Inhibitors of BMP-1/tolloid-like proteinases: efficacy, selectivity and cellular toxicity

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Bone morphogenetic protein-1 (BMP-1) belongs to a small family of zinc-dependent metalloproteinases, known as the BMP-1/tolloid-like proteinases (BTPs), which also includes in humans mammalian tolloid (mTLD, a splice variant of the Bmp1 gene) and two homologous proteins, mammalian tolloid-like 1 and 2 (mTLL-1, mTLL-2) [1]. BTPs are composed of an astacin-like catalytic domain followed by several complement C1r/C1s, Uegf, BMP-1 (CUB) and epidermal growth factor (EGF) domains. They are members of the astacin metalloproteinase subgroup, which also comprises meprin α, meprin β and ovastacin [2]. BMP-1 (also known as procollagen C-proteinase) and related tolloid proteinases were primarily identified as the main enzymes responsible for collagen maturation by excising the C-propeptide of procollagens I-III [3]. Today, around 30 substrates have been described for BTPs [1], turning them into key players in regulating processes such as morphogenesis, tissue repair or tumour progression. These substrates include several fibrillar...
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procollagens (types I, II, III, V, XI), small leucine-rich proteoglycans (decorin, biglycan, osteoglycin), basement membrane components (laminin 332, procollagen VII, perlecan), lysyl oxidases (lysyl oxidase, lysyl oxidase-like) and mineralization factors (dentin matrix protein-1, dentin sialophosphoprotein). BTPs also activate other growth factors and their related components and the activation of growth factors, BTPs have the potential to cleave the same substrates, but their tissue distribution and catalytic efficiencies can differ significantly [4,5]. The catalytic domains of BTPs are very similar [6], and previous studies have shown that comparable results were found when testing inhibitors on several isoforms [7,8]. As the most active and widely distributed isoform, BMP-1 was therefore used in the present study to represent the BTP family.

Fibrotic disorders are forms of defective tissue repair characterized by increased cell proliferation and deposition of extracellular matrix (ECM), particularly fibrillar collagens. They can affect numerous organs such as heart (cardiac fibrosis), liver (liver fibrosis further evolving to cirrhosis), kidneys (renal fibrosis), skin (hypertrophic scars, keloids) and cornea (corneal scarring) and are critical factors in progressive organ dysfunctions, altogether contributing to 45% of all-cause mortality worldwide [9]. Through the maturation of ECM components and the activation of growth factors, BTPs play major roles during tissue remodelling. As such, they are recognized as attractive therapeutic targets to prevent or treat various fibrotic pathologies [10–12].

Inhibitors of zinc metalloproteinases are classically designed as small molecules bearing zinc-binding groups which bind in the catalytic site and behave as competitive inhibitors. Following this strategy, a number of synthetic inhibitors of BTPs have been developed, most of them being hydroxamates [10,13–16]. Some have shown promising activity in cell-based [15,17] or animal [18] models of fibrosis; however, none of them have entered the clinic. Hydroxamates can actually suffer from a set of drawbacks linked to in vivo instability and poor specificity due to their high affinity for metal ions [19]. Other available inhibitors of BTPs include the *Xenopus* protein sizzled, which was previously found to be very potent and selective [7], and a newly developed phosphinic peptide inhibitor which is a broad-spectrum compound targeting both BTPs and meprins. Phosphinic peptide inhibitors have the advantage of showing remarkable in vivo stability and have been successfully used in various animal models to selectively block their targets [20,21]. Though some of these molecules have been designed to spare the activity of some important matrix metalloproteinases (MMPs), their inhibitory activity on other astacins is unknown. This specificity issue is crucial since it has been reported that BTPs and meprins have in common a striking specificity for acidic amino acids in the P1′ position (first residue C-terminal to the scissile bond) [22] and that they share a number of common substrates and a high degree of conservation in their catalytic domains [23]. Importantly, it was previously shown that, similarly to BTPs, meprin α and meprin β are able to perform the C-terminal maturation of procollagens I and III [24,25], making them further potential targets in collagen-related diseases. However, meprins also have distinct functions during various (patho)physiological conditions and have been implicated in inflammation, cancer progression or Alzheimer’s disease [23].

Here, we provide the first systematic comparison of three hydroxamates previously reported as powerful inhibitors of BMP-1 [10,13,16,26], the newly developed phosphinic peptide and sizzled. The broad-spectrum metalloprotease inhibitor GM6001 (also known as galardin or ilomastat), and actinonin, a naturally occurring hydroxamate and effective meprin inhibitor, were also included in the study. We have characterized their cellular toxicity and inhibitory efficacy, both in vitro and in cultures of HT1080 cells and human primary corneal fibroblasts, and have determined their selectivity towards BTPs and related meprins. Altogether, this study will be very useful to guide the choice of suitable tools for the inhibition of BTPs in future studies.

**Materials and methods**

**Proteases**

Recombinant Flag-tagged human BMP-1 (EC 3.4.24.19) was produced in 293-EBNA cells and purified as previously described [27]. Meprin α (EC 3.4.24.18) and meprin β (EC 3.4.24.63) were produced in insect cells, purified and activated according to the protocols described in [28,29].

**Inhibitors (Table 1)**

Recombinant His-tagged sizzled (from *Xenopus laevis*) was produced in 293-EBNA cells and purified as described previously [7] (purity > 95% (SDS/PAGE), ESI-MS m/z calcd 35481.8 found 35483.2). S33A was synthesized as described in [13] (purity > 97% (HPLC); HRMS (high-resolution mass spectrometry; ESI (electrospray ionisation)) m/z: [MH+] calcd for C23H33N4O6S 541.2120; found 541.2119). FG-2575 was designed and prepared by FibroGen, Inc.
(purity > 95% (HPLC); MW = 503.6251; HRMS (ESI) m/z: [MH+] within 1.6 ppm of calcd value). The synthesis of the phosphinic peptide RXP-1001 will be described elsewhere (Lecorché et al., in preparation; purity > 95% (HPLC); MS (matrix-assisted laser desorption ionization) m/z: [MH+] calcd for C_{55}H_{67}N_8O_{14}P 1095.46; found 1095.57). UK383,367 and actinonin were purchased from Sigma-Aldrich (L’Isle d’abreu, France) and GM6001 from Enzo Life Sciences (Villeurbanne, France). All commercial compounds have purity > 98% (HPLC or TLC). Stock

**Table 1.** Protease inhibitors profiled in this study.

| Name      | Type            | Target     | Formula                                      |
|-----------|-----------------|------------|----------------------------------------------|
| FG-2575   | Hydroxamate     | BMP-1      | Undisclosed formula                          |
| UK383,367 | Hydroxamate     | BMP-1      |                                              |
| S33A      | Hydroxamate     | BMP-1      |                                              |
| Sizzled   | Xenopus protein | BMP-1      |                                              |
| RXP-1001  | Phosphinic peptide | BMP-1 |                                              |
| GM6001    | Hydroxamate     | MMP        |                                              |
| Actinonin | Hydroxamate     | Meprin α   |                                              |
solutions of the inhibitors were prepared at 10 mM in DMSO, except for RXP-1001 (3 mM in EtOH 50%) and sizzled (120 μM in HEPES 20 mM NaCl 0.5M pH 7.4).

Cell culture

Human primary keratocytes were obtained from human corneas (collected from three donors aged around 70), harvested at the ‘Banque de Tissus et Cellules’ of the ‘Hospices Civils de Lyon’ in agreement with French ethical regulations. Informed consent was obtained from the donors’ families, and the study conformed to the standards set by the Declaration of Helsinki. Keratocytes were grown in a medium without ascorbate shown to be optimal [30] to maintain their proliferation capacity and prevent their differentiation into myofibroblasts [Dulbecco’s modified Eagle’s medium (DMEM)/HamF12 1 : 1 (GE Healthcare, Velizy, France), 10% iron-supplemented newborn bovine serum (Hyclone, Fisher scientific, Illkirch, France), 1% antibiotic/antimycotic solution (AAS, Sigma-Aldrich) and 5 ng·mL⁻¹ basic fibroblast growth factor (bFGF) (Sigma-Aldrich)]. These culture conditions enabled easy detection of both unprocessed pro-collagen I and released C-propeptide. Cells were used between passage 6 and passage 9.

HT1080 cells (ATCC CCL-121) were grown in DMEM with 10% foetal bovine serum (FBS) (GE Healthcare) and 1% AAS.

Bone morphogenetic protein-1-overexpressing HT1080 cells were obtained as previously described [31] and grown in DMEM with 10% FBS and 500 μg·mL⁻¹ G418 sulphate (GE Healthcare). For inhibitor evaluation, 20 000 cells-well⁻¹ (in 12-well plates) were treated with 2.5 μM of synthetic inhibitors, 0.15 μM of sizzled or with an equal volume of vehicle starting 4 h after seeding. After 2 days, confluent cells were washed with PBS and maintained in serum-free medium (supplemented in inhibitor) for another 24–48 h. Modification of the cell morphology was visualized on a Nikon Eclipse TE300 Inverted Microscope.

Inhibition assay

To determine inhibitor potencies, the fluorogenic peptide substrate Mca-Y-V-A-D-A-P-K-Dnp (20 μM; Enzo Life Sciences), which can be cleaved by both BTPs and meprins [7], was used as a substrate (Km = 7.2, 15 and 9 μM for BMP-1, meprin α and meprin β, respectively). It was incubated with 12 nM BMP-1, 1 nM meprin α or 0.5 nM meprin β, in the presence of increasing concentrations of the inhibitors (0.01–100 μM) in 50 mM HEPES pH 7.4, 0.15 mM NaCl, 5 mM CaCl₂, 0.02% octyl β-D-glucopyranoside. RXP-1001 was also assayed at pH 6.8. The cleavage assays were performed in 96-well nonbinding, flat-bottomed black plates (Corning, Amsterdam, The Netherlands) in 100 μL total reaction volume at 37 °C, and linear increase in fluorescence was monitored during 20 min using an Infinite M1000 Tecan reader (excitation wavelength: 320 nm; emission wavelength: 405 nm). IC₅₀ values were determined by plotting the ratio of the inhibited versus uninhibited enzyme activities against the inhibitor concentration. Nonlinear regression analysis and calculations were performed using PRISM 5 (GraphPad Software, La Jolla, CA, USA) (n = 3).

MTT Assay

HT1080 cells and keratocytes were seeded into 96-well culture plates (Corning) at a density of 1500 and 4000 cells-well⁻¹, respectively. After 48 h, cells were treated with various concentrations of the inhibitors, or with the corresponding vehicle, in serum-free medium (with AAS and bFGF for keratocytes), for 24 or 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich) was then added to each well and incubated with the cells at 37 °C for 3 h. The supernatant was removed and a DMSO:ethanol (1/1) solution added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using the Infinite reader. 100% values were obtained with cells treated with vehicle only.

Procollagen cleavage assay

Keratocytes were seeded in 24-well plates (Corning, 20 000 cells-well⁻¹) and grown for 2 days (until confluence reached 90%). They were then washed three times with PBS followed by addition of serum-free, phenol red-free DMEM supplemented with AAS and bFGF and containing different concentrations of the inhibitors. After 48 h, the conditioned medium of each well was collected, clarified by centrifugation and boiled 5 min in SDS/PAGE sample buffer containing DTT. Equal volumes of each sample were loaded on 4–20% polyacrylamide gradient gels (Bio-Rad, Marnes La Coquette, France), followed by transfer to PVDF membranes (Millipore, St Quentin en Yvelines, France) and analysis by western blotting using the LF-41 rabbit antibody directed against the procollagen I C-propeptide (kind gift of L. Fisher, NIH Bethesda, USA) as primary antibody and goat HRP-linked anti-rabbit antibody (Cell Signaling, Leiden, The Netherlands) as secondary antibody. Protein bands were detected using the Clarity western solution (Bio-Rad, Marnes La Vallée, France) then quantified by densitometry using ImageQuant TL (GE Healthcare).

Results

The molecules selected for this study are as follows (Table 1): hydroxamates FG-2575 (sometimes referred to as BI-1 [8,26]), UK383,367 [16] and S33A [13], the sizzled protein [7] and the recently developed phosphinic peptide RXP-1001. GM6001 and actinonin, whose activity towards BTPs had never been documented, were also included.
In vitro efficacy

The inhibitors were first profiled at pH 7.4 for their ability to block the cleavage of a fluorogenic peptide substrate by the three major human astacins (BMP-1, meprin α and meprin β) [7] and ranked according to their half-maximal inhibitory concentration (IC_{50}, Table 2). For BMP-1, these potencies can be ordered as follows: FG-2575 > S33A ≈ sizzled > UK383,367 > RXP-1001 >> actinonin > GM6001. Thus, the results clearly show that, in these conditions, FG-2575 is the most powerful inhibitor of BMP-1 (IC_{50} = 2.9 ± 0.3 nM). Together with the *Xenopus* protein sizzled (IC_{50} = 8.5 ± 0.5 nM), it also appears to be highly specific for BMP-1 as their IC_{50} values for meprins are more than 1000-fold higher. With 5- to 20-fold higher IC_{50}s, the other hydroxamates remain good inhibitors of BMP-1, but do not demonstrate the same level of specificity as they also inhibit meprin α with an IC_{50} around 300 nM. As it was previously demonstrated that inhibition by phosphinic peptides, in contrast to hydroxamates, involves a proton uptake by a glutamate which is conserved in all metzincins [32,33], RXP-1001 was also tested at pH 6.8. This leads to significantly increased potencies for all 3 enzymes (by a factor between 2.7 and 6.9). Interestingly, RXP-1001 was also the only molecule that showed a high degree of potency against all three enzymes (around micromolar or below at the two tested pH), and thus, it can be viewed as a broad-spectrum inhibitor of human astacins. GM6001 and actinonin were inactive on BMP-1 (IC_{50} > 10 µM), but both appeared as good inhibitors of meprin α, as already reported [34]. Actinonin also displayed some inhibitory activity towards meprin β.

Cellular toxicity of the inhibitors

In order to carefully choose the concentrations to be used in cell-based assays, evaluation of the toxicity of the inhibitors was needed. Viability of human primary keratocytes (corneal stromal cells) and HT1080 cells (fibrosarcoma-derived cancer cells) treated with increasing concentrations of the seven compounds was assessed using the MTT viability assay in serum-free conditions. The two cell types are very different in terms of origin, metabolism and nature of cell–matrix interactions (e.g. no fibrillar collagens are secreted by HT1080 cells) and, as can be seen in Fig. 1, responded

### Table 2. Half-maximal inhibitory concentrations of the inhibitors for meprin α, meprin β and BMP-1 at pH 7.4 or (*6.8. A representative example of data determination (FG-2575 on BMP-1) is given in the Fig. S1.

| Compound  | IC_{50} (nM)ᵃ | Meprin α | Meprin β | BMP-1  |
|-----------|--------------|----------|---------|--------|
| FG-2575   | > 1000       |          | > 10 000| 2.9 ± 0.3 |
| UK383,367 | 249 ± 17     | > 10 000 | 55 ± 15 |
| S33A      | 393 ± 82     |          | 7.9 ± 0.6 |
| Sizzled   | > 1000       |          | 8.5 ± 0.3 |
| RXP-1001  | 245 ± 40 (42 ± 9*) | 1300 ± 100 (484 ± 33*) | 359 ± 20 (52 ± 3*) |
| GM6001    | 185 ± 12     | > 10 000 | 100 000 |
| Actinonin | 55 ± 11      |          | 744 ± 29 |

ᵃMean values ± SD of three independent experiments performed in duplicate.

![Fig. 1. Evaluation of the cellular toxicity of the inhibitors. Viability of human keratocytes (A) or HT1080 cells (B) treated with increasing concentrations of inhibitors was measured using the MTT assay. Cells were seeded and grown for 2 days, washed with PBS and treated with inhibitors in serum-free medium for 48 (keratocytes) or 24 (HT1080) h. 100%: vehicle only. Mean ± SD, n = 3.](image-url)
differently to the addition of the inhibitors. Kerato-
cytes were found to be very resistant, and even when
30 μM of the compounds was added to the cells, their
viability was not significantly reduced (Fig. 1A).
HT1080 cells were more sensitive to the cytotoxic
effects of the molecules (Fig. 1B), at least in the
serum-free conditions of the assay. For this reason,
viability of HT1080 was measured after 24 h only, and
DMSO concentration was kept below 0.3%, since it
started to show some toxicity at around 1%. Whereas
actinonin and GM6001 were essentially harmless to
HT1080 cells, UK383,367 and S33A were strongly
cytoxic at concentrations above 10 μM. FG-2575 was
better tolerated, with a 30% drop in viability above
20 μM, which is a concentration 4 orders of magnitude
above its active concentration. RXP-1001 was the
least toxic compound since even at 30 μM it did not signifi-
cantly modify HT1080 viability. Sizzled could not be
evaluated at concentrations above 15 μM, but even at
this relatively high concentration for a protein, it had
little influence on HT1080 viability.

Effect on BMP-1 activity in human keratocytes

To evaluate the inhibitors in a primary cell culture
assay, we selected human keratocytes which were pre-
viously found to represent a good cellular system to
study substrate processing by physiological levels of
BMP-1 [31] and which are the main cell type involved
in corneal scarring, a condition where BMP-1 is of
therapeutic interest [12]. Adult keratocytes are also
known to deposit large amounts of collagen I, and
they were used in our study to monitor the effect of
the selected molecules on the BMP-1-dependent matu-
rature of procollagen I into pN-collagen and C-pro-
ceptides. Subconfluent keratocytes were treated at the
single concentration of 2.5 μM (synthetic inhibitors) or
0.25 μM (sizzled) for 48 h in serum-free medium and
compared to untreated cells. Media were harvested
and assayed for procollagen processing using western
blotting and an antibody raised against the C-terminal
propeptide of type I procollagen (Fig. 2A). In the
absence of inhibitor or in the presence of actinonin or
GM6001, C-propeptide release was easily detected. In
agreement with the in vitro data, other compounds
were found to efficiently block this processing, as indi-
cated by the decreased amounts of C-propeptide seen
in Fig. 2A. Despite the difference in initial concentra-
tions, the same level of inhibition was obtained with
sizzled and the hydroxamates (around 90%, as quanti-
fied by western blotting, Fig. 2B). In contrast, RXP-
1001, which efficiently inhibited recombinant BMP-1,
required a concentration of 12 μM to reach the same
efficiency (Fig. 2C).

Effect on HT1080 cells overexpressing BMP-1

HT1080 cells do not express fibrillar collagens but
were previously shown to secrete several established
or putative BMP-1 substrates [31]. Also, when BMP-1

Fig. 2. Effects of BTP inhibitors on the
BMP-1-dependent maturation of
procollagen I. (A–C) Subconfluent human
keratocytes were treated with inhibitors or
vehicle for 48 h. Medium was harvested,
assayed by western blotting and probed
with an antibody raised against the C-
terminal propeptide of procollagen type I.
(A) Treatment with 2.5 μM of inhibitors
except sizzled which was assayed at
0.25 μM. (B) Quantification of band
intensities in A. (C) Assay of RXP-1001 at
two higher concentrations. One
representative experiment out of 3
independent experiments is shown in all panels.
is transfected in HT1080 cells, a striking phenotype is induced, with cells losing their rounded shape and starting to detach from the culture plate when reaching high density (Fig. 3, DMSO panel). This change in cell morphology is due to the catalytic activity of BMP-1 since it is not present when a similar construct encoding an inactive BMP-1 mutant (E94A, Anastasi et al., under revision) is used. We took advantage of this marked phenotype, which is a direct readout of BMP-1 activity, to assess the activity of the selected molecules in another type of cell-based assay.

As can be seen in Fig. 3, actinonin and GM6001, which do not inhibit BMP-1 in vitro, had no impact on the phenotype, with cells behaving like untreated (DMSO) cells. In contrast, established inhibitors of BMP-1 had a remarkable effect, leading to the reversal of the adhesion phenotype when cells were grown in the presence of these compounds. Similar to the results obtained with keratocyte cultures, sizzled was efficient at a concentration as low as 0.15 µM, with cells maintaining their normal aspect, while the hydroxamates and RXP-1001 required higher concentrations. Phenotype reversal was observed for hydroxamates at 2.5 µM, whereas, like for keratocytes, RXP-1001 was less efficient at this concentration.

**Discussion**

This work was initiated in the course of our study on the role of BMP-1 in corneal scarring [12], for which we needed to select a potent, selective and nontoxic BMP-1 inhibitor. We wanted to be sure that only BTPs would be targeted by the selected molecule but realized that, except for sizzled, there was no information about the selectivity of BTP inhibitors towards
meprins. This is of utmost importance since BTPs and meprins have both common and specific substrates and, even for common substrates, the cleavage pattern can be significantly different leading to distinct physiological effects [24,25]. Also, previous studies on other metalloproteases such as MMPs have demonstrated that the lack of selectivity of metalloproteinase inhibitors can lead to erroneous conclusions and to dramatic side effects in clinical trials [35]. Noteworthy, FG-2575, S33A, UK383,367, RXP-1001 and sizzled have been tested on representative panels of MMPs and found to have no significant effects [7,13,16]. In contrast, the natural antibiotic actinonin was previously shown to inhibit aminopeptidases or peptide deformylases [36] and GM6001 is a broad-spectrum inhibitor of several metalloproteases [37].

The five established BTP inhibitors showed very different inhibition profiles. One major result of our study is that sizzled and FG-2575 clearly stand out as the most potent and selective compounds with low nanomolar activities on BMP-1 and more than 1000-fold higher IC50s for meprins. Sizzled has previously been shown to efficiently inhibit the cleavage of other BMP-1 substrates such as procollagen III, procollagen V and pro-lysyl oxidase [7]. FG-2575 was also used to inhibit the cleavage of laminin-332 both in vitro and in cultures of human keratinocytes and to prevent pro-lysyl oxidase maturation in 293-EBNA cells over-expressing the protein [8,17]. These two compounds actually behaved very well in our cell-based assays combining efficiency and low cytotoxicity and appear to be the most attractive tools to modulate BTP activity both in vitro and in cellulo. BTPs being known to be important for matrix assembly, their inhibition could interfere with the deposition of the provisional matrix needed to support cell adhesion. It is worth noting that we did not observe any detrimental effect of the addition of the inhibitors in our culture conditions, 4 (HT-1080) or 48 h (MTT assay) after seeding. However, further studies will be needed to obtain information about the usability and pharmacokinetic/biodistribution properties of sizzled and FG-2575 in whole-organism experiments.

In our cell-based assays, we found that the inhibitory capacity of sizzled towards BMP-1 is stronger than that of FG-2575. However, this advantage might be counterbalanced by the increased production cost of sizzled compared to a small chemical compound, by the size of the protein (30 kDa) which may hamper its diffusion within target tissues and by the possible immunogenicity of a protein originating from *Xenopus*. These various parameters will have to be carefully evaluated in the future.

Though slightly less active on BMP-1, RXP-1001 is the only compound tested here to be a broad-spectrum inhibitor of BTPs and meprins, which makes it a very valuable tool to explore redundancy and cross-reactivity of astacins. In particular, as our previous results show that meprins can directly contribute to collagen deposition in tissues [24,25], it could be very informative to evaluate the impact of blocking all procollagen C-proteinase activities simultaneously in fibrosis models. However, RXP-1001 seems somewhat less potent in cell cultures than other synthetic compounds. This can be compensated by the use of higher RXP-1001 concentrations without any obvious toxicity for the two cell types tested here. This apparent loss of inhibitory potency may be explained by its relatively high pH sensitivity around physiological pH or the possible sequestration of RXP-1001 by residual serum proteins present in the medium. As already observed for this type of compounds [38], this sequestration mechanism can be favoured by the hydrophobic residues and negative charges born by the phosphinic peptide. This property can be a real benefit *in vivo*, since it will hamper rapid clearance and help the transport of the molecule to pathological sites. Also, a previous report by Fish *et al.* [15] suggested that ionized BTP inhibitors may have a lower efficacy *in cellulo* compared to neutral compounds, possibly because of repulsive interactions with the biomolecules present in the specific microenvironments where BMP-1 is located.

This study also shows that actinonin can be used to modulate meprin activity with no impact on BTPs, whereas S33A and UK383,367 target both BMP-1 and meprin α, with little effect on meprin β. Finally, the widely used MMP inhibitor GM6001 does not affect BMP-1 and meprin β in the tested concentration range but a clear off-target activity of this molecule on meprin α is observed. This is an illustrative example of why careful comparison is necessary before choosing an inhibitor to probe enzymatic activity.

In summary, this study reveals striking differences in the potency and selectivity of the inhibitors tested. It demonstrates that some of the molecules sometimes claimed to be selective BMP-1 inhibitors by commercial suppliers (UK383,367) actually also inhibit meprin α. Also, the marked differences in inhibition profiles suggest that the full spectrum of activities (from very selective of one family member to family-wide) can be achieved and confirm that there are both common and specific features in the catalytic sites of BTPs and meprins, as previously shown by crystallography [6,39]. The study also highlights for the first time the
advantages and drawbacks of a diverse range of inhibitors that can be picked as required to modulate either one, two or the three proteases. It should help to select the right tool for the right experiment and pave the way towards novel in vivo applications of BTP inhibitors, for which only few examples have been described to date [18].

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Author contributions
SVLG and CM designed and coordinated the study, MT and SVLG performed the experiments, SVLG, PR, FB, WBH, CBP and VD produced and purified the enzymes and/or inhibitors used in this study. OD provided the keratocytes. SVLG, MT and CM analysed and interpreted the data, and wrote the paper. All authors reviewed the results, critically revised the manuscript and approved the final version.

Conflict of interest
WBH is an employee of FibroGen, Inc. The other authors report no conflict of interests.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Representative example of inhibition assay and IC$_{50}$ determination. (A) Inhibition of BMP-1 (12 nM) by increasing concentrations of FG-2575 (0–100 nM, each concentration in duplicate). Linear increase in fluorescence was monitored during 20 min (substrate concentration 20 μM, excitation wavelength 320 nm, emission wavelength 405 nm). Enzyme activity is given by the slope of the curve, determined by linear regression using Excel. One representative experiment out of 3 independent experiments is shown. (B) IC$_{50}$ determination of FG-2575 on BMP-1. Plot of % of residual activity (ratio of inhibited versus uninhibited enzyme activities) against FG-2575 concentration. Mean ± SD of 3 independent experiments performed in duplicate. IC$_{50}$ is determined by nonlinear regression using GraphPad Prism 5.