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Structure-guided discovery of 1,3,5 tri-substituted benzenes as potent and selective matriptase inhibitors exhibiting in vivo antitumor efficacy

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ABSTRACT

Matriptase is a serine protease implicated in cancer invasion and metastasis. Expression of matriptase is frequently dysregulated in human cancers and matriptase has been reported to activate latent growth factors such as hepatocyte growth factor/scatter factor, and proteases such as urokinase plasminogen activator suggesting that matriptase inhibitors could have therapeutic potential in treatment of cancer. Here we report a structure-based approach which led to the discovery of selective and potent matriptase inhibitors with benzene as central core having 1,3,5 tri-substitution pattern. X-ray crystallography of one of the potent analogs in complex with matriptase revealed strong hydrogen bonding and salt-bridge interactions in the S1 pocket, as well as strong CH–π contacts between the P2/P4 cyclohexyl and Trp215 side-chain. An additional interaction of the pendant amine at cyclohexyl with Gln175 side-chain results in substantial improvement in matriptase inhibition and selectivity against other related serine proteases. Compounds **15** and **26** showed tumor growth inhibition in a subcutaneous DU-145 prostate cancer mouse model. These compounds could be useful as tools to further explore the biology of matriptase as a drug target.

1. Introduction

According to the American Cancer Society, cancer is the second principal cause of death in the U.S. with nearly half of all male and one-third of all female population developing some form of cancer during their life spans.[1] Number of cancer deaths worldwide is projected to increase, with an anticipated 13 million deaths in 2030.[2]

Patients with cancer die largely because of metastasis, rapid creation of abnormal cells that grow beyond their usual boundaries and spread from one organ to another. Proteases are involved in cancer spread by mediating the degradation of basement membrane and extracellular matrix constituents thus facilitating migration of cells into the extracellular environment. Matriptase is a type II transmembrane serine protease broadly expressed in epithelial tissues and commonly overexpressed in many tumor types,[3,4] such as in prostate adenocarcinomas.[5,6] The oncogenic and metastatic activities of matriptase derive from its ability to degrade extracellular matrix and to cleave and activate various pro-oncogenic and pro-metastatic factors including inactive precursor of hepatocyte growth factor/scatter factor (pro-HGF/SF).[7] The potent oncogenic activity of matriptase was demonstrated using a transgenic mice model in which mild overexpression of

Abbreviations: Ac, acetyl; Boc, *tert*-butoxycarbonyl; DCM, dichloromethane; DIPEA, di-iso-propylethylamine; DMF, *N*,*N*-dimethylformamide; DMAP, 4-dimethylaminopyridine; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EtOAc, ethyl acetate; EtOH, ethanol; Et_2O, diethyl ether; HOBt, 1-*N*-hydroxybenzotriazole; Py, pyridine; SAR, structure–activity relationship; HPLC, high pressure liquid chromatography; LCMS, liquid chromatography–mass spectrometry; MeOH, methanol; mp, melting point; rt, room temperature; TEA, triethylamine; TLC, thin layer chromatography; THF, tetrahydrofuran; ADME, absorption distribution metabolism and excretion; PPB, plasma protein binding; LLE, ligand-lipophilicity efficiency; CYP, cytochrome P450 enzymes; MLM, mouse liver microsomes; RLM, rat liver microsomes; HLM, human liver microsomes.

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0968-0896/

matriptase in the skin resulted in spontaneous squamous cell carcinoma and a very high susceptibility to carcinogen-induced tumor formation.[8] Interestingly, the oncogenic activity of matriptase was completely negated when HAI-1, the endogenous inhibitor of matriptase, was simultaneously overexpressed in the transgenic mice.[8] Matriptase also activates other serine proteases such as urokinase-type plasminogen activator (uPA) that plays a vital role in angiogenesis, tumor invasion and metastasis. Downregulation of matriptase has also been shown to inhibit tumor invasion through suppression of uPAR activation.[9,10] Taken together, the available data demonstrate that matriptase is functionally engaged in tumor progression and thus represents a potential target for anti-cancer therapy.

In the past decade, potent and selective matriptase inhibitors from diverse chemical classes (Fig. 1) including bis-benzamidines,[11] amidinophenylalanines[12–14] and peptidomimetics[15] have been reported. A simple bis-benzamidine compound such as **1** (Fig. 1) is known to inhibit matriptase with a K_i of ~200 nM and >10 fold selectivity over uPA and thrombin.[11] A very potent tribasic matriptase inhibitor **2** (Fig. 1) with a K_i of 80 pM and >100 fold selectivity over related serine proteases including plasmin, uPA, thrombin and factor Xa was reported by Curacyte.[13] Recently, a peptidomimetic[15] has also been demonstrated to inhibit matriptase with a K_i of 11 nM with several fold selectivity over other tested serine proteases including thrombin. However, only limited information is available on the activity of selective small molecule matriptase inhibitors (**3**,[12] **4**[12] and **5**[16]) in the cellular and in vivo tumor models.

We have previously identified selective matriptase inhibitors[17] through structure-guided design strategies based on the pyridyl bis(oxy) dibenzimidamide scaffold. Notably, these compounds exhibited selective target inhibition, favorable aqueous solubility and microsomal stability along with good potencies on prostate carcinoma cell lines.[17] However, only limited SAR was discussed in that report.[17] In this article, we report extensive SAR of structurally related matriptase inhibitors identified through structure-guided design approach. In addition, we have also discussed about the in vivo efficacies of the two early lead compounds from this series.

During optimization of the chemical series described previously,[17] we were able to solve a high resolution co-crystal structure of one of the preliminary hits (compound **6**, matriptase K_i = 218 nM) in complex with matriptase catalytic domain (PDB ID: 4JZ1).[17] Apart from providing information on the different target recognition moieties, this crystal structure also revealed interesting opportunities for further SAR exploration. The binding mode of compound **6** showed that there is sufficient space available around 4th and 5th positions of the pyridyl core to attempt substitutions (Fig. 2). In addition, since the pyridyl nitrogen was not engaged in any polar contacts, we decided to replace this nitrogen with CH as a benzene core (Fig. 2).

This article describes the synthesis of compounds from a chemical series with benzene as central core having 1,3,5 tri-substitution pattern (prepared as shown in Schemes 1–3) as potent and selective inhibitors. SAR was explored for achieving greater selectivity for matriptase over other trypsin-like serine proteases. Two of the early leads exhibited potent inhibition of matriptase with selectivity over related serine proteases. Furthermore, the lead compounds displayed inhibition of invasion, migration and matrix-independent growth in cellular assays and a desirable DMPK profile, which in turn contributed to significant tumor growth inhibition in a xenograft model of prostate cancer.

2. Chemistry

The synthetic scheme for the preparation of 3,5-bis(4-carbamimidoylphenoxy)phenyl derivatives is outlined in Scheme 1.

The commercially available ethyl 3,5-dihydroxybenzoate (**44**) was treated with 4-fluorobenzonitrile (4 equiv) to afford ethyl 3,5-bis(4-cyanophenoxy)benzoate (**45**), followed by its hydrolysis with LiOH to get 3,5-bis(4-cyanophenoxy)benzoic acid (**46**), which was subjected to *Curtius Rearrangement* using diphenylphosphorylazide[18] to afford 4,4′-((5-amino-1,3-phenylene)bis(oxy))dibenzonitrile (**47**). Acid **46** was coupled with commercially available amines using EDCI to give an amide **48**. Treatment of nitrile groups of **48** with aq NH_2OH[17] in ethanol converted it to *N*-hydroxyamidine (**49**), followed by acetylation and reduction with zinc afforded amidine (**51**). The amino group (if present as Boc-protected) or hydroxyl group (if present as THP-protected) of **51** was deprotected using ethanolic-HCl to obtain **9**–**22**. The inverted-amide analogs (**23**–**27**) were synthesized by the coupling of **47** with a carboxylic acid, followed by its nitrile group conversion to the amidine and then deprotection of amino/hydroxyl group to give the desired compounds.

The sulfonamide analogs (**28**–**29**) were synthesized (Scheme 1) by treating **47** with an arylsulfonyl chloride and then conversion of their nitrile group to amidine provide the 4,4′-((5-(sulfonamido)-1,3-phenylene)bis(oxy))dibenzimidamide derivatives. However, (1*r*,4*r*)-4-aminocyclohexyl 3,5-bis(4-carbamimidoylphenoxy)benzoate (**30**) was prepared by the coupling of **46** with an alcohol, followed by its nitrile groups conversion to the amidine and then Boc-protected amino group was deprotected to give the desired compound. The binding affinity data for selected compounds are summarized in Table 1.

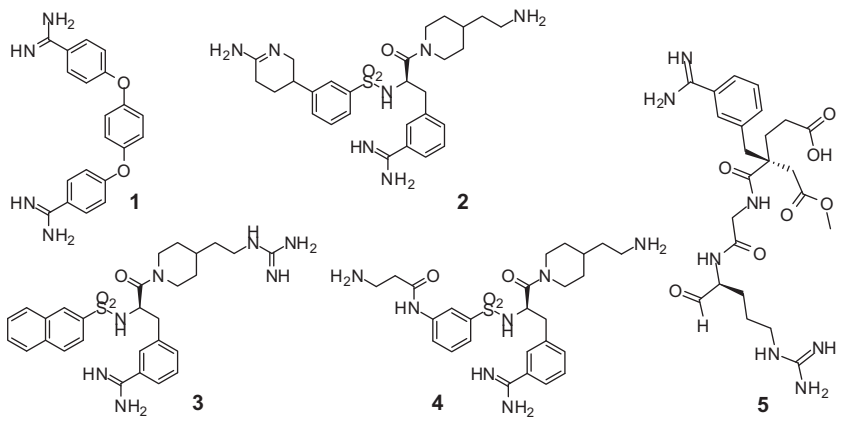


Figure 1. Structures of known matriptase inhibitors.

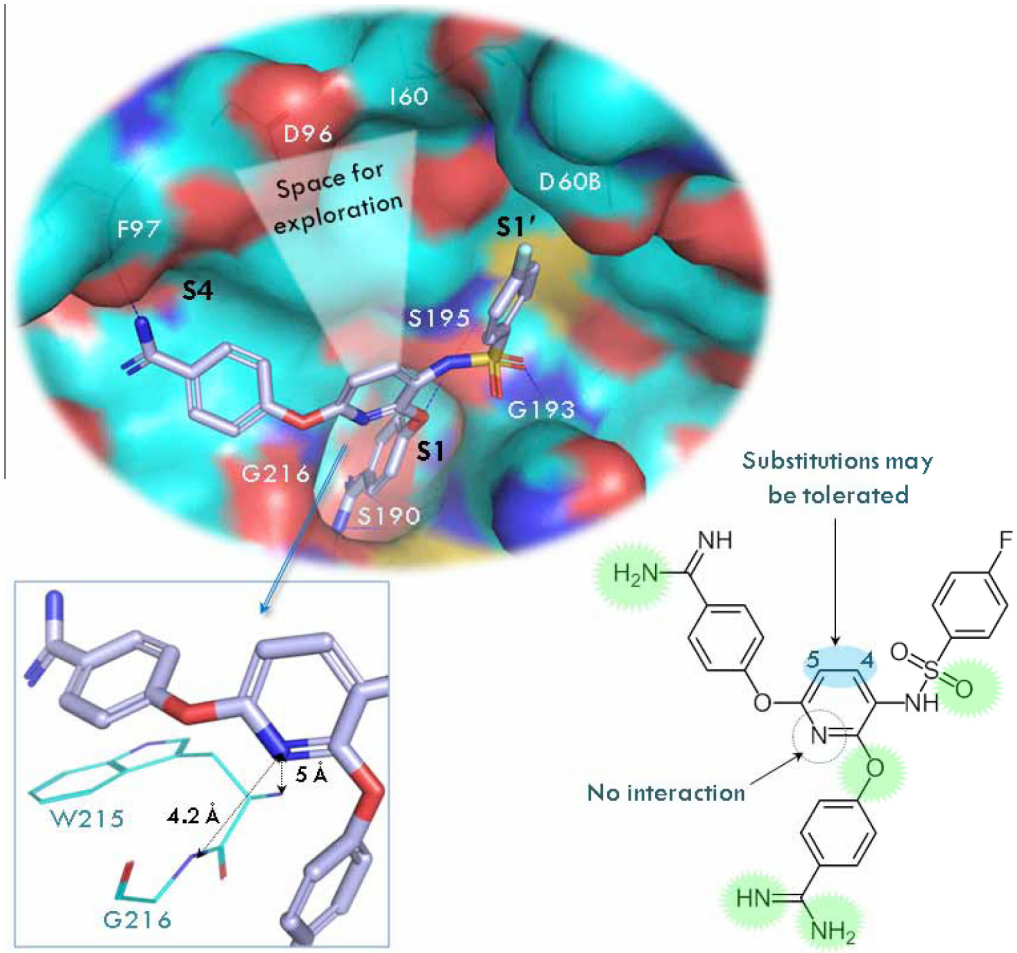


Figure 2. Key learnings from crystallographic binding mode of compound **6** (PDB ID: 4JZ1). Regions highlighted in green indicate moieties engaging polar contacts with different residues.

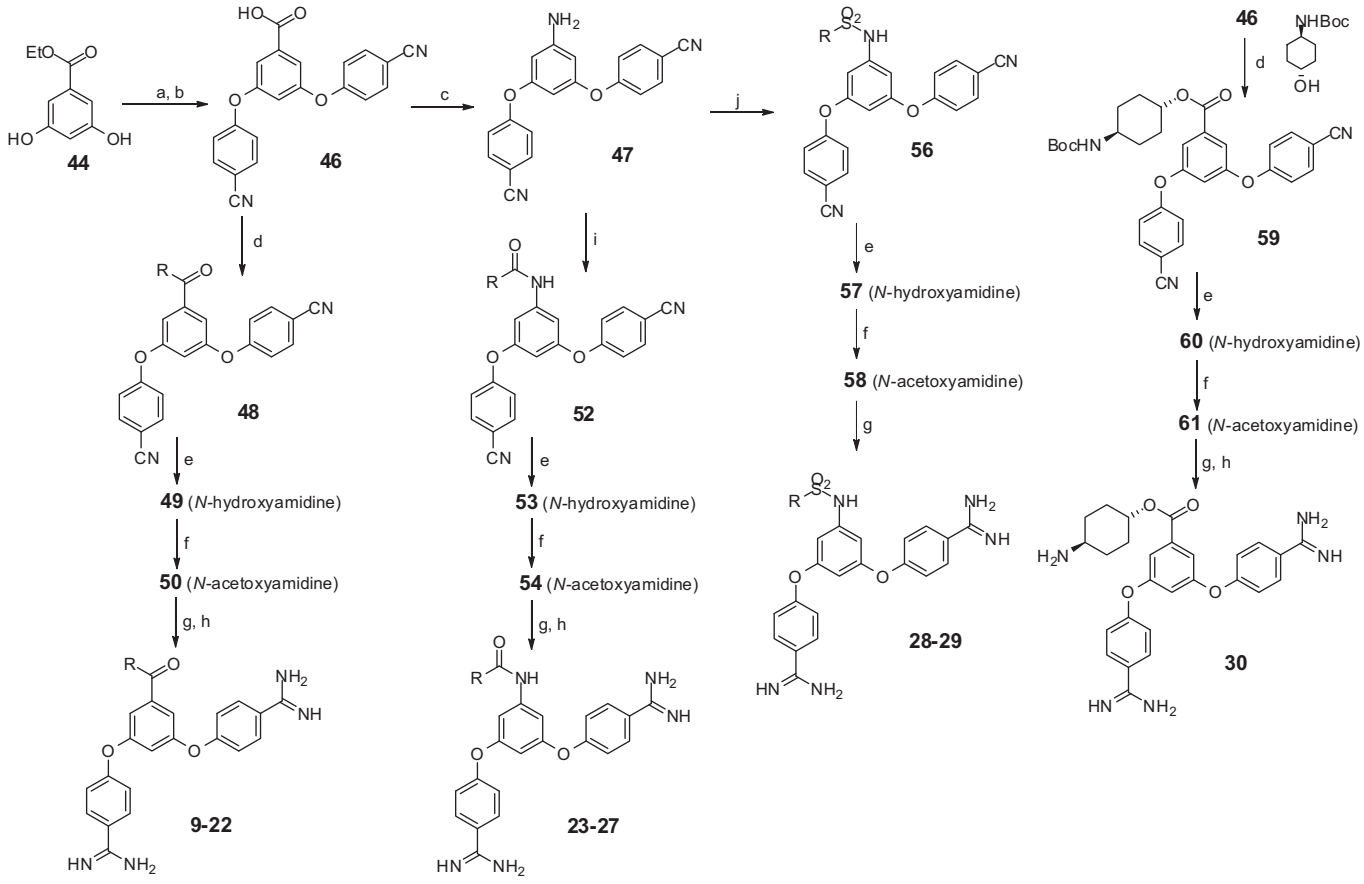
Unsymmetrical compounds were synthesized (Scheme 2) by treating **44** with 4-fluorobenzonitrile (1.5 equiv) to obtain ethyl 3-(4-cyanophenoxy)-5-hydroxybenzoate (**63**), which was hydrolyzed to give an acid **64**, followed by its coupling with *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate resulted in an amide **65**. Using the hydroxyl group of **65** to couple with an alkyl or aryl halide afforded **66**. Using the method described in Scheme 1, nitrile/s of **66** was/were converted to the amidine and then Boc-protected amino group was deprotected using ethanolic-HCl to give **31**–**39**. The nitrile group in **65** was reduced to a Boc-protected aminomethyl[19] group to afford **67**. The free hydroxyl group of **67** was treated with an aryl halide followed by the nitrile group (if present) conversion to the amidine and then deprotection of Boc-protected amino group yielded **40**–**41**.

Compounds with methylene linker were synthesized as outlined in Scheme 3. Compound **44** was treated with 3/4-(bromomethyl)benzonitrile to get an ester (**75**), followed by the ester group hydrolysis to form the acid (**76**), and then coupling with *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate afforded an amide (**77**). Using the hydroxylamine method described in Scheme 1, the nitriles were converted to the amidine, and then deprotection of Boc-protected amino group afforded **42**–**43**.

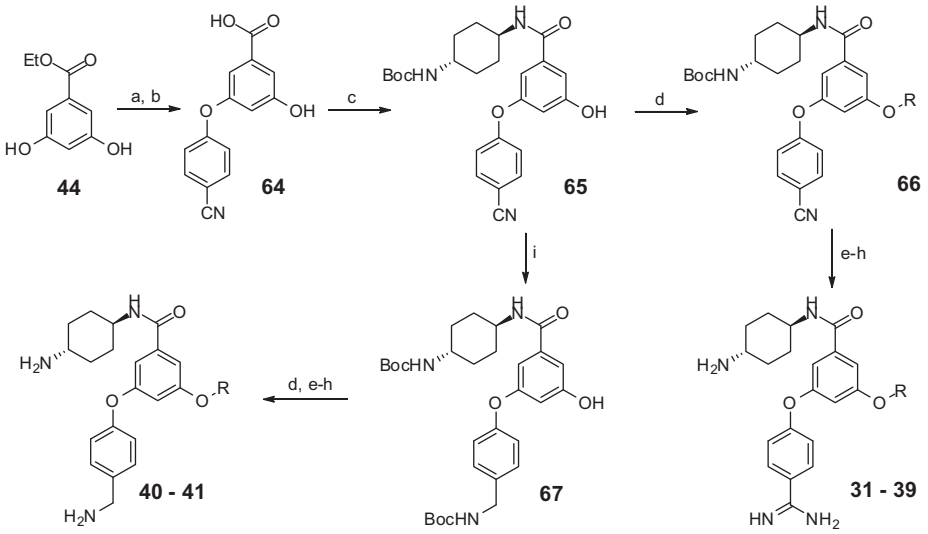
3. Results and discussion

Initially, simple but diverse 5-substituted phenylene1,3-bis(oxy) dibenzimidamide analogs were synthesized, for example, **7**–**11** and **23** (Table 1) to understand the tolerability of different linkers such as amide, inverted-amide, ester and sulphonamide with various cyclic/acyclic groups at the 5-position for matriptase inhibition. Interestingly, all these compounds with such diverse R groups (Table 1) demonstrated moderate inhibition of matriptase with K_i in the range of 410–1600 nM, which indicated that the 5-position is not only amenable to substitution but can also accommodate fairly bulky and conformationally puckered groups.

Additionally, the fact that most of these initial modifications (**7**–**11** and **23**) with diverse R groups (Table 1) exhibited moderate matriptase binding, indicates that the R groups possibly project towards a solvent exposed S2/S4 (spacious) region of the protein. Further molecules in this series were primarily designed through de novo approach based on predicted binding modes of these preliminary hits. Figure 3 illustrates the docked model of one such compound, **11** (Table 1). This model indicated possibilities of salt-bridge interaction in the S1 pocket involving one of the amidines as observed earlier[17,20] and the other amidine orienting towards the S1′ region making hydrogen bonding interaction with the backbone carbonyl of Ile60. The 4-methyl cyclohexyl amide appendage was anticipated to project towards S2/S4 region and the 4-methyl group occupying a region close to the polar side-chain of Gln175. We therefore targeted the region close to the polar side-chain of Gln175 with small hydrophilic groups on the cyclohexyl amide. Additionally, to develop an understanding on potential selectivity hot-spots, we compared the catalytic sites of factor Xa, thrombin, plasmin, trypsin and matriptase (Fig. 4).

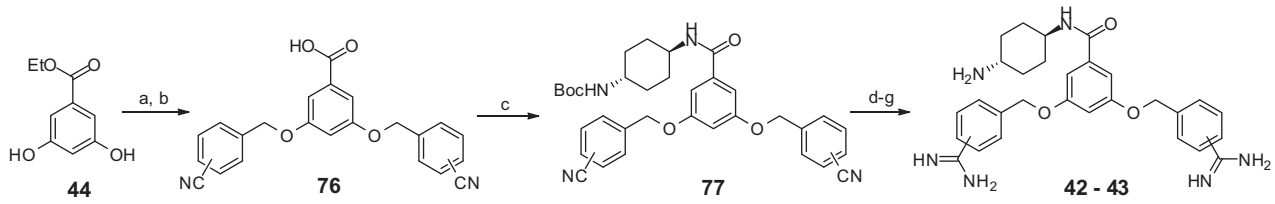


Scheme 1. Synthesis of 3,5-bis(4-carbamimidoylphenoxy)phenyl derivatives. Reagents and conditions: (a) 4-fluorobenzonitrile, K_2CO_3, DMF, 80 °C, 6 h; (b) LiOH, THF, water, 5–10 °C, 2 h; (c) diphenylphosphorylazide, DIPEA, *t*-BuOH, toluene, 80 °C, 12 h; (d) cycloalkyl or aryl amine/alcohol (with or without pendant amine masked with Boc), EDCI, HOBt, DIPEA, DMF, rt, 6 h; (e) aq NH_2OH, EtOH, 75 °C, 6 h; (f) Ac_2O,AcOH, rt, 2 h; (g) Zn, AcOH, rt, 6 h; (h) $HCl_{(g)}$–EtOH, 5–10 °C, 2 h; (i) cycloalkylcarboxylic acid(with or without pendant amine masked with Boc), PyBop, DIPEA, DMF, rt, 6 h; (j) arylsulfonyl chloride, Py, DMAP, DCM, rt, 12 h.



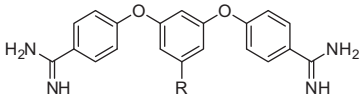
Scheme 2. Synthesis of *N*-(4-aminocyclohexyl)benzamide derivatives. Reagents and conditions: (a) 4-fluorobenzonitrile (1 equiv), K_2CO_3, DMF, 45 °C, 6 h; (b) LiOH, MeOH, water, 10 °C, 2 h; (c) *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate, EDCI, HOBt, DIPEA, DMF, rt, 6–8 h; (d) R-X, K_2CO_3,DMF, 65 °C, 4 h; (e) aq NH_2OH, EtOH, 75 °C, 6 h; (f) Ac_2O, AcOH, RT, 2 h; (g) Zn, AcOH, rt, 3 h; (h) amino group deprotection: $HCl_{(g)}$–EtOH at 5–10 °C, 2 h; (i) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, methanol, 0–10 °C, 4 h.

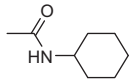
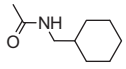
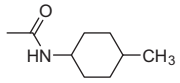
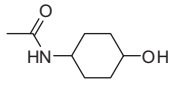
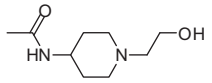
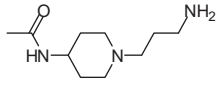
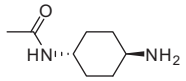
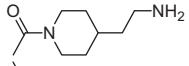
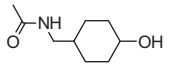
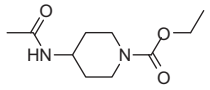
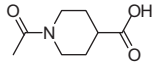
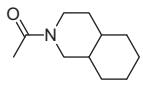
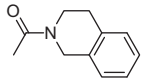
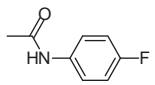
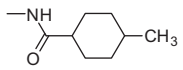
Gln175 in matriptase was observed to be non-conserved and the loop harboring Gln175 (corresponding to Ile in factor Xa, Arg in thrombin and plasmin, Lys in trypsin) as well as the adjoining 90-loop was also found to have different lengths in these proteases, which further indicated that the S4 site might also be different than in all these trypsin-like serine proteases. Therefore, compounds with terminal polar groups which might project towards S4 site and interact with the Gln175 side-chain were synthesized



Scheme 3. Synthesis of *N*-(4-aminocyclohexyl)-3,5-bis((3/4-carbamimidoylbenzyl)oxy)benzamide derivatives. Reagents and conditions: (a) 3/4-(bromomethyl)benzonitrile, K_2CO_3, DMF, 65 °C, 6–7 h; (b) LiOH, THF, MeOH, 10 °C, 2 h; (c) *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate, EDCI, HOBt, DIPEA, DMF, rt, 6–8 h; (d) aq NH_2OH, EtOH, 75 °C, 6 h; (e) Ac_2O, AcOH, rt, 2 h; (f) Zn, AcOH, rt, 3 h; (g) amino group deprotection: $HCl_{(g)}$–EtOH at 5–10 °C, 2 h.

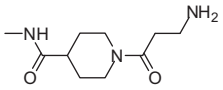
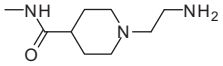
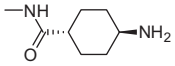
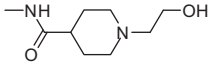
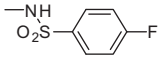
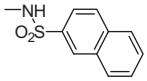
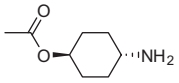
Table 1
SAR of 5-substituted phenylene 1,3-bis(oxy) dibenzimidamide derivatives **7**–**30**



ID	R	K_i[a] (nM)/% inhibition[b] at 5 μM					
		Matriptase	uPA (%)	f Xa	Thrombin	Plasmin	Trypsin
7	$-CONH_2$	1617	*13*	785	1056	*5%*	640
8	$-NH_2$	1060	*22*	851	2816	*6%*	534
9		592	*9*	190	1636	2395	179
10		411	*13*	73	2406	2160	571
11		770	*19*	282	744	3610	253
12		301	*15*	538	1240	*15%*	nd[c]
13		204	*9*	640	1785	*14%*	nd[c]
14		141	*9*	415	1285	*3%*	1035
15		10	*14*	516	2105	*12%*	1625
16		94	*4*	1289	4061	*17%*	545
17		1182	*16*	920	2852	*18%*	1550
18		1850	*6*	1026	*23%*	2630	471
19		*20%*	*12*	*29%*	*5%*	*0%*	718
20		305	*13*	336	501	1530	232
21		730	*12*	273	1562	3855	225
22		591	*20*	1877	2105	2665	1636
23		545	*10*	743	2343	1205	516

(*continued on next page*)

Table 1 (*continued*)

ID	R	K_i[a] (nM)/% inhibition[b] at 5 μM					
		Matriptase	uPA (%)	f Xa	Thrombin	Plasmin	Trypsin
24		61	*9*	95	2680	2590	541
25		18	*12*	62	2530	2410	1338
26		3	*23*	503	2216	*28%*	894
27		18	*17*	1160	4202	*18%*	1468
28		1982	*10*	1164	4850	*7%*	1388
29		1790	*13*	876	*25%*	*0%*	3000
30		1	*25*	44	2467	*28%*	264

[a] K_i: Mean of at least two independent experiments with standard deviation within ±10%.
[b] % Inhibition: K_i was not determined for compounds which show ⩽30% inhibition at 5 μM concentration.
[c] nd: not determined against the mentioned enzyme.

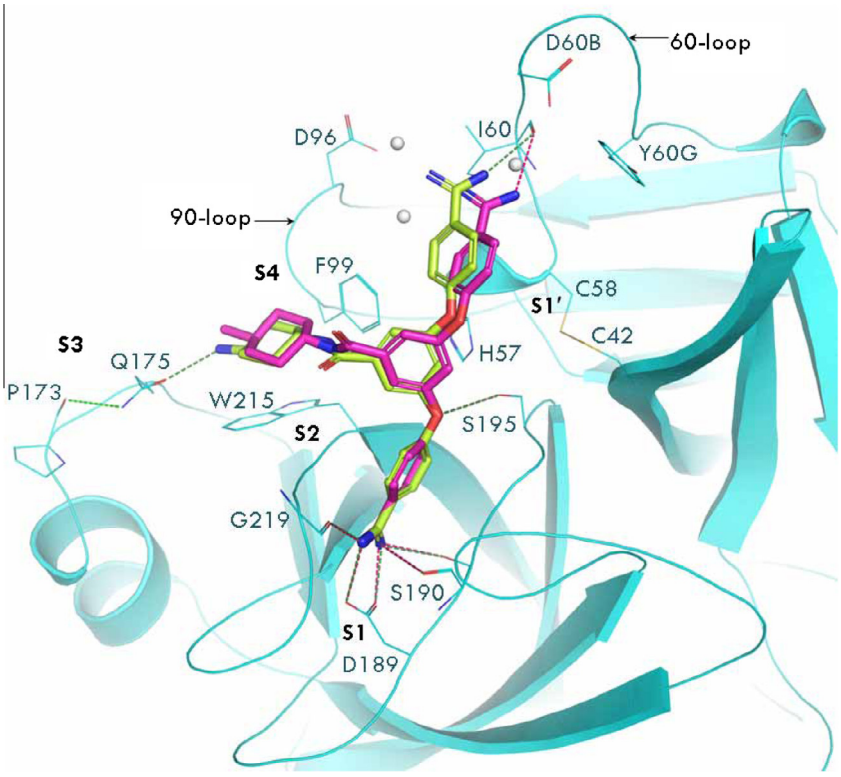


Figure 3. Overlay of the docked model of **11** (pink) on the crystallographic binding mode of **15** (green) in matriptase catalytic domain (PDB ID: 4O9V). Grey spheres indicate the positions of stable water sites (Δ*G* <−1 Kcal/mol) from WaterMap analyses on co-crystal structure of a structurally related matriptase inhibitor (PDB ID: 4JZ1).[17]

to improve matriptase inhibition as well as selectivity over other serine proteases. An improvement in matriptase inhibition was observed by replacing the terminal methyl group with a hydroxyl group (**11** vs **12**). This motivated us to synthesize further analogs including **13**–**16** having different linkers and other terminal polar groups. Interestingly, some of these analogs with 4-aminocyclohexyl appendage, for example, **15**, **26** and **30** exhibited significantly improved matriptase inhibition with the K_i of 10, 3 and 1 nM respectively and also ⩾40 fold selectivity over other serine proteases (Table 1). Improvement in target inhibition and

Figure 4. Sequence comparison of matriptase (m), factor Xa (f), thrombin (th), plasmin (p) and trypsin (tr) catalytic domains.

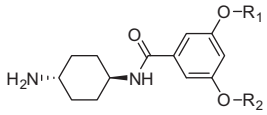
selectivity over other related proteases indicated that different linkers including amide, inverted-amide and ester are well tolerated as these did not exhibit significant difference in matriptase inhibition. Compound **30** with an ester linker showed most potent inhibition of matriptase (K_i = 1 nM). However, because of low metabolic stability, further optimization of compound **30** was discontinued. Compounds **15**, **26** and **30** did not show significant improvement in their Ligand Efficiency (LE) in comparison to compound **7** (LE ~ 0.3 for all 4 compounds) but interestingly these compounds **15**, **26** and **30** exhibited markedly improved Ligand-Lipophilicity Efficiency (LLE) by ~2 units in comparison to **7** (LLE = 3.9), thereby demonstrating that incorporation of the amide/inverted-amide/ester linked cyclohexyl amine not only improved matriptase affinity and selectivity but also 'drug-likeness'.

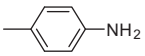
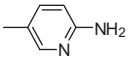
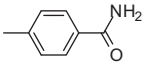
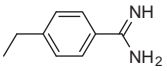
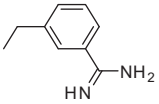
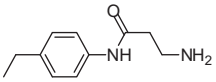
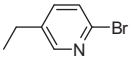
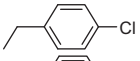
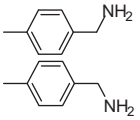
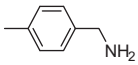
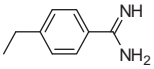
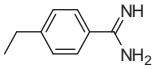
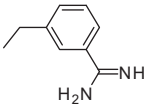
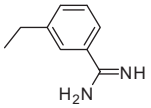
Since compound **15** showed potent matriptase inhibition (K_i = 10 nM) and selectivity (>50 fold) over other related proteases, a high resolution crystal structure of matriptase catalytic domain in complex with **15** was solved (PDB ID: 4O9V). This crystal structure revealed a similar binding mode to the docking model of **11** (Fig. 3), thereby validating the working hypothesis followed during lead identification. Most significantly (and as expected), the 4-aminocyclohexyl was observed to engage in polar contact with the Gln175 side-chain carbonyl.

Owing to the high binding affinities observed with **15** and **26**, further analogs were synthesized replacing the P2/P4 amino cyclohexyl amine of **15** with appendages having terminal carboxylates as in **18** and **19** assuming that the Gln175 might flip and the side-chain NH could make a polar contact with carboxylate oxygen. However, all these analogs surprisingly showed reduced matriptase inhibition. Further analysis of the crystallographic binding mode of **15** with matriptase indicated that the Gln175 side-chain NH makes a long intra-molecular polar interaction with the backbone carbonyl of neighboring Pro173 (Fig. 3) and because of this the Gln175 side-chain likely remains stabilized in only one rotameric state. Such phenomenon could contribute to the lower inhibition observed for compounds with terminal P2/P4 hydrogen bond acceptors, while the presence of a terminal hydrogen bond donor such as primary amine might be critical. Apart from the different hydrogen bonding or salt-bridge interactions observed in the binding mode of **15**, the crystal structure also revealed strong CH–π contacts between P2/P4 cyclohexyl and Trp215 side-chain. Thus, bicyclic analogs **20** and **21** at R (Table 1) were synthesized to test whether incorporation of bicyclic systems can improve matriptase inhibition in comparison to **9**. However, except for **20**, which also showed only moderate matriptase inhibition, none of the other compounds exhibited any improvement in binding. Therefore, 4-aminocyclohexyl with amide linker (**15**) was retained as R for further optimization with an option of introducing the inverted-amide analog (as in **26**). While one of the amidines of **15** was observed to form a strong bidentate salt-bridge in S1 pocket, the other amidine was found to be involved in a relatively weak hydrogen bond with Ile60 backbone in the S1′ region (Fig. 3).

Therefore, we studied the effect of P1′ alterations on matriptase inhibition keeping other features unchanged as in **15** (Table 2). A couple of modifications were tested by synthesizing compounds **31** and **32** with R_1 = 4-aminophenoxy and 6-aminopyridin-3-oxy respectively. These substitutions were not expected to engage in a hydrogen bond with the Ile60 backbone having the P1′ oriented like that in **15**, but the basic objective was to assess whether the P1′ end can adopt a slightly different orientation so that the amine in **31** or **32** might be involved in the same Ile60 backbone contact as it is observed in **15**. However, none of these demonstrated good matriptase binding and in fact showed >1000 fold reduced inhibition. Compound **33** was synthesized by replacing the P1′ 4-carbamimidoylphenoxy of **15** with a 4-carbamoylphenoxy but unexpectedly this also lead to >800 fold reduced potency. We hypothesized that it could be due to orientation of the amide carbonyl towards the Asp96 side-chain carboxylate (Fig. 3), which would create a repulsive interaction. Reduced potency could also be due to the fact that the non Ile60 interacting amidine NH (amidine NH not engaged in polar contact with Ile60 back-bone) in **15** is involved in water-mediated contacts that were not resolved in the crystal structure and replacing this NH with a carbonyl as in **33** likely displace a stable water molecule due to water-amide carbonyl lone pair repulsions. The latter possibility seemed plausible because WaterMap analysis carried out on a co-crystal structure of our earlier compounds[17] predicted stable water molecules (ΔG <−1 Kcal/mol) in the S1′ site (Fig. 3). This rationale was supported by the improved inhibition observed with R_1 (Table 2) as 4-(aminomethyl)phenoxy (**40**), which exhibited improved inhibition possibly because the hypothesized stable water molecule does not get

Table 2
SAR of 5-substituted *N*-((1*r*,4*r*)-4-aminocyclohexyl)-3,5-(bis-alkyl/aryloxy) benzamide derivatives



ID	R_1	R_2	K_i[a] (nM)/% inhibition[b] at 5 μM					
			Matriptase	uPA (%)	f Xa	Thrombin	Plasmin (%)	Trypsin
31		4-Benzimidamide	1131	*4*	*28%*	*18%*	*0*	>3000[d]
32		4-Benzimidamide	2032	*5*	*14%*	*1%*	*0*	>3000[d]
33		4-Benzimidamide	824	*1*	*26%*	3540	*8*	>3000[d]
34		4-Benzimidamide	10	*7*	*25%*	2808	*22*	1868
35		4-Benzimidamide	6	*11*	1140	2677	*13*	1541
36		4-Benzimidamide	116	*2*	3580	3250	*8*	>3000[d]
37		4-Benzimidamide	793	*8*	3685	3985	*5*	>3000[d]
38		4-Benzimidamide	470	*15*	nd[c]	nd[c]	nd[c]	nd[c]
39		4-Benzimidamide	260	*23*	2089	3973	*13*	2064
40		4-Benzimidamide	40	*5*	*21%*	3125	*8*	1541
41			1698	*4*	*21%*	*9%*	*0*	3850
42			23	*15*	1558	1642	*16*	1022
43			3	*11*	157	1289	*10*	901

[a] K_i: Mean of at least two independent experiments with standard deviation within ±10%.
[b] % Inhibition: K_i was not determined for compounds which show ⩽30% inhibition at 5 μM concentration.
[c] nd: not determined against the mentioned enzyme.
[d] Indicates that K_i was not determined because of the poor dose response in the biochemical assay.

displaced from the S1′ site. At the same time, the binding was slightly different that of **15**, likely due to the missing water-mediated interactions. Further modeling studies suggest the possibility of water-mediated interactions of the non Ile60 interacting P1′ amidine NH of **15** with Asp96 in the 90-loop region. The Ile60 interacting P1′ amidine NH_2 of **15** was also anticipated to engage in an additional water-mediated contact with Asp60B in the 60-loop region. Subsequently, additional analogs were designed to interact with Asp96 or Asp60B apart from making the Ile60 backbone contact, like **34**–**35**. Interestingly, most of these inhibitors exhibited similar potency as **15**, which further validated the hypothesis on water-mediated interactions with a 60-loop residue. Figure 5 depict the likely binding modes of **34** and **35**.

To understand the impact of van der Waals (vdW) contacts in the S1′ region, which is slightly hydrophobic in nature due to the presence of a disulfide bridge and a tyrosine residue (Fig. 2), compounds **37**–**39** (Table 2) were synthesized. However, the reduced target inhibition of **37**–**39** suggested that vdW interactions alone in this region might not have significant contribution in binding.

Furthermore, a few symmetric compounds were synthesized to understand the effect of P1 alterations as shown in Table 2. The non-amidine compound **41** resulted in a 40-fold reduced matriptase inhibition in comparison with **40** (R_1 = 4-(aminomethyl)phenoxy, Table 2), thereby signifying the importance of P1 amidine in binding. However, compounds **42** and **43** with P1-benzamidine but with additional methylene spacer showed similar inhibition to **34** and **35** respectively, suggesting that spacers may be tolerated between P1 benzamidine and the ether linker. Among these, **43** showed the highest inhibition of matriptase with the K_i of 3 nM and >50 fold selectivity over other trypsin-like serine proteases.

A crystal structure of matriptase catalytic domain in complex with **43** was solved (PDB ID: 4O97). The binding mode of **43** was different in comparison to **15** because the 4-aminocyclohexyl amide appendage (P2/P4 appendage of **15**) pointed away from

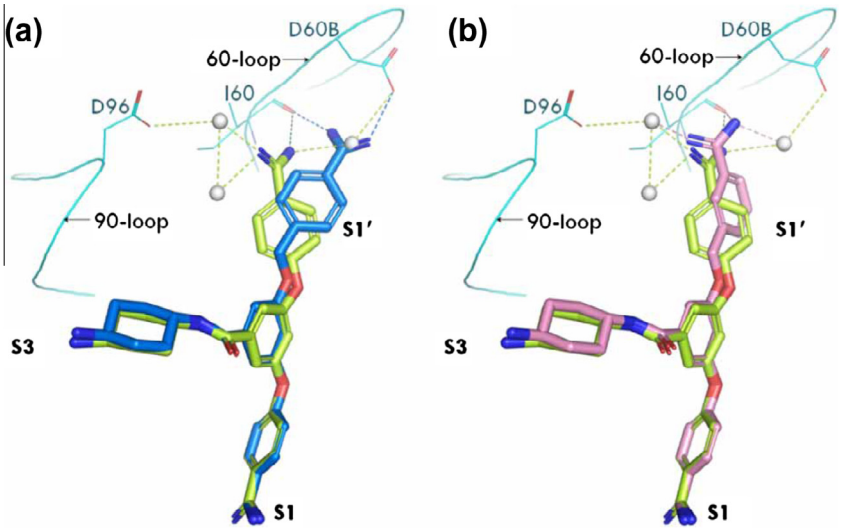


Figure 5. Predicted binding modes of **34** (a, marine blue) & **35** (b, light pink) compared to the crystallographic binding mode of **15** (green) in matriptase catalytic domain (PDB ID: 4O9V). Grey spheres indicate projected distribution of stable water molecules ($\Delta G \sim -1$ Kcal/mol) from WaterMap analyses on co-crystal structure of a structurally related matriptase inhibitor (PDB ID: 4JZ1).[17]

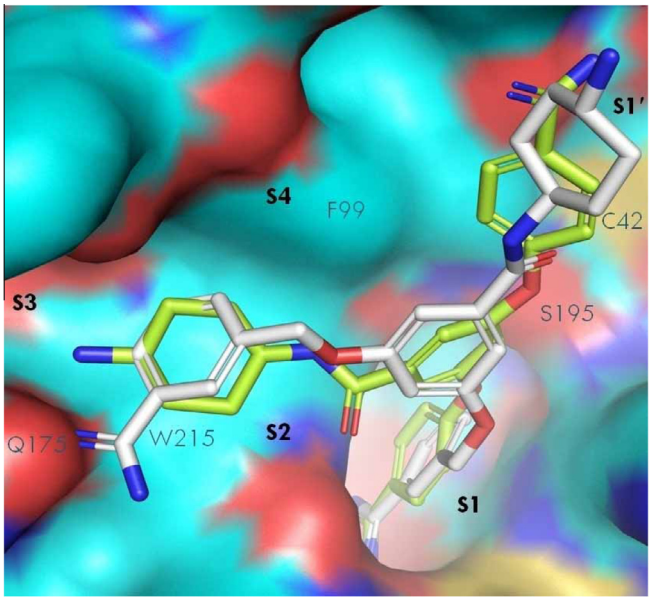


Figure 6. Overlay of **15** (green) and **43** (white) crystallographic binding modes.

the S2/S4 site (Fig. 6). This indicated that the SAR trend observed for the 3,5-bis(4-carbamimidoylphenoxy) analogs may not translate to the 3,5-bis((4-carbamimidoylbenzyl)oxy analogs and hence its further advancement will be discussed separately in due course.

4. Cellular activity and biopharmaceutical properties

After establishing SAR for matriptase inhibition and selectivity against other proteases, we evaluated the effect of selected compounds on migration and invasion of matriptase expressing prostate cancer cell lines[5,21,22] DU-145, PC-3 and LNCaP C42B4 (Table 3). An androgen-sensitive prostate cancer cell line, LNCaP C42B4 was evaluated after confirmation of matriptase protein expression (data not shown). This LNCaP C42B4 is a subline of LNCaP that is more invasive than the parental cells. Compounds that were potent in these assays without exhibiting cytotoxicity were further evaluated for inhibition of soft agar colony formation in matriptase expressing prostate cancer cell lines (Table 4). Importantly, most of the compounds which exhibited good biochemical potency for matriptase also displayed good cellular potency without any signs of cytotoxicity.

The cellular potency for this series of compounds varied depending on the pendant amine at amide appendage and also on P1 amidine/non-amidine motif. At 10 μM concentration, compound **15** (with amide linker) and **30** (with ester linker) exhibited no cytotoxicity and caused >80% inhibition of invasion and

Table 3
Effect of selected compounds on DU145, LNCaP C42B4 and PC-3 cells

ID	% Cell survival at 10 μM			% Inhibition[a] at 10 μM					
	DU-145	LNCaP C42B4	PC-3	DU-145		LNCaPC42B4		PC-3	
				Migration	Invasion	Migration	Invasion	Migration	Invasion
15	100	95	96	88 ± 4	81 ± 1	91 ± 2	84 ± 1	98 ± 2	97 ± 8
16	100	100	93	63 ± 2	62 ± 3	73 ± 1	61 ± 3	48 ± 6	54 ± 3
20	78	79	87	18 ± 2	45 ± 3	19 ± 1	15 ± 1	60 ± 5	47 ± 1
24	98	99	80	98 ± 2	94 ± 2	69 ± 2	60 ± 3	54 ± 2	37 ± 2
26	100	90	94	96 ± 1	98 ± 2	61 ± 9	63 ± 2	84 ± 4	86 ± 1
30	92	95	82	81 ± 2	89 ± 1	98 ± 3	90 ± 1	91 ± 4	95 ± 10
39	78	65	93	50 ± 7	61 ± 4	63 ± 2	76 ± 1	66 ± 3	51 ± 7
40	90	100	100	53 ± 2	49 ± 3	60 ± 4	59 ± 4	73 ± 5	54 ± 2

[a] Values represent mean ± SD.

Table 4
EC_{50}[a] (nM) values for inhibition of soft agar colony formation of DU145, LNCaP C42B4 and PC-3 cells

ID	DU-145	LNCaP C42B4	PC-3
15	200 ± 9	1364 ± 20	48 ± 6
24	130 ± 3	1580 ± 18	580 ± 8
26	41 ± 1	1536 ± 13	105 ± 3
30	152 ± 5	135 ± 9	81 ± 5
40	324 ± 16	1698 ± 19	485 ± 14

[a] Values represent mean ± SD.

migration in both DU-145, LNCaP C42B4 and PC-3 cell lines while compound **26** (inverted-amide) showed good inhibition of invasion and migration in DU-145 and PC-3 cells but moderate inhibition in LNCaP C42B4 cells. The compounds with lower matriptase inhibitory activity in biochemical assays (**16**, **20** and **39**) tend to display reduced activity in migration and invasion assays as compared to the compounds with high activity towards matriptase (**1**, **3**, **10**).

The soft agar colony formation assay measures the long-term survival and anchorage-independent growth of tumor cells. Compounds that were potent inhibitors of invasion and migration were evaluated for inhibition of soft agar colony formation by DU-145, LNCaP C42B4 and PC-3 cells (Table 4). Compound **15** displayed the EC_{50} of 200 nM, 1364 nM and 48 nM in the soft agar colony formation with DU-145, LNCaP C42B4 and PC-3 cells respectively. Compound **30** showed relatively similar potency in these three cell lines. Compound **26** exhibited the EC_{50} of 41 nM and 105 nM with DU-145 and PC-3 cells. Ester analog **30** showed the EC_{50} of 135 nM on LNCaP C42B4 cells in soft agar colony formation, however other analogs (amides and inverted-amides) exhibited only moderate activity.

In vitro ADME properties for compounds **15**, **26** and **30** are shown in Table 5. All three compounds **15**, **26** and **30** showed high aqueous solubility along with a clean CYP inhibition profile. **15** and **26** exhibited high microsomal stability in MLM, RLM and HLM but **30** showed poor microsomal stability in all three species probably due to the presence of an ester group (ester cleavage was indicated by metabolite identification studies). In plasma protein binding studies, surprisingly compound **30** showed >3 fold more free fraction. Therefore **15**, **26** and **30** were selected for in vivo mouse pharmacokinetics studies. Though **30** showed poor in vitro microsomal stability, it was also selected to further validate the in vitro results.

In the subcutaneous PK study (Fig. 7) at 1 mg/kg dose, compounds **15** and **26** showed good exposure and therefore were taken up further for in vivo efficacy studies. As expected **30** exhibited very poor exposure and was discontinued from further profiling.

5. In vivo evaluation of compounds 15 and 26 in a DU-145 prostate cancer model

Since selective matriptase inhibitors **15** and **26** showed significant inhibition of migration, invasion and soft agar colony formation, we investigated the efficacy of these compounds on tumor growth inhibition in SCID mice bearing subcutaneous DU-145 prostate cancer xenograft tumors.

The compounds **15** and **26** were dosed subcutaneously at 1 and 0.5 mg/kg respectively twice daily for 31 days. Both compounds **15** and **26** exhibited significant inhibition of the median primary tumor growth of 48% and 52% respectively (Fig. 8). Furthermore, none of the mice treated with compound **15** and **26** experienced any significant weight loss (<5%) or showed other clinical signs of toxicity during or after the treatment.

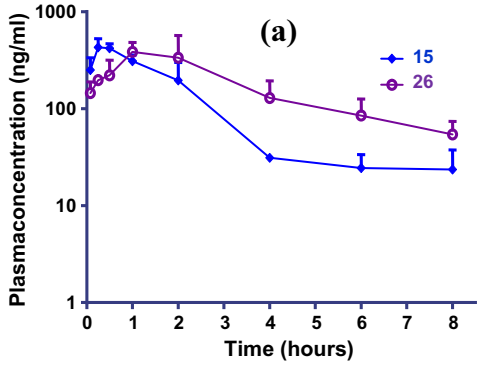
6. Conclusion

In this work, we have identified highly potent phenyl bis(oxy)benzamidine compounds as matriptase inhibitors, which are 20–1000 fold selective against other trypsin-like serine proteases. Replacement of the terminal methyl group (**11**) with an amino group at the 4-position of cyclohexyl (**15**) significantly improved matriptase inhibition along with selectivity against

Table 5
ADME profile of selected compounds (in vitro)

ID	Solubility[a] (μM)	Metabolic stability[a] (% remaining after 20 min)			CYP Inhibition—IC_{50}[b] (μM)				PPB (% free)[a]
		MLM	RLM	HLM	3A4	2D6	2C9	2C19	
15	194 ± 3	81 ± 5	100 ± 1	85 ± 1	>30	>30	>30	>30	8.1 ± 1
26	200 ± 2	83 ± 1	91 ± 4	97 ± 3	>30	>30	>30	>30	7.2 ± 1
30	182 ± 3	24 ± 4	34 ± 5	54 ± 6	>30	>30	>30	>30	26.7 ± 2

[a] Values represent mean ± SD.
[b] SD values for CYP inhibition assays will be 0 since all triplicates showed >30 μM IC_{50}.



(b)

ID	T_{max} (h)	C_{max} (ng/ml)	Beta $t_{1/2}$ (h)	AUC (0-inf) (ng.h/ml)
15	0.27	487	1.6	1000
26	0.75	484	3.3	1852

Figure 7. Pharmacokinetic profile of **15** and **26** in mice. Male NMRI mice were dosed subcutaneous at 1 mg/kg with compound formulated with 100% PBS and time-points were pre-dose, 5, 15, 30 min, 1, 2, 4, 6 and 8 h; (a) graphical presentation and (b) tabular representation of the PK data.

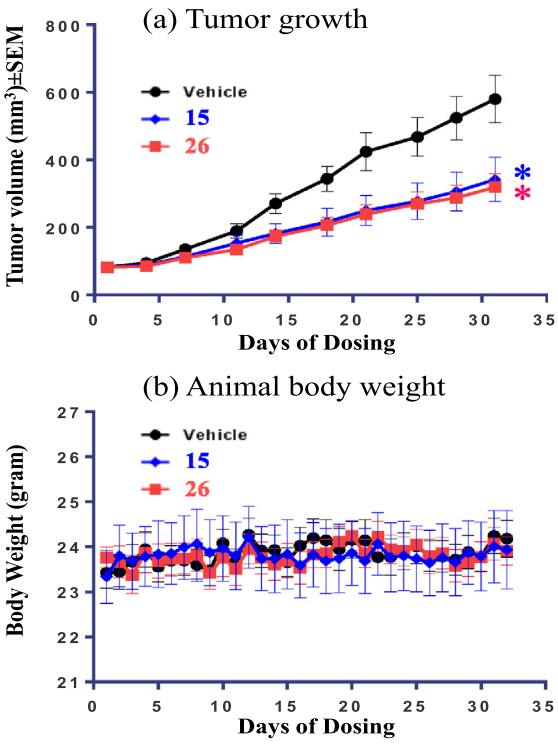


Figure 8. Antitumor activity of compounds **15** and **26** in the DU-145 prostate cancer xenograft model in SCID mice. Tumors were grown to an average of 70 mm^3 and compounds **15** and **26** were administered subcutaneously twice daily for 31 days at 1.0 and 0.5 mg/kg, respectively. (a) Tumor growth. (b) Animal body weight. Values shown are mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test,*p <0.05.

related proteases by projecting towards S4 site and engaging in an additional Gln175 side-chain interaction. Potent inhibition of matriptase by compound **15** could also be due to additional hydrogen bonding or salt-bridge interactions. Potent inhibition and selectivity for matriptase observed for **15** were also supported by strong CH–π contacts between the P2/P4 cyclohexyl and Trp215 side-chain as observed by X-ray crystallography. Among several potent and selective matriptase inhibitors, compound **26** also exhibited excellent activities in several prostate cancer cell lines. Selected compounds exhibited acceptable DMPK properties that allowed for their use as tool compounds after subcutaneous administration. Compound **15** and **26** exhibited primary tumor growth inhibition of 48% (at 1 mg/kg) and 52% (0.5 mg/kg) respectively in a DU-145 prostate cancer xenograft model. In summary, high potency, selectivity and in vivo efficacy of the compounds **15** and **26** support their use as pharmacological tools for understanding the role of matriptase in cancer as well as in inflammatory conditions such as osteoarthritis[23] where matriptase is known to play a key role.

7. Materials and methods

7.1. Experimental section

All commercially available starting materials and solvents were used as received. All anhydrous reactions were performed under a nitrogen atmosphere in oven-dried glassware with magnetic stirring. Column chromatography was performed using silica gel of 60–120 mesh and solvent (hexane/DCM/EtOAc) mixture/gradient. Thin layer chromatography was carried out using silica gel 60 F_{254} plates (Merck, Darmstadt) and developed chromatograms were visualized using UV (254 nm) and/or by staining with potassium permanganate followed by heating. NMR spectra were recorded on a Varian (^{1}H, 300 or 400 MHz; ^{13}C, 75 or 100 MHz) spectrometer, using tetramethylsilane as an internal reference, and chemical shifts were expressed in δ (ppm) units (in ^{1}H NMR description, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br s = broad singlet). Liquid chromatography mass spectra were obtained from Agilent 1100-LC/MSD VL using mobile phase: 0.1% TFA-acetonitrile or water–methanol (either by positive or negative ionization mode). The melting points were determined on a DBK programmed melting point apparatus from Servewell Instruments and were uncorrected. The purity values for all biologically evaluated compounds were >95% (or mentioned otherwise) as determined by the analysis on high performance liquid chromatography (HPLC) performed on Agilent 1100 series HPLC instrument with a Agilent XDB C18 reverse phase column (21.2 × 150 mm, 5micron). The mobile phases were 30% acetonitrile in water (0.1% TFA) to 100% acetonitrile (0.1% TFA). The nomenclature used in this section is based on ChemDraw. Biochemical assays were performed at least in duplicates while functional and ADME assays were performed in triplicates. Matriptase protein expression, purification, crystallization & data collection of crystal structures, aqueous solubility and metabolic stability were determined using the methods as described previously.[17]

7.2. Chemistry

7.2.1. General procedure for synthesis of 3,5-bis(4-carbamimidoylphenoxy)benzamides (9–22)

7.2.1.1. Synthesis of ethyl 3,5-bis(4-cyanophenoxy)benzoate (45). A solution of ethyl 3,5-dihydroxybenzoate (**44**) (1.5 g, 8.23 mmol) in DMF (150 ml) at rt was treated with K_2CO_3 (4.55 g, 32.92 mmol) and stirred for 10 min. A solution of 4-fluorobenzonitrile (4.0 g, 32.92 mmol) in DMF (100 ml) was added drop wise and the resultant solution was stirred for 4 h at 80 °C. The reaction mixture temperature was brought down to rt and quenched with ice-cold water and diluted with EtOAc. The aqueous layer was extracted with EtOAc (3 × 150 ml), and the combined organics were washed with 1 M Na_2CO_3 solution followed by brine. Organic layer was dried over anhydrous sodium sulfate and evaporated to dryness to get crude oily residue, which was purified by column chromatography and eluting with 10–20% EtOAc/hexane to afford ethyl 3,5-bis(4-cyanophenoxy)benzoate as an off-white solid (2.1 g; yield 66%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (t, 3H), 4.32 (q, 2H), 6.92 (s, 1H), 7.18 (d, 4H), 7.45 (s, 2H), 7.84 (d, 4H).

7.2.1.2. Synthesis of 3,5-bis(4-cyanophenoxy)benzoic acid (46). A solution of ester **45** (2.1 g, 5.46 mmol) in THF & water mixture (30 ml, 1:1) at rt was treated with LiOH (0.4 g, 16.38 mmol) and stirred for 2 h. Reaction mixture was washed with ether and the aqueous layer was cooled to 5 °C followed by pH adjusted to 2–3 using 6 N HCl resulted in white precipitate, which was filtered off and dried under vacuum to afford the title compound **46** (1.6 g; yield 82%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.21 (d, 4H), 7.31 (s, 1H), 7.38 (d, 2H), 7.86 (d, 4H), 13.45 (br s, 1H).

7.2.1.3. General procedure for the synthesis of 3,5-bis(4-cyanophenoxy)benzamide derivatives (48). A solution of **46** (0.84 mmol) in DMF (15 ml) at 5 °C was treated with EDCI (0.84 mmol), HOBt (0.84 mmol) and DIPEA (0.84 mmol). The mixture was stirred for 10 min and a solution of an amine or an alcohol (0.84 mmol), dissolved in DMF (3 ml), was added drop wise and stirred at rt for 8 h. Reaction mixture was quenched with ice-cold water and aqueous layer was extracted with ether. Combined organics were washed with 1 M solution of Na_2CO_3 followed with

brine, dried over anhydrous Na_2SO_4 and concentrated to give a semi solid residue, which was purified by CC, eluting with 20% EtOAc/hexane gave pure compound (**48**).

7.2.1.4. General procedure for the conversion of nitrile to *N*-hydroxybenzimidamide derivatives (49). Nitrile **48** (0.81 mmol), dissolved in EtOH (10 ml), at rt was added aq NH_2OH (3.24 mmol). The reaction mixture was stirred at 75 °C for 6 h and then concentrated to afford the *N*-hydroxy compound as a white solid, which was used as such into the next step without further purification.

7.2.1.5. General procedure for the synthesis of *N*-acetoxybenzimidamide derivatives (50). A solution of *N*-hydroxyamidine **49** (0.65 mmol), dissolved in AcOH (3 ml), was treated with Ac_2O (2.6 mmol) and stirred at rt for 2 h. Reaction mixture was concentrated to give acetoxy compound as a white solid (**50**), which was used into the next step as such without further purification.

7.2.1.6. General procedure for the synthesis of dibenzimidamide derivatives (51, 9–11, 18–22). To the solution of *N*-acetoxy benzimidamide **50** (0.57 mmol), in AcOH (5 ml), zinc (1.71 mmol) was added and stirred at rt for 6–8 h. Reaction mixture was filtered through celite and concentrated to give crude solid, which was purified by the reversed-phase preparative HPLC to give the target compounds.

7.2.1.7. General procedure for the deprotection of a Boc-protected amino group or a THP-protected hydroxyl group (12–17). Boc-protected amino group or THP-protected hydroxyl group containing compound **51** (0.34 mmol) was dissolved in 5 ml of ethanol (saturated with HCl gas) and stirred at 5–10 °C for 2 h. Solvent was removed to give the crude solid, which was purified by reversed-phase preparative HPLC to give the target compounds **12**–**17**.

3,5-Bis(4-carbamimidoylphenoxy)-N-cyclohexylbenzamide ***9****:* Yield 74%; mp 148–150 °C; HPLC: 95.03%; LCMS (ES, *m*/*z*): 472.1 $(M+H)^+$; ^1H NMR (400 MHz, DMSO-d_6): δ 1.23 (m, 4H), 1.68 (m, 6H), 3.71 (m, 1H), 7.15 (s, 1H), 7.30 (d, 4H), 7.49 (s, 2H), 7.88 (d, 4H), 8.37 (d, 1H), 9.18 (br s, 4H), 9.28 (br s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 24.82, 25.14, 32.20, 48.60, 114.18, 114.71, 118.11, 122.94, 130.61, 138.31, 156.31, 158.24, 158.57, 160.85, 163.09, 164.76.

3,5-Bis(4-carbamimidoylphenoxy)-N-(cyclohexylmethyl)benzamide ***10****:* Yield 75%; mp 138–140 °C; HPLC: 98.98%; LCMS (ES, *m*/*z*): 485.9 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 0.84 (m, 2H), 1.12 (m, 3H), 1.58 (m, 6H), 3.04 (t, 2H), 7.20 (s, 1H), 7.28 (d, 4H), 7.46 (s, 2H), 7.88 (d, 4H), 8.58 (t, 1H), 9.28 (br s, 8H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.12, 25.78, 30.29, 37.09, 45.38, 113.61, 114.27, 118.08, 122.86, 130.39, 138.13, 156.29, 160.59, 163.97, 164.74.

3,5-Bis(4-carbamimidoylphenoxy)-N-(4-methylcyclohexyl)benzamide ***11****:* Yield 70%; mp 140–142 °C; HPLC: 97.74%; LCMS (ES, *m*/*z*): 486.2 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 0.91 (d, 2H), 1.20-1.81 (m, 9H), 3.78 (m, 1H), 7.12 (s, 1H), 7.26 (d, 4H), 7.48 (s, 2H), 7.84 (d, 4H), 8.34 (d, 1H), 9.13 (br s, 4H), 9.25 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 21.84, 27.47, 29.62, 33.48, 47.00, 48.53, 113.60, 114.49, 114.60, 117.98, 122.81, 130.39, 138.45, 156.15, 160.66, 164.76.

3,5-Bis(4-carbamimidoylphenoxy)-N-(4-hydroxycyclohexyl)benzamide ***12****:* Yield 58%; mp 120–122 °C; HPLC: 97.19%; LCMS (ES, *m*/*z*): 488.1 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.18 (m, 4H), 1.64 (m, 4H), 3.32 (m, 1H), 3.65 (m, 1H), 7.14 (s, 1H), 7.26 (d, 4H), 7.48 (s, 2H), 7.68 (d, 4H), 8.34 (d, 1H), 9.18 (br s, 4H), 9.26 (br s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.14, 34.17, 48.19, 68.27, 114.35, 114.80, 115.32, 118.13, 123.02, 130.65, 138.23, 156.36, 158.45, 158.77, 159.09, 160.91, 163.31, 164.89.

3,5-Bis(4-carbamimidoylphenoxy)-N-(1-(2-hydroxyethyl)piperidin-4-yl)benzamide ***13****:* Yield 62%; mp 134–136 °C; HPLC: 95.44%; LCMS (ES, *m*/*z*): 517.1 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.92 (m, 3H), 3.11 (m, 4H), 3.50–3.78 (m, 6H), 7.14 (s, 1H), 7.28 (d, 4H), 7.51 (s, 2H), 7.86 (d, 4H), 8.62 (d, 1H), 9.16 (br s, 4H), 9.28 (br s, 4H), 9.42 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.30, 44.78, 51.21, 54.98, 57.73, 113.91, 114.67, 117.93, 122.85, 130.37, 137.76, 156.18, 158.13, 158.55, 150.58, 163.79, 164.76.

N-(1-(3-Aminopropyl)piperidin-4-yl)-3,5-bis(4-carbamimidoylphenoxy)benzamide ***14****:* Yield 64%; mp 162–164 °C; HPLC: 95.27%; LCMS (ES, *m*/*z*): 530.2 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.78 (m, 2H), 1.98 (m, 4H), 2.86 (m, 2H), 3.12 (m, 4H), 3.50 (d, 2H), 3.98 (m, 1H), 7.14 (s, 1H), 7.26 (d, 4H), 7.51 (s, 2H), 7.85 (d, 4H), 7.95 (br s, 3H), 8.67 (d, 1H), 9.22 (br s, 4H), 9.29 (br s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.54, 28.35, 36.07, 50.68, 52.68, 114.39, 114.75, 117.89, 122.83, 130.45, 137.51, 156.15, 158.30, 158.73, 160.63, 163.62, 164.62.

N-(4-Aminocyclohexyl)-3,5-bis(4-carbamimidoylphenoxy)benzamide ***15****:* Yield 72%; mp 181–183 °C; HPLC: 99.04%; LCMS (ES, *m*/*z*): 487.2 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.35 (m, 4H), 1.80 (m, 2H), 1.96 (m, 2H), 2.84 (m, 1H), 3.62 (m, 1H), 7.11 (s, 1H), 7.24 (d, 4H), 7.50 (s, 2H), 7.78 (d, 4H), 8.12 (br s, 3H), 8.48 (d, 1H), 9.14 (br s, 4H), 9.36 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.59, 30.14, 48.01, 49.01, 115.31, 118.54, 123.49, 131.07, 138.51, 156.81, 159.41, 159.84, 161.32, 163.87, 165.43.

4,4′-((5-(4-(2-Aminoethyl)piperidine-1-carbonyl)-1,3-phenylene)bis(oxy))dibenzimidamide ***16****:* Yield 55%; mp 145–147 °C; HPLC: 97.85%; LCMS (ES, *m*/*z*): 501.2 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.08 (m, 2H), 1.52 (m, 6H), 2.76 (m, 3H), 2.98 (m, 1H), 3.34 (m, 1H), 4.30 (m, 1H), 6.91 (m, 3H), 7.28 (d, 4H), 7.84 (m, 6H), 9.22 (br s, 4H), 9.31 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 32.56, 33.26, 36.43, 41.82, 45.72, 111.76, 113.67, 115.73, 118.39, 123.10, 130.68, 140.11, 156.59, 158.47, 160.60, 164.77, 166.78.

3,5-Bis(4-carbamimidoylphenoxy)-N-((4-hydroxycyclohexyl)methyl)benzamide ***17****:* Yield 65%; mp 128–130 °C; HPLC: 92.74%; LCMS (ES, *m*/*z*): 502.0 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.38 (m, 6H), 1.56 (m, 3H), 3.12 (t, 2H), 3.62 (m, 1H), 4.32 (br s, 1H), 7.16 (s, 1H), 7.32 (d, 4H), 7.49 (s, 2H), 7.90 (d, 4H), 8.64 (t, 1H), 9.26 (br s, 4H), 9.31 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 24.65, 31.68, 35.94, 44.58, 64.81, 114.17, 114.47, 115.18, 118.29, 123.08, 130.68, 138.12, 156.50, 158.45, 160.80, 164.02, 164.79.

Ethyl 4-(3,5-bis(4-carbamimidoylphenoxy)benzamido)piperidine-1-carboxylate ***18****:* Yield 64%; mp 252 °C; HPLC: 94.24%; LCMS (ES, *m*/*z*): 545.5 $(M+H)^+$; ^1H NMR (400 MHz, DMSO-d_6): δ 1.15 (t, 3H), 1.36 (m, 2H), 1.74 (m, 2H), 2.87 (m, 2H), 3.93 (m, 2H), 4.01 (q, 2H), 7.15 (s, 1H), 7.29 (d, 4H), 7.48 (s, 2H), 7.86 (d, 4H), 8.41 (m, 1H), 9.18 (br s, 4H), 9.26 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.35, 30.82, 42.37, 46.59, 60.39, 114.24, 117.99, 122.85, 130.42, 138.03, 154.50, 156.24, 160.66, 163.35, 164.75.

1-(3,5-Bis(4-carbamimidoylphenoxy)benzoyl)piperidine-4-carboxylic acid ***19****:* Yield 42%, mp 95–97 °C; HPLC: 95.33%; LCMS (ES, *m*/*z*): 501.7 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.34 (m, 2H), 1.70 (m, 2H), 2.83 (m, 1H), 3.05 (m, 1H), 3.51 (m, 1H), 4.16 (m, 2H), 6.92 (s, 2H), 7.00 (s, 1H), 7.29 (d, 4H), 7.85 (d, 4H), 9.08 (br s, 4H), 9.24 (br s, 4H).^{13}C NMR (100 MHz, DMSO-d_6): δ 27.36, 27.94, 46.05, 111.74, 113.43, 118.43, 123.13, 130.61, 139.87, 156.64, 160.52, 164.72, 166.87, 175.32.

4,4′-((5-(Decahydroquinoline-1-carbonyl)-1,3-phenylene)bis(oxy))dibenzimidamide ***20****:* Yield 68%; mp 238–240 °C; HPLC: 93.43%; LCMS (ES, *m*/*z*): 512.2 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 11H), 1.78 (m, 1H), 3.54 (m, 4H), 6.80 (s, 1H), 6.88 (s, 1H), 6.98 (s, 1H), 7.29 (d, 4H), 7.85 (d, 4H), 8.96 (br s, 4H), 9.24 (br s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.47, 24.38, 25.49, 29.03, 33.66, 35.79, 40.94, 45.65, 46.21, 51.35, 111.45, 113.33, 118.54, 123.17, 130.60, 140.14, 156.68, 160.47, 164.70

4,4′-((5-(1,2,3,4-Tetrahydroisoquinoline-2-carbonyl)-1,3-phenylene)bis(oxy))dibenzimidamide ***21****:* Yield 65%; mp 112–114 °C; HPLC: 93.50%; LCMS (ES, *m*/*z*): 506.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.75 (m, 2H), 3.54 (m, 1H), 3.74 (m, 1H), 4.67 (s, 2H), 6.94 (m, 2H), 7.03 (m, 1H), 7.14 (m, 4H), 7.32 (d, 4H), 7.84 (d, 4H), 9.06 (br s, 4H), 9.22 (br s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 28.15, 44.50, 49.87, 111.65, 113.28, 118.39, 123.04, 126.26, 128.31, 130.39, 132.67, 134.08, 139.56, 156.64, 157.96, 158.38, 160.30, 164.72, 167.25

3,5-Bis(4-carbamimidoylphenoxy)-N-(4-fluorophenyl)benzamide ***22****:* Yield 70%; mp 286–288 °C; HPLC: 96.58%; LCMS (ES, *m*/*z*): 484.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.16 (m, 3H), 7.33 (d, 4H), 7.57 (s, 2H), 7.71 (m, 2H), 7.89 (d, 4H), 9.29 (br s, 4H), 9.36 (br s, 4H), 10.41 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 114.11, 114.60, 114.83, 115.13, 118.17, 122.30, 122.40, 123.02, 130.45, 134.77, 138.02, 156.42, 160.48, 163.14, 164.76

4-Aminocyclohexyl 3,5-bis(4-carbamimidoylphenoxy)benzoate ***30****:* Yield 68%; mp 113–115 °C; HPLC: 99.26%; LCMS (ES, *m*/*z*): 488.1 $(M+H)^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.29 (m, 2H), 1.44 (m, 2H), 1.86 (m, 2H), 1.96 (m, 2H), 2.81 (m, 1H), 4.77 (m, 1H), 7.25 (s, 1H), 7.29 (d, 4H), 7.40 (s, 2H), 7.86 (d, 4H), 9.05 (br s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 24.21, 28.75, 29.26, 48.05, 73.02, 115.85, 116.14, 118.40, 124.03, 130.45, 133.59, 156.90, 158.54, 158.75, 160.28, 163.67, 165.04, 175.90

7.2.2. Synthesis of 3,5-bis(4-carbamimidoylphenoxy)benzamide (7)

Acid **46** (0.075 g, 0.21 mmol) was treated with oxalyl chloride (0.32 g, 0.25 mmol), followed with aq NH_3 to yield its amide analog and then by converting its nitrile groups to amidine afforded crude solid, which was purified by reverse-phase preparative HPLC and afforded 0.04 g of **7**. Yield 62%; mp 240–242 °C; HPLC: 98.09%; LCMS (ES, *m*/*z*): 390.2 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.15 (s, 1H), 7.32 (d, 4H), 7.46 (s, 2H), 7.61 (br s, 1H), 7.89 (d, 4H), 8.12 (br s, 1H) 9.18 (br s, 3H), 9.26 (br s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 113.70, 114.36, 118.24, 122.96, 130.42, 137.85, 156.41, 160.53, 164.78, 165.72.

7.2.3. Synthesis of 4,4′-((5-amino-1,3-phenylene)bis(oxy))dibenzonitrile (47)

A solution of acid **46** (1.0 g, 2.8 mmol), diphenylphosphorylazide (0.6 ml, 2.8 mmol) and DIPEA (0.5 ml, 2.8 mmol) in DMF (50 ml) at rt *t*-BuOH (15 ml) was added and stirred at 80 °C for 12 h. Reaction mixture was cooled to rt, diluted with 50 ml of saturated solution of $NaHCO_3$ and extracted with EtOAc. The combined organics were washed with brine, dried over Na_2SO_4 and concentrated to give a yellowish residue, which was purified by CC and eluted with 10–20% EtOAc/hexane to afford a Boc-protected amine. Thus obtained product was treated with 6 N HCl (1 ml) in EtOH (5 ml) at rt and stirred for 2 h. Solvent was evaporated and residue was dissolved in ice-cold water, which was basified with Na_2CO_3 solution to pH 7–8 and then extracted with EtOAc. Combined organics were washed with brine, dried over Na_2SO_4 and concentrated to afford the title compound **47** as a light-yellow solid (0.51 g, yield 55%). LCMS (ES, *m*/*z*) 328.10 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.52 (br s, 2H), 5.94 (t, 1H), 6.18 (d, 2H), 7.11 (d, 4H), 7.28 (d, 4H).

7.2.4. Synthesis of 4,4′-((5-amino-1,3-phenylene)bis(oxy))dibenzimidamide 8

Following the procedure of nitrile group conversion to amidine (**48**→**51**), nitrile groups of **47** were converted to amidine to give the title compound **8**. Yield 45%; mp 256–258 °C; HPLC 96.69%; LCMS (ES, *m*/*z*): 362.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.68 (br s, 3H), 5.95 (s, 1H), 6.12 (s, 2H), 7.22 (d, 4H), 7.84 (d, 4H), 9.09 (br s, 4H), 9.23 (br s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 98.18, 101.06, 117.86, 122.19, 130.38, 151.92, 156.98, 161.28, 164.71.

7.2.5. General procedure for the synthesis of *N*-(3,5-bis(4-cyanophenoxy)phenyl)carboxamide derivatives (52)

A solution of a carboxylic acid (1.07 mmol) in DMF (15 ml) at 5 °C was treated with PyBop (1.07 mmol) and DIPEA (1.07 mmol). The reaction mixture was stirred for 10 min and a solution of amino compound **47** (1.07 mmol) dissolved in DMF (5 ml) was added drop wise. Resulting mixture was stirred at RT for 6 h, quenched with ice-cold water and extracted with EtOAc. Combined organics were washed with 1 M solution of Na_2CO_3 followed with brine, dried over Na_2SO_4 and concentrated to give oily residue, which was purified by CC using hexane–EtOAc (10:2) to give the desired compound **52**.

Further, following the procedure of nitrile group conversion to amidine (**48**→**51**), the nitrile groups of **52** were converted to amidine to give *N*-(3,5-bis(4-carbamimidoylphenoxy)phenyl)carboxamide derivatives (**55** and **23**). Boc-protected amino group or THP-protected hydroxyl group of compound (**55**) was deprotected, following the procedure of Example **12**, to give the free amino or the hydroxyl group derivatives (**24**–**27**).

N-(3,5-bis(4-carbamimidoylphenoxy)phenyl)-4-methylcyclohexanecarboxamide ***23****:* Yield 74%; mp 146–148 °C; HPLC: 98.43%; LCMS (ES, *m*/*z*): 486.2 $(M+H)^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 5H), 1.31 (m, 3H), 1.67 (m, 4H), 2.16 (m, 1H), 6.64 (s, 1H), 7.24 (m, 6H), 7.86 (d, 4H), 9.08 (br s, 4H), 9.24 (br s, 4H), 10.18 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 22.87, 29.37, 31.97, 34.31, 45.15, 106.02, 106.28, 118.77, 123.27, 131.01, 142.83, 157.09, 161.31, 165.22, 175.28.

1-(3-Aminopropanoyl)-N-(3,5-bis(4-carbamimidoylphenoxy)phenyl)piperidine-4-carboxamide ***24****:* Yield 52%; mp 142–144 °C; HPLC: 97.97%; LCMS (ES, *m*/*z*): 544.2 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 3H), 1.74 (m, 3H), 2.11 (m, 3H), 2.95 (m, 3H), 4.36 (m, 1H), 6.60 (s, 1H), 7.24 (s, 2H), 7.31 (d, 4H), 7.64 (br s, 3H), 7.86 (d, 4H), 9.22 (br s, 4H), 9.32 (br s, 4H), 10.44 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 27.62, 29.47, 35.18, 42.25, 43.86, 105.46, 105.98, 118.07, 122.63, 130.29, 141.99, 156.45, 157.94, 158.36, 160.65, 164.71, 167.79, 173.10.

1-(2-Aminoethyl)-N-(3,5-bis(4-carbamimidoylphenoxy)phenyl)piperidine-4-carboxamide ***25****:* Yield 56%; mp 122–124 °C; HPLC: 98.91%; LCMS (ES, *m*/*z*): 516.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.74 (m, 2H), 1.85 (m, 2H), 2.41 (m, 2H), 2.56 (m, 2H), 3.18 (m, 5H), 6.61 (s, 1H), 7.25 (m, 6H), 7.86 (d, 4H), 8.08 (br s, 3H), 9.13 (br s, 4H), 9.22 (br s, 4H), 10.40 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.75, 33.90, 51.39, 53.21, 58.74, 105.74, 106.13, 118.09, 122.69, 130.38, 141.88, 156.49, 158.62, 160.70, 164.71, 172.36.

(1r,4r)-4-Amino-N-(3,5-bis(4-carbamimidoylphenoxy)phenyl)cyclohexanecarboxamide ***26****:* Yield 68%; mp 118–120 °C; HPLC: 99.18%; LCMS (ES, *m*/*z*) 487.2$(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.28 (m, 4H), 1.80 (m, 2H), 1.92 (m, 2H), 2.21 (m, 1H), 2.93 (m, 1H), 6.60 (s, 1H), 7.24 (m, 6H), 7.85 (d, 7H), 9.16 (m, 8H), 10.26 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.90, 29.29, 43.46, 48.56, 105.71, 105.88, 118.08, 118.30, 122.85, 130.57, 142.22, 156.66, 158.26, 158.59, 160.83, 164.76, 173.89

N-(3,5-Bis(4-carbamimidoylphenoxy)phenyl)-1-(2-hydroxyethyl)piperidine-4-carboxamide ***27****:* Yield 60%; mp 128–130 °C; HPLC: 97.18%; LCMS (ES, *m*/*z*): 517.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.84 (m, 4H), 2.78-3.21 (m, 5H), 3.52-3.78 (m, 5H), 6.60 (s, 1H), 7.25 (m, 6H), 7.85 (d, 4H), 9.18 (br s, 4H), 9.26 (br s, 4H), 10.41 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.31, 42.22, 51.25, 54.91, 57.91, 105.67, 106.06, 118.06, 122.66, 130.31, 141.78, 156.46, 158.05, 158.48, 160.63, 164.70, 172.01.

7.2.6. General procedure for the synthesis of *N*-(3,5-bis(4-cyanophenoxy)phenyl)sulfonamide derivatives (56)

To the solution of **47** (1.68 mmol), pyridine (3.36 mmol), DMAP (0.35 mmol) in 50 ml of DCM, arylsulfonyl chloride (1.68 mmol, dissolved in 5 ml of DCM) was added under N_2 atmosphere and resulting mixture was stirred at rt for 12 h. After the reaction completion, mixture was concentrated and thus obtained residue was dissolved in 100 ml of EtOAc. Organic layer was washed with 1 N HCl (50 ml) followed by brine (2 × 50 ml) and dried over anhydrous Na_2SO_4. Solvent was evaporated to give crude solid, which was purified by CC (silica gel, 10% ethyl acetate in hexane) to give the title compound **56**.

Further, following the procedure of nitrile group conversion to amidine (**48**→**51**), nitrile groups of **56** were converted to amidine and afforded the 4,4′-((5-(arylsulfonamido)-1,3-phenylene)bis(oxy))dibenzimidamide derivatives **28**–**29**.

*4,4′-((5-(4-Fluorophenylsulfonamido)-1,3-phenylene)bis(oxy))dibenzimidamide **28**:* Yield 47%; mp 210–212 °C; HPLC: 95.72%; LCMS (ES, *m*/*z*): 520.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.58 (s, 1H), 6.63 (s, 2H), 7.15 (d, 4H), 7.45 (t, 2H), 7.76 (m, 2H), 7.82 (d, 4H), 9.12 (br s, 3H), 9.25 (br s, 3H), 10.68 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 106.31, 116.57, 116.80, 118.28, 123.12, 129.74, 129.84, 130.55, 135.18, 140.64, 156.94, 160.29, 164.67

*4,4′-((5-(Naphthalene-2-sulfonamido)-1,3-phenylene)bis(oxy))dibenzimidamide **29**:* Yield 52%; mp 232–232 °C; HPLC: 96.10%; LCMS (ES, *m*/*z*): 551.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.48 (s, 1H), 6.65 (s, 2H), 7.06 (d, 4H), 7.68 (m, 7H), 8.02 (d, 1H), 8.12 (t, 2H), 8.39 (s, 1H), 8.82 (br s, 4H), 9.18 (br s, 4H), 10.80 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 105.92, 106.19, 118.08, 121.68, 122.94, 127.70, 127.88, 129.05, 129.50, 130.33, 131.40, 134.24, 140.72, 156.77, 160.17, 164.66.

7.2.7. Synthesis of 3-(4-cyanophenoxy)-5-hydroxybenzoic acid (64)

A solution of **44** (2.25 g, 12.4 mmol) in DMF (50 ml) at rt was treated with K_2CO_3 (2.57 g, 18.6 mmol) and stirred for 10 min. A solution of 4-fluorobenzonitrile (2.25 g, 18.6 mmol) in DMF (100 ml) was added drop wise and the resultant solution was stirred at 45 °C for 6 h. The reaction mixture was quenched with ice-cold water and extracted with EtOAc. The organic layer was washed with 1 M Na_2CO_3 solution, followed with brine and then dried over anhydrous sodium sulfate. Solvent was evaporated to dryness to get crude oily residue, which was purified by CC and eluted with 30% EtOAc/hexane to afford the white solid ester (1.57 g; yield 45%). The resulted ester was hydrolyzed, following the procedure of Example **46**, to give the title compound **64** (1.1 g; yield 70%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.95 (s, 1H), 7.21 (d, 2H), 7.56 (s, 2H), 7.88 (d, 2H), 9.28 (br s, 1H), 13.6 (br s, 1H).

7.2.8. Synthesis of *tert*-butyl ((1r,4r)-4-(3-(4-cyanophenoxy)-5-hydroxybenzamido)cyclohexyl)carbamate (65)

Following the procedure of Example **48**, the product of Example **64** (0.34 g, 1.35 mmol) was coupled with *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate (0.29 g, 1.35 mmol) to afford 0.41 g (yield 55%) of **65**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.23 (m, 4H), 1.37 (s, 9H), 1.78 (m, 4H), 3.12 (m, 1H), 3.61 (m, 1H), 6.68 (br s, 1H), 7.01 (s, 1H), 7.18 (d, 2H), 7.31 (s, 2H), 7.85 (d, 2H), 8.28 (br s, 1H).

7.2.9. General procedure for the coupling of alkyl/aryl halide with *tert*-butyl ((1r,4r)-4-(3-(4-cyanophenoxy)-5-hydroxybenzamido)cyclohexyl)carbamate (66)

Following the general procedure for the synthesis of **46** except heating the reaction mixture at 65 °C, the alkoxy analogs **66** were synthesized (yield 45-65%).

Further, following the general procedure of nitrile group conversion to amidine (**48**→**51**), nitrile group/s of **66** were converted to amidine and then amino group deprotection afforded **31**–**39**.

*N-((1r,4r)-4-Aminocyclohexyl)-3-(4-aminophenoxy)-5-(4-carbamimidoylphenoxy)benzamide **31**:* Yield 38%; mp 95–97 °C; HPLC: 97.05%; LCMS (ES, *m*/*z*): 460.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.36 (m, 4H), 1.80 (m, 2H), 1.94 (m, 2H), 3.00 (m, 1H), 3.68 (m, 1H), 6.85 (s, 1H), 7.01 (s, 4H), 7.25 (d, 2H), 7.63 (s, 2H), 7.84 (m, 5H), 8.38 (d, 1H), 9.08 (br s, 2H), 9.25 (br s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.00, 29.49, 47.40, 48.55, 111.62, 112.72, 112.84, 114.28, 117.82, 118.19, 119.88, 120.10, 122.68, 130.37, 137.86, 150.27, 155.90, 158.50, 158.75, 160.78, 163.75, 164.84.

*N-((1r,4r)-4-Aminocyclohexyl)-3-((6-aminopyridin-3-yl)oxy)-5-(4-carbamimidoylphenoxy)benzamide **32**:* Yield 48%; mp 113–115 °C; HPLC: 95.48%; LCMS (ES, *m*/*z*): 461.2 $(M+H)^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.38 (m, 4H), 1.85 (m, 2H), 1.96 (m, 2H), 2.95 (m, 1H), 3.67 (m, 1H), 6.94 (d, 1H), 7.02 (s, 1H), 7.20 (d, 2H), 7.33 (d, 2H), 7.77 (dd, 1H), 7.85 (d, 2H), 7.93 (br s, 3H), 7.98 (s, 1H), 8.40 (d, 1H), 9.27 (br s, 2H), 9.34 (br s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.14, 29.66, 47.53, 48.56, 111.71, 112.35, 113.72, 113.86, 117.87, 122.82, 129.90, 130.57, 136.76, 137.89, 142.42, 153.70, 156.04, 158.54, 158.66, 159.19, 160.94, 163.64, 164.84.

*N-((1r,4r)-4-Aminocyclohexyl)-3-(4-carbamimidoylphenoxy)-5-(4-carbamoylphenoxy)benzamide **33**:* Yield 42%, mp 114–116 °C; HPLC: 97.22%; LCMS (ES, *m*/*z*): 488.2 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 4H), 1.84 (m, 4H), 2.86 (m, 1H), 3.61 (m, 1H), 7.01 (s, 1H), 7.08 (d, 2H), 7.25 (m, 3H), 7.40 (d, 2H), 7.74 (br s, 2H), 7.82 (d, 2H), 7.91 (d, 2H), 8.37 (d, 1H), 8.88 (br s, 2H), 9.21 (br s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.03, 29.53, 47.45, 48.54, 113.31, 113.98, 114.17, 117.61, 118.00, 122.83, 129.63, 130.44, 137.97, 156.15, 157.03, 158.51, 160.72, 163.52, 164.80, 166.92.

*N-((1r,4r)-4-Aminocyclohexyl)-3-((4-carbamimidoylbenzyl)oxy)-5-(4-carbamimidoylphenoxy)benzamide **34**:* Yield 72%, mp 263–265 °C; HPLC: 91.03%; LCMS (ES, *m*/*z*): 501.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.40 (m, 4H), 1.82 (m, 4H), 2.86 (m, 1H), 3.60 (m, 1H), 5.32 (s, 2H), 7.04 (s, 1H), 7.21 (m, 3H), 7.44 (s, 1H), 7.68 (d, 2H), 7.88 (m, 7H), 8.37 (d, 1H), 9.20 (br s, 7H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.68, 30.21, 48.02, 49.22, 69.53, 109.88, 111.02, 111.69, 115.54, 118.39, 123.14, 128.16, 128.73, 131.02, 138.09, 143.11, 156.43, 158.56, 158.98, 159.97, 161.62, 164.66, 165.38, 166.02.

*N-((1r,4r)-4-Aminocyclohexyl)-3-((3-carbamimidoylbenzyl)oxy)-5-(4-carbamimidoylphenoxy)benzamide **35**:* Yield 65%; mp 96–98 °C; HPLC: 96.67%; LCMS (ES, *m*/*z*): 501.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.39 (m, 4H), 1.86 (m, 2H), 1.95 (m, 2H), 2.99 (m, 1H), 3.70 (m, 1H), 5.27 (s, 2H), 7.06 (s, 1H), 7.22 (m, 3H), 7.47 (s, 1H), 7.66 (d, 1H), 7.80 (t, 2H), 7.81 (m, 5H), 8.38 (d, 1H), 9.23 (br s, 2H), 9.29 (br s, 2H), 9.35 (br s, 2H), 9.40 (br s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.00, 29.53, 47.36, 48.57, 69.06, 109.21, 110.40, 111.06, 117.72, 118.57, 122.47, 126.84, 127.42, 128.39, 128.99, 130.31, 132.48, 137.44, 155.77, 158.15, 159.38, 160.97, 164.03, 164.79, 165.65.

*N-((1r,4r)-4-Aminocyclohexyl)-3-(4-(3-aminopropanamido)phenoxy)-5-(4-carbamimidoylphenoxy)benzamide **36**:* Yield 52%; mp 110–112 °C; HPLC: 96.79%; LCMS (ES, *m*/*z*): 531.2 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 4H), 1.82 (m, 4H), 2.71 (t, 2H), 2.95 (m, 2H), 3.12 (q, 2H), 3.65 (m, 1H), 6.89 (s, 1H), 7.08 (d, 2H), 7.22 (d, 2H), 7.31 (d, 2H), 7.62 (d, 2H), 7.82 (m, 8H), 8.38 (d, 1H), 9.16 (br s, 2H), 9.25 (br s, 2H), 10.25 (br s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.11, 29.63, 33.09, 34.90, 47.44, 48.48, 112.06, 112.85, 117.91, 119.84, 120.85, 122.79, 130.61, 135.39, 137.72, 150.89, 155.97, 157.95, 158.27, 158.80, 160.91, 163.69, 164.67, 168.11.

*N-((1r,4r)-4-Aminocyclohexyl)-3-((6-bromopyridin-3-yl)methoxy) -5-(4-carbamimidoylphenoxy)benzamide **37**:* Yield 64%; mp 192–194 °C; HPLC: 95.49%; LCMS (ES, *m*/*z*): 538.1 & 540.1 (Bromo pattern, M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.38 (m, 4H), 1.88 (m, 2H), 1.95 (m, 2H), 2.99 (m, 1H), 3.69 (m, 1H), 5.20 (s, 2H), 7.02 (s, 1H), 7.17 (s, 1H), 7.19 (d, 2H), 7.42 (s, 1H), 7.70 (d, 1H), 7.85 (m, 6H), 8.35 (d, 1H), 8.50 (s, 1H), 9.16 (br s, 2H), 9.28 (br s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.16, 29.70, 47.44, 48.59, 66.69, 109.35, 110.42, 111.24, 117.89, 122.68, 127.95, 130.54, 132.07, 137.42, 139.06, 140.91, 149.71, 155.92, 159.30, 161.07, 164.02, 164.83.

*N-((1r,4r)-4-Aminocyclohexyl)-3-(4-carbamimidoylphenoxy)-5-((4-chlorobenzyl)oxy)benzamide **38**:* Yield 75%; mp 252–254 °C; HPLC: 95.85%; LCMS (ES, *m*/*z*): 493.2 (M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.35 (m, 4H), 1.87 (m, 2H), 1.94 (m, 2H), 2.97 (m, 1H), 3.67 (m, 1H), 6.97 (s, 1H), 7.14 (s, 1H), 7.17 (d, 2H), 7.40 (s, 1H), 7.45 (s, 4H), 7.84 (m, 4H), 8.34 (d, 1H), 9.17 (br s, 2H), 9.25 (br s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.13, 29.69, 47.39, 48.53, 68.91, 109.28, 110.39, 110.96, 115.40, 117.86, 122.67, 128.44, 129.58, 130.53, 132.55, 135.46, 137.29, 155.86, 158.44, 159.49, 161.06, 164.03, 164.85.

*N-((1r,4r)-4-Aminocyclohexyl)-3-((4-bromobenzyl)oxy)-5-(4-carbamimidoylphenoxy)benzamide **39**:* Yield 68%; mp 211–213 °C; HPLC: 96.42%; LCMS (ES, *m*/*z*): 537.1 & 539.1 (Bromo pattern,M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 4H), 1.81 (m, 2H), 1.95 (m, 2H), 2.92 (m, 1H), 3.64 (m, 1H), 5.15 (s, 2H), 6.99 (s, 1H), 7.16 (s, 1H), 7.21 (d, 2H), 7.44 (m, 3H), 7.61 (d, 2H), 7.78 (d, 2H), 8.04 (br s, 3H), 8.38 (d, 1H), 9.05 (br s, 2H), 9.30 (br s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.00, 29.53, 47.34, 48.56, 68.99, 109.08, 110.41, 110.90, 117.76, 120.88, 122.49, 129.56, 130.31, 131.17, 135.80, 137.38, 155.78, 159.37, 160.93, 164.02, 164.80.

7.2.10. General procedure for the conversion of nitrile group to Boc-protected aminomethyl group (67)

A solution of **65** (1.6 g, 3.53 mmol) in MeOH (100 ml) at 0 °C was added nickel(II) chloride hexahydrate (0.046 g, 0.02 mmol), di-*tert*-butyl dicarbonate (1.15 g, 5.29 mmol), followed by sodium borohydride (0.94 g, 24.72 mmol) in portions during 15 min and then resulting mixture was stirred at rt for 1 h. Reaction mixture was cooled to 0 °C and added diethylenetriamine (3.64 g, 35.32 mmol) drop wise and then stirred at rt for 2 h. Solvent was evaporated and residue was dissolved in water and then extracted with EtOAc. Combined organics were washed with water, followed with brine and dried over anhydrous sodium sulfate. Solvent was evaporated to yield crude solid, which was purified by CC and eluted with 20–30% EtOAc/hexane to give the off-white compound **67** (0.8 g; yield 45%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 4H), 1.36 (s, 18H), 1.74 (m, 4H), 3.14 (m, 1H), 3.58 (m, 1H), 4.46 (d, 2H), 6.75 (br s, 1H), 6.94 (s, 1H), 7.26 (d, 2H), 7.34 (m, 4H), 8.05 (br s, 2H), 8.28 (br s, 1H).

Further, following the general procedure of alkylation of Example **66**, the hydroxyl group of **67** was alkylated with an aryl halide, followed by the nitrile group (if present) conversion to amidine (like **48**→**51**) and then Boc-protected amino group deprotection to give compounds **40**–**41**.

*N-((1r,4r)-4-Aminocyclohexyl)-3-(4-(aminomethyl)phenoxy)-5-(4-carbamimidoylphenoxy)benzamide **40**:* Yield 56%; mp 192–194 °C; HPLC: 96.64%; LCMS (ES, *m*/*z*): 474.2 (M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (m, 4H), 1.79 (m, 2H), 1.81 (m, 2H), 2.84 (m, 1H), 3.62 (m, 1H), 4.01 (d, 2H), 6.95 (t, 1H), 7.15 (d, 2H), 7.22 (d, 2H), 7.34 (s, 1H), 7.38 (s, 1H), 7.48 (d, 2H), 7.84 (d, 4H), 8.16 (br s, 2H), 8.38 (d, 1H), 9.14 (br s, 2H), 9.26 (br s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.19, 29.73, 41.69, 47.56, 48.58, 113.59, 115.06, 118.00, 119.25, 122.95, 129.96, 130.68, 137.89, 156.05, 156.13, 158.09, 158.50, 161.00, 163.68, 164.91.

*N-((1r,4r)-4-Aminocyclohexyl)-3,5-bis(4-(aminomethyl)phenoxy) benzamide **41**:* Yield 48%; mp 93–95 °C; HPLC: 98.87%; LCMS (ES, *m*/*z*): 461.2 (M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.31 (m, 4H), 1.85 (m, 2H), 1.94 (m, 2H), 2.99 (m, 1H), 3.65 (m, 1H), 4.05 (s, 4H), 6.79 (s, 1H), 7.14 (d, 4H), 7.26 (s, 2H), 7.48 (d, 4H), 7.82 (br s, 3H), 8.18 (br s, 6H), 8.38 (d, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.95, 29.45, 41.57, 47.34, 48.49, 111.32, 112.28, 118.75, 129.41, 130.71, 137.69, 156.00, 157.51, 163.80.

7.2.11. General procedure for the coupling of an alkyl halide with ethyl 3,5-dihydroxybenzoate (75)

Following the procedure of Example **66**, ethyl 3,5-dihydroxybenzoate (2.4 mmol) was coupled with 3 or 4-(bromomethyl)benzonitrile (2.4 mmol) to give ethyl 3,5-bis((3 or 4-cyanobenzyl)oxy)benzoate analogs (yield 75%).

7.2.12. General procedure for the synthesis of 3,5-bis((3 or 4-cyanobenzyl)oxy)benzoic acid (76)

Following the procedure of Example **46**, ethyl 3,5-bis((3 or 4-cyanobenzyl)oxy)benzoate (1.8 mmol) was hydrolyzed to give 3,5-bis((3 or 4-cyanobenzyl)oxy)benzoic acid derivatives (yield 85%).

7.2.13. General procedure for the synthesis of *tert*-butyl ((*1r*,*4r*)-4-(3,5-bis((3 or 4-cyanobenzyl)oxy)benzamido)cyclohexyl) carbamate (77)

Following the procedure of Example **48**, acid **76** (1.5 mmol) was coupled with *tert*-butyl ((1*r*,4*r*)-4-aminocyclohexyl)carbamate (1.5 mmol) to give amide **77** (yield 65–70%).

Further, following the procedure of nitrile group conversion to amidine (**48**→**51**), nitrile groups of **77** were converted to amidine and then Boc-protected amino group deprotection afforded *N*-3,5-bis((4-cyanobenzyl)oxy)benzamide derivatives (**42**–**43**).

*N-((1r,4r)-4-Aminocyclohexyl)-3,5-bis((4-carbamimidoylbenzyl) oxy)benzamide **42**:* Yield 72%; mp 171–173 °C; HPLC: 98.01%; LCMS (ES, *m*/*z*): 515.2 (M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.41 (m, 4H), 1.88 (m, 2H), 1.98 (m, 2H), 3.01 (m, 1H), 3.71 (m, 1H), 5.28 (s, 4H), 6.88 (s, 1H), 7.14 (s, 2H), 7.67 (d, 4H), 7.85 (d, 4H), 7.92 (br s, 3H), 8.28 (d, 1H), 9.29 (br s, 4H), 9.38 (br s, 4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.01, 29.57, 47.25, 48.60, 68.63, 104.60, 106.69, 127.38, 127.99, 136.82, 142.75, 158.58, 158.90, 164.69, 165.46.

*N-((1r,4r)-4-Aminocyclohexyl)-3,5-bis((3-carbamimidoylbenzyl) oxy)benzamide **43**:* Yield 68%; mp 210–212 °C; HPLC: 98.63%; LCMS (ES, *m*/*z*): 515.2 (M+H)$^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.41 (m, 4H), 1.89 (m, 4H), 2.92 (m, 1H), 3.61 (m, 1H), 5.22 (s, 4H), 6.91 (s, 1H), 7.18 (s, 2H), 7.67 (d, 2H), 7.80 (m, 4H), 7.92 (m, 5H), 8.29 (d, 1H), 9.26 (br s, 4H), 9.38 (br s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.21, 29.79, 47.39, 48.66, 68.85, 104.54, 106.70, 115.59, 118.56, 127.04, 127.61, 128.61, 129.21, 132.68, 136.84, 137.82, 158.67, 158.99, 159.14, 164.84, 165.80.

7.3. Molecular modeling

Publicly accessible matriptase co-crystal structures (viz., PDB IDs: 2GV6 & 1EAX) were employed for setting-up protocols for molecular docking. Prior to this, the protein coordinates were optimized through assignment of suitable bond-orders, water molecules elimination, addition of hydrogen atoms, internal hydrogen bonds refinement and restrained minimization through OPLS 2005 force-field. Hydrogen bonding constraints were applied to Asp189 and Ser195 side-chains during receptor grid generation so that they can be used in docking (if required). The molecules were energy minimized through LigPrep and then docked in to generated grid (matriptase active-site) employing standard precision mode using Glide software. The matriptase crystal structure in complex with compound **6** was employed for WaterMap estimations,

as described previously.[17] Briefly, the calculation was done in default mode which included a simulation run of 2.0 ns at 300 K for computing the hydration site statistics. All software names mentioned in this section are products of Schrödinger Inc and all images were prepared using PyMOL.[24]

7.4. Cytochrome P450 inhibition

Cytochrome p450 inhibition assay was done using the probe-substrate method.[25–28] A mixture of five substrates was prepared by pooling caffeine (CyP1A2), tolbutamide (CyP2C9), dextromethorphan (CyP2D6), midazolam (CyP3A4) and testosterone (CyP3A4).[25,26] Serial dilutions of the test compounds ranging from 100 to 0.005 μM were incubated with human liver microsomes in the presence of a pool of probe substrates specific for five major isoforms. The reaction was initiated by the addition of NADPH and terminated by adding acetonitrile. The supernatant samples were analyzed for the metabolites of the probe substrates by LCMS:MS and the IC_{50} for selected compounds screened was determined using the software Graphpad Prism.

7.5. Plasma protein binding

Plasma protein binding assay was carried out by the ultra-filtration method using the Microcon-10 assembly.[29,30] 10 μM solutions of test compounds were incubated in human plasma for 1 h and the protein free fraction was obtained by centrifugation and the compound concentrations in the samples were analyzed by LCMS:MS and percent bound was calculated.

7.6. Pharmacokinetic profiling

To determine the pharmacokinetic parameters, male CD-1 mice were given a single administration of test compound by subcutaneous route. Mice aged 8-10 weeks weighing 30–40 g were used for the studies. The dose was 1 mg/kg and dose volume 10 mL/kg. The test compounds were formulated in the vehicle comprising of 1% *N*-methyl-2-pyrrolidone (NMP), 5% Tween80, 5% Glycofurol and saline. The test compounds were dissolved in NMP with sonication for 1 min, followed by addition of Tween80, Glycofurol and finally made up to 100% with saline. Animals were dosed within 1 h of preparation and the blood and tissue samples were collected at predetermined time points. Blood samples were collected by cardiac puncture under carbon dioxide anesthesia into pre-cooled K_2EDTA polypropylene tubes and kept cooled until the separation of plasma by centrifugation. Plasma samples processing was done as per standard acetonitrile precipitation method and drug levels were quantified using LC–MS/MS. Plasma concentration is expressed as ng/ml, tissue concentrations expressed as ng/g and the PK parameters calculated using WinNonlin 5.1 software.[31,32]

7.7. Establishment of the DU-145 prostate cancer xenograft tumor model

Severe combined immunodeficiency (SCID) mice (6–8 weeks old) breeding pairs were procured from Charles River Laboratories and bred to obtain sufficient number of animals for efficacy experiment. DU-145 cells (5×10^6 cells) were injected subcutaneously into the male SCID mice. When the tumors reached 60–80 mm^3, animals were randomized into groups of ten for initiation of therapy. Compounds were administered in a vehicle comprising of 2% ethanol, 10% hydroxyl cyclodextrin in 0.9% saline and dosed twice a day subcutaneously for 31 days as indicated in Figure 8. Tumors size was measured every alternate day using Vernier calipers. Tumor volume, body weight and clinical symptoms were monitored. Tumor volumes were calculated assuming tumors to be ellipsoid using the formula $V = (D \times d^2)/2$ where V (mm^3) is tumor volume, D is longest diameter in mm, and d is shortest diameter in mm.

Accession codes

Protein crystal structure coordinates and structure factors are deposited in the RCSB Protein Data Bank with the accession numbers 4O9V and 4O97.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.04.013.

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