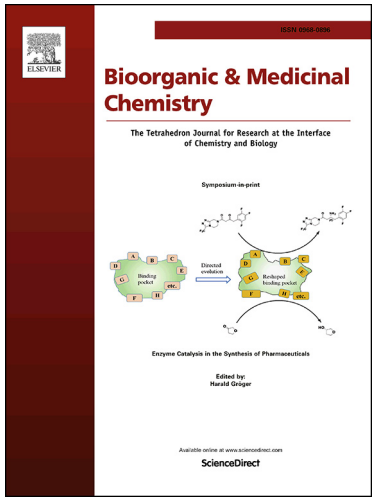
Accepted Manuscript



Structure-Activity Relationship Investigation of Phe-Arg Mimetic Region of Human Glutaminyl Cyclase Inhibitors

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Structure-Activity Relationship Investigation of Phe-Arg Mimetic Region of Human Glutaminyl Cyclase Inhibitors

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ABSTRACT

Glutamyl cyclase (QC) is a promising therapeutic target because of its involvement in the pathogenesis of Alzheimer's disease. In this study, we developed novel QC inhibitors that contain 3-aminoalkyloxy-4-methoxyphenyl and 4-aminoalkyloxyphenyl groups to replace the previously developed pharmacophore. Several potent inhibitors were identified, showing IC_{50} values in a low nanomolar range, and were further studied for *in vitro* toxicity and *in vivo* activity. Among these, inhibitors **51** and **53** displayed the most potent $A\beta_{N3pE-40}$-lowering effects in *in vivo* acute model with reasonable BBB penetration, without showing cytotoxicity and *h*ERG inhibition. The molecular modeling analysis of **53** indicated that the salt bridge interaction and the hydrogen bonding in the active site provided a high potency. Given the potent activity and favorable BBB penetration with low cytotoxicity, we believe that compound **53** may serve as a potential candidate for anti-Alzheimer's agents.

1. Introduction

β-Amyloid (Aβ) is a main component of the amyloid plaques found in Alzheimer's disease (AD), and the elevated levels of Aβ peptides and amyloid plaques are thought to be the hallmarks of AD pathology. The brain Aβ peptides are present as a mixture of various isoforms, among which $Aβ_{1-40}$ and $Aβ_{1-42}$, are most abundant in AD patients. Several recent studies found that pyroglutamyl-β-amyloid (pE-Aβ) peptides, the less abundant but more hydrophobic form of Aβ, appeared to be more neurotoxic than $Aβ_{1-40}$ and $Aβ_{1-42}$ and to promote the formation of amyloid and tau plaques.[1-3] In particular, pE-Aβ levels are highly upregulated in the brains of patients and animal models with AD,[4,5] and are shown to be the major constituents of Aβ deposit in sporadic and familial Alzheimer's disease (AD).[6-8]

Pyroglutamyl-β-amyloid (pE-Aβ) peptides are a type of the *N*-truncated Aβ forms containing an *N*-terminal pyroglutamate at positions 3 or 11 in Aβ. Due to their increased hydrophobicity, pE-Aβ peptides are prone to rapid aggregation and are much more resistant to proteolytic degradation, which explains their exacerbated neurotoxicity. The formation of the pyroglutamate from the *N*-terminal glutamate of Aβ is catalyzed by glutamyl cyclase (QC), which has been implicated in the pathogenesis of AD. Moreover, the administration of QC inhibitors reduced the brain pGlu-Aβ levels and Aβ plaques[9,10] and decreased gliosis and recovered memory deficits in AD mice.[4] Given that most of the Aβ-lowering drugs that were advanced to clinical trials have not yet proven to be effective,[11,12] blocking the formation of neurotoxic Aβ species such as pE-Aβ can provide a promising alternative to current therapeutic approaches.

Previously reported QC inhibitors were developed by a pharmacophore design based on the *N*-terminal structure of its substrate $Aβ_{3-42}$. The representative inhibitors developed by Probiodrug (**1**, **2**),[13-15] Wu and colleagues (**3**)[16] and our research group (**4**)[17-19] are shown in **Figure 1**. Naturally occurring inhibitors[20,21] and other small molecule inhibitors developed by the fragment-based approach[22] were also reported; however, these inhibitors demonstrated only modest activity.

Currently, PQ912, developed by Probiodrug, is undergoing a clinical trial, and it has exhibited favorable safety and tolerability. More importantly, PQ912 demonstrated a slight improvement in synaptic and neurological functions in patients with AD in a recently completed phase IIa clinical trial,[23,24] supporting that QC is a potential therapeutic target for the treatment of AD.

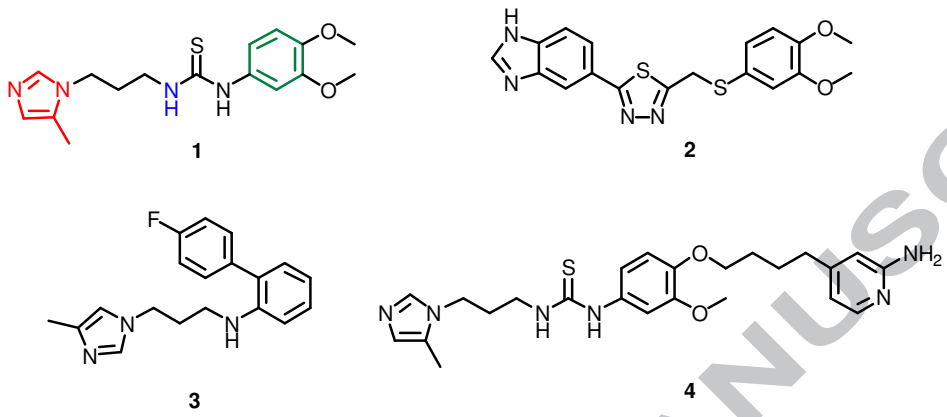


Figure 1. Representative QC inhibitors.

The Probiodrug compound **1** contains three key pharmacophores derived from the *N*-terminal Glu-Phe moiety of $A\beta_{3E\text{-}42}$.[14,15] The 5-methylimidazole ring (red) mimics the *N*-terminal carboxylic acid as a zinc-binding motif. The distal NH of thiourea (blue) serves as a hydrogen bond donor, mimicking the first peptide bond from the *N*-terminus. The phenyl ring (green) mimics the Phe side chain at the penultimate position to the *N*-terminus. Inspired by this approach, we had previously developed a series of QC inhibitors (template **A**), with an extended scaffold as described in **Figure 2**.[18,19] The scaffold contains an additional pharmacophore that mimics the binding interaction of the guanidine side chain of Arg at the antepenultimate position to the *N*-terminus. The newly developed QC inhibitors displayed much improved potency with a range of 5 to 40-fold more potent inhibition than **1**. Specifically, compound **4** not only exhibited potent inhibition without cytotoxicity but also significantly reduced the brain concentrations of pE-Aβ and total Aβ while restoring cognitive functions in an AD animal model. The molecular modeling analysis of **4**

indicated that the 2-aminopyridine moiety showed strong interactions with the carboxylate group of Glu327 in the QC binding site.

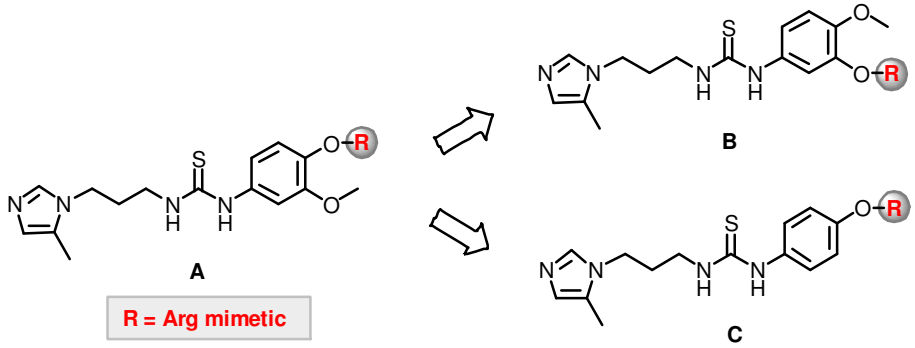


Figure 2. Design rationale for QC inhibitors with novel templates

In this study, we investigated a series of 3-aminoalkyloxy-4-methoxyphenyl (template B) and 4-aminoalkyloxyphenyl (template C) surrogates as variants of template A. We anticipate that these templates would provide useful information to optimize the position of Arg-mimetic region (from template B) and to identify the significance of the 3-methoxy group (from template C) for QC inhibition. We evaluated the human QC inhibitory activity of the synthesized compounds *in vitro* and selected several potent inhibitors (IC_{50} < 10 nM). We further tested these compounds for *in vitro* toxicity/permeability and *in vivo* activity in acute AD model mice. Finally, a molecular modeling study was performed to analyze the specific binding interactions in the QC active site.

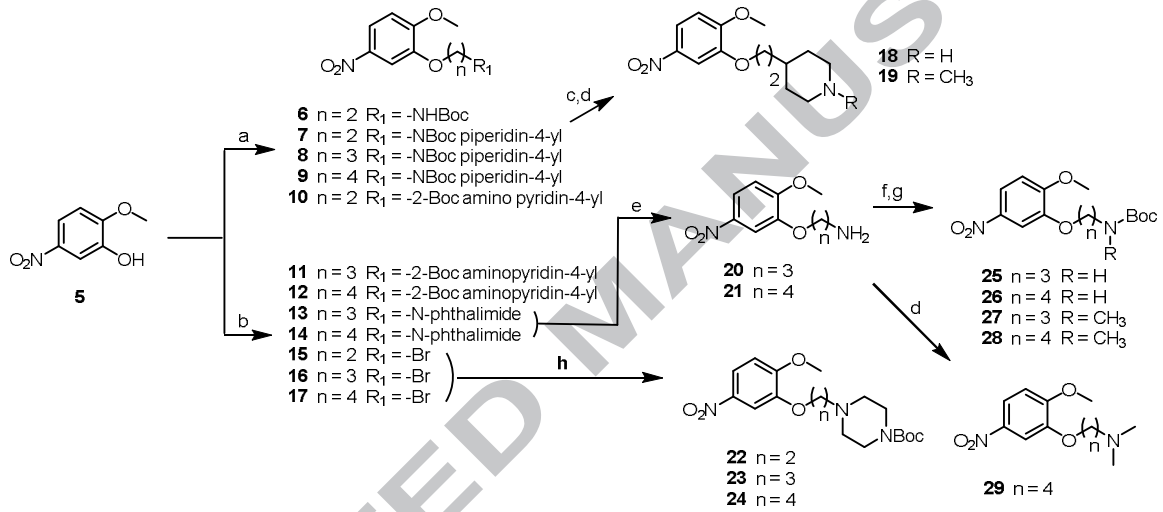
2. Results and discussion

2.1. Chemistry

In general, the final thiourea compounds were synthesized by the coupling reaction between 3-(5-methyl-1*H*-imidazol-1-yl)propan-1-amine and aniline intermediates obtained by the reduction of nitro fragments prepared in **Schemes 1** and **2**.

The synthesis of the 3-aminoalkyloxy-4-methoxy-1-nitrobenzene fragments is described in **Scheme 1**. Starting from 5-nitroguaiacol (**5**), the Mitsunobu reaction or Williamson reaction

incorporated *N*-protected aminoalkyl moieties into the phenolic position to provide **6**-**17**. Among them, the *N*-Boc protected amino (**6**-**12**) and phthalimide protected amino (**13**, **14**) intermediates were directly employed for the thiourea coupling. The bromides **15**-**17** were reacted with *N*-Boc piperazine to give **22**-**24**. After deprotection, the free amines (**18**, **21**) were converted to the *N*-methylpiperidine (**19**) and dimethylamino (**29**) analogues by reductive amination, respectively. Meanwhile, the amines (**20**, **21**) were protected with a Boc group and then *N*-methylated to afford the corresponding *N*-Boc methylamino (**27**, **28**) analogues.

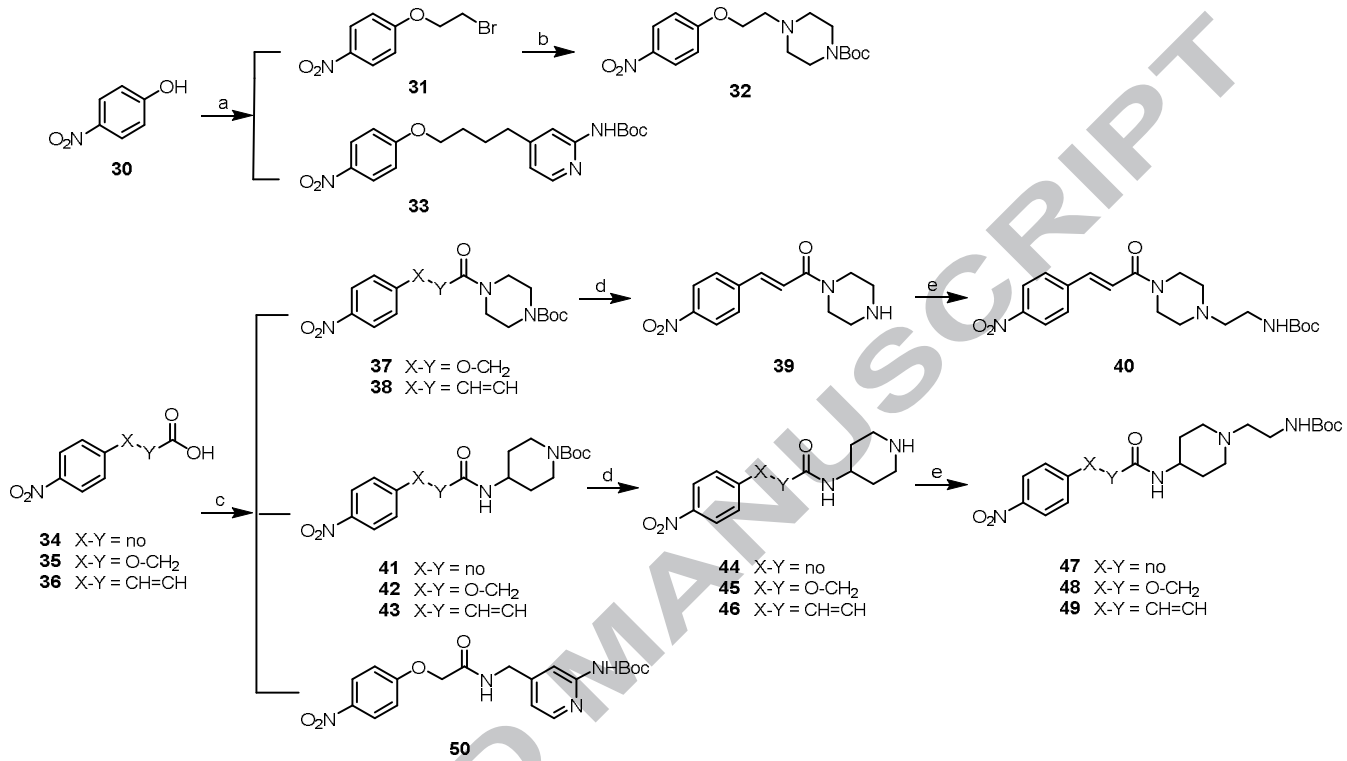


Scheme 1. Reagents and conditions: (a) *N*-Boc ethanolamine, *N*-Boc piperidine derivatives or *tert*-butyl (4-(2-hydroxyethyl)pyridin-2-yl)carbamate, DEAD, PPh_3, DCM, r.t., overnight; (b) alkyl halides, K_2CO_3, DMF, 100 °C, 1 h; (c) TFA, DCM; (d) $ZnCl_2$, HCHO, $NaBH_3CN$, MeOH, r.t., overnight; (e) $N_2H_4.H_2O$, EtOH, r.t., overnight; (f) Boc_2O, TEA, DMC; (g) CH_3I, NaH, THF, 0 °C for **27**, **28**; (h) *N*-Boc piperazine, DMF, TEA, 60 °C, 2 h.

The synthesis of 4-aminoalkyloxy-1-nitrobenzene fragment is shown in **Scheme 2**. The *N*-Boc aminoalkyloxy analogues (**32**, **33**) were prepared from 4-nitrophenol (**30**) by alkylation reactions.

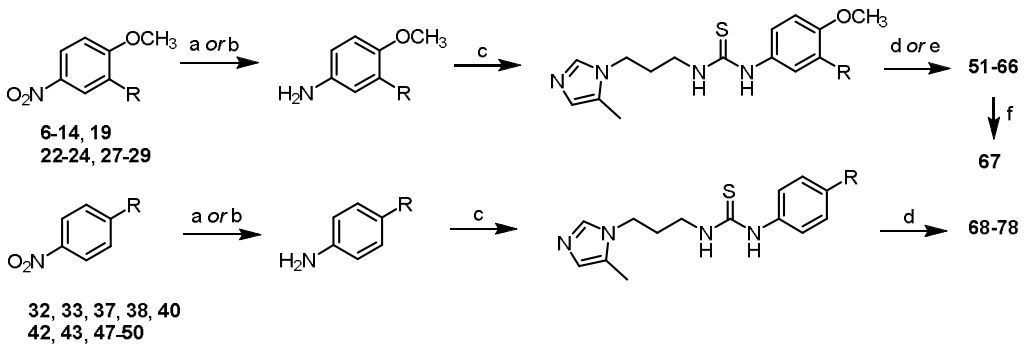
The *N*-Boc amido analogues (**37**, **38**, **42**, **43**, **50**) were synthesized from 4-nitrobenzoic acid (**34**), 2-(4-nitrophenoxy)acetic acid, (**35**) or 4-nitro cinnamic acid (**36**), by coupling with the corresponding amines. The *N*-aminoethyl piperazinyl (**40**) and piperidinyl (**47**-**49**) analogues were prepared from

the corresponding piperazine (**39**) and piperazine (**44**-**46**) precursors by alkylation with *tert*-butyl (2-iodoethyl)carbamate.



Scheme 2. Reagents and conditions: (a) alkyl halide, Cs_2CO_3, DMF, heat; (b) *N*-Boc piperazine, Cs_2CO_3, DMF, heat; (c) RNH_2, EDC, HOBt, DMC; (d) TFA, DMC; (e) *tert*-Butyl (2-iodoethyl)carbamate, NaH, DMF, 0 °C to r.t., 2 h.

The synthesis of the final thiourea compounds is illustrated in **Scheme 3**. The synthesized anilines were reduced either by hydrogenation or by zinc in acetic acid to provide the corresponding amines, which were then coupled with 3-(5-methyl-1*H*-imidazol-1-yl)propan-1-amine by previous method[18] to afford the corresponding thioureas. Finally, the deprotection of *N*-Boc and *N*-phthalimide provided the final 3-aminoalkyloxy-4-methoxyphenyl (**51**-**66**) and 4-aminoalkyloxyphenyl (**68**-**78**) derivatives, respectively. The pyrimidine **67** was synthesized from amine **53** by reacting with 2-chloropyrimidine.



Scheme 3. Reagents and conditions: (a) Pd, H_2, MeOH; (b) Zn, AcOH, MeOH; (c) 3-(5-methyl-1H-imidazol-1-yl)propan-1-amine, TCDI, Et_3N, DMC, r.t., overnight; (d) TFA, DMC, 0 °C to r.t., overnight; (e) $N_2H_4.H_2O$, EtOH, r.t., overnight; (f) 2-chloropyrimidine, TEA, EtOH, reflux, 2 days.

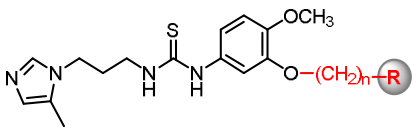
2.2. In vitro QC Inhibition

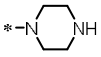
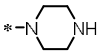
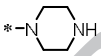
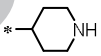
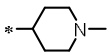
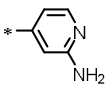
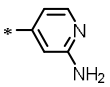
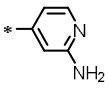
As previously reported, to evaluate the human QC inhibition of the synthesized compounds we performed the QC activity assays employing a fluorogenic substrate, Gln-AMC (L-glutamine 7-amido-4-methylcoumarin), and pyroglutamyl peptidase (pGAP) as an auxiliary enzyme. [25]

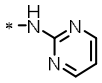
First, we investigated a series of 3-aminoalkyloxy-4-methoxyphenyl analogues represented as template B (**Table 1**). The primary amine derivatives (**51**-**53**) exhibited similarly potent inhibition regardless of the length of linkers with a range of IC_{50} = 7.9-9.0 nM, which were 3.5-fold more potent than the parent **1**. The secondary amine derivatives (**54**, **55**) were found approximately 3-fold less active than the corresponding primary amines (**52**, **53**). The tertiary amine derivative (**56**) showed poor inhibition compared to the corresponding secondary derivative (**55**). Next, we examined the cyclic amine derivatives. The piperazine derivatives (**57**-**59**) also displayed reasonable inhibitory effect regardless of the linker length. The piperidine derivatives (**60**-**62**) exhibited better activity than those of piperazine derivatives, and they showed similar IC_{50} values compared to the primary amine derivatives (**51**-**53**). However, the *N*-methylation of the piperidine (**63**) resulted in the loss of activity. Because the previously developed 2-aminopyridine derivatives (template A, **Figure 2**) showed promising activities both in *in vitro* (IC_{50} = 4.5 nM for **4**) and *in vivo*, we also examined the 2-aminopyridine analogues (**64**-**66**). Unfortunately, these compounds were found to

be less potent than their 3-methoxyphenyl-4-aminoalkyloxy counter parts (template A). When the aminopyridine group was replaced with a 2-aminopyrimidine (**67**), the compound showed similar inhibition as the aminopyridine analogue **66**.

Table 1. IC_{50} values for the inhibition of *h*QC by template B compounds



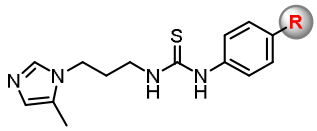
Cpd#	R	n	IC_{50} (nM)[a]
1	*–CH_3		29.2[b]
51	*–NH_2	2	7.9 (±0.7)
52	*–NH_2	3	9.0 (±2.3)
53	*–NH_2	4	8.8 (±0.6)
54		3	26.8 (±4.7)
55		4	22.0 (±6.8)
56		4	46.8 (±10.8)
57		2	18.4 (±1.9)
58		3	15.8 (±2.8)
59		4	15.0 (±0.5)
60		2	7.3 (±6.3)
61		3	8.8 (±2.2)
62		4	7.9 (±2.1)
63		2	20.6 (±7.4)
64		2	26.4 (±8.8)
65		3	15.0 (±2.9)
66		4	15.9 (±7.4)

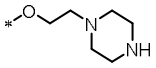
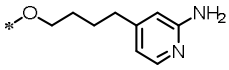
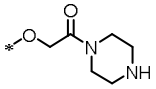
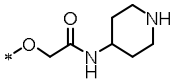
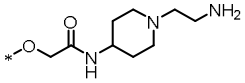
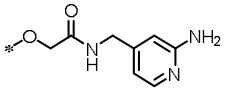
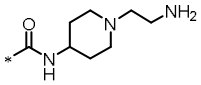
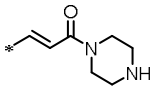
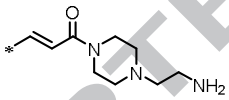
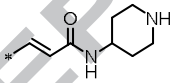
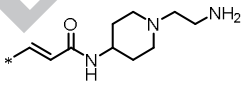
67		4	15.2 (±2.0)

[a] The values indicate the mean of at least three experiments. [b] Refs. 14 and 18

Next, we examined a series of 4-aminoalkyloxyphenyl analogues, in which the 3-methoxy group was removed and only contained the substituent at the 4-position and was represented as template C (**Table 2**). We first tested compounds **68** and **69**, which were most potent when they contained the 3-methoxy group.[18] However, it was found that compounds **68** and **69** were much less active, by 27- and 5.5-fold respectively, than their previously reported 3-methoxy counterparts, suggesting that the 3-methoxy group represents an important binding interaction for inhibitory effect. We also examined inhibition of compounds **70**-**72** with an amido linker to compare the structure-activity relationship of the previously developed derivatives containing a 3-methoxy group.[19] These compounds appeared to maintain similar activity compared to the previously reported compounds,[19] while the *N*-aminoethyl piperidine derivative (**72**) was found to be more potent than that of **70**, suggesting that the presence of an additional amide group may aid extra binding interactions and that the terminal amino group may serve as an Arg-mimetic. However, shortening or rigidifying the linker group mostly resulted in the loss of activity, as demonstrated in **73** and **74** (a 2-fold reduction than compounds **69** and **72**, respectively). As a bioisostere of methyleneoxy group in the linker, we also tested the cinnamic linker surrogates (**75**-**78**). Although compounds containing the cinnamic linker appeared to be slightly less active than the corresponding alkyloxy derivatives, the addition of an *N*-aminoethyl group to compound **75** increased the activity 3-fold to give potent inhibitor **76** with an IC_{50} = 6.4 nM, again suggesting that the terminal amino group likely served as an Arg-mimetic group to interact with QC.

Table 2. IC_{50} values for inhibition of *h*QC by template C compounds



Cpd#	R	IC_{50} (nM)[a]
68		18.7 (±5.8)
69		24.7 (±6.5)
70		11.4 (±2.0)
71		12.9 (±5.9)
72		7.9 (±7.3)
73		56.5 (±12)
74		16.2 (±4.0)
75		19.4 (±9.9)
76		6.4 (±0.6)
77		26.0 (±9.6)
78		11.2 (±3.2)

[a] The values indicate the mean of at least three experiments.

2.3. In vivo activity

Based on the *in vitro* QC inhibition of the synthesized compounds, we selected eight of the most potent inhibitors with IC_{50} values less than 10 nM for further evaluation. We first examined cytotoxicity by incubating HT-22 cells, an immortalized hippocampal neuronal cell line, with 10

μM of each compound, and performed MTT assays. All tested compounds were not cytotoxic, demonstrating normal cell viability. We also evaluated the ability of *h*ERG channel blocking for all compounds to assess drug toxicity. All compounds showed moderate (35.1%, **62**) to low inhibition (2.8%, **53**) at 10 μM, indicating that they pose a marginal to low risk for cardiotoxicity. To evaluate the ability of the compounds to penetrate the blood-brain barrier (BBB), we carried out a parallel artificial membrane permeability assay (PAMPA). Six compounds, **51**-**53** and **60**-**62**, showed reasonable permeability, with a range of 5.0-5.9 for –logPe. Interestingly, all of the primary amine (**51**-**53**) and piperidine (**60**-**62**) derivatives showed reasonable values for BBB penetration regardless of the length of the carbon linker, whereas the *N*-aminoethyl derivatives, **72** and **76**, exhibited very low permeability (–logPe = 10 and 9.0).

Finally, we tested each compound in acute AD model mice to evaluate QC inhibition *in vivo*. We first injected human $A\beta_{3\text{-}40}$ (5 μg) and each compound (25 mg/kg) successively into deep cortical/hippocampal tissues of ICR mice (male, six weeks old) by intracerebroventricular (icv) administration. On the next day, we measured the levels of human $A\beta_{N3pE\text{-}40}$ in the brain extracts of each mouse to determine the QC inhibitory activity. As described in **Table 3**, compounds **51**-**53**, **62** and **76** suppressed the formation of $A\beta_{N3pE\text{-}40}$ by 12.8% to 36.6% compared to the vehicle control. In particular, compounds **51** and **53**, with *in vitro* IC_{50} values of 7.9 and 8.8 nM, exhibited the most potent $A\beta_{N3pE\text{-}40}$-lowering effects by 35.3 and 36.6% reduction, respectively, indicating that the specific inhibition of QC resulted in the reduced brain levels of $A\beta_{N3pE\text{-}40}$. Overall, these two compounds exhibited potent *in vitro* and *in vivo* QC inhibitory activities and good brain penetration without potential toxicity.

Table 3. Studies of *in vitro* toxicity, permeability, and *in vivo* QC inhibition in acute model

	in vitro IC_{50} (nM)	cytotoxicity at 10 μM (% of control)	*h*ERG assay (10 μM, % inhibition)	PAMPA (-logPe)	% inhibition of human $A\beta_{N3pE\text{-}40}$ formation (icv)[a]
51	7.9	~100	15.3	5.7	35.3

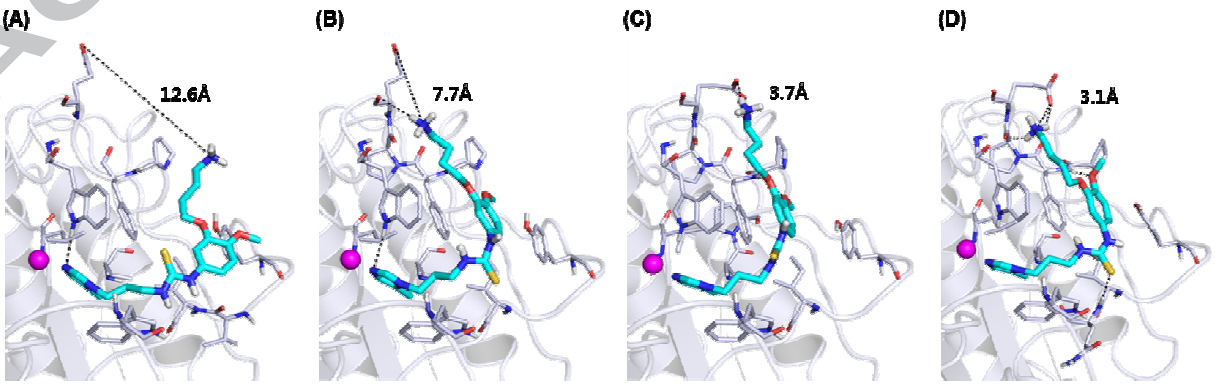
52	9.0	~100	8.8	5.6	12.8
53	8.8	~100	2.8	5.9	36.6
60	7.3	~100	14.9	5.3	1.8
61	8.8	~100	25.6	5.0	5.5
62	7.9	~100	35.1	5.7	14.4
72	7.9	~100	19.9	10.0	NE
76	6.4	~100	7.2	9.0	22.9

[a] 5 μL of human $A\beta_{3-40}$ in PBS (1 μg/μL) was injected into the deep cortical/hippocampus to 5 weeks old ICR mice (25 g, n = 4, male) using a stereotaxic frame to induce acute Aβ toxicity. Test compounds were administrated via icv injection. Sandwich ELISA was performed for the quantification of the brain $A\beta_{N3pE-40}$

2.4. Molecular modeling

To evaluate the binding interactions between the *h*QC and the potent inhibitor **53**, we carried out the sequential docking studies using X-ray crystal structure of *h*QC (PDB id: 3PBB).[26] For the initial docking study, the protonated amine form of **53** at pH 7.4 was used and placed into a Glide SP (Standard Precision). The result exhibited that the *N*-3 nitrogen of the 5-methyl imidazole chelated with zinc and formed a hydrogen bonding with Trp329. Additionally, the 5-methyl group occupied a hydrophobic pocket composed of Trp207, Leu249, Ile303, Ile321, and Phe325. The thiourea group caused the appropriate positioning of the phenyl ring for the hydrophobic interaction with Ile303, while the methoxy oxygen on the phenyl ring formed a hydrogen bond with Tyr299 (**Figure 3A**).

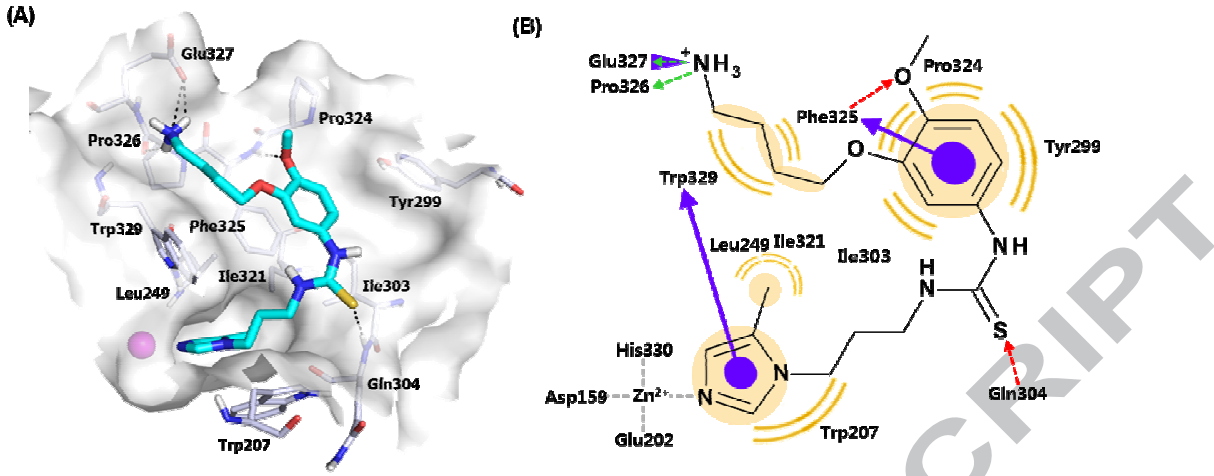
Figure 3. Sequential docking and refinement of **53** in *h*QC.



(A) Glide SP docking, (B) QPLD, (C) Local optimization, and (D) Monte Carlo minimization. Binding modes of **53** in protonated form were shown in each step. The distances between Glu327 and the terminal *N* from D-region of the ligands were revealed in black dashed lines.

Afterwards, Glide QM-Polarized Ligand Docking (QPLD) in Maestro was implemented, and the result showed the amino group in the side chain shifted toward Glu327 of the *h*QC active site (**Figure 3B**). The local optimization refinement was conducted, and the result displayed the bended Glu327 side chain, and constituted a salt bridge with the amino group of **53** (**Figure 3C**). To search for the global minimum, we performed a Monte Carlo minimization (**Figure 3D**). This type of sequential optimization for the protein-ligand complexes formed hydrogen bonding as well as salt bridge interactions, along with the H-bonding with the phenyl ring of **53** (**Figure 4**). Accordingly, the imidazole ring formed π-π interactions with Trp329, and the thiourea group showed a hydrogen bond with Gln304 while the dimethoxyphenyl group formed a H-bonding interaction and π-π interaction with Phe325. Moreover, the protonated amine group located in the side chain displayed a salt bridge interaction with Glu327 and H-bonding with Pro326 and Glu327. Overall, we believe that switching the substituents in the 3-and 4-positions did not alter the binding interactions significantly, partly due to the flexibility of the 4-aminoalkoxy chain, which is also supported by the SAR found in the analogues with rigid and short linkers.

Figure 4. Docked and refined structure of **53** in *h*QC.



(A) Binding interactions of **53** at the active site of the *h*QC. Ligand is displayed as sticks with cyan carbon atoms, and Zn^{2+} is in purple ball. The interacted residues are shown in light blue sticks. Hydrogen bonds are described as black dashed lines. (B) 2D illustration of the binding interactions between **53** with *h*QC. Hydrophobic interactions are indicated in light brown. Hydrogen bonds are exposed as red- and green-dotted point with the directionality. The π-π stacking interaction is signified in purple disc and arrow, and the salt bridge interaction is displayed as purple wedge line.

3. Conclusion

In this study, we investigated a series of QC inhibitors containing 4-aminoalkyloxy-3-methoxyphenyl and 3-aminoalkyloxyphenyl groups as Phe-Arg mimetics of $A\beta_{3\text{-}42}$. The primary amines (**51**-**53**) and 4-piperidinyl (**57**-**59**) derivatives exhibited potent QC inhibition, demonstrating 3-4 fold more potent activity than the parent inhibitor **1** by Probiodrug. Further *in vivo* studies revealed that inhibitors **51** and **53** displayed the most potent $A\beta_{N3pE\text{-}40}$-lowering effects with 35.3 and 36.6% *in vivo*, respectively, with reasonable BBB penetration, which also corresponded to their *in vitro* potency. The molecular modeling analysis of **53** indicated that the salt bridge interaction and the hydrogen bonding of the protonated amine group with Glu327 and Pro326 provided a high potency compared to the parent inhibitor **1**. Given the potent QC inhibitory effect, favorable BBB penetration, and the toxicity profile, we believe that compound **53** may serve as a potential candidate for anti-Alzheimer's agents.

4. Experimental

4.1. Chemistry

4.1.1. General

All chemical reagents were commercially available. Melting points were determined on a melting point Buchi B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230-400 mesh, Merck. ^{1}H NMR spectra were recorded on a a JEOL JNM-LA 300 at 300 MHz, Bruker Analytik, DE/AVANCE Digital 400 at 400 MHz, a Bruker Analytik, DE/AVANCE Digital 500 at 500 MHz, and a JEOL JNM-ECA-600 at 600 MHz. Mass spectra were recorded on a VG Trio-2 GC–MS instrument and a 6460 Triple Quad LC–MS instrument. All final compounds were assessed for purity by high performance liquid chromatography (HPLC) on Agilent 1120 Compact LC (G4288A) system via the following conditions. Column: Agilent TC-C18 column (4.6 mm × 250 mm, 5 μm). Mobile phase A: MeOH, Mobile phase B: 0.1% TFA in water (v/v) in 30 min. Wavelength: 254 nM. Flow: 0.7 mL/min. According to the HPLC analyses, all final compounds showed a purity of ≥95%.

4.1.2. General procedure

4.1.2.1. Mitsunobu reaction (Procedure 1)

Triphenylphosphine (1.3 eq) was added under nitrogen to a solution of 5-nitroguaiacol (1.0 eq) in CH_2Cl_2, followed by adding alcohol (1.2 eq) and a solution of diethyl azodicarboxylate (1.3 eq) in CH_2Cl_2. After the solution was stirred for 30 min at room temperature, the reaction was poured onto a column of silica and was eluted with EtOAc/*n*-hexane to give desired product.

4.1.2.2. Williamson reaction (Procedure 2)

Alkyl halide (4.0 eq) was added to a suspension of 5-nitroguaiacol or 4-nitrophenol (1.0 eq) and Cs_2CO_3 (2.0 eq) in anhydrous DMF. The reaction mixture was heated to 100 °C for 1 h and then cooled to room temperature before quenched by H_2O. The mixture was extracted with EtOAc (2 x

50 mL). The organic layer was washed by H_2O three times, dried by $MgSO_4$ and concentrated. This concentration was then purified by column chromatography to get product.

4.1.2.3. Deprotection of phthalimide group (Procedure 3)

Hydrazine monohydrate was added to a solution of phthalimide compound in ethanol and stirred at room temperature overnight. The precipitate was filtered and washed with EtOH. The filtrate was collected and concentrated in vacuo. The residue was then purified by PLC ($MeOH/CH_2Cl_2$) to give product.

4.1.2.4. N-Alkylation (Procedure 4)

A mixture of alkyl halide, amine, and excess base in DMF was stirred at 60 °C for 30 min. The mixture reaction was quenched by H_2O and extracted by EtOAc several times. The combined organic layer was washed with H_2O and brine and concentrated in vacuo. The residue was purified by column chromatography.

4.1.2.5. Boc protection (Procedure 5)

To a suspension of starting material amine (1.0 eq) in CH_2Cl_2 was added triethylamine (1.2 eq) and di-*tert*-butyl dicarbonate (2.5 eq) in CH_2Cl_2 under ice bath. The mixture was stirred at room temperature until starting material was consumed, by checking with TLC. The mixture was diluted with H_2O and extracted with CH_2Cl_2 several times. The combined organic layers were washed with 10% aqueous $NaHCO_3$ solution, H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography to get the desired product.

4.1.2.6. Boc deprotection (Procedure 6)

Trifluoroacetic acid (10 eq) was added to the solution of *Boc*-protected compound (1.0 eq) in CH_2Cl_2 (DCM:TFA = 1:1 (v/v)). Then, the mixture was stirred at room temperature until the starting material consumed and evaporated. The residue was dissolved in MeOH and purified by ion-exchange column to get desired product or carried to the next step without further purification.

4.1.2.7. Reductive methylation of amine (Procedure 7)

To a stirred solution of amine (1 eq) in MeOH (5 mL) containing 37% aqueous formaldehyde (3 eq) at room temperature was added a solution of sodium cyanoborohydride (1 eq) and zinc chloride (0.5 eq) in MeOH (5 mL). After the reaction mixture was stirred at room temperature for overnight, the solution was taken up in 0.1 N NaOH (10 mL), and most of the MeOH was evaporated under reduced pressure. After the aqueous solution was extracted with EtOAc (20 mL x 3), the combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and evaporated until dry . The residue was distilled in vacuo to give the desired product.

4.1.2.8. EDC coupling (Procedure 8)

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDC-HCl, 1.1 eq) and *N,N*-diisopropylethylamine (2.2 eq) were added to a solution of amine (1.0 eq), acid (1.0 eq) and 1-hydroxybenzotriazole (HOBt, 1.1 eq) in CH_2Cl_2. The mixture was stirred for 24 h at room temperature under nitrogen. The solvent was removed in vacuo and the residue purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH to provide the desired compound.

4.1.3. Intermediate compounds

4.1.3.1. tert-Butyl (2-(2-methoxy-5-nitrophenoxy)ethyl)carbamate ***(6)***. The title compound was prepared from 5-nitroguaiacol (**5**) according to procedure 1 as a yellow solid in 99% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 (dd, J = 8.97, 2.55 Hz, 1H), 7.76 (d, J = 2.58 Hz, 1H), 6.93 (d, J = 8.97

Hz, 1H), 5.07 (br, NH), 4.15 (t, *J* = 5.31 Hz, 2H), 3.96 (s, 3H), 3.61 (q, *J* = 5.49 Hz, 2H), 1.45 (s, 9H).

4.1.3.2. tert-Butyl 4-(2-(2-methoxy-5-nitrophenoxy)ethyl)piperidine-1-carboxylate ***(7)***. The title compound was prepared from compound **5** according to procedure 1 as a yellow oil in 63% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (dd, *J* = 8.97, 2.58 Hz, 1H), 7.73 (d, *J* = 2.55 Hz, 1H), 6.92 (d, *J* = 8.97 Hz, 1H), 4.35-4.17 (m, 3H), 3.96 (s, 3H), 2.72 (t, *J* = 11.73 Hz, 2H), 1.85-1.80 (m, 2H), 1.75-1.72 (m, 2H), 1.46 (s, 9H), 1.46-1.45 (m, 2H), 1.36-1.30 (m, 2H).

4.1.3.3. tert-Butyl 4-(3-(2-methoxy-5-nitrophenoxy)propyl)piperidine-1-carboxylate ***(8)***. The title compound was prepared from compound **5** according to procedure 1 as a yellow oil in 76% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (dd, *J* = 8.97, 2.73 Hz, 1H), 7.73 (d, *J* = 2.73 Hz, 1H), 6.92 (d, *J* = 8.97 Hz, 1H), 4.15-4.05 (m, 4H), 3.96 (s, 3H), 3.48 (m, 1H), 2.72 (t, *J* = 12.06 Hz, 2H), 1.95 (p, *J* = 6.60 Hz, 2H), 1.72 (d, *J* = 13.53 Hz, 2H), 1.45 (s, 9H), 1.45-1.39 (m, 2H), 1.17-1.11 (m, 2H).

4.1.3.4. tert-Butyl 4-(4-(2-methoxy-5-nitrophenoxy)butyl)piperidine-1-carboxylate ***(9)***. The title compound was prepared from compound **5** according to procedure 1 as a brown oil in 63% yield. ^{1}H NMR (300 MHz, CDCl3) δ 7.92 (dd, *J* = 8.79, 2.55 Hz, 1H), 7.73 (d, *J* = 2.76 Hz, 1H), 6.91 (d, *J* = 8.79 Hz, 1H), 4.15-4.06 (m, 4H), 3.96 (s, 3H), 2.71 (t, *J* = 12.09 Hz, 2H), 1.91 (p, *J* = 6.96 Hz, 2H), 1.68-1.64 (m, 2H), 1.51-1.47 (m, 3H), 1.45 (s, 9H), 1.35-1.28 (m, 2H), 1.15-1.02 (m, 2H).

4.1.3.5. tert-Butyl (4-(2-(2-methoxy-5-nitrophenoxy)ethyl)pyridin-2-yl)carbamate ***(10)***. The title compound was prepared from compound **5** according to procedure 1 as a yellow solid in 59% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (d, *J* = 5.13 Hz, 1H), 7.94-7.90 (m, 2H), 7.73 (d, *J* = 2.55 Hz, 1H), 7.43 (br, NH), 6.94 (dd, *J* = 5.13, 1.47 Hz, 1H), 6.92 (d, *J* = 8.97 Hz, 1H), 4.33 (t, *J* = 6.78 Hz, 2H), 3.96 (s, 3H), 3.19 (t, *J* = 6.78 Hz, 2H), 1.53 (s, 9H).

*4.1.3.6. tert-Butyl (4-(3-(2-methoxy-5-nitrophenoxy)propyl)pyridin-2-yl)carbamate **(11)**.*

The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 65% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (d, J = 5.10 Hz, 1H), 7.93 (dd, J = 8.79, 2.55 Hz, 1H), 7.84 (s, 1H), 7.70 (d, J = 2.58 Hz, 1H), 7.64 (s, 1H), 6.92 (d, J = 8.79 Hz, 1H), 6.84 (dd, J = 5.13, 2.28 Hz, 1H), 4.11 (t, J = 6.24 Hz, 2H), 3.97 (s, 3H), 2.86 (t, J = 7.14 Hz, 2H), 2.27 (p, J = 6.24 Hz, 2H), 1.52 (s, 9H).

*4.1.3.7. tert-Butyl (4-(4-(2-methoxy-5-nitrophenoxy)butyl)pyridin-2-yl)carbamate **(12)**.* The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 65% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (d, J = 5.31 Hz, 1H), 7.92 (dd, J = 8.97, 2.76 Hz, 1H), 7.80 (br, NH), 7.72 (d, J = 2.55 Hz, 1H), 7.21 (br, NH), 6.91 (d, J = 8.79 Hz, 1H), 6.83 (dd, J = 4.95, 1.47 Hz, 1H), 4.11 (t, J = 6.06 Hz, 2H), 3.96 (s, 3H), 2.72 (t, J = 7.68 Hz, 2H), 1.89-1.81 (m, 4H), 1.52 (s, 9H).

*4.1.3.8. 2-(3-(2-Methoxy-5-nitrophenoxy)propyl)isoindoline-1,3-dione **(13)**.*

The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 61% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90-7.81 (m, 3H), 7.75-7.61 (m, 3H), 6.84 (d, J = 8.97 Hz, 1H), 4.18 (t, J = 6.03 Hz, 2H), 3.96 (d, J = 6.60 Hz, 2H), 3.73 (s, 3H), 2.31 (p, J = 6.24 Hz, 2H).

*4.1.3.9. 2-(4-(2-Methoxy-5-nitrophenoxy)butyl)isoindoline-1,3-dione **(14)**.* The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 94% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (dd, J = 8.97, 2.55 Hz, 1H), 7.86-7.81 (m, 2H), 7.75-7.69 (m, 3H), 6.90 (d, J = 8.97 Hz, 1H), 4.14-4.10 (m, 2H), 3.94 (s, 3H), 3.81-3.77 (m, 2H), 1.94-1.91 (m, 4H).

4.1.3.10. 2-(2-Bromoethoxy)-1-methoxy-4-nitrobenzene ***(15)***. The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 73% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (dd, J = 8.76, 2.55 Hz, 1H), 7.77 (d, J = 2.58 Hz, 1H), 6.95 (d, J = 8.97 Hz, 1H), 4.42 (t, J = 6.42 Hz, 2H), 3.98 (s, 3H), 3.72 (t, J = 6.21 Hz, 2H).

4.1.3.11. 2-(3-Bromopropoxy)-1-methoxy-4-nitrobenzene ***(16)***. The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 71% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (dd, J = 8.97, 2.58 Hz, 1H), 7.78 (d, J = 2.58 Hz, 1H), 6.93 (d, J = 8.76 Hz, 1H), 4.28 (t, J = 7.14 Hz, 2H), 3.96 (s, 3H), 3.80 (t, J = 6.21 Hz, 2H), 2.37 (p, J = 6.06 Hz, 2H).

4.1.3.12. 2-(4-Bromobutoxy)-1-methoxy-4-nitrobenzene ***(17)***. The title compound was prepared from compound **5** according to procedure 2 as a yellow solid in 63% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (dd, J = 8.97, 2.55 Hz, 1H), 7.74 (d, J = 2.58 Hz, 1H), 6.92 (d, J = 8.97 Hz, 1H), 4.14 (t, J = 5.85 Hz, 2H), 3.96 (s, 3H) 3.53 (t, J = 6.24 Hz, 2H), 2.10-2.05 (m, 4H).

4.1.3.13. 4-(4-(2-Methoxy-5-nitrophenoxy)butyl)piperidine ***(18).*** The title compound was prepared from compound **7** according to procedure 6 as a red solid in 75% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 7.91 (dd, J = 8.97, 2.76 Hz, 1H), 7.76 (d, J = 2.58 Hz, 1H), 7.09 (d, J = 8.97 Hz, 1H), 4.14 (t, J = 5.85 Hz, 2H), 3.93 (s, 3H), 3.06-3.02 (m, 2H), 2.65-2.56 (m, 2H), 1.80-1.74 (m, 5H), 1.29-1.18 (m, 2H).

4.1.3.14. 4-(2-(2-Methoxy-5-nitrophenoxy)ethyl)-1-methylpiperidine ***(19)***. The title compound was prepared from compound **18** according to procedure 7 as a white solid in 45% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (dd, J = 8.40, 2.58 Hz, 1H), 7.73 (d, J = 2.73 Hz, 1H), 6.92 (d, J = 8.97 Hz, 1H), 4.14 (t, J = 6.60 Hz, 2H), 3.96 (s, 3H), 3.12 (d, J = 11.88 Hz, 2H), 2.45 (s, 3H), 2.26 (t, J = 9.90 Hz, 2H), 1.89-1.83 (m, 4H), 1.56-1.47 (m, 3H)

*4.1.3.15. 3-(2-Methoxy-5-nitrophenoxy)propan-1-amine **(20)**.* The title compound was prepared from compound **13** according to procedure 3 as a light yellow solid in 94% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (dd, J = 8.43, 2.43 Hz, 1H), 7.72 (d, J = 2.43 Hz, 1H), 6.88 (d, J = 8.46 Hz, 1H), 4.13 (t, J = 5.82 Hz, 2H), 3.93 (s, 3H), 2.62 (t, J = 6.12 Hz, 2H), 1.98-1.88 (m, 2H).

*4.1.3.16. 4-(2-Methoxy-5-nitrophenoxy)butan-1-amine **(21)**.* The title compound was prepared from compound **14** according to procedure 3 as a white solid in 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (dd, J = 8.40, 2.40 Hz, 1H), 7.71 (d, J = 2.40 Hz, 1H), 6.88 (d, J = 8.88 Hz, 1H), 4.13 (t, J = 5.80 Hz, 2H), 3.93 (s, 3H), 2.79 (t, J = 6.68 Hz, 2H), 2.00-1.83 (m, 4H)

*4.1.3.17. tert-Butyl 4-(2-(2-methoxy-5-nitrophenoxy)ethyl)piperazine-1-carboxylate **(22)**.* The title compound was prepared from compound **15** according to procedure 4 as a white solid in 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (dd, J = 8.40, 2.58 Hz, 1H), 7.80 (d, J = 2.58 Hz, 1H), 6.92 (d, J = 8.97 Hz, 1H), 4.24 (t, J = 5.85 Hz, 2H), 3.95 (s, 3H), 3.47 (t, J = 4.95 Hz, 4H), 2.90 (t, J = 5.85 Hz, 2H), 2.56 (t, J = 4.74 Hz, 4H), 1.46 (s, 9H).

*4.1.3.18. tert-Butyl 4-(3-(2-methoxy-5-nitrophenoxy)propyl)piperazine-1-carboxylate **(23)**.* The title compound was prepared from compound **16** according to procedure 4 as a white solid in 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (dd, J = 9.15, 2.55 Hz, 1H), 7.77 (d, J = 2.19 Hz, 1H), 6.91 (d, J = 9.15 Hz, 1H), 4.18 (t, J = 6.57 Hz, 2H), 3.95 (s, 3H), 3.44 (t, J = 4.95 Hz, 2H), 2.57 (t, J = 6.60 Hz, 4H), 2.41 (t, J = 4.95 Hz, 4H), 2.08 (p, J = 6.78 Hz, 2H), 1.46 (s, 9H).

*4.1.3.19. tert-Butyl 4-(4-(2-methoxy-5-nitrophenoxy)butyl)piperazine-1-carboxylate **(24)**.* The title compound was prepared from compound **17** according to procedure 4 as a white solid in 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (dd, J = 8.97, 2.55 Hz, 1H), 7.73 (d, J = 2.55 Hz, 1H), 6.92 (d, J

= 8.79 Hz, 1H), 4.15 (d, *J* = 6.96 Hz, 2H), 3.95 (s, 3H), 3.44 (t, *J* = 5.13 Hz, 4H), 2.45-2.37 (m, 6H), 1.96 (p, *J* = 6.75 Hz, 2H), 1.74 (p, *J* = 7.89 Hz, 2H), 1.46 (s, 9H).

4.1.3.20. tert-Butyl (2-(2-methoxy-5-nitrophenoxy)ethyl)carbamate ***(25)***. The title compound was prepared from compound **20** according to procedure 5 as an opaque semi-solid in 72% yield, which was used for the next step without further purification.

4.1.3.21. tert-Butyl (3-(2-methoxy-5-nitrophenoxy)propyl)carbamate ***(26)***. The title compound was prepared from compound **21** according to procedure 5 as a white solid in 62% yield, which was used for the next step without further purification.

4.1.3.22. tert-Butyl (2-(2-methoxy-5-nitrophenoxy)ethyl)(methyl)carbamate ***(27)***. The title compound was prepared from compound **25** according to procedure 4 as a light yellow solid in 51% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (dd, *J* = 8.46, 2.40 Hz, 1H), 7.72 (d, *J* = 2.43 Hz, 1H), 6.87 (d, *J* = 8.61 Hz, 1H), 4.14 (t, *J* = 6.03 Hz, 2H), 3.93 (s, 3H), 2.92 (t, *J* = 6.12 Hz, 2H), 2.78 (s, 3H), 2.01-1.90 (m, 2H), 1.44 (s, 9H).

4.1.3.23. tert-Butyl (3-(2-methoxy-5-nitrophenoxy)propyl)(methyl)carbamate ***(28)***. The title compound was prepared from compound **26** according to procedure 4 as a light yellow solid in 55% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (dd, *J* = 8.79, 2.73 Hz, 1H), 7.72 (d, *J* = 2.73 Hz, 1H), 6.89 (d, *J* = 8.97 Hz, 1H), 4.14 (t, *J* = 6.21 Hz, 2H), 3.94 (s, 3H), 2.65 (t, *J* = 6.12 Hz, 2H), 2.80 (s, 3H), 2.01-1.87 (m, 4H), 1.44 (s, 9H).

4.1.3.24. 4-(2-Methoxy-5-nitrophenoxy)-N,N-dimethylbutan-1-amine ***(29)***. The title compound was prepared from compound **21** according to procedure 7 as a red semi-solid in 21% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (dd, *J* = 8.97, 2.73 Hz, 1H), 7.74 (d, *J* = 2.58 Hz, 1H), 6.92 (d, *J* = 8.97

Hz, 1H), 4.14 (t, *J* = 6.24 Hz, 2H), 3.96 (s, 3H), 2.62 (t, *J* = 8.61 Hz, 2H), 2.40 (s, 6H), 1.95 (p, *J* = 6.60 Hz, 2H), 1.82 (p, *J* = 7.32 Hz, 2H).

4.1.3.25. 1-(2-Bromoethoxy)-4-nitrobenzene ***(31).*** The title compound was prepared from compound **30** according to procedure 2 as a yellow solid in 64% yield. ^{1}H NMR (300 MHz, CDCl3) δ 8.20 (m, 2H), 6.97 (m, 2H), 4.36 (d, *J* = 6.03 Hz, 2H), 3.68 (d, *J* = 6.24 Hz, 2H).

4.1.3.26. tert-Butyl 4-(2-(4-nitrophenoxy)ethyl)piperazine-1-carboxylate ***(32)***. The title compound was prepared from compound **31** according to procedure 4 as a white solid in 61% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (2H, m), 6.97 (2H, m), 4.19 (t, *J* = 5.67 Hz, 2H), 3.45 (m, 4H), 2.85 (t, *J* = 5.70 Hz, 2H), 2.52 (m, 4H), 1.45 (s, 9H).

4.1.3.27. tert-Butyl (4-(4-(4-nitrophenoxy)butyl)pyridin-2-yl)carbamate ***(33)***. The title compound was prepared from compound **30** according to procedure 2 as a white solid in 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, *J* = 9.15 Hz, 2H), 8.11-8.09 (m, 2H), 7.86 (s, 1H), 6.94 (d, *J* = 9.15 Hz, 2H), 6.82 (dd, *J* = 5.73, 1.47 Hz, 1H), 4.06 (t, *J* = 5.67 Hz, 2H), 2.68 (t, *J* = 6.06 Hz, 2H), 1.86-1.81 (m, 4H), 1.51 (s, 9H).

4.1.3.28. tert-Butyl 4-(2-(4-nitrophenoxy)acetyl)piperazine-1-carboxylate ***(37)***. The title compound was prepared from compound **35** according to procedure 8 as a yellow solid in 81% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (d, *J* = 9.33 Hz, 2H), 6.99 (d, *J* = 9.33 Hz, 2H), 4.75 (s, 2H), 3.53-3.49 (m, 2H), 3.46-3.43 (m, 2H), 3.40-3.34 (m, 4H), 1.39 (s, 9H).

4.1.3.29. tert-Butyl (E)-4-(3-(4-nitrophenyl)acryloyl)piperazine-1-carboxylate ***(38)***. The title compound was prepared from commercial available **36** according to procedure 8 as a white solid in 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25 (d, *J* = 8.79 Hz, 2H), 7.70 (d, *J* = 15.36 Hz, 1H),

7.65 (d, *J* = 8.61 Hz, 2H), 7.02 (d, *J* = 15.57 Hz, 1H), 3.72-3.64 (m, 4H), 3.42-3.36 (m, 4H), 1.48 (s, 9H).

4.1.3.30. (E)-3-(4-Nitrophenyl)-1-(piperazin-1-yl)prop-2-en-1-one ***(39)***. The title compound was prepared from compound **38** according to procedure 6 as a yellow oil in 94% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (d, *J* = 8.79 Hz, 2H), 7.89 (d, *J* = 8.58 Hz, 2H), 7.71 (d, *J* = 15.57 Hz, 1H), 7.39 (d, *J* = 15.57 Hz, 1H), 4.00-3.95 (m, 4H), 3.39-3.22 (m, 4H).

4.1.3.31. tert-Butyl (E)-(2-(4-(3-(4-nitrophenyl)acryloyl)piperazin-1-yl)ethyl)carbamate ***(40)***. The title compound was prepared from compound **39** according to procedure 4 as a pale yellow solid in 54% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (d, *J* = 8.79 Hz, 2H), 7.72-7.65 (m, 3H), 7.03 (d, *J* = 15.57 Hz, 1H), 3.76-3.66 (m, 4H), 3.27 (q, *J* = 5.31 Hz, 2H), 2.53-2.47 (m, 6H), 1.46 (s, 9H).

4.1.3.32. tert-Butyl 4-(4-nitrobenzamido)piperidine-1-carboxylate ***(41)***. The title compound was prepared from compound **34** according to procedure 8 as a white solid in 80% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (d, *J* = 8.79 Hz, 2H), 7.95 (d, *J* = 8.79 Hz, 2H), 6.18 (d, *J* = 7.50 Hz, NH), 4.15-4.10 (m, 3H), 2.94 (t, *J* = 12.24 Hz, 2H), 2.06 (t, *J* = 4.59 Hz, 2H), 1.46 (s, 9H), 1.42-1.39 (m, 2H).

4.1.3.33. tert-Butyl 4-(2-(4-nitrophenoxy)acetamido)piperidine-1-carboxylate ***(42)***. The title compound was prepared from compound **35** according to procedure 8 as a white solid in 82% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25 (d, *J* = 9.15 Hz, 2H), 7.02 (d, *J* = 9.33 Hz, 2H), 6.32 (br, NH), 4.54 (s, 2H), 4.13-4.09 (m, 3H), 2.88 (t, *J* = 12.09 Hz, 2H), 1.93-1.90 (m, 2H), 1.43 (s, 9H), 1.38-1.33 (m, 2H).

*4.1.3.34. tert-Butyl (E)-4-(3-(4-Nitrophenyl)acrylamido)piperidine-1-carboxylate **(43)**.* The title compound was prepared from compound **36** according to procedure 8 as a white solid in 83% yield. ^{1}H NMR (300 MHz, $CDCl_3$) 8.25 (d, *J* = 8.79 Hz, 2H), 7.70 (d, *J* = 15.36 Hz, 1H), 6.65 (d, *J* = 8.61 Hz, 2H), 6.54 (d, *J* = 15.57 Hz, 1H), 5.76 (d, *J* = 7.89 Hz, NH), 4.13 (m, 3H), 2.93 (t, *J* = 11.73 Hz, 2H). 2.05-1.98 (m, 2H), 1.46 (s, 9H), 1.36-1.32 (m, 2H).

*4.1.3.35. 4-Nitro-N-(piperidin-4-yl)benzamide (**44**).* The title compound was prepared from compound **41** according to procedure 6 as a pale yellow oil in 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (d, *J* = 8.79 Hz, 2H), 7.94 (d, *J* = 8.79 Hz, 2H), 6.05 (d, *J* = 7.89 Hz, NH), 4.14-4.04 (m, 1H), 3.16 (td, *J* = 12.45, 2.55 Hz, 2H), 2.81 (dt, *J* = 12.27, 2.55 Hz, 2H), 2.08-2.08 (m, 2H), 1.51-1.38 (m, 2H).

*4.1.3.36. 2-(4-Nitrophenoxy)-N-(piperidin-4-yl)acetamide **(45)**.* The title compound was prepared from compound **42** according to procedure 6 as a a brown semi-solid in 92% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25 (d, *J* = 8.97 Hz, 2H), 7.02 (d, *J* = 9.03 Hz, 2H), 6.32 (br, NH), 4.54 (s, 2H), 4.13-4.09 (m, 1H), 3.16-3.10 (m, 2H), 2.78 (t, *J* = 12.09 Hz, 2H), 2.00-1.95 (m, 2H), 1.48-1.39 (m, 2H).

*4.1.3.37. (E)-3-(4-Nitrophenyl)-N-(piperidin-4-yl)acrylamide **(46)**.* The title compound was prepared from compound **43** according to procedure 6 as a yellow solid in 94% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (d, *J* = 8.79 Hz, 2H), 7.80 (d, *J* = 8.79 Hz, 2H), 7.65 (d, *J* =15.93 Hz, 1H), 6.77 (d, *J* = 15.90 Hz, 1H), 4.08-4.01 (m, 1H), 3.53-3.43 (m, 2H), 3.19 (dt, *J*= 12.99, 3.12 Hz , 2H), 2.18-2.14 (m, 2H), 1.79-1.65 (m, 2H).

*4.1.3.38. tert-Butyl (2-(4-(4-nitrobenzamido)piperidin-1-yl)ethyl)carbamate **(47)**.* The title compound was prepared from compound **44** according to procedure 4 as an opaque semi-solid in 51%

yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (d, J = 8.61 Hz, 2H), 7.93 (d, J = 8.79 Hz, 2H), 6.07 (d, J = 8.07 Hz, NH), 4.98 (br, NH), 4.02-3.95 (m, 1H), 3.24 (q, J = 6.69 Hz, 2H), 2.93-2.90 (m, 2H), 2.51 (t, J = 6.06 Hz, 2H), 2.26 (t, J = 11.55 Hz, 2H), 2.08-2.04 (m, 2H), 1.63-1.59 (m, 2H), 1.45 (s, 9H).

4.1.3.39. tert-Butyl (2-(4-(2-(4-nitrophenoxy)acetamido)piperidin-1-yl)ethyl)carbamate ***(48)***. The title compound was prepared from compound **45** according to procedure 4 as a red semi-solid in 55% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 9.15 Hz, 2H), 7.01 (d, J = 9.15 Hz, 2H), 6.38 (br, NH), 5.12 (br, NH), 4.53 (s, 2H), 3.94-3.90 (m, 1H), 3.25-3.23 (m, 2H), 2.93-2.91 (m, 2H), 2.54-2.51 (m, 2H), 2.24-2.20 (m, 2H), 1.96-1.94 (m, 2H), 1.62-1.58 (m, 2H), 1.42 (s, 9H).

4.1.3.40. tert-Butyl (E)-(2-(4-(3-(4-nitrophenyl)acrylamido)piperidin-1-yl)ethyl)carbamate ***(49)***. The title compound was prepared from compound **46** according to procedure 4 as a pale yellow solid in 21% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (d, J = 8.97 Hz, 2H), 7.80 (d, J = 8.79 Hz, 2H), 7.60 (d, J =15.90 Hz, 1H), 6.79 (d, J = 15.72 Hz, 1H), 4.08-4.01 (m, 1H), 3.53-3.43 (m, 2H), 2.93-2.87 (m, 2H), 2.55-2.51 (m, 2H), 2.42-2.39 (m, 2H), 1.96-1.89 (m, 2H), 1.65-1.55 (m, 2H), 1.42 (s, 9H).

4.1.3.41. tert-Butyl (4-((2-(4-nitrophenoxy)acetamido)methyl)pyridin-2-yl)carbamate ***(50)***. The title compound was prepared from compound **35** according to procedure 8 as a white solid in 65% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 9.15 Hz, 2H), 8.19 (d, J = 5.31 Hz, 1H), 7.88 (s, 1H), 7.06 (d, J = 9.15 Hz, 2H), 6.87 (dd, J = 5.31, 1.47 Hz, 1H), 4.64 (s, 2H), 4.53 (d, J = 6.03 Hz, 2H), 1.50 (s, 9H).

4.1.4. Final compounds

4.1.4.1. General procedure for final compound

All nitro compounds were reduced by either hydrogenation using 10% Pd/C or zinc powder in acidic medium to obtain the corresponding amines, respectively. The amines were converted into the isothiocyanates by 1,1'-thiocarbonyldiimidazole (1.02 eq) in anhydrous CH_2Cl_2, and then coupled with 3-(5-methyl-1*H*-imidazol-1-yl)propan-1-amine (1.1 eq) to afford the corresponding thiourea, respectively. The Boc deprotection by following the general procedure 6 provided the final compounds.

4.1.4.2. N-(3-(2-Aminoethoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(51)***. mp = 58-59 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.37 (s, 1H), 6.91 (d, J = 8.43 Hz, 1H), 6.78-6.72 (m, 3H), 5.93 (br, 1H), 4.02 (t, J = 5.13 Hz, 2H), 3.91 (t, J = 7.14 Hz, 2H), 3.88 (s, 3H), 3.69 (q, J = 6.24 Hz, 2H), 3.14 (t, J = 5.13 Hz, 2H), 2.17 (d, J = 0.93 Hz, 3H), 2.09 (p, J = 7.14 Hz, 2H). MS (FAB) *m/z* 364 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{17}H_{25}N_5O_2S$ $[M + H]^+$ 364.1807, found: 364.1818. Anal. HPLC 99.33% (R_t = 3.723 min).

4.1.4.3. N-(3-(3-Aminopropoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(52)***. mp = 154-155 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (br, 1H), 7.38 (s, 1H), 6.88-6.85 (m, 1H), 6.76-6.72 (m, 3H), 6.10 (br, 1H), 4.09 (t, J = 6.03 Hz, 2H), 3.91 (t, J = 7.14 Hz, 2H), 3.86 (s, 3H), 3.68 (q, J = 6.24 Hz, 2H), 2.93 (t, J = 6.60 Hz, 2H), 2.17 (d, J = 0.90 Hz, 3H), 2.09-1.92 (m, 4H). MS (FAB) *m/z* 378 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{18}H_{27}N_5O_2S$ $[M + H]^+$ 378.1964, found: 378.1972. Anal. HPLC 99.53% (R_t = 3.641 min)

4.1.4.4. N-(3-(4-Aminobutoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(53)***. mp = 55-56 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (br, 1H), 7.43 (s, 1H), 6.80 (d, J = 8.43 Hz, 1H), 6.76-6.70 (m, 3H), 6.00 (br, 1H), 4.00 (t, J = 6.60 Hz, 2H), 3.91 (t, J = 7.14 Hz, 2H), 3.87 (s, 3H), 3.68 (q, J = 6.42 Hz, 2H), 2.79 (t, J = 6.78 Hz, 2H), 2.17 (d, J = 0.90 Hz, 3H), 2.09 (p, J = 7.14 Hz, 2H), 1.92 (p, J = 6.78 Hz, 2H), 1.66-1.59 (m, 2H). MS (FAB) *m/z* 392

$[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{19}H_{29}N_5O_2S$ $[M + H]^+$ 392.2120, found: 392.2127. Anal. HPLC 100.00% (R_t = 3.199 min).

4.1.4.5. N-(4-Methoxy-3-(3-(methylamino)propoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(54)***. mp = 88-89 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (s, 1H), 6.86 (d, *J* = 8.43 Hz, 1H), 6.78 (dd, *J* = 8.43, 2.37 Hz, 1H), 6.73 (d, *J* = 2.19 Hz, 1H), 6.68 (s, 1H), 6.09 (br, 1H), 4.07 (t, *J* = 6.24 Hz, 2H), 3.89 (t, *J* = 6.96 Hz, 2H), 3.87 (s, 3H), 3.66 (q, *J* = 6.60 Hz, 2H), 2.83 (t, *J* = 6.57 Hz, 2H), 2.47 (s, 3H), 2.17 (s, 3H), 2.09-2.02 (m, 4H). MS (ESI) *m/z* 392 $[M+H]^+$. HRMS (ESI) calcd for $C_{19}H_{29}N_5O_2S$ $[M + H]^+$ 392.2115, found 392.2097. Anal. HPLC 98.5% (R_t = 3.222 min).

4.1.4.6. N-(4-Methoxy-3-(4-(methylamino)butoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(55)***. mp = 76-77 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.43 (d, *J* = 0.93 Hz, 1H), 6.85 (d, *J* = 8.43 Hz, 1 H), 6.84 (d, *J* = 2.40 Hz, 1H), 6.69 (dd, *J* = 8.61, 2.37 Hz, 1H), 6.57 (s, 1H), 3.92-3.85 (m, 4H), 3.73 (s, 3H), 3.49 (t, *J* = 6.96 Hz, 2H), 2.67 (t, *J* = 7.32 Hz, 2H), 2.37 (s, 3H), 2.12 (d, *J* = 0.90 Hz, 3H), 1.93 (p, *J* = 7.14 Hz, 2H), 1.75-1.58 (m, 4H). MS (ESI) *m/z* 406 $[M+H]^+$. HRMS (ESI) calcd for $C_{20}H_{31}N_5O_2S$ $[M + H]^+$ 406.2271, found 406.2262. Anal. HPLC 97.6% (R_t = 3.012 min).

4.1.4.7. N-(3-(4-(Dimethylamino)butoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(56)***. mp = 51-52 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.58 (s, 1H), 6.96 (d, *J* = 8.61 Hz, 1H), 6.91 (d, *J* = 2.40 Hz, 1H), 6.77 (dd, *J* = 8.58, 2.55 Hz, 1H). 6.66 (s, 1H), 4.01 (t, *J* = 6.06 Hz, 2H), 3.97 (t, *J* = 7.32 Hz, 2H), 3.82 (s, 3H), 3.61 (t, *J* = 6.96 Hz, 2H), 2.42 (t, *J* = 7.71 Hz, 2H), 2.25 (s, 6H), 2.21 (d, *J* = 0.90 Hz, 3H), 2.05 (p, *J* = 7.14 Hz, 2H), 1.81-1.75 (m, 2H), 1.72-1.67

(m, 2H). MS (FAB) *m/z* 420 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{21}H_{33}N_5O_2S$ $[M + H]^+$ 420.2428, found: 420.2438. Anal. HPLC 98.23% (R_t = 3.454 min).

4.1.4.8. N-(4-Methoxy-3-(2-(piperazin-1-yl)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(57)***. mp = 71-72 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (s, 1H), 6.79-6.94 (m, 2H), 6.80 (dd, *J* = 8.43, 2.37 Hz, 1H), 6.66 (s, 1H), 4.14 (t, *J* = 5.49 Hz, 2H), 3.99 (t, *J* = 7.14 Hz, 2H), 3.81 (s, 3H), 3.59 (q, *J* = 7.14 Hz, 2H), 2.88 (t, *J* = 4.92 Hz, 4H), 2.82 (t, *J* = 5.49 Hz, 2H), 2.61 (t, *J* = 4.95 Hz, 4H), 2.22 (d, *J* = 1.08 Hz, 3H), 2.05 (p, *J* = 6.78 Hz, 2H). MS (FAB) *m/z* 433 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{21}H_{32}N_6O_2S$ $[M + H]^+$ 433.2380, found: 433.2370. Anal. HPLC 99.30% (R_t = 4.101 min).

4.1.4.9. N-(4-Methoxy-3-(3-(piperazin-1-yl)propoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(58)***. mp = 58-59 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (d, *J* = 0.90 Hz, 1H), 6.96 (d, *J* = 8.58 Hz, 1H), 6.91 (d, *J* = 2.37 Hz, 1H), 6.77 (dd, *J* = 8.40, 2.37 Hz, 1H), 6.66 (s, 1H), 4.05 (t, *J* = 6.03 Hz, 2H), 3.99 (t, *J* = 6.96 Hz, 2H), 3.81 (s, 3H), 3.61 (t, *J* = 6.75 Hz, 2H), 2.87 (t, *J* = 4.95 Hz, 4H), 2.57-2.49 (m, 6H), 2.22 (d, *J* = 1.11 Hz, 3H), 2.07-1.96 (m, 4H). MS (ESI) *m/z* 447 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{22}H_{34}N_6O_2S$ $[M + H]^+$ 447.2537, found: 447.2534. Anal. HPLC 99.32% (R_t = 3.799 min).

4.1.4.10. N-(4-Methoxy-3-(4-(piperazin-1-yl)butoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(59)***. mp = 82-83 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (s, 1H), 6.95-6.92 (m, 2H), 6.77 (dd, *J* = 8.40, 2.37 Hz, 1H), 6.66 (s, 1H), 4.03 (t, *J* = 6.03 Hz, 2H), 3.99 (t, *J* = 7.32 Hz, 2H), 3.82 (s, 3H), 3.61 (t, *J* = 7.14 Hz, 2H), 2.92 (t, *J* = 4.92 Hz, 4H), 2.50-2.41 (m, 6H), 2.22 (d, *J* = 1.11 Hz, 2H), 2.08 (p, *J* = 7.50 Hz, 2H), 1.80 (p, *J* = 6.21 Hz, 2H), 1.69-1.61 (m, 2H). MS (FAB) *m/z* 461 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{36}N_6O_2S$ $[M + H]^+$ 461.2693, found: 461.2705. Anal. HPLC 99.69% (R_t = 3.701 min).

4.1.4.11. N-(4-Methoxy-3-(2-(piperidin-4-yl)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(60)***. mp = 83-84 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (d, J = 1.11 Hz, 1H), 6.96 (d, J = 8.61 Hz, 1H), 6.91 (d, J = 2.19 Hz, 1H), 6.77 (dd, J = 8.43, 2.40 Hz, 1H), 6.67 (s, 1H), 4.04 (t, J = 5.85 Hz, 2H), 3.99 (t, J = 7.14 Hz, 2H), 3.82 (s, 3H), 3.58 (t, J = 6.96 Hz, 2H), 3.07-3.02 (m, 2H), 2.65 (td, J = 10.08, 3.84 Hz, 2H), 2.22 (d, J = 0.93 Hz, 3H), 2.07 (p, J = 7.14 Hz, 2H), 1.80-1.74 (m, 4H), 1.73-1.72 (m, 1H), 1.23-1.11 (m, 2H). MS (FAB) *m/z* 432 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{22}H_{33}N_5O_2S$ $[M + H]^+$ 432.2433, found: 432.2426. Anal. HPLC 95.47% (R_t = 4.070 min).

4.1.4.12. N-(4-Methoxy-3-(3-(piperidin-4-yl)propoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(61)***. mp = 49-50 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.60 (d, J = 1.11 Hz, 1H), 6.95 (d, J = 8.58 Hz, 1H), 6.91 (d, J = 2.40 Hz, 1H), 6.76 (dd, J = 8.43, 2.40 Hz, 1H), 6.66 (s, 1H), 3.98 (t, J = 6.39 Hz, 4H), 3.82 (s, 3H), 3.61 (t, J = 6.96 Hz, 2H), 3.11-3.07 (m, 2H), 2.69 (td, J = 12.27, 2.58 Hz, 2H), 2.22 (d, J = 0.93 Hz, 3H), 2.07 (p, J = 7.14 Hz, 2H), 1.86-1.76 (m, 5H), 1.44-1.37 (m, 2H), 1.25-1.12 (m, 2H). MS (FAB) *m/z* 446 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{35}N_5O_2S$ $[M + H]^+$ 446.2589, found: 446.2595. Anal. HPLC 96.06% (R_t = 4.066 min).

4.1.4.13. N-(4-Methoxy-3-(4-(piperidin-4-yl)butoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(62)***. mp = 64-65 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.50 (s, 1H), 6.87 (d, J = 8.61 Hz, 1H), 6.81 (d, J = 2.19 Hz, 1H), 6.67 (dd, J = 8.40, 2.37 Hz, 1H), 6.57 (s, 1H), 3.90 (t, J = 6.21 Hz, 4H), 3.73 (s, 3H), 3.52 (t, J = 6.96 Hz, 2H), 2.99-2.95 (m, 2H), 2.57 (t, J = 12.45 Hz, 2H), 2.14 (d, J = 0.93 Hz, 3H), 1.98 (p, J = 7.14 Hz, 2H), 1.67-1.63 (m, 5H), 1.40-1.35 (m, 2H), 1.25-1.18 (m, 2H), 1.11-1.03 (m, 2H). MS (FAB) *m/z* 460 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{24}H_{37}N_5O_2S$ $[M + H]^+$ 460.2746, found: 460.2744. Anal. HPLC 98.64% (R_t = 3.851 min).

4.1.4.14. N-(4-Methoxy-3-(2-(1-methylpiperidin-4-yl)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(63)***. mp = 71-72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58 (s, 1H), 6.95-6.91 (m, 2H), 6.77 (dd, *J* = 8.40, 2.55 Hz, 1H), 6.66 (s, 1H), 4.04 (t, *J* = 6.06 Hz, 2H), 3.96 (t, *J* = 7.50 Hz, 2H), 3.81 (s, 3H), 3.58 (t, *J* = 7.14 Hz, 2H), 2.88 (d, *J* = 10.26 Hz, 2H), 2.26 (s, 3H), 2.21 (d, *J* = 0.90 Hz, 3H), 2.08-2.00 (m, 4H), 1.80-1.71 (m, 4H), 1.33-1.29 (m, 3H). MS (FAB) *m/z* 446 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{35}N_5O_2S$ $[M + H]^+$ 446.2584, found: 446.2581. Anal. HPLC 98.99% (R_t = 4.109 min).

4.1.4.15. N-(3-(2-(2-Aminopyridin-4-yl)ethoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(64)***. mp = 66-67 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.77 (d, *J* = 5.31 Hz, 1H), 7.51 (s, 1H), 6.96-6.91 (m, 2H), 6.78 (dd, *J* = 8.61, 2.37 Hz, 1H), 6.65 (s, 1H), 6.60 (dd, *J* = 5.52, 1.47 Hz, 1H), 6.53 (s, 1H), 4.21 (t, *J* = 6.60 Hz, 2H), 3.98 (t, *J* = 7.14 Hz, 2H), 3.80 (s, 3H), 3.60 (t, *J* = 6.21 Hz, 2H), 2.98 (t, *J* = 6.39 Hz, 2H), 2.20 (d, *J* = 0.75 Hz, 3H), 2.06 (p, *J* = 6.93 Hz, 2H). MS (FAB) *m/z* 441 $[M+H]^+$. HRMS (FAB) *m/* calcd for $C_{22}H_{28}N_6O_2S$ *z* $[M + H]^+$ 441.2067, found: 441.2067. Anal. HPLC 99.52% (R_t = 3.967 min).

4.1.4.16. N-(3-(3-(2-Aminopyridin-4-yl)propoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(65)***. mp = 76-77 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 7.75 (d, *J* = 5.24 Hz, 1H), 7.58 (s, 1H). 6.97 (d, *J* = 8.56 Hz, 1H), 6.88 (d, *J* = 2.16 Hz, 1H), 6.78 (dd, *J* = 8.52, 2.36 Hz, 1H), 6.65 (s, 1H), 6.50 (d, *J* = 4.28 Hz, 1H), 6.45 (s, 1H), 3.99-3.94 (m, 4H), 3.84 (s, 3H), 3.60 (t, *J* = 6.88 Hz, 2H), 2.70 (t, *J* = 7.43 Hz, 2H), 2.20 (d, *J* = 0.72 Hz, 3H), 2.08-2.00 (m, 4H). MS (FAB) *m/z* 455 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{30}N_6O_2S$ $[M + H]^+$ 455.2224, found: 455.2223. Anal. HPLC 96.06% (R_t = 3.979 min).

4.1.4.17. N-(3-(4-(2-Aminopyridin-4-yl)butoxy)-4-methoxyphenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(66)***. mp = 63-64 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J* = 5.49 Hz, 1H),

7.60 (s, 1H), 7.39 (s, 1H), 6.89 (d, *J* = 8.43 Hz, 1H), 6.76-6.73 (m, 2H), 6.66 (d, *J* = 2.19 Hz, 1H), 6.51 (d, *J* = 5.49 Hz, 1H), 6.36 (s, 1H), 5.94 (br, NH), 3.98 (t, *J* = 6.06 Hz, 2H), 3.91 (t, *J* = 7.14 Hz, 2H), 3.87 (s, 3H), 3.69 (q, *J* = 6.60 Hz, 2H), 2.60 (t, *J* = 7.32 Hz, 2H), 2.17 (d, *J* = 0.72 Hz, 3H), 2.09 (p, *J* = 7.32 Hz, 2H), 1.85-1.80 (m, 4H). MS (FAB) *m/z* 469 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{24}H_{32}N_6O_2S$ $[M + H]^+$ 469.2380, found: 469.2385. Anal. HPLC 98.26% (R_t = 4.017 min).

*4.1.4.18. N-(4-Methoxy-3-(4-(pyrimidin-2-ylamino)butoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea **(67)**.* To a solution of **53** (1 eq) in EtOH was added 2-chloropyrimidine (2 eq) and triethylamine (2.5 eq). The mixture was refluxed for 2 days, then solvent was removed by evaporation. The residue was purified by column chromatography (MeOH : CH_2Cl_2) to give white solid, 35% yield. mp = 50-51 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (d, *J* = 4.74 Hz, 2H), 7.60 (s, 1H), 7.37 (s, 1H), 6.88 (d, *J* = 8.61 Hz, 1H), 6.76-6.68 (m, 3H), 6.52 (t, *J* = 4.77 Hz, 1H), 5.94 (br, 1H), 5.35 (br, 1H), 4.03 (t, *J* = 6.21 Hz, 2H), 3.91 (t, *J* = 7.14 Hz, 2H), 3.87 (s, 3H), 3.69 (q, *J* = 6.42 Hz, 2H), 3.52 (q, *J* = 6.60 Hz, 2H), 2.17 (d, *J* = 0.93 Hz, 3H), 2.09 (p, *J* = 7.32 Hz, 2H), 1.99 (p, *J* = 7.86 Hz, 2H), 1.85 (p, *J* = 6.78 Hz, 2H). MS (FAB) *m/z* 470 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{31}N_7O_2S$ $[M + H]^+$ 470.2338, found: 470.2340. Anal. HPLC 96.60% (R_t = 4.414 min).

*4.1.4.19. N-(4-(2-(Piperazin-1-yl)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea **(68)**.* mp = 85-86 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.58 (s, 1H), 7.17 (m, 2H), 6.97 (m, 2H), 6.66 (s, 1H), 4.15 (t, *J* = 5.31 Hz, 2H), 3.98 (t, *J* = 6.96 Hz, 2H), 3.60 (t, *J* = 6.96 Hz, 2H), 2.87 (m, 4H), 2.81 (t, *J* = 5.70 Hz, 2H), 2.57 (m, 4H), 2.21 (s, 3H), 2.04 (m, 2H). MS (FAB) *m/z* 403 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{20}H_{30}N_6OS$ $[M + H]^+$ 403.2275, found: 403.2282. Anal. HPLC 98.22% (R_t = 2.893 min).

*4.1.4.20. N-(4-(4-(2-Aminopyridin-4-yl)butoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea **(69)**.* mp = 58-59 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.75 (d, *J* = 5.49 Hz, 1H),

7.59 (s, 1H), 7.14 (d, *J* = 8.79 Hz, 2H), 6.93 (d, *J* = 8.97 Hz, 2H), 6.66 (s, 1H), 6.49 (dd, *J* = 5.31, 1.47 Hz, 1H), 6.44 (s, 1H), 3.99-3.97 (m, 4H), 3.60 (t, *J* = 6.39 Hz, 2H), 2.56 (t, *J* = 6.96 Hz, 2H), 2.21 (s, 3H), 2.04 (p, *J* = 6.96 Hz, 2H), 1.78-1.75 (m, 4H). MS (FAB) *m/z* 439 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{30}N_6OS$ $[M + H]^+$ 439.2275, found: 439.2268. Anal. HPLC 99.67% (R_t = 2.957 min).

4.1.4.21. N-(4-(2-Oxo-2-(piperazin-1-yl)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(70)***. mp = 95-96 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (s, 1H), 7.18 (d, *J* = 8.97 Hz, 2H), 6.69 (d, *J* = 8.97 Hz, 2H), 6.66 (s, 1H), 4.82 (s, 2H), 3.99 (t, *J* = 7.32 Hz, 2H), 3.60-3.50 (m, 6H), 2.85-2.77 (m, 4H), 2.21 (s, 3H), 2.07 (p, *J* = 6.96 Hz, 2H). MS (FAB) *m/z* 417 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{20}H_{28}N_6O_2S$ $[M + H]^+$ 417.2067, found: 417.2066. Anal. HPLC 96.16% (R_t = 2.936 min).

4.1.4.22. N-(4-(2-Oxo-2-(piperidin-4-ylamino)ethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(71)***. mp = 98-99 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 (s, 1H), 7.21 (d, *J* = 8.79 Hz, 2H), 7.01 (d, *J* = 8.97 Hz, 2H), 6.66 (s, 1H), 4.50 (s, 2H), 3.99 (t, *J* = 7.32 Hz, 2H), 3.91-3.84 (m, 1H), 3.58 (t, *J* = 6.06 Hz, 2H), 3.08-3.04 (m, 2H), 2.71 (t, *J* = 12.27 Hz, 2H), 2.22 (s, 3H), 2.07 (p, *J* = 6.96 Hz, 2H), 1.87-1.83 (m, 2H), 1.50-1.45 (m, 2H). MS (FAB) *m/z* 431 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{21}H_{30}N_6O_2S$ $[M + H]^+$ 431.2224, found: 431.2246. Anal. HPLC 100.00 % (R_t = 2.947 min).

4.1.4.23. N-(4-(2-((1-(2-Aminoethyl)piperidin-4-yl)amino)-2-oxoethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(72)***. mp = 73-74 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (s, 1H), 7.21 (d, *J* = 8.76 Hz, 2H), 7.01 (d, *J* = 8.76 Hz, 2H), 6.66 (s, 1H), 4.49 (s, 2H), 3.99 (t, *J* = 7.32 Hz, 2H), 3.79-3.74 (m, 1H), 3.58 (t, *J* = 6.66 Hz, 2H), 2.88-2.85 (m, 2H), 2.75-2.71 (t, *J* = 6.78 Hz, 2H),

2.45 (t, *J* = 6.75 Hz, 2H), 2.22 (s, 3H), 2.14-2.11 (m, 2H), 2.05-2.01 (p, *J* = 6.93 Hz, 2H), 1.86-1.82 (m, 2H), 1.65 (m, 2H). MS (FAB) *m/z* 474 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{35}N_7O_2S$ $[M + H]^+$ 474.2646, found: 474.2662. Anal. HPLC 98.75% (R_t = 2.779 min).

4.1.4.24. N-(4-(2-(((2-Aminopyridin-4-yl)methyl)amino)-2-oxoethoxy)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(73)***. mp = 90-91 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.79 (d, *J* = 5.49 Hz, 1H), 7.58 (s, 1H), 7.20 (d, *J* = 8.98 Hz, 2H), 7.03 (d, *J* = 8.97 Hz, 2H), 6.66 (s, 1H), 6.50 (dd, *J* = 5.31, 1.53 Hz, 1H), 6.45 (s, 1H), 4.60 (s, 2H), 4.34 (s, 2H), 4.00 (t, *J* = 6.96 Hz, 2H), 3.59 (t, *J* = 6.06 Hz, 2H), 2.22 (d, *J* = 0.93 Hz, 3H), 2.05 (p, *J* = 6.66 Hz, 2H). MS (FAB) *m/z* 454 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{22}H_{27}N_7O_2S$ $[M + H]^+$ 454.2020, found: 454.2046. Anal. HPLC 98.24% (R_t = 4.023 min)

4.1.4.25. N-(4-((1-(2-Aminoethyl)piperidin-4-yl)carbamoyl)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(74)***. mp = 91-92 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.81 (d, *J* = 8.61 Hz, 2H), 7.60 (s, 1H), 7.50 (d, *J* = 8.43 Hz, 2H), 6.67 (s, 1H), 4.01 (t, *J* = 7.32 Hz, 2H), 3.87-3.84 (m, 1H), 3.62 (t, *J* = 7.14 Hz, 2H), 2.99-2.95 (m, 2H), 2.75 (t, *J* = 6.96 Hz, 2H), 2.49 (t, *J* = 6.87 Hz, 2H), 2.23 (s, 3H), 2.16-2.14 (m, 2H), 2.10 (t, *J* = 6.93 Hz, 2H), 1.95-1.88 (m, 2H), 1.72-1.65 (m, 2H). MS (FAB) *m/z* 444 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{22}H_{33}N_7OS$ $[M + H]^+$ 444.2540, found: 444.2557. Anal. HPLC 99.61% (R_t = 3.661 min).

4.1.4.26. N-((E)-4-(3-oxo-3-(piperazin-1-yl)prop-1-en-1-yl)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(75)***. mp = 112-113 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.63-7.60 (m, 3H), 7.57 (d, *J* = 15.36 Hz, 1H), 7.41-7.35 (m, 2H), 7.11 (d, *J* = 15.39 Hz, 1H), 6.67 (s, 1H), 4.02 (t, *J* = 7.14 Hz, 2H), 3.68-3.59 (m, 6H), 2.84-2.80 (m, 4H), 2.23 (d, *J* = 0.93 Hz, 3H), 2.09 (p, *J* = 6.75 Hz, 2H),

MS (FAB) *m/z* 413 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{21}H_{28}N_6OS$ $[M + H]^+$ 413.2118, found: 413.2111. Anal. HPLC 96.61% (R_t = 2.938 min).

4.1.4.27. N-((E)-4-(3-(4-(2-aminoethyl)piperazin-1-yl)-3-oxoprop-1-en-1-yl)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(76)***. mp = 66-67 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.63 (m, 3H), 7.57 (d, *J* = 15.36 Hz, 1H), 7.41 (d, *J* = 8.61 Hz, 2H), 7.11 (d, *J* = 15.39 Hz, 1H), 6.67 (s, 1H), 4.02 (t, *J* = 7.14 Hz, 2H), 3.68-3.64 (m, 4H), 3.61 (t, *J* = 6.57 Hz, 2H), 2.84 -2.81 (m, 4H), 2.52-2.48 (m, 4H), 2.23 (s, 3H), 2.09 (p, *J* = 6.96 Hz, 2H). MS (FAB) *m/z* 456 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_7OS$ $[M + H]^+$ 456.2540, found: 456.2518. Anal. HPLC 98.74% (R_t = 2.943 min).

4.1.4.28. N-((E)-4-(3-oxo-3-(piperidin-4-ylamino)prop-1-en-1-yl)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(77)***. mp = 76-77 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.59 (s, 1H), 7.55-7.46 (m, 3H), 7.40 (d, *J* = 8.43 Hz, 2H), 6.67 (s,1H), 6.57 (d, *J* = 15.70 Hz, 1H), 4.02 (t, *J* = 7.50 Hz, 2H), 3.78-3.72 (m, 1H), 3.61 (t, *J* = 6.69 Hz, 2H), 3.07-3.03 (m, 2H), 2.70-2.63 (m, 2H), 2.23 (d, *J* = 0.93 Hz, 3H), 2.09 (p, *J* = 6.96 Hz, 2H), 1.93-1.90 (m, 2H), 1.59-1.55 (m, 2H)**.** MS (FAB) *m/z* 427 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{22}H_{30}N_5OS$ $[M + H]^+$ 427.2275, found: 427.2277. Anal. HPLC 98.19% (R_t = 2.602 min).

4.1.4.29. N-(E)-(4-(3-(4-(2-Aminoethyl)piperazin-1-yl)-3-oxoprop-1-en-1-yl)phenyl)-N'-(3-(5-methyl-1H-imidazol-1-yl)propyl)thiourea ***(78)***. mp = 80-81 °C. ^{1}H NMR (500 MHz, CD_3OD) δ 7.62 (d, *J* = 8.45 Hz, 2H), 7.59 (s, 1H), 7.56 (d, *J* = 15.40 Hz, 1H), 7.42 (d, *J* = 8.50 Hz, 2H), 7.11 (d, *J* = 15.40 Hz, 1H), 6.67 (s, 1H), 4.02 (t, *J* = 7.15 Hz, 2H), 3.76-3.72 (m, 5H), 3.62-3.55 (m, 2H), 2.83 (t, *J* = 6.25 Hz, 2H), 2.52-2.40 (m, 6H), 2.23 (d, *J* = 1.00 Hz, 3H), 2.10 (p, *J* = 7.05 Hz, 2H). MS (FAB) *m/z* 456 $[M+H]^+$. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_7OS$ $[M + H]^+$ 456.2540, found: 456.2518. Anal. HPLC 97.30% (R_t = 2.863 min).

4.2. Molecular Modeling

The X-ray crystal structure of the human glutaminyl cyclase (PDB ID: 3PBB)[26] was prepared via the Protein Preparation Wizard in Maestro v.10.2 (Schrödinger, LLC, New York, NY, USA). During the preparation process, bond orders were assigned, zero-order bonds to Zn^{2+} were generated, and hydrogen atoms were added. The entire hydrogen atoms were energy minimized with the optimized potential for liquid simulation (OPLS) 2005 force field. The protonation states of the ligand molecules were forecasted by the pKa prediction module in ADMET Predictor™ (Simulations Plus, Lancaster, CA, USA). The 3D structure of **53** was created by LigPrep v.3.4 in Maestro and the resulting structure was energy minimized in implicit solvent with OPLS 2005 force field in Maestro. The prepared ligand molecules were docked to the *h*QC with Glide v.6.7 in Maestro. The grid for the active site was generated through the centroid of the co-crystallized ligand, PBD150, and the grid box size was selected as default. Metal coordination constraint was set as tetrahedral geometry for the Zn^{2+}. Glide SP docking was completed with the maximum number of 30 poses per ligand. The resulting best pose of **53** were chosen and conducted for the subsequent QM-Polarized Ligand Docking (QPLD) procedure. The partial charges of the docked ligands were analyzed by Jaguar with the option of accurate QM level. Then, the ligands accompanied with the updated charges were re-docked using Glide extra precision (XP). The protein-ligand complex obtained from the QPLD was taken for further optimization by Refine Protein-Ligand Complex module in Prime v.4.0 in Maestro. Protein residues within 5 Å of the docked ligand were minimized by local optimization refinement. The side chain conformations of the selected protein residues were predicted and minimized along with the docked ligand during this process. The results were further energy minimized using Monte Carlo sampling algorithm in 2500 steps in Maestro.

All the molecular graphic figures were generated by PyMOL software (http://www.pymol.org). All computational studies were undertaken on an Intel Xeon Octa-Core 2.67 GHz workstation with Linux CentOS release 6.7.

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Structure-Activity Relationship Investigation of Phe-Arg Mimetic Region of Human Glutaminyl Cyclase Inhibitors

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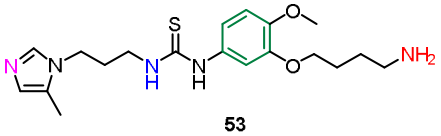
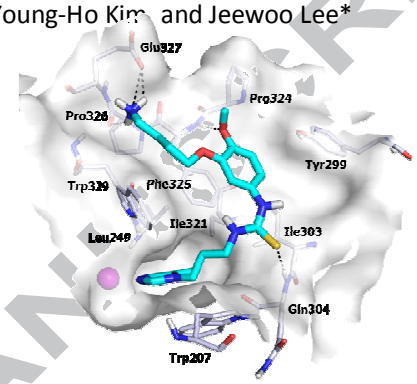


53



A : zinc binding motif
B : H-bonding donor
C : Phe mimetic
D : Arg mimetic

- Glu-Phe-Arg mimetic of $A\beta_{42}$
- hQC IC_{50} = 8.8 nM
- in vivo (icv) : 36.6% inh. of $hA\beta_{N3pE-42}$