

# Towards targeting overactive BMP signaling in Fibrodysplasia Ossificans Progressiva

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## ABSTRACT

Fibrodysplasia Ossificans Progressiva (FOP) is a rare monogenetic disorder in which patients develop heterotopic ossification (HO). A heterozygous mutation in BMP type I receptor ALK2 results in hyper-sensitized BMP signaling. The aim of this study is to identify small molecules which can selectively inhibit this overactive BMP pathway. Thirteen FDA-approved small molecules were tested on their effect on BMP6-induced target gene expression, alkaline phosphatase activity and mineralization in KS483 cells. We identified cryptotanshinone as a small molecule able to inhibit BMP signaling. In conclusion, cryptotanshinone could be a novel small molecule inhibitor of the overactive BMP signaling pathway in FOP.

## Keywords

Fibrodysplasia Ossificans Progressiva, FOP, ALK2, small molecules, cryptotanshinone, heterotopic ossification

## INTRODUCTION

Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disease affecting only 1 in 2 million people worldwide. Trauma, injury or other inflammatory-related events induce so-called 'flare-ups' during which FOP patients develop extra-skeletal bone in connective tissues, a process called heterotopic ossification (HO).<sup>1</sup>

The clinical presentation of FOP patients is caused by a heterozygous mutation in the *ACVR1* gene which encodes for the BMP type I receptor ALK2 (**Figure 1**).<sup>2</sup> This mutation is located in the 'GS-domain' of the ALK2 receptor and causes the receptor to be no longer inhibited, but stimulated by Activin A.<sup>3</sup> This genetic deficit thus leads to overactive BMP signaling which in combination with inflammation results in heterotopic ossification.

Since surgical resection of extra skeletal bone lead to counterproductive results, current therapies focus on the prevention of the harmful flare-up periods by education, *influenza* vaccination and glucocorticosteroids administration. Recently, several other therapeutic strategies have been examined for the treatment of FOP.

Small interfering RNAs and Antisense oligonucleotides

(AONs) have been developed to specifically target the mutated ALK2.<sup>4</sup> The feasibility of safe transfer of these RNAs in humans, however, is still under debate. A curative therapy for the heterotopic ossification has thus not been identified yet.

Treatment of heterotopic ossification could be established by novel small molecule inhibitors. Palovarotene, a small molecule RAR- $\gamma$  agonist, is currently being evaluated in a clinical trial for the treatment of FOP. Major concerns about this treatment, however, are the long-lasting effects of RAR- $\gamma$  agonists on growth plates of children with FOP.<sup>5</sup>

Also, several small molecule inhibitors of the ALK2 receptor have already been developed. LDN-193189 targets ALK2 and has a higher specificity for this receptor than his precursor dorsomorphin.<sup>6</sup> Further optimization of the dorsomorphin-derivatives resulted in the identification of the third generation ALK2 inhibitor LDN-212854. LDN-212854 has a much higher specificity for ALK2 in comparison with other BMP and TGF- $\beta$  receptors than LDN-193189.<sup>7</sup> The major disadvantage of the dorsomorphin-like small molecule kinase inhibitors, however, is their lack of specificity.<sup>8</sup> Small molecule inhibitors that target kinases downstream of ALK2 could potentially circumvent these harmful off-target effects.

In conclusion, the aim of this study is to identify a novel small molecule BMP inhibitor for the treatment of HO in FOP. By only screening small molecules which clinical use is approved by the Food and Drug Administration (FDA) identified small molecules could be readily translated into the clinic of FOP patients.

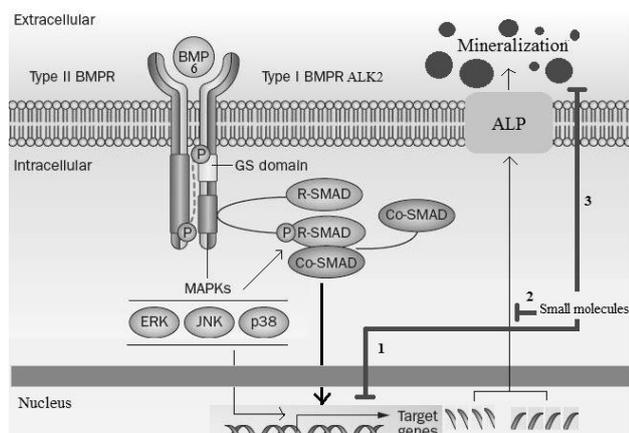
## METHODS

All experiments were performed on KS483 mouse bone-forming precursor cells (osteoblasts). For the last experiment, endothelial precursor cells obtained from FOP patients were used. Cells treated with only solvent DMSO (10  $\mu$ M) were used as a positive control. Small molecule ALK2 inhibitor LDN-193189 (120 nM) treated cells functioned as a negative control.

## Cell culture

Mouse osteoblast progenitor cells KS483 were cultured in Alpha Minimal Essential Medium ( $\alpha$ -MEM) + GlutaMAX<sup>TM</sup> (Gibco) with 10% fetal bovine serum (Gibco) and 0.4% penicillin/streptomycin (Invitrogen). EBM-2 (Lonza) supplemented with 10% FBS (Gibco)

and 0.4% penicillin/streptomycin (Invitrogen) was used to culture FOP-patient-derived endothelial precursor cells. Cells were grown at 37°C and 5% CO<sub>2</sub>. Medium was changed every three or four days.



**Figure 1** An overview of the used methods: qPCR [1], ALP assay [2] and Mineralization assay [3].<sup>9</sup>

### Quantitative PCR [1]

BMP6 (50 ng/mL), a ligand for ALK2, was used to stimulate the BMP signaling pathway in KS483 cells for 6 hours. As a result of BMP receptor stimulation, BMP target genes (*Id1*, *Id3*) are more transcribed into mRNAs. We measured the effect of half an hour pretreatment with small molecules (10 μM) on the abundance of *Id1* and *Id3* mRNAs in a quantitative PCR. The housekeeping gene HPRT1 was used to normalize the *Id1* and *Id3* gene expression data.

### Alkaline phosphatase (ALP) assay [2]

By stimulating the osteoblast precursor cells with BMP6 (50 ng/mL) for three days, these KS483 cells can change (differentiate) into bone forming cells. An early marker of osteoblast differentiation, is the formation of phosphates by an enzyme called alkaline phosphatase. By adding p-nitrophenyl phosphate (PNPP) solution (Thermo Scientific™ Pierce™) the effect on small molecules on BMP6-induced ALP activity was measured at 405 nm.

### Mineralization assay [3]

The actual formation of calcium deposits (bone) can be measured with a Mineralization assay. KS483 cells were stimulated with BMP6 (50 ng/mL), small molecules and ALP substrates, ascorbic acid and β-glycerolphosphate for six days. An Alizarin Red (Sigma) staining was performed to visualize the calcium deposits. Cetylpyridinium chloride was used to extract the staining in a quantifiable solution. Absorbance was measured at 545 nm.

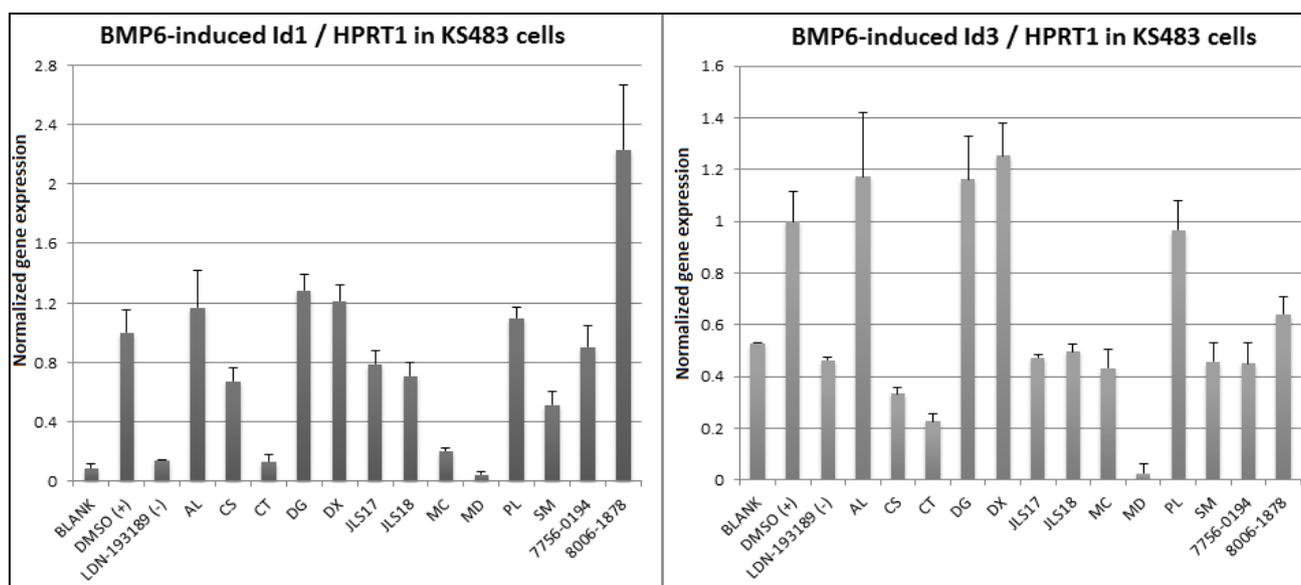
### Quantification and statistical methods

For staining quantification of the mineralization and alkaline phosphatase assay the absorption of the cell lysate was measured by a plate reader. All statistical analyses were performed using Student's t-tests. A p-value of less than 0.05 was considered significant.

## RESULTS

### Seven small molecules inhibited BMP target gene expression without cell toxicity

Thirteen FDA-approved small molecules listed in **Table 1** were selected by a pre-screening and included in this screening. First, the effect of the small molecules on BMP-target gene expression was measured in a quantitative PCR.



**Figure 2** The effect of compounds on BMP6-induced target gene expression in an initial screening of KS483 cells. KS483 cells were pretreated with 10 μM compound for half an hour and stimulated with BMP6 (50 ng/mL) for six hours. CS, CT, JLS-17, JLS-18, MC, MD and SM inhibited *Id1* and *Id3* gene expression. (n=1)

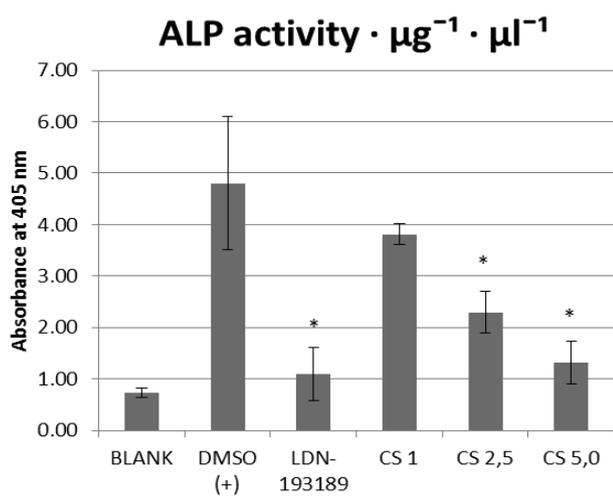
All KS483 cells were stimulated with BMP6 (50 ng/mL), except the non-treated BLANK condition. DMSO was used as a vehicle control and ALK2 inhibitor, LDN-193189 as a negative control. As Figure 2 shows, seven of the thirteen compounds (e.g., CS, CT, JLS17, JLS18, MC, MD, SM) potently inhibited both BMP6-induced *Id1* and *Id3* gene expression in mouse osteoblast progenitor KS483 cells. Cryptotanshinone (CS), cantharadin (CT), miconazole nitrate (MC), moxidectin (MD), selamectin (SM), JLS-17 and JLS-18 also did not show a major effect on KS483 cell proliferation in a MTS assay (Data not shown).

**Table 1** FDA-approved small molecules included in the initial screening

Alantolactone (AL)	Piplartine (PL)
Cantharadin (CT)	Selamectin (SM)
Cryptotanshinone (CS)	JLS17
Digoxigenin (DG)	JLS18
Digoxin (DX)	7756-0194
Miconazole nitrate (MC)	8006-1878
Moxidectin (MD)	

#### Cryptotanshinone dose-dependently inhibits osteoblast differentiation

Because of its potency of inhibiting BMP6 target gene expression and BMP6-induced ALP activity and mineralization (Data not shown) without cell toxicity, we selected cryptotanshinone (CS) as the most promising candidate. As Figure 4 shows, CS dose-dependently inhibited ALP activity in KS483 cells. ALK2 inhibitor LDN-193189, 2.5 and 5.0  $\mu\text{M}$  of CS significantly inhibited ALP activity in comparison to vehicle control DMSO. Also, mineralization was potently inhibited by CS on a dose-dependent manner; 5.0 and 10.0  $\mu\text{M}$  of CS reduced mineralization significantly (Figure 5).



**Figure 4** The effect of CS on ALP activity in KS483 cells. 2.5 and 5.0  $\mu\text{M}$  significantly inhibited ALP activity. 10  $\mu\text{M}$  was cell-toxic in this assay. (n = 3) p < 0.05 = \*, p < 0.01 = \*\*, p < 0.001 = \*\*\*

#### The effect of cryptotanshinone on FOP patient-derived cells

In order to assess the effect of CS on FOP patient-derived cells, endothelial precursor cells obtained from patients were tested in a mineralization assay. In an experimental design, comparable to the KS483 cells, the endothelial precursor cells were stimulated with low dose CS (1.0, 2.5 or 5.0  $\mu\text{M}$ ), BMP6 (50 ng/ml) and osteogenic medium for seven days. Unexpectedly, cryptotanshinone was cell-toxic in these FOP patient-derived endothelial precursor cells. The cells cultured in normal medium and the cells treated with BMP6, osteogenic medium and ALK2 inhibitor LDN-193189 didn't show cell death.

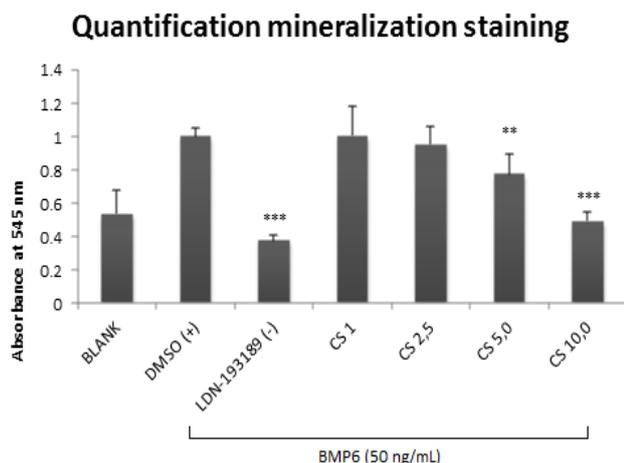
#### CONCLUSION

Because small molecules could be attractive therapeutics for inhibiting the overactive BMP signaling pathway in FOP, we performed a screening of thirteen FDA-approved small compounds. From this screening cryptotanshinone (CS) was selected as the best candidate based on its inhibiting effects on osteoblast differentiation without cell-toxic effects on KS483 cells. Cryptotanshinone decreased BMP6-induced target gene expression, ALP activity and mineralization. Low dose CS was also able to induce to induce cell-death in endothelial precursor cells derived from FOP patients.

CS, a tanshinone isolated from the traditional Chinese herb *Salvia miltiorrhiza*, has been reported to have several effects on major cell processes. It has been shown to induce apoptosis in multiple tumor cell lines *in vitro*.<sup>10</sup> Also, several studies suggest that patients with cognitive impairment (Alzheimer's disease, insomnia e.g.) might benefit from treatment with CS because of its acetylcholine esterase inhibiting properties.<sup>11</sup>

Furthermore, CS inhibits the inflammatory related TNF- $\alpha$  and LPS-induced signal transduction pathways in several cell types.<sup>12</sup> If CS simultaneously inhibits inflammatory pathways and the BMP signaling pathway, CS treatment could potentially act as a double-edge sword by reducing the inflammatory-related flare-ups and inhibiting the overactive BMP signaling pathway in FOP patients.

We show that CS inhibited the overactive BMP signaling pathway in a mouse cell line with osteogenic properties. However, endothelial cells have been shown to give rise to the largest portion of cells infiltrating heterotopic ossification sites in a lineage tracing study of a FOP mouse model.<sup>13</sup> Therefore, we believe that the use of unique FOP patient-derived endothelial cells in long term differentiation assays is an excellent *in vitro* model for the assessment of BMP-inhibiting properties of small molecules. CS induced apoptosis in FOP patient-derived endothelial precursor cells which can thus reduce the amount of infiltrate on the heterotopic ossification sites. It is, however, likely that the induction of apoptosis by CS is not limited to these harmful infiltrating cells. By restricting the CS treatment to topical administration during flare-ups, we could diminish the side-effects of the CS treatment. Nevertheless, the cell-toxicity of CS should be assessed in multiple human cell lines in future research.



**Figure 5** Titrate curve of CS in a mineralization assay in KS483 cells. Figure 5 shows the quantification of the Alizarin Red staining. CS 5.0 and 10  $\mu$ M concentrations significantly inhibited mineralization. (n = 3 for LDN-193189, CS 2.5 and CS 5.0, n = 6 for BLANK, DMSO, CS 1.0 and CS 10.0)

CS might be more specific than ALK2 inhibitors and the RAR- $\gamma$  agonist currently evaluated in a clinical trial, because it has the potential to specifically inhibit mineralization induced by flare-ups. However, the exact mechanism via which CS exerts its function is unclear. In conclusion, we identified cryptotanshinone as a novel BMP inhibitor for the treatment of FOP.

#### ROLE OF THE STUDENT

This study was performed as a Student Research Project of the BSc Biomedical Sciences at the LUMC. The prescreening was performed by M. van Dinther. The screening was designed by Prof. Dr. P. Ten Dijke and executed by Bachelor Student J. van Staalduinen. CS was selected by J. van Staalduinen and Dr. G. Sánchez-Duffhues. The data analysis, statistical analyses and writing was performed by J. van Staalduinen and discussed with Dr. G. Sánchez-Duffhues and Prof. Dr. P. Ten Dijke. The authors declare no conflicts of interest.

#### ACKNOWLEDGMENTS

I would like to thank Dr. Gonzalo Sánchez-Duffhues for his study supervision, technical support and discussion and Prof. Dr. Peter ten Dijke for his study supervision and discussion. Furthermore, I would like to thank Maarten van Dinther for performing the prescreening and Dr. A. García de Vinuesa for discussion of the results. Also, I thank M. Thorikay for her technical support.

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