Published in final edited form as:

J Med Chem. 2015 August 13; 58(15): 5808–5824. doi:10.1021/acs.jmedchem.5b00354.

SAR Studies of Exosite-Binding Substrate-Selective Inhibitors of A Disintegrin And Metalloprotease 17 (ADAM17) and Application as Selective in Vitro Probes

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Abstract

ADAM17 is implicated in several debilitating diseases. However, drug discovery efforts targeting ADAM17 have failed due to the utilization of zinc-binding inhibitors. We previously reported discovery of highly selective nonzinc-binding exosite-targeting inhibitors of ADAM17 that exhibited not only enzyme isoform selectivity but synthetic substrate selectivity as well (*J. Biol. Chem.* **2013**, *288*, 22871). As a result of SAR studies presented herein, we obtained several highly selective ADAM17 inhibitors, six of which were further characterized in biochemical and cell-based assays. Lead compounds exhibited low cellular toxicity and high potency and selectivity for ADAM17. In addition, several of the leads inhibited ADAM17 in a substrate-selective manner, which has not been previously documented for inhibitors of the ADAM family. These findings suggest that targeting exosites of ADAM17 can be used to obtain highly desirable substrate-

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Supporting Information: Molecular formula strings (CSV). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00354.

Author Contributions: The manuscript was written by D.M. and edited by G.B.F. All authors have given approval to the final version of the manuscript.

Notes: The authors declare no competing financial interest.

selective inhibitors. Additionally, current inhibitors can be used as probes of biological activity of ADAM17 in various in vitro and, potentially, in vivo systems.

Graphical abstract

Introduction

ADAM17 is a prototypical member of a disintegrin and metalloproteinase family of metzincin proteases¹ implicated in several aggressive forms of cancer,^{2,3} rheumatoid arthritis,² and Alzheimer's disease.⁴ Not surprisingly, multiple pharmaceutical companies attempted to develop inhibitors of ADAM17 as potential drug candidates for the above-mentioned therapeutic indications. From 2001 to 2009, more than a hundred ADAM17 inhibitors were patented.⁵ Even though some of these molecules inhibit ADAM17 with subnanomolar potency and several of them reached clinical trials,² they ultimately failed as drugs. It is believed that the main reason for these failures was the zinc-binding mechanism of action leading to a broad-spectrum inhibition of many zinc-dependent metalloproteases leading to in vivo side effects most frequently manifesting as a musculoskeletal syndrome (MSS).⁶ Additionally, many of the zinc-binding moieties, such as hydroxamates, are metabolically unstable, which limits their bioavailability.⁷ There are several comprehensive reviews available on collagenase inhibitors,⁸ TACE inhibitors,⁹ metzincin inhibitors,¹⁰ and metalloenzyme inhibitors^{11,12} that address the drawbacks of zinc-binding inhibitors.

To circumvent the problems of zinc-binding inhibitors of ADAM17 and metzincins in general, several groups, including ours, pursued discovery and development of inhibitors that act via a nonzinc-binding mechanism and target secondary substrate binding sites (also known as exosites).^{13–16} For example, Aventis discovered a pyrimidine dicarboxamide that had low nanomolar potency for MMP-13 and no activity against other MMPs when tested at 100 μ M.¹³ Pfizer reported discovery of highly selective nanomolar range MMP-13 inhibitors based on pyrimidinedione and quinazolinone scaffolds acting via binding to the S₁'

exosite.^{14,17} Similarly, Alantos Pharmaceuticals identified a new class of highly selective nonzinc-binding MMP-13 inhibitors.^{15,16} Most recently, Takeda Pharmaceutical Company reported yet another nonzinc-binding inhibitor of MMP-13 that acts via binding to the S₁' site.¹⁸ The lead from the Takeda series, compound 26c, exhibited subnanomolar activity against MMP-13 and good oral bioavailability. Our group reported the discovery of a MMP-13 exosite inhibitor that binds to two different exosites.^{19–21} Several of these inhibitors were efficacious in in vivo models of arthritis without causing MSS,^{14,15,22} suggesting that nonzinc-binding inhibition can indeed overcome the drawbacks of zinc-binding inhibitors of metzincins.

In addition to potentially being drug candidates, selective inhibitors of ADAM17 could represent extremely useful tools for dissecting the highly complex role of ADAM17 in various diseases and normal processes. Such probes have become the staple of chemical biology for studying cellular processes.²³ The development of exosite inhibitors of ADAM17 and ADAM in general have been hampered by the lack of knowledge about exosites in the structures of ADAM. However, development of an ADAM17-selective low nanomolar inhibitory antibody²⁴ was reported in 2011. This antibody was shown to work via binding across the catalytic and noncatalytic domains of ADAM17, thereby obstructing access of the substrate to the catalytic cleft of ADAM17.

Our group has previously reported the discovery of the first small molecule exosite-binding selective inhibitor of ADAM17.²⁵ In the present study, we report results of structure–activity relationship studies and cellular characterization of this novel class of ADAM17 inhibitors that could potentially be used as probes for the biological activity of ADAM17.

Results

SAR Studies

We previously conducted initial exploration of structure–activity relationship of pyrrolidine diketopiperazine analogues using *S*-4-hydroxybenzyl and *S*-propyl in the R1 position.²⁵ In both cases, we tried *S*- and *R*-hydroxybenzyl and *S*-2-naphthylmethyl in position R2, *S*- and *R*-hydroxybenzyl in position R3, and 2-phenylbutyl, 2-adamantan-1-yl-ethyl, and cyclopentyl-methyl in position R4. Analogues with *S*-propyl in R1 position did not produce highly potent compounds (best compound IC₅₀ value 12 μ M) but were selective for ADAM17 as compared to ADAM10. In the present study, we expanded the SAR of the R1 *S*-propyl analogues in order to determine whether greater activity can be achieved without loss of selectivity. Thus, compounds **1–7** have *S*-propyl in the R1 position, *R*-hydroxybenzyl in position R2, and *S*-hydroxybenzyl in position R3, whereas residues in position R4 are varied (Table 1). All compounds of this series produced complete inhibition at 100 μ M, with IC₅₀ values ranging from 94 to 5.9 μ M. Two out of three best compounds (**2** and **6**, IC₅₀ = 10 $\pm$ 1.3 and 5.9 $\pm$ 0.9 μ M, respectively) contained 4-adamantan-1-yl-methyl and 2-adamantan-1-yl-ethyl in the R4 position, respectively. From our previous study, the best compound reported by us (compound **15** in ref 25 and compound **17** in present work) had 2-adamantan-1-yl-ethyl moiety in the R4 position. Overall, when 2-adamantan-1-yl-ethyl was present in position R4, it produced the greatest inhibition without loss of selectivity regardless of which functionalities were present in positions R1–3. On the basis of this

consideration, the next series of analogues all have *S*-propyl in the R1 position, *R*-hydroxybenzyl in R2, and 2-adamantan-1-yl-ethyl in R4, while position R3 is varied to produce compounds **8–11** (Table 2). The functionalities used in position R3 were selective in the positional scan study during the deconvolution stage as described previously.²⁵ Stereoisomers with *S*- and *R*-hydroxybenzyl in position R3, compounds **6** and **8**, exhibited similar IC₅₀ values (IC₅₀ = 5.9 ± 0.9 and 5.4 ± 0.5 μM for **6** and **8**, respectively). *R*- and *S*-Naphthalen-2-ylmethyl (compounds **9** and **10**) were also equipotent (IC₅₀ = 7.9 ± 0.8 and 10 ± 0.8 μM for **10** and **9**, respectively). Finally, compound **11** with *S*-butyl in R3 had IC₅₀ of 6.5 ± 0.9 μM. These results suggested that the potency of compounds of this scaffold is largely determined by the presence of 2-adamantan-1-yl-ethyl in R4. To confirm this hypothesis, we introduced *R*-6-naphthalen-2-ylmethyl in position R1. Activity of *R*-6-naphthalen-2-ylmethyl was greater than *S*-propyl based on the positional scan.²⁵ Additionally, positions R4 and R2 were fixed with 2-adamantan-1-yl-ethyl and *S*-4-hydroxybenzyl, respectively, while R3 was varied (Table 3). Introduction of *R*-6-naphthalen-2-ylmethyl in position R1 resulted in 2–3-fold loss of potency (Table 3, IC₅₀ compound **8** = 5.4 ± 0.5 μM versus 15 ± 1.2 μM for compound **13**; 5.9 ± 0.9 μM for **6** and 8.1 ± 0.9 μM for compound **12**) when hydroxybenzyl was present in position R3. When 5-naphthalen-2-ylmethyl was present in R3, introduction of *R*-6-naphthalen-2-ylmethyl into R1 did not have a significant effect (Table 3, IC₅₀ compound **9** = 10 ± 0.8 μM versus 8.4 ± 1.0 μM for **14**; 7.9 ± 0.8 μM for **10** versus 5.7 ± 1.0 μM for **15**). Similarly, the presence of *S*-5-butyl in R3 resulted in comparable IC₅₀ values whether R1 contained either *S*-propyl or *R*-6-naphthalen-2-ylmethyl (Table 3, IC₅₀ compound **11** = 6.5 ± 0.9 μM versus 5.7 ± 0.8 for **16**).

As a result of the structure–activity relationship study, several compounds with low micromolar IC₅₀ values were identified. We chose compounds exhibiting the greatest potency for ADAM17 containing 2-adamantan-1-yl-ethyl in R4 (**20**, **16**, and **17** described in ref 25) to be tested against an extended panel of zinc metalloproteinases. Additionally, we chose the most potent compounds without 2-adamantan-1-yl-ethyl (**18**, **19**, and **21**) for comparison in selectivity testing and cell-based studies.

Compounds **21**, **19**, **20**, and **16** exhibited the best selectivity profile, inhibiting only ADAM17 and sparing five other metzincins. Compound **17** exhibited limited inhibition of MMP-2, MMP-9, and MMP-14/MT1-MMP (Table 4) at 100 μM, most likely due to nonspecific binding. This biochemical inhibition profile suggested that compounds of this series could potentially be used as selective in vitro and in vivo probes of ADAM17.

Cell-Based Studies: Cell Toxicity and Cell Surface Targets

Before testing the ADAM17 inhibitors for abrogation of shedding of cell surface proteins that are generally considered to be physiologically relevant substrates of ADAM17, the compounds were tested for toxicity in cell viability assays. All six leads were tested with healthy and lung cancer cell lines (CHO-K1 and A549, respectively). Additionally, **18**, **17**, and **20** were tested with liver and breast cancer cell lines (HEPG2 and MDA-MB-231, respectively). CHO-K1 and A549 cells were viable for 72 h in the presence of up to 33 μM of lead compounds (Figure 1). **18**, **17**, and **20** did not inhibit viability of HEPG2 and MDA-

MB-231 cells for 72 h up to 33 μ M (data not shown). These results suggested that the lead compounds could be utilized as probes of ADAM17 activity in cell-based assays without compromising the health of the cellular system under investigation.

ADAM17 has been implicated as either the main or, in some cases, the only protease responsible for the cleavage of more than 70 cell surface proteins.²⁶ To assess activity of the lead compounds against ADAM17 in a cell-based setting, several cell lines and cell surface proteins were examined.

Cytokines

The ability of compounds to protect of TNF α from shedding by ADAM17 was assessed. TNF α is a canonical substrate of ADAM17.^{27,28} The present ADAM17 inhibitors were discovered using substrate based on the TNF α cleavage sequence;²⁵ therefore, we were interested to see whether TNF α shedding would be inhibited in a cell-based assay. We tested compounds at 25 μ M and used Marimastat (a broad spectrum metzincin inhibitor) at 10 μ M as a control. Five out of six test compounds and Marimastat inhibited approximately 30–50% of TNF α cleavage in THP1 cells (Figure 2A). To assess the potency of this inhibition, we performed a dose–response study with compounds **17** and **19**, whereby they exhibited $\sim$ 100 and 28 μ M IC₅₀ values, respectively (Figure 2B).

PMA - or LPS-induced production of chemokine IL-8 by epithelial cells of human airway (NCI-H292) or human tracheal smooth muscle cells (HTSMC) was reported to be mediated by ADAM17's cleavage of TGF α and subsequent trans-activation of ErbB/EGFR²⁹ and was inhibited by ADAM17 siRNA. In human tracheal smooth muscle cells (HTSMC), IL-8 was shed in response to LPS, acid, and IL-1 stimulation³⁰ and was decreased as a result of ADAM17 shRNA application. Thus, IL-8 production by these cells can be used as read out for TGF α shedding. We tested compounds **18**, **17**, **20**, and **16** for inhibition of LPS- and IL-1-stimulated IL-8 release from HTSMCs. Ten μ M of compounds **18**, **17**, **20**, and **16** inhibited 18–62% of LPS-stimulated IL-8 shedding (Figure 3A). Interestingly, none of the tested compounds were able to inhibit IL-1-mediated production of IL-8 (Figure 3B). It is known that IL-1 stimulates production of IL-8 via inducing cleavage of neuregulins, which can be cleaved by multiple proteases of which ADAM17 is the critical sheddase in the cell system that was used here.^{29,30} Lack of inhibition of IL-1-induced IL-8 release in combination with inhibition of LPS-induced IL-8 release suggests that our lead compounds target TGF α cleavage by ADAM17 but not cleavage of neuregulins. Our compounds may have significant utility for dissecting of complex proteolytic pathways where ADAM17 is implicated in a substrate-specific manner.

To test for selectivity of compounds, we conducted assays for inhibition of shedding of fractalkine/CX3CL1 and CXCL16 using HTSMC. Fractalkine/CX3CL1 is shed both constitutively and in response to PMA stimulation. ADAM17 was shown to be responsible for PMA-induced cleavage,³¹ whereas constitutive shedding is ascribed to ADAM10.³² None of the lead compounds were able to abrogate constitutive ADAM10-mediated release of fractalkine/CX3CL1 (Figure 3C), suggesting that the compounds are selective for ADAM17. Additionally, compounds **18**, **17**, **20**, and **16** did not inhibit the shedding of

CXCL16 ascribed to ADAM10,³³ further suggesting their selectivity for ADAM17 in HTSMC (data not shown).

Receptors

Shedding of discoidin domain receptor 1 (DDR1) in HCC1806 breast cancer cells is mediated by cell surface-bound metalloproteases.³⁴ Membrane type 1 matrix metalloprotease (MT1-MMP, also known as MMP-14) was determined to be one of the enzymes responsible for the constitutive cleavage of DDR1. However, MT1-MMP knockdown or inhibition by pharmacological agents did not result in the abrogation of DDR1 shedding. These data suggested the existence of a compensatory mechanism whereby another metalloprotease cleaves DDR1 in the event of MT1-MMP inhibition. Because ADAM10 and ADAM17 mRNAs were detected in HCC1806 cells,³⁴ the hypothesis of ADAM proteases involvement in DDR1 cleavage was formed and it was decided to investigate whether shedding of DDR1 ectodomain could be affected by the application of ADAM17 selective inhibitors. Compound **17** was tested using 10, 20, and 40 μ M concentrations. DDR1 shedding was inhibited in a dose-dependent manner (Figure 4), however, even at the highest tested dosage (40 μ M), soluble DDR1 was detectable. This suggests that both ADAM17 and MT1-MMP have to be inhibited to completely abrogate the shedding of DDR1. Additionally, compound **17** did not inhibit ADAM10 in the in vitro assay (Table 4), suggesting that this enzyme does not appear to be involved in constitutive shedding of DDR1 despite the presence of its mRNA in HCC1806 cells.

Another cell surface protein with importance in cancer progression that is cleaved by MT1-MMP and ADAM17 is protein tyrosine kinase 7 (PTK7). PTK7 proteolysis controls cell migration in early embryogenesis and regulates cancer cell directional motility, invasion, and metastasis.^{35–39} The full-length PTK7 efficiently inhibits cell invasion and downregulates myosin light chain (MLC) phosphorylation, a downstream event in the Wnt/PCP pathway.³⁶ Proteolysis of PTK7 reverses the inhibitory signal of the full-length PTK7 and subsequently promotes cell invasion³⁵ and metastasis.³⁸ Thus, PTK7 proteolysis by MT1-MMP and ADAM17 is a proteolytic master switch that turns on the motility of cells. We tested lead compounds (**18**, **17**, **19**, and **16**) at 25, 50, and 100 μ M concentrations using a human fibrosarcoma cell line that expressed a PTK7 mutant uncleavable by MT1-MMP (HT1080-L622D) and colorectal cancer cells (SW480) expressing wild-type PTK7 endogenously (wtPTK7). Compound **17** inhibited shedding of L622D-PTK7 and wtPTK-7 at 50 and 100 μ M (Figure 5A) when both HT1080-L622D and SW480 cells were stimulated with PMA. **18**, **19**, and **16** did not exhibit inhibition of L622D-PTK7 and wtPTK-7 up to 100 μ M (data not shown).

A dual inhibitor of ADAM10 and ADAM17, INCB3619 (ADAM17 IC₅₀ = 14 nM, ADAM10 IC₅₀ = 22 nM, MT1-MMP IC₅₀ = 772 nM)⁴⁰ was able to inhibit both shedding of PTK7 and invasion of HT1080 cells.³⁷ On the basis of this evidence, we hypothesized that selective inhibition of ADAM17 should result in a decrease of invasiveness of HT1080 cells. Indeed, we observed a dose-dependent inhibition of invasion of HT1080 cells in a Transwell assay (Figure 5B).

Growth Factors

ADAM10 and ADAM17 are believed to be the main sheddases of EGFR ligands.⁴¹ Moreover, the expression of ADAM17 was shown to be up-regulated in lung cancer,⁴² where it has been shown to shed heregulin⁴³ leading to activation of EGFR/ERK/Akt pathways and resulting in cancer cell proliferation,⁴⁴ resistance to EGFR-based therapies,⁴⁰ and poor prognosis.^{40,42} On the basis of these considerations, we were interested to see whether exosite inhibition of ADAM17 can be an effective tool to study the role of ADAM17 in lung cancer. We used A549 nonsmall cell lung cancer cells, which were shown to have high expression levels of heregulin⁴⁰ to determine the ability of our lead compounds to inhibit its shedding. Heregulin is specifically cleaved by ADAM17⁴⁵ and therefore represents a suitable end point in determining cellular efficacy of ADAM17 inhibitors. Interestingly, despite very similar IC₅₀ values for ADAM17 inhibition in the biochemical assay (Table 4), only compound **17** completely inhibited shedding of heregulin in the cell-based assay (Figure 6A, all leads tested at 40 μ M). To assess enzyme selectivity of compound **17**, we tested its ability to inhibit shedding of another EGFR ligand, betacellulin, which is cleaved exclusively by ADAM10.^{41,45} Marimastat and compounds **21**, **18**, and **16** were also tested. None of tested selective ADAM17 inhibitors affected the shedding of betacellulin from A549 cell surface at 40 μ M, whereas Marimastat exhibited almost complete inhibition at 10 μ M. This suggests that **17** selectively inhibits ADAM17 not only in biochemical but in a cell-based environment and could be a useful tool to study the role of ADAM17 in cells.

We previously reported that **17** was able to preferentially inhibit ADAM17 hydrolysis of a glycosylated synthetic substrate based on the TNF α sequence (**15** in ref 46). ADAM17 is known to be able to cleave more than 70 cell surface proteins,⁴⁷ creating concern that indiscriminate inhibition of all ADAM17-mediated shedding could have unpredictable side effects. In turn, inhibitors that can selectively inhibit hydrolysis of certain substrates or a subset of substrates could be of interest for drug discovery. To assess whether **17** inhibits heregulin with some degree of selectivity, we tested it for the inhibition of shedding of TGF α , another EGFR ligand with importance in lung cancer. Indeed, **17** did not affect shedding of TGF α in A549 cells up to 100 μ M, whereas 10 μ M Marimastat decreased soluble TGF α in the supernatant of A549 cells to the level of non-PMA-treated control (Figure 6C).

Synergy Studies

It was shown that ADAM17 inhibition by broad spectrum metalloprotease inhibitor INCB3619 led to abrogation of heregulin shedding in A549 cells, which potentiated the activity of the EGFR tyrosine kinase inhibitor gefitinib.⁴⁰ As mentioned above, **17** inhibited shedding of heregulin in A549 cells (Figure 6A); therefore, we used it to test for synergy with gefitinib. Then 40 μ M compound **17** potentiated gefitinib activity against A549 cells in the viability assay (Figure 1). The IC₅₀ value for gefitinib inhibition of A549 cell growth was 8.4 μ M, but in the presence of 40 μ M compound **17** the IC₅₀ value decreased 4-fold (1.9 μ M). For comparison, 10 μ M INCB3619 shifted gefitinib's IC₅₀ value from ~8 to ~1 μ M in A549 cells.⁴⁰ Because **17** exhibited no intrinsic activity against A549 cell in viability assay (Figure 1), the improvement of gefitinib's potency can be ascribed to the synergy between **17**

and gefitinib. For comparison, compound **17** did not potentiate the activity of two other FDA-approved lung cancer drugs etoposide or doxorubicin (Figure 7B,C). Etoposide and doxorubicin act via topoisomerase inhibition, distinct from the inhibition of EGFR tyrosine kinase activity of gefitinib.

Discussion

Despite the large number of ADAM17 inhibitors developed by various academic and industrial entities, there is a paucity of ADAM17 inhibitors that work by binding to so-called exosites. Therefore, there is limited understanding of the biological and biochemical effects of inhibition by such molecules. Indeed, what can be expected from molecules that bind to an exosite rather than to an active site of ADAM17 or any other enzyme? For example, the anti-BACE1 exosite-binding antibody inhibited hydrolysis of a longer APP-based synthetic substrate 10-fold more potently than a shorter version of the same substrate,⁴⁸ suggesting that exosite-based inhibition can lead to substrate selectivity. Substrate-selective inhibition is much more rare than enzyme-selective inhibition but has been demonstrated for different classes of enzymes. In the case of COX-2 substrate-selective inhibitors, binding to an allosteric subunit results in noncompetitive inhibition of endocannabinoid oxygenation, but no inhibition of arachidonic acid oxygenation was observed.⁴⁹ Substrate-selective inhibition of protein kinase PDK1 was also achieved as a result of binding of small molecule to an allosteric site.⁵⁰

To our knowledge, substrate-selective inhibition of ADAM17 has not been reported so far. While metzincin isoform selectivity was addressed in many studies, the possibility of substrate selectivity of metzincin inhibitors still remains largely unexplored. It is now understood that some metzincins should not be inhibited in certain disease scenarios (i.e., MMP-8 and MMP-14 in skin and breast cancer^{51–53}). However, it is much less known about whether hydrolysis of any of the cognate substrates should be spared from inhibition. As demonstrated in the case of γ -secretase inhibitor discovery for Alzheimer's disease, inhibition of γ -secretase abrogated release of β -amyloid but also prevented a cleavage of Notch leading to toxicity (reviewed in ref 54). It is well-known that metzincins have broad substrate repertoires, therefore, it is possible that total abrogation of activity of a target enzyme might be unwanted.

Our data suggest that exosite inhibition of ADAM17 can lead to a substrate-selective inhibition (Table 5). Data presented herein demonstrate that compound **17** inhibits ADAM17 selectively in cell-based assays, which in turn suggests that it can be used as a probe of ADAM17 activity in in vitro and, potentially, in vivo, systems. In addition to enzyme selectivity, compound **17** also exhibited unusual substrate selectivity by sparing ADAM17-mediated cleavage of TGF α . For comparison, both Marimastat (a broad spectrum ADAM and MMP zinc-binding inhibitor) and INCB84298 (a moderately ADAM17-selective zinc-binding inhibitor) inhibited shedding of each tested EGFR ligand (heregulin, TGF α , HB-EGF, amphiregulin, and EGF) in A549 cells almost equipotently.⁴⁰ Even though limited enzyme selectivity might be achieved by targeting the zinc of the ADAM17 active site, the zinc-binding inhibitors cannot selectively inhibit proteolysis of subset of ADAM17 substrates. Further studies characterizing the substrate inhibition profile of exosite inhibitor

17 are needed to determine which substrates of ADAM17 are protected from shedding and what is the underlying mechanism of this unusual substrate selectivity. One possibility is that different ADAM17 substrates interact with different exosites or sets of exosites in ADAM17, while compound **17** potentially interacts with just one exosite and, therefore, cannot inhibit binding of substrates that interact with exosites different from the one that compound **17** binds to. This possible explanation is consistent with the fact that ADAM17-selective exosite-binding antibody D1(A12)²⁴ inhibits shedding of all tested ADAM17 substrates (i.e., TNF α , TGF α , amphiregulin, HB-EGF-AP, and TNFR1a) while sparing ADAM10. D1(A12) interacts with a significantly greater surface area of ADAM17 as compared to **17** bridging catalytic and noncatalytic domains and, therefore, potentially interacting with many more exosites than **17**. The limited exosite interaction is possibly due to the fact that inhibitors of the **17** chemotype were discovered using a TNF α -based glycosylated substrate, creating a bias toward inhibition of TNF α (Table 5) over other ADAM17 substrates (Table 5, TNF α versus TGF α , heregulin). It will be interesting to see whether a similar bias toward inhibiting certain other ADAM17 substrates can be achieved by using HTS substrates derived from different cognate substrates of these adamalysins or other metzincins. Conversely, this ability to “program” or bias discovery toward substrate-selective inhibitors can be highly desirable for drug discovery for enzymes with complex substrate repertoires.

Conclusion

The findings presented herein suggest that targeting exosites of ADAM17 can be used to obtain highly desirable enzyme isoform and substrate-selective inhibitors. Additionally, current inhibitors can be used as probes of the biological activity of ADAM17 in various in vitro and, potentially, in vivo systems.

Experimental Procedures

General Synthesis Procedure for Pyrrolidine-bis-diketopiperazine

All compounds were synthesized via solid-phase methodology (Scheme 1) on 4-methylbenzhydrylamine hydrochloride resin (MBHA) (1.1 mmol/g, 100–200 mesh) using the “tea-bag” approach⁵⁵ as previously described elsewhere.⁵⁶ Boc-amino acids were coupled utilizing standard coupling procedures (6 equiv) with hydroxybenzotriazole hydrate (HOBt, 6 equiv) and *N,N'*-diisopropylcarbodiimide (DIC, 6 equiv) in dimethylformamide (DMF, 0.1 M) for 120 min. Boc protecting groups were removed with 55% trifluoroacetic acid (TFA)/45% dichloromethane (DCM) (1 $\times$, 30 min) and subsequently neutralized with 5% diisopropylethylamine (DIEA)/95% DCM (3 $\times$, 2 min). Carboxylic acids (10 equiv) were coupled utilizing standard coupling procedures with HOBt (10 equiv) and DIC (10 equiv) in DMF (0.1 M) for 120 min. Completion of all couplings was monitored with a ninhydrin test. Compounds were reduced to polyamines (Scheme 1) using a 40 $\times$ excess of borane (1.0 M in tetrahydrofuran (THF)) over each amide bond in a glass vessel under nitrogen at 65 °C for 72 h. The solution was then poured off, the reaction was quenched with methanol (MeOH), and the bags were washed with THF (1 $\times$, 1 min) and MeOH (4 $\times$, 1 min) and allowed to air-dry. Once dry, the bags were treated with piperidine overnight at 65 °C in a glass vessel. The solution was poured off, and the bags were washed with DMF (2 $\times$, 1 min), DCM (2 $\times$, 1

min), MeOH (1×, 1 min), DMF (2×, 1 min), DCM (2×, 1 min), and MeOH (1×, 1 min) and allowed to air-dry. Completion of reduction was checked by cleaving a control sample and analyzing using LCMS. As previously reported by our group and others, the reduction of polyamides with borane is free of racemization (refs 1–3). Diketopiperazine cyclization (Scheme 1) was performed under anhydrous conditions (<22% humidity). The dry bags were washed with anhydrous DMF (2×, 1 min), then added to a solution of 1,1'-oxalyldiimidazole (5-fold excess for each cyclization site) in anhydrous DMF (0.1 M) and shaken at room temperature overnight. The solution was poured off, and the bags were rinsed with DMF (3×, 1 min) and DCM (3×, 1 min). Completion of cyclization was checked by cleaving a control sample and analyzing by LCMS. The compounds were then cleaved from the resin with hydrofluoric acid (HF) in the presence of anisole in an ice bath at 0 °C for 90 min (Scheme 1) and extracted using 95% acetic acid (AcOH)/5% H₂O (2×, 5 mL). Final crude products were purified using HPLC as described below. All chirality was generated from the corresponding amino acids. Under the reaction conditions described, no epimerization was observed, and for those compounds with multiple chiral centers, a single diastereomer was obtained.

Compound Purification and Characterization

The final compounds were purified using preparative HPLC with a dual pump Shimadzu LC-20AB system equipped with a Luna C18 preparative column (21.5 mm × 150 mm, 5 μm) at λ = 214 nm, with a mobile phase of (A) H₂O (+0.1% formic acid)/(B) acetonitrile (ACN) (+0.1% formic acid) at a flow rate of 13 mL/min; gradients varied by compound based on hydrophobicity. ¹H NMR and ¹³C NMR spectra were recorded in DMSO-*d*₆ on a Bruker Ascend 400 MHz spectrometer at 400.14 and 100.62 MHz, respectively, and MALDITOF mass spectra were recorded using an Applied Biosystems Voyager DE-PRO Biospectrometry workstation. The purities of synthesized compounds were confirmed to be greater than 95% by liquid chromatography and mass spectrometry on a Shimadzu LCMS-2010 instrument with ESI Mass Spec and SPD-20A liquid chromatograph with a mobile phase of (A) H₂O (+0.1% formic acid)/(B) ACN (+0.1% formic acid) (5–95% over 6 min with a 4 min rinse).

(S)-4-((4-(tert-Butyl)cyclohexyl)methyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (1)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **1** was synthesized using the following reagents: Boc-L-norvaline-OH (R₁), Boc-L-tyrosine(2-Br-*Z*)-OH (R₂), Boc-L-tyrosine(2-Br-*Z*)-OH (R₃), and 4-(*tert*-butyl)-cyclohexanecarboxylic acid (R₄). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ¹H NMR (400 MHz, DMSO-*d*₆): (400 MHz, DMSO-*d*₆): δ ppm 8.5 (br s, 1 H) 7.6 (br s, 1 H) 7.0–7.1 (m, 1 H) 6.9 (br s, 1 H) 6.7 (br s, 1 H) 6.5 (br s, 1 H) 6.3 (br s, 1 H) 3.6 (d, *J* = 12.5 Hz, 1 H) 3.5 (br s, 1 H) 3.3 (br s, 19H) 3.2 (br s, 1 H) 2.9 (br s, 1 H) 2.7–2.8 (m, 1 H) 2.7 (br s, 13 H) 2.3 (br s, 4 H) 2.1 (br s, 1 H) 1.9 (br s, 1 H) 1.8 (br s, 1 H) 1.7 (br s, 1 H) 1.5 (br s, 1 H) 1.4 (d, *J* = 12.3 Hz, 1 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H) 0.7–0.9 (m, 2 H). *m/z* calcd C₄₃H₆₁N₅O₆ [M + H]⁺, 744.46; found (MS ESI), 744.35. Purity LCMS: 95.5% (254 nm, peak height).

(S)-4-((3S,5S,7S)-Adamantan-1-ylmethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (2)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **2** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-tyrosine(2-Br-*Z*)-OH (R_3), and 1-adamantanecarboxylic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, DMSO- d_6): δ ppm 8.5 (br s, 1 H) 7.3 (d, $J = 6.8$ Hz, 1 H) 7.1 (br s, 1 H) 7.0 (br s, 1 H) 6.9 (br s, 1 H) 6.7 (br s, 1 H) 6.5 (br s, 1 H) 6.3 (br s, 1 H) 3.6 (br s, 3 H) 3.3 (br s, 18 H) 3.1 (br s, 2 H) 2.8 (d, $J = 11.7$ Hz, 2 H) 2.7 (br s, 9 H) 2.3 (br s, 5 H) 2.0 (br s, 1 H) 1.9 (br s, 1 H) 1.6 (br s, 1 H) 1.5 (br s, 2 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H). m/z calcd $\text{C}_{43}\text{H}_{57}\text{N}_5\text{O}_6$ [$\text{M} + \text{H}$] $^+$, 740.43; found (MS ESI), 740.40. Purity LCMS: 95.1% (254 nm, peak height).

(S)-4-(Cyclohexylmethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (3)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **3** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-Tyrosine-(2-Br-*Z*)-OH (R_3), and cyclohexanecarboxylic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, DMSO- d_6): δ ppm 8.4 (br s, 1 H) 7.6–7.7 (m, 2 H) 7.3 (d, $J = 5.8$ Hz, 1 H) 7.1 (br s, 1 H) 7.0 (br s, 1 H) 6.9 (br s, 1 H) 6.6 (br s, 1 H) 6.5 (br s, 1 H) 4.8 (br s, 1 H) 3.8 (br s, 2 H) 3.6 (br s, 3 H) 3.3 (br s, 13 H) 3.0 (br s, 3 H) 2.8 (d, $J = 11.7$ Hz, 2 H) 2.7 (br s, 6 H) 2.2–2.5 (m, 3 H) 2.0 (br s, 1 H) 1.8 (br s, 1 H) 1.7 (br s, 2 H) 1.5 (br s, 2 H) 1.2 (br s, 1 H) 1.1 (d, $J = 16.9$ Hz, 1 H) 0.9 (br s, 1 H) 0.8 (br s, 1 H). m/z calcd $\text{C}_{39}\text{H}_{53}\text{N}_5\text{O}_6$ [$\text{M} + \text{H}$] $^+$, 688.40; found (MS ESI), 688.20. Purity LCMS: 96.1% (254 nm, peak height).

(S)-4-(Cyclobutylmethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (4)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **4** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-tyrosine-(2-Br-*Z*)-OH (R_3), and cyclobutanecarboxylic acid (R_4). ^1H NMR (400 MHz, DMSO- d_6): 8.5 (br s, 1 H) 6.9–7.0 (m, 5 H) 6.6–6.7 (m, 4 H) 3.6 (d, $J = 12.47$ Hz, 1 H) 3.3 (br s, 13 H) 2.8 (d, $J = 10.86$ Hz, 2 H) 2.7 (br s, 8 H) 2.3 (br s, 4 H) 1.9 (br s, 1 H) 1.8 (br s, 1 H) 1.7 (br s, 2 H) 1.6 (d, $J = 11.30$ Hz, 4 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H). m/z calcd $\text{C}_{37}\text{H}_{49}\text{N}_5\text{O}_6$ [$\text{M} + \text{H}$] $^+$, 660.37; found (MS ESI), 660.30. Purity LCMS: 97.3% (254 nm, peak height).

(5S)-1-((S)-1-((S)-2-(((S)-2,3-Dioxo-6-propylpiperazin-1-yl)-methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)-4-(2-phenylbutyl)piperazine-2,3-dione (5)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **5** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-Tyrosine(2-Br-

Z)-OH (R_3), and 2-phenylbutanoic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ ppm 8.5 (br s, 1 H) 7.6 (br s, 1 H) 6.9 (br s, 1 H) 6.7–6.8 (m, 1 H) 6.5 (br s, 1 H) 6.3 (br s, 1 H) 3.3 (br s, 17 H) 2.8 (br s, 2 H) 2.7 (br s, 9 H) 2.3 (br s, 1 H) 2.1–2.2 (m, 1 H) 2.1 (br s, 1 H) 2.0 (br s, 1 H) 1.9 (br s, 1 H) 1.8 (br s, 1 H) 1.7 (br s, 2 H) 1.5 (br s, 2 H) 1.5–1.6 (m, 3 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H) 0.7 (br s, 1 H). m/z calcd $\text{C}_{44}\text{H}_{59}\text{N}_5\text{O}_6$ $[\text{M} + \text{H}]^+$, 724.40; found (MS ESI), 724.30. Purity LCMS: 97.0% (254 nm, peak height).

(S)-4-(2-((3S,5S,7S)-Adamantan-1-yl)ethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (6)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **6** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-tyrosine(2-Br-*Z*)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ ppm 8.5 (br s, 1 H) 7.0 (br s, 1 H) 6.9 (br s, 1 H) 6.7 (br s, 1 H) 6.5 (br s, 1 H) 6.3 (br s, 1 H) 3.4 (br s, 22 H) 2.8 (br s, 2 H) 2.7 (br s, 14 H) 2.3 (br s, 3 H) 2.1 (br s, 1 H) 2.0 (br s, 1 H) 1.9 (br s, 1 H) 1.8 (br s, 1 H) 1.7 (br s, 1 H) 1.6 (br s, 1 H) 1.4 (br s, 1 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H). m/z calcd $\text{C}_{44}\text{H}_{59}\text{N}_5\text{O}_6$ $[\text{M} + \text{H}]^+$, 754.45; found, (MS ESI), 754.30. Purity LCMS: 99.3% (254 nm, peak height).

(S)-4-(Cyclopentylmethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (7)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **7** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-tyrosine(2-Br-*Z*)-OH (R_3), and cyclopentylcarboxylic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ ppm 8.4 (br s, 1 H) 7.00 (br s, 1 H) 6.9 (d, $J = 6.28$ Hz, 2 H) 6.7–6.8 (m, 1 H) 6.5 (br s, 1 H) 3.6 (br s, 3 H) 3.3 (br s, 25 H) 2.8 (br s, 2 H) 2.7 (br s, 13 H) 2.3 (br s, 3 H) 1.4–1.6 (m, 1 H) 1.3 (br s, 1 H) 1.2 (br s, 1 H) 0.9 (br s, 1 H). m/z calcd $\text{C}_{38}\text{H}_{51}\text{N}_5\text{O}_6$ $[\text{M} + \text{H}]^+$, 674.38; found (MS ESI), 674.25. Purity LCMS: 95.8% (254 nm, peak height).

(R)-4-(2-((3R,5R,7R)-Adamantan-1-yl)ethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (8)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **8** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-D-tyrosine(2-Br-*Z*)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ ppm 8.4 (br s, 1 H) 7.3 (d, $J = 5.8$ Hz, 1 H) 6.9–7.0 (m, 1 H) 6.9 (br s, 1 H) 6.6–6.7 (m, 3 H) 6.5 (br s, 1 H) 3.8 (d, $J = 10.64$ Hz, 1 H) 3.5–3.7 (m, 3 H) 3.4–3.5 (m, 3 H) 3.3 (br s, 7 H) 3.1–3.3 (m, 3 H) 3.0 (br s, 2 H) 2.7–2.9 (m, 4 H) 2.3–2.5 (m, 2 H) 2.2 (d, $J = 7.8$ Hz, 1 H) 1.8–2.0 (m, 3 H) 1.8 (br s, 1 H) 1.7 (d, $J = 11.74$ Hz, 4 H) 1.8

(d, $J = 11.00$ Hz, 4 H) 1.4 (br s, 5 H) 1.3 (br s, 1 H) 1.1–1.3 (m, 2 H) 0.8–1.0 (m, 3 H). m/z calcd $C_{44}H_{59}N_5O_6$ $[M + H]^+$, 754.45; found (MS ESI), 754.30. Purity LCMS: 96.3% (254 nm, peak height).

(S)-4-(2-((3S,5S,7S)-Adamantan-1-yl)ethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(naphthalen-2-ylmethyl)piperazine-2,3-dione (9)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **9** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-Ala(2-naphthyl)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.4 (br s, 1 H) 7.8–8.0 (m, 2 H) 7.7 (br s, 1 H) 7.5 (br s, 1 H) 7.4 (br s, 1 H) 6.9 (d, $J = 6.4$ Hz, 2 H) 6.6 (d, $J = 7.5$ Hz, 2 H) 6.5 (br s, 1 H) 4.0 (br s, 1 H) 3.8 (d, $J = 12.8$ Hz, 1 H) 3.5 (d, $J = 13.4$ Hz, 4 H) 3.3 (br s, 6 H) 3.0–3.2 (m, 3 H) 2.9 (d, $J = 14.1$ Hz, 2 H) 2.8 (br s, 2 H) 2.7 (d, $J = 12.8$ Hz, 4 H) 2.2–2.5 (m, 3 H) 1.9 (br s, 1 H) 1.8 (br s, 3 H) 1.7–1.8 (m, 2 H) 1.6 (d, $J = 11.9$ Hz, 3 H) 1.4–1.5 (m, 4 H) 1.3 (br s, 4 H) 1.0–1.2 (m, 4 H) 0.7–1.0 (m, 2 H). m/z calcd $C_{48}H_{61}N_5O_5$ $[M + H]^+$, 788.47; found (MS ESI), 788.30. Purity LCMS: 98.9% (254 nm, peak height).

(R)-4-(2-((3R,5R,7R)-Adamantan-1-yl)ethyl)-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)-5-(naphthalen-2-ylmethyl)piperazine-2,3-dione (10)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **10** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-D-Ala(2-naphthyl)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.4 (br s, 1 H) 7.7–7.9 (m, 2 H) (br s, 1 H) 7.5 (br s, 1 H) 7.3 (d, $J = 5.75$ Hz, 1 H) 7.0 (br s, 1 H) (d, $J = 7.0$ Hz, 1 H) 3.9 (br s, 1 H) 3.8 (d, $J = 9.0$ Hz, 1 H) 3.6 (d, $J = 14.9$ Hz, 4 H) 3.4 (br s, 9 H) 3.2 (br s, 3 H) 3.0 (br s, 2 H) 2.9 (br s, 2 H) 2.8 (d, $J = 11.9$ Hz, 2 H) 2.7 (br s, 3 H) 2.4 (br s, 2 H) 2.2 (d, $J = 6.7$ Hz, 1 H) 2.1 (br s, 1 H) 1.9 (br s, 1 H) 1.8 (br s, 3 H) 1.7 (br s, 2 H) 1.5–1.6 (m, 3 H) 1.4–1.5 (m, 3 H) 1.3 (br s, 5 H) 1.2 (br s, 1 H) 1.1 (br s, 1 H) 0.9 (br s, 2 H). m/z calcd $C_{48}H_{61}N_5O_5$ $[M + H]^+$, 788.47; found (MS ESI), 788.35. Purity LCMS: 98.3% (254 nm, peakheight).

(S)-4-(2-((3S,5S,7S)-adamantan-1-yl)ethyl)-5-butyl-1-((S)-1-((S)-2-(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-(4-hydroxyphenyl)propan-2-yl)piperazine-2,3-dione (11)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **11** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-L-tyrosine(2-Br-*Z*)-OH (R_2), Boc-L-norleucine-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. 1H NMR (400 MHz, DMSO- d_6): δ ppm 9.2 (br s, 1 H) 8.4 (br s, 1 H) 6.9 (d, $J = 7.3$ Hz, 1 H) 6.6 (d, $J = 7.3$ Hz, 1 H) 3.1 (br s, 1 H) 3.8 (d, $J = 12.8$ Hz, 1 H) 3.5–3.7 (m, 3 H) 3.3 (br s, 12 H) 3.2 (d, $J = 10.9$ Hz, 1 H) 2.9 (d, $J = 11.6$ Hz, 2 H) 2.8 (br s, 1 H) 2.8 (br s, 1 H) 2.7 (br s, 3 H) 2.4 (br s, 1 H) 2.3 (d, $J = 7.8$ Hz, 1 H) 1.9 (br s, 3 H) 1.7–1.9 (m, 1 H) 1.5–1.7 (m, 6 H)

1.5 (br s, 6 H) 1.4 (br s, 5 H) 1.3 (br s, 6 H) 0.9 (d, $J = 8.6$ Hz, 2 H). m/z calcd $C_{41}H_{61}N_6O_5$ $[M + H]^+$, 704.47; found (MS ESI), 704.35. Purity LCMS: 99.8% (254 nm, peak height).

(S)-4-(2-((3S,5S,7S)-Adamantan-1-yl)ethyl)-5-(4-hydroxybenzyl)-1-((S)-1-(4-hydroxyphenyl)-3-((S)-2-(((R)-6-(naphthalen-2-ylmethyl)-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)-piperazine-2,3-dione (12)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **12** was synthesized using the following reagents: Boc-D-Ala(2-naphthyl)-OH (R_1), Boc-L-tyrosine(2-Br-Z)-OH (R_2), Boc-L-tyrosine(2-Br-Z)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.5 (br s, 1 H) 7.9–7.9 (m, 3 H) 7.6–7.7 (d, $J = 6.5$ Hz, 1 H) 7.5 (br s, 2 H) 7.0 (d, $J = 7.4$ Hz, 1 H) 6.9 (d, $J = 7.3$ Hz, 2 H) 6.6–6.7 (m, 1 H) 6.5 (br s, 1 H) 3.8 (br s, 4 H) 3.7 (br s, 1 H) 3.4 (br s, 19 H) 23.0 (d, $J = 11.5$ Hz, 3 H) 2.8 (br s, 2 H) 2.7 (br s, 8 H) 2.3 (br s, 2 H) 1.9 (br s, 1 H) 1.6 (br s, 1 H) 1.6 (br s, 1 H) 1.4 (br s, 1 H) 1.2 (br s, 2 H) 1.0 (br s, 2 H). m/z calcd $C_{52}H_{61}N_5O_6$ $[M + H]^+$, 852.46; found (MS ESI), 852.25. Purity LCMS: 95.1% (254 nm, peak height).

(R)-4-(2-((3R,5R,7R)-Adamantan-1-yl)ethyl)-5-(4-hydroxybenzyl)-1-((S)-1-(4-hydroxyphenyl)-3-((S)-2-(((R)-6-(naphthalen-2-ylmethyl)-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)-piperazine-2,3-dione (13)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **13** was synthesized using the following reagents: Boc-D-Ala(2-naphthyl)-OH (R_1), Boc-L-tyrosine(2-Br-Z)-OH (R_2), Boc-D-tyrosine(2-Br-Z)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.6 (br s, 1 H) 7.8–7.9 (m, 3 H) 7.6 (d, $J = 7.6$ Hz, 1 H) 7.6 (br s, 2 H) 7.0 (d, $J = 7.5$ Hz, 1 H) 6.9 (d, $J = 7.3$ Hz, 2 H) 6.5–6.7 (m, 3 H) 3.8 (br s, 1 H) 3.7 (br s, 1 H) 3.4–3.6 (m, 4 H) 3.2–3.4 (m, 3 H) 3.2 (br s, 2 H) 2.9–3.1 (m, 3 H) 2.8 (br s, 1 H) 2.5–2.7 (m, 5 H) 2.3–2.5 (m, 3 H) 2.2 (br s, 1 H) 2.4 (br s, 1 H) 1.9 (br s, 3 H) 1.6 (br s, 5 H) 1.6 (d, $J = 11.0$ Hz, 4 H) 1.4 (br s, 5 H) 1.3 (br s, 1 H) 1.2 (br s, 2 H) 1.0 (br s, 1 H). m/z calcd $C_{52}H_{61}N_5O_6$ $[M + H]^+$, 852.46; found (MS ESI), 852.35. Purity LCMS: 96.6% (254 nm, peak height).

(S)-4-(2-((3S,5S,7S)-Adamantan-1-yl)ethyl)-1-((S)-1-(4-hydroxyphenyl)-3-((S)-2-(((R)-6-(naphthalen-2-ylmethyl)-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)-5-(naphthalen-2-ylmethyl)piperazine-2,3-dione (14)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **14** was synthesized using the following reagents: Boc-D-Ala(2-naphthyl)-OH (R_1), Boc-L-tyrosine(2-Br-Z)-OH (R_2), Boc-L-Ala(2-naphthyl)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.0 (br s, 1 H) 7.9–8.1 (m, 2 H) 7.8 (d, $J = 13.0$ Hz, 1 H) 7.6–7.7 (m, 2 H) 7.5–7.6 (m, 2 H) 7.4–7.5 (m, 2 H) 7.4 (br s, 1 H) 6.9–7.1 (m, 1 H) 6.7–6.8 (m, 1 H) 6.6 (d, $J = 7.5$ Hz, 2 H) 4.1 (br s, 1 H) 3.4–3.7 (m, 5 H) 3.3 (br s, 8 H) 3.1–3.3 (m, 3 H) 3.0–3.1 (m, 2 H) 2.9 (br s, 1 H) 2.8 (br s, 1 H) 2.6 (br s, 4 H) 2.4 (d, $J = 8.4$ Hz, 2 H) 2.2 (br s, 1 H) 2.1 (d, $J = 11.3$ Hz, 1 H) 1.2 (br s, 1 H) 1.99 (br s, 2 H) 1.7–1.8 (m, 2 H) 1.6 (d, $J = 11.4$ Hz, 3 H) 1.5 (d, $J = 11.1$ Hz, 3 H) 1.3 (br s, 4 H) 1.3

(d, $J = 14.6$ Hz, 1 H) 1.2 (br s, 1 H). m/z calcd $C_{56}H_{63}N_5O_5$ $[M + H]^+$, 886.48; found (MS ESI), 886.40. Purity LCMS: 99.8% (254 nm, peak height).

(R)-4-(2-((3R,5R,7R)-Adamantan-1-yl)ethyl)-1-((S)-1-(4-hydroxyphenyl)-3-(((R)-6-(naphthalen-2-ylmethyl)-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)-5-(naphthalen-2-ylmethyl)piperazine-2,3-dione (15)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **15** was synthesized using the following reagents: Boc-D-Ala(2-naphthyl)-OH (R_1), Boc-L-tyrosine(2-Br-Z)-OH (R_2), Boc-D-Ala(2-naphthyl)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. 1H NMR (400 MHz, DMSO- d_6): δ ppm 8.6 (br s, H) 7.8–7.9 (m, 6 H) 7.5–7.6 (m, 2 H) 7.5 (br s, 2 H) 7.4 (br s, 1 H) 7.3 (d, $J = 8.2$ Hz, 1 H) 7.0 (d, $J = 6.7$ Hz, 2 H) 6.7 (d, $J = 7.2$ Hz, H) 4.0 (br s, 1 H) 3.8 (br s, 1 H) 3.5–3.7 (m, 1 H) 3.5 (d, $J = 12.0$ Hz, 3 H) 3.3 (d, $J = 12.2$ Hz, 3 H) 3.2 (br s, 2 H) 2.8–3.1 (m, 3 H) 2.8 (br s, 3 H) 2.7 (br s, 2 H) 2.6 (d, $J = 14.31$ Hz, 3 H) 2.4–2.5 (m, 1 H) 2.1–2.4 (m, 1 H) 1.8 (br s, 3 H) 1.9 (br s, 1 H) 1.5–1.7 (m, 5 H) 1.4–1.5 (m, 5 H) 1.3 (br s, 5 H) 1.2 (d, $J = 11.7$ Hz, 1 H) 1.1 (br s, 1 H). m/z calcd $C_{56}H_{63}N_5O_5$ $[M + H]^+$, 886.48; found (MS ESI), 886.30. Purity LCMS: 99.6% (254 nm, peak height).

(S)-4-(2-(Adamantan-1-yl)ethyl)-5-butyl-1-((S)-1-(4-hydroxyphenyl)-3-(((S)-2-(((R)-6-(naphthalen-2-ylmethyl)-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)piperazine-2,3-dione (16)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **16** was synthesized using the following reagents: Boc-D-Ala(2-naphthyl)-OH (R_1), Boc-L-tyrosine(2-Br-Z)-OH (R_2), Boc-L-norleucine-OH (R_3), and 1-adamantaneacetic Acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. 1H NMR (400 MHz, DMSO- d_6): δ 8.56 (d, $J = 5.01$ Hz, 1 H) 7.8–8.0 (m, 3 H) 7.5–7.6 (m, 2 H) 7.0 (m, $J = 8.1$ Hz, 2 H) 6.6 (m, $J = 8.1$ Hz, 2 H) 3.8 (br s, 1 H) 3.6–3.8 (m, 3 H) 3.5 (d, $J = 10.4$ Hz, 3 H) 3.2–3.5 (m, 8 H) 2.9–3.2 (m, 4 H) 2.7–2.9 (m, 4 H) 2.7 (d, $J = 6.5$ Hz, 1 H) 2.6 (d, $J = 11.6$ Hz, 1 H) 2.2–2.5 (m, 2 H) 1.9–2.1 (m, 3 H) 1.5–1.8 (m, 10H) 1.5 (br s, 5 H) 1.3 (br s, 5 H) 0.9 (br s, 3 H) ^{13}C NMR (100 MHz, DMSO- d_6) δ 158.3, 157.9, 1557.5, 156.4, 156.1, 135.7, 133.6, 132.4, 129.9, 128.7, 128.5, 128.3, 128.2, 127.9, 126.7, 126.2, 115.5, 56.1, 54.8, 53.7, 42.1, 41.5, 40.7, 40.5, 37.0, 34.7, 32.0, 30.8, 28.4, 23.4, 22.5, 14.3. m/z calcd $C_{49}H_{63}N_5O_5$ $[M + H]^+$, 802.49; found (MS ESI), 802.3. Purity LCMS: 99% (254 nm, peak height)

(5R)-4-(2-((1R,3R)-Adamantan-1-yl)ethyl)-5-(4-hydroxybenzyl)-1-((2R)-1-(2-(((R)-6-(4-hydroxybenzyl)-2,3-dioxopiperazin-1-yl)-methyl)pyrrolidin-1-yl)-3-(naphthalen-2-yl)propan-2-yl)piperazine-2,3-dione. (17)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **17** was synthesized using the following reagents: Boc-D-tyrosine(2-Br-Z)-OH (R_1), Boc-D-2-naphthylalanine-OH (R_2), Boc-D-tyrosine(2-Br-Z)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. 1H NMR (400 MHz, DMSO- d_6): δ 8.5 (br s, 2 H) 7.8–7.9 (m, 5 H) 7.4–7.5 (m, 5 H) 7.2 (d, $J = 8.1$ Hz, 2H) 7.0 (br s, 2H) 6.9 (d, $J = 7.2$ Hz, 4 H) 6.7 (d, $J = 6.5$ Hz, 7H) 3.9

(d, $J = 13.6$ Hz, 2 H) 3.8 (br s, 2 H) 3.7 (br s, 2 H) 3.5 (s, 3 H) 3.5 (s, 2 H) 3.4 (br s, 3 H) 3.3 (br s, 2 H) 3.2 (br s, 3 H) 3.0 (t, $J = 14.6$ Hz, 4 H) 2.9 (d, $J = 13.1$ Hz, 3 H) 2.7–2.9 (m, 7 H) 2.3–2.5 (m, 3 H) 2.3 (d, $J = 8.19$ Hz, 2 H) 1.8 (br s, 7 H) 1.6–1.8 (m 10 H) 1.4–1.6 (m, 6 H) 1.3 (br s, 10 H) 1.2 (br s, 2 H) 1.1 (br s, 2 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 158.4, 157.9, 157.1, 156.8, 156.4, 133.5, 132.2, 130.7, 130.4, 128.3, 127.9, 127.7, 127.7, 127.1, 126.5, 125.9, 116.0, 115.9, 64.2, 58.7, 56.3, 54.8, 53.6, 41.9, 41.1, 40.7, 40.5, 37.0, 35.9, 34.7, 31.8, 28.3, 27.8, 23.3. m/z calcd $\text{C}_{44}\text{H}_{65}\text{N}_5\text{O}_5$ $[\text{M} + \text{H}]^+$, 744.51; found (MS ESI), 744.25. Purity LCMS: 98% (254 nm, peak height).

(S)-1((R)-1((S)-2(((S)-6-Benzyl-2,3-dioxopiperazin-1-yl)methyl)-pyrrolidin-1-yl)-3-cyclohexylpropan-2-yl)-5-(4-hydroxybenzyl)-4-phenethylpiperazine-2,3-dione (18)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic

diketopiperazines, compound **18** was synthesized using the following reagents: Boc-L-phenylalanine-OH (R_1), Boc-D-cyclohexylalanine-OH (R_2), Boc-L-tyrosine(2-Br- Z)-OH (R_3), and phenylacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.80–1.05 (m, 2 H) 1.08–1.20 (m, 3 H) 1.24 (br s, 2 H) 1.50–1.71 (m, 6 H) 1.78–1.96 (m, 2 H) 1.97–2.13 (m, 2 H) 2.55–2.65 (m, 2 H) 2.67–2.88 (m, 6 H) 2.90–3.04 (m, 3 H) 3.11 (d, $J = 12.96$ Hz, 1 H) 3.19–3.32 (m, 2 H) 3.37 (br s, 1 H) 3.52 (dd, $J = 13.08$, 3.30 Hz, 2 H) 3.65 (d, $J = 10.03$ Hz, 2 H) 3.70–3.94 (m, 2 H) 6.73 (m, $J = 8.07$ Hz, 2 H) 7.01 (m, $J = 8.07$ Hz, 2 H) 7.15–7.31 (m, 9 H) 8.50 (d, $J = 5.01$ Hz, 1 H) ^{13}C NMR (101 MHz, DMSO- d_6) δ ppm 22.74 (s, 1 C) 26.13 (s, 1 C) 26.30 (s, 1 C) 26.56 (s, 1 C) 29.54 (s, 1 C) 32.83 (s, 1 C) 33.55 (s, 1 C) 33.63 (s, 1 C) 34.51 (s, 1 C) 37.14 (s, 1 C) 37.51 (s, 1 C) 39.40 (s, 1 C) 39.61 (s, 1 C) 39.82 (s, 1 C) 40.03 (s, 1 C) 40.23 (s, 1 C) 40.45 (s, 1 C) 40.66 (s, 1 C) 48.41 (s, 1 C) 55.86 (s, 1 C) 58.51 (s, 1 C) 62.76 (s, 1 C) 115.89 (s, 1 C) 126.79 (s, 1 C) 127.11 (s, 1 C) 127.76 (s, 1 C) 128.81 (s, 1 C) 128.98 (s, 1 C) 129.13 (s, 1 C) 129.77 (s, 1 C) 130.35 (s, 1 C) 137.72 (s, 1 C) 139.29 (s, 1 C) 156.65 (s, 1 C) 156.72 (s, 1 C) 157.34 (s, 1 C) 157.80 (s, 1 C) 157.95 (s, 1 C). m/z calcd $\text{C}_{44}\text{H}_{55}\text{N}_5\text{O}_5$ $[\text{M} + \text{H}]^+$, 734.42; found, 734.15 (MALDI), 734.15 (MS ESI) Purity LCMS: 99.0% (254 nm, peak height).

(S)-5-Benzyl-1-((R)-1-cyclohexyl-3-((S)-2(((S)-2,3-dioxo-6-propylpiperazin-1-yl)methyl)pyrrolidin-1-yl)propan-2-yl)-4-phenethylpiperazine-2,3-dione. (19)—

Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **19** was synthesized using the following reagents: Boc-L-norvaline-OH (R_1), Boc-D-cyclohexylalanine-OH (R_2), Boc-L-phenylalanine-OH (R_3), and phenylacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, DMSO- d_6): δ 8.4 (br s, 1 H) 7.3 (br s, 2 H) 7.3 (br s, 3 H) 7.2 (s, 2 H) 7.2 (s, 3 H) 3.7 (br s, 3 H) 3.4–3.7 (m, 2 H) 3.2 (d, $J = 11.86$ Hz, 2 H) 3.0 (br s, 1 H) 2.9 (br s, 1 H) 2.8 (br s, 1 H) 2.7 (br s, 3 H) 2.6 (br s, 2 H) 2.1 (br s, 2 H) 1.9 (br s, 2 H) 1.6 (br s, 5 H) 1.6 (br s, 4 H) 1.3 (br s, 4 H) 1.2 (br s, 4 H) 1.0 (br s, 1 H) 0.8–1.0 (m, 4 H) 0.8 (br s, 1 H) ^{13}C NMR (100 MHz, DMSO- d_6) δ 158.0, 157.4, 156.8, 139.3, 137.9, 129.4, 129.1, 128.8, 127.2, 126.7, 62.9, 56.8, 55.6, 53.9, 51.2, 48.3, 41.0, 40.7, 38.0, 37.7, 34.5, 33.7, 33.6, 32.8, 29.6, 26.6, 26.3, 26.1, 22.9, 19.4, 14.2. m/z calcd $\text{C}_{40}\text{H}_{55}\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$, 670.43; found (MS ESI), 670.20. Purity LCMS: 99% (254 nm, peak height).

(S)-4-(2-(Adamantan-1-yl)ethyl)-1-((S)-1-((S)-2-(((S)-6-benzyl-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-phenylpropan-2-yl)-5-(4-hydroxybenzyl)piperazine-2,3-dione (20)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **20** was synthesized using the following reagents: Boc-L-phenylalanine-OH (R_1), Boc-L-phenylalanine-OH (R_2), Boc-L-tyrosine(2-Br-Z)-OH (R_3), and 1-adamantaneacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/30, 40/65. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 8.54 (d, $J = 5.13$ Hz, 1 H) 7.09–7.34 (m, 6 H) 7.02 (d, $J = 6.97$ Hz, 3 H) 6.76 (d, $J = 8.07$ Hz, 2 H) 3.88 (d, $J = 10.51$ Hz, 1 H) 3.66–3.83 (m, 2 H) 3.42–3.63 (m, 4 H) 3.29–3.42 (m, 7 H) 3.22 (d, $J = 12.10$ Hz, 2 H) 3.12 (br s, 1 H) 2.75–3.02 (m, 6 H) 2.61 (br s, 2 H) 2.38 (d, $J = 7.46$ Hz, 2 H) 2.25 (q, $J = 8.15$ Hz, 1 H) 1.86–2.02 (m, 3 H) 1.74–1.86 (m, 1 H) 1.61–1.73 (m, 4 H) 1.48–1.60 (m, 3 H) 1.38 (br s, 5 H) 1.06–1.32 (m, 2 H). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ ppm 176.94 (s, 1 C) 176.50 (s, 1 C) 174.17 (s, 1 C) 165.03 (s, 1 C) 158.36 (s, 1 C) 157.84 (s, 1 C) 156.77 (s, 1 C) 156.40 (s, 1 C) 144.44 (s, 1 C) 138.69 (s, 1 C) 137.91 (s, 1 C) 137.20 (s, 1 C) 130.46 (s, 1 C) 129.78 (s, 1 C) 129.08 (s, 1 C) 128.79 (s, 1 C) 128.72 (s, 1 C) 127.73 (s, 1 C) 127.20 (s, 1 C) 126.74 (s, 1 C) 115.97 (s, 1 C) 114.23 (s, 1 C) 63.97 (s, 1 C) 58.48 (s, 1 C) 56.35 (s, 1 C) 54.85 (s, 1 C) 41.96 (s, 1 C) 41.13 (s, 1 C) 40.66 (s, 1 C) 40.45 (s, 1 C) 40.23 (s, 1 C) 40.03 (s, 1 C) 39.82 (s, 1 C) 39.61 (s, 1 C) 39.40 (s, 1 C) 36.98 (s, 1 C) 36.89 (s, 1 C) 31.86 (s, 1 C) 28.37 (s, 1 C) 23.36 (s, 1 C). m/z calcd $\text{C}_{48}\text{H}_{59}\text{N}_5\text{O}_5$ $[\text{M} + \text{H}]^+$, 786.46; found, 787.0037 (MALDI), 786.15 (MS ESI). Purity LCMS: 98.0% (254 nm, peak height).

(S)-5-Benzyl-1-((R)-1-((S)-2-(((S)-6-benzyl-2,3-dioxopiperazin-1-yl)methyl)pyrrolidin-1-yl)-3-cyclohexylpropan-2-yl)-4-phenethylpiperazine-2,3-dione (21)—Using general scheme (Scheme 1) for the synthesis of bis-cyclic diketopiperazines, compound **21** was synthesized using the following reagents: Boc-L-phenylalanine-OH (R_1), Boc-D-cyclohexylalanine-OH (R_2), Boc-L-phenylalanine-OH (R_3), and phenylacetic acid (R_4). The final crude product was purified using HPLC as described above, with a gradient of (B) 0/5, 2/5, 4/20, 40/55. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 8.5 (br s, 2 H) 7.4 (d, $J = 7.1$ Hz, 4 H) 7.2–7.3 (m, 17 H) 7.2 (d, $J = 7.2$ Hz, 6 H) 3.7–3.9 (m, 5 H) 3.7 (d, $J = 9.4$ Hz, 2 H) 3.5 (d, $J = 12.7$ Hz, 3 H) 3.3 (d, $J = 12.6$ Hz, 4 H) 3.1 (d, $J = 12.8$ Hz, 2 H) 2.9–3.0 (m, 7 H) 2.7–2.9 (m, 11 H) 2.6 (br s, 4 H) 2.0–2.2 (m, 4 H) 1.9 (d, $J = 11.74$ Hz, 4 H) 1.5–1.7 (m, 12 H) 1.2 (br s, 5 H) 1.2 (br s, 6 H) 0.8–1.1 (m, 4 H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 158.0, 157.8, 157.3, 156.8, 139.2, 137.9, 137.7, 129.8, 129.4, 129.1, 129.0, 128.8, 127.3, 127.1, 126.8, 62.8, 58.5, 55.6, 53.8, 51.2, 48.4, 40.7, 40.5, 37.9, 37.8, 37.5, 34.5, 33.6, 33.5, 32.8, 29.6, 26.6, 26.3, 26.1, 22.8; m/z calcd $\text{C}_{44}\text{H}_{55}\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$, 718.43; found (MS ESI), 718.20. Purity LCMS: 98.0% (254 nm, peak height).

SAR Studies

Test compounds^{57,58} were solubilized in 3% DMSO/ H_2O and added to polypropylene 384-well plates (Greiner catalogue no. 781280). ADAM10 and 17 glycosylated substrate assays followed the same general protocol. Then 5 μL of $3\times$ enzyme solution (30 nM) in assay buffer (10 mM Hepes, 0.001% Brij-35, pH 7.5) were added to solid bottom white 384 low volume plates (Nunc catalogue no. 264706). Test compounds and pharmacological assay control were prepared as 10-point, 1:3 serial dilutions starting at 300 μM , then added to the

cells (5 μL per well) using the Biomek NX^P. After 30 min incubation at RT the reactions were started by addition of 5 μL of 3 $\times$ solutions of substrate (30 μM). Fluorescence was measured every 30 min for 2 h using the multimode microplate reader Synergy H4 (Biotek Instruments, Winooski, VT) using $\lambda_{\text{excitation}} = 360 \text{ nm}$ and $\lambda_{\text{emission}} = 460 \text{ nm}$. Rates of hydrolysis were obtained from plots of fluorescence versus time, and inhibition was calculated using rates obtained from wells containing substrates only (100% inhibition) and substrates with enzyme (0% inhibition). Three parameters were calculated on a per-plate basis, (a) the signal-to-background ratio (S/B), (b) the coefficient for variation [CV; CV = (standard deviation/mean) $\times$ 100] for all compound test wells, and (c) the Z' -factor.⁵⁹ The IC₅₀ value of the pharmacological control ((*N*-hydroxy-1-(4-methoxyphenyl)sulfonyl-4-(4-biphenylcarbonyl)piperazine-2-carboxamide, Calbiochem catalogue no. 444252) was also calculated to ascertain the assay robustness.

MMP assays

MMP assays followed the same general protocol. First 5 μL of 3 $\times$ enzyme solution (3 nM for MMP-8 and -14, 6 nM for MMP-2 and -9) in TCB assay buffer (50 mM Tricine, 50 mM NaCl, 10 mM CaCl₂, 0.005% Brij-35, pH 7.5) were added to solid bottom white 384 low volume plates (Nunc catalogue no. 264706). Test compounds and pharmacological assay control (marimastat) were prepared as 10-point, 1:3 serial dilutions starting at 300 μM , then added to the cells (5 μL per well) using the Biomek NX^P. After 30 min incubation at RT, the reactions were started by addition of 5 μL of 3 $\times$ solutions of Mca-KLPGL-Dnp-AR substrate (30 μM , R&D Systems catalogue no. ES010). Fluorescence was measured after 1 h incubation at RT using the multimode microplate reader Synergy H4 (Biotek Instruments, Winooski, VT) using $\lambda_{\text{excitation}} = 324 \text{ nm}$ and $\lambda_{\text{emission}} = 393 \text{ nm}$. Inhibition was calculated using RFU values obtained from wells containing substrate only (100% inhibition) and substrates with enzyme (0% inhibition). Three parameters were calculated on a per-plate basis: (a) the signal-to-background ratio (S/B), (b) the coefficient for variation [CV; CV = (standard deviation/mean) $\times$ 100] for all compound test wells, and (c) the Z' -factor.⁵⁹ The IC₅₀ value of the pharmacological control marimastat ((2*S*,3*R*)-*N*4-[(1*S*)-2,2-dimethyl-1-[(methylamino)carbonyl]propyl]-*N*1,2-dihydroxy-3-(2-methylpropyl)butanediamide, Tocris catalogue no. 2631) was also calculated to ascertain the assay robustness.

CellTiter Glo Cell Viability Assay

Compounds were solubilized in 3% DMSO/H₂O and added to polypropylene 384-well plates (Greiner catalogue no. 781280). Then 1250 of CHO-K1, HEPG2, MDA-MB-438, M14, or A549 cells were plated in 384-well plates in 5 μL of serum-free media (EMEM for HEPG2, DMEM/F12 for MDA-MB-438, F12 for CHO-K1 and A549). Test compounds and gefitinib (pharmacological assay control) were prepared as 10-point, 1:3 serial dilutions starting at 300 μM , then added to the cells (5 μL per well) using the Biomek NX^P. Plates were incubated for 72 h at 37 °C, 5% CO₂, and 95% RH. After incubation, 5 μL of CellTiter-Glo (Promega catalogue no. G7570) were added to each well and incubated for 15 min at room temperature. Luminescence was recorded using a Biotek Synergy H4 multimode microplate reader. Viability was expressed as a percentage relative to wells containing cells treated with 100 μM gefitinib (0%) and wells containing cells treated with 1% DMSO only (100%). Three parameters were calculated on a per-plate basis: (a) the

signal-to-background ratio (S/B), (b) the coefficient for variation [CV; CV = (standard deviation/mean) $\times$ 100)] for all compound test wells, and (c) the Z' -factor (18). The IC₅₀ value of the pharmacological control (gefitinib, LC Laboratories no. G-4408) was also calculated to ascertain the assay robustness.

Synergy Studies

First, 1250 of A549 cells were plated in 384-well plates in 5 μ L of serum-free F12 media. Next, compound 17 was added in 5 μ L of serum-free F12 media such that its final concentration in the assay was 40 μ M and incubated for 1 h at 37 °C, 5% CO₂ and 95% RH. Gefitinib, etoposide, and doxorubicin were prepared as 10-point, 1:3 serial dilutions starting at 300 μ M, then added to the cells (5 μ L per well) using the Biomek NX^P. Plates were incubated for 72 h at 37 °C, 5% CO₂ and 95% RH. After incubation, 5 μ L of CellTiter-Glo (Promega catalogue no. G7570) were added to each well and incubated for 15 min at room temperature. Luminescence was recorded using a Biotek Synergy H4 multimode microplate reader. Viability was expressed as a percentage relative to wells containing cells treated with 100 μ M gefitinib (0%) and wells containing cells treated with 1% DMSO only (100%). Three parameters were calculated on a per-plate basis: (a) the signal-to-background ratio (S/B), (b) the coefficient for variation [CV; CV = (standard deviation/mean) $\times$ 100)] for all compound test wells, and (c) the Z' -factor (18). The IC₅₀ value of the pharmacological control (gefitinib, LC Laboratories no. G-4408) was also calculated to ascertain the assay robustness.

Inhibition of TNF α Shedding

THP-1 cells were cultured in RPMI 1640 (ATCC) medium supplemented with 10% fetal bovine serum (FBS, Thermo Scientific HyClone, Logan, UT, USA), 50 U/mL penicillin, and 50 μ g/mL streptomycin (Invitrogen, CA), and 0.05 mM 2-mercaptoethanol (Invitrogen). The cell cultures were maintained in a 37 °C incubator with 5% CO₂ and under a humidified atmosphere. Protocol described in⁶⁰ was followed. Briefly, THP-1 cells were dispensed at 3000 cells/4 μ L/well in 384-well white solid bottom plates, followed by an addition of 4 μ L compound in 3% DMSO solution or 3% DMSO only. After 1 h incubation, 1 μ L LPS in assay medium at 5 μ g/mL LPS (final concentration of 1 μ g/mL) was dispensed into the assay plates and plates were incubated overnight at 37 °C with 5% CO₂ and under a humidified atmosphere. 1.5 μ L of the mixture containing acceptor beads conjugated with anti-TNF α antibody was added into each well. The assay plates were incubated in the dark at RT for 1 h, followed by the addition of 1.5 μ L of donor bead coated with streptavidin which captures the biotinylated anti-TNF α antibody. After the assay plates were incubated in the dark at RT for another 1 h, the assay plates were read in the AlphaScreen mode using Biotek Synergy H4 multimode microplate reader (Biotek Inc., VT).

Inhibition of DDR1 Shedding

Briefly, HCC1806 cells, seeded in 6-well plates, were treated overnight without (control) or with various concentrations of inhibitor **17** in serum-free media. Then 400 μ L/well of conditioned media was collected, TCA precipitated, and the resultant pellets were resolved

by reducing 12% SDS-PAGE gel followed by immunoblot analyses. The blot was probed with N-terminal DDR1 antibody (R&D Systems no. AF2396).

Inhibition of PTK7 Shedding

Cells were grown to reach a 60–90% confluence in wells of a 12-well tissue culture plate (BD Biosciences). Cells were washed with and then transferred to fresh serum-free DMEM (0.5 mL/well). PMA (50 ng/mL) and the inhibitors were added to the cells at the indicated concentrations, and incubation was continued for an additional 16 h. The medium aliquots were then withdrawn and centrifuged to remove cell debris. The supernatant was concentrated until dryness using a Speed-Vac. To remove salt, the pellet was washed using acetone, dissolved in 1% SDS, and centrifuged to remove insoluble material (14000g, 10 min). The supernatant aliquots were separated by SDS-gel electrophoresis in 4–12% NuPAGE gels (Invitrogen). The shed PTK7 was detected by Western blotting with a goat polyclonal PTK7 antibody.

Western Blot Densitometry

The immunoblots were scanned and digitized. Pixel density of the protein bands was quantified using the ImageJ software. Inhibition of shedding (%) with compound was calculated.

Inhibition is demonstrated as a percentage relative to the density of protein bands of untreated samples containing no compound (0%) and the density of background intensity (no protein band, complete inhibition, 100%).

Inhibition of Shedding of Cytokines

Tracheal smooth muscle cells (HTSMC, Promocell no. C-12565) were grown to 70% confluency in 24 well-plates. Cells were starved with growth factor free medium (smooth muscle cell basal medium, C-22262, PromoCell, Germany) supplemented with 0.5% FBS and 1% penicillin/streptavidin 24 h before stimulation. Cells were washed twice to remove growth factors and preincubated with 10 μ M inhibitor or DMSO (0.1%) or 10 μ M of GI254023X⁶¹ as inhibition control for 30 min. Supernatant was removed, and cells were washed once and then stimulated with or without 100 ng/mL LPS or 10 ng/mL of IL-1 in the absence or presence of inhibitors. Release of cytokines was measured 4 and 24 h after stimulation using ELISA kits for IL-8, FKN, and CXCL16 (R&D Systems, D8000C, DX310, and DCX160, respectively). Percent inhibition was calculated as a percentage relative to the untreated control samples containing no compound (0%).

Inhibition of Heregulin Shedding

Briefly, A549 cells were cultured in T175 flasks in F12K media supplemented by 10% FBS. For assay, cells were passaged and seeded at 5×10^6 A549 cells in T175 flasks in 20 mL of serum-free F12K media. All test and control inhibitors were added and incubated for 1 h, after which PMA was added at 500 ng/mL final concentration to stimulate heregulin shedding. All leads were tested at 40 μ M, and marimastat was tested at 5 μ M. A549 cells were incubated with PMA for 1 h, after which supernatant was collected and concentrated using Amicon Ultra-15. Cells were harvested and lysed using mammalian protein extraction

buffer (GE Healthcare no. 28-9412-79). Protein concentration was determined in both supernatants and lysates using NanoDrop (ThermoFisher), and all samples were run on SDS-PAGE using recombinant Heregulin (R&D Systems no. 5898-NR) as a control. Gels were transferred and probed by Heregulin mAb (R&D Systems no. MAB377). Lysates were probed for actin to control for protein concentration.

Inhibition of TGF α and Betacellulin Shedding

A549 cells were cultured in T175 flasks in F12K media supplemented by 10% FBS. For assay, cells were passaged and seeded at 1.3×10^4 in 24-well plates in serum-free F12K media. All test and control inhibitors were added and incubated for 1 h, after which PMA was added at 500 ng/mL final concentration to stimulate TGF α shedding. In the case of betacellulin, PMA was not added because betacellulin is cleaved constitutively. All leads were tested at 40 μ M, and marimastat was tested at 10 μ M. A549 cells were incubated with PMA for 1 h, after which supernatant was collected and concentrated using Amicons. TGF α and betacellulin were detected in supernatant by ELISA (R&D Systems catalogue nos. DTGA00 and DY261, respectively).

Invasion Studies

Invasion assay protocol has been extensively published.^{35,36,62} Briefly, we used 6.5 mm, 24-well Transwell plates with the 8 μ m pore size membranes. The membrane was coated with Matrigel. Cells (1×10^5 /well) were seeded in the serum-free media (0.1 mL) into the upper chamber. The 10% serum-containing media (0.6 mL) was added to the lower chamber as a chemoattractant. Serum-free media in both upper and lower chambers was used as a negative control. Compound **17** was added to both chambers. Cells were allowed to invade for 5–24 h. The cells on the upper membrane surface were removed with a cotton swab. The cells that migrated onto the membrane's lower surface were stained for 10 min using 0.2% crystal violet in 20% methanol/water solution (0.3 mL). The dye was extracted using 1% SDS (0.25 mL). The OD_{570 nm} values were measured using a plate reader.

Statistics

One-way analysis of variance (ANOVA) was used followed by Dunnett posthoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the James and Esther King Biomedical Research Program (2KN05 to D.M.), the National Institutes of Health (DA033985-01 to D.M., CA098799 to G.B.F., and DA031370 to R.A.H.), and by the State of Florida, Executive Office of the Governor's Office of Tourism, Trade, and Economic Development. We thank Angela Morales for her technical help.

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Abbreviations Used

ADAM	a disintegrin and metalloprotease
MMP	matrix metalloprotease
TACE	TNF α converting enzyme
DDR1	discoidin domain receptor 1

PTK7	protein tyrosine kinase 7
PMA	phorbol myristic acid
LPS	lipopolysaccharide

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Figure 1.
Results of cell viability assay with ADAM17 inhibitors. (A) Viability assay with CHO-K1 cells. (B) Viability assay with A549 cells.

Figure 2.

Results of TNF α shedding assay in THP-1 cells with ADAM17 inhibitors. (A) Single point assay. One-way analysis of variance (ANOVA) was used followed by Dunnett posthoc test. The data shown are the mean $\pm$ SEM, $n = 5$. ***** $p < 0.0001$; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (B) Dose-response assay with compounds **17** and **19**.

Figure 3. Effect of ADAM17 inhibitors on cytokine shedding in HTMS cells. (A) LPS-stimulated IL-8 shedding inhibition assay. (B) IL-1-stimulated IL-8 shedding inhibition assay. (C) Fractalkine constitutive shedding inhibition assay. One-way analysis of variance (ANOVA) was used followed by Dunnett posthoc test. The data shown are the mean $\pm$ SEM, $n = 3$ from three independent experiments. **** p value <0.0001 , *** p value <0.001 , ** p value <0.01 , * p value <0.05 . GI: control ADAM10 selective inhibitor GI254023X.

Figure 4. Results of DDR1 release assay in HCC1806 breast cancer cells with compound **17**. One, untreated control; 2, 10 μ M compound **17**; 3, 20 μ M compound **17**, 4–40 μ M compound **17**.

Figure 5. Effect of compound **17** on PTK7 shedding and invasion. (A) PTK7 shedding assay in HT1080 (fibrosarcoma) and SW480 (colorectal cancer) cells. (B) Invasion assay in HT1080 cells. sPTK7: soluble PTK7.

Figure 6.

Effect of ADAM17 inhibitors on growth factors shedding in A549 cells. (A) Effect on shedding of heregulin. M-stat: marimastat. (B) Effect on shedding of betacellulin. One-way analysis of variance (ANOVA) was used followed by Dunnett posthoc test. The data shown are the mean $\pm$ SEM, $n = 3$. *** $p < 0.001$. (C) Effect on shedding of TGF α . One-way analysis of variance (ANOVA) was used followed by Dunnett posthoc test. The data shown are the mean $\pm$ SEM, $n = 3$. *** $p < 0.001$.

Figure 7. Results of synergy studies of compound **17** with FDA-approved lung cancer drugs. (A) Synergy study with gefitinib. (B) Synergy study with etoposide. (C) Synergy study with doxorubicin.

Scheme 1. General Synthesis Procedure for Leads of Pyrrolidine-bis-diketopiperazine Series Used in Present Studies

Table 1
SAR Study Results of Individual Compounds Synthesized Based on Kinetic Studies of
Mixture Samples^a

Sample #	STRUCTURE	R ₄	ADAM10 IC ₅₀ , μM	ADAM17 IC ₅₀ , μM
Core		N/A	N/A	N/A
1		4-tert-Butyl-Cyclohexyl	>100	10 ± 1.0
2		4-adamantan-1-yl-methyl	>100	10 ± 1.3
3		Cyclohexylmethyl	>100	29 ± 2.0
4		4-Cyclobutylmethyl	>100	94 ± 8.1

Sample #	STRUCTURE	R ₄	ADAM10 IC ₅₀ , μM	ADAM17 IC ₅₀ , μM
5		2-Phenylbutyl	>100	15 ± 2.1
6		2-adamantan-1-yl-ethyl	>100	5.9 ± 0.9
7		4-Cyclopentylmethyl	>100	35 ± 3.3

^a Positions R1-3 are fixed, R4 is scanned. IC₅₀ values reported as a mean of three experiments ± standard deviation.

Table 2
SAR Study Results of Individual Compounds Synthesized Based on Positional Scan of Mixture Samples^a

Sample #	STRUCTURE	R ₃	ADAM10 IC ₅₀ , μM	ADAM17 IC ₅₀ , μM
Core		N/A	N/A	N/A
6		S-4-hydroxybenzyl	>100	5.9 ± 0.9
8		R-4-hydroxybenzyl	>100	5.4 ± 0.5
9		S-5-naphthalen-2-ylmethyl	>100	10 ± 0.8
10		R-5-naphthalen-2-ylmethyl	>100	7.9 ± 0.8

Sample #	STRUCTURE	R ₃	ADAM10 IC ₅₀ , μM	ADAM17 IC ₅₀ , μM
11		S-5-butyl	>100	6.5 ± 0.9

^aR₃ is scanned, and R₁, R₂, and R₄ are fixed. IC₅₀ values data reported as a mean of three experiments ± standard deviation.

Table 3
SAR Study Results of Individual Compounds Synthesized Based on Positional Scan of Mixture Samples^a

Sample #	STRUCTURE	R ₃	ADAM10 IC ₅₀ , μM	ADAM 17 IC ₅₀ , μM
Core		N/A	N/A	N/A
6		S-4-hydroxybenzyl	>100	5.9 ± 0.9
8		R-4-hydroxybenzyl	>100	5.4 ± 0.5
12		S-4-hydroxybenzyl	>100	8.1 ± 0.9
13		R-4-hydroxybenzyl	>100	15 ± 1.2

Sample #	STRUCTURE	R ₃	ADAM10 IC ₅₀ , μM	ADAM 17 IC ₅₀ , μM
9		S-5-naphthalen-2-ylmethyl	>100	10 ± 0.8
10		R-5-naphthalen-2-ylmethyl	>100	7.9 ± 0.8
14		S-5-naphthalen-2-ylmethyl	>100	8.4 ± 1.0
15		R-5-naphthalen-2-ylmethyl	>100	5.7 ± 1.0
11		S-5-butyl	>100	6.5 ± 0.9
16		S-5-butyl	>100	5.7 ± 0.8

^aR3 is scanned, and R1, R2, and R4 are fixed. IC₅₀ values data reported as a mean of three experiments $\pm$ standard deviation.

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Table 4
Summary of Selectivity Testing of Novel ADAM17 Inhibitors against a Panel Zinc of Metalloproteases^a

ID	Structure	MMP2	MMP8	MMP9	MMP14	ADAM10	ADAM17
16		>100	>100	>100	>100	>100	5.7 (100)
17		NT	>100 (13)	NT	>100 (25)	>100	4.2 (95)
18		>100	>100 (28)	>100 (13)	>100 (27)	>100	5.4 (100)

ID	Structure	MMP2	MMP8	MMP9	MMP14	ADAM10	ADAM17
19		>100	>100	>100	>100	>100	4.3 (100)
20		>100	>100	>100	>100	>100	11 (100)
21		>100	>100	>100	>100	>100	8.5 (100)

^a All results are IC₅₀ (μM) (%inhibition at 100 μM). No parentheses indicates 0% inhibition at 100 μM. NT: not tested.

Table 5
Summary of Cell-Based Testing of Novel ADAM17 Inhibitors for Suppression of Shedding of Cell-Surface Proteins^a

target ID	cell line	[C] tested (μ M)	16	17	18	19	20	21
TNF α	THP1	25	57	43	36	60	44	0
PTK7	HT1080	25–100	0	25–50	0	0	NT	NT
PTK7	SW480	25–100	0	0–82	0	0	NT	NT
DDR1	HCC1806	10–40	NT	59–81	NT	NT	NT	NT
IL-8	HTSMC	10	62	36	18	NT	62	NT
FKN	HTSMC	10	0	0	0	NT	0	NT
CXCL16	HTSMC	10	0	0	0	NT	0	NT
Heregulin	A549	40	0	100	0	28	0	34
TGF α	A549	40	0	0	0	0	0	0
BTC	A549	40	0	0	0	NT	0	0
Notch ^b	SKMEL-28	25	NT	0	NT	NT	NT	NT
Neprilysin ⁶³	Ea.hy926	15	NT	NT	62	38	NT	NT

^aData reported as inhibition, %. NT: not tested.

^bPersonal communication from Dr. Kaushik (Kansas University Medical Center).