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RESEARCH PAPER

Differential effects of p38MAP kinase inhibitors on the expression of inflammation-associated genes in primary, interleukin-1β-stimulated human chondrocytes

H Joos[1], W Albrecht[2], S Laufer[3] and RE Brenner[1]

[1]*Division for Biochemistry of Joint and Connective Tissue Diseases, Department of Orthopedics, University of Ulm, Ulm, Germany,* [2]*c-a-i-r biosciences GmbH, Tübingen, Germany, and* [3]*Institute of Pharmacy, Department of Pharmaceutical and Medicinal Chemistry, Eberhard-Karls University Tübingen, Tübingen, Germany*

Background and purpose: A main challenge in the therapy of osteoarthritis (OA) is the development of drugs that will modify the disease. Reliable test systems are necessary to enable an efficient screening of therapeutic substances. We therefore established a chondrocyte-based *in vitro* cell culture model in order to characterize different p38MAPK inhibitors.
Experimental approach: Interleukin-1β (IL-1β)-stimulated human OA chondrocytes were treated with the p38MAPK inhibitors Birb 796, pamapimod, SB203580 and the new substance CBS-3868. Birb 796- and SB203580-treated cells were analysed in a genome-wide microarray analysis. The efficacy of all inhibitors was characterized by quantitative gene expression analysis and the quantification of PGE_2 and NO release.
Key results: Microarray analysis revealed inhibitor-specific differences in gene expression. Whereas SB203580 had a broad effect on chondrocytes, Birb 796 counteracted the IL-1β effect more specifically. All p38MAPK inhibitors significantly inhibited the IL-1β-induced gene expression of COX-2, mPGES1, iNOS, matrix metalloproteinase 13 (MMP13) and TNFRSF11B, as well as PGE_2 release. Birb 796 and CBS-3868 showed a higher efficacy than SB203580 and pamapimod at inhibiting the expression of COX-2 and MMP13 genes, as well as PGE_2 release. In the case of mPGES1 and TNFRSF11B gene expression, CBS-3868 exceeded the efficacy of Birb 796.
Conclusions and implications: Our test system could differentially characterize inhibitors of the same primary pharmaceutical target. It reflects processes relevant in OA and is based on chondrocytes that are mainly responsible for cartilage degradation. It therefore represents a valuable tool for drug screening in between functional *in vitro* testing and *in vivo* models.
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Keywords: chondrocytes; interleukin 1β; *in vitro* model; osteoarthritis; p38MAPK inhibition; whole-genome array; Birb 796; pamapimod; SB203580

Abbreviations: IL-1β, interleukin-1β; iNOS, inducible NOS; MAPK, mitogen-activated protein kinase; MMP13, matrix metalloproteinase 13; mPGES1, prostaglandin E synthase; PGE_2, prostaglandin E_2; TNFRSF11B, osteoprotegerin

Introduction

The central role of p38MAP kinases (p38MAPK), foremost the α-isoform, in the production of inflammatory response proteins such as TNF-α, interleukin-1β (IL-1β), COX-2 and microsomal prostaglandin E synthase (mPGES1) is well documented (Masuko-Hongo *et al.*, 2004; Schieven, 2005). Activated p38α MAPK up-regulates cytokine production by several independent mechanisms, including direct phosphorylation of transcription factors, and direct or indirect stabilization and increased translation of mRNAs by phosphorylation of adenylate/uridylate-rich element-binding proteins (Ashwell, 2006). Since its identification as a protein that binds cytokine-suppressing anti-inflammatory molecules (Lee *et al.*, 1994), p38α MAPK has been considered to be an attractive target for drug-mediated modulation of inflammatory processes. Many small molecules have been described in the scientific literature and in patent application, and a few have been clinically developed as a treatment for conditions such as rheumatoid arthritis (RA), Crohn's disease or psoriasis

Correspondence: Dr Rolf E. Brenner, Division for Biochemistry of Joint and Connective Tissue Diseases, Department of Orthopedics, University of Ulm, Oberer Eselsberg 45, 89081 Ulm, Germany. E-mail: rolf.brenner@uni-ulm.de
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(Dominguez *et al*., 2005; Genovese, 2009). Most drug discovery programmes have focused on the inhibition of the α-form, but essentially all p38α MAPK inhibitors also interact with the β-isoform. However, recently published results of clinical studies, which investigated the efficacy of pamapimod (Cohen *et al*., 2009; Alten *et al*., 2010) and VX-702 (Damjanov *et al*., 2009) for treatment of RA, were disappointing. During a 12 week treatment of patients with p38α/β MAPK inhibitor either alone or in combination with methotrexate, a significant benefit was not observed. The reasons for this failure of p38α/β MAPK inhibitors in clinical studies are unknown and somehow surprising as they generally show good efficacy in experimental models of arthritis and in clinical pharmacodynamic studies (Sweeney, 2009). Systemically, after intravenous LPS stimulation in healthy subjects, a dose-dependent inhibition of TNF-α release following a single administration of the earlier clinical candidates doramapimod (Birb 796) and RWJ-67657 was observed (Fijen *et al*., 2001; Branger *et al*., 2002).

Based on the outcome of the RA proof-of-concept studies, it was hypothesized that biological adaptations allow the re-constitution of the inflammatory process by bypassing the p38α-signalling pathway (Genovese, 2009). Another not-yet-explored explanation relates to different cell- and tissue-specific potencies of drugs. For example, the p38α/β MAPK inhibitors SB239063 and SD-282 (Smith *et al*., 2006), as well as RWJ-67657 (Westra *et al*., 2004), exhibited different potencies regarding the inhibition of LPS-induced cytokine release in monocytes and macrophages (Smith *et al*., 2006). Similar results were obtained when the efficacy of p38α/β MAPK inhibitors was investigated by high-content analysis in SW1353 chondrocytes and baby hamster kidney cells (Ross *et al*., 2006).

Tissue-specific differences may play an important role in diseases such as RA and osteoarthritis (OA), where articular chondrocytes significantly contribute to the overall pathophysiology. A potent and sustained inhibition of inflammatory processes in this compartment might be pivotal for the efficacy of p38α/β MAPK inhibitors, and therefore, a suitable and reliable *in vitro* chondrocyte model may deliver important information for defining the molecular properties required of clinical candidates.

The relevance of p38α MAPK signalling in chondrocytes is well documented. Experimental data on the effect of extracellular stimuli such as IL-1β or TNF-α, however, indicate that the other members of the MAP kinase family, the extracellular regulated kinases ERK1/2 and the c-Jun terminal kinases JNK1/2, become activated and contribute to the release of pro-inflammatory mediators (Nieminen *et al*., 2005). To address the complex interactions in chondrocyte signalling and its assumed relevance for the anti-arthritic efficacy of p38α/β MAPK inhibitors, a global gene expression analysis in primary human chondrocytes after stimulation with IL-1β, in the absence and presence of SB203580 (Joos *et al*., 2009) or Birb 796, was performed. Many genes that were up-regulated by IL-1β and counter-regulated by the inhibitors were identified (Joos *et al*., 2009). To characterize the pharmacological profile of different p38α/β inhibitors in IL-1β-stimulated chondrocytes, based on the microarray analysis, a panel of genes was selected and quantitative real-time PCR assays were developed. In the present paper, the effects of different p38α/β inhibitors on the expression of selected genes are presented, and the potential relevance of this model as a screening tool that specifically addresses OA-relevant processes is discussed.

Methods

Cartilage samples

Human osteoarthritic cartilage was obtained from donors undergoing total knee joint replacement due to OA; informed consent was obtained from the patients according to the terms of the Ethics Committee of the University of Ulm. Overall, tissue samples from 30 patients were included in the study; the mean age of the donors was 66 ± 8 years.

Cell culture

Well-preserved cartilage from femoral condyles was used for chondrocyte isolation as described previously (Joos *et al*., 2008). The cartilage was minced and digested for 45 min with 9 U·mL^{-1} Pronase (Sigma-Aldrich, Munich, Germany), and for 14 h with 80 U·mL^{-1} Collagenase type IV (Sigma-Aldrich). After being washed and filtered, the isolated cells were cultivated in complete medium consisting of 1:1 DMEM/Hams F12 supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 0.5% L-glutamine and 10 μg·mL^{-1} 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Fluka, Seelze, Germany). After 24 h incubation, adhered chondrocytes were treated with trypsin (trypsin/EDTA 0.05%/0.02% in PBS) and frozen in complete medium containing 5% dimethyl sulphoxide (DMSO, Roth, Karlsruhe, Germany). All chemicals were obtained from Biochrom, Berlin, Germany, unless indicated otherwise.

Cell stimulation and treatment with inhibitor

For all experiments, thawed cells were cultivated for 1–3 days, treated with trypsin and pooled as indicated below and seeded at a density of 5×10^4 cells cm^{-2} in complete medium. For the microarray analysis, 3.8×10^6 cells were used per experiment, for the other experiments, 1.8×10^5 cells per batch were applied. After 24 h of adherence, they were silenced for 24 h in serum-free medium [DMEM containing 0.5% penicillin/streptomycin, 0.5% L-glutamine, 1% non-essential amino acids 100-fold concentrate, 1 mM pyruvate, 0.1% SES1 solution A (supplement for serum-free media) and 0.1% freshly added SES1 solution B (supplement containing insulin for serum-free media); all from Biochrom]. Cells were stimulated for the indicated time with 10 ng·mL^{-1} rhIL-1β (tebu-bio, Offenbach, Germany) in serum-free medium. Inhibitor-treated cells were incubated for 15 min prior to stimulation and subsequently co-incubated with 10 ng·mL^{-1} rhIL-1β and inhibitor, with a final DMSO concentration of 0.1% in the cultivation medium, for the indicated time span. For comparability, the same amount of DMSO was added to control cells. The inhibitors used were CBS-3868 ([4-[6-(4-fluoro-phenyl)-1-oxo-2,3-dihydro-1H-1lambda*4*-imidazo [2,1-b]thiazol-5-yl]-pyridin-2-yl]-((*S*)-1-phenyl-ethyl)-amine),

Table 1 Specificities of the inhibitors for p38α MAPK and other kinases

Gene symbol	*Kinase target*	K_d *(nM)*			*CBS-3868**
		Birb 796	*SB203580*	*Pamapimod*	
ABL1	ABL1(T315I)	42			
CSNK1D	CSNK1D		37		
CSNK1E	CSNK1E		100	260	
DDR1	DDR1	1.9	1000		
DDR2	DDR2	33	5000		
GAK	GAK		19		
MAPK8	JNK1		1100	190	
MAPK9	JNK2	7.3	130	16	×
MAPK10	JNK3	110	35	19	×
MAP4K4	MAP4K4	90	3700		
MAPK11	p38 Beta	1500	70	120	×
MAPK12	p38 Gamma	19	1500		
MAPK14	p38 alpha	0.37	12	1.3	×
NLK	NLK	1000	25	170	
RIPK2	RIPK2		24		
STK10	LOK	12			
TEK	TIE2	20			
TIE1	TIE1	8.3			

K_d values are according to Karaman *et al.* (2008; Birb 796 and SB203580) and Hill *et al.* (2008; pamapimod). Only those kinases at which at least one inhibitor shows a K_d < 100 nM are listed.
*For CBS-3868, no K_d values were determined, but the inhibitor shows a relevant affinity for the indicated kinases (personal communications).

Birb 796 (1-(5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)-naphthalen-1-yl]urea), pamapimod (pyrido[2,3-d]pyrimidin-7(8H)-one, 6-(2,4-difluorophenoxy)-2 - [[3 - hydroxy - 1 - (2 - hydroxyethyl)propyl] amino]-8-methyl-) and SB203580 (4 - (4 - fluorophenyl) - 2 - (4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) (all provided by c-a-i-r biosciences GmbH, Tübingen, Germany). The kinase interactions of these p38 inhibitors are given in Table 1. At the end of the stimulation period, cells were washed twice in sterile PBS (PAA Laboratories, Egelsbach, Germany) and lysed in 600 µL lysis buffer RLT (Qiagen, Hilden, Germany) per 10^6 cells.

Microarray experiment
To obtain enough RNA for the microarray experiment, cells of six different donors were pooled after they had been thawed and treated as described above. After cell lysis, a whole human genome oligo-microarray (Human Genome Oligo-Set-Version 2.0, Operon, Ebersberg, Germany), representing 21 329 genes, was conducted at the Chip Facility of Ulm according to Buchholz *et al*. (2005). The experiment was performed in triplicate with six different donors each. By the use of this experimental design with biological replication, we could assess biological variation in spite of the need for pooling different donors (Kendziorski *et al*., 2005).

GoMiner analysis
Genes that showed at least a twofold regulation and a significance level of $P < 0.05$ in the microarray analysis were assigned to Gene Ontologies by an analysing tool called GoMiner (http://discover.nci.nih.gov/gominer/) (Zeeberg *et al*., 2003). The Gene Ontology (GO) consortium offers three main ontologies, namely 'biological process', 'cellular component' and 'molecular function', subsuming subsequent terms that are organized in a tree-like structure. We used the ontology 'biological process'. In brief, given a set of regulated genes, the set of all unique GO terms within the ontology was first identified that was associated with one or more of these genes. Next, the number of the regulated genes and the number of the genes that were assayed (all the genes represented on the microarray) were annotated at each term (Zeeberg *et al*., 2003).

Isolation of mRNA and cDNA synthesis
For isolating RNA from cultured cells, the RNeasy mini kit (Qiagen) was used according to the manufacturer's instructions. Briefly, the cell lysate was mixed 1:1 with 70% ethanol, loaded on a mini column and, after several washing steps and DNase digestion, the RNA was eluted in 30 µL of RNase free water. cDNA was synthesized with Omniscript RT (Qiagen) in accordance with the manufacturer's instructions using 12 µL of RNA solution.

Real-time PCR
Quantitative real-time PCR was used to detect human COX-2, mPGES1, iNOS, matrix metalloproteinase 13 (MMP13) and TNFRSF11B mRNA in human articular chondrocytes. The primers (Table 2) were designed using Primer 3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) except for the MMP13 primer which was assumed from literature (Bau *et al*., 2002). A gene-specific cDNA fragment was amplified for each gene, using the specified primers (Table 2), and sequenced with the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions, on a fluorescent automated DNA sequencer (ALFexpress, Amersham Bio-

Table 2 Sequences of primers used for quantitative real-time PCR

Gene	*GenBank*	*Amplification*	*Forward*	*Reverse*
symbol	*accession no*	*length (bases)*	*5′ 3′*	*5′ 3′*
18SrRNA	X03205	65	cgcagctaggaataatggaatagg	catggcctcagttccgaaa
COX-2	NM_000963	123	cccttgggtgtcaaaggtaa	ggcaaagaatgcaaacatca
MMP13	NM_002427	97	tcctcttcttgagctggactcatt	cgctctgcaaactggaggtc
iNOS	NM_000625	117	attcactcagctgtgcatcg	tcaggtgggatttcgaagag
TNFRSF11B	NM_002546	117	ggcaacacagctcacaagaa	cgctgttttcacagaggtca
mPGES1	NM_004878	155	cccccagtattgcaggag	ggaagaccaggaagtgcatc

sciences) to confirm the correct amplification products of the specific primers. Amplifications were done with an AbiPrism 7000 system (Applera, Darmstadt, Germany). For all the genes analysed, the Power SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used according to the manufacturer's instructions, except for MMP13, for which Invitrogen Platinum SYBR qPCR SuperMix UDG (Invitrogen, Karlsruhe, Germany) was utilized. 18S-rRNA was used as endogenous control; the concentration of primers applied was 1 μM (18S-rRNA, COX-2, MMP13 and TNFRSF11B) or 0.3 μM (mPGES1 and iNOS).

PGE_2 and NO assays

Absolute concentrations of nitrite, a stable end product of NO metabolism, were determined in the media of the cell culture using a spectrophotometric method based on the Griess assay (Griess Reagent System, Promega, Mannheim, Deutschland) according to the manufacturer's instructions. Absorbance was measured at 550 nm, and nitrite concentration was determined by comparison with standard solutions of sodium nitrite.

PGE_2 production was measured in the media by a high sensitivity, commercially available enzyme immunoassay kit (PGE_2 ELISA Correlate EIA, Biotrend, Assay Designs, Köln, Germany) according to the manufacturer's instructions. The sensitivity was 13 $pg \cdot mL^{-1}$. PGE_2 concentration was determined in duplicate and was read against a standard curve.

Determination of % inhibition and IC_{50} values

The results of quantitative real-time PCR and metabolite determination were used to calculate % inhibition normalized to stimulatability of the cells according to the following formula:

$$H_{pn} = 100 - 100\,(v_I - v_C)/(v_S - v_C)$$

with H_{pn} = normalized percentage inhibition, v_C = value of control, v_S = value of stimulation and v_I = value of inhibition probe.

The IC_{50} values were determined by the two-point form of a linear equation. Given the lowest inhibitor concentration (x_1) with which inhibition of more than 50% was achieved, and the highest inhibitor concentration (x_2) with which an inhibition of less than 50% was achieved, the IC_{50} value was calculated accordingly as follows:

$$IC_{50} = (x_1 - x_2)(50\% - y_1)/(y_1 - y_2) + x_1$$

with $y_1 = H_{pn}$ in x_1 and $y_2 = H_{pn}$ in x_2.

Nomenclature

The nomenclature of genes and molecular targets conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Statistical analysis

For the microarray results, a print-tip loess normalization according to Buchholz *et al*. (2005) and a moderated *t*-test (Smyth, 2004) were performed at the Chip Facility of Ulm. In the GoMiner analysis, the two-sided Fisher's exact test evaluated whether there were more genes of interest at the term than one might expect by chance (Zeeberg *et al*., 2003). For standardization of the gene expression levels determined by TaqMan analysis, mRNA expression was normalized to 18SrRNA expression. Differential regulation was determined by calculating the ratios of gene expression in different treatments. A two-tailed paired *t*-test of the calculated ratios was performed to evaluate significant differences from the relative control treatment; as always, identical donors were compared.

Results

Control of cell phenotype and viability

The OA cartilage used for the experiments, macroscopically, had a smooth surface and no severe osteoarthritic changes. As described elsewhere (Joos *et al*., 2008), analysis of collagen type II, aggrecan and cartilage oligomeric matrix protein expression confirmed a stable differentiation stage of the chondrocytes during the experimental period. Trypan blue staining showed comparable cell viability with and without IL-1β stimulation.

Microarray experiment

The first objective was to characterize the effects of p38MAPK inhibition on global gene expression in IL-1β-stimulated human chondrocytes. To this end, three chondrocyte cultures, each composed of cells from six different donors, were stimulated with 10 $ng \cdot mL^{-1}$ IL-1β in the presence or absence of 10 μM SB203580 and 10 μM Birb 796 (Doramapimod) respectively. After 24 h of stimulation, RNA was isolated and

subjected to a whole human genome oligo-microarray analysis (Human Genome Oligo-Set-Version 2.0, Operon) as described previously (Joos *et al*., 2008). All microarray data have been deposited on ArrayExpress (http://www.ebi.ac.uk/microarray-as/aer; Accession number: E-MEXP-1434).

Effects of SB203580 and Birb 796 on whole-genome gene expression

Stimulation of chondrocytes with IL-1β resulted in an up- or down-regulation of the expression of 1141 genes when compared to controls. This IL-1β-induced gene expression profile was used as a reference to analyse the effects of the p38α/β MAPK inhibitors on gene expression. In the presence of SB203580, 646 genes were modulated and 116 thereof (=18%) were co-regulated by IL-1β and SB203580. Most of the co-modulated genes (87%) were regulated in opposite directions, 13% moved unidirectional, whereas most genes were up-regulated. Co-incubation with Birb 796 affected 503 genes with 208 genes (=41%) co-regulated by Birb 796 and IL-1β; 98% of these co-modulated genes were regulated in opposite directions. Among the genes analysed on the microarray, some are hypothetical or unknown. The list of known co-regulated genes with their accession numbers, fold change and *P* values for IL-1β and Birb 796 regulation is shown in Supporting Information Table S1. The genes that were co-regulated by IL-1β and SB203580 have been presented in a previous study (Joos *et al*., 2009).

Effects of SB203580 and Birb 796 on biological processes

For the identification of the biological processes regulated, the genes regulated by IL-1β, SB203580 or Birb 796 were analysed with the GoMiner software tool and classified into biological coherent categories. A Fisher's exact test evaluated GO terms with a significant accumulation of changed genes on all levels of the hierarchical GO tree structure. In the GO 'biological processes', 215, 145 and 58 processes were found to be regulated by IL-1β, Birb 796 and SB203580, respectively (see Supporting Information Tables S2–S4). A comparison of the regulated processes revealed 27 terms that were co-regulated by IL-1β and Birb 796 (including 19% of all Birb 796-regulated genes), and five terms that were co-regulated by IL-1β and SB203580 (including 9% of all SB203580-regulated genes). The terms of the subcategories were topically allocated to main fields. IL-1β mainly affected genes in the field 'response to stimulus' that involved 43 subterms. The main topics in this field were 'immune response', 'response to oxidative stress' and 'cytokine production'. In addition, eight cytoskeleton-associated terms were affected. In contrast, only a few of the SB203580-affected processes could be allocated to main fields. Thirteen terms could be assigned to the field 'response to stimulus', involving three oxidative stress processes. Five terms were associated with the cytoskeleton. Birb 796 influenced the main term 'response to stimulus' with 26 subterms like 'immune response' and 'response to oxidative stress', but revealed no impact on the cytoskeleton. Furthermore, Birb 796 affected 12 processes involved in apoptosis and cell death. A closer look at the genes involved revealed that, for example, death-associated protein kinase 3 and programmed cell death 2 were up-regulated by Birb 796, whereas the anti-apoptotic gene baculoviral IAP repeat-containing 3 was down-regulated.

Cluster analysis of regulated genes

Cluster analysis of the microarray data was used to study the gene expression patterns of IL-1β- and p38MAPK inhibitor-treated chondrocytes. Microarray data were assigned to the software tool 'Genesis' in order to perform a hierarchical clustering. Tools for visualization of the gene expression data allowed us to identify 334 genes that were up-regulated by IL-1β and differentially regulated by SB203580 and/or Birb 796. A possible role in RA and OA was ascribed to 35 of those genes, which are listed in Supporting Information Table S5. In order to investigate pathophysiological parameters of OA with widely accepted relevance for *in vivo* models, COX-2, MMP13, inducible NOS (iNOS) and TNFRSF11B (osteoprotegerin) were chosen as panel of genes for further quantitative analyses. They are all actively involved in the pathogenesis of OA and RA, and are expected to correlate with the course of the disease. COX-2 and iNOS are involved in the synthesis of inflammatory mediators, MMP13 is a major catabolic protease in OA and osteoprotegerin has been shown to play a role in the progression of OA (Schieven, 2005; Goldring and Goldring, 2007; Schett *et al*., 2008; Kwan Tat *et al*., 2009). The expression of these genes may be used to distinguish different p38α MAPK inhibitors and may form a suitable test system for inhibitor characterization.

Quantitative characterization of p38α/β MAPK inhibitors

For inhibitor characterization, the gene expression of COX-2, MMP13, iNOS and TNFRSF11B was quantitatively analysed. In addition to the gene expression of mPGES1, the release of prostaglandin E_2 (PGE_2) was measured as an indicator of the activity of COX-2 and mPGES1 on protein levels. The inhibition of the NO synthesis pathway was further analysed by determination of NO release. To evaluate this test system, several inhibitors were administered to IL-1β-stimulated chondrocytes, and the specified outcome parameters were determined. The tested substances included the three established p38MAPK inhibitors Birb 796, SB203580 and pamapimod, as well as a new p38α/β selective agent under development (CBS-3868).

Effects of p38MAPK inhibitors on PGE_2 synthesis pathway

The effects of the inhibitors on the PGE_2 synthesis pathway are shown in Figure 1. The stimulation of OA chondrocytes increased gene expression of COX-2 after 4 and 24 h by a factor of 30 and 150 respectively. The p38 inhibitors repressed this stimulation, in a concentration-dependent manner, up to 90%. The IC_{50} values were all below 0.1 μM, except for that of SB203580 (IC_{50} = 0.9 μM). The IC_{50} values for all measurements are given in Table 3.

The gene expression of mPGES1 was augmented threefold after 4 h ($P = 0.001$) and 11-fold after 24 h ($P < 0.001$) by IL-1β, respectively. As seen in Figure 1B, co-incubation with p38α/β MAPK inhibitors resulted in an approx. 50% inhibition of the

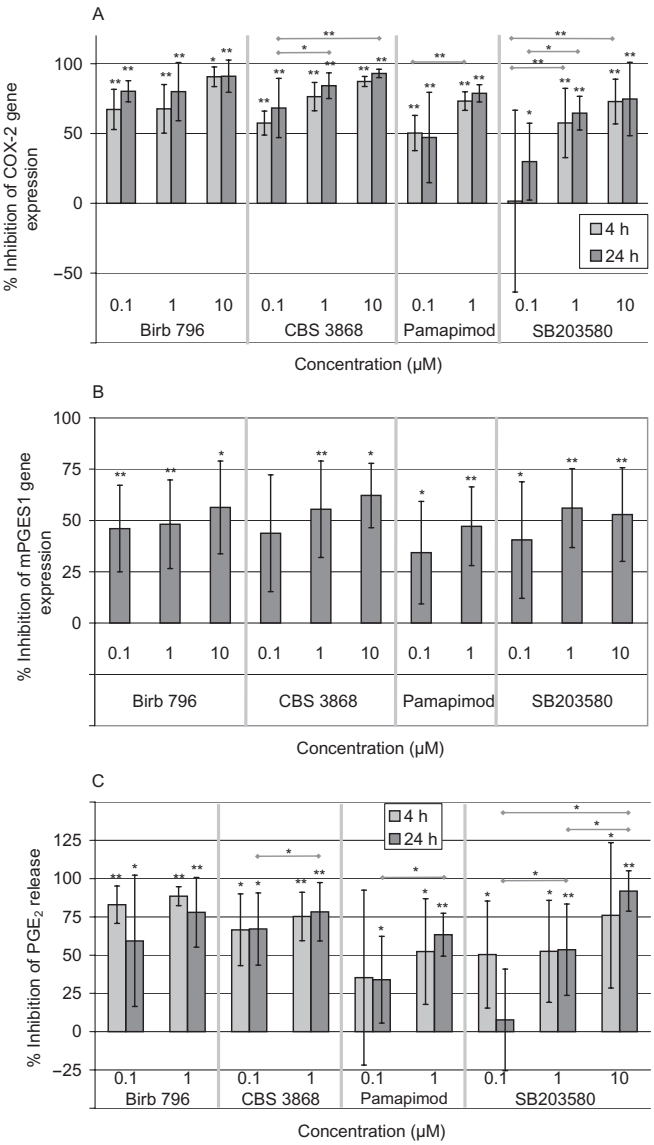

Figure 1 Inhibition of IL-1β-induced PGE_2 synthesis, and gene expression of the enzymes involved, by anti-inflammatory substances. Chondrocytes from two donors were pooled and cultivated with inhibitor and 10 ng·mL^{-1} IL-1β. mRNA was isolated for quantitative gene expression analysis, and PGE_2 levels in the supernatant were determined by ELISA. Data were obtained from at least six independent experiments with 12 different donors, except for 10 μM Birb 796 and 10 μM CBS-3868 where three independent experiments with six different donors were used. (A) % Inhibition of IL-1β-induced COX-2 gene expression after 4 and 24 h. (B) % Inhibition of IL-1β-induced mPGES1 gene expression after 24 h. (C) % Inhibition of IL-1β-induced PGE_2 release after 4 and 24 h. Significant inhibition and concentration-related dependence is marked (*P < 0.05; **P < 0.01).

IL-1β-induced expression with IC_{50} values between 0.6 and 3 μM. The inhibitory effect on mPGES1 gene expression, determined 4 h after chondrocyte stimulation was statistically not significant.

To estimate the activity of the enzymes COX-2 and mPGES1 in IL-1β-treated chondrocytes, the release of their product PGE_2 was measured in the presence and absence of p38 α/β inhibitors. IL-1β stimulation augmented the PGE_2 concentration in the supernatant from 0.9 to 6.0 ng·mL^{-1} after 4 h, and from 1.3 to 11.6 ng·mL^{-1} after 24 h. All tested substances acted as strong inhibitors (Figure 1C) with IC_{50} values below or around 0.1 μM; only pamapimod and SB203580 showed IC_{50} values up to 0.9 μM (Table 3). The effects of all the inhibitors, except for Birb 796, were concentration dependent.

Effects of p38MAPK inhibitors on NO synthesis pathway

To examine the effect of the pharmaceutical agents on the NO synthesis pathway, modulation of iNOS gene expression and NO release was analysed. The results are shown in Figure 2. As NO is rapidly oxidized, nitrite concentration was determined in the supernatant of treated chondrocytes as an indicator for NO production. IL-1β stimulation caused a 250- and 370-fold increase in iNOS gene expression after 4 and 24 h respectively. No significant down-regulation could be detected after 4 h incubation with inhibitor. After 24 h, Birb 796, CBS-3868 and SB203580 caused a significant repression of iNOS gene expression of 50–70% with IC_{50} values between 2 and 10 μM. Nitrite release was increased by IL-1β after 24 h, but not after 4 h from 1.2 to 6.2 μM (P < 0.01). This IL-1β-induced increase in NO was inhibited by high concentrations of the inhibitors, but the effects were not statistically significant. The IC_{50} values were 6 μM except for SB203580 where the IC_{50} > 10 μM (Table 3).

Effects of p38MAPK inhibitors on MMP13 and TNFRSF11B gene expression

In addition, the impact of the anti-inflammatory substances on MMP13 and TNFRSF11B gene expression was examined. MMP13 was threefold (P = 0.08) and 43-fold (P = 0.014) up-regulated by IL-1β after 4 and 24 h respectively. In samples analysed 4 h after IL-1β stimulation, in the presence of CBS-3868, the up-regulation of MMP13 gene expression was significantly inhibited, whereas the other inhibitors had no significant effect. The drug-mediated effects, determined after 24 h, are shown in Figure 3A. At 10 μM, the test compounds inhibited MMP13 expression by 80% to almost 100%. The IC_{50} values of Birb 796 and CBS-3868 were below 0.1 μM, and the IC_{50} values of SB203580 and pamapimod were 0.6 and 0.7 μM, respectively (Table 3). TNFRSF11B was increased three- and fivefold by IL-1β after 4 and 24 h respectively. CBS-3868 (1 μM, P = 0.0103) and pamapimod (1 μM, P = 0.037) inhibited the increase in TNFRSF11B after 4 h significantly. CBS-3868 and SB203580 significantly down-regulated TNFRSF11B gene expression after 24 h (Figure 3B). Birb 796 did not show a significant inhibitory effect at either time-point. IC_{50} values are given in Table 3. In addition, IC_{75} values were determined from all the concentration–effect curves and included in Supporting Information Table S6).

Discussion and conclusions

In the present study, the effect of p38α/β MAPK inhibitors on the expression of inflammation-related genes in IL-1β-stimulated, primary human chondrocytes was investigated. Initially, a genome-wide microarray analysis was performed

Table 3 IC_{50} values of the inhibitors tested for inhibition of gene expression and metabolite release in μM

		Time (h)	*Birb 796*	*CBS-3868*	*Pamapimod*	*SB203580*
Gene expression	COX-2	4	<0.1	<0.1	0.1	0.9
	COX-2	24	<0.1	<0.1	0.2	0.6
	mPGES1	24	3	0.6	1	0.7
	iNOS	24	7	2	>1	10
	MMP13	24	<0.1	<0.1	0.7	0.6
	TNFRSF11B	24	>10	<0.1	>1	0.9
Release	PGE_2	4	<0.1	<0.1	0.9	0.1
	PGE_2	24	<0.1	<0.1	0.6	0.9
	NO	24	6	6	>1	>10

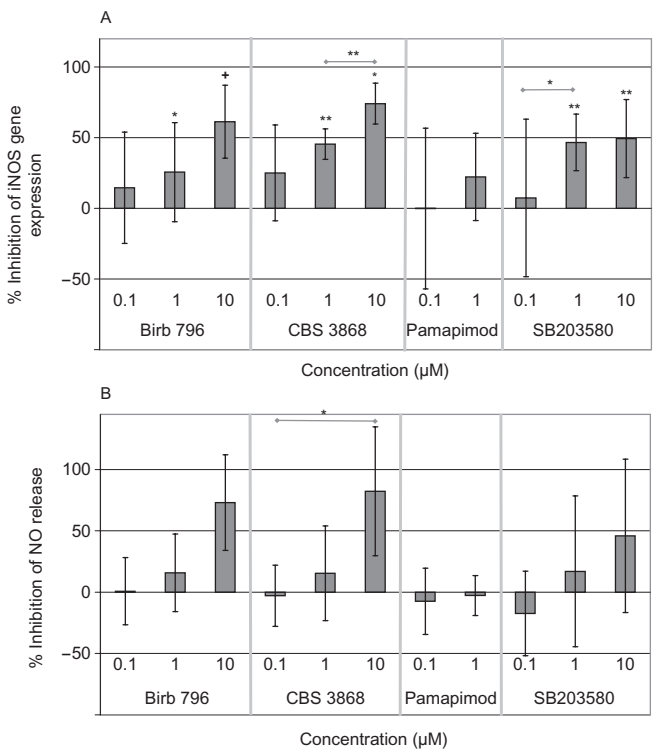

Figure 2 Inhibition of IL-1β-induced iNOS gene expression and NO synthesis by anti-inflammatory substances. Chondrocytes from two donors were pooled and cultivated with inhibitor and 10 ng·mL^{-1} IL-1β. mRNA was isolated for quantitative gene expression analysis, and NO levels in the supernatant were obtained by determination of nitrite concentration with the Griess assay. Data were obtained from at least six independent experiments with 12 different donors, except for 10 μM Birb 796 and 10 μM CBS-3868 where three independent experiments with six different donors were used. (A) % Inhibition of IL-1β-induced iNOS gene expression after 24 h. (B) % Inhibition of IL-1β-induced nitrite release after 24 h. Significant inhibition and concentration-related dependence is marked (+P = 0.054; *P < 0.05; **P < 0.01).

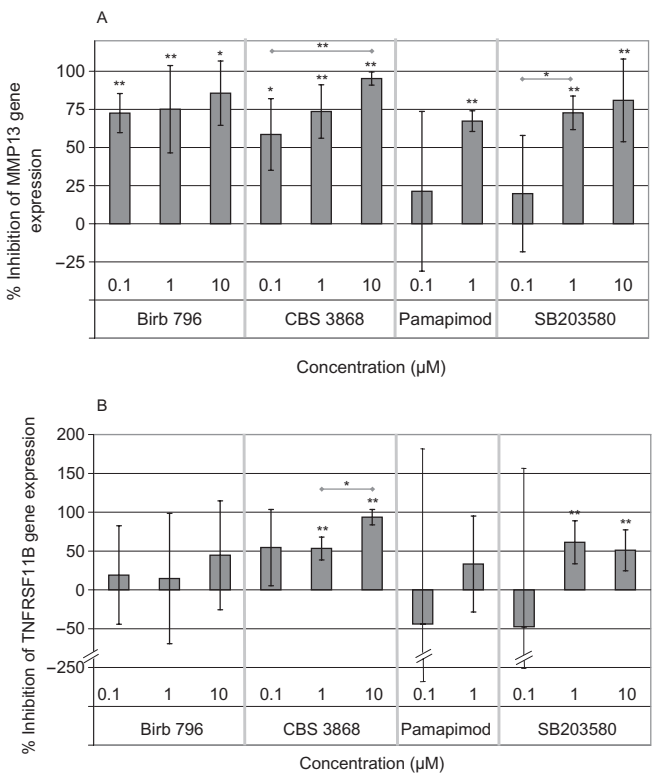

Figure 3 Inhibition of IL-1β-induced MMP13 and TNFRSF11B gene expression by anti-inflammatory substances. Chondrocytes from two donors were pooled and cultivated with inhibitor and 10 ng·mL^{-1} IL-1β, and then mRNA was isolated for quantitative gene expression analysis. Data were obtained from at least six independent experiments with 12 different donors, except for 10 μM Birb 796 and 10 μM CBS-3868 where three independent experiments with six different donors were used. (A) % Inhibition of IL-1β-induced MMP13 gene expression after 24 h. (B) % Inhibition of IL-1β-induced TNFRSF11B gene expression after 24 h. Significant inhibition and concentration-related dependence is marked (*P < 0.05; **P < 0.01).

with stimulated chondrocytes in the presence or absence of the test article Birb 796, and the results were compared with those obtained with SB203580. The objective of this analysis was to identify genes, which are related to the IL-1β-induced inflammatory processes in chondrocytes and, which are suitable to qualitatively and quantitatively determine the effects of anti-inflammatory drugs on the expression of these genes. A rather high inhibitor concentration of 10 μM was chosen to detect the effects of all relevant inhibitors, as chondrocytes are less sensitive than blood cells (unpublished observation). As published, the results of IL-1β-mediated induction of gene expression and the effect of SB203580 demonstrated that the model produces reliable and reproducible results (Joos *et al*., 2008; 2009). Many of the IL-1β-regulated genes (e.g. MMP13, SOD2 or GADD45 β) have also been shown to be differentially expressed in OA tissue (Aigner *et al*., 2006; Sato *et al*., 2006; Ijiri *et al*., 2008). These findings support the relevance of the *in vitro* microarray results to *in vivo* processes. The lists of genes regulated that were obtained also represent a valuable

database for the selection of further OA-related genes that could extend the set of parameters used for the testing of anti-inflammatory substances.

The comparison of effects of Birb 796 and SB203580 (10 μM each) demonstrated that the number of genes modulated by SB203580 was higher than that of Birb 796. Quantitative evaluation of global expression analysis also demonstrated that SB203580 and Birb 796 counter-regulated 87 and 98%, respectively, of those genes that were regulated by IL-1β and the test articles. This analysis indicates that both compounds may modulate additional targets at the concentration used, 10 μM, in line with the knowledge that both compounds are not absolutely selective for p38α/β MAPK (Table 1). Therefore, it is possible that off-target effects contribute to the results of this study. In addition, the analysis of regulated biological processes would allow for the screening for undesired side effects of the test articles. Apoptosis of chondrocytes for example plays an important role in OA (Kuhn *et al*., 2004). While the application of SB203580 did not reveal alarming gene regulation with respect to pro-apoptotic genes (Joos *et al*., 2009), Birb 796 influenced apoptotic processes in the GoMiner analysis. The direction of regulation indicated a pro-apoptotic effect, which might represent a possible negative side effect and warrants further investigation.

Based on the results of global gene expression analysis, a set of genes involved in inflammatory processes (PGE_2 and NO pathway), matrix degradation (MMP13) and bone metabolism (TNFRSF11B) were selected to investigate the effect of different p38α/β MAPK inhibitors on gene expression. Analysis of the PGE_2 synthesis pathway included the specification of COX-2 and mPGES1 gene expression, and release of PGE_2 into the supernatant of chondrocyte cultures. All inhibitors significantly abolished IL-1β-induced up-regulation of COX-2 after 4 and 24 h incubation. This effect is in accordance with another work and indicates that p38α MAPK inhibition directly blocks COX-2 transcription (Cieslik *et al*., 2002). When compared to the potency of Birb 796, CBS-3868 and pamapimod, SB203580 was less efficient at inhibiting COX-2 expression. This outcome is in line with the relative potencies of the compounds regarding p38α MAPK inhibition. In an enzyme assay, the IC_{50} of SB203580 was 19.5-, 14.1- and 2.2-fold higher than that of Birb 796, CBS-3868 and pamapimod, respectively (unpublished results, c-a-i-r biosciences GmbH).

IL-1β-induced increase in mPGES1 gene expression was less pronounced than that of COX-2, and a significant inhibition of gene expression was observed only after 24 h. With an IC_{50} value of 3 μM, Birb 796 was the weakest inhibitor of mPGES1 expression, while CBS-3868 and SB203580 showed a somewhat higher potency with IC_{50} values of 0.7 and 0.6 μM. Pamapimod inhibited the IL-1β-induced mPGES1 expression with an IC_{50} value of 1 μM. Masuko-Hongo *et al*. investigated the regulation of mPGES1 expression based on the effects of the p38α/β MAPK inhibitor SB203580 and a known p38α-selective inhibitor SC-906. The authors concluded that the expression of mPGES1 is regulated by p38β rather than p38α (Masuko-Hongo *et al*., 2004). The results of the present study support the concept of an involvement of p38β MAPK, as the published K_d values of the ligand/p38β MAPK affinity determined for SB203580 (70 nM), pamapimod (120 nM) and Birb 796 (1500 nM) (Hill *et al*., 2008; Karaman *et al*., 2008) correlate with the inhibition of mPGES1 expression. Yet, other mechanisms like ERK1/2 signalling may contribute to our findings (Masuko-Hongo *et al*., 2004).

Birb 796 and CBS-3868, according to their effect on COX-2 gene expression, exerted the strongest effect on PGE_2 synthesis with an IC_{50} of <0.1 μM. The efficacy of pamapimod and SB203580 was weaker by a factor of 10, and correlates with their IC_{50} values of COX-2 gene expression. Consequently, the drug-mediated effect on COX-2 expression was thought to be more relevant for the inhibition of PGE_2 synthesis than their effect on mPGES1.

The effects of the inhibitors on the NO synthesis pathway were examined by the analysis of iNOS gene expression and nitrite release as an indicator of NO formation. Although it has been suggested that a p38-dependent mechanism is involved in the regulation of iNOS expression and NO synthesis (Mendes *et al*., 2002), the p38α/β MAPK inhibitors tested did not seem to directly prevent the induction of iNOS. A significant inhibition of iNOS expression was achieved with SB203580 (50% at 10 μM) and CBS-3868 (70% at 10 μM) only after 24 h. Both the modest-to-moderate extent of inhibition and the time-course are in agreement with the multiple mechanisms of regulation for iNOS gene expression described previously, and confirm that p38α MAPK is neither an immediate nor the only regulator of iNOS expression and NO synthesis (Otero *et al*., 2005; Chockalingam *et al*., 2007; Chowdhury *et al*., 2008). The IL-1β-mediated induction of MMP13 gene expression was efficiently and concentration dependently inhibited by all test compounds. At concentrations of 1 and 10 μM, the extent of inhibition was similar. At 0.1 μM, the high degree of inhibition, which was achieved with Birb 796 and CBS-3868 compared to pamapimod and SB203580, correlated with the inhibitory potencies of the test compounds on p38α MAPK activity. A promising new approach for inhibition of cartilage degradation was recently introduced by Kimura *et al*. (2009) who presented a new inhibitor that, in contrast to SB203580, inhibited MMP13 expression, but not the expression of other MMPs.

The effects of the p38MAPK inhibitors on TNFRSF11B gene expression were divergent. *In vivo*, the so-called decoy receptor TNFRSF11B interferes with RANK/RANKL signalling, thereby preventing the RANKL-mediated osteoclastogenesis (Schett *et al*., 2003). An up-regulation of TNFRSF11B protein has not only been described in IL-1β-treated chondrocytes (Kwan Tat *et al*., 2009), but also in OA cartilage and in the synovium of RA patients (Komuro *et al*., 2001). The low extent of induction, three- and fivefold up-regulation after 4 and 24 h, made it difficult to detect the drug-mediated effects unequivocally. Birb 796 was the weakest inhibitor of TNFRSF11B gene expression, indicating the contribution of another mechanism rather than p38α-mediated signalling. However, in osteosarcoma cells, p38α/β, but not JNK, ERK or NFκB, inhibition was shown to influence IL-1β-induced TNFRSF11B gene expression (Lambert *et al*., 2007). It is possible that a comparable mechanism exists in chondrocytes, and therefore an effect mediated by p38β could play a role in the regulation of TNFRSF11B expression.

In summary, in the present study, a reliable *in vitro* model using IL-1β-stimulated human primary chondrocytes was

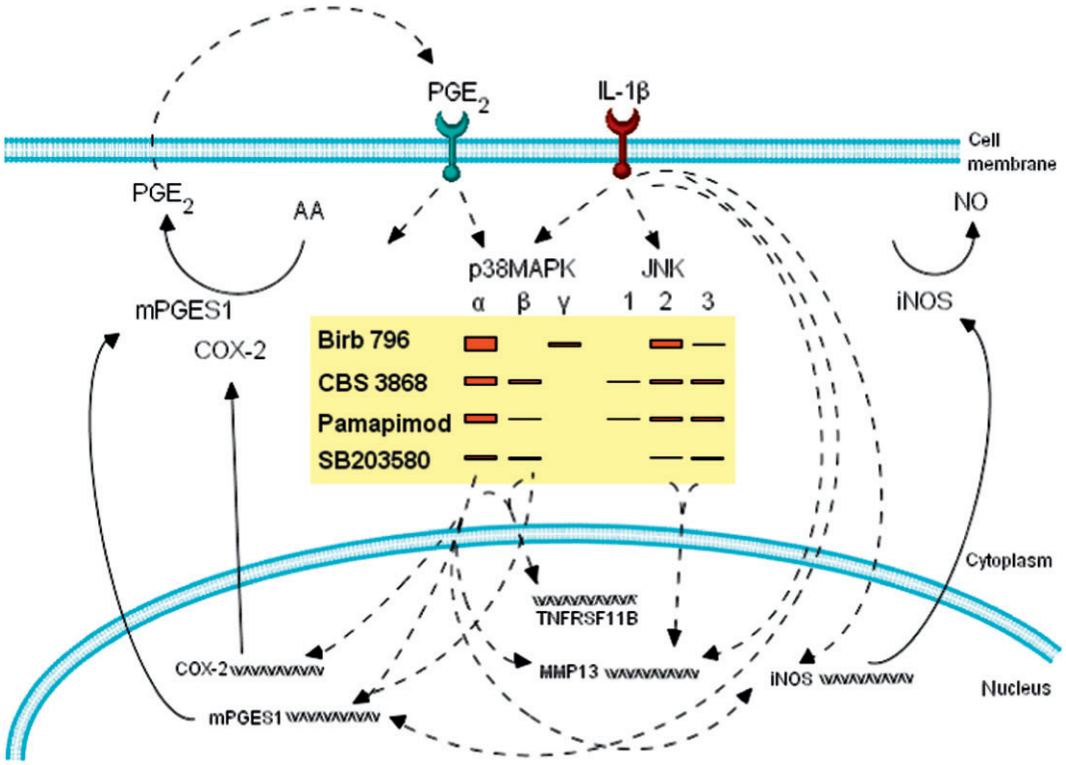

Figure 4 Schematic presentation of inhibitor effects on the expression of the gene analysed and on metabolite release. The size of the blocking bar indicates the affinity of the inhibitor for the respective target according to their K_d values (Hill *et al.*, 2008; Karaman *et al.*, 2008). The estimation of CBS-3868 affinities is based on IC_{50} determinations (personal communication, c-a-i-r biosciences GmbH, Tübingen, Germany). Dashed arrows indicate signalling pathways with intermediate steps. AA, arachidonic acid; IL-1β, interleukin 1β; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TNFRSF11B, osteoprotegerin. The scheme gives an overview of regulatory mechanisms discussed in this paper. The different target affinities and the different off-target specificities of the inhibitors are a possible explanation for their different impacts described in the presented study.

established with the objective to investigate and compare the effects of different p38α/β MAPK inhibitors on gene expression. The role of p38MAPK and JNK isoforms in the regulation of the analysed biomarkers is illustrated in Figure 4. It was demonstrated that the effects of the test compounds on COX-2 and MMP13 expression, as well as on PGE_2 release, correlated well with their potency at inhibiting p38α MAPK. In contrast, their effect on mPGES1 and TNFRSF11B expression appeared to be associated with the affinity of the test compounds for p38β rather than the α-form of MAPK. These observations shed new light on the role of p38β MAPK in chondrocytes and on the required α/β-specificity of p38MAPK inhibitors. Although in the case of mPGES1, they confirm those obtained in a previous study (Masuko-Hongo *et al.*, 2004). Undoubtedly, further studies are required to unequivocally verify these findings. iNOS expression and NO release appear to be useful, as biomarkers of inflammation, for differentiating the efficacy of p38α/β MAPK inhibitors. Marked differences were observed with the inhibitors tested, especially at low concentrations, which may be more relevant *in vivo* because of their limited bioavailability within cartilage tissue. Despite the selection of candidate genes for differential analysis of test substances with respect to well-known relevance to the *in vivo* situation, the correlation of our results with *in vivo* models remains to be determined. Overall, our tissue-specific test system could be successfully applied for differential characterization of inhibitors with the same primary pharmaceutical target. It therefore represents a valuable tool for drug screening between functional *in vitro* testing and *in vivo* models in the field of OA.

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Conflicts of interest

The authors declare that they have no competing interests.

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Supporting information

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

Table S1 Co-regulated genes by IL-1beta and Birb 796.
Table S2 Regulated biological processes by IL-1beta.
Table S3 Regulated biological processes by Birb 796.
Table S4 Regulated biological processes by SB203580.
Table S5 Microarray data of differentially regulated genes selected by hierarchical clustering and associated with OA.
Table S6 IC_{75} values of the tested inhibitors for inhibition of gene expression and metabolite release.

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