Generation of selective anti-BMP VHHs

Effective inhibition of Bone Morphogenetic Protein function

by highly specific llama-derived antibodies

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*Running title: Generation of selective anti-BMP VHHs

Keywords: VHH; BMP; structure; inhibitor;

Total words: 6,612

No potential conflicts of interest were disclosed in this manuscript. This work was supported by the European Research Council starting grant Targets4Barrett (S.Calpe and K.K.Krishnadath).
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ABSTRACT

Bone Morphogenetic Proteins (BMPs) have important but distinct roles in tissue homeostasis and disease, including carcinogenesis and tumor progression. A large number of BMP inhibitors are available to study BMP function; however, since most of these antagonists are promiscuous, evaluating specific effects of individual BMPs is not feasible. Because the oncogenic role of the different BMPs varies for each neoplasm, highly selective BMP inhibitors are required. Here we describe the generation of three types of llama-derived heavy chain variable domains (VHHs) which selectively bind to either BMP4, to BMP2 and 4, or to BMP2,4,5 and 6. These generated VHHs have high affinity to their targets and are able to inhibit BMP signalling. Epitope binning and docking modelling have shed light into the basis for their BMP-specificity. As opposed to the wide structural reach of natural inhibitors, these small molecules target the grooves and pockets of BMPs involved in receptor binding. In organoid experiments, specific inhibition of BMP4 does not affect the activation of normal stem cells. Furthermore, in vitro inhibition of cancer derived BMP4 non-canonical signals results in an increase of chemosensitivity in a colorectal cancer cell line. Therefore, due to their high specificity and low off-target effects these VHHs could represent a therapeutic alternative for BMP4+ malignancies.

INTRODUCTION

BMPs are growth factors that belong to the TGFβ superfamily. They consist of around 20 members, classified into distinct subfamilies, depending on their sequence homology and functionality. BMP2, BMP4 and BMP5, BMP6, BMP7 form two subgroups that have been extensively studied (1). They mediate their function by binding to two molecules of type I...
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(BMPR1A, BMPR1B or ACVR1) and two molecules of type 2 (BMPR2, ACVRL1, ActRII or ActRIIB) serine/threonine kinase receptors (2). Depending on the presence or absence of pre-formed BMPR complexes, the signalling is mediated via the Signalling Mothers Against Decapentaplegic (SMAD) family of transcription factors following a defined canonical pathway or by less defined non-canonical intracellular effectors (3,4). Translocation to the nucleus of these transcription factors, initiates transcription of BMP-target genes (5,6).

BMPs are emerging as attractive therapeutic targets due to their multiple roles in a variety of diseases, in particular cancer. For many years, the BMP field has been dogged by the assumption that many BMPs are functionally interchangeable. However, there is increasing evidence indicating the disparate actions of BMPs (7). For instance, whereas BMP4 and BMP2 seem to facilitate metastasis and invasion in several cancers, BMP6 and BMP7 present a suppressive role in metastatic breast cancer and melanomas (8). Most intriguing is the role of BMP4, which presents opposing roles depending on the neoplasm (7,9). In certain malignancies, such as glioblastoma (10) and myeloma (11), high levels of BMP4 are associated with less malignant features while in breast (12), ovarian (13), lung (14) and prostate (15) BMP4 is involved in Epithelial Mesenchymal Transition (EMT) and metastatic behaviour. In gastrointestinal cancers such as pancreas (16), colon (17) and gastric (18), BMP4 has been associated with tumour progression and chemotherapy resistance. Therefore, to resolve and inhibit the individual action of BMP4 in tumours, the generation of potent and specific BMP4 inhibitors is warranted.

Targeting and regulating BMP signalling at the extracellular receptor level is clinically highly attractive (19). Neutralization of BMP by several natural antagonists and chemical inhibitors has already been proven successful for the suppression of metastasis in prostate (15), lung (14) as well as breast cancer (20). Albeit significant progress has been made in generating highly specific
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and less toxic small molecule inhibitors that target BMP type I receptors (21–23), these inhibitors are still non selective. Therefore the clinical use of current BMP inhibitors is questionable (24). For instance, in vivo treatment with the natural antagonist Coco or the chemical inhibitor LDN-193189 results in reactivation of breast cancer (25) or colon tumour burden (26) in mice with a particular mutational phenotype, respectively. Therefore, these off-target effects could lead to undesirable side effects if used in the clinic.

Llama-derived antibodies are small proteins of about 15kDa that, as opposed to conventional antibodies, their antigen binding fragment is formed by just the variable domain of the heavy chain, hence their denomination as VHHs (27). Because of their unique structure, VHHs can bind specifically and with high affinity to their antigens, often due to their ability to bind to hidden epitopes within grooves or cavities. This peculiar characteristic was leveraged to design specific and highly effective inhibitors of BMP4. In the present study, we describe the characterization of five anti-BMP4 llama-derived VHHs. Specificity to different BMPs allowed their classification into 3 groups: VHHs specific for BMP4; BMP2,4; and BMP2,4,5,6. We demonstrate that their binding affinities are greater than those published for Noggin, a natural BMP antagonist, and for conventional anti-BMP4 antibodies. We show that the VHHs effectively inhibit BMP downstream signalling and transcriptional activation of BMP target genes. Our epitope and docking experiments, as well as mutagenesis analyses, provide structural evidence for their affinity and specificity. These studies show that each VHH binds to a different molecular interface within the BMP molecule and, subsequently, unveil the binding regions that shape BMP specificity. Finally, we show that our VHHs inhibit endogenous BMP function as they can replace Noggin in ex vivo organoid cultures as well as increase in vitro chemosensitivity of BMP4+ colorectal cancer cells.
MATERIALS AND METHODS

Generation of llama-derived anti-BMP4 VHHs

To generate VHHs specific for BMP4 we immunized two llamas with 100µg of recombinant human BMP4 (R&D Systems) on day 0, 14, day 28 and day 35. At day 44 peripheral blood lymphocytes were purified for RNA extraction and library construction. Fragments of ~350 bp in size, were cloned in a phage-display plasmid and transferred to the *Escherichia coli* strain TG1. Two rounds of phage selection were performed to select for VHH binders to BMP4, which were confirmed by ELISA. Eight high binders to BMP4 were then purified from the soluble periplasmic fractions using Talon beads (Clontech). Fractions containing purified VHHs were pooled, and their purity analysed by SDS-PAGE and Coomassie staining. Binding to BMP4 was again confirmed by ELISA. PCR and site-directed mutagenesis were used to generate the biheads and the mutant monoheads, respectively. Specific details on the generation, isolation, purification and modification of VHHs can be found in Supplementary Materials and Methods.

Cell culture

The human hTERT-immortalized esophageal cell line, EPC2-hTERT, was a kind gift of Prof. A. Rustgi, University of Pennsylvania, Philadelphia, Pennsylvania, USA (28) and was cultured as previously described (29). To detect phosphorylation of SMAD1/5/8 *in vitro*, EPC2-hTERTs were cultured in 24-wells plates and activated with 100ng/ml of BMP2, BMP4, BMP5, BMP6 or BMP7 (R&D Systems) for 4 hours with or without VHHs or Noggin at the indicated concentrations. C2C12 mouse myoblasts (stably transfected with a reporter plasmid for BMP activity) were a kind gift of Dr. L. Zilberberg and Dr. D. Rifkin (30) (New York University,
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School of Medicine, New York, NY, USA). CL-11 and Colo-678 were obtained from the COSMIC project at the Sanger Institute, UK. The rest of the cell lines were obtained from ATCC (American Type Culture Collection). All cell lines were obtained more than 6 months ago and no authentication has been performed by the authors.

**Western Blot**

Protein isolation and SDS-PAGE separation were performed as described previously (31). Briefly, EPC2-hTERT and organoids were lysed with 100µl of M-PER buffer (Sigma-Aldrich) whereas the mouse organoids were lysed at a ratio of 1:20 with T-PER buffer (Sigma-Aldrich). Cell debris were pelleted and supernatant was collected. For protein detection the following antibodies were used: antiphospho-SMAD1/5/8 at 1:500; p-p38 (T180/Y182) at 1:500, pAkt (ser473) at 1:1000, total Akt at 1:500, total p38 at 1:500; (Cell Signalling); anti-βactin at 1:1000, anti-SMAD4 at 1:500, anti-pERK (Y204) at 1:1000 (Santa Cruz); anti-GAPDH at 1:500 (Millipore); anti-BMP-7 MAB3541 (R&D Systems) and anti-BMP6 AF507 (R&D Systems) at 1:1000; and anti-BMP5 ab10858 (Abcam), anti-BMP2 500-P195 (Prepotech) and anti-BMP4 MAB757 (R&D Systems) all at 1:500.

**BMP activity luciferase reporter assay**

C2C12 cells were plated in 96-well plates at 5x10^3 cells/well and cells were allowed to attach overnight. 100µl of DMEM with 0.1% BSA were added in each well. Cells were treated in triplicate with BMPs, VHHs or controls at the indicated concentrations for 16 hours. Wells with unstimulated cells or no cells were added as controls. Luciferase activity was measured by adding 100µl of luciferase substrate solution from the Bright-Glo™ Luciferase Assay System (Promega Benelux). After 3 minutes of incubation, luciferase activity was measured with Synergy HT.
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Multi-Mode Microplate Reader (Biotek). Luciferase values were normalized to background activity (activity of wells with no cells) and represented as ratio to the activity of BMP-stimulated cells.

ELISA

Concentrations of BMP present in the conditioned mouse organoid media were evaluated via ELISA, according to the manufacturers’ protocol: BMP2 (RAB0028), BMP-4 (RAB0029), BMP-5 (RAB0031) and BMP-6 (RAB0032), from Sigma-Aldrich.

Surface plasmon resonance (SPR)

Serial dilutions (2.0-10 μg/ml) of purified VHHs were spotted on an amine-specific SensEye gold-film gel-type SPRchip (Ssens), using a Continuous Flow Microspotter (Wasatch Microfluidics) as described (32). BMP binding was analyzed on an IBIS MX96 (IBIS Technologies) instrument by performing injections with dilution series (0.05 - 2.0μg/ml) of recombinant BMPs on the VHH-coated chip. In each injection, BMPs were injected and incubated for 8 min, followed by 15 min thorough washing with binding buffer (PBS + 0.05 % Tween20 + 0.05 % sodium azide) to measure dissociation. Epitope binning was done by injecting VHH dilutions (2.0μg/ml) over the chip, immediately after the dissociation step. Injections with blank binding buffer were used as reference. After each concatenated injection, the chip was regenerated with 10mM glycine-HCl, pH 2.0. Experimental data were processed with SPRintX software (IBIS Technologies) and kinetic parameters were determined using Scrubber2 software (BioLogic Software). Binding constants were obtained by global fitting to a one-site binding model.

HADDOCK Modelling
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HADDOCK software (High Ambiguity Driven protein-protein DOCKing) (33) was used to model BMP4 binding to the different VHHS. A 3D homology model of BMP4 was generated with BMP2 (PBD 1ES7) as a template using Modeller9.9. Structures of the different VHHS were modelled based on crystal structures of other VHHS, such as 4B5E (for C4) and SJX (for E7 and C8) based on their similarities at the sequence level (~74%). A detailed description of the residues chosen for docking BMP4 to VHHS can be found in Supplementary Materials and Methods.

Organoid cultures

Crypts were isolated from small intestines of wt mice as previously described (34). After isolation, 500 crypts were resuspended in 50µl of polymerized Matrigel per well, in a 24-well plate. 500µl Advanced DMEM/F12 (Invitrogen) medium containing 50ng/ml EGF (Tebu-BIO), Fc-Noggin (10%) and Fc-Rspondin (20%), both generated in house, were added per well. Where indicated, VHHS were used in place of Fc-Noggin (C4C4 at 100ng/ml, C8C8 at 500ng/ml, C8C8 at 5µg/ml, E7 at 5µg/ml). As controls, some crypts were cultured in the absence of Noggin or VHHS. At day 3, the supernatant was collected and medium was replaced. Lysates for protein extraction were collected at day 5.

Chemosensitivity

HT29 cells were plated in 96-well plates at a density of 10,000 cells per well. After 24 hours they were stimulated with the indicated concentrations of 5’Fluorouracil (5’FU), Oxaliplatin and Cisplatin (Sigma-Aldrich) for 48 hours. The VHHS or Noggin were added at the same time at a concentration of 500ng/ml. Cell viability was measured by adding the Presto Blue Reagent (Invitrogen) for 2 hours at 37°C, after which absorbance was measured at 520nm.
RESULTS

Specificity and functional activity of the anti-BMP4 VHHs

The immune phage display libraries constructed from peripheral blood lymphocytes (PBLs) of llamas immunized with BMP4 resulted in the isolation of five VHHs with high affinity for BMP4 binding. In BMP responsive EPC2-hTERT cells (29), a normal human squamous esophageal cell line with no detectable basal levels of phosphorylated SMAD1/5/8, all VHHs were able to inhibit BMP4-mediated SMAD1/5/8 phosphorylation (Fig. 1A).

Due to the high similarities (~60-80%) between the mature regions of BMP4 and other members of the BMP family, we tested the specificity of the VHHs with regard to BMP2, BMP5, BMP6 and BMP7. We found that whereas A1, C3, and C4 were strictly BMP4 specific, C8 also inhibited BMP2-mediated signalling (Fig. 1A). To determine whether our VHHs would also inhibit BMP-mediated transcriptional activity, we employed the BMP-responsive C2C12 cells which are stably transfected with the BRE-luciferase vector containing the mouse DNA-binding protein inhibitor 1 (ID1) promoter, an immediate early gene that is activated by binding of the phosphorylated SMAD to its promoter region (30). As shown in Fig. 1B, all VHHs were able to inhibit BMP4-mediated transcriptional activity to the same level as Noggin. Both C8 and E7 exerted an inhibitory effect in BMP2-transcriptional activation. E7 also inhibited BMP5 and BMP6, albeit to a lesser extent than the other BMPs.

Bivalent VHHs lead to increased functional activity

Hetero- and homodimeric VHHs have superior antigen neutralizing activities compared to monomers (35). We constructed three bivalent VHHs with two consecutive peptide spacers (GGGGS) as a linker. In EPC2-hTERT cells the dimers inhibited BMP-mediated pSMAD1/5/8
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activation at much lower concentration compared to the monomers (Supplementary Fig. S1). To evaluate the extent of this increase in potency, the IC\textsubscript{50} of the different VHHs were determined in a dose-dependent inhibition assay using C2C12 cells (Fig.2). C4C4 displayed a 9-fold increase at inhibiting BMP4-mediated transcriptional activation as compared to monomer C4 and the lowest IC\textsubscript{50} amongst all tested VHHs (Fig. 2A). Similarly, bivalent C8 also resulted in a more potent dimer (Fig. 2B-C), an effect more striking for BMP2 rather than BMP4 inhibition. Albeit better than E7, bihead C8E7 proved to be similar at inhibiting both BMP2 and BMP4 as compared to C8 (3D-E), suggesting that C8 might be the dominant molecule in this bihead. The specificity of the biheads is retained, except for C8C8, which surprisingly, weakly inhibits BMP5-mediated transcriptional activity at higher doses (Supplementary Fig. S2). The remaining members of the BMP subfamily have low sequence similarities to BMP4 (~30% identities). Nevertheless, they were tested to confirm the specificity of the monoheads and biheads. As expected, no inhibition of BMP9, BMP10 or BMP12 was observed by any of the VHHs tested (Supplementary Fig.S3A-C). Other members of the TGF-β superfamily were also tested to further confirm the BMP-specificity of the VHHs. No inhibition of SMAD2 phosphorylation was observed after TGF-β or Activin A activation (Supplementary Fig. S3D-F).

Affinity analysis of the VHHs

BMP specificity and binding affinities of the VHHs was determined by surface plasmon resonance (SPR) analysis. Noggin and BMPR1\textsubscript{a} were used as controls and bound all BMPs, concurring with published observations (Table 1) (36,37). Amongst all measured monomers, C8 and E7 were the only ones binding to BMP2 (Fig.3A), with slightly higher affinity for BMP4 (Table 1), explaining the lower IC\textsubscript{50} values of C8 and E7 for BMP4 than for BMP2 (Fig.2). The affinities of E7 and C8 to BMP2 are similar but their binding kinetics differ. Whereas E7 binds to
BMP2 much faster, C8 dissociates from BMP2 at a much slower pace than E7. This could explain why C8 is better than E7 at inhibiting BMP2-mediated signalling and transcriptional activation (Fig. 1). Due to poor coupling to the plate, binding affinities for monomer C4 could not be obtained.

The C8 dimers had increased affinity for BMP2 and BMP4 (Table 1). The lower K_Ds observed for C8C8 and C8E7 result mainly from lower dissociation rates as compared to the monomers (Table 1). Interestingly, the association rate for C8E7 to both BMP2 and BMP4 is similar to the one observed for C8, confirming the assumption that C8 is the prevalent molecule in this bihead. The BMP binding affinities of biheads C4C4 and C8C8 were higher than those found for Noggin or for a commercially available conventional anti-BMP4 antibody from R&D systems (Table 1).

In keeping with the functional experiments, E7, C8C8 and C8E7 bound to BMP5. However, the affinities for these interactions are low, which might explain why functional inhibition of BMP5 by these VHHs is not as striking as the one for BMP4. Notably, and in contrast to their monomers, unspecific binding of the bivalents to diverse BMPs were observed. C4C4 was shown to bind BMP2; C8C8 to BMP5 and C8E7 to BMP7 (Fig. 3A). Because of the presence of two binding sites per bihead, the change for re-binding BMP after initial dissociation is higher, explaining the increase in potency but the loss in specificity. These equilibrium dissociation constants, however, befall within the ranges of 2-123nM, three hundred times higher than the ones observed for BMP4 binding (~10-100pM), which might explain the lack of functional implications for these interactions (Table 1).

**Epitope binning**
The solved crystal structures of BMP2 and BMP7 with their receptors have provided the basis of receptor binding to BMP ligands (37–39). Two epitopes are involved in the binding of BMPs to their receptors (Supplementary Fig. S4): the ‘wrist epitope”, a large concave area involved in the binding to type 1 receptors (40) and the “knuckle epitope”, a convex molecular interface involved in type 2 receptor binding (41).

In an effort to determine the binding epitopes and explain the different BMP specificities between C4, C8 and E7, we employed an SPR sandwich cross-binding or “epitope binning” assays (Supplementary Table S1). Notably, besides BMPR1a, all molecules showed weak self-binding in the cross-binding assay (Fig. 3B). C8 and E7 do not share the same epitope, as they could cross-bind with each other in both directions. Further, whereas both E7 and C8 compete with Noggin for BMP4 binding, E7 but not C8 could still bind to BMPR1a-bound BMP4. This suggests that the epitope of C8 maps to the wrist epitope, whereas the E7 epitope maps to a region different to that of BMPR1a, but targeted by Noggin. Because Noggin binds to both BMPR1a and BMPR2 epitopes (42), it is most likely that the E7 epitope therefore maps to the BMPR2 binding site.

Due to technical difficulties of coating C4 into the SPR chip, we decided to use C4C4 for these experiments. C8 and E7, but not Noggin or BMPR1a, were able to weakly bind BMP4 bound to C4C4 (Fig. 3B). Therefore, C4C4 binds an epitope that is different from C8 and E7, but overlaps with that of Noggin and BMPR1a. These results suggest that C4 and C8 target a different area within the wrist epitope, a not surprising finding due to the small nature of the VHHs and the large contact area of the this epitope. Cross-binding with a commercial anti-BMP4 (R&D) was detected on all VHHs and controls, demonstrating that the epitope of this anti-BMP4 is remarkably different than that of the VHHs and it resides in an area non-overlapping Noggin or BMPR1a (Fig. 3B).
Docking models of C4, C8 and E7

To identify putative binding sites of the VHHs to BMP4 modelling using HADDOCK software (High Ambiguity Driven protein-protein DOCKing) was performed (33). Modelling of C4 to BMP4 showed that C4 interacts with the “hydrophobic groove” of the wrist epitope of BMP4 (Fig. 4). In this model, loop2 of BMP4 packs mainly hydrophobically against residues from both CDR2 and CDR3 of C4. P50 of BMP4 sustains hydrophobic interactions with I57 of VHH C4; and D46 of BMP4 forms an hydrogen bond with S102 of C4 (Fig. 4A). In contrast, the preβ1 and β1 region mainly interact with CDR3 residues. Of note is D99, that forms salt bridges with both K12 and R15 of BMP4, a similar interaction was observed between K15 of BMP2 and D46 of BMPR1α (37).

Modelling of C8 to BMP4 revealed that C8 interacts with the other contact point of the wrist epitope. In this model, C8-BMP4 binding appears to be driven by interactions between residues in the CDR3 region of C8 and the “hydrophobic pocket” of the wrist epitope of BMP4 (Fig. 4B). In the hydrophobic surface of the α-helix of BMP4A, L66 sticks out to interact with T105 of C8. Residues in loop1 of BMP4B provide a hydrophobic area allowing for interactions like P109 with W31 of BMP4 and the double salt bridge between D30 of BMP4 and R106 of C8 (Fig. 4B). Also F110 and F102 of C8 make multiple contacts with the area of BMP4 containing D30. Y107 of C8 forms a hydrogen bond with K101 located at the inner side of the β8 strand of BMP4.

HADDOCK modelled both CDR1 and CDR2 residues of E7 to be involved in binding to the knuckle epitope of BMP4 (Fig. 4C). The nitrogen atom from the main chain of T56 in E7 forms a hydrogen bond with S88 of BMP4. L57 from E7 is also in close proximity to S88. V32 from the CDR1 of E7 is in close proximity to a hydrophobic area formed by the residues located in the
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outer sides of the β3 (A34) and β8 strands (L90) of BMP4. Finally, W47 from E7 interacts with Q39 of BMP4 forming a hydrogen bond. This interaction is reminiscent of the one in type 2 receptor ActR-IIIB and BMP2, in which W60 (a highly conserved residue amongst type 2 receptors) extends to the hydrophobic core of the knuckle epitope of BMP2 (43).

Mutation analysis was performed to confirm the HADDOCK models. Some of the residues shown to be involved in VHH-BMP4 binding were substituted singly by in vitro mutagenesis. Except for I57 of C4, all mutations affected the potency of the different VHHs (Figure 4D-E), suggesting that the mutated residues are important binding determinants.

**VHHs inhibit endogenous BMP activity in ex vivo organoid cultures**

The ability of our VHHs to provide functional inhibition of endogenous BMP was tested in mouse intestinal cultures of stem cells. Inhibition of BMP activity is one of the requirements to maintain intestinal stem cells in ex vivo cultures of “organoids”, structures that mirror the intestinal crypt-villus units. In these cultures, BMP inhibition is usually provided by the addition of the natural BMP-inhibitor Noggin to the media (34). ELISA (Fig. 5A) and western blot (Fig. 5B) analysis revealed that BMP2, BMP4, BMP5 and BMP6 are expressed and secreted in organoids cultured for three days in the absence of Noggin. To check the ability of the VHHs to provide inhibition of these endogenous BMPs and to sustain organoid cultures ex vivo, freshly isolated crypts were cultured with the different VHHs. Analysis of the BRE-luciferase activity in the supernatants of these cultures confirmed the functionality of these VHHs (Fig. 5C). Whereas specific BMP4 inhibition provided by C4C4 resulted in a partial decrease in the BRE-luciferase activity, concomitant inhibition of BMP2 and BMP4 was sufficient to result in an almost complete blockage of BRE-luciferase activity. Further inhibition of BMP5 and BMP6 in these...
cultures, by the remaining VHHs (or by Noggin), did not result in a further decrease in BRE-luciferase activity, revealing a minor role for other BMPs in these cultures (Fig. 5C). Although these results manifest the capacity of C4C4 to inhibit endogenous BMP4 function, they suggest that inhibition of BMP4 is not sufficient to maintain intestinal crypt cultures. Indeed, the number of organoids counted from the crypts cultured for five days with C4C4, was as low as the crypts cultured without Noggin (Fig. 5D). Furthermore, the average size of these organoids was small, and presented features comparable to the ones observed for the organoids grown in the absence of Noggin (Fig. 5E). In contrast, inhibition of BMP2 and BMP4 provided by the addition of C8C8 (at 500ng/ml) was enough to sustain self-renewal of intestinal crypts. These organoids presented the same morphological characteristics as the ones cultured with Noggin, manifested by the presence of several crypt-like budding structures, where the intestinal stem cells together with the Paneth cells reside (Fig. 5E). Additional inhibition of BMP5 provided by C8C8 at higher doses (5µg/ml) or BMP5,6 by E7 did not provide superior growth advantage to the cultures when compared to C8C8 at lower dose. Similar findings were observed when cultured with Noggin (Fig. 5D,E). In sum, these experiments show that inhibition of both endogenous BMP2 and BMP4 is required to sustain self-renewal of small intestine stem cells in vitro and this can effectively and selectively be provided by C8C8, the BMP2,4-specific VHH.

**Inhibition of BMP4 by the VHHs increase the chemosensitivity of BMP4+ colorectal cancer cells**

BMP signalling has been shown to be implicated in chemoresistance in colorectal cancer cell lines (44). We wanted to determine whether this effect could be attributable to BMP4 being secreted by the cancer cells, and if therefore, could be inhibited by our BMP4-specific VHHs. A panel of colorectal cancer cells was tested for the presence of active BMPs in their conditioned
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media (Fig. 6A). To that end, the C2C12 cells were cultured with supernatants from different cancer cell lines. When normalized for the BMPs present in normal fetal calf serum, we found a high variability in BRE-luciferase activity across cell lines, indicating differences in the amounts of BMPs being secreted by the cell lines. Incubation of the conditioned media of the cancer cell lines with the different VHHs, showed that certain lines were secreting exclusively BMP4 and not BMP2 or other BMPs, as C4C4 was already sufficient to completely inhibit this signal (Fig. 6B). One of those cell lines, HT29, was used to test the effect of BMP4 inhibition in terms of proliferation. No effect was observed when cultured with the VHHs or Noggin alone (data not shown). However, both VHHs C4C4 and C8C8 proved to increase the chemosensitivity when combined with different types of chemotherapeutics. This is demonstrated by the fact that combining the VHHs with Carboplatin, 5’FU or Oxaliplatin had a significant stronger effect on cell viability as compared to chemotherapy alone (Figure 6C,D and E). The effect on cell viability by Noggin was not as effective as observed for C4C4 or C8C8. As SMAD-independent signalling has been shown to mediate BMP-induced chemoresistance (44), we next studied wether the VHHs would inhibit non-canonical BMP4 signals in HT29 cells. Figure 6F shows that C4C4 and C8C8 inhibit the phosphorylation of the kinases p38, Akt as well as ERK. As HT29 cells harbour mutations in the SMAD4 gene, the SMAD pathway is inactive in these cells and therefore the inhibition of pSMAD by the VHHs has no functional consequences (Figure 6F). Together, these results show that the VHHs increase chemosensitivity of HT29 cells through the inhibition of non-canonical BMP4 signals.

DISCUSSION

VHHs are emerging as promising clinical tools with a growing number of benefits compared to conventional antibodies (45) such as low immunogenicity, cost-effective production, high
stability, consistent activity and ease of manipulation. Their advantageous characteristics have prompted their usage for research, diagnostic and therapeutic applications (46,47). Their small size and structural properties make VHHs better adapted to bind to epitopes in hidden grooves, such as the ones present in the homodimeric BMP structures. In our study, we have generated and characterized three types of BMP-specific inhibitors and compared their activity to that of the most potent and generally expressed natural BMP inhibitor Noggin (Figure 6G).

One highly interesting and unique group of VHHs that we classified as the C4-like antibodies inhibits BMP4-mediated function only, in contrast to Noggin, that also inhibits BMP2,5,6, and 7. The homo-bihead C4C4 proved to be the VHH with the highest affinity to BMP4, 10 times higher than that of Noggin. To our knowledge these are the first VHHs which can effectively inhibit BMP4 function, while leaving important homeostatic functions of the other BMPs intact. Unselective inhibition of BMP signalling in mice, has been shown to increase tumor burden (26) and activation of metastatic dormancy of breast cancer cells (25). Thus, when using BMP inhibition as therapy, avoiding the deleterious side effects of unchecked cancer stem cell proliferation is of major importance. In the intestinal organoid cultures inhibition of BMP4 alone by C4C4 does not affect stem cell proliferation. We also found that in SMAD4− colorectal cancer cells inhibition of the BMP4 non-canonical pathway by C4C4 increases the chemosensitivity to several chemotherapeutic agents. As this mutational profile has also been linked to other BMP-mediated oncogenic functions in colorectal cancer such as migration and invasion (48), it is tempting to speculate that those features would also be inhibited by C4C4 treatment. Therefore, C4-like antibodies have an important potential for clinical, diagnostic and therapeutic use in a subset of colorectal cancers. As BMP4 has also been shown to contribute to carcinogenesis in
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breast (12), lung (14), prostate (15), and gastric (18) cancers, the oncologic applicability of C4-
like antibodies is considerable.

C8C8 belongs to a second group of inhibitors (C8-like), which inhibit both BMP2 and BMP4
action, with affinities higher to those found for Noggin. At lower doses C8C8 can be used as a
potent BMP2,4 specific inhibitor. This effect is clearly exemplified in our organoids experiments,
in which C8C8 was as effective in maintaining intestinal organoid cultures as Noggin. Since
recombinant Noggin is expensive, bihead C8C8 offers an easy accessible and cheaper alternative
for ex vivo stem cell cultures.

E7 is the most promiscuous of the studied VHHs as its specificity is extended to BMP2,4,5 and 6,
sharing a similar pattern of inhibition with Noggin (49), albeit presenting a much lower potency.
Because homobivalency of C8 and C4 has resulted in higher efficacy for binding and potency, it
is tempting to speculate that the generation of E7E7 might result in a homo-bihead that will
closely resemble and share the same BMP-inhibitory capabilities of Noggin.

Indiscriminate binding for both receptors and antagonists, is one of the most puzzling features of
the BMP family. While previous studies hypothesized that only a small subset of residues might
shape binding specificity and affinity (40), our results have provided important evidence of the
location of such residues. Our epitope binning experiments and subsequent HADDOCK
modelling have shown that each VHH binds to a different BMP4-BMPR binding region (Fig.
6G). Because the other BMPR receptors might share similar binding mechanisms as BMPR1a and
BMPR2, it is tempting to speculate that the VHHs would also inhibit their binding to BMP4.

C4-like VHHs target the hydrophobic groove of the wrist epitope of BMP4, a BMPR binding
interface comprised by one BMP4 monomer and presenting differences in residues between
Generation of selective anti-BMP VHHs

BMP2 and BMP4 (Supplementary Fig. S4B). The docking model showed that these non-conserved residues are involved in binding with residues from the CDR2 and CDR3 of C4, therefore validating its BMP4-specificity. Interestingly, residues located in loop2 of the groove epitope represent a hot spot of binding to BMPR1a (36), which could explain the high affinity binding observed for C4C4. C8-like VHHs target the hydrophobic pocket of the wrist epitope of BMP2 and BMP4, an epitope formed by residues from both monomers. This is an area in which non-identical and non-isofunctional residues between BMP2,4 and BMP5,6,7 reside (Supplementary Fig. S4B), which explains why C8 binds only to one BMP subgroup. Finally, E7 targets the knuckle epitope, comprised of residues from one monomer. Interestingly, all the binding determinants located at the core of the knuckle epitope are invariant between the BMPs, in particular, S88 at the center is highly conserved (43). This explains why targeting this region renders E7 nonselective to BMPs. Moreover, the knuckle epitope exhibits a concave hydrophobic area with no deep pockets that perfectly complements the convex nature of E7. Due to the structural conformation of the knuckle epitope, binding of type 2 receptors to BMPs is characterized as a low affinity binding (43), a notion that might explain the lower affinity of E7 in comparison to the other VHHs. Albeit present, the binding of C4 on C8-or E7-bound BMP4 coated chips was not as striking as the one observed between C8 and E7 (Fig. 3B). This could be explained by the effect of a small allosteric competition between C4 and the other two VHHs, as the hydrophobic groove of the wrist epitope is in close proximity to the other two binding interfaces targeted by C8 and E7 (Fig. 6G).

One of the limitations of our HADDOCK modelling is the lack of crystal structures for BMP4 and the VHHs. Nevertheless, mutational analysis of the paratopes of the VHHs supports our modelling data (Fig. 4D-E). Furthermore, experiments with heterodimer BMP4 and BMP2
confirm that whereas C4 and E7 target an epitope formed by one BMP monomer only, C8 binds to an epitope formed by two monomers as it is unable to inhibit heterodimers of BMP4 or BMP2 (Supplementary Fig S5A-B).

A notable result of our studies is the fact that all VHHs compete for binding with Noggin. This is explained by the wide reach of contact points of Noggin, which simultaneously masks both BMPR1a and BMPR2 epitopes (42). This remarkable structural reach is conserved amongst BMP antagonists and might explain their lack of BMP-specificity (50). While the binding interface of Noggin to BMP is about 1,400 Å² (42), the size of a VHH paratope is usually 700 Å² (51). The generation of highly effective VHHs that target small areas within the epitopes for BMPR binding, therefore demonstrate that neutralization of small areas are enough to antagonize ligand activity. An interesting result is the fact that the commercial anti-BMP4 antibody (R&D systems) presents lower affinity than our antibodies. This antibody targets a BMP4 specific area not recognized by Noggin or BMPR1a. Therefore, the N-terminal heparin binding domain (52,53) (highly different between BMP2 and BMP4) represents a likely region to be targeted by this antibody (Supplementary Fig. S4B). Indeed, functional experiments with a BMP4 lacking the heparin-binding domain, confirms this assumption (Supplementary Fig. S5C). These data therefore seem to indicate that targeting small BMPR binding area would provide higher affinity than targeting the heparin binding domain.

There is now overwhelming evidence supporting the notion that large functional differences between highly similar BMPs exist. Besides cancer, BMPs are also involved in a variety of diseases, including bone abnormalities, cardiovascular and metabolic disorders. Because BMPs present opposing functions, the importance of specific inhibition for each individual BMP extends beyond the oncology field. We have generated small, potent and specific inhibitors of
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BMP molecules which target the contact points involved in binding to the receptors. In particular, our studies have underscored a previously unappreciated molecular interface that ultimately dictates BMP-specificity. By targeting a small region within the large wrist epitope, namely the hydrophobic groove, we have developed a highly potent BMP4-specific antibody that could represent a promising therapeutic strategy for sensitizing BMP4+ colorectal cancer cells to chemotherapy. Its novel structural format makes it remarkably suited to overcome the limitations that affect the clinical and research applications of current BMP inhibitors.

ACKNOWLEDGMENTS

We thank lab members of the K. Krishnadath’s laboratory for reading the manuscript. We thank colleagues at the LEXOR lab for providing the supernatants of the cancer cell lines.

REFERENCES


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Table 1: Kinetic constants for BMP binding

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VHHs and controls are immobilized on a chip. $k_a$ in $10^4$ sec$^{-1}$M$^{-1}$, $k_d$ in $10^{-5}$ sec$^{-1}$, $K_D$ in pM - : No binding detected. ND: Not Determined (VHH could not be immobilized).

FIGURE LEGENDS

Figure 1: anti-BMP4 VHHs specificity to the different BMPs.
Generation of selective anti-BMP VHHs

(a) Phosphorylation of SMAD1/5/8 in EPC2-hERT cells after activation with 100ng/ml of human BMP2,4,5, 6 or 7 in the presence of 5μg/ml VHHs. Error bars represent standard deviations of the mean, calculated from 3 independent experiments. (b) C2C12 cells were activated with human BMPs in the presence or absence of 5μg/ml of VHHs or human Fc-Noggin. After 16 hours incubation, luciferase activity was assayed. Error bars represent standard deviations of the mean, calculated from three independent experiments, with experimental triplicates each. ***p= <0.0001. Statistical analysis was done using a two-tailed P-test.

Figure 2: IC₅₀ curves of VHH biheads and monoheads.

C2C12 cells were activated with human BMP4 at 5ng/ml (A, B, D) or BMP2 at 50ng/ml (C, E) with increasing concentrations of VHHs. Data is representative from two experiments and with experimental triplicates each.

Figure 3: Kinetic analysis for BMP binding.

A) Binding of VHHs to the different BMPs was tested in a direct SPR assay. The graphs show two replicate ligand injections at just one concentration of ligand (0.4μg/ml for BMP2 and BMP4; and 2.0μg/ml for BMP5, BMP6, and BMP7). For clarity just one replication is shown. B) “Epitope binning” of VHH-coated chip plates. BMP4 (1.0μg/ml) was injected on the chip, followed by the injection of a second VHH (2.0μg/ml) or the corresponding controls.

Figure 4: HADDOCK docking of the VHHs to BMP4 binding.

Ribbon diagrams of the docking of A) C4, B) C8 and C) E7 binding to BMP4. Hydrogen bonds and salt bridges are represented as dashed lines. The enlarged ribbon diagrams show the residues constituting the molecular interfaces. Residues corresponding to the VHH paratope are
Generation of selective anti-BMP VHHs

represented as magenta sticks. Residues forming the BMP4 epitope are represented as yellow sticks and labelled in green (A), orange (B) or red (C). Function of the generated mutant variants in inhibiting ID1 promoter activity in C2C12 cells after stimulation with (D) BMP2 (50ng/ml) or (E) BMP4 (5ng/ml) for 16 hours. Uns= unstimulated C2C12 cells. Values calculated from three independent experiments, with experimental triplicates each.

**Figure 5: VHH biheads inhibit endogenous BMP function.**

A) ELISA determinations of BMP secretion in mouse intestinal organoids cultured in the absence of Noggin for three days. BMP5 levels were not detected (N.D). B) Western blot analysis of lysates of mouse intestinal organoids grown under No Noggin conditions for three days. Lysates from mouse duodenum, jejunum and ileum, were used as positive controls. C) BMP activity of conditioned media of organoids cultured at the indicated conditions for 3 days. Error bars represent standard deviations of the mean as compared to No Noggin (black bar). Data collected from at least five experiments with experimental triplicates each. ***p= <0.0001, **p=<0.01, *p=<0.1. Statistical analysis was done using a two-tailed P-test. D) Number of organoids formed in cultures of mouse intestinal organoids grown for five days at the indicated conditions. E) Representative bright field images of mouse intestinal organoids grown under the indicated conditions for five days.

**Figure 6: anti-BMP4 VHHs inhibit cancer derived BMP signals.**

A) C2C12 cells were incubated with conditioned media from a panel of different colon cancer cell lines. B) C2C12 cells were incubated with conditioned media of the indicated cancer cell lines. C4C4, C8C8 or human Fc-Noggin were added at the same time at a concentration of 500ng/ml for 16 hours. HT29 cells were treated with Carboplatin (C), 5’FU (D) or Oxaliplatin
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(E) at the indicated concentrations with or without VHHs or Noggin. After 48 hours the viability was measured. Experiments were repeated at least three times with experimental triplicates each. **p=<0.01, *p=<0.1. Statistical analysis was done using a two-tailed P-test. F) HT29 cells were starved overnight and treated with the VHHs or Noggin at a concentration of 500 ng/ml for 1 hour. SMAD4 expression was tested on lysates of resting HT29 and HCT116. Blots are representative of at least three experiments. G) Space filled view of BMP4 binding to BMPR1a and BMPR2 where the proposed model of BMP4-VHH binding is shown.
Figure 3

A

**BMP2**

- **monoheads**
- **biheads**

**BMP4**

- **monoheads**
- **biheads**

**BMP5**

- **mono- + biheads**

**BMP6**

- **mono- + biheads**

**BMP7**

- **mono- + biheads**

B

**C8**

- **oBMP4**
- **C4C4 + C8**

**E7**

- **oBMP4**

**C4C4**

- **oBMP4**

**mNoggin**

- **oBMP4**

**BMPR1a**

- **oBMP4**

**αBMP4**

- **C8**
Figure 4

A

BMP4A  BMP4B  C8

Wrist Epitope Hydrophobic Groove

BMP4A  BMP4B

Wrist Epitope Hydrophobic Pocket

CDR3

C

BMP4A  BMP4B

Knuckle Epitope

D

E7

Relative ID1 Promoter Activity

Uns  BMP2  C4  C4 L57  C4 D09  C8  C8 P109  ET  ET V32  ET E7 L31 Y

E

Relative ID1 Promoter Activity

Uns  BMP2  C4  C4 L57  C4 D09  C8  C8 P109  ET  ET V32  ET E7 L31 Y

BMP4
Molecular Cancer Therapeutics

Effective inhibition of Bone Morphogenetic Protein function by highly specific llama-derived antibodies

Silvia Calpe, Koen Wagner, Mohamed El Khattabi, et al.

*Mol Cancer Ther* Published OnlineFirst September 8, 2015.

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