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Targeting Zymogen Activation To Control the Matriptase-Prostasin Proteolytic Cascade

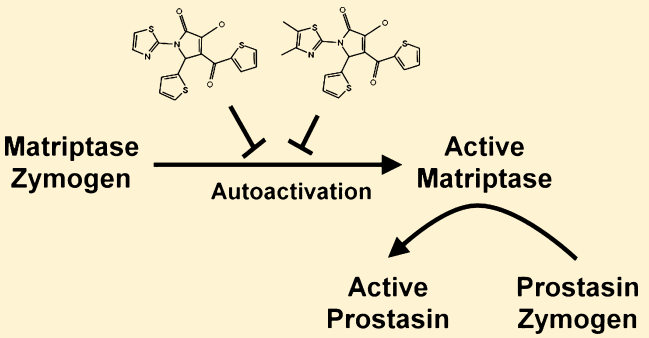
Zhenghong Xu,[†] Ya-Wen Chen,[†,‡] Aruna Battu,[†] Paul Wilder,[§] David Weber,[§] Wenbo Yu,[∥] Alexander D. MacKerell, Jr.,[∥] Li-Mei Chen,[⊥] Karl X. Chai,[⊥] Michael D. Johnson,[#] and Chen-Yong Lin*[,†,§]

[†]Greenebaum Cancer Center, [‡]Graduate Program in Life Science, [§]Department of Biochemistry and Molecular Biology, and [∥]Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201, United States

[⊥]Department of Molecular Biology and Microbiology, Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, Orlando, Florida 32816-2364, United States

[#]Lombardi Cancer Center, Georgetown University, Washington, DC, 20007, United States

ABSTRACT: Membrane-associated serine protease matriptase has been implicated in human diseases and might be a drug target. In the present study, a novel class of matriptase inhibitors targeting zymogen activation is developed by a combination of the screening of compound library using a cell-based matriptase activation assay and a computer-aided search of commercially available analogues of a selected compound. Four structurally related compounds are identified that can inhibit matriptase activation with IC_{50} at low micromolar concentration in both intact-cell and cell-free systems, suggesting that these inhibitors target the matriptase autoactivation machinery rather than the intracellular signaling pathways. These activation inhibitors can also inhibit prostasin activation, a downstream event that occurs in lockstep with matriptase activation. In contrast, the matriptase catalytic inhibitor CVS-3983 at a concentration 300-fold higher than its K_i fails to inhibit activation of either protease. Our results suggest that inhibiting matriptase activation is an efficient way to control matriptase function.



INTRODUCTION

Inappropriate proteolysis has been found to be a critical component of various pathological processes, such as carcinogenesis, metastasis, inflammation, hypertension, skin disease, and osteoarthritis. Proteolytic activities are, therefore, considered to be promising targets for drug development.[1] Matriptase, a type II transmembrane serine protease,[2] has been implicated in many disease processes, and this enzyme may be an attractive drug target for treating various human diseases. Increased matriptase expression and an imbalance between matriptase and its endogenous inhibitor hepatocyte growth factor activator inhibitor 1 (HAI-1), a Kunitz type serine protease inhibitor, are commonly observed in a wide variety of primary human carcinomas.[3,4] In some cases, this dysregulation is associated with poor patient outcome.[5−7] Evidence for the oncogenic potential and a pro-metastatic role of matriptase has been provided by studies using animal models, including matriptase transgenic mice and tumor xenograft studies in nude mice.[8,9] Increased matriptase zymogen activation has also been seen in several distinct human skin diseases, and keratinocytes exhibiting increased matriptase activation have been observed in close proximity to areas of inflammation.[10] In addition to expression in epithelial and carcinoma cells, matriptase is also expressed by chondrocytes,[11] monocytes,[12−14] and mast cells.[15] Matriptase activity in chondrocytes may play an important role in the degradation of cartilage matrix and contribute to osteoarthritis.[11] In atherosclerotic lesions, monocytic matriptase may stimulate endothelial cells to release pro-inflammatory cytokines and thereby contribute to the disease.[14] Expression of matriptase in mast cells that play important roles in allergy-related diseases, such as asthma, suggests that the membrane protease may have the potential to contribute to these diseases as well.[15]

Traditionally, strategies to control protease activity have been targeted directly against the proteolytic mechanism of the enzymes using synthetic inhibitors. Several catalytic inhibitors of matriptase have been developed, including small molecule and peptide-based inhibitors, which exhibit great potency against matriptase in in vitro assays that, in most cases, have utilized a recombinant serine protease domain of matriptase.[16−21] The specificity of these inhibitors has generally been investigated by testing them against a relatively small number of commonly available serine proteases. Targeting matriptase activity through the use of catalytic inhibitors presents several challenges, some of which relate to this approach in general and some of which derive from the unique dynamics of the matriptase activation system. There are a great number of

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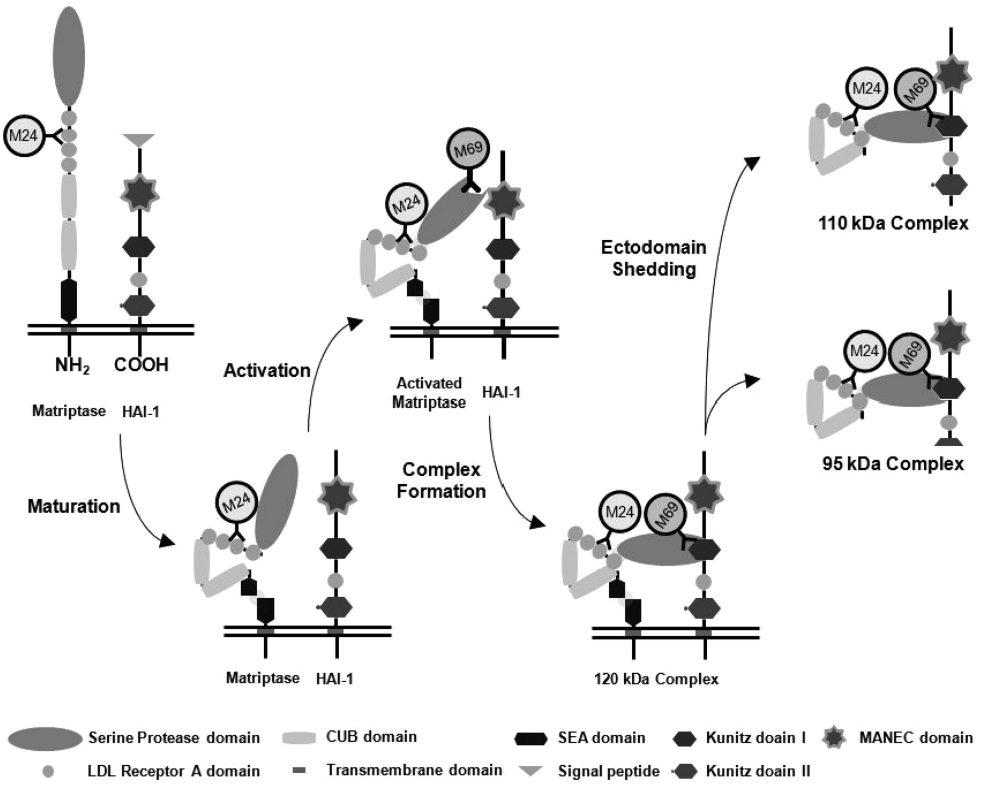

Figure 1. Matriptase−HAI-1 life cycle and the specificity of the various matriptase mAbs. Both matriptase and HAI-1 are membrane-bound proteins with multiple distinct domains and modules, as indicated. Matriptase is synthesized as a full length zymogen, which is rapidly converted into an activation-competent, mature form through a cleavage within the SEA domain (N-terminal process).[26,49] The signal peptide of HAI-1 is removed during the maturation process. Through autoactivation, latent matriptase is converted into active matriptase with full proteolytic activity. The active matriptase is rapidly inactivated by binding to its endogenous inhibitor HAI-1. Matriptase−HAI-1 complexes then are shed as complexes of two different sizes, depending on where the HAI-1 is cleaved. The mAb M24 recognizes a noncatalytic domain of matriptase, although the exact location of the epitope has not been determined. This matriptase mAb can detect both latent and activated forms of matriptase, as indicated. The mAb M69 recognizes an epitope on the serine protease domain that is only present in activated matriptase. This mAb is, therefore, able to distinguish activated matriptase from latent matriptase.

serine proteases, and there is significant structural homology between the catalytic domains of these enzymes, many of which exhibit overlapping specificity. This makes the task of designing truly specific catalytic inhibitors very difficult and the goal of demonstrating that they are indeed specific almost impossible. The unusually tight control of the cellular matriptase proteolytic activity presents additional challenges. Matriptase, like most serine proteases, is synthesized as a zymogen and acquires its full proteolytic activity only after undergoing zymogen activation through a cleavage of the enzyme at the canonical activation motif.[22−25] Instead of relying on other active proteases for the activating cleavage, as occurs during the activation of most serine proteases, matriptase undergoes autoactivation.[26] This autoactivation process requires interactions between the matriptase zymogen molecules and the HAI-1 and probably involves other proteins yet to be identified. The zymogen form of matriptase possesses unusually high intrinsic activity, which is characterized by a maximal activity at pH 6.0, and the inhibition by increased concentrations of sodium chloride.[27] Both of these biochemical features mirror the key features of the induction of matriptase activation in cells, suggesting that this intrinsic activity is responsible for matriptase zymogen activation.[28] The endogenous matriptase inhibitor HAI-1 is involved in matriptase autoactivation and has direct access to the nascent active matriptase.[29] The tight coupling of matriptase zymogen activation with HAI-1-mediated inhibition means that the uncomplexed, free active matriptase is an extremely short-lived species.[30] The events associated with matriptase processing, activation, and inhibition are summarized in Figure 1. Interestingly, even with such rapid inhibition, matriptase is still able to activate its physiological substrate, prostasin, a glycosylphosphatidylinositol (GPI)-anchored serine protease.[10] It seems that the activation of matriptase zymogen, the activation of prostasin by active matriptase, and the inhibition of active matriptase by HAI-1 take place at essentially the same time.[10] As a result, the scarcity of free active matriptase as a target for catalytic inhibitors represents a major challenge that will limit the potential utility of matriptase catalytic inhibitors as a means to control matriptase function. The catalytic inhibitors may not find the desired target, the scarce active matriptase, and significant dose escalation may be required to produce an effect, which might produce off-target effects on other serine proteases. For example, the matriptase inhibitor CJ-730 was tested for its ability to inhibit cellular activation of pro-hepatocyte growth factor (HGF), a well-known matriptase substrate.[31,32] In spite of the high potency of this inhibitor, which has a K_i of 40 nM against matriptase,[19] inhibition of pro-HGF activation required an inhibitor concentration of 50 μM, more than 1000-fold higher than the K_i.[33] Likewise, in the case of a matriptase inhibitory antibody, which has a K_i of 15 pM against matriptase, more than 10000-fold of the K_i (200 nM) was used to inhibit

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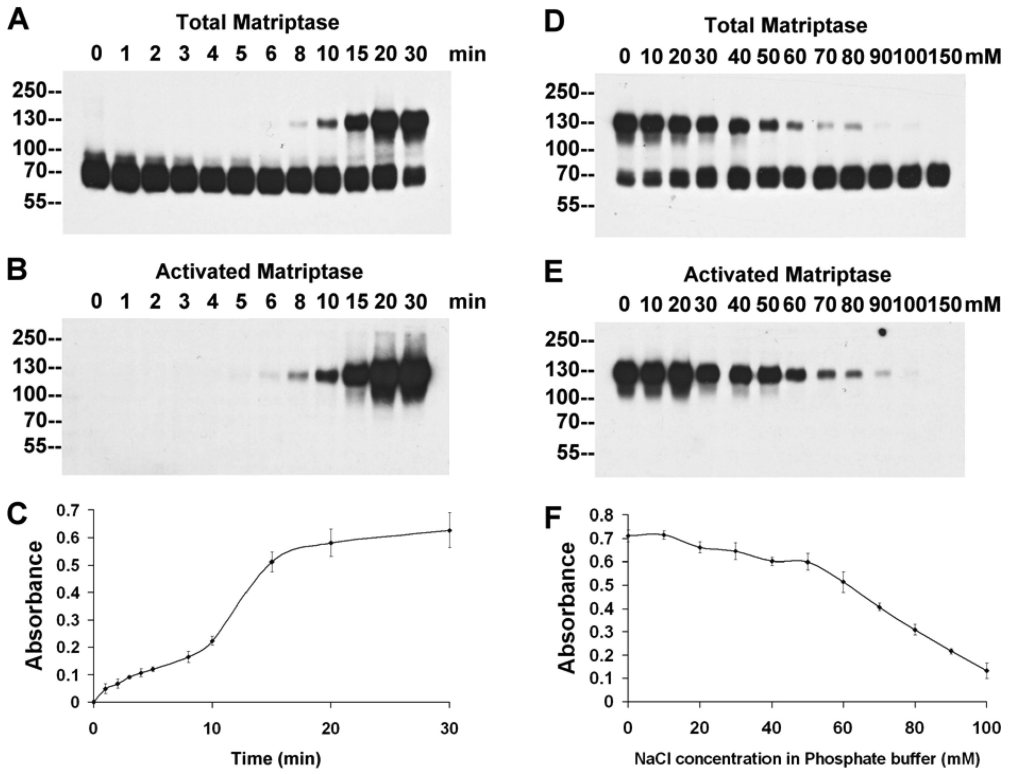

Figure 2. Development of the cell-based, ELISA-like assay for matriptase activation. The kinetics of matriptase activation: Mammary epithelial 184 A1N4 cells were incubated with a pH 6.0 buffer at room temperature for the indicated times, and the kinetics of the acid-induced matriptase activation were analyzed by immunoblotting with the mAb M24 (against total matriptase, A) and the mAb M69 (against activated matriptase, B) and using the cell-based, ELISA-like assay with the mAb M69 (C). The dose-dependent inhibition of matriptase activation by NaCl: 184 A1N4 cells were incubated with pH 6.0 buffers containing the indicated concentrations of NaCl at room temperature for 20 min, and matriptase activation was analyzed by immunoblotting with the mAb M24 (against total matriptase, D) and the mAb M69 (against activated matriptase, E) and using the cell-based, ELISA-like assay with the mAb M69 (F).

the P1-Arg proteolytic activity on the cell surface associated with several matriptase-expressing cancer cells.[34] Furthermore, the inhibition of active matriptase by catalytic inhibitors may be too late to effectively suppress matriptase's biological action, since much of the function of active matriptase, such as the activation of prostasin, occurs at essentially the same time as the generation of active matriptase and its inhibition through binding to HAI-1.

In light of the apparent disadvantages of matriptase catalytic inhibitors, which have to not only compete with matriptase substrates but also with the endogenous matriptase inhibitors, such as HAI-1, which is present at high concentrations in close proximity to matriptase and which has a Ki for active matriptase as good as, if not better, than the synthetic inhibitors,[35] we intend to develop a novel strategy to control matriptase activity with the goal of translating it into effective interventions in human diseases in the future. In the present study, a novel scheme, consisting of a cell-based, enzyme-linked immunosorbent assay (ELISA)-like screening assay and a computational search approach, was established to identify novel small molecule inhibitors of matriptase, targeting its zymogen activation rather than the catalytic activity. Four lead compounds were identified and validated to target the activation mechanism directly, rather than the intracellular signaling that triggers activation. These matriptase activation inhibitors effectively inhibit not only matriptase activation but also matriptase-mediated prostasin activation. In contrast, the matriptase catalytic inhibitor CVS-3983[17] inhibits neither event. These data support our hypothesis that inhibition of matriptase activation would be a more efficient approach to suppress the actions of matriptase than directly inhibiting matriptase catalytic activity. Our compounds may have potential for development into drugs for the treatment of human diseases associated with dysregulated matriptase.

RESULTS

Cell-Based, ELISA-Like Matriptase Activation Assay.

To identify small molecule inhibitors that can suppress matriptase zymogen activation, a cell-based, ELISA-like matriptase activation assay was developed. This activation assay combines a system in which robust matriptase zymogen activation is induced in whole cells by exposing them to a pH 6.0 buffer followed by formalin fixation, with a highly specific and sensitive method for detecting the level of activated matriptase, based on the ability of a unique matriptase monoclonal antibody M69 that specifically recognizes activated matriptase, but not latent matriptase. The optimal conditions for the whole-cell activation assay were first assessed using immunoblot assays. As shown in Figure 2, prior to induction of matriptase zymogen activation in the 184 A1N4 cells, matriptase is detected as a 70 kDa latent form in immunoblots of cell lysates probed with the total matriptase monoclonal antibody (mAb) (Figure 2A, 0 min), and the cells are devoid of the activated form of matriptase, as no protein band is detected by the activated matriptase-specific mAb M69 (Figure 2B, 0 min). When the cells are exposed to a pH 6.0 buffer and

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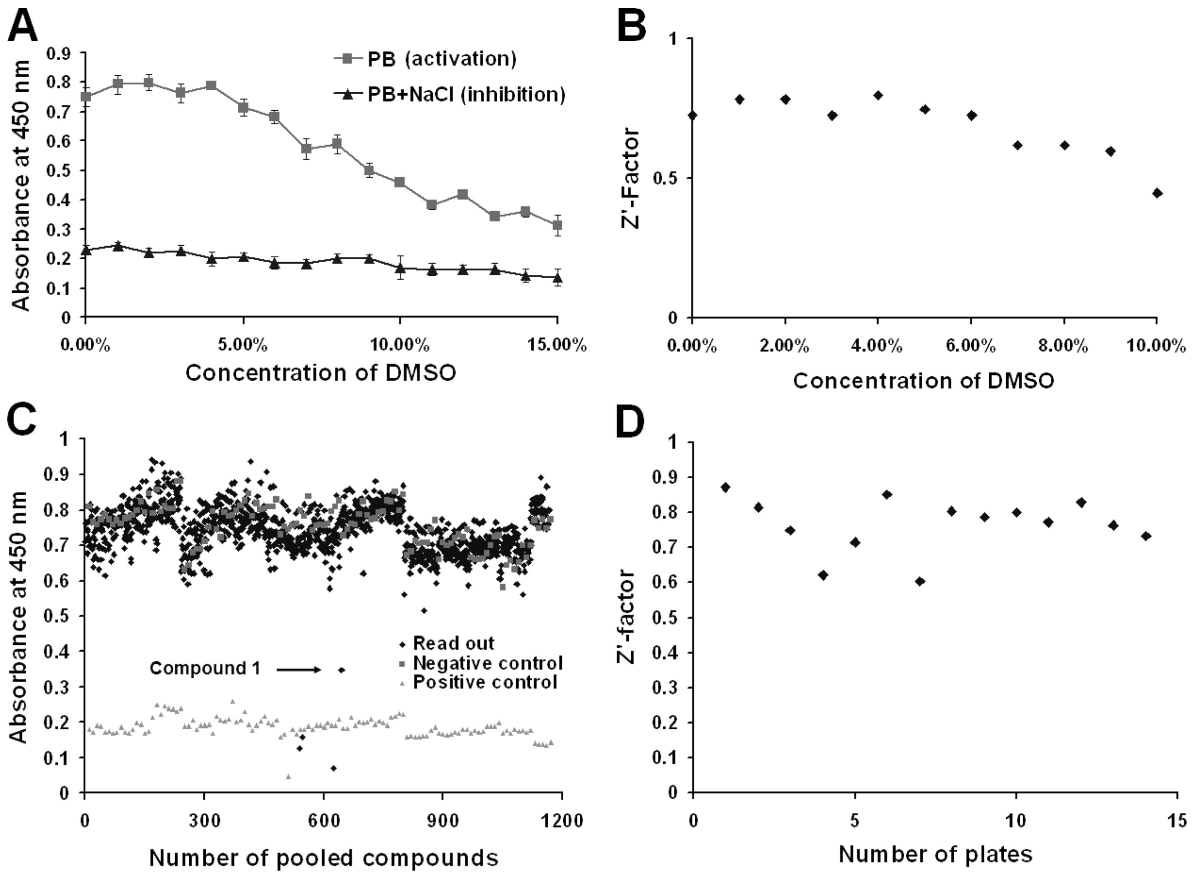

Figure 3. Screening for small molecule inhibitors of matriptase activation. The effect of DMSO on the ELISA-like assay: 184 A1N4 cells were seeded in 96-well plates, and matriptase activation was induced by exposure to phosphate buffer (PB), pH 6.0, containing increasing concentrations of DMSO, in the presence or absence of 150 mM NaCl. The levels of activated matriptase were determined by the ELISA-like assay (A). The *Z*′ factors were also calculated as described in the Experimental Procedures and plotted with their corresponding concentrations of DMSO (B). Screening of the compound libraries. Chemical libraries were screened using the ELISA-like assay for inhibitors of matriptase activation, and the readouts of each test sample from a random selection of 14 plates of the 96-well are displayed (C). The pH 6.0 buffer alone was used as negative inhibition control, while the buffer containing 150 mM NaCl was used as a positive control for inhibition. One lead compound **1** was identified as indicated. *Z*′ factors of these selected plates were calculated to evaluate for the quality of the screening (D).

incubated at room temperature, matriptase activation is rapidly initiated within few minutes, and 70 kDa latent matriptase is rapidly converted into 120 kDa activated matriptase−HAI-1 complexes, in a time-dependent manner (Figure 2A,B). The appearance of the 120 kDa complexes and disappearance of the 70 kDa matriptase can be detected by the total matriptase mAb (Figure 2A). The activation and the appearance of 120 kDa matriptase−HAI-1 complex can also be detected by the activated matriptase mAb M69 (Figure 2B). After 20 min, a large portion of the latent matriptase had been converted into activated matriptase, detected in the 120 kDa complexes. The magnitude and the rapidness of matriptase activation observed in these immunoassays ensure the likelihood of being able to specifically determining the matriptase activation event in a 96-well plate assay with a high signal-to-noise ratio.

We then set out to convert the immunoblot-based activation assay into an ELISA-like assay, which can be further configured into a high-throughput screening format. The assay capitalizes on the ability of the mAb M69 to specifically detect activated matriptase in formalin-fixed cells. This allows us to build an assay that does not require cell lysis, with all of the technical challenges that this process would present for the development of a high-throughput assay. The cells were grown in 96-well plates, and following the induction of matriptase activation by exposure to the pH 6.0 buffer, the cells were washed and fixed using formaldehyde. The level of activated matriptase was determined using the M69 mAb as the primary antibody, HRP-labeled antimouse IgG as the secondary, and the chromogenic peroxidase substrate 3,3′,5,5′-tetramethyl benzidine (TMB). Cellular levels of matriptase activation are reported as the absorbance at 450 nm. Quantitation of acid-induced matriptase activation using this ELISA-like assay exhibits the same kinetic patterns as demonstrated in the Western blot-based assays (Figure 2B,C), indicating the successful adaptation of the assay system.

One of the unique features of the acid-induced matriptase activation is its sensitivity to sodium chloride.[28] The NaCl-mediated inhibition of acid-induced matriptase activation was initially observed in a cell-free, in vitro activation system and may be due to the suppression of the intrinsic matriptase zymogen activity.[27] Results from experiments performed with immunoblots showed that the NaCl-mediated inhibition of matriptase activation also occurred in the whole living cells activation system and that the inhibition occurred in a dose-dependent manner (Figure 2D,E). Using the ELISA-like assay, the NaCl-mediated inhibition showed the same dose-dependent profile as seen with the immunoblot analysis (Figure 2F).

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Sodium chloride (150 mM) was, therefore, used as the positive control for inhibition in our screening.

Characterization of the ELISA-Like Assay. The successful development of the cell-based, ELISA-like assay enables us to conduct screenings for small molecule inhibitors of matriptase activation using a random compound library (ChemDiv, San Diego, CA). Because the compounds in the library were dissolved in dimethyl sulfoxide (DMSO), the effects of DMSO on the assay and matriptase activation were determined by incorporating 0−15% DMSO into the activation induction buffer. The impact of DMSO on matriptase activation was evaluated using Z' factor as an indicator. As shown in Figure 3A,B, DMSO had no significant influence on the detection of either the matriptase activation or the sodium chloride-mediated inhibition when present at concentrations below 4%, and the Z' factors were all above 0.5, indicating an excellent high-throughput screening capability in this range.[37]

Screening and Computational Search for Inhibitors of Matriptase Activation. A screen for small molecule inhibitor of matriptase activation was then performed using the cell-based, ELISA-like assay described above. To increase the efficiency of screening, four different compounds were pooled as a testing sample, with an approximate final concentration for each compound in the assay of about 5 μM in 1% DMSO. A total of 20000 compounds from the ChemDiv 40000 library were tested, and a selection of the readouts of these assays and the negative and positive controls are presented in Figure 3C. The 450 nm absorbance readouts for the negative controls fluctuated between 0.6 and 0.9 absorbance units, while that of the positive controls with 0.15 M NaCl were approximately 0.2 (Figure 3A). The vast majority of the test compounds showed no inhibition of matriptase activation and yielded readouts similar to that of the negative controls. Several compounds that caused the detachment of the cells from the plates gave false positive readouts giving absorbance values even lower than the positive controls. The Z' factors of randomly selected plates were calculated based on the positive and negative controls and all were above 0.6, further confirming the quality of this high-throughput screening assay (Figure 3D). One pooled compound mixture with inhibitory activity of matriptase activation was identified (Figure 3C) and after testing the individual compounds making up the pool, the active compound 3-hydroxy-1-(thiazol-2-yl)-5-(thiophen-2-yl)-4-(thiophene-2-carbonyl)-1*H*-pyrrol-2(5*H*)-one (A2844/119997) was identified.

To identify additional matriptase activation inhibitors, a chemical fingerprint-based similarity search was performed against the structure of the active compound **1** (A2844/119997). One hundred forty compounds with the structures similar to **1** were identified based on a Tanimoto index of 0.86 or greater in combination with the BIT-MACCS fingerprints.[38,39] Of these compounds, 61 were purchased based on the availability and tested in a follow-up screen. Three more active compounds were identified, two of which exhibit improved inhibitory potency when compared with **1**. The IC_{50} values of these four compounds were determined using the ELISA-like assay to be 2.6 μM for 1-(4,5-dimethylthiazol-2-yl)-3-hydroxy-5-(thiophen-2-yl)-4-(thiophene-2-carbonyl)-1*H*-pyrrol-2(5*H*)-one (F3226-1198), 7.0 μM for 1-(4,5-dimethylthiazol-2-yl)-3-hydroxy-5-phenyl-4-(thiophene-2-carbonyl)-1*H*-pyrrol-2(5*H*)-one (F3226-1197), 9.8 μM for **1**, and 21.8 μM for 4-benzoyl-1-(4,5-dimethylthiazol-2-yl)-3-hydroxy-5-(thiophen-2-yl)-1*H*-pyrrol-2(5*H*)-one (STOCK3S-92907) (Figure 4).

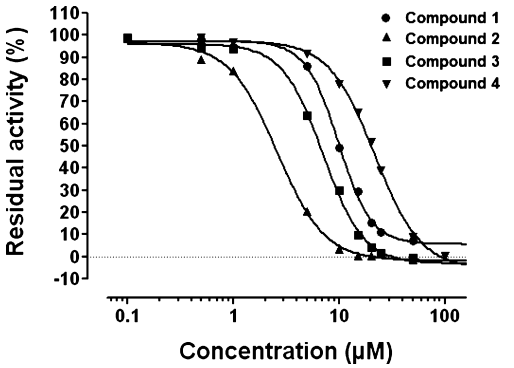

Figure 4. Determination of the IC_{50} for the lead compounds. 184 A1N4 cells were seeded in 96-well plates and treated with varying concentrations of the four matriptase activation inhibitors. The levels of matriptase activation were determined by the ELISA-like assay. The inhibition of matriptase activation by these inhibitors is presented as the residual activity vs their concentrations, and IC_{50} values were calculated using Graphpad Prism 5 (Graphpad Software Inc.).

As shown in Figure 5, these four compounds share the same 3-hydroxy-1*H*-pyrrol-2(5*H*)-one scaffold. Notably, modifications on the substituents on this scaffold affect their ability to inhibit matriptase activation. The initial hit compound **1** with 1-thiazol, 4-thiophen, and 5-thiophen substituents exhibited a micromolar inhibition. Introduction of two methyl groups onto the 1-thiazol moiety [compound **2** (F3226-1198)] improves the inhibitory capacity; alternatively increasing the size of the 1-position substituent diminished the potency. On the basis of the structure of compound **2**, replacing either of the 4- or the 5-thiophen substituents with a benzene group, yielding compounds **3** (F3226-1197) and **4** (STOCK3S-92907), respectively, affected the inhibitory activity. Compounds with a benzene group on both of the 4- and the 5-position or with even larger substituents on either position totally lost their inhibitory potency at the tested concentration. These results suggest that both the size and the polarity of the 1-, 4-, and 5-substituents are crucial toward optimizing the inhibitory effect of the compounds.

Validation of the Active Compounds. Although the amount of activated matriptase detected by the M69 mAb in the ELISA-like assay is a good indication for the levels of activation, a decrease in the readout, independent of the effects on matriptase activation, might result from the loss of total matriptase due to enhanced shedding. One way to control for this potential confounding effect is to assess the total matriptase, which easily can be achieved using immunoblot assays. All of the compounds that scored positively for the suppression of matriptase activation in the ELISA-like screens were, therefore, validated using immunoblot assays. As shown in Figure 6A, prior to the induction of activation, all of the matriptase is in the 70 kDa latent form (Figure 6A, total matriptase, lane 1). After the induction of matriptase activation but in the absence of the compound **2**, the majority of matriptase was detected in the 120 kDa complex (Figure 6A, total matriptase, lane 2). With increasing concentrations of the compound, the levels of 120 kDa matriptase−HAI-1 complex gradually decreased (Figure 6, total matriptase, lanes 3−6), and the levels of 70 kDa latent matriptase increased. In the presence of 5 μM compound **2**, there was no detectable 120 kDa

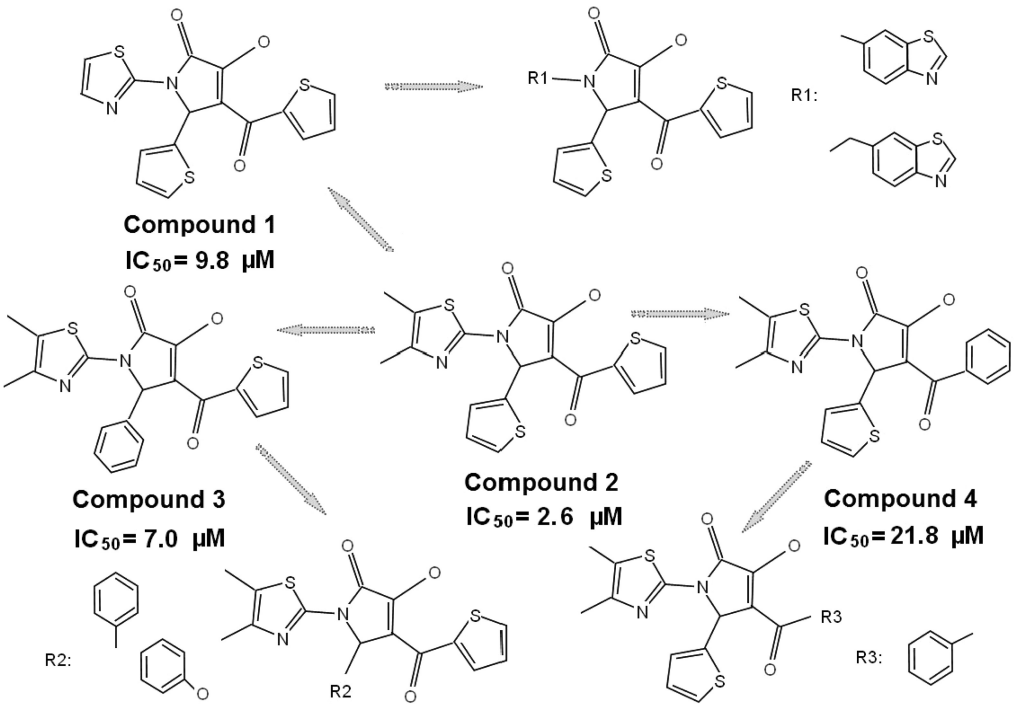

Figure 5. Structural and functional relationships among the matriptase activation inhibitors. The chemical structures of the four matriptase activation inhibitors are presented and compared in relation to their IC_{50} values. Some of the analogues that showed no inhibition of matriptase activation (without measurable IC_{50} values) are also presented and compared to show the negative impact of the modifications in the surrounding rings (R1, R2, and R3) on the inhibitory activity. The arrows indicate a decreasing in inhibition potency with the structure changes.

complex (Figure 6A, total matriptase, lane 6), and the level of 70 kDa latent matriptase became comparable to the control (Figure 6A, total matriptase, lane 1). The same pattern of inhibition was observed with increasing concentrations of compounds **3** (Figure 6B), **1**, and **4** (data not shown). The inhibition of matriptase activation by these compounds was also confirmed by the reduced signal from the mAb M69 specific for activated matriptase (Figure 6, lower panels). These data confirmed that all four active compounds are inhibitors of matriptase activation.

Mechanistic Study. As described above, the inhibitory effect of the lead compounds on the acid-induced matriptase activation was identified and validated using intact cells. A question remains as to whether the inhibition results from a direct action against the matriptase activation process or is mediated indirectly through the blocking of some intracellular signal pathways. While it is very likely that the activation of matriptase caused by mild acid exposure bypasses the intracellular signal pathways and directly acts on the activation machinery through the significant enhancement of the intrinsic catalytic activity of the matriptase zymogen under acidic conditions, the possibility remains that the compounds may suppress signal pathways involved in the control of matriptase activation. We previously demonstrated that the acid-induced matriptase activation also occurs in a cell-free setting, in which the insoluble fractions of the cell homogenates are used in the absence of soluble cytosolic factors.[28] In such a cell-free setting, any intracellular signaling pathways are likely either absent or incomplete. Thus, to determine whether the inhibitory effect of the active compounds was the result of directly targeting the activation machinery or involved in inhibiting the intracellular signaling pathways, the ability of the compounds to inhibit matriptase activation was tested using the cell-free system. The insoluble fractions of 184 A1N4 cell homogenates were preincubated with the compounds, and then, activation of matriptase was induced. Immunoblot analyses using the M24 and M69 mAbs show that these compounds can suppress matriptase activation in a dose-dependent manner (**2** and **3**, Figure 7; **1** and **4** not shown). In spite of the fact that the activation is less robust in the cell-free setting when compared with that in the intact-cell setting, the inhibitory efficiency achieved was almost the same in both systems (Figures 6 and 7), suggesting that all of the inhibitions are likely to be directly against the activation machinery, rather than signal pathways.

Inhibition of the Activation of a Matriptase Substrate. One of the most remarkable features of matriptase regulation is that the activation of the enzyme is rapidly followed by HAI-1-mediated inhibition. It is this rapid HAI-1-mediated inhibition of active matriptase that may reduce the effectiveness of the catalytic inhibitors for the control of matriptase function and that led to our attempts to develop the activation inhibitors. With the activation inhibitors available, we set out to test our hypothesis that inhibition of matriptase activation would prove to be a more effective means of controlling matriptase function than the catalytic inhibitors. In our previous study,[10] we demonstrated that even in the face of the rapid HAI-1-mediated inhibition of matriptase immediately following activation, activated matriptase is still able to activate a physiological substrate, prostasin, a GPI-anchored serine protease. When HaCaT human keratinocytes were exposed to a pH 6.0 buffer, activation of matriptase was induced, followed by the rapid HAI-1-mediated inhibition of active matriptase and the formation of 120 kDa matriptase−HAI-1 complexes, similar to what is seen with 184 A1N4 cells. The activation of matriptase and the appearance of 120 kDa matriptase−HAI-1 complex in the keratinocytes can be demonstrated using the

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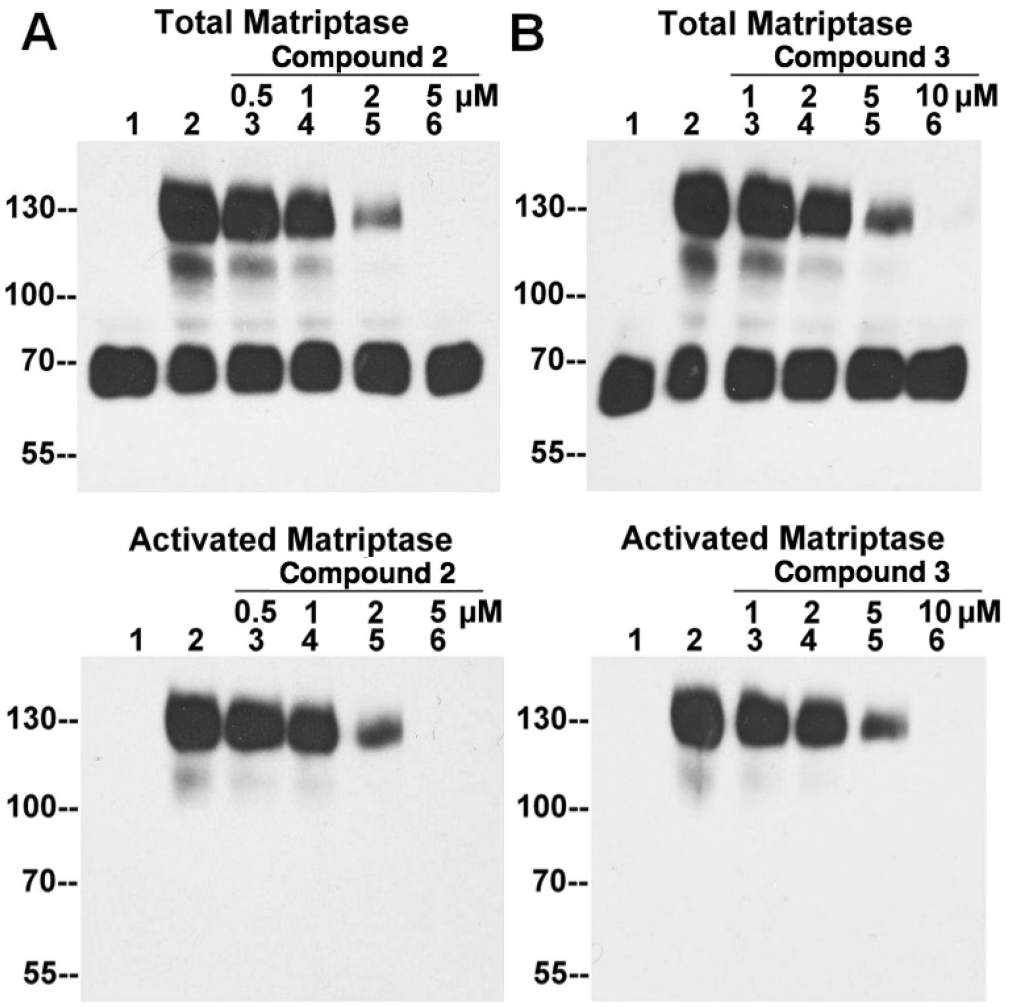

Figure 6. Validation of lead compounds **2** and **3** by immunoblot assay. 184 A1N4 cells were incubated with PBS (lanes 1, as a nonactivation control) or phosphate buffer, pH 6.0, alone (lanes 2, as an activation control), or phosphate buffer, pH 6.0, with the matriptase inhibitors **2** (A) and **3** (B) at the indicated concentrations (lanes 3–6) for 30 min at 4 °C, followed by the induction of matriptase activation at room temperature for 20 min. Cell lysates were subjected to immunoblot analyses for total matriptase with the mAb M24 and activated matriptase with the mAb M69.

total matriptase mAb, the activated matriptase mAb, and the HAI-1 mAb (Figure 8, comparing lanes 2 with lanes 1). Activation of prostasin also occurred during the course of matriptase activation and inhibition. Because HAI-1 also rapidly inhibits newly activated prostasin, as we have previously shown,[10] prostasin activation can be assessed by the appearance of the prostasin–HAI-1 complexes near the 100 kDa molecular mass marker using the HAI-1 mAb M19 (Figure 8, HAI-1, comparing lanes 2 with lanes 1) and a prostasin antibody (Figure 8, prostasin, comparing lanes 2 with lanes 1). The acid-induced activation of both matriptase and prostasin in the keratinocytes was clearly inhibited by the activation inhibitor **2** in a dose-dependent manner as shown in Figure 8A. In contrast, acid-induced activation of matriptase and prostasin was not inhibited by the matriptase catalytic inhibitor CVS-3983 even at concentrations as high as 1 μM, 60-fold higher than the IC_{50} (17 nM) or 300-fold higher than its K_i (3.3 nM)[17] against matriptase proteolytic activity (Figure 8B). The same pattern of inhibition was obtained using the compounds **3**, **1**, and **4** (data not shown). The inhibition of prostasin activation by the inhibitors of matriptase activation and the inactivity of the catalytic inhibitor suggest that matriptase activation would be a better target than its active site in controlling matriptase function.

DISCUSSION AND CONCLUSIONS

The proteolytic activity can be controlled by a variety of different strategies. The use of small molecule and peptide-based inhibitors directly targeting the catalytic site of a given protease has, for many years, been the mainstream strategy. Several matriptase inhibitors of such kind have been developed with excellent potency and good specificity. With an understanding of the complex regulatory mechanisms governing matriptase activation and inhibition, it has become clear that matriptase catalytic inhibitors may, however, not be efficient in the control of matriptase function. In the current study, a new strategy of targeting matriptase zymogen activation rather than its proteolytic activity to control matriptase function is established. This proof-of-concept study encompasses the development and optimization of an approach involving compound screening, computational search for the analogues of the screening hits, and the validation of the inhibitors identified using an alternative assay. This successful scheme led to the identification and validation of four inhibitors of matriptase activation with the same backbone structure. This

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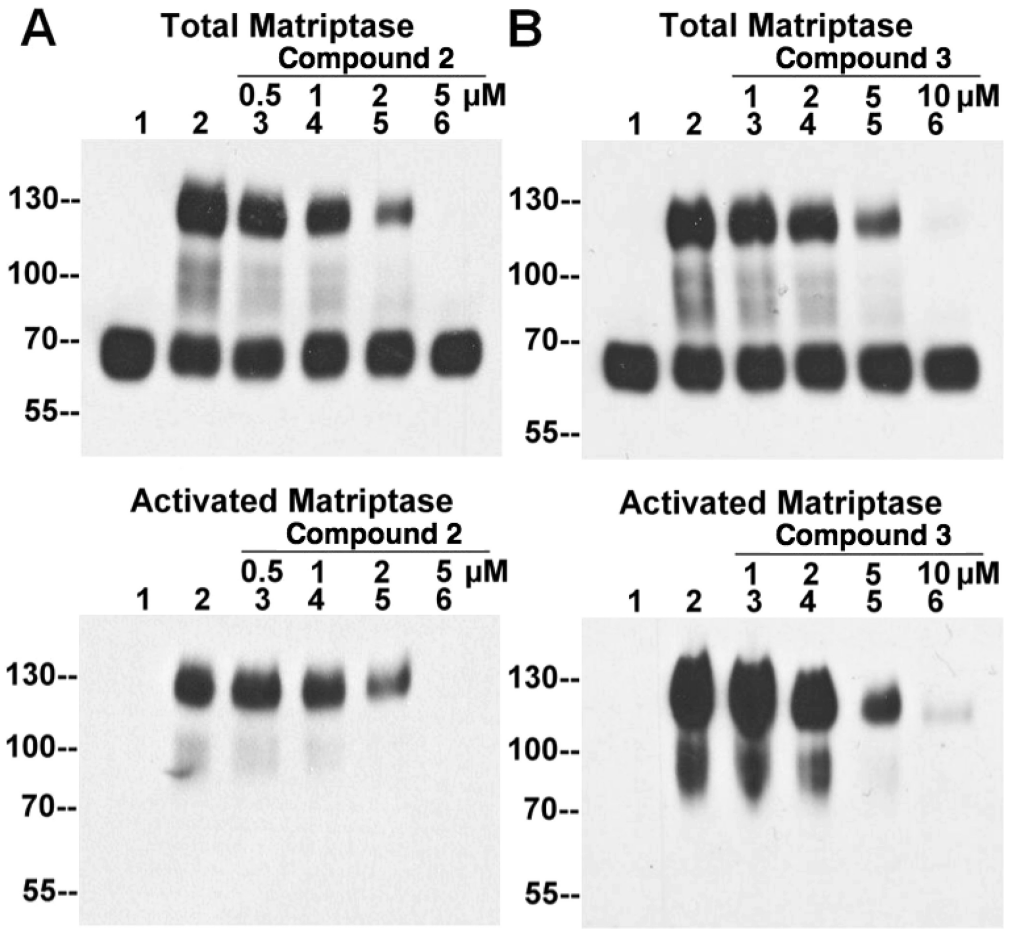

Figure 7. Inhibition of matriptase activation by the inhibitors using an in vitro activation assay. 184 A1N4 cells were homogenized in PBS, and the insoluble fractions were collected by centrifugation. The insoluble fractions were incubated with PBS (lanes 1, as nonactivation control) or phosphate buffer (PB), pH 6.0, alone (lanes 2, as activation control), or PB, pH 6.0, with the compounds **2** (A) and **3** (B) at the indicated concentrations (lanes 3–6) for 30 min at 4 °C, followed by induction of matriptase activation at room temperature for 20 min. The insoluble fractions were lysed and subjected to immunoblot analyses for total matriptase using the mAb M24 and activated matriptase using the mAb M69.

novel class of matriptase inhibitor can successfully suppress not only the acid-induced matriptase activation but also the activation of the physiologically relevant downstream matriptase substrate prostasin, in an assays system using living cells under conditions wherein free active matriptase is a very short-lived species due to its rapid inhibition by HAI-1. These matriptase activation inhibitors appear to be much more efficient than matriptase catalytic inhibitors in terms of their ability to suppress matriptase activation and prostasin activation by active matriptase.

The ability to efficiently control matriptase function has potentially important clinical implications, and although matriptase is broadly expressed by epithelial tissues throughout the body,[40] there is reason to believe that inhibiting the enzyme is feasible from a toxicity stand point. Individuals with inherited matriptase frame-shift mutations at the splice site appear to be healthy with clinical consequences mainly for the skin but that are not serious or life-threatening.[41] Blockage of matriptase would not, therefore, be expected to cause catastrophic mechanism-based toxicity. With respect to efficacy, because of the complexity of protease signaling pathways, blockade of a protease cascade at an early stage should be more efficient than blocking one of the later stages. Our previous discoveries have demonstrated that matriptase is at the pinnacle of protease cascades based on our observations that matriptase undergoes autoactivation in response to nonprotease stimuli[26,42] and that several downstream protease substrates have been identified, including uPA and prostasin.[10,31,43] Therefore, targeting matriptase would lead to more effective blockade of the entire protease cascade than could be achieved by targeting the individual downstream proteases and would likely require a lower dose of an inhibitor to achieve comparable effectiveness, which should reduce safety and toxicity concerns.

In general, proteases and their cognate inhibitors are typically expressed by different cell types, with the result that it is likely that active proteases will have enough time to act on their substrates prior to being inactivated by their inhibitors. For example, in colorectal and breast carcinomas, uPA is expressed by fibroblasts[44–46] and the uPA inhibitor PAI-1 is expressed by endothelial cells.[47] In contrast, the relationship between matriptase and its endogenous inhibitor HAI-1 is relatively unusual, since both matriptase and HAI-1 are found to be almost ubiquitously coexpressed and codistributed along the secretory pathway in many cells.[48] Furthermore, previous studies have indicated that HAI-1 plays important roles in the regulation of matriptase at multiple levels. The interaction between matriptase and HAI-1 is crucial for matriptase expression and intracellular trafficking.[29] HAI-1 is also involved in matriptase zymogen activation[26] and is responsible for the rapid inhibition of both matriptase and its substrate prostasin.[10]

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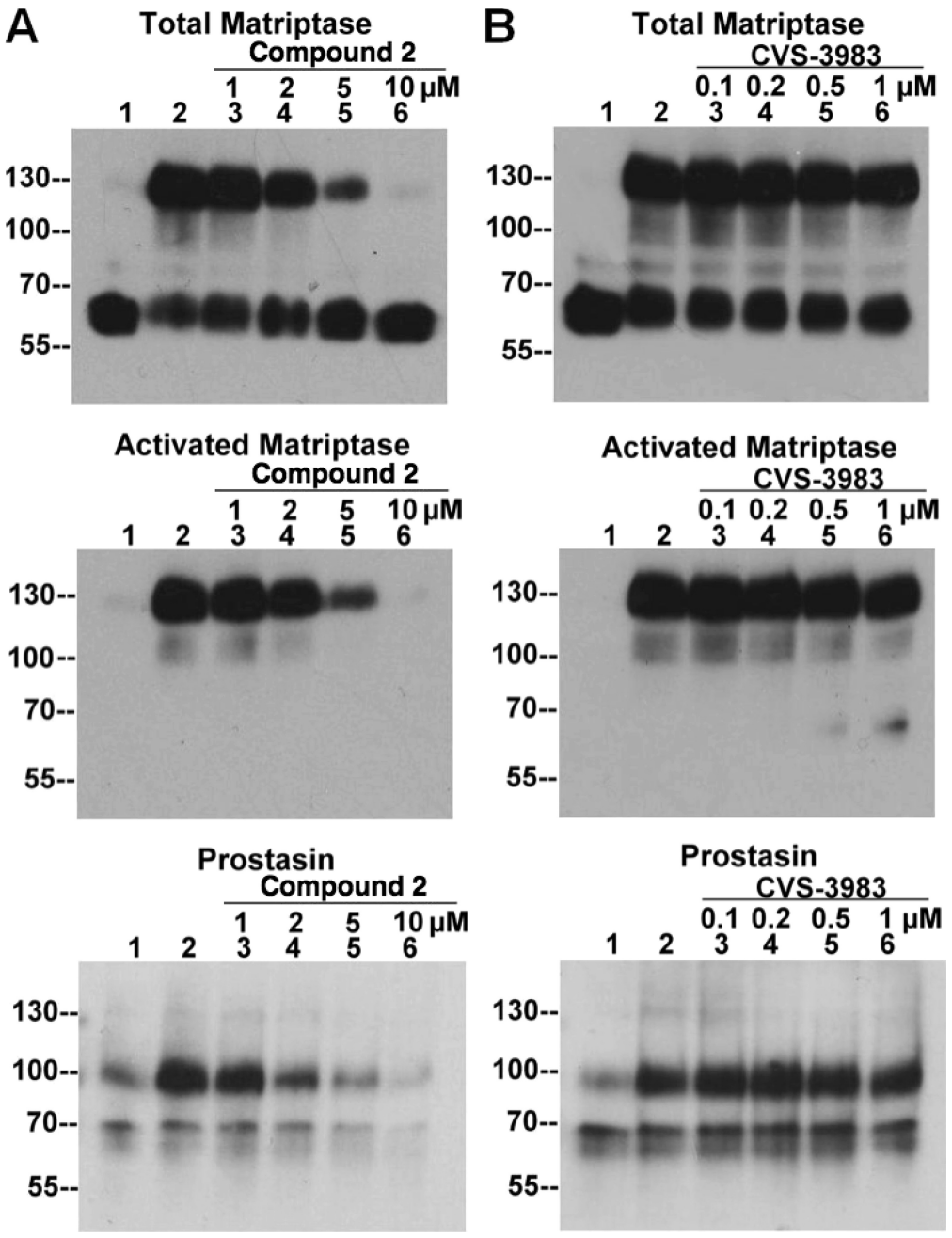

Figure 8. Inhibition of the matriptase−prostasin cascade by the matriptase activation inhibitors but not by the catalytic inhibitor CVS-3983. HaCaT cells were incubated with PBS (lanes 1, as the nonactivation control), or phosphate buffer (PB), pH 6.0, alone (lanes 2, as activation control), or PB, pH 6.0, with matriptase activation inhibitor compound **2** or catalytic inhibitor CVS-3983 at the indicated concentrations (lanes 3−6, μM) for 30 min at 4 °C followed by the induction of matriptase activation at room temperature for 20 min. Cell lysates were subjected to immunoblot analyses for total matriptase using the mAb M24, activated matriptase using the mAb M69, HAI-1 using the mAb M19, and prostasin with a prostasin polyclonal antibody.

This tight relationship among matriptase zymogen activation, prostasin activation, and their inhibition by HAI-1 represents a major challenge for the development of catalytic inhibitors that are able to effectively control matriptase function. This unique feature of matriptase regulation may also provide an explanation for the inefficiency of the catalytic inhibitor CVS-3983 in controlling matriptase function (Figure 8), as there is very limited free active matriptase or limited time for CVS-3983 to exhibit its inhibitory effect.

In summary, by directly targeting the matriptase zymogen activation machinery, a novel class of small molecule inhibitors was developed and validated in both cell-based and cell-free systems. The correlation between the structures and the activities of these compounds suggested that their inhibition likely results from specific interactions with the matriptase zymogen activation machinery. As compared with the catalytic inhibitor CVS-3983, these compounds showed profound inhibitory effects not only on matriptase activation but also on the matriptase-mediated activation of prostasin. These results demonstrate that these small molecule inhibitors may be more potent and specific than classic protease inhibitors in blocking both the matriptase activity and the downstream

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consequences of matriptase activation. Considering the important role that matriptase plays in human carcinomas, skin disorders, and other diseases, these inhibitors have the potential to serve as lead compounds of an important new class of therapeutic agents.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Chemical compounds were obtained from Ambinter (Paris, France). The selected compounds are ≥95% purity by HPLC analysis conducted by the Biopolymer/Genomics Core Facility, University of Maryland School of Medicine, with the help of Dr. Pat Campbell. Media, supplements, buffers, and general chemicals used in these experiments were purchased from Sigma, unless otherwise specified.

Cell Lines and Culture Conditions. The immortalized human mammary epithelial cells 184 A1N4 were cultured in a 50:50 mixture of Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM-F12 50/50, Mediatech Inc.) supplemented with 0.5% fetal bovine serum (FBS) (Gemini Bio-Products), 5 μg/mL recombinant human insulin (rh-insulin) (Invitrogen), 0.5 μg/mL hydrocortisone, 10 ng/mL recombinant human epidermal growth factor (rhEGF) (Promega), 100 U/mL penicillin, and 100 μg/mL streptomycin (1% pen-strep) (Mediatech Inc.). The immortalized human keratinocytes HaCaT were cultured in DMEM (Mediatech Inc.) supplemented with 10% FBS (Gemini Bio-Products) and 1% pen-strep (Mediatech Inc.).

Monoclonal Antibodies. Human matriptase was detected using the mAb M24, which recognizes both latent and activated matriptase. The epitope recognized by the M24 mAb likely lies in the noncatalytic domains of matriptase. The mouse mAb M69 recognizes an epitope present only in the activated form of matriptase and can specifically detect activated matriptase without cross-reaction with latent matriptase.[25] In Figure 1, we summarize the interactions of the matriptase antibodies with various matriptase species. Prostasin was detected using a polyclonal antibody recognizing both activated prostasin in complexes with HAI-1 and uncomplexed prostasin. Interestingly, this antibody appears to recognize the prostasin−HAI-1 complex much better than latent prostasin.[10]

Acid-Driven Matriptase Activation. Acid-induced matriptase activation using intact cells and cell-free extracts was carried out as described previously.[28,36] Briefly, cells or the insoluble fractions of cell homogenates were exposed to 150 mM sodium phosphate, pH 6.0, for 20 min at room temperature to induce matriptase activation. Phosphate-buffered saline (PBS) containing 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1% triton X-100 was then used to lyse the cells. For the inhibition tests, cells or the insoluble fractions of cell homogenates were pretreated with indicated compounds at 4 °C, and then subjected to acid-driven matriptase activation.

Immunoblotting. The protein concentration was determined using the Bradford protein assay reagents according to the manufacturer's protocol. Protein samples for Western blotting were diluted in 5× sample buffer. The sample buffer did not contain a reducing agent, and the samples were not boiled. Proteins were resolved by electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), transferred to nitrocellulose membranes (Pall Corp., Pensacola, FL), and probed with the antibodies described above. Binding of the primary antibody was detected with the use of horseradish peroxidase-conjugated secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and visualized using the Western Lightening Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA). All experiments were performed in triplicate.

Cell-Based, ELISA-Like Assay. The 184 A1N4 cells were seeded at 8×10^4 cells/well in 96-well plate 24 h prior to the treatment. After the induction of matriptase activation, the cells were washed with PBS and fixed with 4% formaldehyde in 0.1% Triton for 20 min. The cellular levels of activated matriptase were determined using mAb M69 as the primary antibody, HRP-labeled antimouse IgG as the secondary antibody, and the chromogenic peroxidase substrate TMB. Signals were measured as the absorbance at 450 nm. All experiments were performed in triplicate.

Screening of Compound Libraries. The compounds screened were obtained from the ChemDiv 40000 library, which contains 40000 diverse chemical compounds purchased from Chemical Diversity Laboratories (ChemDiv). The compounds in the library were selected from ChemDiv's ∼600000 compound collection to represent maximal chemical and structural diversity. Compound libraries were stored as 10 mM stock solutions in DMSO obtained from the High-Throughput Screening Center, University of Maryland at Baltimore. Mixtures of four individual compounds were used as single samples in the screening assay, and the final concentration of each compound was 5 μM in a buffer containing 1% DMSO and 150 mM sodium phosphate, pH 6.0. The pH 6.0 buffer alone was used as negative control for inhibition, while the pH 6.0 buffer with 150 mM NaCl was used as a positive control. Each 96-well working plate contained 80 wells for samples, eight wells for the positive control and eight wells for the negative control. Briefly, 184 A1N4 cells were seeded at 8×10^4 cells/well in 96-well plate 24 h prior to the treatment with the chemicals. The cells were incubated with control or compound-containing buffers at 4 °C for 30 min and then at room temperature for 20 min. The level of matriptase activation in each well was then determined as described above. The inhibition of matriptase activation caused by the compounds was calculated as a percentage of the absorbance in the test compound wells relative to that in the positive controls after subtracting the absorbance of the negative control wells. Compound mixtures showing greater than 50% inhibition were selected, and the individual compounds were then assessed independently using the same cell-based ELISA-like assay.

***Z′* Factor.** The *Z′* factor was calculated for assay optimization and quality assessment, which is defined by four parameters: the means and standard deviations of both the positive (p) and the negative (n) controls (μ_p, σ_p, μ_n, and σ_n). Given these values, the *Z′* factor was calculated as: $1 - 3(\sigma_p + \sigma_n)/|\mu_p - \mu_n|$.[37]

AUTHOR INFORMATION

Corresponding Author

*Tel: 410-706-3261. E-mail: cylin@som.umaryland.edu.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; HAI-1, hepatocyte growth factor activator inhibitor 1; HGF, hepatocyte growth factor; mAb, monoclonal antibody; PBS, phosphate-buffered saline; rhEGF, recombinant human epidermal growth factor; TMB, 3,3′,5,5′-tetramethyl benzidine

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