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Characterization of the Catalytic Properties of the Membrane-anchored Metalloprotease ADAM9 in Cell-based assays

Thorsten Maretzky[1], **Steven Swendeman**[1,2], **Elin Mogollon**[1], **Gisela Weskamp**[1], **Umut Sahin**[1,3], **Karina Reiss**[4], **Carl P. Blobel**[1,5,6]

[1]Arthritis and Tissue Degeneration Program, Hospital for Special Surgery at Weill Cornell Medicine, New York, NY, 10021, USA

[2]Boston Children's Hospital, Boston, MA, 02115, USA

[3]EMBO Young Investigator Program, Center for Life Sciences and Technologies, Bogazici University, Istanbul, 34342 Turkey

[4]Dept. of Dermatology, University Hospital Schleswig-Holstein, Campus Kiel, 24105 Kiel, Germany

[5]Department of Physiology, Biophysics and Systems Biology, Weill Cornell Medicine, New York, NY, USA

[6]Institute for Advanced Study, Technical University Munich, 85748 Garching, Germany

Abstract

ADAM9 (a disintegrin and metalloprotease9) is a membrane-anchored metalloproteinase that has been implicated in pathological retinal neovascularization and in tumor progression. ADAM9 has constitutive catalytic activity in both biochemical and cell-based assays and can cleave several membrane proteins, including Epidermal-Growth-Factor and Ephrin receptor B4. Yet, little is currently known about the catalytic properties of ADAM9 and its posttranslational regulation and inhibitor profile in cell-based assays. To address this question, we monitored processing of the membrane-anchored EphB4 by co-expressing ADAM9, with the catalytically inactive ADAM9 E>A mutant serving as negative control. We found that ADAM9-dependent shedding of EphB4 was not stimulated by three commonly employed activators of ADAM-dependent ectodomain shedding, phorbol esters, pervanadate or calcium ionophores. With respect to the inhibitor profile, we found that ADAM9 was inhibited by the hydroxamate-based metalloprotease inhibitors marimastat, TAPI-2, BB94, GM6001, GW280264X and by 10 nM of the tissue inhibitor of metalloproteinases (TIMP)-3, but not by up to 20 nM of TIMP-1 or −2. Additionally, we screened a non-hydroxamate small molecule library for novel ADAM9 inhibitors and identified four compounds that selectively inhibited ADAM9-dependent proteolysis over ADAM10- or ADAM17-dependent processing. Taken together, this study provides new information about the molecular fingerprint of ADAM9 in cell-based assays by showing that it is not stimulated by strong activators of ectodomain shedding, and by defining a characteristic inhibitor profile. The identification of novel non-hydroxamate inhibitors of ADAM9 could provide the basis for

Address correspondence to: Dr. Carl P. Blobel, Arthritis and Tissue Degeneration Program, Hospital for Special Surgery at Weill Cornell Medicine, 535 E 70th Street, New York, NY, 10021, phone: 212-606-1429, fax: 212-774-2560, blobelc@hss.edu.

designing more selective compounds that block the contribution of ADAM9 to pathological neovascularization and cancer.

Introduction

ADAMs (a disintegrin and metalloproteinase) are a family of membrane-anchored metalloproteinases with critical roles in cell-cell communication, growth factor signaling and in the shedding of various membrane bound proteins, including cytokines, growth factors, and adhesion molecules [1-4]. ADAMs 10 and 17 have emerged as key modulators of EGFR signaling, regulating the availability of soluble EGFR ligands through proteolytic cleavage of the transmembrane precursors of these growth factors [1, 5-9]. ADAM9 is markedly upregulated in several human carcinomas including liver [10, 11], pancreas [12, 13], and gastric cancer [14, 15]. Increased expression levels of ADAM9 are also associated with cancer progression in breast carcinoma [16, 17] as well as in renal cancer, where ADAM9 expression is significantly correlated with markers of poor prognosis [18, 19]. Additionally, ADAM9 plays a role in tumorigenesis in mouse models for prostate, breast and gastrointestinal cancers [20-23]. It was also found to be upregulated both transcriptionally and functionally in response to oxidative stress [22, 23] and has been implicated as a regulator of bone formation [24].

ADAM9 is a membrane-anchored metalloproteinase containing an N-terminal prodomain, followed by a metalloprotease domain, a disintegrin domain and cysteine-rich region, an EGF-repeat, a transmembrane domain and a cytoplasmic tail with potential SH3-ligand domains [25]. ADAM9 is catalytically active in both biochemical [26] and cell-based assays [23], and can cleave several membrane proteins (Table 1) [9, 20, 23, 26-45], including Epidermal-Growth-Factor (EGF), the Fibroblast Growth Factor Receptor (FGFR)2iiib and the Ephrin receptor B4 (EphB4), when it is overexpressed together with these substrates [20, 23, 24]. Yet little is known about its catalytic properties or its response to activators and inhibitors of metalloproteases in cell-based assays.

Previous efforts to characterize the catalytic properties of ADAM9 have focused on biochemical assays with the recombinantly expressed soluble extracellular catalytic domain of ADAM9 to determine its cleavage sites in peptide substrates and its inhibitor profile for hydroxamate-type metalloprotease inhibitors [26]. As an extension to the biochemical characterization of the catalytic activity of purified ADAM9, we initiated the current study to establish a “fingerprint” of the catalytic properties of ADAM9 in cell-based assays, which is defined as its response to activators and inhibitors of metalloproteases in cells [46]. The fingerprint of ADAM9 in cell-based assays is likely to be more physiologically relevant than its inhibitor profile in biochemical assays. It can serve to facilitate future functional studies on ADAM9 by providing information to link this enzyme to relevant substrates in cells and in tissues, such as collagen XVII in skin [32]. Moreover, the molecular fingerprint can be used to identify selective inhibitors for treatment of ADAM9-dependent pathologies.

In order to characterize an enzyme such as ADAM9 in cell-based assays it is crucial to have an assay that can serve as a selective readout for the enzyme of interest. Since we have not identified a substrate whose processing is completely abolished in “loss of function studies”

using ADAM9-deficient cells, we decided instead to use “gain of function” overexpression experiments with ADAM9 compared to overexpression of a catalytically inactive mutant as an assay for its catalytic activity in cells. As substrate, we used EphB4, that is processed by ADAM17 in “loss of function” experiments [33], but that can be processed by ADAM9 upon over-expression, or when its expression is upregulated by reactive oxygen species in mouse embryonic fibroblasts (mEFs) [23]. Here, we present a systematic evaluation of how known activators of ectodomain shedding, the phorbol ester PMA, the phosphatase inhibitor pervanadate (PV) or calcium influx triggered by the ionophore ionomycin (IO), affect the activity of ADAM9. Moreover, we determined how ADAM9 responds to inhibitors of ectodomain shedding including small molecule hydroxamates and tissue inhibitors of matrix metalloproteases (TIMPs). Finally, we screened a small-molecule library in order to identify novel potential inhibitors of ADAM9.

Materials & Methods

Cell lines and reagents

Simian virus large T-antigen-immortalized *Adam9*−/−, *Adam10/17*−/− and the respective wild type mouse embryonic fibroblasts (mEFs) were generated and characterized previously [43, 47, 48]. COS7 cells were from ATCC. All cells were grown in DMEM supplemented with 1% penicillin/streptomycin and 5% fetal calf serum. All reagents were from Sigma-Aldrich unless otherwise indicated. Pervanadate (PV) was prepared immediately prior to each experiment by mixing sodium vanadate and hydrogen peroxide to a final concentration of 100 μM each. Ionomycin (IO) and the hydroxamate inhibitors GM6001 and TAPI-2 were obtained from Calbiochem (San Diego, CA). Batimastat (BB94), GI254023X (GI) and GW280264X (GW) were kindly provided by Dr. D. Becherer (GlaxoSmithKline, Research Triangle Park). Marimastat was synthesized using a slightly modified version of the two previously described procedures [49, 50]. Anti-ADAM9, ADAM15, and ADAM17 antibodies have been described previously [25, 51-53]. TIMP-1 and TIMP-2 were purchased from Calbiochem or R&D systems. TIMP-3 was a gift from Dr. R. Black (Amgen, Seattle, WA). The primary small molecule library was purchased from Telik Inc. (Palo Alto, CA). This library was designed to employ only a limited number of compounds (96) to identify small molecule candidates in cell-based assays that are not well suited for high throughput screening.

Expression vectors

The expression vectors for the AP-tagged EphB4, TGFα and BTC and plasmids encoding for murine ADAM9 E>A (EA9) and WT ADAM9 (A9), have been described previously [9, 23, 33, 54].

Overexpression of ADAM9 in cell-based assays and Western blot analysis

Mouse embryonic fibroblasts or COS7 cells were transiently transfected with Lipofectamine2000, according to the manufacturer’s protocol, using the A9 or EA9 plasmids at a concentration of 1.5 μg per well of a 6-well plate, unless otherwise indicated, and Ephb4-AP at 1 μg. For shedding assays, the cells were washed with OptiMEM medium for 1 hour, which was then replaced by fresh OptiMEM with or without the indicated

inhibitors or stimuli, and incubated for 30 minutes to 2 hours as indicated (see legends of individual figures for details). The AP-activity in the supernatant and the cell lysates (Supplementary Figure 1) was determined by colorimetric assays as described previously [9, 23, 34, 55]. Briefly, three identical wells were prepared, and the ratio between the total AP activity in the supernatant and the total AP activity in the cell lysate plus supernatant were calculated for normalization. The ratio reflects the relative shedding activity of a given sheddase towards a given AP-tagged ligand. No AP activity was present in conditioned media of non-transfected cells. Western blot analysis for ADAM9, ADAM15, and ADAM17 was performed as described previously [25, 51-53].

Determination of IC50 values

IC50 values were calculated using Microsoft Excel software. All samples were first corrected by the ADAM9 E>A measurements. The data points were plotted (concentration vs. corrected measurement) and third-degree polynomial regression curves were fit through at least 5 data points (average of no drug wells and at least 4 dilution points). The concentration of the IC50 was determined from the resulting fit.

Statistical analysis

All values are expressed as means ± standard error of the mean (SEM). The standard error values indicate the variation between mean values obtained from at least three independent experiments. The assumptions for normality (Kolmogorov-Smirnov test) and equal variance (Levene mediantest) were verified with SigmaStat 3.1 software (Erkrath, SYSSTAT, Germany). The analysis of variance was performed with one-way analysis of variance. The comparison of the means between two groups was performed with the Student's t-test. Multiple parametric statistical comparisons between experimental groups versus a control group were accomplished by Dunnett's method. P values of <0.05 in a student's t-test were considered statistically significant.

Results

Full-length membrane-anchored ADAM9 cleaves alkaline phosphatase-tagged EphB4 in cell-based assays.

The main goal of the current study was to learn more about the catalytic properties of ADAM9 by establishing its molecular fingerprint, defined as its response to activators and inhibitors of metalloproteases in cells [46]. When ADAM9 is expressed in COS7 cells together with an alkaline phosphatase-tagged EphB4, this leads to significantly increased release of EphB4-AP compared to cells co-expressing an inactive mutant of ADAM9 in which the catalytic glutamate was mutated to alanine (ADAM9 E>A, EA9) [23, 26]. To corroborate that ADAM9 can shed EphB4-AP from different cell types, we expressed ADAM9 or ADAM9 E>A together with EphB4-AP in COS7 cells, *Adam9*−/−, *Adam10/17*−/− or wild type (WT) mEFs (Figure 1A-D). In each case, overexpression of ADAM9 reproducibly increased EphB4-AP shedding compared with cells overexpressing ADAM9 E>A. Western blots analysis confirmed that ADAM9 and ADAM9 E>A were expressed at similar levels in these experiments (Figure 1A-D, lower panels). Moreover, the level of alkaline phosphatase activity was also comparable in the lysates of cells

co-transfected with ADAM9 or ADAM9E>A, demonstrating similar expression levels of EphB4-AP (Supplementary Figure 1). The ADAM9-dependent shedding of EphB4-AP could be further enhanced by increasing the amount of ADAM9 plasmid used to transfect cells, which also led to increased ADAM9 levels, as demonstrated by Western blot analysis (Figure 1E). These experiments confirm that overexpression of wild-type ADAM9 increases shedding of EphB4-AP in a dose-dependent manner compared to the inactive control, thereby providing a robust readout for the catalytic activity of ADAM9 in several different cell lines. We therefore took advantage of this "gain-of-function" system where EphB4-AP shedding mediated by ADAM9 was used to evaluate this protease's response to various stimulators or inhibitors.

Effect of tissue inhibitors of metalloproteases (TIMPs) on ADAM9-mediated EphB4-AP shedding.

TIMPs-1, −2 and −3 are known to inhibit most MMPs [56], and TIMP-3 is a potent inhibitor of ADAM10 and ADAM17, whereas TIMP-1 inhibits ADAM10, but not ADAM17 [43, 54, 57-59]. Previous *in vitro* studies have demonstrated that recombinantly expressed ADAM9 is not inhibited by TIMPs 1, 2 or 3 at concentrations up to 200 nM [60]. When TIMPs-1 or −2, were added to *Adam10/17*−/− mEFs over expressing EphB4-AP together with ADAM9 or its inactive E>A mutant, the increase in shedding seen in cells transfected with ADAM9 compared to the ADAM9 E>A control was not significantly affected by concentrations of TIMP-1 or TIMP-2 up to 20 nM (Figure 2A, B), whereas TIMP-3 inhibited ADAM9 partially at 10 nM, and strongly at 20 nM (Figure 2C). These results suggest that ADAM9 is sensitive to inhibition by TIMP-3 in cell-based assays.

Effect of hydroxamate-type metalloprotease inhibitors on ADAM9-mediated EphB4-AP shedding.

ADAMs and related metzincin metalloproteases can usually be inhibited by hydroxamic acid-type metalloprotease inhibitors [26, 34, 61-63]. Therefore we tested how six hydroxamate metalloprotease inhibitors affected ADAM9-dependent shedding of EphB4-AP. TAPI-2, GM6001, marimastat, and BB94 inhibited the ADAM9-dependent release of EphB4-AP in a dose-dependent manner (Figure 3A-D). The apparent IC_{50} value of EphB4-AP shedding was 13.52 μM for TAPI-2, 0.72 μM for GM6001, 0.13 μM for marimastat, and 0.05 μM for BB94. We also performed inhibitor studies with two hydroxamate-based compounds that differ in their capacity to block ADAM10 or ADAM17 [63]. The inhibitor GW280264X has been shown to preferentially block ADAM17 and, to a lesser extent, ADAM10 [63-65]. The compound GI254023X blocks ADAM10, but not ADAM17 at a concentration of 1 μM in cell-based assays, providing a means to distinguish between the activity of ADAM10 and ADAM17 in cells at this concentration [66]. GW280264X effectively inhibited ADAM9 activity at concentrations as low as 0.5 μM and increasing inhibition was observed at concentrations between 1 and 10 μM (Figure 3E). Recently, the ADAM10-specific inhibitor GI254023X was reported to affect the catalytic activity of ADAM9 in biochemical assays, [67]. When we overexpressed ADAM9 in *Adam10/17*−/− mEFs together with EphB4-AP, GI254023X did not block ADAM9-mediated shedding of EphB4-AP from *Adam10/17*−/− mEFs at concentrations of up to 10 μM GI254023X (Figure

3F), even though it inhibited shedding of the ADAM10 substrate betacellulin-AP in WT mEFs at concentrations as low as 0.04 μM (Supplementary Figure 2).

ADAM9 does not respond to stimulation with phorbol esters, calcium ionophore or phosphatase inhibitors.

In principal, shedding of membrane proteins can proceed in a constitutive or regulated fashion [43, 68, 69]. Stimulation of protein kinase C (PKC) using the phorbol ester phorbol-12 myristate 13-acetate (PMA) is frequently used to stimulate ADAM17-dependent ectodomain shedding, whereas ionomycin (IO), which promotes shedding through stimulation of calcium influx, can activate shedding by ADAM10 and ADAM17 [9, 43, 54, 69, 70]. Addition of 20 ng/ml of the phorbol ester PMA, which stimulates ADAM17-dependent shedding of proteins such as TGFα or TNFα, did not enhance ADAM9-dependent shedding of EphB4-AP in *Adam10/17*−/− mEFs (Figure 4). ADAM9-mediated EphB4-AP release was also not responsive to 100 μM of the phosphatase inhibitor pervanadate (PV) or 2.5 μM of ionomycin (IO), two other commonly used stimuli of ectodomain shedding (Figure 4). These results indicate that ADAM9 does not respond to stimulation with PMA, PV or IO, at least under the conditions tested here, which are known to strongly stimulate ADAM17 (PMA, PV) or both ADAM10 and ADAM17 (IO) [43, 54, 71].

Identification of four ADAM9 inhibitors based on a screen of a small molecule library.

Given the observations that loss of ADAM9 decreases retinal pathological neovascularization and that ADAM9 is potential marker for poor prognosis in human prostate cancer, and is up-regulated in other carcinomas, it would be important to identify novel potential ADAM9 selective inhibitors. For this purpose, we used the cell-based assay described above (*Adam10/17*−/− mEFs expressing ADAM9 or ADAM9 E>A together with EphB4-AP) to screen a chemical compound library with 96 distinct core compounds for their inhibitory potential towards ADAM9. The approach was to identify molecules which inhibited the ADAM9-dependent shedding but did not affect basal shedding in cells expressing ADAM9 E>A. The benchmark for maximal inhibition was 5 μM marimistat for a 2 hour shedding assay. Each molecule was resuspended in DMSO and used at a final concentration of 10 μM in three separate assays, each performed in duplicates. Of the 96 molecules screened, four compounds (#28, #41, #50 and #67; Figure 5A-D) fit the criteria of inhibiting ADAM9-dependent shedding, while having little or no effect on basal shedding. These were then assayed in a titration curve and showed dose-dependent inhibition of ADAM9-dependent shedding (Figure 5E-H). Since the EphB4-AP was previously shown to be shed by ADAM17 and modestly by ADAM10, the four compounds were also tested for the ability to inhibit ADAM10 (Figure 5I-L) or ADAM17 (Figure 5M-P) in WT mEFs. For each compound, only ADAM9-dependent inhibition of shedding was observed. The results of this assay using core structures should provide an initiation point for the development of ADAM9 selective inhibitors as potential therapeutics for cancer and pathological angiogenesis in retinal diseases.

Discussion

The main goal of this study was to determine the molecular fingerprint of ADAM9 by analyzing how commonly used activators and inhibitors of protein ectodomain shedding affect the catalytic properties of ADAM9 in cell-based assays [46]. The main approach was to use *Adam10/17*−/− deficient mEFs where ADAM9 and its substrate EphB4-AP were ectopically expressed. This allowed us to monitor the activity of ADAM9 in the absence of ADAM17, which is otherwise a major sheddase for EphB4 in mEFs [33]. As a control, we expressed a mutant ADAM9 carrying an inactivating point mutation in its catalytic site (HELGH to HALGH, ADAM9E>A) that abolishes the catalytic activity of ADAM9 without affecting its export from the endoplasmic reticulum to the cell surface or the removal of its pro-domain [26].

Our results demonstrate that ADAM9-dependent processing of EphB4-AP in cell-based assays depends on the expression level of ADAM9, with higher concentrations of transfected expression constructs leading to higher levels of EphB4-AP processing. However, the ADAM9-dependent processing of EphB4-AP was not further enhanced by several commonly used stimulators of ectodomain shedding, the phorbol ester PMA, the phosphatase inhibitor pervanadate, and the Calcium ionophore ionomycin. This suggests that other stimuli like pro-inflammatory cytokines or LPS could be slow activators of ADAM9, presumably inducing transcriptional upregulation rather than posttranslational modifications of the protease [29]. In this regard, ADAM9 differs from ADAM17, which is highly responsive to all three stimuli, and from ADAM10 whose activity is strongly enhanced after treatment with ionomycin. Moreover, recent studies have shown that microRNAs, a family of small non-coding RNA molecules, can affect the progression of cancer cells by post-transcriptional regulating of ADAM9 expression. Inhibition of microRNA-590 increases ADAM9-mediated migration and invasiveness of non-small lung cancer cells [72], while microRNA-140 inhibits proliferation, migration and invasion of glioma cells by targeting ADAM9 [73]. Furthermore, microRNA-203 plays a tumor suppressive role by downregulating ADAM9 expression in hepatocellular carcinoma [74]. Additionally, microRNA-33a and microRNA-126 regulate breast cancer cell proliferation and metastasis by targeting ADAM9 and ROS1 [75, 76].

These studies cannot rule out that there are conditions under which the activity of ADAM9 can be further enhanced by posttranslational stimuli, but they provide useful information about the molecular fingerprint of ADAM9 that can help distinguish its activity from that of other ADAMs such as ADAM10 and ADAM17 in cell-based assays. Moreover, they suggest that higher levels of ADAM9 should lead to higher activity, which could be important in the context of diseases such as breast, prostate, or pancreatic cancer in which ADAM9 is overexpressed, for example by increasing its expression through reactive oxygen species. With respect to the inhibitor profile of ADAM9-dependent processing of EphB4-AP in cell-based assays, we found that the tissue inhibitor of matrix metalloproteinases TIMP-1 and TIMP-2 did not affect the activity of ADAM9 at up to 20 nM, whereas a concentration of 10 nM TIMP-3 reduced the activity of ADAM9 by about 40-50%, whereas 20 nM of TIMP-3 reduced the activity of ADAM9 by 75%. Thus the TIMP inhibitor profile of ADAM9 in cell-based assays differs from its profile in biochemical assays in that the full

length ADAM9 is sensitive to TIMP-3 in cell based assays, whereas the recombinantly expressed soluble ADAM9 is not [60]. The difference in TIMP-3 inhibitor profile could be caused by a different conformation of full length membrane-anchored ADAM9 in cells compared to the soluble purified protein, or by the presence of other molecules on the cell surface that somehow affect the ability of TIMP-3 to block ADAM9.

Interestingly, it is well known that hydroxamate inhibitors usually have a several orders of magnitude higher IC50 in cell-based assays than in biochemical studies using recombinantly expressed metalloproteinases such as ADAM17. When we tested the hydroxamate inhibitor profile for ADAM9-dependent processing of EphB4-AP, we found that marimastat and BB94 had a similar inhibitor profile towards ADAM9 in biochemical assays as in cell-based assays (K_i recombinant soluble ADAM9, marimastat: 0.274 μM, BB94: 0.014 μM [26], IC50 full length ADAM9, marimastat 0.13 μM, BB94: 0.05 μM). On the other hand, the IC50 for TAPI-2 was higher in cell based assays (TAPI-2: IC50 13.52 μM) than in biochemical studies (TAPI-2: K_i 0.017 μM) [26]. We also found that the ADAM10-selective hydroxamate GI254023X did not block the ADAM9-dependent processing of EphB4-AP at concentrations up to 5 μM, although it has been reported to block soluble ADAM9 with an IC50 of 280 ± 110 nM [67]. Even though we are not aware of an IC50 for GM6001 or GW280264X towards soluble ADAM9, the IC50 towards ADAM9 established here helps to further define the molecular fingerprint of ADAM9 in cell-based assays (A comparison of the potency of metalloprotease inhibitors toward ADAM proteases is summarized in Table 2) [34, 54, 64, 77-83].

Finally, we screened a library of small molecule compounds for novel ADAM9-selective inhibitors and identified four potential candidates that selectively blocked the activity of ADAM9 compared to ADAM10 or ADAM17. As ADAM9 overexpression is critical for pathogenesis of several human carcinomas, these compounds could serve as basis for development of ADAM9-selective therapeutics that are not hydroxamates that could be used to block the catalytic activity of ADAM9 activity in these cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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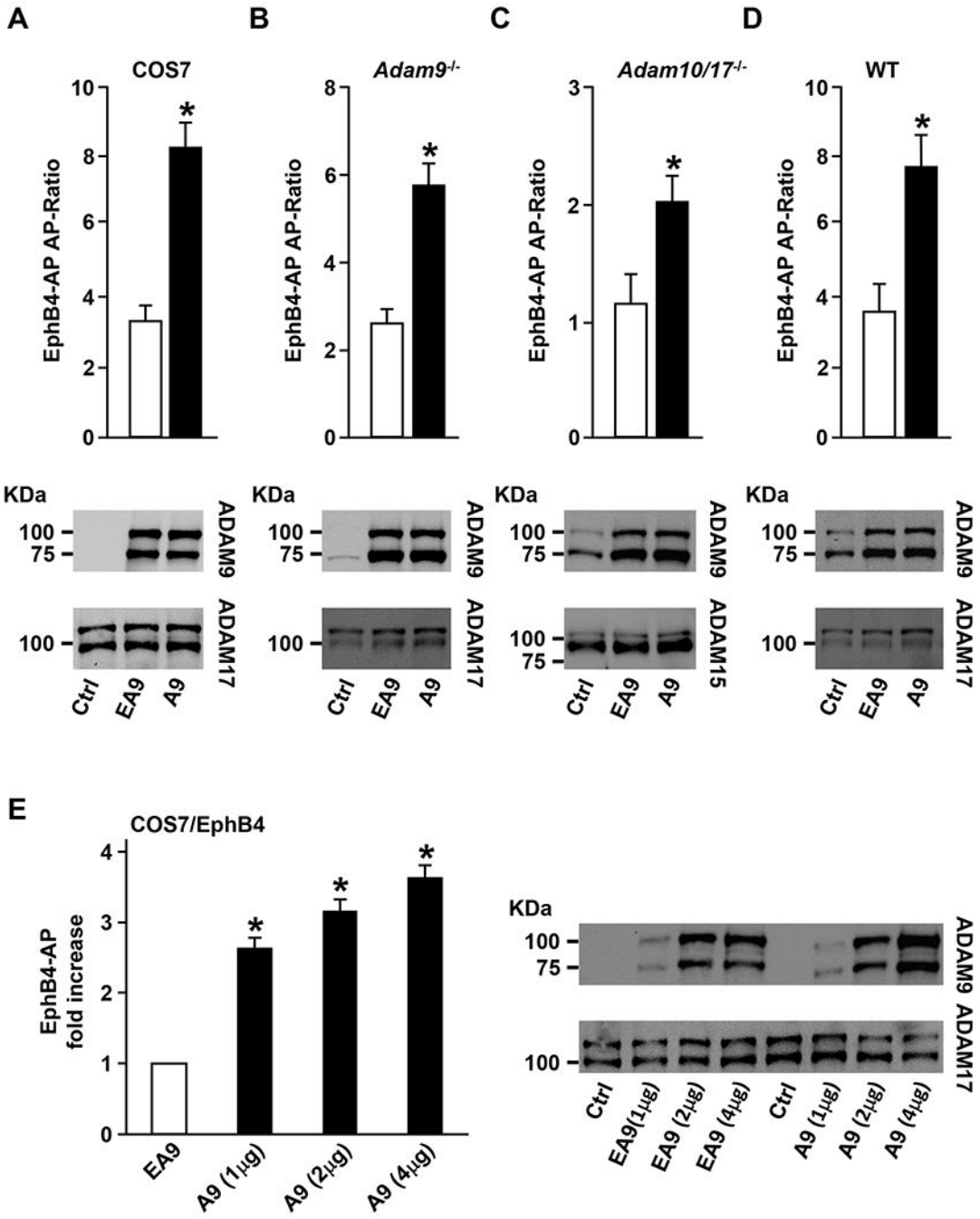

Figure 1. Overexpression of ADAM9 results in increased release of EphB4-AP.

COS7 cells (A, E), *Adam9*−/− (B), *Adam10/17*−/− (C) and wild type (WT)-mEFs (D) were co-transfected with EphB4-AP and WT ADAM9 (A9) or the inactive ADAM9 E>A control (EA9), in which the catalytic site consensus sequence HEXXH was mutated to HAXXH. EphB4 was tagged with an alkaline phosphatase (AP) moiety to allow detection of the cleaved ectodomain in the cell supernatant. Then the cells were washed and incubated in conditioned medium for 2 hours. (A-D) In all cases, there was a significant increase in EphB4-AP shedding from cells overexpressing A9 compared to cells overexpressing

EA9. Western blot analysis confirmed similar levels of overexpressed A9 or the EA9 control in the different cell lines shown here. Please note that the anti-mouse ADAM9 cyto antibodies used here do not cross-react with endogenous ADAM9 in COS7 cells (derived from monkey kidney), but recognize a minor non-specific band in *Adam9*−/− mEFs, and detect endogenous ADAM9 in wild type and *Adam10/17*−/− mEFs. Student's t-test, *P ≤ 0.05; ±SEM. (E) Increasing amounts of transfected A9 cDNA increased the shedding of co-transfected EphB4-AP from COS7 cells. Dunnett's test, *P ≤ 0.05; ±SEM. Increased levels of transfected A9 or EA9 cDNA correlated with increased levels of A9 or EA9 protein, as confirmed by Western blot analysis. All Western blots and graphs are representative of at least 3 separate experiments.

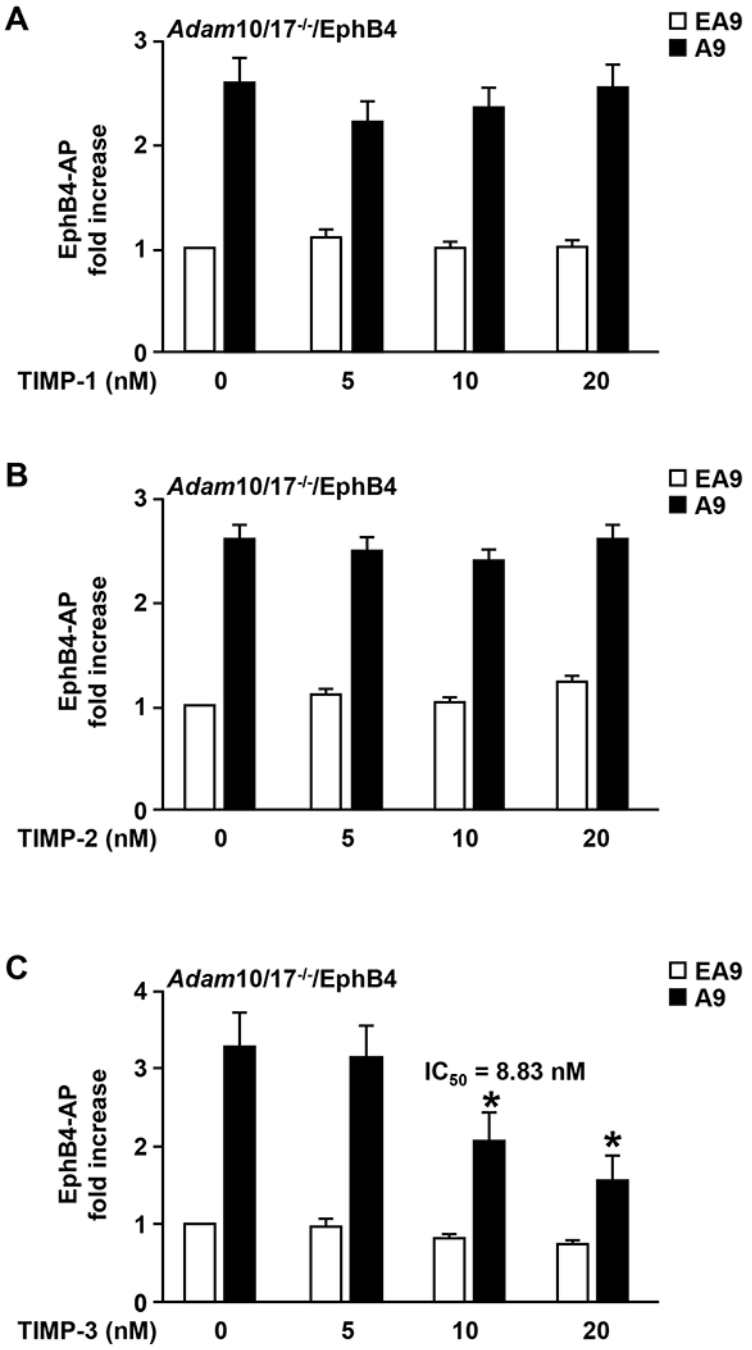

Figure 2. TIMP-inhibitor profile of ADAM9 in cell-based assays.

The effect of 5, 10 or 20 nM TIMP-1 (A), TIMP-2 (B), or TIMP-3 (C) was tested on *Adam10/17*−/− mEFs expressing EphB4-AP together with ADAM9 (A9) or catalytically inactive ADAM9 E>A (EA9). The numbers indicate the fold increase in shedding after 2 hours of incubation under different conditions, using untreated cells expressing EA9 as a reference for background shedding, which is set to 1. TIMP-1 had no significant effect on background shedding in the EA9 expressing cells. In cells expressing A9, there was an increase in EphB4 shedding of approximately 250%, and this was not significantly affected

by up to 20 nM TIMP-1. Concentrations of up to 20 nM TIMP-2 also did not affect EphB4 background shedding in the presence of EA9. TIMP-3 partially blocked EphB4 shedding at concentrations of 10 nM and 20 nM. These results are representative of at least 3 separate experiments. Dunnett's test, *$P \leq 0.05$; ±SEM

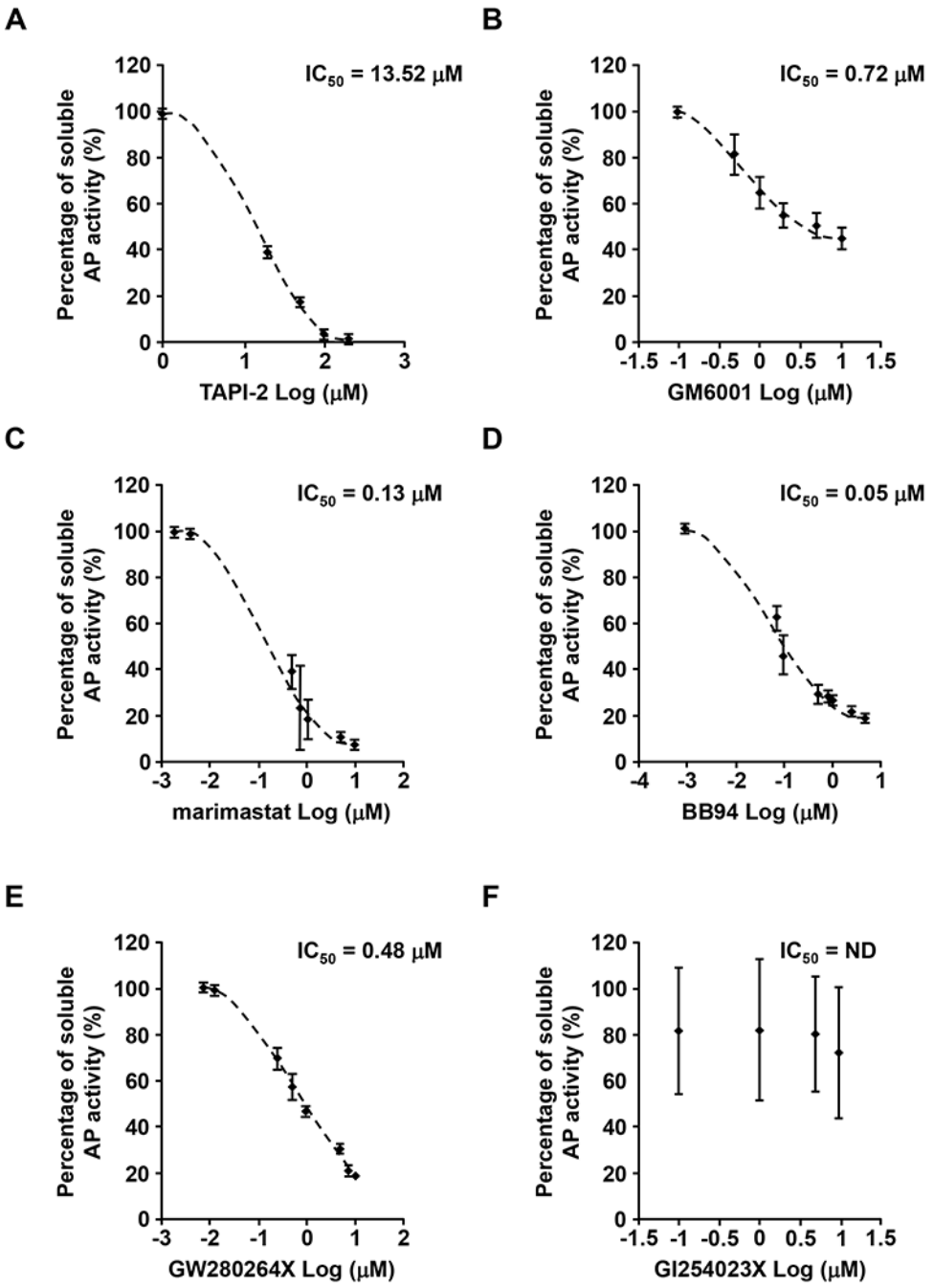

Figure 3. The catalytic activity of ADAM9 in cell-based assays is sensitive to the hydroxamates TAPI-2, GM6001, marimastat, BB94 and GW280264X but not to the ADAM10 inhibitor GI254023X.

Panels A – F show the effect of different concentrations of the hydroxamates TAPI-2 (A), GM6001 (B), marimastat (C), BB94 (D), GW280264X (E) and GI254023X (F) on the increase in EphB4-AP shedding after 2 hours of incubation in *Adam10/17*−/− mEFs overexpressing ADAM9 compared to cells expressing ADAM9 E>A. Each panel is representative of at least 3 separate experiments. Dunnett's test, *P ≤ 0.05; ±SEM

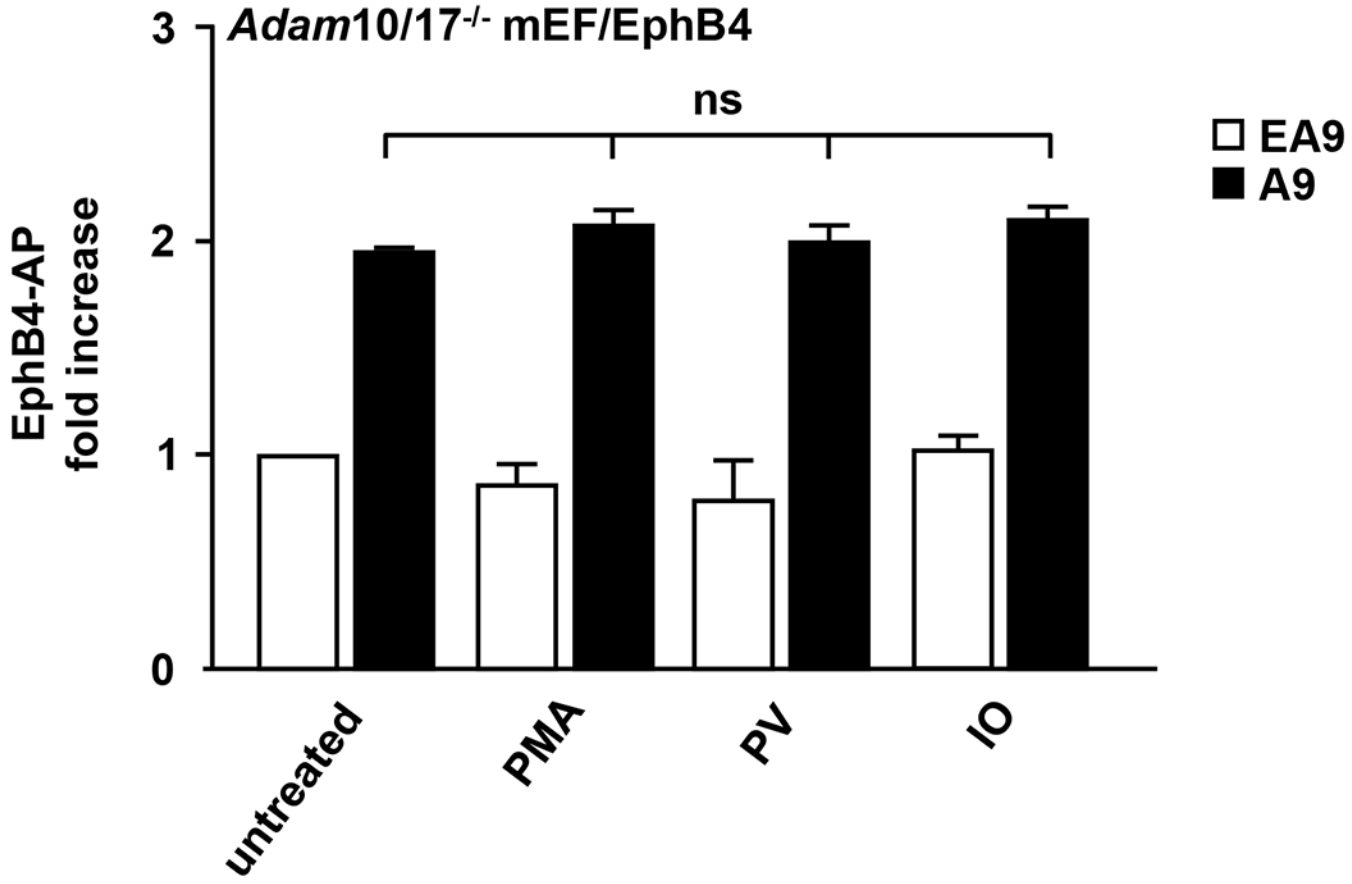

Figure 4. Evaluation of the response of ADAM9 to stimuli of ectodomain shedding in cell-based assays.

The effect of 20 ng/ml PMA, 100 μM of the phosphatase inhibitor pervanadate (PV), or 2.5 μM Ionomycin (IO) on ADAM9 (A9)-dependent shedding of EphB4-AP from *Adam10/17*−/− mEFs is shown after 30 minutes of stimulation. The numbers in each bar represent the percent shedding compared to cells expressing ADAM9 E>A (EA9), where shedding in the absence of treatment was used as a reference, and set to 1. Treatment with these compounds had no effect on EphB4-AP shedding in the presence or absence of active A9. These results are representative of at least 3 separate experiments. Dunnett's test, ns = not significant; ±SEM

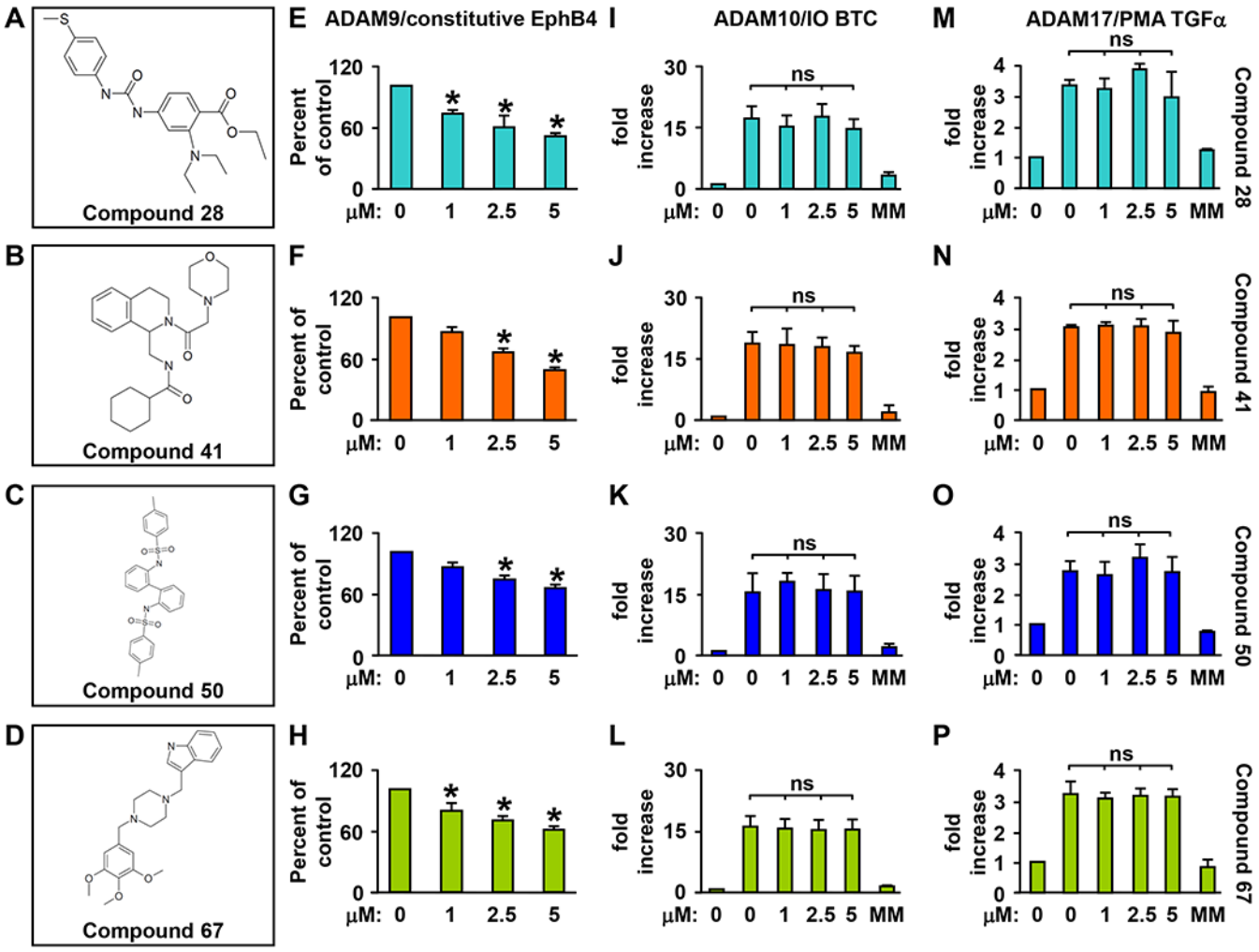

Figure 5. Identification of four ADAM9 inhibitors based on a screen of a small molecule library.

The structures of compounds 28, 41, 50, and 67 are shown in A-D. The effect of different compound concentrations on the increase in EphB4 shedding in *Adam10/17*−/− mEFs overexpressing ADAM9 compared to cells expressing ADAM9 E>A after 2 hours is shown for compound 28 (E), compound 41 (F), compound 50 (G), and compound 67 (H). In wild type mEFs, none of the inhibitors had an effect on the IO-stimulated shedding of BTC-AP (2.5 μM, 30 minutes), which depends on ADAM10 (I-L) or the PMA-stimulated shedding of TGFα–AP (20 ng/ml, 30 minutes), which depends on ADAM17 (M-P) respectively. MM, marimastat (5 μM). Dunnett's test, *P ≤ 0.05; ±SEM

Table 1

ADAM9-mediated shedding events

Substrate name	other proteases	References
ADAM10	ADAM15	27
Amyloid-Precursor-Protein (APP)	ADAMs10, 17	26, 28
Angiotensin-I converting enzyme (ACE)	-	29, 30
CD40	ADAM17	23, 31
Collagen XVII/BP180	ADAM10	32
Ephrin receptor B4 (EphB4)	ADAM17	23, 33
Epidermal-Growth-Factor (EGF)	ADAM10	9, 20
Fibroblast-Growth-Factor 2iiib (FGFR)	ADAMs10, 15	20, 34
Fibronectin	ADAM8	35, 36
Gelatin	ADAM15	36, 37
Heparin-Binding EGF-Like-Growth Factor (HB-EGF)	ADAMs12, 17	9, 38-40
Neuregulin 1β (NRG)	ADAM17	41, 42
TIE2	ADAM17	23, 42
Tumor Necrosis Factor a (TNFα)	ADAM17	25, 43
p75 neutrophin receptor (p75NTR)	ADAM17	26, 44
Vascular-Endothelial-Cadherin (VE-CAD)	ADAMs10, 17	23, 45, 33
Vascular-Endothelial-Growth-Factor-Receptor −2 (FLK1)	ADAM17	23, 42
Vascular-Cell-Adhesion-Molecule 1 (VCAM1)	ADAM17	23, 33

Table 2

Potency of metalloproteinase inhibitors (IC50/inhibition constants) for inhibition of ADAMs9, 10, 12, 15, and 17 in cell-based assays

Compound	ADAM9	ADAM10	ADAM12	ADAM15	ADAM17	References
GM6001	1.0 nM */0.72 μM	8.1-110 nM *	>10 μM */5.93 μM	0.24 μM	1.3-7.5 nM *	33, 77, 78
TAPI-2	13.52 μM	$3x10^3$ nM *	>100 μM *	8.17 μM	120 nM */<0.1μM *	33, 78, 79
Batimastat/BB94	0.05 μM	~0.2 μM	NS	NS	0.019 μM	80, 81
Marimastat	0.13 μM	78 nM *	NS	0.17 μM	12 nM *	33, 82
GI254023X	ND	5.3 nM *	NS	NS	541.0 nM *	64
GW280264X	0.48 μM	11.5 nM *	NS	NS	8.0 nM *	64
TIMP1	ND	<1 nM	NS	ND	ND	33, 54, 83
TIMP2	ND	<1 nM	NS	>100 nM	ND	33, 54, 83
TIMP3	~10 nM	<1 nM	NS	~20 nM	~0.1-0.4 μM	33, 54, 83

Abbreviations: NS = not specified; ND = not detected;

* **= Inhibition of recombinant ADAMs in *In vitro* assays.** Please note that the IC50 of hydroxamic acid type metalloproteinase inhibitors for ADAMs is usually 10 – 1000-fold higher in cell based assays than in biochemical assays