Primary evaluation of potential small molecule inhibitors of the astacin metalloproteinase ovastacin, a novel drug target in female infertility treatment

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ABSTRACT

Despite huge progress in hormonal therapy and improved *in vitro* fertilization methods, the success rates in infertility treatment are still limited. A recently discovered mechanism revealed the interplay between the plasma protein fetuin-B and the cortical granule-based proteinase ovastacin as novel key-mechanism in the regulation of fertilization. Upon sperm-egg fusion, cleavage of a distinct zona pellucida component by ovastacin destroys the sperm receptor, enhances zona robustness and eventually provides a definitive block against polyspermy. An untimely onset of this zona hardening prior to fertilization would consequently result in infertility. Physiologically, this process is controlled by fetuin-B, an endogenous ovastacin inhibitor. Here we aimed at the discovery of small molecular inhibitors of ovastacin that could mimic the effect of fetuin-B. Hence, these compounds could be useful lead structures for the development of specific ovastacin inhibitors that can be utilized in infertility treatment or *in vitro* fertilization.

KEYWORDS

ovastacin, astacins, metalloproteinase, metzincins, fetuin-B, infertility, in vitro fertilization, hydroxamate
INTRODUCTION

During mammalian fertilization, sperm entry into the ovum is tightly regulated. Dysregulation of this fine-tuned process promotes infertility. An essential mechanism at this point is the inhibition of ovastacin, a member of the astacin-family and the metzincin superfamily of metalloproteinases\(^1\). Upon fertilization, the release of ovastacin (encoded by the gene astl) from the oocyte’s cortical granules into the perivitelline space leads to remodeling of the zona pellucida (ZP), a glycoprotein matrix surrounding the oocyte, by cleavage of the zona pellucida protein 2 (ZP2) at a distinct site. This cleavage abolishes sperm binding, renders the ZP impermeable and thus blocks further sperm to enter the oocyte\(^2\). However, even before fertilization, during oocyte maturation, small amounts of ovastacin are seeping out of the oocyte to cause zona pellucida hardening\(^3\). Under physiological conditions, pre-fertilization cleavage of ZP2 is inhibited by the plasma protein fetuin-B, a very potent and specific endogenous inhibitor of ovastacin\(^4,5\). As shown recently, the absence of fetuin-B causes infertility in knock-out mice due to this pre-fertilization cleavage\(^6\). Hence, this proteolytic network comprising zona pellucida components (i.e. ZP2), ovastacin and the inhibitor fetuin-B is an important mechanism in the regulation of female fertility. About 5% of all couples are affected by infertility and the unfulfilled desire of having children\(^7\). Current treatment usually involves several hormones or other peptidic drugs and in vitro fertilization (IVF). Addition of fetuin-B increases the fertilization rate in vitro in mice\(^8\) and even in human serum fetuin-B levels correlate with the outcome of IVF\(^9\). This points out the importance of the regulation of ovastacin during fertilization. However, despite the high inhibitory potency of fetuin-B, its application in infertility treatment or IVF might be limited due to its proteinogenic origin, as well as the lack of well-defined fetuin-B containing IVF media. In this setting, the inhibition of ovastacin by synthetic small molecules could be a valuable non-
hormonal treatment option of female infertility or an alternative supplement to facilitate IVF. Ovastacin is a member of the astacin-family within the metzincin superfamily of zinc-metalloproteinases, comprising ovastacin, meprin α/β and BMP-1/tolloid-like proteinases (BTPs) in humans\(^1\). The BTPs have been in focus of drug development for fibrotic diseases and also the meprins are emerging drug targets, which led to the development of potent inhibitors, recently\(^{10–14}\). However, except the endogenous inhibitor fetuin-B no specific compounds that modulate ovastacin activity have been reported to date\(^{4,5,15}\). Due to the unmet medical need of non-hormonal infertility treatment, also accompanied with a high social impact, we aimed at the discovery of small molecule inhibitors of ovastacin that could serve as starting point or lead compounds for the development of such a treatment or as additives for culture-media that could facilitate IVF.

As mentioned above, inhibitors of other human astacin proteinases have already been reported and also the structures of mature BMP-1 and meprin β have been elucidated earlier\(^{16,17}\). However, no structural data of meprin α and ovastacin is available to date. Thus, a homology model of human ovastacin was generated to compare the active sites of all human astacin proteinases to gain insight into structural similarities and differences that could guide compound selection for an inhibitor screening against ovastacin (Figure 1).
Figure 1: Comparison of the protease domains of human ovastacin (A, homology model), human meprin α (B, homology model), human meprin β (C, pdb: 4GWN) and human BMP-1 (D, pdb: 3EDG).

Figure 2: Detailed view of the active sites: human ovastacin (A), hmeprin α (B), hmeprin β (C) and hBMP-1 (D). The side chains of amino acids within the active sites that are potentially involved in ligand binding are highlighted.
Although all astacin proteinases share a common fold, distinct differences within the active sites, i.e. in the S₁, S₁´ and S₂´-subsites lead to altered substrate specificities \(^{(1)}\). Vice versa, these small structural differences could also lead to different preferred interactions that could be addressed for the design of selective inhibitors (Figure 2). The S₁-subsite is different in all the compared human astacin proteases. Thus, interactions with these residues, as proposed for the reported meprin inhibitors \(^{(12,13)}\), might be a major determinant of inhibitor activity and particularly their selectivity. While the S₁-site is shaped by Phe\(^{214}\) in ovastacin, the respective residue is Tyr\(^{187}\) in meprin α, Arg\(^{184}\) in meprin β and Gln\(^{124}\) in BMP-1, respectively. Hence, the S₁-site of ovastacin is much more lipophilic compared to the polar environment in meprin β or BMP-1 and thus more similar to the physicochemical character of tyrosine, as found in meprin α. The amino acids shaping the S₁´-subpocket are highly conserved among the astacins. The respective arginine residues, Arg\(^{264}\) in ovastacin, Arg\(^{242}\) in meprin α, Arg\(^{238}\) in meprin β and Arg\(^{176}\) in BMP-1 are the major contributors to the preference for acidic amino acids in P₁´-position of the astacin proteinase substrates \(^{(18)}\). However, some amino acids flanking the S₁´-site are different. While Gln\(^{215}\) in meprin α or Ser\(^{212}\) in meprin β could be involved in hydrogen bonds contributing to ligand binding, the respective residues – Gly\(^{239}\) in ovastacin and Ala\(^{150}\) in BMP-1 are ruling out electrostatic sidechain interactions. The S₂´-site is again quite similar among the compared proteinases, featuring a hydrophilic, basic environment created by Arg\(^{177}\) in ovastacin, Arg\(^{146}\) in meprin β and Lys\(^{86}\) in BMP-1. Only meprin α exhibits a different pattern, with Tyr\(^{149}\) creating a more lipophilic environment in the S₂´-pocket. Taken together, the residues within the active site of ovastacin share some features of meprin α as well as meprin β, but differ slightly from BMP-1.
Due to the structural similarities that could contribute to inhibitor interactions, in particular within the S₁, S₁′ and S₂′-subsites of ovastacin with either meprin α and β, we assumed that the recently reported tertiary amine based hydroxamate inhibitors might also be able to inhibit the activity of ovastacin. Hence, selected potent meprin α and meprin β inhibitors, covering a range of different structural features, were screened against murine ovastacin to probe their potential as lead compounds for drug development and further compound optimization (table 1). Murine and human ovastacin share an overall sequence identity of ~68%. However, within the active site cleft the residues shaping the subsites that are assumed to be involved in inhibitor binding, i.e. the S₁, S₁′ and S₂′ subsite, are virtually identical with a sequence identity of ~97%. Just one residue adjacent to the S₂′-pocket differs significantly, i.e. Arg¹⁷⁴ in murine ovastacin vs. Gln¹⁷⁴ in the human enzyme (see sequence alignment and structural comparison in the SI). However, this might not contribute to the binding of small molecule inhibitors targeting the catalytic Zn²⁺-ion as primary interaction, which for astacin metalloproteinases was first studied with transition state analog inhibitors directed against prototypal astacin¹⁹,²⁰ and hydroxamates targeting meprins²¹. Small structural differences within the active sites of murine and human ovastacin revealed by the homology models might be attributed to the individual model quality, albeit both used the same structural template and thus might be negligible. However, in the present model of murine ovastacin, Ile¹⁷⁹ is swung out towards the upper rim of the active site cleft. In the model of human ovastacin, Phe²⁴³ is turned away from the catalytic center, although in all available structures of astacins and the remaining homology models, the respective residue is involved in shaping the lower rim of the active site cleft. Thus, the orientation of the sidechains of some active site residues in murine and human ovastacin remains elusive and could be solved by X-ray crystallography in the future.
Table 1 Inhibition of murine ovastacin by tertiary amine hydroxamic acids

| R₁ | R² | R³ | R⁴ | R⁵ | R⁶ | n   | IC₅₀ [nM]¹ | IC₅₀ [nM]¹ | RA [%]² (Kᵢ(app) [nM]) |
|----|----|----|----|----|----|-----|-----------|-----------|--------------------------|
| 1  | H  | COOH | H | H | COOH | H | 1 | 16050 ± 212 | 49 ± 11 | 109 ± 4 |
| 2  | H  | COOH | H | H | COOH | H | 2 | 5030 ± 57 | 495 ± 1 | 86 ± 4 |
| 3  | -OCH₂O- | H | H | -OCH₂O- | 1 | 1915 ± 120 | 6555 ± 509 | 85 ± 5 |
| 4  | -OCH₂O- | H | H | -OCH₂O- | 2 | 157 ± 1 | 2945 ± 346 | 63 ± 5 (1150 ± 71) |
| 5  | Cl | OH | F | F | OH | Cl | 1 | 368 ± 8 | 24 ± 1 | 72 ± 4 |
| 6  | Cl | OH | F | F | OH | Cl | 2 | 186 ± 1 | 28 ± 1 | 46 ± 3 (494 ± 55) |
| 7  | H  | COOH | H | H | H | H | 1 | n.d. | 1285 ± 15 | 96 ± 8 |
| 8  | H  | COOH | H | H | OCH₃ | 1 | 3420 ± 325 | 76 ± 17 | 102 ± 5 |
| 9  | H  | COOH | H | H | -OCH₂O- | 1 | 1910 ± 70 | 285 ± 94 | 96 ± 4 |
| 10 | Cl | H  | F | F | H | Cl | 2 | 1001 ± 41 | 19200 ± 990 | 78 ± 6 |
| 11 | Cl | OCH₃ | F | F | OCH₃ | Cl | 2 | 495 ± 33 | 19550 ± 919 | 62 ± 5 (1130 ± 45) |
| 12 | Cl | H  | F | F | OH | Cl | 2 | 215 ± 18 | 274 ± 21 | 57 ± 5 (749 ± 45) |
| 13 | Cl | OH | F | F | H | -OCH₂O- | 2 | 159 ± 11 | 146 ± 11 | 80 ± 6 (730 ± 27) |
| 14 | Cl | H  | F | F | H | -OCH₂O- | 2 | 186 ± 33 | 960 ± 38 | 83 ± 2 |
| 15 | H  | N(Pyridine) | H | H | -OCH₂O- | 2 | 464 ± 11 | 9650 ± 297 | 82 ± 5 |
| 16 | H  | N(Pyridine) | H | F | OH | Cl | 2 | 1105 ± 50 | 1220 ± 113 | 75 ± 5 |
| 17 | N(Pyridine) | H | H | H | -OCH₂O- | 2 | 474 ± 4 | 8175 ± 106 | 116 ± 5 |
| 18 | N(Pyridine) | H | H | F | OH | Cl | 2 | 646 ± 6 | 703 ± 30 | 77 ± 7 |
| 19 | H  | N(Pyridine) | H | H | N(Pyridine) | H | 2 | 7680 ± 410 | 53900 ± 3111 | 98 ± 5 |
| 20 | CN | H  | H | H | H | CN | 2 | 3415 ± 50 | 68250 ± 1061 | 88 ± 4 |

¹ IC₅₀-values from Ramsbeck et al.¹² and Tan et al.¹³, respectively. Meprin α and β IC₅₀-values of 12-18 were determined as described therein; ² inhibitor concentration 250 nM
The evaluation of the inhibitory potency against murine ovastacin revealed a dose dependent reduction of ovastacin activity by all of the tested compounds. Furthermore, the majority of compounds exhibited a significant inhibition, i.e. a relative activity below fifty percent at a compound concentration of 5 µM (see supporting information). Obviously, compounds that are preferably inhibitors of meprin β (1, 5, 8, 9) exhibited less inhibitory activity against murine ovastacin at 0.25 µM compared to compounds with higher activity against meprin α (4 & 6). Notably, the latter two are derivatives of β-alanine, while the remaining compounds are glycine hydroxamates, which exhibit shorter spacers. The same influence of the spacer length was already observed for meprin α vs. meprin β activity and is further corroborated by compound 2, exhibiting also a slightly higher activity compared to 1$^{(13)}$. Albeit the homology models do not reveal differences in the overall geometry of the active site, there seem to exist slight differences in the native conformation of ovastacin compared to meprin β, that lead to the same preference of β-alanine derived inhibitors as for meprin α, but remains elusive with the present models. Furthermore, compounds 1, 2, 7, 8 & 9 are bearing at least one carboxylic acid moiety. Although the S₁´ and S₂´-pockets of murine ovastacin are shaped by arginines and almost all astacins exhibit the same preference for acidic P₁´-residues, this moiety is obviously less favorable for inhibitor binding. This also corroborates the structure-activity relationships found for meprin α$^{(13)}$. This reduced activity could be due to the lack of a hydrogen bond donor within the S₁´-site of ovastacin, i.e. Gly$^{239}$ vs. Ser$^{212}$ in meprin β, that additionally contributes to ligand binding, rather than solely a charged interaction with the arginine. The most effective inhibition of ovastacin was observed for compound 6, exhibiting a K_i(app)-value of 494 ± 55 nM. Thus, this compound turned out to be a pan-specific astacin inhibitor, balancing lipophilicity with reduced acidity caused by the halophenol moieties, thereby enabling interactions with either lipophilic or basic S₁, S₁´or S₂´-
subpockets found in ovastacin, meprin α or meprin β, respectively. This was corroborated by in silico docking. The majority of found docking solutions revealed an orientation of the two halophenol moieties towards the S₁ and S₁´-pockets (Figure 3, shown in transparent purple). Albeit, a clearly preferred conformation could not be observed, most likely due to the flexibility of the tertiary amines. Nevertheless, the top ranked docking solution (Figure 3, shown in orange) suggests potential direct interactions with F₂₁₄, F₂₄₃ and R₂₆₄ in the S₁ and S₁´-site, respectively.

Based on these initial findings, further β-alanine hydroxamates (10-20) were evaluated with regard to their ovastacin inhibition. The depletion of the acidic phenols of 6, leaving just an electron-deficient aryl moiety (10) led to a strong decrease in activity, while it was less decreased by etherification of the phenol (11, \(K_{i^{(app)}} = 1130 \pm 45 \text{nM}\)). However, the combination of an acidic halophenol with the electron-deficient chloro-fluoroaryl residue (12) again led to increased inhibition of ovastacin, exhibiting a \(K_{i^{(app)}}\)-value of 749 ± 45 nM. Also, the combination of the halophenol with an electron-rich benzodioxolane moiety (13, \(K_{i^{(app)}} = 730 \pm 27 \text{nM}\)) led to pronounced inhibition of murine ovastacin. The depletion of the halophenol again led to a reduced activity of 14, underpinning the importance of this polar substructure for favorable inhibition of murine ovastacin. The introduction of electron-deficient moieties, i.e. pyridyl-residues (15-19), as
well as cyanoaryl (20), also led to significant decreased inhibition of ovastacin. This first insight into the structure-activity relationships of small molecule ovastacin inhibitors exemplified the requirement of distinct features for specific and potent ovastacin inhibition. This preliminary data resembles more the subsite specificity and structure-activity relationships found for meprin α, i.e. a preference for lipophilic substituents and moieties with reduced acidity, rather than the polar carboxylic acid found to be important for potent and selective inhibition of meprin β. Assuming a similar binding mode as postulated for meprin α and β, this might be due to the higher similarity of ovastacin compared to meprin α within the respective binding pockets: the S₁′-pocket, shaped by Arg²⁶⁴ and lacking an additional H-bond donor, like Ser²¹² in meprin β, and the S₁-pocket, shaped by Phe²¹⁴ and Phe²⁴³, that creates a lipophilic binding site like Tyr¹⁸⁷ in meprin α, rather than the polar cationic Arg¹⁸⁴ in meprin β. Hence, the interaction with this sub-pocket might be the key element that could be addressed for the design of selective inhibitors of the individual proteinases. However, the structural elucidation of ovastacin in complex with an inhibitor will shed light on the true binding mode and potentially enable a structure-guided design of novel inhibitors.

In summary, this primary evaluation of tertiary amine hydroxamate based compounds came up with nanomolar inhibitors of the astacin metalloproteinase ovastacin. Ovastacin catalyzes a unique and essential proteolytic cleavage within the egg envelope, which under physiological conditions is not performed by any other proteinase, since ZP2 remains non-cleaved in ovastacin deficient mice(2). Absence of the natural ovastacin antagonist fetuin-B causes female infertility. Hence, this study may pave the way for the development of a novel treatment strategy for female infertility utilizing small molecule inhibitors based on an elaborated peptidomimetic scaffold.
METHODS

Homology Modelling

Human ovastacin: Chain A of the Danio rerio hatching enzyme (PDB ID: 3LQB) and the FASTA sequence of human ovastacin (Q6HA08) were aligned with MOE (v2016.0802; Protein Align 2016.11), resulting in a sequence identity of 46.7%. During the modeling process the number of independent main-chain models was set to 100. Electrostatic solvation energy (Generalized Born/Volume Integral, GB/VI) was calculated and scored for refinements and the subsequent selection of the final model. Finally, the template structure was superposed to the ovastacin model and the Zn$^{2+}$ ion was copied to the model structure as new chain. For comparisons, pairwise RMSD values were calculated and for examination of the model geometry, Ramachandran plots ($\Phi$-$\Psi$ plots) were visualized.

For murine ovastacin the FASTA sequence Q6HA09 was employed and also aligned to PDB ID: 3LQB with MOE (v2018.01; Protein Align 2018.01). The resulting pairwise identity equals 44.5%. The final murine ovastacin model was generated and selected according to the remaining modeling steps described for human ovastacin.

Docking

For the molecular docking of compound 6 the homology model of murine ovastacin was used as receptor. The docking procedure was set up in MOE (v2019.0102) and performed with GOLD (v5.7.1). The active site was defined by the Zn$^{2+}$-ion. The ligand was utilized in its deprotonated form. The search efficacy was set to 100%, ChemScore was used as scoring function and 30 docking runs were performed. The resulting solutions were visually inspected and solutions lacking an interaction between hydroxamate and the zinc ion were discarded. The remaining 19
solutions were subjected to Postdock\textsuperscript{(22)} (postdock-1.2.svl script available via the CCG svl exchange server - svl.chemcomp.com) to create the figures and visualize the docking solutions according to their RMSD from the top ranked solution and their respective docking score.

Inhibition assay

The inhibitory activity of tertiary amine hydroxamate inhibitors against mouse ovastacin was determined \textit{in vitro} by means of a fluorogenic enzyme activity assay as previously described\textsuperscript{(5)}. Ovastacin was expressed as previously described\textsuperscript{(6)} and activated by human plasmin (Haematologic Technologies Inc., Essex Junction, USA) for 30 min in a molar ratio of 10:1. Concentration of active ovastacin (1 nM) was determined via titration and IC\textsubscript{50} calculation with heterologously expressed murine fetuin-B\textsuperscript{(4)}. All assays were performed as independent double measurements in triplicate at 37°C in 100 µl final volume, buffered with 150 mM NaCl 50 mM Tris/HCl, pH 7.4, 0.01% Brij-35. All hydroxamate inhibitors were dissolved in dimethyl sulfoxide. Enzyme activity measurements were started by addition of 25-30 µM Ac-R-E(Edans)-D-R-Nle-V-G-D-D-P-Y-K(Dabcyl)-NH\textsubscript{2}, dissolved in dimethyl sulfoxide (final concentration 1.4%). Initial velocities were recorded for at least 1000 s (50 times for 100 ms at intervals of 20 s). Thereafter, 1.5 µl of proteinase K (at 20 mg/ml; Sigma-Aldrich, Taufkirchen, Germany) were added to reach complete substrate turnover, which was monitored and subsequently calculated using the formula $v = [S] \times m / \Delta F$, where $[S]$ is the substrate concentration, $m$ the $[F/t]$ slope of initial linear substrate turnover, and $\Delta F$ the maximal fluorescence intensity corresponding to complete turnover. Kinetic parameters of inhibition (K\textsubscript{i}(app)) were determined using Morrison’s equation\textsuperscript{(23)}. 
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