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TGF β 1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity

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Abstract

Background: Dysregulated transforming growth factor β (TGF β) signaling is implicated in osteoarthritis development, making normalizing TGF β signaling a possible therapy. Theoretically, this can be achieved with small molecule inhibitors specifically targeting the various TGF β receptors and downstream mediators. In this study we explore in primary chondrocytes the use of small molecule inhibitors to target TGF β -induced pSmad1/5/9-, pSmad2/3- and TGF β -activated kinase 1 (TAK1)-dependent signaling.

Method: Primary bovine chondrocytes and explants were isolated from metacarpophalangeal joints. To modulate TGF β signaling the activin receptor-like kinase (ALK)1/2/3/6 inhibitor LDN-193189, the ALK4/5/7 inhibitor SB-505124 and the TAK1 inhibitor (5Z)-7-Oxozeaenol were used. pSmad1/5 and pSmad2 were measured using western blot analysis and TGF β 1-induced gene expression was measured using quantitative real time PCR (qPCR).

Results: In chondrocytes, TGF β 1 strongly induced both pSmad1/5 and pSmad2. Remarkably, LDN-193189 did not inhibit TGF β -induced pSmad1/5. In contrast, SB-505124 did inhibit both TGF β -induced Smad2 and Smad1/5 phosphorylation. Furthermore, (5Z)-7-Oxozeaenol also profoundly inhibited TGF β -induced pSmad2 and pSmad1/5. Importantly, both SB-505124 and (5Z)-7-Oxozeaenol did not significantly inhibit constitutively active ALK1, making an off-target effect unlikely. Additionally, LDN-193189 was able to potently inhibit BMP2/7/9-induced pSmad1/5, showing its functionality. On gene expression, LDN-193189 did not affect TGF β 1-induced regulation, whereas both SB-505124 and (5Z)-7-Oxozeaenol did. Similar results were obtained in cartilage explants, although pSmad1/5 was not strongly induced by addition of TGF β 1.

Conclusion: Our data suggest that ALK5 kinase activity plays a central role in both TGF β -induced Smad1/5 and Smad2/3 phosphorylation, making it difficult to separate both pathways with the use of currently available small molecule inhibitors. Furthermore, our data regarding (5Z)-7-Oxozeaenol suggest that TAK1 facilitates Smad-dependent signaling.

Keywords: TGF β , Smad, Small molecule inhibitor, ALK1, ALK5, TAK1

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Background

Transforming growth factor β (TGF β) plays a crucial role in regulation of tissue homeostasis via its control over diverse cellular processes such as proliferation, differentiation and matrix formation. By binding to different receptors of the activin-receptor like kinase (ALK) family, TGF β induces intracellular carboxy-terminal (C-terminal) phosphorylation of receptor-regulated Smad (R-Smad) proteins. Phosphorylated R-Smads form complexes with the common-mediator Smad; Smad4, and these complexes translocate to the nucleus where they bind DNA to regulate gene transcription via recruitment of transcription factors [1, 2]. Use of different ALKs can result in activation of different R-Smads; activation of ALK5 (TGF β R1) induces phosphorylation of Smad2 and Smad3, whereas activation of an ALK1 (or 2 or 3) complex mediates phosphorylation of Smad1, Smad5 and Smad9 [3, 4]. However, TGF β signaling is not limited to R-Smads as Smad-independent signaling can occur by activation of e.g. TGF β -activated kinase 1 (TAK1 or MAP3K7). Smad-independent signaling results in activation of various downstream signaling pathways, including the JUN N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways [5].

In many tissues, e.g. in cartilage, phosphorylated Smad2/3 and Smad1/5 have opposing functions [6, 7]. In cartilage, phosphorylated Smad3 guards chondrocyte phenotype against deleterious hypertrophy and production of catabolic enzymes like matrix metalloproteinases (MMPs) [8–10]. In contrast, phosphorylated Smad1/5 is essential for chondrocyte hypertrophy [11, 12], and is associated with expression of matrix metalloproteinase 13 (MMP13), the main cartilage-degrading enzyme [13]. Therefore, balancing TGF β signaling via Smad2/3 or Smad1/5 is important for chondrocytes to maintain cellular homeostasis and deregulation of this balance has been proposed as a cause of disease, e.g. osteoarthritis (OA) [4, 13]. It would be of great benefit if cellular responses to TGF β could be directed towards either the Smad1/5 or Smad2/3 pathway; this would enable blockade of deleterious pathways while maintaining beneficial signaling.

Inhibiting Smad phosphorylation can be achieved by blocking the kinase domain of ALK receptors. Small molecules have been developed that specifically inhibit the kinase activity of ALK4, ALK5 and ALK7, the receptors that phosphorylate Smad2 and Smad3, or ALK1, ALK2, ALK3 and ALK6, the receptors that phosphorylate Smad1, Smad5 and Smad9 [14, 15]. In this study we investigated in chondrocytes and cartilage explants the use of SB-505124 [15, 16], an inhibitor of ALK4/5/7 kinase activity and LDN-193189, an inhibitor of the ALK1/2/3/6 kinase activity [14, 16] to study their ability to direct R-Smad signaling and downstream effects.

Furthermore, we used (5Z)-7-Oxozeaenol as a selective inhibitor of TAK1 kinase activity to evaluate the importance of TAK1 in TGF β signaling in chondrocytes [17, 18], because of the ability of TAK1 to induce the JNK and p38 MAPK pathways and MMP production in cartilage [19, 20].

In this study we show that inhibition of ALK4/5/7 kinase activity prevented TGF β 1-induced C-terminal phosphorylation of both Smad2 and Smad1/5. Furthermore, treatment with (5Z)-7-Oxozeaenol, a selective TAK1 kinase inhibitor, attenuated phosphorylation of both Smad2/3 and Smad1/5 and expression of Smad target genes. Use of the ALK1/2/3 inhibitor LDN-193189 did not affect TGF β 1-induced R-Smad phosphorylation or gene expression. In conclusion, our data show that in cartilage the activity of the ALK5 kinase domain is essential for TGF β 1 signaling, whereas ALK1 kinase activity is not. Furthermore, our results obtained with (5Z)-7-Oxozeaenol underline that TAK1 kinase activity is facilitating R-Smad phosphorylation. Our study indicates that TGF β 1-induced Smad1/5 and Smad2/3 phosphorylation cannot be selectively targeted by using the small molecule inhibitors SB-505124 and LDN-193189.

Methods

Chondrocyte culture

Primary bovine chondrocytes were isolated from the metacarpophalangeal joint of cows (2–5 years old) obtained from a slaughterhouse within 3 hours post mortem. Chondrocytes were obtained by incubating cartilage slices overnight in Collagenase B (for details see [21]). Chondrocytes were seeded in DMEM/F12 1:1 containing 10% fetal calf serum (Thermo Scientific UK) at a density of 0.5×10^5 cells per cm^2 in 6-well plates (Greiner Bio-one International, the Netherlands) for protein studies, or in 24-well plates (Bio-one International, the Netherlands) for mRNA experiments. Cells were cultured for 1 week at 37 °C and 5% CO₂ before the start of the experiments. For explant studies, four 7-mm² explants per condition were pooled in 1 ml of medium. Both monolayer and explant studies were repeated in different donors.

Adenoviral transfection of primary chondrocytes

Adenoviruses were used to induce expression of constitutively active (ca)ALK1 [4], constitutively active ALK5 [4] and the CAGA₁₂-luciferase reporter construct [22] in primary chondrocytes. Cells were rinsed twice with saline, and adenoviruses were added in a multiplicity of infection of 200 for 3 h at 37 °C in a minimal volume of DMEM/F12 1:1. Hereafter, cells were washed twice with saline, and DMEM/F12 1:1 was added. Cells were used 48 h after transfection.

Inhibition of TGF β 1 signaling

Before stimulation, cells were deprived of serum for 24 h and thereafter stimulated with 1 ng/ml recombinant human TGF β 1 (Biolegend, the Netherlands) for 2 or 24 h. In experiments where inhibitors were used, DMSO was used as vehicle control. To block TAK1 activity, we used (5Z)-7-Oxozeaenol [17] (Tocris Bioscience) in a concentration of 0.5 μ M. To inhibit ALK5 kinase, we used SB-505124 [15] (Sigma Aldrich) in a concentration of 5 μ M. For inhibition of ALK1 kinase, LDN-193189 [14] (Axon Medchem) was used in a concentration of 0.05 μ M. This concentration of LDN-193189 is well above its reported half maximal inhibitory concentration (IC₅₀), 0.8 nM for ALK1, but far below its IC₅₀ for ALK5 of 350 nM [16, 23]. Cells were pre-incubated with the inhibitors for 1 h prior to addition of TGF- β 1. Either 2 or 24 h after addition TGF- β 1, medium was removed and TRI-reagent was added for RNA isolation.

Protein isolation

To isolate proteins from monolayer cultures, cells were lysed on ice using lysis buffer (Cell signaling, USA) containing a protease inhibitor cocktail (complete, Roche Diagnostics, Germany) and subsequently sonicated on ice using a Bioruptor (Diagenode, USA). With a BCA-assay (Thermo Scientific, USA) protein concentration was measured and after addition of Laemmli sample buffer, samples were boiled for 5 minutes at 99 °C.

To isolate proteins from explants, four 7-mm² explants were homogenized using a Mikro-dismembrator (B. Braun, Germany) and dissolved in 1 ml ice cold radioimmunoprecipitation assay (RIPA) buffer with added 1 mM Na₃VO₄ and protease inhibitor cocktail (Roche Diagnostics, Germany). After 1 h incubation on a roller bench at 4 °C, samples were spun down for 3 minutes at 10⁴ \times g and the pellet was discarded. Cetylpyridinium chloride was added up to a concentration of 1% (m/v) and samples were incubated on a roller bench for 1 h at 4 °C. Hereafter, samples were spun down twice for 15 minutes at 10⁴ \times g at 4 °C and the pellet was discarded. Using 10 kDa centrifugal filter units (Millipore, USA) the supernatant was concentrated to a volume of 50 μ l. Subsequently, proteins were precipitated by addition of 950 μ l of 20% m/v trichloroacetic acid and 0.1% m/v dithiothreitol in acetone at -20 °C and spinning samples down for 15 minutes at 6700 \times g at 4 °C. Next, the pellets were washed three times with 0.1% m/v dithiothreitol in acetone at -20 °C. Finally, the pellets were dried under vacuum for 20 minutes and dissolved in 100 μ l 1% m/v sodium dodecyl sulfate in 100 mM tris(hydroxymethyl)aminomethane in H₂O pH 9.0.

Detection of proteins using SDS-PAGE and western blot

Proteins were separated on a 7.5% Bisacrylamide gel, and transferred to a nitrocellulose membrane using wet

transfer (Towbin buffer, 2.5 h 275 mA at 4 °C). After overnight incubation at 4 °C with 1:1000 polyclonal Rabbit anti P-Smad1/5 (S463/465)/Smad8 (S426/428) (Cell signaling, USA) or anti P-Smad2 (S465/467) (Cell signaling, USA), membranes were incubated with 1:1500 polyclonal Goat anti Rabbit labeled with horseradish peroxidase (HRP) (DAKO, Belgium) for 1 h. Hereafter, enhanced chemiluminescence using ECL plus (GE Healthcare, UK) was used to visualize the proteins. To visualize overexpression of constitutively active ALKs, a rabbit polyclonal antibody directed against their internal HA tag was used: HA-probe Antibody (Y-11) (Santa Cruz, USA) (1:1000). As loading control either Gapdh was stained with an anti-Gapdh mouse mAb (1G5) (Sigma Aldrich, Germany) (1:10 000) in combination with IRDye 680RD Donkey anti mouse (1:10 000) (Licor, USA) using the Odyssey detection system (Licor, USA) or Vinculin with a rabbit pAb (H300) (Santa Cruz, USA) (1:1000) in combination with HRP-labeled Goat-anti Rabbit (1:2000) (DAKO, Denmark) using ECL. Finally, blots were quantified using ImageJ.

Detection of gene expression

TRI-reagent (Sigma-Aldrich, Germany) was used for RNA isolation according to the manufacturer's protocol (for details see [21]). RNA concentration was measured using a Nanodrop photospectrometer (Thermo Scientific, USA). Per sample, 1 μ g of RNA was treated with DNase (Life Technologies, USA), which was subsequently inactivated at 65 °C with 1 μ l 25 mM EDTA (Life Technologies, USA). To perform reverse transcriptase reaction, 1.9 μ l ultra pure water, 2.4 μ l 10 \times DNase buffer, 2.0 μ l 0.1 M DTT, 0.8 μ l 25 mM dNTP, 0.4 μ g oligo dT primer, 1 μ l 200 U/ μ l M-MLV Reverse transcriptase (all Life Technologies, USA) and 0.5 μ l 40 U/ μ l RNasin (Promega, the Netherlands) was added, and samples were incubated for 5 minutes at 25 °C, 60 minutes at 39 °C, and 5 minutes at 95 °C using a thermo cycler. The obtained cDNA was diluted 10 times in ultra pure water, and gene expression was measured using 1 μ M of validated cDNA-specific primers (Biolegio, the Netherlands; see Table 1) in a quantitative real-time polymerase chain reaction (qPCR) using SYBR green master mix (Applied Biosystems). The following protocol was used: after 10 minutes at 95 °C, 40 cycles of 15 sec at 95 °C and 1 minute at 60 °C each were run. Hereafter a melting curve was made to verify gene-specific amplification. For calculations of the $-\Delta$ Ct, two reference genes were used: *glyceraldehyde 3-phosphate dehydrogenase* and *ribosomal protein S14*.

Statistics

All quantitative data are expressed as a mean of multiple repeats \pm SD. For every analysis data were checked for

Table 1 Sequence and efficiency of the primers used in this study

Gene	NCBI reference sequence	P (bp)	E (%)	Forward primer	Reverse primer
<i>bGapdh</i>	NM_001034034.2	90	101	CACCCACGGCAAGTTC AAC	TCTCGCTCCTGGAAGATGGT
<i>bRps14</i>	NM_001077830.2	125	105	CATCACTGCCCTCCACATCA	TTCCAATCCGCCCAATCTTCA
<i>bAlk1</i>	NM_001083479.1	109	100	ACAACACAGTGCTGCTCAGACA	TGCTCGTGGTAGTGCGTGAT
<i>bAlk2</i>	NM_176663.3	61	105	CGTTGGAGACAGCACTTTAGCA	AGAGCCGCTTCCCGATGTA
<i>bAlk3</i>	NM_001076800.1	142	96	GAGCAAGATGAAGCATTATTCCA	CAACTGCCGAACCATCTG
<i>bAlk5</i>	NM_174621.2	75	94	CAGGACCACTGCAATAAAATAGA ACTT	TGCCAGTTC AACAGGACCAA
<i>bSerpine1</i>	NM_174137.2	55	99	CGAGCCAGGCGGACTTC	TGCGACACGTACAGAACTCTTGA
<i>bld1</i>	NM_001097568.2	73	107	GCTCCGCTCAGCACTCTCAA	GATCGTCCGCTGGAACACA
<i>bMmp3</i>	NM_001206637.1	130	106	GAATCTGTGCTCCCGAAC C	TCCTGAAAGATTTCCGCCAA
<i>bSmad7</i>	NM_001192865.1	72	98	GGGCTTTCAGATTC CCAACTT	CTCCAGTATGCCACCACG
<i>bTgfb1</i>	NM_001166068.1	80	107	GGTGG AATACGGCAACAAATCT	GCTCGACGTGTTGAAGAAC
<i>bJunb</i>	NM_001075656.1	139	97	CCTTCTACCACGACGACTCA	CCGGGTGCTTTGAGATTTCCG
<i>bNgf</i>	NM_001099362.1	110	85	CAGCTCTTTGATCGGCATACA	TGTGTCAAGGGAATGCTGAAGT
<i>bFn1</i>	NM_001163778.1	95	96	GCACCACTCCCGACATTACT	CTGATCGGCATGGACCACTT
<i>bCol2a1</i>	NM_001001135.2	60	97	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG

P is product length in base pairs, E is efficiency in percentage. *Alk1* is also known as *Acvr11*, *Alk2* as *Acvr1*, *Alk3* as *Bmpr1a* and *Alk5* as *Tgfb1*

normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) with Tukey multiple comparison post-hoc test was used to determine the significance. The statistical analyses were performed using Graphpad Prism 5.0 software.

Results

SB-505124 and (5Z)-7-Oxozeaenol both inhibit TGF β 1-induced Smad1/5 and Smad2/3 phosphorylation whereas LDN-193189 does not

To begin, we confirmed in primary bovine chondrocytes the expression of the receptors that TGF β can use to induce Smad2/3 phosphorylation i.e. ALK5 or Smad1/5 phosphorylation, i.e. ALK1, ALK2 and ALK3 [3, 4]. All four receptors were readily detected using qPCR (Additional file 1: Figure S1), and indeed confirmed that stimulation of chondrocytes with TGF β 1 results in both Smad2 and Smad1/5 phosphorylation (Fig. 1a).

Subsequently, we analyzed the effects of the ALK4/5/7-kinase inhibitor SB-505124, the ALK1/2/3-kinase inhibitor LDN-193189 and the TAK1-kinase inhibitor (5Z)-7-Oxozeaenol on TGF β 1-induced R-Smad phosphorylation (Fig. 1a, b and c). Strikingly, addition of 5 μ M SB-505124 not only inhibited Smad2 phosphorylation, but also pSmad1/5 phosphorylation. Remarkably, treatment with 0.5 μ M (5Z)-7-Oxozeaenol also resulted in inhibition of TGF β 1-induced Smad1/5 and Smad2 phosphorylation. Noteworthy, addition of 0.05 μ M LDN-193189 did not affect TGF β 1-induced Smad1/5 phosphorylation but did significantly enhance TGF β -induced pSmad2.

To investigate if the striking effect of SB-505124 on Smad1/5 phosphorylation was a dose-dependent effect, we performed a dose-response experiment (Fig. 1d and e). This experiment showed that at all tested doses (0.1, 0.5, 1, 5, 10 and 25 μ M), SB-505124 inhibited TGF β 1-induced Smad1/5 phosphorylation, and that Smad1/5 phosphorylation was inhibited more potently than that of Smad2. Therefore, to address if ALK1 inhibition was a possible off-target effect of SB-505124, this compound was added to cells over-expressing constitutively active (ca)ALK1 or caALK5. This experiment showed that SB-505124 did not affect caALK1-induced Smad1/5 phosphorylation (Fig. 2a and b), but did inhibit caALK5-induced Smad2 phosphorylation (Fig. 2a and b). Therefore, SB-505124 is specific for ALK5 kinase and does not inhibit ALK1 kinase. The same experimental setup was used to address the specificity of (5Z)-7-Oxozeaenol, and no significant inhibitory effect of this compound on either caALK1 or caALK5 function was detected, making it unlikely that either the ALK1 or ALK5 kinase domain is an off-target of (5Z)-7-Oxozeaenol.

To investigate if the lack of effect of LDN-193189 on TGF β 1-induced Smad1/5 was maybe dose-dependent, we added a 10 times higher dose of LDN-193189 (0.5 μ M) to the cells, but were still unable to inhibit TGF β 1-induced Smad1/5 phosphorylation (Fig. 2c). Therefore we sought to validate the function of LDN-193189. To do this, we added 0.05 μ M LDN-193189 to chondrocytes over-expressing caALK1 and observed that this blocked caALK1-induced Smad1/5 phosphorylation (Fig. 2a and b), Furthermore, the effect of 0.05 μ M LDN-193189 on bone morphogenic protein (BMP) signaling was also investigated (Fig. 2d) by stimulating primary chondrocytes for 1 h with either

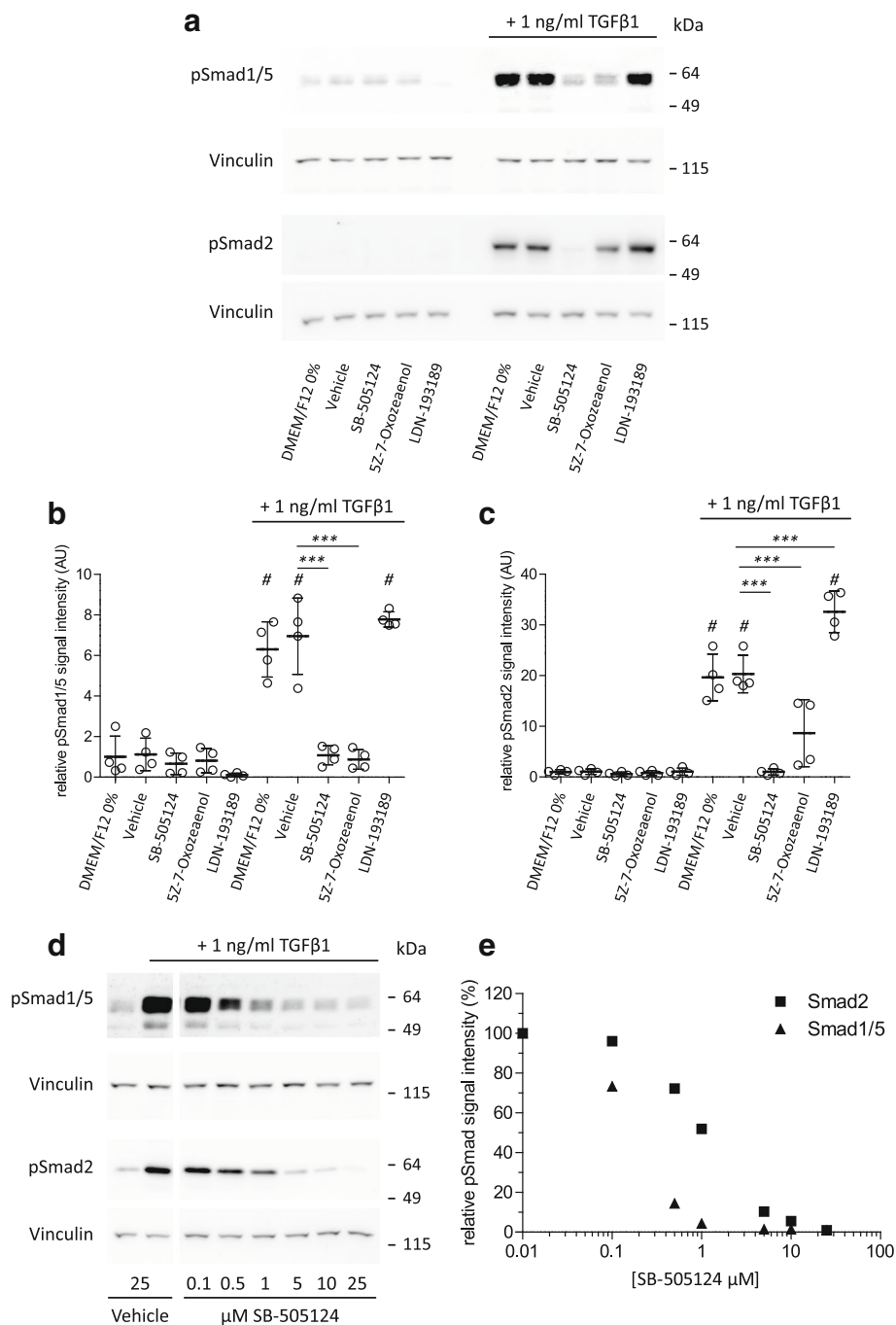


Fig. 1 SB-505124 and (SZ)-7-Oxozaeanol inhibit both transforming growth factor β1 (TGFβ1)-induced Smad1/5 and Smad2/3 phosphorylation whereas LDN-193189 does not. **a** Primary bovine chondrocytes were pre-incubated with either 5 μM SB-505124, 0.5 μM (SZ)-7-Oxozaeanol or 0.05 μM LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGFβ1 for 1 h, after which phosphorylated Smads were visualized using western blot by specific antibodies. kDa, kilodalton. **b** Quantification of the pSmad1/5 signal (as shown in **a**) in four experiments. pSmad1/5 levels were normalized to vinculin levels and plotted as a relative amount in arbitrary units (AU) compared to the control group: #p ≤ 0.001 compared to unstimulated; ***p ≤ 0.001. **c** Quantification of the pSmad2 signal (as shown in **a**) in four experiments. pSmad2 levels were normalized to vinculin levels and plotted as a relative amount in AU compared to the control group: #p ≤ 0.001 compared to unstimulated; ***p ≤ 0.001. **d** Dose-response effect of SB-505124 on TGFβ1 signaling in primary chondrocytes. **e** Quantification of pSmad levels (shown in **d**); levels were normalized to vinculin levels and plotted as a relative amount in percentage compared to the control group

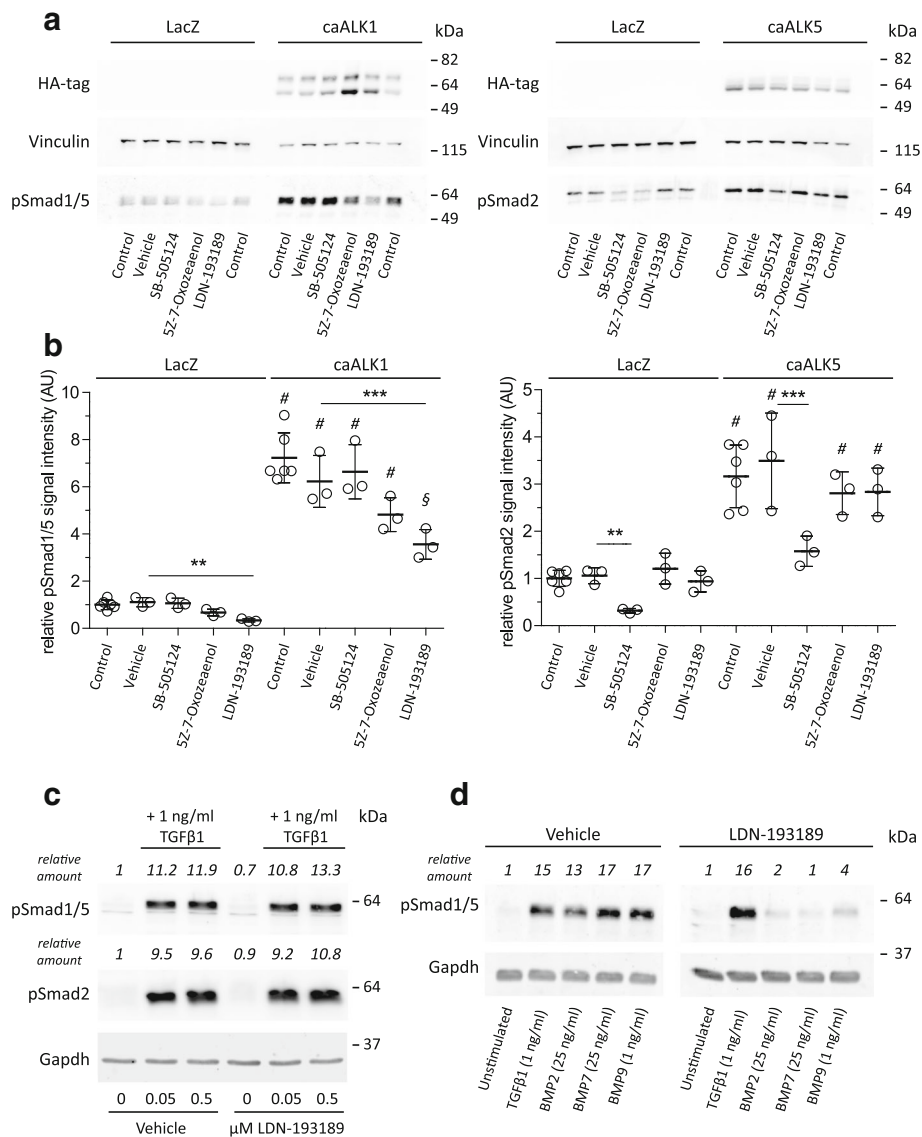


Fig. 2 SB-505124 and (5Z)-7-Oxozeaenol do not affect constitutively active activin receptor-like kinase 1 (ALK1) whereas LDN-193189 does. **a** Primary bovine chondrocytes were transfected with either constitutively active ALK1 (caALK1) or caALK5 and 2 days later treated with inhibitors for 1 h. A virus overexpressing LacZ was used as control. Efficiency of overexpression was visualized on western blot by staining for the HA-tag attached to the caALKs. **b** Quantification of the pSmad1/5 and pSmad2 signal (as shown in **a**) in three experiments; #*p* ≤ 0.001 compared to unstimulated; [§]*p* ≤ 0.01 compared to unstimulated; ***p* ≤ 0.01; ****p* ≤ 0.001. **c** Dose-response effect of LDN-193189 on transforming growth factor β1 (TGFβ1) signaling in primary chondrocytes. pSmad levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) levels and indicated as a relative amount in AU compared to the control group. **d** Primary chondrocytes were incubated for 1 h with LDN-193189 and subsequently stimulated for 1 h with 1 ng/ml (78 pM) TGFβ1, 25 ng/ml (1.94 nM) recombinant human bone morphogenic protein 2 (rhBMP2), 25 ng/ml (3.06 nM) bone morphogenic protein 7 (BMP7) or 1 ng/ml (83 pM) bone morphogenic protein 9 (BMP9). pSmad levels were normalized to Gapdh levels and indicated as a relative amount in AU compared to the control group

the ALK2/3 ligands BMP2 or BMP7 [24] or the ALK1/2 ligand BMP9 [25, 26]. We observed that BMP-induced Smad1/5 phosphorylation was clearly inhibited by 0.05 μM LDN-193189. Together, these experiments strongly confirm the function of 0.05 μM LDN-193189 in primary chondrocytes.

In conclusion, in primary chondrocytes, TGFβ1-induced Smad1/5 phosphorylation is unaffected by LDN-193189,

whereas both Smad2/3 and Smad1/5 phosphorylation are inhibited by SB-505124 and (5Z)-7-Oxozeaenol.

SB-505124 fully and (5Z)-7-Oxozeaenol partly inhibit TGFβ1-induced Smad-dependent gene expression whereas LDN-193189 does not

Next, we analyzed the effects of TGFβ1 signaling inhibitors on TGFβ1-induced Smad-dependent gene expression.

For this, *bId1* and *bSerpine1* were used as markers of pSmad1/5 and Smad3 signaling, respectively [4, 27] [22, 28]. Expression of *bId1* was profoundly upregulated by stimulation with 1 ng/ml of TGF β 1 (Fig. 3a). In accordance with our western blot data on pSmad1/5, expression of *bId1* was inhibited by both SB-505124 and (5Z)-7-Oxozeaenol, and was unaffected by LDN-193189. Expression of *bSerpine1* was upregulated by 1 ng/ml of TGF β 1 and fully inhibited by SB-505124, but was only partly inhibited by (5Z)-7-Oxozeaenol (Fig. 3b). To confirm these effects on pSmad3-dependent gene expression, we used the CAGA₁₂-luc construct, which produces luciferase specifically in response to pSmad3. After 8 h, TGF β -induced CAGA₁₂-luc activity was significantly inhibited by (5Z)-7-Oxozeaenol and SB-505124, showing that pSmad3-signaling is indeed lowered in these conditions. When the effect of the inhibitors on multiple TGF β 1-induced (Fig. 3c), genes like *bSmad7*, *bMmp3*, *bCol2a1*, *bNgf*, *bAlk5*, *bJunb*, *bFN1* and *bTgfb1* was measured, none of these genes was significantly downregulated by LDN-193189, unlike SB-505124, which profoundly downregulated *bSmad*, *bNgf*, *bAlk5*, *bJunb*, *bFN1* and *bTgfb1*, or unlike treatment with (5Z)-7-Oxozeaenol, which significantly lowered *bNgf*, *bTgfb1* and *bMmp3* expression (Fig. 3d). Because a role for pSmad1/5 in the regulation of e.g. *Smad7*, *Junb* and *Col2a1* has been established [29–31], these data indicate that the ALK5 kinase inhibitor SB-505124 inhibits gene expression downstream of both pSmad2/3 and pSmad1/5, whereas (5Z)-7-Oxozeaenol does so to a lesser extent and in a more limited amount of genes. No effect of LDN-193189 was observed on TGF β 1-induced gene expression.

In intact cartilage, SB-505124 and-Oxozeaenol inhibit TGF β 1-induced pSmad1/5 and pSmad2

As TGF β 1 signaling is known to be affected by cellular context, we sought to validate our observations in monolayer chondrocytes in intact cartilage *ex vivo*. Therefore we stimulated cartilage explants with 1 ng/ml TGF β 1 with or without inhibitors, and investigated Smad phosphorylation. Explants were stimulated for 2 h to allow for diffusion of TGF β 1 through the cartilage matrix. In explants, 1 ng/ml TGF β 1 induced pSmad2, but Smad1/5 phosphorylation was more difficult to detect (Fig. 4a), in contrast to in monolayer cultures. Therefore BMP9 was used as positive control, which, in comparison to TGF β , resulted in far stronger induction of pSmad1/5 (for quantification see Additional file 2: Figure S3). A smaller amount of pSmad1/5 was detected in explants treated with TGF β 1 and SB-505124, (5Z)-7-Oxozeaenol or LDN-193189 compared to TGF β 1-treated samples alone. TGF β -induced phosphorylation of Smad2 was clearly inhibited by SB-505124 and partly by (5Z)-7-Oxozeaenol (Fig. 4a), but on gene expression

bSerpine1 was only significantly inhibited by SB-505124 and not by (5Z)-7-Oxozeaenol (Fig. 4b). In explants, expression of *bId1* could not be used as a read-out for pSmad1/5 signaling, because TGF β 1 downregulated its expression, in contrast to its induction in monolayer experiments. This is a well-known Smad3-dependent cytostatic effect of TGF β 1 [32], and further indicates that TGF β 1 signaling differs between chondrocytes in monolayer and chondrocytes in intact cartilage. Notably, LDN-193189 did downregulate *bId1* expression, even in absence of TGF β 1 (Additional file 3: Figure S2), demonstrating the bioactivity of this compound in cartilage explants. Again, when multiple, TGF β 1-induced, Smad-dependent, genes were measured (Fig. 4c) SB-505124 inhibited TGF β 1-induced expression of *bSmad7*, *bNgf*, *bFn1* and *bTgfb1*, and (5Z)-7-Oxozeaenol inhibited TGF β 1-induced expression of *bFn1*, *bTgfb1*, whereas no effect of LDN-193189 was observed. Therefore, SB-505124 also inhibits both TGF β 1-induced Smad1/5 and Smad2 phosphorylation and gene expression in intact cartilage, whereas LDN-193189 does not. Furthermore, (5Z)-7-Oxozeaenol partly inhibited both TGF β 1-induced Smad1/5 and Smad2 phosphorylation.

Discussion

TGF β 1 signaling is a crucial regulator of chondrocyte homeostasis. We investigated the impact of small molecule inhibitors on TGF β 1 signaling in primary chondrocytes both *in vitro* and *ex vivo*. We report that inhibition of ALK4/5/7 kinase with SB-505124 lowered both TGF β 1-induced Smad1/5 and Smad2/3 phosphorylation and downstream gene expression, whereas inhibition of ALK1/2/3/6 kinase with LDN-193189 did not affect TGF β 1-induced Smad1/5 phosphorylation or transcriptional activity. In addition, we showed that treatment with (5Z)-7-Oxozeaenol, a selective TAK1 kinase inhibitor, attenuated TGF β 1-induced R-Smad phosphorylation and downstream gene expression.

In many tissues, including cartilage, TGF β 1 induces pSmad1/5 and pSmad2/3 [3, 4, 7]. ALK5 is essential for TGF β 1-induced Smad2/3 phosphorylation, whereas a role for ALK1 [4], ALK2 and ALK3 [3] has been demonstrated in TGF β -induced Smad1/5 phosphorylation (see Fig. 5). Remarkably, we found that specific inhibition of ALK4/5/7 kinase with SB-505124 was sufficient to inhibit both TGF β 1-induced Smad2/3 and Smad1/5 phosphorylation and transcriptional activity. In contrast, we were unable to block TGF β 1-induced Smad1/5 phosphorylation with LDN-193189, even though we used a dose (0.05 μ M) well above the reported IC₅₀ of this inhibitor for ALK1 (0.8 nM), ALK2 (0.8 nM) and ALK3 (5.3 nM) [23]. Additionally, even a 10 times higher dose of LDN-193189 (0.5 μ M) was unable to block TGF β 1-induced Smad1/5 phosphorylation. LDN-193189 has

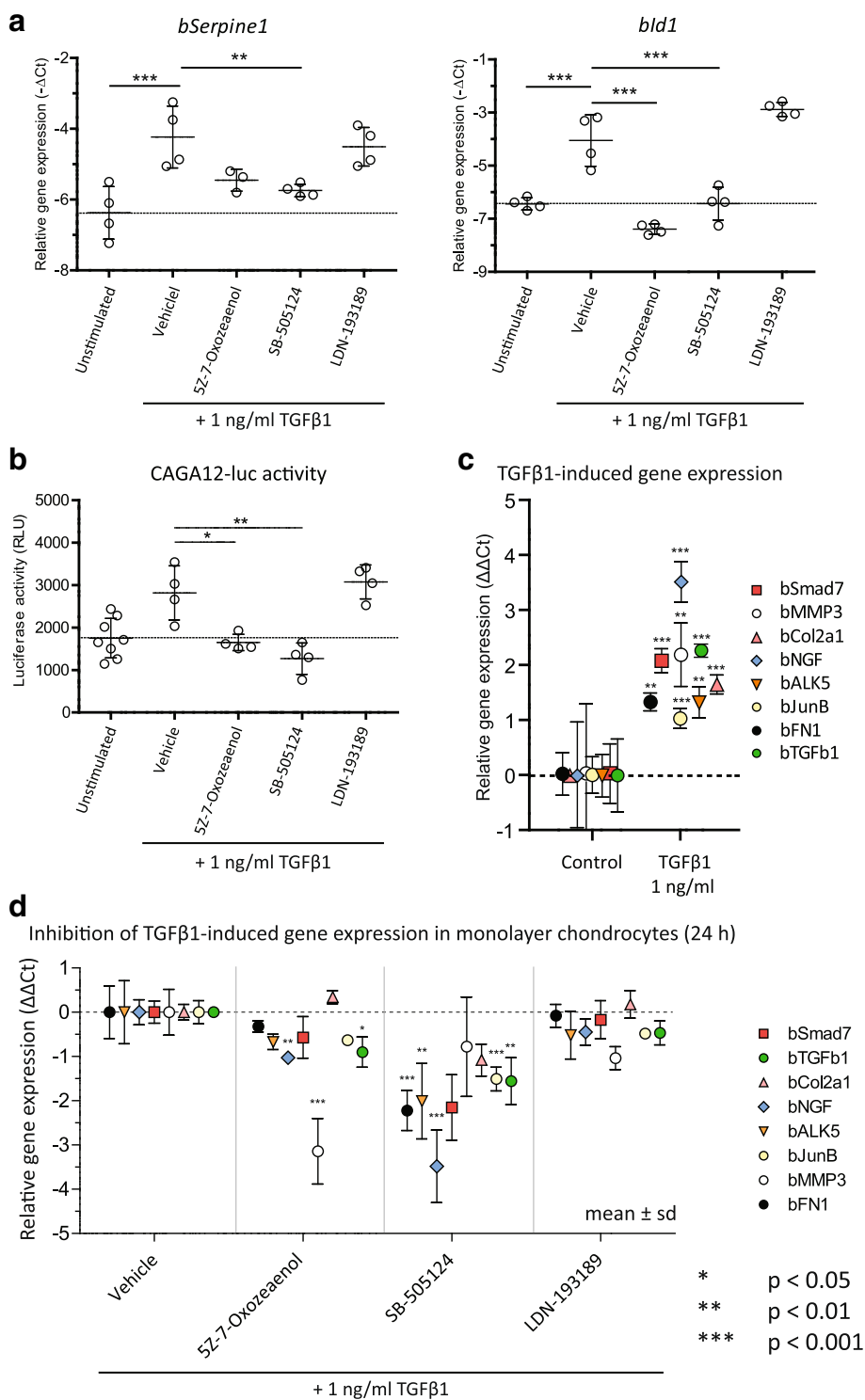


Fig. 3 (See legend on next page.)

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Fig. 3 SB-505124 fully and (5Z)-7-Oxozeaenol partly inhibits transforming growth factor β 1 (*TGF β 1*)-induced Smad-dependent gene expression whereas LDN-193189 does not. **a** Primary bovine chondrocytes were pre-incubated with 5 μ M SB-505124, 0.5 μ M (5Z)-7-Oxozeaenol or 0.05 μ M LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGF β 1 for 2 h and 24 h. With the use of quantitative (q)PCR, expression of *bSerpine1* was measured as response gene for pSmad3 signaling after 2 h, and *blcl1* for pSmad1/5 signaling after 2 h. **b** The pSmad3 responsive CAGA₁₂-luciferase (*CAGA₁₂-luc*) construct was placed in primary chondrocytes with the use of an adenovirus, and cells were stimulated with 1 ng/ml TGF β 1 for 8 h with or without inhibitors after 2 days. **c** Primary chondrocytes were stimulated with 1 ng/ml TGF β 1 for 24 h and gene expression was measured using validated cDNA-specific primers and qPCR. *MMP* matrix metalloproteinase, *NGF* nerve growth factor, *ALK5* activin receptor-like kinase 5. **d** Gene expression was measured after pre-incubation of chondrocytes for 1 h with inhibitors followed by stimulation with 1 ng/ml TGF β 1 for 24 h. For qPCR data, average \pm sd (*mean \pm sd*) was plotted, with each *dot* representing the average of one donor (**a**) or four donors (**c** and **d**). Analysis was performed using one-way analysis of variance with Tukey's post-hoc test: **p* \leq 0.05; ***p* \leq 0.01; ****p* \leq 0.001. A change of 1 $\Delta\Delta C_t$ equals twofold upregulation

been established as an efficient inhibitor of BMP type I receptors, i.e. ALK1/2/3 and ALK6, both in vitro and in vivo [14, 23, 33]. We were able to confirm this inhibitory effect of LDN-193189 on BMP-induced Smad1/5 phosphorylation in primary chondrocytes by using BMPs that signal via ALK1, ALK2 and/or ALK3, i.e. BMP2, BMP7 and BMP9 [24, 25]. Furthermore, we were also able to block caALK1 activity with LDN-193189 in our primary cell cultures. Together, these experiments indicate that LDN-193189 is functional in primary bovine chondrocytes and blocks the ALK1, ALK2 and ALK3 kinase activity at a dose of 0.05 μ M. Therefore, our data indicate that ALK1, ALK2 or ALK3 kinase activity is not required for TGF β 1-induced Smad1/5 in primary chondrocytes and cartilage. Notably, various studies using the LDN-193189 precursor dorsomorphin also show that this BMP type I receptor blocker is unable to block TGF β 1-induced Smad1/5 phosphorylation, supporting our observations [34, 35].

Based on structural analyses of R-Smad-ALK interactions, the ability to phosphorylate Smad1/5 is thought to be limited to BMP type I receptors [36, 37]. Our data indicate that ALK5 kinase activity is crucial, and possibly sufficient, for TGF β 1-induced Smad1/5 phosphorylation. In line with our data, it has previously been demonstrated that inhibition of ALK5-kinase with SB-431542 in endothelial cells [6] or with SB-505124 in synovial fibroblasts [34] attenuates TGF β 1-induced phosphorylation of Smad1/5. Furthermore, by knocking down ALK5, it has been shown by others that this receptor is necessary for TGF β -induced Smad1/5 phosphorylation [3, 6]. Possibly, ALK5 can directly phosphorylate Smad1/5 independently of BMP type I receptors in cartilage. Direct phosphorylation of Smad1 by caALK5 has been demonstrated by in vitro kinase assays [35, 38], and it has been shown that TGF β 1-induced Smad1 phosphorylation is inhibited in cells expressing a kinase defective ALK5 variant: ALK5KR [38]. Together, these studies indicate a direct induction of pSmad1/5 by ALK5, which would be blocked by SB-505124, but challenge the dogma that ALK5 cannot directly interact with Smad1 [36, 37]. In contrast, multiple studies show that in the absence of BMP type I receptors, TGF β 1-induced

pSmad1/5 is reduced [3, 6] indicating at least a role for these receptors.

Apart from R-Smad phosphorylation, TGF β 1 also induces TAK1 activation, a key component of Smad-independent TGF β signaling [5]. Noteworthy, with the use of the specific TAK1 kinase inhibitor (5Z)-7-Oxozeaenol [17] at 0.5 μ M, we showed that TAK1 kinase activity facilitates canonical C-terminal R-Smad phosphorylation in chondrocytes and cartilage. Previous studies support a role for TAK1 in R-Smad-dependent signaling; with the use of cartilage-specific TAK1 knockout animals, it has been shown that loss of TAK1 greatly diminishes BMP-induced C-terminal Smad1/5 phosphorylation while not affecting total R-Smad levels [39, 40]. Furthermore, tissue specific knockout of TAK1 in mesenchymal cells of neural crest origin shows that also C-terminal Smad2/3 phosphorylation is inhibited in the absence of TAK1 [41]. The pronounced effects of TAK1 on canonical C-terminal Smad phosphorylation have been ascribed to TAK1-induced Smad-linker phosphorylation, a post-translational Smad modification [41, 42].

Linker modification of R-Smads is an essential aspect of TGF β 1-signaling (reviewed in [43, 44]). The linker region of R-Smads contains threonine and serine residues that can be phosphorylated by various intracellular kinases like extracellular signal-regulated kinases (ERK), cyclin-dependent kinases (CDKs) and JNKs [43, 45]. Degradation, nuclear transport and interaction of R-Smads with other proteins are regulated by phosphorylation of this region, enabling context-dependent TGF β signaling [43, 44]. TAK1 can directly interact with R-Smads via the R-Smad MH2 domain [42], and phosphorylate, e.g. Smad2 at threonine 220 (Thr-220). Phosphorylation of this threonine greatly affects cellular localization and transcriptional activity of this Smad [41, 42]. TAK1 kinase activity is essential for this interaction of TAK1 with R-Smads [42]. As a consequence of this kinase dependency, it has been reported that the use of (5Z)-7-Oxozeaenol results in similar effects on C-terminal Smad1/5 and Smad2/3 phosphorylation as knockout of TAK1 [41]. We did not have access to cartilage-specific TAK1 knockout animals, but with the use of (5Z)-7-Oxozeaenol, we show for the first time

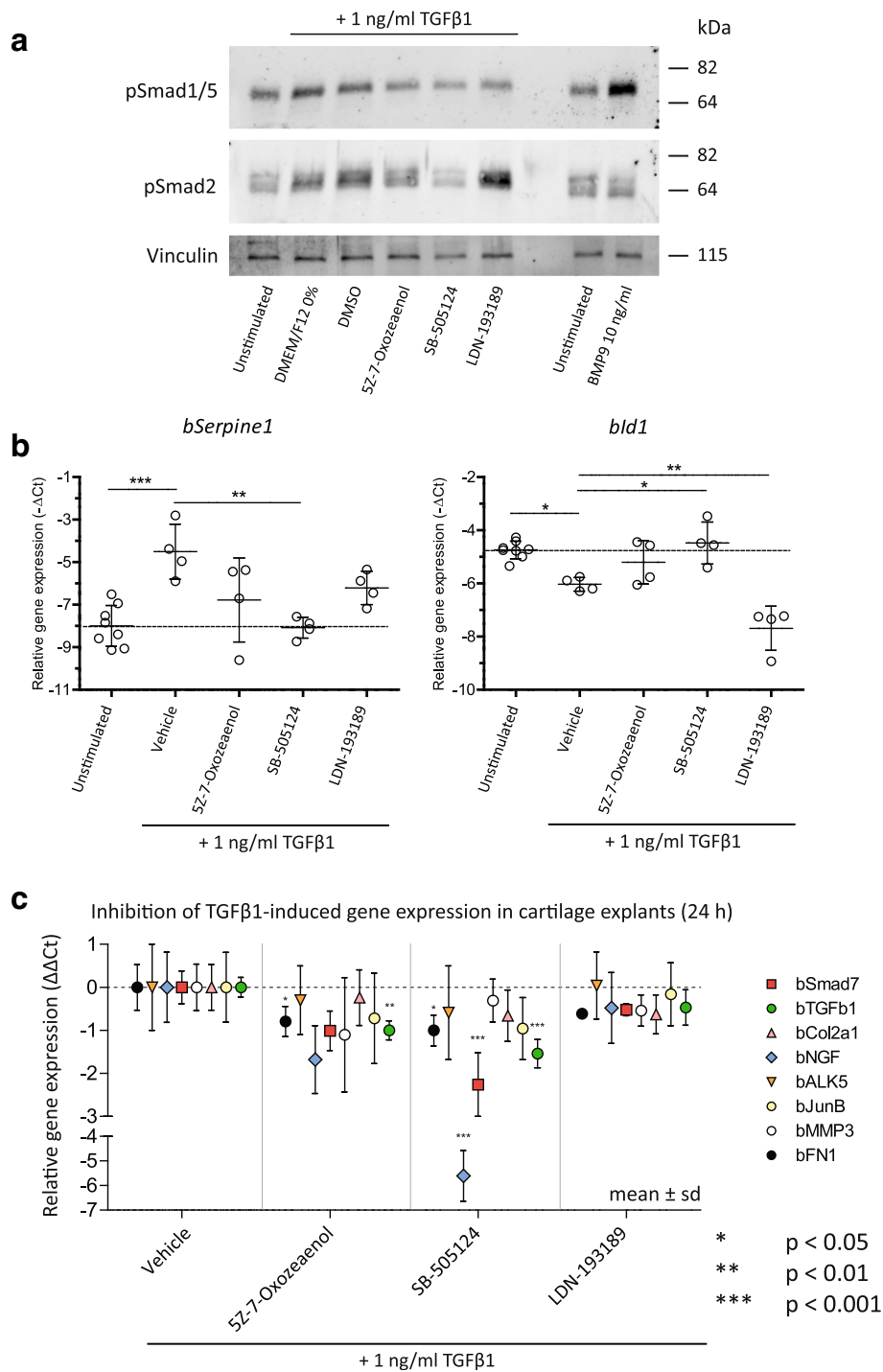
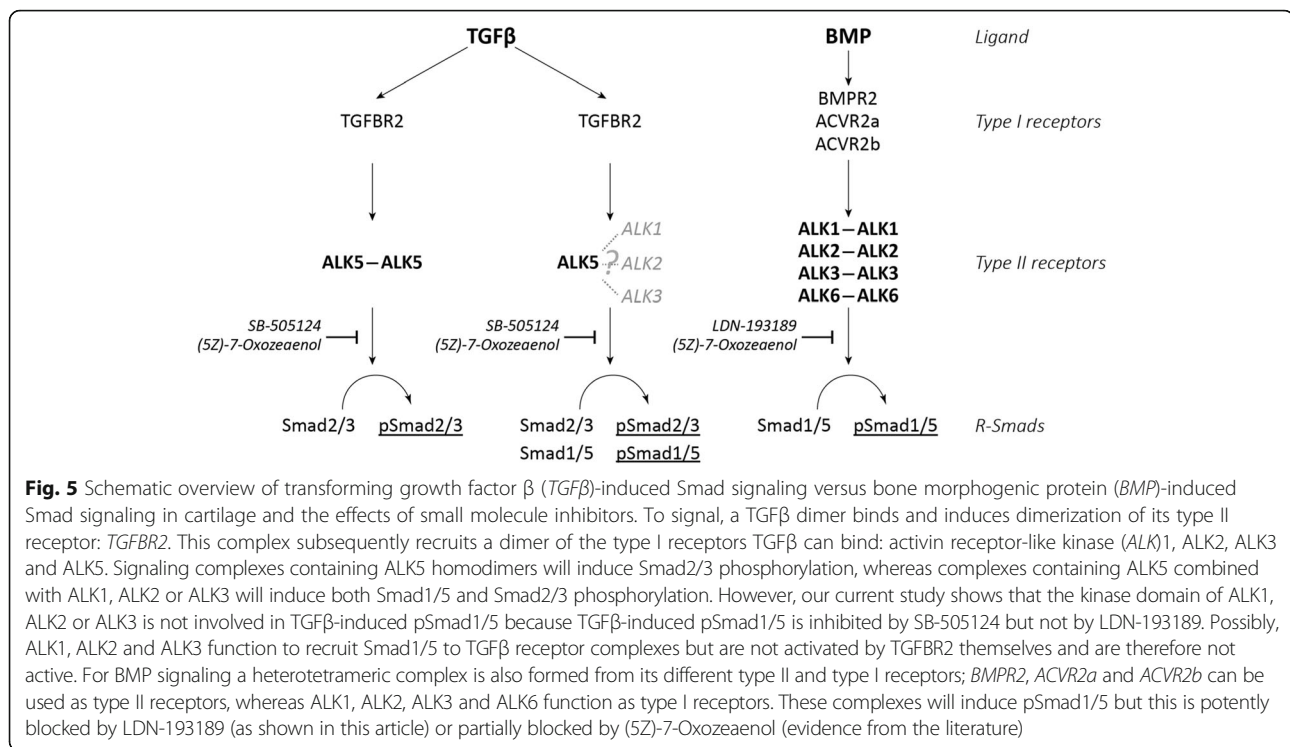


Fig. 4 In cartilage, SB-505124 fully inhibits and (5Z)-7-Oxozeaenol partly inhibits transforming growth factor β1 (*TGFβ1*)-induced Smad2/3 phosphorylation and gene expression, whereas LDN-193189 does not. **a** Bovine cartilage explants were pre-incubated ex vivo with 5 μM SB-505124, 0.5 μM (5Z)-7-Oxozeaenol or 0.05 μM LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGFβ1 for 2 h, after which phosphorylated Smads were visualized by western blot using specific antibodies. *kDa* kilodalton. **b** With the use of quantitative (q)PCR, expression of *bSerpine1* was measured as response gene for pSmad3 signaling after 24 h, and *bld1* for pSmad1/5 signaling after 24 h. **c** Gene expression was measured after pre-incubation of explants for 1 h with inhibitors followed by stimulation with 1 ng/ml TGFβ1 for 24 h. For qPCR data, average ± sd (*mean ± sd*) was plotted, with each dot representing the average of one donor (**b**) or four donors (**c**). Analysis was performed using one-way analysis of variance with Tukey's post-hoc test. **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001. A change of 1 ΔΔC_t equals twofold upregulation or downregulation. *TGF* transforming growth factor, *COL2A1* = collagen type II alpha 1 chain *MMP* matrix metalloproteinase, *NGF* nerve growth factor, *ALK5* activin receptor-like kinase, *JUNB* = JunB proto-oncogene, *FN1* = fibronectin 1



that this selective TAK1 kinase inhibitor greatly affects $TGF\beta$ -induced C-terminal R-Smad phosphorylation both in vitro and ex vivo in articular cartilage.

A limitation of our study is the possibly imperfect specificity of the used compounds, making it possible that off-target effects can explain our observations and interfere with our conclusions. However, in the experiments with the caALKs, we did not observe a significant effect of $SB-505124$ or $(5Z)-7-Oxozeaenol$ on caALK1-induced $pSmad1/5$, or an effect of $(5Z)-7-Oxozeaenol$ on caALK5-induced $pSmad2$. Therefore we do not think that these compounds have off-target effects on $ALK1$. Furthermore, a dose-response curve showed that $SB-505124$ inhibited $TGF\beta$ -induced $Smad1/5$ phosphorylation more potently than $Smad2/3$ phosphorylation, demonstrating that this effect of $SB-505124$ on $TGF\beta$ signaling is not an off-target effect obtained at high dosage, and this compound therefore cannot be used to specifically inhibit $TGF\beta$ -induced $Smad2$ in chondrocytes.

Another limitation of our study is that we did not include in vivo data. Unfortunately, the in vivo use of both $SB-505124$ and $LDN-193189$ is difficult because of their unfavorable pharmacokinetic properties, resulting in the need for daily re-administration via injection [33]. Up to now, only one study has used $SB-505124$ as a $TGF\beta$ blocker in vivo in a model of OA; i.e. in anterior cruciate ligament transection in mice [46]. In this study, inhibition of $TGF\beta$ signaling with 1 mg/kg/day of $SB-505124$ attenuated OA development, confirming that inhibition

of $TGF\beta$ signaling with a small molecule inhibitor is a feasible and promising approach to treat OA. However, the use of a higher dose of $SB-505124$ (2.5 mg/kg/day) was detrimental for cartilage because this resulted in proteoglycan depletion, indicating that total $TGF\beta$ inhibition comes with a risk. Possibly, a small molecule inhibitor that prevents $TGF\beta$ -induced $pSmad1/5$ but leaves $pSmad2$ unaffected will not have this risk. To our knowledge, both $LDN-193189$ and $(5Z)-7-Oxozeaenol$ have not been used in vivo to study their effects on OA development. They have been used in other applications, e.g. $LDN-193189$ in atherosclerosis research and $(5Z)-7-Oxozeaenol$ in cancer studies; however, this was not to specifically modulate $TGF\beta$ signaling but to inhibit BMP and $TAK1$ signaling, respectively. Therefore it is as yet unknown how these inhibitors modulate $TGF\beta$ signaling in vivo or in OA.

Conclusions

In conclusion, with the use of the small molecule inhibitors $SB-505124$ and $LDN-193189$ we were unable to direct $TGF\beta$ -signaling towards either $Smad1/5$ or $Smad2/3$ phosphorylation in cartilage. Our data suggest that in cartilage, $ALK5$ plays a central role in both $TGF\beta$ -induced $Smad1/5$ and $Smad2/3$ phosphorylation, making it difficult to separate the Smad pathways with the use of currently available intracellular small molecule inhibitors of the ALK receptors. Furthermore, we showed that treatment with the TAK1 inhibitor $(5Z)-7-Oxozeaenol$

inhibits TGF β -induced C-terminal phosphorylation of both Smad1/5 and Smad2/3, suggesting a link between the non-canonical and the canonical TGF β pathway in cartilage.

Additional files

Additional file 1: Figure S1. Expression of ALK1, ALK2, ALK3 and ALK5 mRNA in primary bovine cartilage and chondrocytes. **a** With the use of qPCR, expression of ALK1, ALK2, ALK3 and ALK5 was measured in both freshly isolated cartilage explants and in primary chondrocytes after 1 week of cell culture in DMEM/F12 supplemented with 10% non-heat-inactivated FCS without passage. All four ALKs were readily detected in both groups, but expression of all the receptors was higher in freshly isolated tissue. For calculations of the $-\Delta\Delta C_t$, two reference genes were used: *bGapdh* and *bRps14*. (PDF 2065 kb)

Additional file 2: Figure S3. Quantification of TGF β -induced pSmad2 and pSmad1/5 in cartilage explants. Quantification of the western blot as shown in Fig. 4a. The experiment was repeated three times. Significance was not obtained due to variation between experiments. pSmad levels were normalized to vinculin levels and plotted as a relative amount in arbitrary units (AU) compared to the control group. (PDF 2278 kb)

Additional file 3: Figure S2. LDN-193189 in a concentration of 0.05 μ M inhibits basal *ID1* expression in cartilage explants. Primary chondrocytes were incubated with LDN-193189 for 2 h in a dose of 0.05 μ M and *bd1* expression was measured using qPCR. LDN-193189 significantly inhibited *bd1* expression showing the bioactivity of this compound in cartilage explants. (PDF 2122 kb)

Abbreviations

μ l: Microliter; μ M: Micromolar (10^{-6} mol per liter); ALK: Activin receptor-like kinase; BCA: Bicinchoninic acid assay; BMP: Bone morphogenetic protein; caALK: Constitutively active activin receptor-like kinase; CDK: Cyclin-dependent kinase; cDNA: Complementary deoxyribonucleic acid; DMEM: Dulbecco's modified Eagle's medium; DNase: Deoxyribonuclease; dNTP: Deoxynucleoside triphosphate; dT: Deoxythymidine; DTT: Dithiothreitol; EDTA: Ethylenediaminetetraacetic acid; ERK: Extracellular signal-regulated kinase, also known as mitogen-activated protein kinase (MAPK1); g: Gravitational force; Gapdh: Glyceraldehyde-3-phosphate dehydrogenase; h: Hour; HRP: Horseradish peroxidase; IC $_{50}$: Half maximal inhibitory concentration; JNK: JUN N-terminal kinase; kDa: Kilodalton; LacZ: β -galactosidase; m/v: Mass per volume; mAb: monoclonal antibody; MAPK: Mitogen-activated protein kinase; min: Minute; ml: Milliliter; mm: Millimeter; M-MLV: Moloney murine leukemia virus; MMP: Matrix metalloproteinase; (m)RNA: (messenger) Ribonucleic acid; Ng: Nanogram; OA: Osteoarthritis; PCR: Polymerase chain reaction; R-Smad: receptor-regulated Smad; TAK1: TGF β -activated kinase 1, also known as: mitogen-activated protein kinase kinase 7 (MAP3K7); TGF β : Transforming growth factor beta; U: units

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Authors' contributions

Conception and design: AC, EBD and PK. Collection and assembly of data: AC and WM. Analysis and interpretation of data: AC, WM, AGV, MJG, PD, EBD and PK. Drafting of the manuscript: AC, EBD and PK. Critical revision: AC, WM, AGV, MJG, PD, EBD and PK. Final approval of the article: AC, WM, AGV, MJG, PD, EBD and PK.

Authors' information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

Not applicable. No live animals were used for this study, nor were animals specifically killed for this study to obtain material; all animals were slaughtered for their meat. Therefore ethical approval of an ethics committee was not required, nor was it applicable for this study.

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