DMEM containing 10% fetal calf serum (from Invitrogen). Human bronchial epithelial cell line CFBE41o-, derived from a CF patient and stably expressing wild-type CFTR (CFBE-WT) and F508del CFTR (CFBE-F508del) (Kunzelmann *et al.*, 1993; Gruenert *et al.*, 2004; Illek *et al.*, 2008). CFBE cells were obtained and used following a special agreement with UCSF. CFBE cells were grown on Transwell inserts (Costar) in MEM containing 10% FCS (Kunzelmann *et al.*, 1993; Gruenert *et al.*, 2004). Cells seeded at 2.5×10^5 cells cm^{-2} were placed at an air-liquid interface after 24h and allowed to polarize for at least 6 days. MucilairTM epithelium is a 3D human airway epithelia reconstituted *in vitro* obtained from primary human bronchial cells from a single CF donor, homozygous for F508del (Epithelix, Geneva, Switzerland). All cells were grown at 37°C with 5% CO₂. For treatment, drugs were added in DMEM with 1% FBS.

Plasmid construction and luciferase reporter assay. To assay the transcriptional activity of NF- κ B, a luciferase reporter plasmid, NF- κ B-Luc (Stratagene, California, USA), containing five sequential NF- κ B binding sites upstream of a

minimum promoter element, was used. To assay the transcriptional activity of IL-8, a luciferase reporter plasmid containing the IL-8 promoter (-133 bp from the transcription start site) fused to the Luciferase gene was used (Edwards *et al.*, 2005). Cells were transfected with the Turbofect™ *In Vitro* Transfection Reagent (Fermentas, Villebon, France), according to the manufacturer's protocol. A *Renilla* luciferase reporter construct (pRL-TK, Promega, San Luis Obispo, CA, USA) was co-transfected with each firefly luciferase construct. After 48 hours of transfection, cells were exposed to 10 ng·mL⁻¹ TNF- α for 1 hour. Cells were pre-treated and treated with sulindac, ibuprofen or vehicle control 1 hour before and 4 hours after induction with TNF- α . Cells were lysed with the Passive Lysis Buffer (Promega, San Luis Obispo, CA, USA), and luciferase activity was determined with the Dual Luciferase Assay System (Promega, San Luis Obispo, CA, USA) using a luminometer (Tristar; Berthold, Thoiry, France). Relative luciferase activity was calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity.

Protein extraction and Western blots. HeLa-F508del cells were treated 24 h with VX-809 (10 μ M). Total protein fraction was extracted with RIPA buffer and Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). For Western blots, equal amounts of proteins were resolved by 7% SDS-PAGE and transferred onto polyvinylidene fluoride membrane (GE Healthcare, LittleChalfont, UK). The membranes were probed with specific antibodies anti-CFTR 24-1 (1:200) (R&D Systems, Minneapolis, MN, USA) and anti- β actin (A5441; 1/10,000) (Sigma-Aldrich, St-Louis, USA) and detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:50,000) (Sigma-Aldrich, St-Louis, USA). The signal was detected using the enhanced chemiluminescence ECL Advance kit (GE Healthcare) and visualized using the G:BOX-iChemi (Syngene, SynopticsLTD, UK).

Cytokine quantification

Levels of IL-8 in the cell media were measured with the IMMULITE 1000 Automated Analyzer (Diagnostic Products Corp., Los Angeles, CA, USA) and commercially available chemiluminescent enzyme immunometric assay (Immulite®, DPC, Los Angeles, CA, USA), according to the manufacturer's instructions. In CFBE cells and bronchoalveolar lavage fluid (BALF), cytokines were quantified by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Animals models and ethical statement

All animal care and experimental protocols complied with INSERM guidelines and were approved by the Regional Ethical Committee C2EA-16 (n°12.074-11/12/12-17). The experimental procedures used in the work were as humane as possible. The animal studies are reported as recommended by the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015).

Adult male C57BL/6J mice (8 weeks old; average weight 23–26 g; Janvier, Le Genest-Saint-Isle, France) were fed normal mouse chow and water *ad libitum*. Adult (8–16 weeks old) CF

male mice homozygous for the F508del-CFTR mutation (F508del/F508del) in the 129/FVB outbred background (cftr^{tm1Eur}) (van Doorninck *et al.*, 1995) (average weight 22 to 29 g) and their normal homozygous WT littermates (+/+) (average weight 22 to 33 g) were obtained from the "Centre de Distribution, de Typage et d'Archivage Animal" UPS44 CNRS (Orleans, France). To prevent CF-induced intestinal obstruction, Movicol (55.4 g·L⁻¹) was added in drinking water and animals were fed with 2918 Teklad global 18% protein Rodent Diet (Harlan, Gannat, France) *ad libitum*. All mice were reared and housed under standard conditions with air filtration (21±2°C; 12h Light/12h dark).

Sulindac quantification in mice

To optimize the timing of treatment of mice with sulindac, we developed a mass spectrometry-based analysis technique to quantify sulindac in mouse serum and lung samples. C57BL/6J mice were injected i.p. with 40 mg·kg⁻¹ sulindac. At several time points, serum and lungs were collected and stored at -80°C. Sulindac was quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS), performed by electrospray ionization in positive mode using a triple quadrupole mass spectrometer.

Stock and working solutions, internal standard, calibration standards. For each drug, a primary stock solution was prepared at 5 mg·mL⁻¹ in methanol or DMSO/methanol (v/v, 50/50) for sulindac and then stored at -80°C. Stock solutions were mixed together in order to get a methanolic working solution containing drug at 1 mg·mL⁻¹. It was used for the preparation of the calibration standards ranging from 39 to 10,000 ng·mL⁻¹. A 1 mg·mL⁻¹ diclofenac stock solution was prepared and used as internal standard. The calibration standard plasma samples were stored at -80°C prior to analysis.

Ten-point calibration standard curves have been calculated and fitted by least-squares linear regression of the peak-area ratio of each drug to IS against the concentrations of the corresponding drug in each standard. The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the three standard curves.

LC-MS/MS conditions. The liquid chromatography consisted of a Thermo Scientific (Courtaboeuf, France) Accela® autosampler and a quaternary pump. Separation was performed on an Hypersil Gold® (2.1 x 100 mm; pore size 1.9 μ m) analytical column placed in a thermostated column heater at 50°C. The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, equipped with an Ion Max electrospray ionization (ESI) interface and operated with XCalibur 2.07 software (Thermo Fisher Scientific Inc., Courtaboeuf France).

The mobile phase used for chromatography was 10 mM ammonium formate buffer containing 0.1% (v/v) formic acid (solution A), and acetonitrile with 0.1 % (v/v) formic acid (solution B). The mobile phase was delivered using the following stepwise gradient elution program: run from 50:50 (A:B) to obtain 20:80 (A:B) at 13 minutes, conditions 50:50 (A:B) maintained from 13.1 to 15 minutes for equilibration.

The thermostated column heater was set at 50°C and the autosampler was maintained at 4°C. The MS conditions were as follows: ESI in positive mode, capillary temperature: 325 °C: 10V, tube lens voltages were 110 and 240 for sulindac and diclofenac; collision energies (50 and 15 V); spray voltage: 3500 V; sheath and auxiliary gas (nitrogen) flow-rate: 45 psi and 25 (arbitrary units), respectively. The Q2 collision gas (argon) pressure was 1.5 mTorr. Data were acquired in selected reaction monitoring (SRM) mode. Two transitions were selected for each analyte in order to improve the specificity of the assay: one was used for the quantitation 357.1 - 233 for sulindac and 339.97 - 321.9 for diclofenac and the other one for confirmation 357.1 - 248 for sulindac (ion ratio of 80%).

Quadrupole resolution on Q1 and Q3 were set to 0.7 FWHM. Scan width was 0.05 amu, scan time 0.04 ms. Data acquisition and processing were performed using the Xcalibur® (version 2.07) (ThermoScientific, San Jose, CA, USA).

The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the twenty standard curves. The LLOQ was established at 39 ng·mL⁻¹ for sulindac. Mean intra-day and inter-day precisions were good with CVs within 4.3 and 9.8%. Matrix effects and extraction yields ranged from 84.6 to 109 % and 84.0 to 101.2% respectively.

Sample extraction procedures. Methanol (100µL for serum or 300µL for lung) containing 10 ng·mL⁻¹ diclofenac was added to each experimental sample. Standards were extracted in the same conditions. After vortexing, each sample was centrifuged at 6,000 x g at 4°C for 15 minutes. Samples of supernatant (50µL) were diluted two-fold in the mobile phase A and 25 µL were injected into the HPLC system.

Model of acute lung injury (ALI) in mice

CF mice and WT littermates received intra-tracheal instillation of 400 µg·kg⁻¹ LPS from *P.aeruginosa* (Sigma, St. Louis, MO) or an equivalent volume of saline, 1 h after i.p injection of 40 mg·kg⁻¹ sulindac. Three hours later, mice were anesthetized with 110 mg·kg⁻¹ ketamine and 4.8 mg·kg⁻¹ xylazine and bronchoalveolar lavage was performed by cannulating the trachea and lavaging with 4 x 0.5 mL sterile saline.

RNA extraction and mRNA quantification

RNA from lungs were extracted with Trizol® Reagent (Invitrogen), according to the manufacturer's instructions. The mRNA was DNase treated for 30 min at 37°C before heat denaturation of the enzyme (Fermentas). The treated RNA (300 ng) was reverse transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas). Resulting cDNA was then diluted by 5 in sterile distilled water and subsequently used as a template. Quantitative PCR was performed using SYBR Green Taq Ready Mix (Qiagen). Samples were amplified in a LightCycler® 480 System (Roche). Results were normalized to the amount of 18S RNA. RT and Q-PCR analyses were performed in duplicate. The 2^{-ΔΔCt} method was applied to estimate relative transcript levels. The primer sequences were as follows: 18S_forward 5'-AACTTTCGATGGTAGTCGCCGT-3', 18S_reverse 5'-TCCTTGGATGTGGTAGCCGTTT-3', KC_forward 5'-AAGCTC-CCTTGGTTCAGAAA -3', KC_reverse 5'-TCAGAAGCCAGCGT-TCAC-3', CXCL2_forward 5'-GCCAAGGGTTGACTTCAAGA-3', CXCL2_reverse 5'-CTTCAGGGTCAAGGCAAAC-3'.

Data and statistical analysis

These studies comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The experimental procedures or treatment and data analyses were carried out with randomization and blinding. For *in vivo* studies, all groups were initially designed to contain 10 mice by group for C57BL/6J background and 6 mice for 129/FVB background. Sizes of the mice groups were different due to early death or technical difficulties in BALF procedure. For statistical analyses, we used raw data except for mRNA quantification by RT qPCR which have been normalized to the amount of 18S RNA. We used non parametric methods (Wilcoxon-Mann-Whitney for two groups' comparison or Kruskal-Wallis for ≥3 groups comparison followed by Dunn's post hoc tests when F reached significance).

For *in vitro* studies, we performed a minimum of 5 independent experiments, where individual data points were based on at least technical duplicates each. For statistical analysis, we used normalized data to reduced variability of baseline between independent experiments. Data were normalized in fold over vehicle without TNF-α mean. For two group's comparison, we used unpaired Student's t-test. When we compared ≥3 different groups, we first used one-way ANOVA, and if F reached significance, two post hoc tests were used: the Dunnett post hoc test when comparing each group with Veh., or the Sidak post hoc test whenever multiple groups comparison was necessary. All analyses were performed on GraphPad Prism® version 6.0 software and P-values of <0.05 were considered significant. For graphic representation, experimental values are expressed as a percentage of the mean value from the WT cells treated with Veh + TNF-α.

Materials

Sulindac, sulindac sulfone, ibuprofen, human TNF-α, NS-398 and CFTR(inh)-172 (inh172) were purchased from Sigma (St. Louis, MO) and VX-809 (lumacaftor) was purchased from AbMol BioScience (Houston, USA).

Results

Effect of sulindac on the NF-κB pathway

To demonstrate the effects of sulindac on NF-κB activity, HeLa cells were transfected with an NF-κB luciferase reporter plasmid and NF-κB transcription activity was stimulated by 10 ng·mL⁻¹ TNF-α. Cells were treated with various concentrations of NSAIDs. As shown in Fig. 1A, compared with vehicle, ibuprofen significantly inhibited TNF-α-induced NF-κB activities at 500 µM and EC₅₀ was extrapolated to be 1,813 µM. Sulindac caused a dose-dependent decrease in TNF-α-induced luciferase activity in HeLa cells (Fig. 1B) and in the lung epithelial cell line, BEAS-2B (Fig. 1C). Sulindac significantly inhibited NF-κB activities at concentrations equal or greater than 300 µM and EC₅₀ values were 420 µM in HeLa cells and 347 µM in BEAS-2B cells. Sulindac is a prodrug that can be metabolized to sulindac sulfone or sulindac sulfide (Fig. 2A). From studies in cancer research, sulindac is known

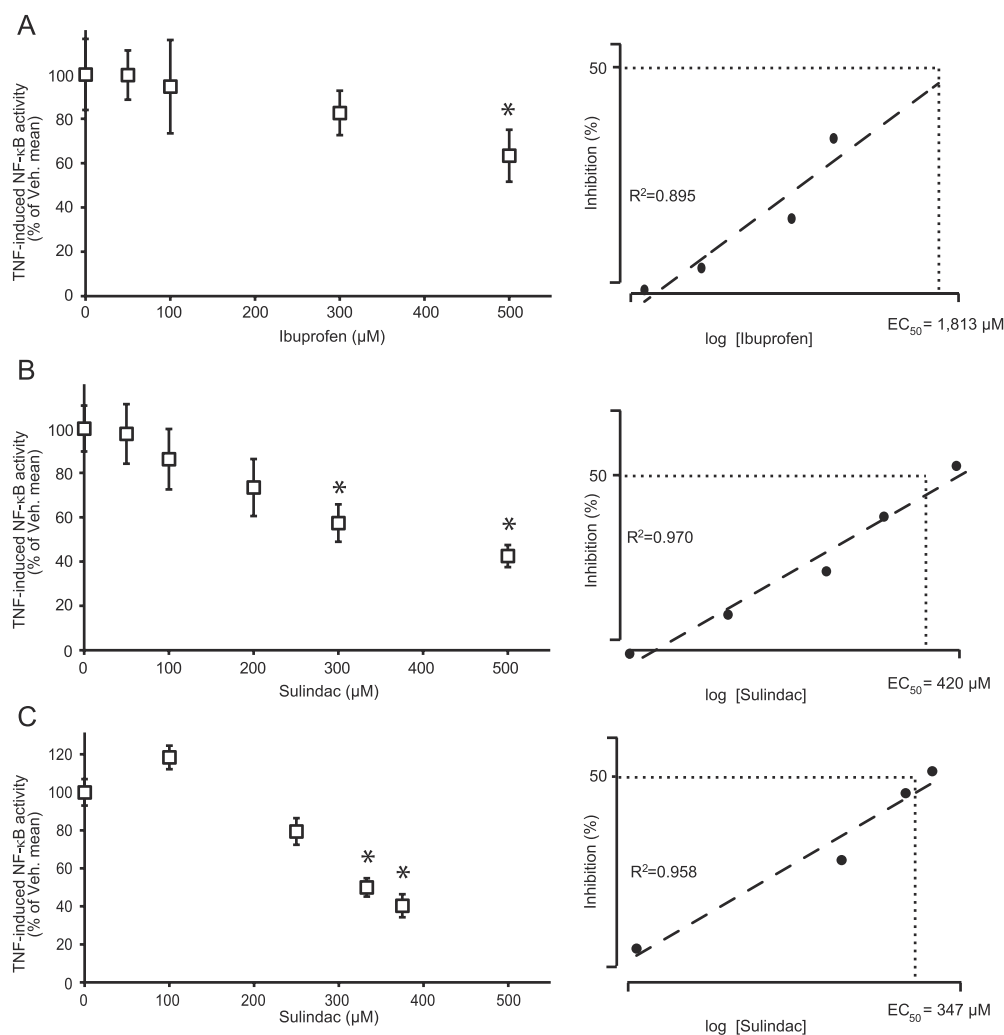


Figure 1

Concentration-dependent inhibition of sulindac and ibuprofen on NF- κ B transcriptional activity. Concentration-response data for the effects of ibuprofen ($n=6$) (A), sulindac ($n=7$) on HeLa cells (B) and sulindac ($n=7$) on airway epithelial cells BEAS-2B (C), on NF- κ B activity. Left panels represent TNF-induced NF- κ B transcriptional activity expressed as a percentage of vehicle (Veh.) +TNF- α , at doses ranging from 0 μ M to 500 μ M of NSAIDs. Right panels represent linear regression of percentage of inhibition of NF- κ B activity in function of log[dose] to determine EC₅₀ values for ibuprofen and sulindac. * $P < 0.05$, significantly different from Veh. +TNF- α .

to inhibit NF- κ B activity at concentrations between 200 and 1,000 μ M, without toxicity (Yamamoto *et al.*, 1999; Loveridge *et al.*, 2008; Mackenzie *et al.*, 2010). We chose an intermediate dose of 500 μ M of the prodrug to evaluate its anti-NF- κ B activity on HeLa cells stably transfected with CFTR wild type (HeLa-WT) and CFTR F508del (HeLa-F508del). To evaluate NF- κ B activity, cells were transfected with an NF- κ B luciferase reporter plasmid and pretreated with sulindac, ibuprofen or vehicle for 30 minutes. NF- κ B activity was then stimulated by 10 ng·mL⁻¹ TNF- α for 1h and cells were treated with or without sulindac or ibuprofen for 4h as shown in Fig. 2B. NF- κ B activity was greater in the HeLa-F508del cells, both under basal conditions and in response to TNF- α , than in the HeLa-WT cells under TNF- α stimulation (Fig. 2C, left panel). To compare the effects of drugs in both cell types, the NF- κ B activities were normalized and expressed as the percentage of induction in

HeLa-WT cells treated with vehicle and induced by TNF- α (Fig. 2C, right panel). Basal NF- κ B activity was not significantly modified by ibuprofen or sulindac (Figure S1). NF- κ B activity induced by TNF- α was similarly decreased by ibuprofen and sulindac in HeLa-WT and in HeLa-F508del cells (Fig. 2C).

In order to explore whether these sulindac effects were dependent on its known anti-COX activity, we tested effects of another COX inhibitor, NS-398 (Zhu *et al.*, 2008) and the COX-inactive metabolite, sulindac sulfone. Treatment by NS-398 did not significantly modify TNF- α -induced NF- κ B activity with or without sulindac in HeLa-WT and HeLa-F508del cells (Fig. 2D). Moreover sulindac sulfone decreased NF- κ B activity similarly to sulindac (Fig. 2E).

To investigate the mechanism of action of sulindac on the NF- κ B pathway, we analysed subcellular localizations of p65, an NF- κ B subunit, and I κ B. The cytosolic proteins were used

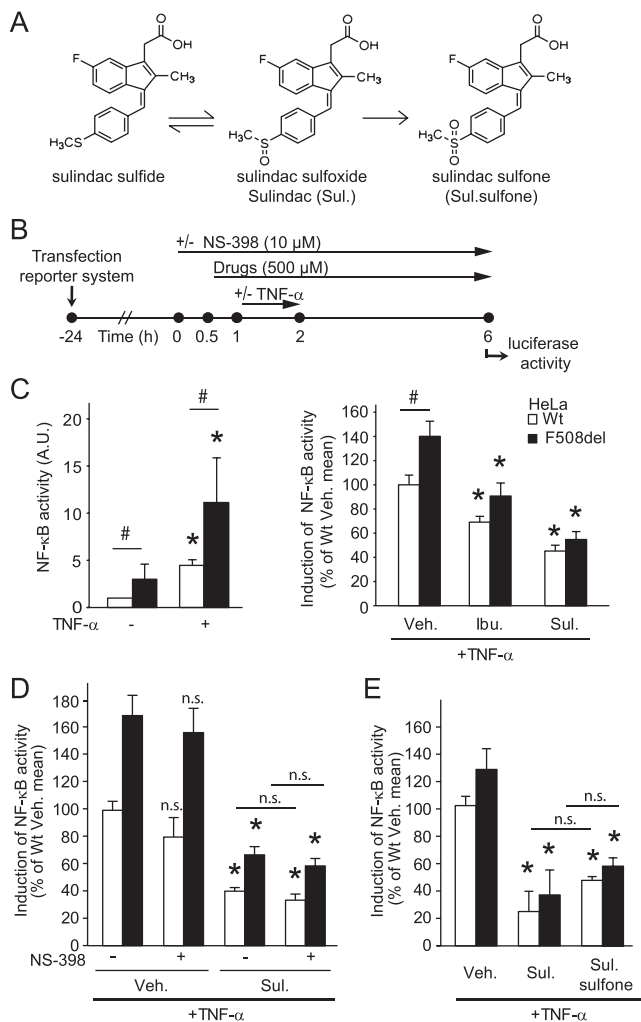


Figure 2

to detect I κ B by Western blot. TNF- α induced a rapid degradation of more than 90% of I κ B, and this was not modified by sulindac pretreatment (Figure S2). We then checked the sub-cellular distribution of p65 by immunofluorescence and by quantitative Western blot assay after cell fractionation. In control cells, p65 had a cytosolic distribution and TNF- α induced a rapid and full nuclear translocation. Treatment with sulindac did not modify p65 localization, either qualitatively or quantitatively, under either in basal or induced conditions (Figure S2).

Effect of sulindac on the IL-8 pathway

To investigate whether the inhibition of NF- κ B activity by sulindac had a downstream effect on the IL-8 promoter, we used a luciferase reporter assay (de Becdelievre *et al.*, 2013). HeLa cells transiently transfected with this construct were treated with vehicle, sulindac or ibuprofen and inflammation was induced by TNF- α as shown Fig. 3A. As previously described in other cells, ibuprofen did not affect IL-8 promoter activity (Daultebaeve *et al.*, 2010). However, sulindac significantly decreased IL-8 promoter activity in HeLa-WT and in HeLa-F508del cells (Fig. 3B).

Treatment with NS-398 did not modify TNF- α -induced IL-8 promoter activity in HeLa cells treated with vehicle or sulindac (Fig. 3C). Lastly, the sulfone metabolite of sulindac inhibited IL-8 promoter activity, as effectively as sulindac (Fig. 3D).

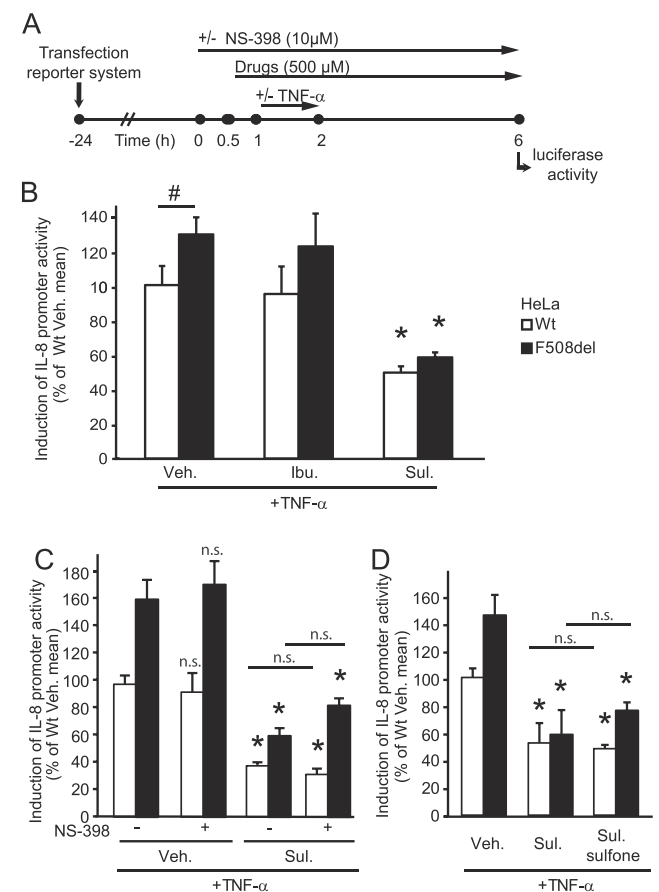


Figure 3

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We then tested the effect of sulindac on NF- κ B and IL-8 promoter activity, in combination with VX-809 (lumacaftor), a CFTR corrector that can rapidly improve CFTR maturation in F508del cells (Van Goor *et al.*, 2011) (Fig. 4). HeLa-F508del cells transiently transfected with the reporter system

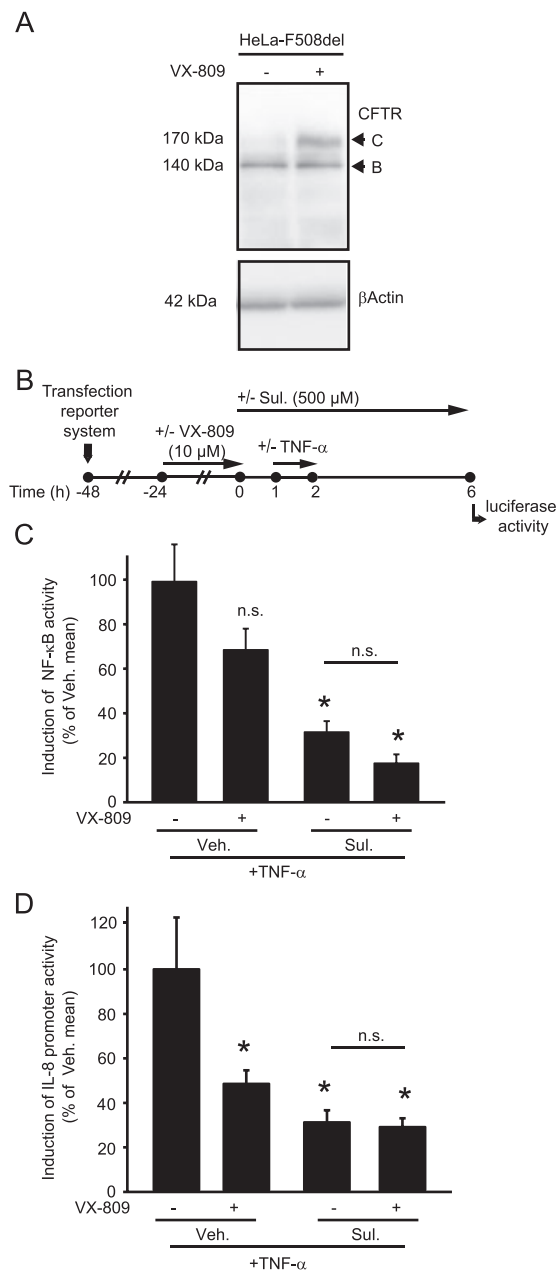


Figure 4 CFTR correction by VX-809 in HeLa-F508del cells has no effect on inhibition by Sulindac of NF- κ B and IL-8 promoter activities. (A) Representative immunoblot of CFTR pattern of HeLa-F508del cells after 24 hours of treatment with VX-809. (Band B: immature CFTR; band C: mature CFTR) (B) Treatment protocol used to study the effect of sulindac with the CFTR corrector VX-809 on HeLa-F508del cells. (C) Effect of sulindac with or without VX-809 on the transcriptional NF- κ B activity (n=9) and (D) on the transcriptional activity of the IL-8 promoter (n=9). * $P < 0.05$, significantly different from Veh. +TNF- α , without VX-809.

were treated with VX-809, then treated with vehicle or sulindac and inflammation was induced by TNF- α , as shown Fig. 4B. VX-809 treatment induced maturation of CFTR F508del (Fig. 4A) and inhibited NF- κ B activity and IL-8 promoter activity (Fig. 4C and 4D). However, adding VX-809 to sulindac had no significant additive effect on NF- κ B activity and IL-8 promoter activity although levels of NF- κ B activity tended to be lower with the combination (Fig. 4C and 4D).

To explore if this transcriptional effect was reproduced at the protein level, we assayed IL-8 protein secretion into the culture medium (Fig. 5A). HeLa-F508del cells secreted twice as much IL-8 as the HeLa-WT cells under basal conditions, and three-fold more IL-8 than HeLa-WT cells following upon TNF- α stimulation (i.e., approximately 32-fold and 48-fold respectively) (Fig. 5B, left panel). The preliminary data indicated that sulindac, but not ibuprofen, reduced this stimulation by TNF- α , decreasing IL-8 secretion similarly in HeLa-WT and in F508del cells (Fig. 5B, right panel).

These effects were also demonstrated in monolayers of polarized human bronchial epithelial cells, expressing recombinant CFTR (CFBE cells; Fig. 6). In these cells, IL-8 secretion was induced by apical addition of 40 ng·mL⁻¹ TNF- α , 5 μ g·mL⁻¹ LPS and 2 ng·mL⁻¹ IL-1 β (referred to as CytoMix). In CFBE-F508del cells, the CytoMix induced apical IL-8 secretion up to four-fold (Fig. 6A, left panel) and sulindac significantly reduced this secretion (Fig. 6A, right panel). In order to test this effect in a CF model which mimics all classes of CFTR mutations (Fanen *et al.*, 2014), we inhibited CFTR activity with 10 μ M inh172. At this concentration, inh172 is a potent and specific reversible allosteric inhibitor for CFTR function (Ma *et al.*, 2002; Kopeikin *et al.*, 2010; Melis *et al.*, 2014). Inh172 treatment mimics the CF inflammatory profile and can be used to create a CF like model (with its own control) (Perez *et al.*, 2007). In CFBE-WT cells, the CytoMix induced apical IL-8 secretion up to nine-fold, relative to basal secretion level (Fig. 6B, left panel) and this secretion of IL-8 was attenuated by sulindac. CFTR inhibition (with inh172) had no effect under basal conditions and the CytoMix still significantly increased IL-8 secretion (Fig. 6B, left panel). Under sulindac treatment, this increased secretion induced by the CytoMix was effectively blocked (Fig. 6B, right panel). In a final series of experiments, inhibition of IL-8 secretion by sulindac was demonstrated in primary human bronchial epithelial cells derived from a CF donor, homozygous for F508del (MucilairTM, Epithelix) (Fig. 7). In these cell cultures, basal IL-8 secretion was significantly decreased by 100 μ M and by 500 μ M of sulindac (Fig. 7).

Effect of sulindac on lung inflammation

To test this pharmacological effect *in vivo*, we chose a mouse model of pulmonary inflammation commonly used in pharmacological studies. Inflammation limited to the lungs is caused by intra-tracheal instillation of lipopolysaccharide (LPS) in WT and CF mice. This methodology provides a good model to monitor pro-inflammatory cytokine secretion and neutrophil recruitment to the lungs (Matute-Bello *et al.*, 2008). Under our conditions, instillation of 400 μ g·kg⁻¹ LPS induced a rapid and transient secretion of the murine chemokines that are functional rodent homologues of human IL-8. These chemokines are CXCL2 (MIP-2) and keratinocyte-derived chemokine (KC).

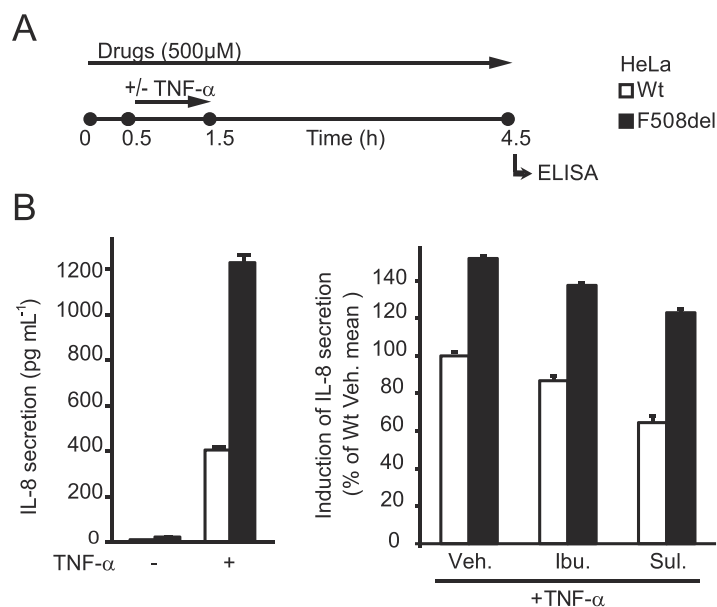


Figure 5

Sulindac reduces IL-8 secretion in HeLa cells. (A) Treatment protocol used to study IL-8 secretion in the cell media on HeLa-WT and HeLa-F508del cells. (B) Left panels represent the IL-8 secretion in the vehicle condition. In right panels, IL-8 secretion was normalized and expressed as a percentage of induction in WT Veh. +TNF- α (n=3).

Sulindac has a long half-life in humans, with 7 hours for the original sulfoxide form, and 15 hours for the metabolites (Huang *et al.*, 2014). Bioavailability and pharmacokinetic studies on animal models have shown that: (i) sulindac was present in tissues of multiple organs, including the lungs (in lesser amounts than in the liver and kidneys), (ii) its half-life was variable and depended on the species studied, (iii) the method of administration had important effects on its pharmacokinetics and pharmacodynamics (Duggan *et al.*, 1978; Duggan *et al.*, 1980; Kapetanovic *et al.*, 2006). We first assessed sulindac distribution after i.p. administration of an intermediate dose (40 mg·kg⁻¹) (Yip-Schneider *et al.*, 2007; Wentz *et al.*, 2009) in C57BL/6 mice. Lung and serum sulindac concentrations were quantified using an LC-MS/MS approach developed in our laboratory. Following i.p. injection in mice, sulindac appeared rapidly in both serum and lung (Fig. 8A). The highest concentration was measured at the first sampling at 30 minutes post-injection with 24,980±1,150 ng·mL⁻¹ of sulindac in serum and 21±6 ng·mg⁻¹ in lung tissue. In mice, sulindac has a rapid bioavailability and a very short half-life of about 1 hour. In order to study the effect of sulindac on the secretion of CXCL2 and KC in BALF, mice were injected with sulindac or vehicle 1h before intra-tracheal LPS instillation and BALF was collected 6h after instillation (Fig. 8B). In absence of LPS stimulation, there was no CXCL2 or KC detectable in BALF but, following LPS, both chemokines were clearly present in BALF. Sulindac treatment decreased these stimulated levels of both chemokines (Fig. 8C and 8D).

The effect of sulindac on the mRNA for pro-inflammatory chemokines was confirmed in CF mice (cfr^{tm1Eur}, F508del/F508del) and not in their littermates (+/+) (Fig. 9). Mice were injected with sulindac or vehicle 1h before intra-tracheal LPS instillation, BALF and lung were collected three hours after

instillation. mRNA levels and cytokine secretion were monitored by RT-qPCR and ELISA respectively (Fig. 9A). LPS increased the mRNA for CXCL2 in both strains (+/+ and F508del/F508del). Sulindac treatment significantly reduced the induction by LPS of the mRNA for CXCL2 (Fig. 9B) or for KC (Fig. 9C) in CF mice. We then tested LPS-induced mRNA expression of other pro-inflammatory cytokines in the lung. After sulindac treatment we also observed a reduction of IL-6 and TNF- α mRNA in CF and WT (+/+) littermate mice (Figure S3).

All groups were initially designed to contain 6 mice on the 129/FVB background but due to technical difficulties in the BALF procedure, the final sizes of the groups were different. Therefore, no statistical analysis could be performed for the levels of chemokine protein secreted into BALF (Curtis *et al.*, 2015). In absence of LPS stimulation, CXCL2 secretion in BALF was 72±50 pg·mL⁻¹ and 25±15 pg·mL⁻¹ for +/+ and F508del/F508del mice respectively, with no effect of sulindac (data not shown). Sulindac treatment tended to decrease secretion of CXCL2 (Fig. 9D) and of KC (Fig. 9E) in +/+ and F508del/F508del mice. Despite the apparent reduction of the KC and CXCL2 levels in BALF, the decrease was clearly greater in the WT littermates.

Discussion

The present study was designed to test if sulindac could be valuable at replacing ibuprofen to diminish lung inflammation in CF patients. The pulmonary pathology of CF is distinguished by the hyperactivity of transcription factors involved in inflammation, more particularly of NF- κ B, which is associated with an increased expression of pro-inflammatory

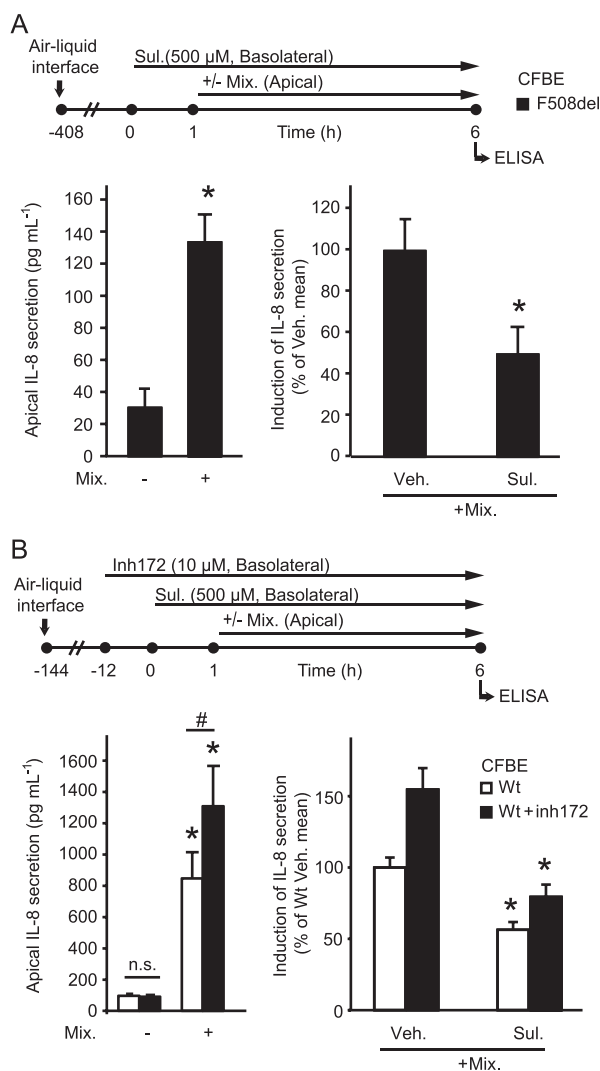


Figure 6

Sulindac reduces IL-8 secretion in polarized CFBE cells. Treatment protocol used to study IL-8 secretion on polarized (A) CFBE-F508del cells (n=6) and (B) CFBE-WT cells without or with inhibition of CFTR function by inh172 (n=8), at an air-liquid interface. IL-8 secretion was measured in the apical media at the end of the treatment. (A) and (B) Upper panels represent treatment protocol. Left panels represent the IL-8 secretion in the diluent condition. In right panels, IL-8 secretion was normalized and expressed as a percentage of induction in control cells with diluent and stimulated condition (Mix.). * $P < 0.05$, significantly different from Veh. + CytoMix in the same cells. # $P < 0.05$, WT significantly different from WT+inh172.

cytokines, such as IL-8. Dysregulation of NF- κ B signaling in CF appears to have multiple origins and remains controversial. Whether there is constitutive activation of pro-inflammatory signalling directly linked to CFTR dysfunction in humans is still unresolved and is a difficult issue to address experimentally. Results obtained with *in vivo* models of CF have concluded that infection precedes inflammation. Indeed, at birth, CF piglets lack inflammation in their airways but harbor more bacteria than the WT littermates and they fail to eradicate bacteria and develop lung disease characterized by airway inflammation, remodeling, mucus

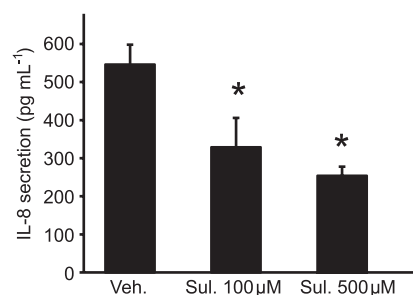


Figure 7

Sulindac reduces IL-8 secretion in primary human bronchial epithelium from CF donor (F508del/F508del) (MucilairTM). IL-8 secretion was measured in basolateral media after treatment with vehicle (Veh.), 100 μ M of sulindac (Sul. 100 μ M) or 500 μ M of sulindac (Sul. 500 μ M) (n=6). * $P < 0.05$, significantly different from Veh. +TNF- α .

accumulation and infection within the first few months of life (Rogers *et al.*, 2008; Stoltz *et al.*, 2010). CF ferrets have also exhibit defective eradication of lung bacteria, but demonstrate an abnormally elevated inflammatory response at birth (Keiser *et al.*, 2015). In addition, although infections precede inflammation, subsequent inflammatory responses and resolution of inflammation is abnormal. Interestingly, recent *in vitro* studies have indicated an intrinsic anti-inflammatory activity of WT CFTR (Hunter *et al.*, 2010; Veit *et al.*, 2012), and have shown that its loss of function could lead to the activation of the NF- κ B pathway (Perez *et al.*, 2007).

Whatever the cause of this increased NF- κ B activation may be, it is a clear target for new anti-inflammatory drugs in the context of CF. In order to compare our work with the current standard therapy (high-dose ibuprofen), we have used a similar methodological approach to that established by Dauletbaev *et al.* in their study on the mechanism underlying the action of ibuprofen in CF cells (Dauletbaev *et al.*, 2010). Although we have used a heterologous model of HeLa epithelial cells stably expressing WT or F508del CFTR, the latter presenting CF characteristics such as enhanced NF- κ B activity and IL-8 secretion, we have obtained similar results for ibuprofen, thus validating our research model (Clain *et al.*, 2001; Jungas *et al.*, 2002).

We first showed that sulindac inhibited TNF- α -induced NF- κ B activity in a concentration-dependent manner. Sulindac was more efficient than ibuprofen with an EC₅₀ value four-fold lower in HeLa cells. Anti-NF- κ B activity of sulindac was confirmed in other cell lines, including bronchial epithelial cells with the same efficiency as shown by similar EC₅₀ values in these cells. Studies on the anti-cancer properties of sulindac highlighted its ability to inhibit NF- κ B activation by inhibiting I κ B degradation, and consequently preventing nuclear translocation of NF- κ B (Yamamoto *et al.*, 1999; Li *et al.*, 2012). Under our experimental conditions, sulindac did not modify the TNF- α -induced I κ B degradation and did not inhibit nuclear translocation of p65. Although I κ B degradation is critical for NF- κ B activation, other processes such as phosphorylation of its subunits or binding of co-activators may also be necessary. It can therefore be hypothesized that sulindac affects NF- κ B nuclear activity by modifying

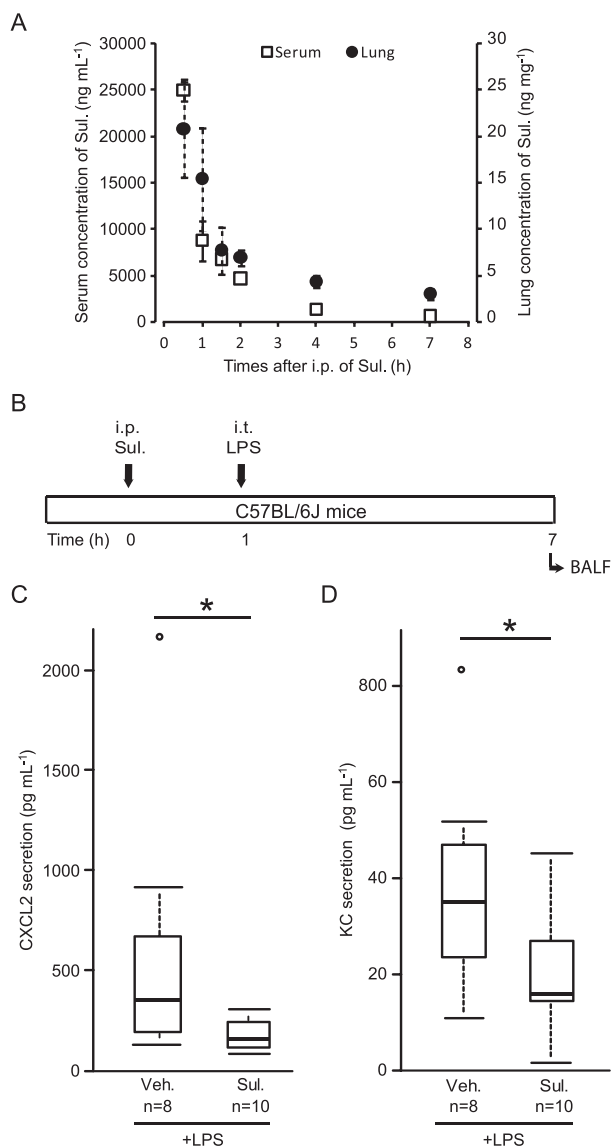


Figure 8

Sulindac decreases LPS-induced pro-inflammatory cytokines in BALF of C57Bl/6J mice. (A) Sulindac distribution in serum and lung after i.p. injection. C57Bl/6J male mice were injected i.p. with sulindac (40 mg·kg⁻¹). Lung and serum were collected at different times and sulindac was quantified by mass spectrometry (n=4). Sulindac distribution in serum (left axis) and in lung (right axis) is shown as the means ± SEM. (B) *In vivo* experimental protocol of lung inflammation. Levels of CXCL2 (C) and KC (D) were quantified in BALF. Graphs represent box plot of raw data showing median, inter quartile range, minimum and maximum values. **P* < 0.05, Veh. significantly different from Sul.

its interactions either with protein partners and/or with DNA. Sulindac may also modify NF-κB localization within the nucleus. In SW480 cells, inhibition of NF-κB activity followed sequestration of an inactive form of NF-κB in the nucleolus, after 5 h of incubation with sulindac (Loveridge *et al.*, 2008). Although we have not observed any modification of p65 localization within the nucleus after 30 minutes, we cannot exclude a similar mechanism in our cell model.

We then investigated if the inhibition of NF-κB activity led to a decrease in the production of the main inflammatory marker in CF airways, IL-8. Previous studies of the IL-8 promoter have shown that its -1 to -133 region was essential and sufficient for the regulation of gene transcription, and that this region contains an NF-κB binding site that is necessary for the induction of IL-8 expression (Edwards *et al.*, 2005). Interestingly, we found that, in contrast to ibuprofen, sulindac reduced the activity of this IL-8 promoter as assessed by a reporter system. This difference between ibuprofen and sulindac suggests that these two NSAIDs have different mechanisms of inhibition of the NF-κB pathway and/or on pathways regulating IL-8 expression. In fact, the IL-8 promoter contains at least two other binding sites for NF-IL6 and AP-1 transcription factors. The functionality of these binding sites depends on cell type and pro-inflammatory signals, probably due to the formation of transcriptional enhanceosome containing various transcription factors and co-activators (Hoffmann *et al.*, 2002).

At the protein level, and in contrast to ibuprofen, sulindac was able to inhibit IL-8 secretion. This effect was confirmed in several human epithelial cell line models: HeLa-WT and -F508del cells heterologously expressing CFTR, Calu-3 bronchial secretory cells (Figure S4), CFBE- WT bronchial cells with or without inh172 treatment, CFBE-F508del cells and primary human bronchial cells polarized at air-liquid interface. Overall, these results suggest a greater therapeutic capacity of sulindac compared to ibuprofen.

In order to test if the anti-NF-κB and anti-IL-8 activities of sulindac were dependent on its effects on the COX pathway, we used another COX inhibitor NS-398 or the sulindac sulfone metabolite that is inactive on COX. Our results showed that effects of sulindac, NS-398 and sulindac sulfone on both activities were very similar, demonstrating a COX-independent mechanism for sulindac. From a pharmacological standpoint, these are key results, as most side effects associated with NSAID treatment, notably those on the GI tract, are due to COX inhibition.

Importantly, in the context of therapeutic applications, we have tested the anti-IL-8 effect of sulindac on an *in vivo* model of pulmonary inflammation. There are two functional homologues of IL-8 that have been identified in mice: CXCL2 and KC. Pulmonary inflammation is characterized by a strong increase in the level of these chemokines in murine BALF 3 hours after LPS instillation. Treatment by sulindac induced a significant decrease in CXCL2 and KC secretion in C57Bl/6 mice, validating its *in vivo* anti-IL-8 effect. In CF mice, treatment by sulindac induced a non-significant decrease in CXCL2 and KC concentrations, whereas we observed a marked and significant inhibition of CXCL2 and KC mRNA induced by LPS in the lung of CF mice. These results indicate a different response time in CF mice and that a longer exposure time will be necessary to see an effect not only at the transcriptional level but also at the protein level.

We then explored the anti-inflammatory effect of sulindac on CF cells treated with the CFTR corrector VX-809. Treatment with VX-809 alone inhibited the NF-κB and IL-8 promoter activity but there was no additive effect in combination with sulindac. We also observed that sulindac was able to fully correcting the pro-inflammatory effect induced by inhibition of CFTR with inh172 in the CFBE-Wt

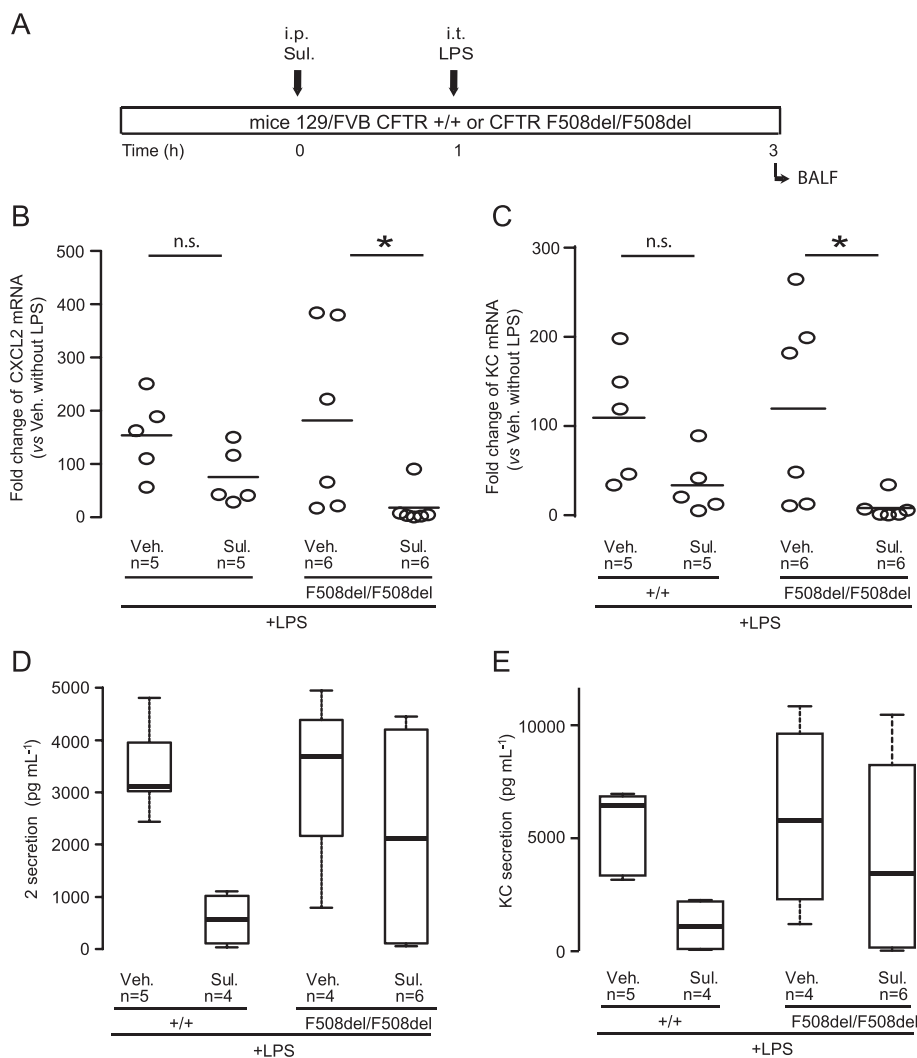


Figure 9

Sulindac decreases LPS-induced pro-inflammatory cytokines in BALF of CF mice. (A) *In vivo* experimental protocol of lung inflammation. (B) CXCL2 and (C) KC mRNA were quantified in lung. Results were normalized to the amount of 18S mRNA. Bars represent the mean. (D) CXCL2 and (E) KC protein were quantified in BALF. Graphs represent box plot of raw data showing median, inter quartile range, minimum and maximum values. * $P < 0.05$, Veh. significantly different from Sul.

cells treated with the CytoMix. This may suggest an overlap between the signaling pathways involved in CFTR anti-inflammatory activity and the MOA of sulindac. Ibuprofen has been shown to either inhibit or activate CFTR channel activity (Devor and Schultz, 1998; Tondelier *et al.*, 1999; Li *et al.*, 2008) and more recently to rescue F508del-CFTR trafficking (Carlile *et al.*, 2015). It thus would be interesting to investigate the effect of sulindac on CFTR processing and activity.

Altogether, our data demonstrate a COX-independent, anti-inflammatory effect of sulindac. Taken together, these features demonstrate that sulindac is a good therapeutic candidate for CF therapy. Furthermore, recent studies have made it possible to consider a real improvement of drugs through a lead optimization phase. The objective of this phase is to synthesize lead compounds, new analogues with improved potency, physicochemical/metabolic properties and reduced

off-target activities. As already performed in cancer research studies (Zhou *et al.*, 2010), a screen of sulindac analogues could permit the identification of molecules that are safer and more potent in their effect on the NF- κ B pathway and/or IL-8, in the context of CF therapeutic applications. In addition, the lung bioavailability of sulindac could be improved via an optimized mode of administration such as inhalation, an approach that has recently been validated for other NSAIDs (Stigliani *et al.*, 2013).

In conclusion, our results have shown that sulindac inhibited NF- κ B and IL-8 by a COX-independent mechanism and that this drug could be beneficially employed in CF. Sulindac may be chemically improved for this new application. If further clinical studies support our conclusions, and because the molecule has already been approved by the Food and Drug Administration, we should expect its use to be more readily applicable to the treatment of patients.

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Author contributions

J.R., A.T. conceived and designed the experiments; J.R., A.H., S.M., W.V.-M., A.T. performed the experiments; A.H., S.M., A.T., VPE provided new techniques; A.A., J.R., A.T., analysed the data; J.R., A.T., P.F. drafted the manuscript; A.W.-H, R.E., A.A., A.T. revised the paper; A.T. and P.F. supervised the project.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

References

- Alexander SPH, Peters JA, Kelly E, Marrion N, Benson HE, Faccenda E *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: Other ion channels. *Br J Pharmacol* 172: 5942–5955.
- Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015b) The Concise Guide to PHARMACOLOGY 2015/16: Enzymes. *Br J Pharmacol* 172: 6024–6109.
- Babbar N, Ignatenko NA, Jr. Casero RA, Gerner EW (2003). Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J Biol Chem* 278: 47762–47775.
- Carlile GW, Robert R, Goepf J, Matthes E, Liao J, Kus B *et al.* (2015). Ibuprofen rescues mutant cystic fibrosis transmembrane conductance regulator trafficking. *J Cyst Fibros* 14: 16–25.
- Chan TA, Morin PJ, Vogelstein B, Kinzler KW (1998). Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc Natl Acad Sci U S A* 95: 681–686.
- Clain J, Fritsch J, Lehmann-Che J, Bali M, Arous N, Goossens M *et al.* (2001). Two mild cystic fibrosis-associated mutations result in severe cystic fibrosis when combined in cis and reveal a residue important

for cystic fibrosis transmembrane conductance regulator processing and function. *J Biol Chem* 276: 9045–9049.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. *Br J Pharmacol* 172: 3461–3471.

Dauletbaev N, Lam J, Eklove D, Iskandar M, Lands LC (2010). Ibuprofen modulates NF- κ B activity but not IL-8 production in cystic fibrosis respiratory epithelial cells. *Respiration* 79: 234–242.

de Becdelievre A., Rocca J., Aissat A., Drevillon L, Moutereau S, Le Gouvello S *et al.* (2013). COMMD1 modulates noxious inflammation in cystic fibrosis. *Int J Biochem Cell Biol* 45: 2402–2409.

Devor DC, Schultz BD (1998). Ibuprofen inhibits cystic fibrosis transmembrane conductance regulator-mediated Cl⁻ secretion. *J Clin Invest* 102: 679–687.

Duggan DE (1981). Sulindac: therapeutic implications of the prodrug/pharmacophore equilibrium. *Drug Metab Rev* 12: 325–337.

Duggan DE, Hooke KF, Hwang SS (1980). Kinetics of the tissue distributions of sulindac and metabolites. Relevance to sites and rates of bioactivation. *Drug Metab Dispos* 8: 241–246.

Duggan DE, Hooke KF, Noll RM, Hucker HB, Van Arman CG (1978). Comparative disposition of sulindac and metabolites in five species. *Biochem Pharmacol* 27: 2311–2320.

Edwards MR, Mukaida N, Johnson M, Johnston SL (2005). IL-1 β induces IL-8 in bronchial cells via NF- κ B and NF-IL6 transcription factors and can be suppressed by glucocorticoids. *Pulm Pharmacol Ther* 18: 337–345.

Fanen P, Wohlhuter-Haddad A, Hinzpeter A (2014). Genetics of cystic fibrosis: CFTR mutation classifications toward genotype-based CF therapies. *Int J Biochem Cell Biol* 52: 94–102.

Gruenert DC, Willems M, Cassiman JJ, Frizzell RA (2004). Established cell lines used in cystic fibrosis research. *J Cyst Fibros* 3 Suppl 2: 191–196.

Han EK, Arber N, Yamamoto H, Lim JT, Delohery T, Pamukcu R *et al.* (1998). Effects of sulindac and its metabolites on growth and apoptosis in human mammary epithelial and breast carcinoma cell lines. *Breast Cancer Res Treat* 48: 195–203.

Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M (2002). Multiple control of interleukin-8 gene expression. *J Leukoc Biol* 72: 847–855.

Huang WH, Shao L, Li SX, Guo D, Wang LS, Zhi Li Z *et al.* (2014). Simultaneous determination of sulindac and its metabolites sulindac sulfide and sulindac sulfone in human plasma by a sensitive UPLC-PDA method for a pharmacokinetic study. *Anal Meth* 6: 4679–4685.

Hunter MJ, Treharne KJ, Winter AK, Cassidy DM, Land S, Mehta A (2010). Expression of wild-type CFTR suppresses NF- κ B-driven inflammatory signalling. *PLoS One* 5: e11598.

Illek B, Maurisse R, Wahler L, Kunzelmann K, Fischer H, Gruenert DC (2008). Cl transport in complemented CF bronchial epithelial cells correlates with CFTR mRNA expression levels. *Cell Physiol Biochem* 22: 57–68.

Jacquot J, Tabary O, Le Rouzic P, Clement A (2008). Airway epithelial cell inflammatory signalling in cystic fibrosis. *Int J Biochem Cell Biol* 40: 1703–1715.

Jarvis MC, Gray TJ, Palmer CN (2005). Both PPAR γ and PPAR δ influence sulindac sulfide-mediated p21WAF1/CIP1 upregulation in a human prostate epithelial cell line. *Oncogene* 24: 8211–8215.

Jungas T, Motta I, Duffieux F, Fanen P, Stoven V, Ojcius DM (2002). Glutathione levels and BAX activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic

- fibrosis transmembrane conductance regulator. *J Biol Chem* 277: 27912-27918.
- Kapetanovic IM, Krishnaraj R, Martin-Jimenez T, Yuan L, van Breemen RB, Lyubimov A. (2006). Effects of oral dosing paradigms (gavage versus diet) on pharmacokinetics and pharmacodynamics. *Chem Biol Interact* 164: 68-75.
- Keiser NW, Birket SE, Evans IA, Tyler SR, Crooke AK, Sun X *et al.* (2015). Defective innate immunity and hyperinflammation in newborn cystic fibrosis transmembrane conductance regulator-knockout ferret lungs. *Am J Respir Cell Mol Biol* 52: 683-694.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. *Br J Pharmacol* 160:1577-1579.
- Koehler DR, Downey GP, Sweezey NB, Tanswell AK, Hu J (2004). Lung inflammation as a therapeutic target in cystic fibrosis. *Am J Respir Cell Mol Biol* 31: 377-381.
- Konstan MW, Hoppel CL, Chai BL, Davis PB (1991). Ibuprofen in children with cystic fibrosis: pharmacokinetics and adverse effects. *J Pediatr* 118: 956-964.
- Konstan MW, Skchluchter MD, Xue W, Davis PB (2007). Clinical use of Ibuprofen is associated with slower FEV1 decline in children with cystic fibrosis. *Am J Respir Crit Care Med* 176: 1084-1089.
- Kopeikin Z, Sohma Y, Li M, Hwang TC (2010). On the mechanism of CFTR inhibition by a thiazolidinone derivative. *J Gen Physiol* 136: 659-671.
- Kunzelmann K, Schwiebert EM, Zeitlin PL, Kuo WL, Stanton BA, Gruenert DC (1993). An immortalized cystic fibrosis tracheal epithelial cell line homozygous for the delta F508 CFTR mutation. *Am J Respir Cell Mol Biol* 8: 522-529.
- Lands LC, Stanojevic S (2007). Oral non-steroidal anti-inflammatory drug therapy for cystic fibrosis. *Cochrane Database Syst Rev*: CD001505.
- Li J, Xiang YY, Ye L, Tsui LC, Macdonald JF, Hu J *et al.* (2008). Nonsteroidal anti-inflammatory drugs upregulate function of wild-type and mutant CFTR. *Eur Respir J* 32: 334-343.
- Li X, Gao L, Cui Q, Gary BD, Dyess DL, Taylor W *et al.* (2012). Sulindac inhibits tumor cell invasion by suppressing NF-kappaB-mediated transcription of microRNAs. *Oncogene* 31: 4979-4986.
- Loveridge CJ, MacDonald AD, Thoms HC, Dunlop MG, Stark LA (2008). The proapoptotic effects of sulindac, sulindac sulfone and indomethacin are mediated by nucleolar translocation of the RelA (p65) subunit of NF-kappaB. *Oncogene* 27: 2648-2655.
- Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galiotta LJ *et al.* (2002). Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 110: 1651-1658.
- Mackenzie GG, Sun Y, Huang L, Xie G, Ouyang N, Gupta RC *et al.* (2010). Phospho-sulindac (OXT-328), a novel sulindac derivative, is safe and effective in colon cancer prevention in mice. *Gastroenterology* 139: 1320-1332.
- Matute-Bello G, Frevert CW, Martin TR (2008). Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 295: L379-399.
- McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br J Pharmacol* 172: 3189-3193
- Melis N, Tauc M, Cougnon M, Bendahhou S, Giuliano S, Rubera I *et al.* (2014). Revisiting CFTR inhibition: a comparative study of CFTRinh-172 and GlyH-101 inhibitors. *Br J Pharmacol* 171: 3716-3727.
- Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SPH, Buneman OP *et al.*, NC-IUPHAR(2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. *Nucl Acids Res* 42 (Database Issue): D1098-D1106.
- Perez A, Issler AC, Cotton CU, Kelley TJ, Verkman AS, Davis PB (2007). CFTR inhibition mimics the cystic fibrosis inflammatory profile. *Am J Physiol Lung Cell Mol Physiol* 292: L383-395.
- Piazza GA, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T *et al.* (1997). Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 57: 2909-2915.
- Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ *et al.* (2008). Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* 321: 1837-1841.
- Soh JW, Mao Y, Kim MG, Pamukcu R, Li H, Piazza GA *et al.* (2000). Cyclic GMP mediates apoptosis induced by sulindac derivatives via activation of c-Jun NH2-terminal kinase 1. *Clin Cancer Res* 6: 4136-4141.
- Stigliani M, Aquino RP, Del Gaudio P, Mencherini T, Sansone F, Russo P (2013). Non-steroidal anti-inflammatory drug for pulmonary administration: design and investigation of ketoprofen lysinate fine dry powders. *Int J Pharm* 448: 198-204.
- Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ *et al.* (2010). Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* 2: 29ra31.
- Taylor-Cousar JL, Von Kessel KA, Young R, Nichols DP (2010). Potential of anti-inflammatory treatment for cystic fibrosis lung disease. *J Inflamm Res* 3: 61-74.
- Tondelier D, Brouillard F, Lipecka J, Labarthe R, Bali M, Costa de Beauregard MA *et al.* (1999). Aspirin and some other nonsteroidal anti-inflammatory drugs inhibit cystic fibrosis transmembrane conductance regulator protein gene expression in T-84 cells. *Mediators Inflamm* 8: 219-227.
- van Doorninck JH, French PJ, Verbeek E, Peters RH, Morreau H, Bijman J *et al.* (1995). A mouse model for the cystic fibrosis delta F508 mutation. *EMBO J* 14: 4403-4411.
- Van Goor F, Hadida S, Grootenhuis PD, Burton B, Stack JH, Straley KS *et al.* (2011). Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A* 108: 18843-18848.
- Veit G, Bossard F, Goepf J, Verkman AS, Galiotta LJ, Hanrahan JW *et al.* (2012). Proinflammatory cytokine secretion is suppressed by TMEM16A or CFTR channel activity in human cystic fibrosis bronchial epithelia. *Mol Biol Cell* 23: 4188-4202.
- Verma R, Brahmankar M, Kushwah L, Suresh B (2013). Evaluating the inhibitory potential of sulindac against the bleomycin-induced pulmonary fibrosis in wistar rats. *Environ Toxicol Pharmacol* 36: 769-778.
- Wentz SC, Yip-Schneider MT, Gage EA, Saxena R, Badve S, Schmidt CM (2009). Sulindac prevents carcinogen-induced intrahepatic cholangiocarcinoma formation in vivo. *J Surg Res* 157: e87-95.
- Yamamoto Y, Yin MJ, Lin KM, Gaynor RB (1999). Sulindac inhibits activation of the NF-kappaB pathway. *J Biol Chem* 274: 27307-27314.
- Yip-Schneider MT, Wu H, Ralstin M, Yiannoutsos C, Crooks PA, Neelakantan S *et al.* (2007). Suppression of pancreatic tumor growth by combination chemotherapy with sulindac and LC-1 is associated with cyclin D1 inhibition in vivo. *Mol Cancer Ther* 6: 1736-1744.

Zhou H, Liu W, Su Y, Wei Z, Liu J, Kolluri SK *et al.* (2010). NSAID sulindac and its analog bind RXRalpha and inhibit RXRalpha-dependent AKT signaling. *Cancer Cell* 17: 560-573.

Zhu YM, Azahri NS, Yu DC, Woll PJ (2008). Effects of COX-2 inhibition on expression of vascular endothelial growth factor and interleukin-8 in lung cancer cells. *BMC Cancer* 8: 218.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13464>

Figure S1 Sulindac and ibuprofen did not inhibit NF- κ B transcriptional activity in HeLa-WT and HeLa-F508del cells under basal conditions.

Figure S2 Sulindac reduces NF- κ B activity after I κ B degradation.

Figure S3 Sulindac decreases LPS-induced pro-inflammatory cytokine mRNA in the lung of CF mice.

Figure S4 Sulindac inhibits IL-8 expression.