Bioorganic & Medicinal Chemistry 21 (2013) 3821–3830

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure–activity relationship of human glutaminyl cyclase inhibitors having an *N*-(5-methyl-1*H*-imidazol-1-yl)propyl thiourea template

Phuong-Thao Tran [a], Van-Hai Hoang [a], Shivaji A. Thorat [a], Sung Eun Kim [a], Jihyae Ann [a], Yu Jin Chang [b], Dong Woo Nam [b], Hyundong Song [b], Inhee Mook-Jung [b], Jiyoun Lee [c], Jeewoo Lee [a,*]

[a] *Laboratory of Medicinal Chemistry, Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*
[b] *Department of Biochemistry and Biomedical Sciences, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea*
[c] *Department of Global Medical Science, Sungshin Women's University, Seoul 142-732, Republic of Korea*

ARTICLE INFO

Article history:
Received 18 March 2013
Revised 6 April 2013
Accepted 8 April 2013
Available online 17 April 2013

Keywords:
Glutaminyl cyclase
QC
Alzheimer's disease
Beta amyloid
Pyroglutamate
Enzyme inhibitor

ABSTRACT

In an effort to design inhibitors of human glutaminyl cyclase (QC), we have synthesized a library of *N*-aryl *N*-(5-methyl-1*H*-imidazol-1-yl)propyl thioureas and investigated the contribution of the aryl region of these compounds to their structure–activity relationships as cyclase inhibitors. Our design was guided by the proposed binding mode of the preferred substrate for the cyclase. In this series, compound **52** was identified as the most potent QC inhibitor with an IC_{50} value of 58 nM, which was two-fold more potent than the previously reported lead **2**. Compound **52** is a most promising candidate for future evaluation to monitor its ability to reduce the formation of pGlu-Aβ and Aβ plaques in cells and transgenic animals.

1. Introduction

Glutamyl cyclases (QCs) catalyze the intramolecular cyclization of N-terminal glutaminyl and glutamyl residues to form pyroglutamic (pGlu) peptides or proteins. Importantly, the post-translational formation of N-terminal pGlu is known to play a crucial role in maturation of various hormones and cytokines.[1–3] QCs have been identified in both plants and animals and are most abundant in mammalian secretory and neuronal tissues such as the pituitary gland and the hypothalamus.[3–5] In humans, QCs have been implicated in several pathological conditions including amyloidosis,[6] osteoporosis,[7] rheumatoid arthritis,[8] and melanoma.[9] Recently, it was reported that human QC catalyzes cyclization of the N-terminal glutamate of β-amyloid peptides (Aβ) into pGlu.[10] The resulting pGlu-Aβ peptides become more hydrophobic and resistant to proteolysis. Therefore, they rapidly aggregate and accumulate into neuritic plaques causing neurotoxicity.[11,12] It was demonstrated that pGlu-Aβ peptides are more toxic than other Aβ species such as $Aβ_{1-42}$ and $Aβ_{1-40}$, and they may act as initiators of Alzheimer's disease.[13] Finally, inhibition of QC reduced the amount of both pGlu-Aβ and Aβ plaques in transgenic mouse models of Alzheimer's disease indicating that QC is a therapeutically important target in Alzheimer's disease.[14]

Despite the significant potential role of QC in Alzheimer's disease pathology, only a few QC-specific inhibitors have been developed thus far.[15,16] These QC-specific inhibitors have been designed based on two distinct structural characteristics of human QCs. First, the structure of human QC is closely related to that of aminopeptidases and has one zinc ion in the active site.[17] Second, human QC prefers substrates with a large hydrophobic side chain on the position penultimate to the N-terminal glutamine.[5] Therefore, previously reported QC-specific inhibitors contain an aromatic ring tethered to an imidazole as a zinc-binding moiety. Based on these findings, we have divided the common structural features of the reported QC-selective inhibitors (**1**, **2**) into three regions as shown in Figure 1. The A-region represents a zinc-binding imidazole with a methyl substituent on the 5-position to enhance potency.[15] The B-region contains a hydrogen bond donor that matches the C-terminal amide nitrogen of glutamine. The C-region accommodates the hydrophobic side chain penultimate to the N-terminus of the preferred substrate, H-Gln-Phe-Ala-NH_2. Among these three regions, the C-region appears to be the most promising for further modification, because it accommodates various functional groups implying great structural flexibility in that region. Therefore, we

* Corresponding author. Tel.: +82 2 880 7846; fax: +82 2 888 0649.
E-mail address: jeewoo@snu.ac.kr (J. Lee).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.04.005

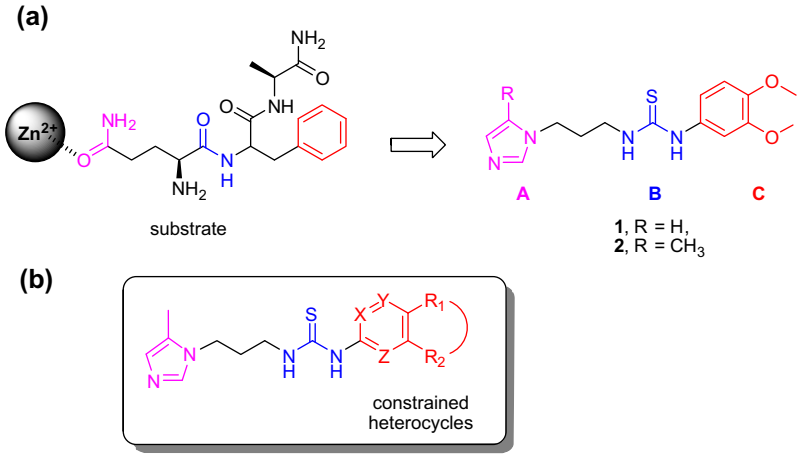


Figure 1. (a) Structures of the previously reported hQC inhibitors; (b) Newly designed scaffold based on the proposed binding mode of the preferred substrate shown in (a).

decided to focus on modifying the C-region to investigate the structure–activity relationships of QC inhibitors.

In this report, we have developed a new series of QC inhibitors with various constrained analogs of heterocycles and aromatic rings in the C-region. We evaluated their enzyme inhibitory activities and investigated the structure–activity relationships of these derivatives.

2. Result and discussion

2.1. Chemistry

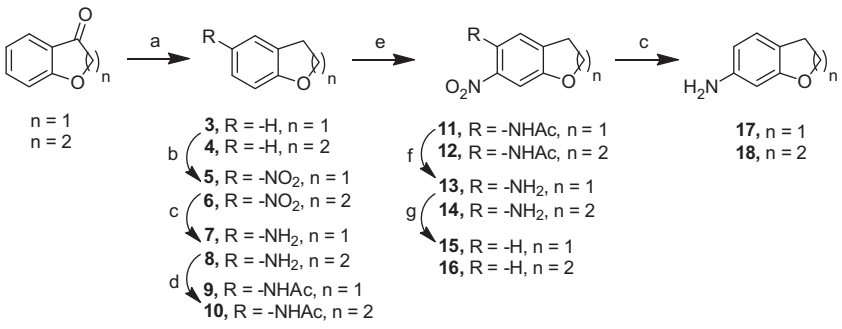
The C-region fragments with various aromatic rings and heterocycles were synthesized as shown in Scheme 1 through Scheme 4. All other amine fragments not listed in the scheme were obtained commercially. Benzofuran and chromane fragments were synthesized from commercially available coumaran and chromanone, respectively, as shown in Scheme 1. Each ketone was reduced by using $LiAlH_4$ and $AlCl_3$ and then nitrated. Subsequent reduction to the amine yielded fragments **7** and **8**, which were further subjected to another round of acetylation, nitration, deacetylation, deamination and reduction to provide fragments **17** and **18**. Benzoxazine fragments were constructed from 4- or 5-nitro-2-aminophenol as shown in Scheme 2. Each aminophenol was cyclized by using chloroacetyl chloride in biphasic media to obtain benzoxazinone intermediates in high yields (95–55%). Subsequent reduction of the ketone and nitro group yielded benzoxazine fragments **25** and **26**. Indazole and imidazole fragments were synthesized from commercially available starting material as shown in Schemes 3 and 4.

The A-region fragment with 5-methyl imidazole was synthesized by applying an optimized Gabriel synthesis based on the previously reported procedure[15] as described in Scheme 5. The isothiocyanate fragment **41** was synthesized from commercially available 4(5)-methylimidazole in 5 steps to give an overall yield of 36%. Final coupling steps with all the C-region fragments and isothiocyanate **41** were carried out as demonstrated in Scheme 6 by using an optimized co-solvent system to obtain thiourea compounds **42–47**, **52–55, 58–60**, and **65–85** as final products. Boc-protected thiourea compounds **48**, **49**, **61**, and **63** were further subjected to deprotection in 6 N HCl to obtain final compounds **50**, **51**, **62**, **64**. Final products containing tetralone, **54** and **55**, underwent another reduction step with sodium borohydride to afford compounds **56** and **57**.

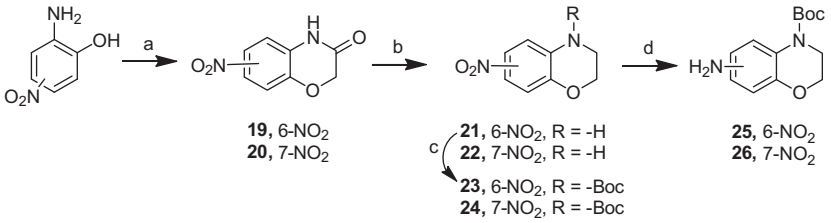
Finally, a reverse quinazolinedione derivative **88** was synthesized from commercially available 2*H*-1,3-benzoxazine-2,4-(1*H*)-dione as shown in Scheme 7. The 5-methyl imidazole fragment **40** was added first, and then the quinazolinedione core was cyclized in two steps to provide **88** as final product.

2.2. Biological activity

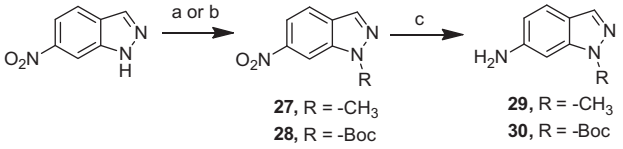
Glutaminyl cyclase is expressed in various cell lines including HEK293.[18] In this study, we lysed HEK293 cells expressing endogeous QC with RIPA-buffer and used the lysates as the source of QC for our in vitro assays.[18] QC activity was assayed as described by Schilling et al.[19] and IC_{50} values (Tables 1 and 2) were determined from the inhibitory dose response curves. The IC_{50} value of the previously reported compound **2** was measured for comparison.



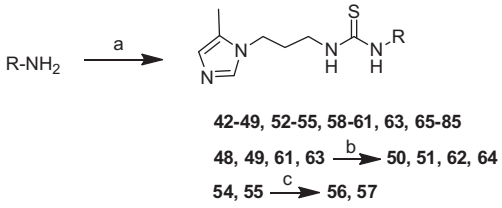
Scheme 1. Reagents and conditions: (a) $LiAlH_4$, $AlCl_3$, diethyl ether, 0 °C to reflux, 1 h; (b) $AgNO_3$, acetyl chloride, MeCN, 1 h, 0 °C for **6**, or HNO_3 63%, −10 °C, 1 h for **7**; (c) 10% Pd/C, H_2, MeOH/THF (1:1), 2 h, rt; (d) Ac_2O, 1,4-dioxane, pyridine, 50 °C, 2 h, rt; (e) concd HNO_3, AcOH, 1.5 h, rt; (f) concd HCl, EtOH, 2 h, reflux; (g) isoamylnitrite, THF, 3 h, reflux.



Scheme 2. Reagents and conditions: (a) chloroacetyl chloride, isobutylmethyl ketone/water (1:1), $NaHCO_3$, 0 °C (30 min) to reflux (7 h); (b) BH_3, THF, reflux, 2 h, MeOH, reflux, 1 h; (c) $(Boc)_2O$, $NaHCO_3$, CH_2Cl_2, rt, 12 h; (d) H_2, 10% Pd/C, MeOH, rt, 2 h.



Scheme 3. Reagents and conditions: (a) 60% NaH, iodomethane, DMF, rt, 1 h; (b) Boc_2O, $NaHCO_3$, THF, rt, 2 h, (c) 10% Pd/C, H_2, MeOH/THF (2:1), 2 h, rt.



Scheme 6. Reagents and conditions: (a) **41**, NEt_3, CH_2Cl_2/MeCN (1:1), 2 h, 0 °C to rt; (b) 6 N HCl, EtOH, 100 °C, overnight; (c) $NaBH_4$, MeOH, rt, 3 h.

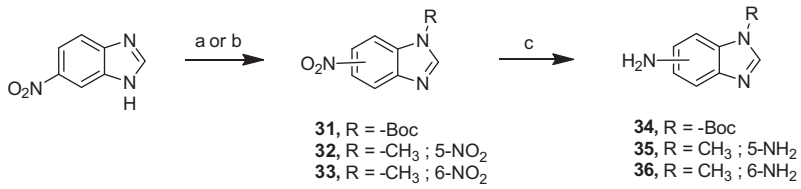
Compounds **42–57** and compound **88** containing various 5- and 6-membered ring constrained analogs exhibited IC_{50} values ranging from 58 nM to low micromolar concentrations (Table 1). Compound **52** was the most potent compound reported to date being twice as potent as compound **2**, the best QC inhibitor reported so far.[15,16] When one of the oxygen atoms in the fused dioxane was replaced with either nitrogen or carbon, as shown with compounds **46–51**, the IC_{50} value was significantly increased. Additionally, when the ether group was replaced with a ketone, inhibitory potency decreased slightly (**54** and **55**), and reduction of the ketone to an alcohol reduced potency even further (**56** and **57**), yielding IC_{50} values 10-fold weaker than that of **52**. Furthermore, when the position of the dioxane ring was moved as shown in **53**, the compound completely lost its activity. Based on these observations, possible hydrogen bonding interactions exist in this specific region, and the presence of hydrogen bond acceptors appears to be crucial for binding.

When the fused dioxane was replaced with a dioxolane (compound **42**), the IC_{50} value increased up to three-fold. Again, inhibitory effects decreased further when the oxygen atoms were replaced with carbon (**43**–**45**) as was observed with the dioxane analogs. Interestingly, a reverse quinazolinedione analog **88** demonstrated comparable potency (IC_{50} = 123 nM), which warrants further investigation with various analogs. Significant activity changes within this series indicate that the electronic properties and spatial arrangements in the A-region are critical for inhibitory activity.

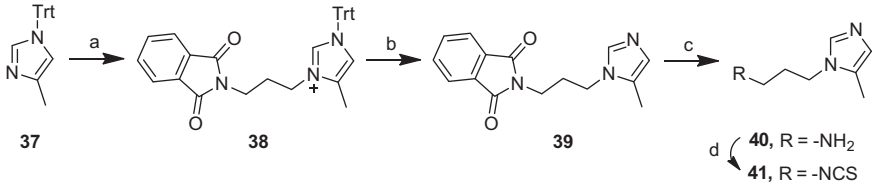
IC_{50} values of compounds with various 5- and 6-membered aromatic heterocycles are presented in Table 2. Compounds **60** and **78** demonstrated comparable potency having IC_{50} values of 239 and 158 nM, respectively. Compounds in this series showed slightly weaker inhibitory potencies compared to the non-aromatic heterocycles in Table 1, suggesting that these planar aromatic rings may not fit well inside the active site. Additionally, compounds lacking hydrogen-bond acceptors demonstrated poor inhibition whereas compounds having more electron-withdrawing atoms at specific positions appeared to exhibit higher inhibitory effects. Again, these findings support our initial observations that hydrogen-bonding interactions are critical in the A-region for specific inhibition.

3. Conclusion

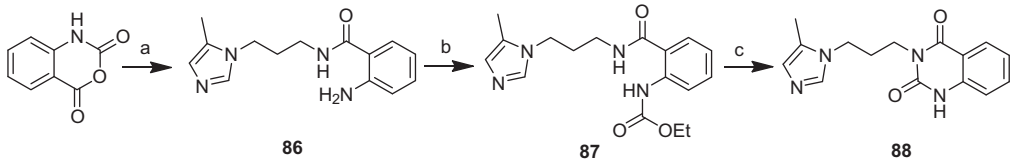
We have developed a series of potent hQC inhibitors and determined IC_{50} values against HEK293 cell lysates expressing QC.



Scheme 4. Reagents and conditions: (a) Boc_2O, $NaHCO_3$, THF, rt, 2 h; (b) 60% NaH, iodomethane, DMF, rt, 1 h; (c) 10% Pd/C, H_2, MeOH, 2 h, rt.

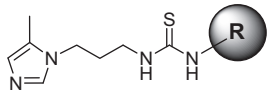


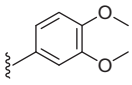
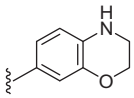
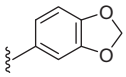
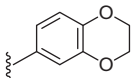
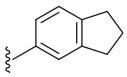
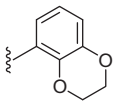
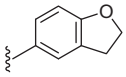
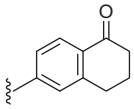
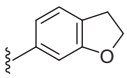
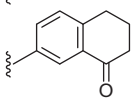
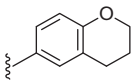
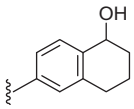
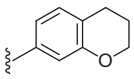
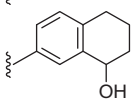
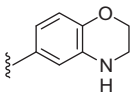
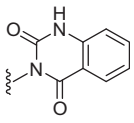
Scheme 5. Reagents and conditions: (a) *N*-(3-bromopropyl)phthalimide, MeCN, overnight, reflux; (b) TFA, MeOH, 3 h, reflux; (c) hydrazine monohydrate, EtOH, rt, 3 h; (d) di-2-pyridyl-thionocarbonate, CH_2Cl_2, 3 h, rt.



Scheme 7. Reagents and conditions: (a) **40**, THF rt, overnight; (b) EtOCOCl, EtOH, reflux, 3 h; (c) KOH, EtOH, reflux.

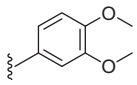
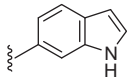
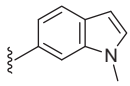
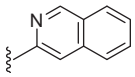
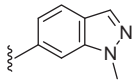
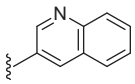
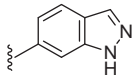
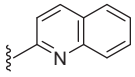
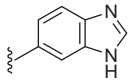
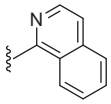
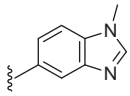
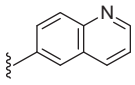
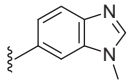
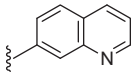
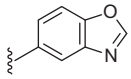
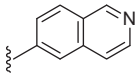
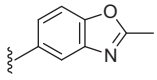
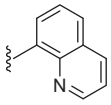
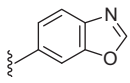
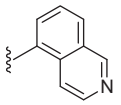
Table 1
IC_{50} values for inhibition of hQC by benzocyclic compounds



	R	IC_{50} (nM)[a]		R	IC_{50} (nM)
2		119	**51**		1070
42		185	**52**		58
43		WE[b]	**53**		NE[b]
44		437	**54**		482
45		405	**55**		788
46		208	**56**		519
47		479	**57**		812
50		492	**88**		123

[a] The values indicate the mean of at least three experiments.
[b] WE: weakly effective, NE: not effective.

Table 2
IC_{50} values for inhibition of hQC by heterocyclic compounds

	R	IC_{50} (nM)[a]		R	IC_{50} (nM)
2		119	**73**		453
58		WE[b]	**74**		WE[b]
59		1613	**75**		518
60		239	**76**		WE[b]
62		381	**77**		337
64		463	**78**		158
65		711	**79**		293
66		461	**80**		768
67		475	**81**		WE[b]
68		524	**82**		WE[b]
69		1070	**83**		546
70		329	**84**		375
71		1220	**85**		3050
72		488			

[a] The values indicate the mean of at least three experiments.
[b] WE: weakly effective.

Among the compounds of this series, compound **52** is the most potent hQC inhibitor developed to date, two-fold more potent than the previously reported inhibitor. Several other compounds, **42**, **78** and **88**, demonstrated potencies close to the reference compound **2**. Compounds with hydrogen-bond acceptors such as oxygen and nitrogen atoms appeared to be more potent than compounds lacking hetero atoms or containing delocalized aromatic rings. Our results indicate that having hydrogen-bond acceptors within the A-region is essential for inhibitory effect. Further exploration of structure–activity relationships looking at non-aromatic heterocycles should help to identify the crucial interactions within the active site. Compound **52** emerged as the most promising candidate for future evaluation to monitor its ability to reduce the formation of pGlu-Aβ and Aβ plaques in cells and transgenic animals.

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. Proton NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz, Bruker Analytik, DE/AVANCE Digital 400 at 400 MHz, and Bruker Analytik, DE/AVANCE Digital 500 at 500 MHz. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC–MS.

4.1.2. General procedure for thiourea coupling

Procedure A: The isothiocyanate compound (1 equiv) was added to the solution of amine (1 equiv) in 5 ml of a mixture of dichloromethane and acetonitrile (1:1, v/v). The mixture was cooled to 0 °C. Then, triethylamine (2 equiv) was added gradually. The mixture was stirred at 0 °C for 15 min, after which stirring was continued at room temperature for 2–10 h. The reaction mixture was concentrated, extracted with dichloromethane, and washed with brine. The organic layer was dried over $MgSO_4$ and purified by column chromatography ($MeOH/CH_2Cl_2$) or by preparative TLC ($MeOH/CH_2Cl_2$) to afford the desired product.

4.1.3. General procedure for reduction of the nitro group to the amine

Procedure B: The nitro-group containing compound was dissolved in MeOH (or a mixture of MeOH and tetrahydrofuran) and then 10% Pd/C was added. The mixture was stirred at room temperature under hydrogen gas until the starting material had been consumed. The crude mixture was filtered through Celite, washed with methanol, and then concentrated. The product was carried on to the next step without further purification or was purified by column chromatography.

4.1.4. General procedure for Boc-protection

Procedure C: $NaHCO_3$ (3.3 equiv) was added to the solution of the amine (1 equiv) at 0 °C, stirred for 30 min, and then di-*tert*-butyl dicarbonate (1.1 equiv) was added to the reaction mixture. The reaction was continued at room temperature for 2 h and then diluted with ethyl acetate. It was washed with H_2O and then with brine. Finally, it was dried over $MgSO_4$, concentrated, and purified by column chromatography to give the desired product.

4.1.5. General procedure for Boc-deprotection

Procedure D: The Boc-protected compound was treated in ethanol with 6 N HCl (4 equiv). The reaction mixture was heated at 100 °C until the starting material was consumed. It was then basified with K_2CO_3 to pH >8 and extracted with ethyl acetate. The organic layers were combined and concentrated under reduced pressure. The crude residue was purified by column chromatography or PLC to give the desired product.

4.1.6. General procedure for N-methylation

Procedure E: NaH (1.1 equiv) was added to the solution of amine (1 equiv) in anhydrous *N*,*N*-dimethylformamide. Iodomethane (1.1 equiv) was added dropwise to the reaction mixture at 0 °C. The reaction was continued at room temperature until the starting material was consumed. The resulting mixture was diluted with ethyl acetate and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The product was carried on to the next reaction without further purification or was isolated by column chromatography.

4.1.7. Experimental procedure

4.1.7.1. Chroman (4). To a solution of chroman-4-one (10 g, 1 equiv) in diethyl ether was slowly added aluminum chloride (31.5 g, 3.5 equiv) at 0 °C, followed by lithium aluminium hydride (4.35 g, 1.75 equiv) added portionwise. The reaction mixture was stirred for 1 h at boiling temperature. The reaction was cooled, and a solution of NH_4HCO_3 was added slowly, after which the solution was filtered through a plug of Celite and extracted with ethyl acetate (3 × 200 mL). The combined organic extracts were washed with water (3 × 100 mL), and brine (100 mL), dried over $MgSO_4$, and purified by chromatography on a silica gel column (EA/*n*-hexane = 1:9) to give **4** (4.88 g, 54%) as a light yellow oil.

4.1.7.2. 5-Nitro-2,3-dihydrobenzofuran (5). Silver nitrate (1.41 g, 8.32 mmol) and coumaran **3** (0.59 mL, 8.32 mmol) were dissolved in acetonitrile (10 mL) and placed in a 100 mL three-necked flask equipped with a dropping funnel, condenser and drying tube, and thermometer. 0.59 mL (8.32 mmol) of acetyl chloride was added to the reaction mixture at 0 °C. As the quantity of silver chloride increased, further dilution with acetonitrile and more vigorous stirring were used to help maintain reactant contact for 1 h at 0–5 °C and for 4 h at room temperature. 20 mL of water was added to the reaction flask at 0 °C and then an additional 10 mL of water was added when the mixture was at room temperature. Exhaustive treatments of solid and liquid materials with ethyl acetate were used to extract the products. The organic layer was concentrated, dried over $MgSO_4$, and then purified by column chromatography (EA/*n*-hexane) to give 740 mg of **5** (54%) as a yellow solid.

4.1.7.3. 6-Nitrochroman (6). To chroman **4** (4.88 g) was added dropwise a solution of HNO_3 (8.5 mL, 63%) at −10 °C for 1 h. After the reaction was completed, the mixture was basified with a 10% solution of NaOH and extracted with dichloromethane (3 × 100 mL). The combined organic extracts were washed with water (3 × 50 mL) and brine, dried over $MgSO_4$, and purified by chromatography on a silica gel column (EtOAc/*n*-hexane gradient) to give **6** (1.35 g, 19%) as a yellow solid.

4.1.7.4. 5-Amino-2,3-dihydrobenzofuran (7) and 6-aminochroman (8). Prepared from compound **5** or **6** respectively by following the general procedure B described above.

4.1.7.5. *N*-(2,3-Nihydrobenzofuran-5-yl)acetamide (9) and *N*-(chroman-6-yl)acetamide (10). Ac_2O (2 equiv) and pyridine (1 equiv) were added dropwise to a stirred solution of compound **7** or **8**, respectively, (1 equiv) in dioxane (4 mL) at 0 °C and the solution was stirred at 20 °C for 16 h. The solution was diluted with water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with brine, dried over $MgSO_4$, and then evaporated to give **9** (99%) as a brown solid or **10** (86%) as a white solid

4.1.7.6. *N*-(6-Nitro-2,3-dihydrobenzofuran-5-yl)acetamide (11) and *N*-(7-nitrochroman-6-yl)acetamide (12). A solution of concd HNO_3 (1.4 equiv) in HOAc (1 mL) was added dropwise to a stirred solution of acetamide **9** or **10** (1 equiv) in HOAc (10 mL) at 15 °C. The mixture was stirred at 15 °C for 1 h and then poured into ice/water (80 mL) and stirred for 30 min. The combined solvent was extracted with ethyl acetate, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel (EtOAc/*n*-hexane) to give **11** in 64% yield as a brown solid or **12** in 33% yield as a yellow solid.

4.1.7.7. Nitroaniline (13) and 7-nitrochroman-6-amine (14). A suspension of **11** or **12** (2.07 mmol) and conc. HCl (6.5 mL) in EtOH (25 mL) was heated at reflux temperature for

2 h. The solution was cooled, carefully neutralized with aqueous NH_3 solution, and the resulting precipitate was filtered and dried to give **13** (227 mg, 61%) as an orange solid or **14** (368.5 mg, 99%) as a brown solid.

4.1.7.8. 6-Nitro-2,3-dihydrobenzofuran (15) and 7-nitrochroman (16). Isoamylnitrite (3 equiv) was added dropwise to a solution of **13** or **14** (1 equiv) in anhydrous THF at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then refluxed for 3 h. The solution was cooled to room temperature, water (50 mL) was added, and the solution was extracted with ethyl acetate (3×50 mL). The organic layer was washed with brine, dried over $MgSO_4$, then evaporated and purified by column chromatography with a gradient eluent of EA/*n*-hexane (2–8%) to give **15** (70%) as a yellow solid or **16** (74%) as a yellow solid.

4.1.7.9. 6-Amino-2,3-dihydrobenzofuran (17) and 7-aminochroman (18). Prepared from nitro compounds **15** and **16** by following the general reduction procedure B with 56% and 83% yield, respectively.

4.1.7.10. 6-Nitro-2*H*-benzo[*b*][1,4]oxazin-3(4*H*)-one (19) and 7-nitro-2*H*-benzo[*b*][1,4] oxazin-3(4*H*)-one (20). To a solution of the 4- or 5-nitro-2-aminophenol (400 mg, 1 equiv) in isobutyl methylketone (4 mL) was added $NaHCO_3$ (522 mg, 2.4 equiv) and water (4 mL) and then the resulting mixture was cooled in an ice-bath. Chloroacetyl chloride (0.24 mL, 1.15 mL) was added dropwise with stirring and the cold mixture was set aside until it reached ambient temperature; it was then refluxed for 7 h. The mixture was extracted with ethyl acetate (3×30 mL), washed with water (2×30 mL), brine (30 mL) and dried over $MgSO_4$, and then it was concentrated under vacuum to give **19** (95% yield) or **20** (99% yield) as a brown solid.

4.1.7.11. 6-Nitro-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine (21) and 7-nitro-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine (22). To a solution of **19** or **20** (500 mg) in anhydrous THF was added dropwise boran tetrahydrofuran complex solution (1.0 M, 5.2 mL). The reaction mixture was heated to reflux for 2 h and then 4 mL MeOH was added. After 1 h, NH_4HCO_3 solution was added to the reaction mixture. The mixture was extracted with dichloromethane, washed with water, dried over $MgSO_4$, and concentrated under vacuum to give compound **21** or **22** with 53% or 62% yield, respectively, as an orange solid.

4.1.7.12. *tert*-Butyl 6-nitro-2*H*-benzo[*b*][1,4]oxazine-4(3*H*)-carboxylate (23) and *tert*-butyl 7-nitro-2*H*-benzo[*b*][1,4]oxazine-4(3*H*)-carboxylate (24). Prepared by following the general Boc-protection (procedure C) from the corresponding compounds **21** and **22**. Products **23** and **24** were obtained with 50% and 90% yields, respectively, as yellow solids.

4.1.7.13. *tert*-Butyl 6-amino-2*H*-benzo[*b*][1,4]oxazine-4(3*H*)-carboxylate (25) and *tert*-butyl 7-amino-2*H*-benzo[*b*][1,4] oxazine-4(3*H*)-carboxylate (26). Synthesized from nitro compounds **23** or **24** by following the general procedure B.

4.1.7.14. 1-Methyl-6-nitro-1*H*-indazole (27). Prepared from commercially available 6-nitroindazole by following the general procedure E. Two isomers were obtained, 1-methyl-6-nitro-1*H*-indazole (**27**) as a yellow solid (59% yield) and 1-methyl-5-nitro-1*H*-indazole as an orange solid (38% yield).

4.1.7.15. *tert*-Butyl 6-nitro-1*H*-indazole-1-carboxylate (28). Prepared from commercially available 6-nitroindazole by following the general procedure C. Product as a yellow solid (99% yield) was carried on to the next step without further purification.

4.1.7.16. 1-Methyl-6-amino-1*H*-indazole (29). Synthesized from **27** by following the general procedure B.

4.1.7.17. *tert*-Butyl 6-amino-1*H*-indazole-1-carboxylate (30). Prepared from nitro compound **28** by following the general procedure B and purified by column chromatography with an EtOAc/*n*-hexane (1:1) system to give the desired product as a yellow solid (67% yield).

4.1.7.18. *tert*-Butyl 5-nitro-1*H*-benzo[*d*]imidazole-1-carboxylate or *tert*-butyl 6-nitro-1*H*-benzo[*d*]imidazole-1-carboxylate (31). Prepared from commercially available 6-nitrobenzimidazole by following the general procedure C. The mixture of 5- and 6-nitro compounds was not separated.

4.1.7.19. 1-Methyl-5-nitro-1*H*-benzo[*d*]imidazole (32) and 1-methyl-6-nitro-1*H*-benzo[*d*]imidazole (33). Prepared from commercially available 6-nitrobenzimidazole by following the general procedure E. The mixture of **32** and **33** was obtained as an orange solid (99% yield).

4.1.7.20. *tert*-Butyl 5-amino-1*H*-benzo[*d*]imidazole-1-carboxylate or *tert*-butyl 6-amino-1*H*-benzo[*d*]imidazole-1-carboxylate (34). Compound **34** was prepared from **31** by following the general procedure B to give the corresponding amine in 62% yield, as a yellow oil.

4.1.7.21. 1-Methyl-5-amino-1*H*-benzo[*d*]imidazole (35) and 1-methyl-6-amino-1*H*-benzo[*d*]imidazole (36). Prepared from the mixture of **32** and **33** by following the general procedure B to give amines **35** (45% yield) and **36** (40% yield) as brown solids.

4.1.7.22. 4-Methyl-1-trityl-1*H*-imidazole (37). 4(5)-Methylimidazole (5 g, 60.94 mmol) was dissolved in 20 mL of *N*,*N*-dimethylformamide, and triethylamine (17 mL, 121.88 mmol) and trityl chloride (18.69 g, 67 mmol) were added. The mixture was stirred for 2 h. The precipitate was filtered off and then washed with ice-cooled *N*,*N*-dimethylformamide (3×50 mL) and water (3×50 mL). After the solvent was removed, the remaining product was dissolved in dichloromethane and then was washed with water. The organic layer was dried over $MgSO_4$ and was concentrated by rotary evaporation. Compound **37** as a white solid (18 g, 92%) was used without further purification.

4.1.7.23. 2-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)isoindoline-1,3-dione (39). Compound **37** (3 g, 9.26 mmol) was suspended in 30 mL acetonitrile and 2-(3-bromopropyl)phthalimide (1.48 g, 9.26 mmol) was added. The mixture was kept under reflux overnight and concentrated to give **38**. Crude mixture **38** was dissolved in a stirred solution containing methanol (20 mL) and trifluoroacetic acid (3 mL). The mixture was kept under reflux overnight. The solvent was removed, and the remaining oil was purified by flash chromatography using silica gel and a MC/MeOH gradient. Yield: 1.52 g (61%).

4.1.7.24. 3-(5-Methyl-1*H*-imidazol-1-yl)propan-1-amine (40). Compound **39** (300 mg, 1.11 mmol) was dissolved in ethanol (2 mL) and hydrazine monohydrate (0.27 ml, 5.55 mmol) was added dropwise. The mixture was stirred at room temperature for 2 h. The formed precipitate was filtered off and washed with EtOH. The filtrate was collected and concentrated by rotary evaporation. The yellow oil product 3-(5-methyl-1*H*-imidazol-1-yl) propan-1-amine **40** (144 mg, 93%) was carried on to the next step

without further purification. ^{1}H NMR (300 MHz, CD_3OD): δ 7.56 (d, J = 0.9 Hz, 1H), 6.67 (s, 1H), 4.01 (t, J = 7.14 Hz, 2H), 2.65 (t, J = 7.14 Hz, 2H), 2.22 (d, J = 0.93 Hz, 3H), 1.92 (quintet, J = 7.32 Hz, 2H). MS (FAB) m/z: 140 $[M+H]^+$.

4.1.7.25. 1-(3-Isothiocyanatopropyl)-5-methyl-1*H*-imidazole (41). Di-2-pyridylthionocarbonate (DPT) (834 mg, 3.59 mmol) in anhydrous dichloromethane was added to a solution of compound **40** (500 mg, 3.59 mmol) in anhydrous dichloromethane. The mixture was stirred at room temperature for 2 h until the starting material was consumed. The reaction mixture was evaporated and purified by column chromatography ($MeOH/CH_2Cl_2$) to yield the product as an orange oil (418 mg, 64%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.43 (d, J = 0.93 Hz, 1H), 6.8 (s, 1H), 4.04 (t, J = 6.78 Hz, 2H), 3.55 (t, J = 6.21 Hz, 2H), 2.23 (d, J = 0.93 Hz, 3H), 2.13 (quintet, J = 6.21 Hz, 2H). MS (FAB) m/z: 182 $[M+H]^+$.

4.1.7.26. 1-(Benzo[*d*][1,3]dioxol-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (42). Prepared from commercially available 3,4-(methylenedioxy)aniline by following the general thiourea coupling procedure: 50% yield, white solid, mp = 92–93 °C; ^{1}H NMR (500 MHz, CD_3OD): δ 7.59 (s, 1H), 6.81(d, J = 8.2 Hz, 1H), 6.78 (d, J = 1.8 Hz, 2H), 6.67–6.65 (m, 2H), 5.96 (s, 2H), 4.54 (br, NH), 3.98 (t, J = 4.1 Hz, 2H), 3.60 (t, J = 6.8 Hz, 2H), 2.21 (s, 3H), 2.06 (quintet, J = 7.1 Hz, 2H). MS (FAB) m/z: 319 $[M+H]^+$.

4.1.7.27. 1-(2,3-Dihydro-1*H*-inden-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (43). Prepared from commercially available 5-aminoindan by following the general thiourea coupling procedure: 65% yield, white solid, mp = 94–95 °C; ^{1}H NMR (500 MHz, CD_3OD): δ 7.59 (s, 1H), 7.21 (d, J = 7.9 Hz, 1H), 7.09 (s, 1H), 6.97 (d, J = 7.85 Hz, 1H), 6.66 (s, 1H), 3.98 (t, J = 7.25 Hz, 2H), 3.61 (t, J = 6.8 Hz, 2H), 2.91–2.86 (m, 4H), 2.21 (s, 3H), 2.11–2.00 (m, 4H). MS (FAB) m/z: 315 $[M+H]^+$.

4.1.7.28. 1-(2,3-Dihydrobenzofuran-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (44). Prepared from amine **7** by following the general thiourea coupling procedure: 81% yield, white solid, mp = 151–152 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.59 (d, J = 1.08 Hz , 1H), 7.07 (s, 1H), 6.93 (dd, J_1 = 2.37 Hz, J_2 = 8.4 Hz, 1H), 6.74 (d, J = 8.43 Hz, 1H), 6.66 (s, 1H), 4.58 (t, J = 8.79 Hz, 2H), 3.96 (t, J = 7.32 Hz, 2H), 3.58 (t, J = 6.96 Hz, 2H), 3.20 (t, J = 8.61 Hz, 2H), 2.22 (d, J = 1.08 Hz, 3H), 2.07 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 317 $[M+H]^+$.

4.1.7.29. 1-(2,3-Dihydrobenzofuran-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (45). Prepared from amine **17** by following the general thiourea coupling procedure: 72% yield, white solid, mp = 60–61 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.59 (d, J = 1.08 Hz, 1H), 7.20 (d, J = 8.25 Hz, 1H), 6.69–6.66 (m, 3H), 4.59 (t, J = 8.61 Hz, 2H), 3.99 (t, J = 7.14 Hz, 2H), 3.20 (t, J = 6.78 Hz, 2H), 2.79 (t, J = 8.58 Hz, 2H), 2.22 (d, J = 0.93 Hz, 3H), 2.06 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 317 $[M+H]^+$.

4.1.7.30. 1-(Chroman-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (46). Prepared from amine **8** by following the general thiourea coupling procedure: 62% yield, white solid, mp = 171–172 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.59 (s, 1H), 6.91–6.88 (m, 2H), 6.75–6.72 (m, 2H), 6.66 (s, 1H), 4.17 (t, J = 5.31 Hz, 2H), 3.96 (t, J = 7.14 Hz, 2H), 3.61 (t, J = 6.78 Hz, 2H), 2.79 (t, J = 6.39 Hz, 2H), 2.22 (d, J = 0.9 Hz, 3H), 2.07–1.93 (m, 4H). MS (FAB) m/z: 331 $[M+H]^+$.

4.1.7.31. 1-(Chroman-7-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (47). Prepared from amine **18** by following the general thiourea coupling procedure: 30% yield, white solid, mp = 90–91 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.56 (s, 1H), 7.37 (s, 1H), 7.26 (d, J = 2.67 Hz, 1H,), 6.73 (s, 1H), 6.63 (s, 1H), 6.63 (d, J = 7.71 Hz, 2H), 6.60 (s, 1H), 4.21 (t, J = 5.49 Hz, 2H), 3.92 (t, J = 7.32 Hz, 2H), 3.69 (q, J = 6.96 Hz, 2H), 2.80 (t, J = 6.42 Hz, 2H), 2.18 (s, 3H), 2.11–2.00 (m, 4H). MS (ESI) m/z: 331 $[M+H]^+$.

4.1.7.32. 1-(3,4-Dihydro-2*H*-benzo[*b*][1,4]oxazin-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (48) and 1-(3,4-dihydro-2*H*-benzo[*b*][1,4]oxazin-7-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (49). Prepared from **25** or **26** by following the general thiourea coupling procedure: 40% and 65% yield, respectively, white solid.

4.1.7.33. 1-(3,4-Dihydro-2*H*-benzo[*b*][1,4]oxazin-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (50). Prepared from **48** by following the general Boc-deprotection (procedure D), white solid, 40% yield, mp = 230–231 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.50 (s, 1H), 6.60–6.56 (m, 2H), 6.37 (d, J = 2.19 Hz, 1H), 6.29 (dd, J_1 = 2.37 Hz, J_2 = 8.4 Hz, 1H), 4.08 (t, J = 4.38 Hz, 2H), 3.88 (t, J = 7.14 Hz, 2H), 3.51 (t, J = 6.78 Hz, 2H), 3.25–3.24 (m, 2H), 2.12 (t, J = 1.08 Hz, 3H), 1.94 (quintet, J = 7.14 Hz, 2H) . MS (FAB) m/z: 330 $[M-H]^+$.

4.1.7.34. 1-(3,4-Dihydro-2*H*-benzo[*b*][1,4]oxazin-7-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (51). Prepared from **49** by following the general Boc-deprotection (procedure D), white solid, 49% yield, mp = 94–95 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.50 (s, 1H), 7.40 (s, 1H), 6.74 (s, 1H), 6.62–6.58 (m, 3H), 5.97 (s, 1H), 4.27 (t, J = 4.59 Hz, 2H), 3.90 (t, J = 7.32 Hz, 2H), 3.68 (q, J = 6.60 Hz, 2H), 3.46 (t, J = 4.23 Hz, 2H), 2.18 (s, 1H), 2.09 (quintet, J = 6.75 Hz, 2H), 1.86 (s, 1H). MS (ESI) m/z: 332 $[M+H]^+$.

4.1.7.35. 1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (52). Prepared from commercially available 6-amino-1,4-benzodioxane by following the general thiourea coupling procedure: 50% yield, white solid, mp = 73–74 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63 (s, 1H), 7.37 (s, 1H), 6.91 (d, J = 8.4 Hz, 1H), 6.72 (d, J = 8.4 Hz, 2H), 6.67 (dd, J_1 = 2.37 Hz, J_2 = 8.4 Hz, 1H), 6.00 (br, 1H), 4.27 (s, 4H), 3.91 (t, J = 7.14 Hz, 2H), 3.68 (q, J = 6.24 Hz, 2H), 2,18 (d, J = 0.93 Hz, 3H), 2.10 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 333 $[M+H]^+$.

4.1.7.36. 1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (53). Prepared from commercially available 5-amino-1,4-benzodioxane by following the general thiourea coupling procedure: 25% yield, white solid, mp = 94–95 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.48 (s, 1H), 6.60–6.56 (m, 2H), 6.37 (d, J = 2.19 Hz, 1H), 6.29 (dd, J_1 =2.37 Hz, J_2 = 8.4, 1H), 4.08 (t, J = 4.41 Hz, 2H), 3.85 (t, J = 7.14 Hz, 2H), 3.48 (t, J = 6.18 Hz, 2H), 3.25–3.24 (m, 2H), 2.25 (d, J = 1.08 Hz, 3H), 1.92 (t, J = 7.14 Hz, 2H). MS (FAB) m/z: 334 $[M+H]^+$.

4.1.7.37. 1-(3-(5-Methyl-1H-imidazol-1-yl)propyl)-3-(5-oxo-5,6,7,8-tetrahydronaphthalen-2-yl)thiourea (54). Prepared from commercially available 6-amino-1-tetralone by following the general thiourea coupling procedure: 40% yield, white solid, mp = 67–68 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.93 (d, J = 8.61 Hz, 1H), 7.63 (s, 1H), 7.47 (s, 1H), 7.35 (dd, J_1 = 2.01 Hz, J_2 = 8.43, 1H), 6.69 (s, 1H), 4.05 (t, J = 7.14 Hz, 2H), 3.65 (t, J = 6.96 Hz, 2H), 2.98 (t, J = 6.03 Hz, 2H), 2.63 (t, J = 5.85 Hz, 2H), 2.24 (d, J = 0.93 Hz, 3H), 2.15–2.04 (m, 4H). MS (ESI) m/z: 344 $[M+H]^+$.

4.1.7.38. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(8-oxo-5,6,7,8-tetrahydronaphthalen-2-yl)thiourea (55). Prepared from commercially available 7-amino-1-tetralone by following

the general thiourea coupling procedure: 48% yield, white solid, mp = 165–166 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.86 (d, J = 2.37 Hz, 1H), 7.61 (d, J = 1.29 Hz, 1H), 7.54 (dd, J_1 = 2.37 Hz, J_2 = 8.04 Hz, 1H), 7.34 (d, J = 8.22 Hz, 1H), 6.67 (s, 1H), 4.03 (t, J = 7.14 Hz, 2H), 3.63 (t, J = 6.6 Hz, 2H), 2.99 (d, J = 5.88 Hz, 2H), 2.66 (d, J = 6.03 Hz, 2H), 2.24 (d, J = 1.11 Hz, 3H), 2.16–2.01 (m, 4H). MS (ESI) m/z: 343 $[M+H]^+$.

4.1.7.39. 1-(5-Hydroxy-5,6,7,8-tetrahydronaphthalen-2-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (56). $NaBH_4$ (16.6 mg, 3 equiv) was added slowly to a solution of ketone compound **54** in MeOH (5 mL). The reaction mixture was stirred for 3 h at room temperature. The reaction was quenched with saturated NH_4Cl, and the mixture was extracted with dichloromethane, washed with water (2 × 30 mL), dried over $MgSO_4$, and purified by column chromatography (MeOH/MC = 1:9) to give **56** (21 mg, 45%) as a white solid, mp = 90–91 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.62 (s, 1H), 7.42 (d, J = 8.43 Hz, 1H), 7.08 (dd, J_1 = 2.19 Hz, J_2 = 8.25 Hz, 1H), 7.00 (s, 1H), 6.68 (s, 1H), 4.69 (br, 1H), 4.01 (t, J = 7.14 Hz, 2H), 3.63 (t, J = 7.14 Hz, 2H), 2.78–2.73 (m, 2H), 2.23 (d, J = 0.99 Hz, 3H), 2.09–1.96 (m, 5H), 1.86–1.81 (m, 2H). MS (ESI) m/z: 345 $[M+H]^+$.

4.1.7.40. 1-(8-Hydroxy-5,6,7,8-tetrahydronaphthalen-2-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (57). Prepared from ketone compound **55** by following the method described for the synthesis of compound **56**, 23.7 mg, 47% yield, white solid, mp = 95–96 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.60 (s, 1H), 7.28 (d, J = 2.0 Hz, 1H), 7.13 (d, J = 8.25 Hz, 1H), 7.07 (dd, J_1 = 8.22 Hz, J_2 = 2.19 Hz, 1H), 6.67 (s, 1H), 4.71 (m, 1H), 4.01 (t, J = 7.14 Hz, 2H), 3.62–3.61 (m, 2H), 2.78–2.73 (m, 2H), 2.23 (d, J = 0.93 Hz, 3H), 2.09–2.02 (m, 4H), 1.85–1.83 (m, 2H). MS (ESI) m/z: 345 $[M+H]^+$.

4.1.7.41. 1-(1*H*-Indol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (58). Prepared from commercially available 6-aminoindole by following the general thiourea coupling procedure: 48% yield, white solid, mp = 177–178 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.59–7.56 (m, 2H), 7.28 (s, 1H), 7.27 (d, J = 3.12 Hz, 1H), 6.87 (dd, J_1 = 2.01 Hz, J_2 = 8.4 Hz. 1H), 6.64 (s, 1H), 6.46 (d, J = 3.3 Hz, 2H), 3.97 (t, J = 7.5 Hz, 2H), 3.62 (t, J = 6.96 Hz, 2H), 2.20 (d, J = 0.9 Hz, 3H), 2.06 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 314 $[M+H]^+$.

4.1.7.42. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(1-methyl-1*H*-indol-6-yl)thiourea (59). Prepared from commercially available 1-methyl-1*H*-indol-6-amine by following the general thiourea coupling procedure: 40% yield, white solid, mp = 159–160 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.58 (d, J = 8.61 Hz, 2H), 7.29 (s, 1H), 7.20 (d, J = 3.12 Hz, 1H), 6.89 (dd, J_1 = 1.83 Hz, J_2 = 8.25 Hz, 1H), 6.64 (s, 1H), 6.44 (d, J = 1.17 Hz, 1H), 3.98 (t, J = 7.32 Hz, 2H), 3.78 (s, 3H), 3.63 (t, J = 6.96 Hz, 2H), 2.20 (d, J = 0.93 Hz, 3H), 2.04 (p, J = 7.14 Hz, 2H). MS (FAB) m/z: 328 $[M+H]^+$.

4.1.7.43. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(1-methyl-1*H*-indazol-6-yl)thiourea (60). Prepared from amine **29** by following the general thiourea coupling procedure: 73% yield, white solid, mp = 175–176 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.96 (s, 1H), 7.74 (d, J = 8.61 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.03 (d, J = 8.43 Hz, 2H), 6.67 (s, 1H), 4.03 (s, 3H), 4.00 (t, J = 7.32 Hz, 2H), 3.66 (t, J = 6.96 Hz, 2H), 2.23 (d, J = 0.9 Hz, 3H), 2.09 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 329 $[M+H]^+$.

4.1.7.44. *tert*-Butyl 6-(3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thioureido)-1*H*-indazole-1-carboxylate (61). Compound **30** was treated with isothiocyanate compound **41** by following the general thiourea coupling procedure to give **61** as a white solid, 59% yield.

4.1.7.45. 1-(1*H*-Indazol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (62). Prepared from compound **61** by following the general procedure D. The crude residue was purified by PLC to give **62** as a white solid (38% yield), mp = 240–241 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.01 (d, J = 0.9 Hz, 1H), 7.76 (d, J = 8.58 Hz, 1H), 7.62–7.60 (m, 2H), 7.02 (dd, J_1 = 1.65 Hz, J_2 = 8.61 Hz, 1H), 6.66 (s, 1H), 4.02 (t, J = 7.14 Hz, 2H), 3.64 (t, J = 6.96 Hz, 2H), 2.23 (d, J = 0.9 Hz, 3H), 2.11 (quintet, J = 6.96 Hz, 2H). MS (ESI) m/z: 315 $[M+H]^+$.

4.1.7.46. *tert*-Butyl 5-(3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thioureido)-1*H*-benzo[*d*]imidazole-1-carboxylate or *tert*-butyl 6-(3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thioureido)-1*H*-benzo[*d*]imidazole-1-carboxylate (63). Compound **34** was treated with isothiocyanate compound **41** by following the general thiourea coupling procedure to give **63**: 51% yield, yellow solid.

4.1.7.47. 1-(1*H*-Benzo[*d*]imidazol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea or 1-(1H-benzo[*d*]imidazol-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (64). Prepared from compound **63** by following the general procedure D. white solid (15% yield), mp = 190–191 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.1 (s, 1H), 7.58–7.57 (m, 3H), 7.16 (d, J = 8.61 Hz, 1H), 6.65 (s, 1H), 3.97 (t, J = 7.14 Hz, 2H), 3.60 (t, J = 6.96 Hz, 2H), 2.22 (d, J = 1.08 Hz, 3H), 2.06 (quintet, J = 6.96 Hz, 2H). MS (ESI) m/z: 316 $[M+H]^+$.

4.1.7.48. 1-(1-Methyl-1*H*-benzo[*d*]imidazol-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl) thiourea (65). Prepared from amine **35** by following the general thiourea coupling procedure: 50% yield, white solid, mp = 200–201 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.15 (s, 1H), 7.58–7.56 (m, 3H), 7.24 (dd, J_1 = 2.01 Hz, J_2 = 8.43 Hz, 1H), 6.65 (s, 1H), 4.00 (t, J = 7.32 Hz, 2H), 3.95 (s, 3H), 3.63 (t, J = 6.75 Hz, 2H), 2.22 (d, J = 0.93 Hz, 3H), 2.09 (quintet, J = 6.96 Hz, 2H). MS (ESI) m/z: 329 $[M+H]^+$.

4.1.7.49. 1-(1-Methyl-1*H*-benzo[*d*]imidazol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl) thiourea (66). Prepared from amine **36** by following the general thiourea coupling procedure: 50% yield, white solid, mp = 181–182 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.14 (s, 1H), 7.67 (d, J = 8.61 Hz, 1H), 7.60 (d, J = 1.11 Hz, 1H), 7.56 (d, J = 1.44 Hz, 1H), 7.14 (dd, J_1 = 1.83 Hz, J_2 = 8.43 Hz, 1H), 6.66 (s, 1H), 4.01 (t, J = 7.14 Hz, 2H), 3.88 (s, 3H), 3.64 (t, J = 6.96 Hz, 2H), 2.22 (d, J = 1.08 Hz, 3H), 2.09 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 329 $[M+H]^+$.

4.1.7.50. 1-(Benzo[*d*]oxazol-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (67). Prepared from commercially available 5-aminobenzoxazole by following the general thiourea coupling procedure: 27% yield, pink solid, mp = 74–75 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.49 (s, 1H), 7.74 (d, J = 2.01 Hz, 1H), 7.68 (d, J = 8.61 Hz, 1H), 7.60 (s, 1H), 7.36 (dd, J_1 = 2.19 Hz, J_2 = 8.79 Hz, 1H), 6.67 (s, 1H), 4.02 (t, J = 7.14 Hz, 2H), 3.64 (t, J = 6.96 Hz, 2H), 2.23 (d, J = 0.93 Hz, 3H), 2.11 (quintet, J = 6.96 Hz, 2H). MS (ESI) m/z: 316 $[M+H]^+$.

4.1.7.51. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(2-methylbenzo[*d*]oxazol-5-yl)thiourea (68). Prepared from commercially available 2-methylbenzo[*d*]oxazol-5-amine by following the general thiourea coupling procedure: 57% yield, white solid, mp = 58–59 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.59 (s, 1H), 7.58 (d, J = 2.04 Hz, 1H), 7.56 (d, J = 8.58 Hz, 1H), 7.26 (dd,

J_1 = 8.61 Hz, J_2 = 2.04 Hz, 1H), 6.67 (s, 1H), 4.01 (t, J = 6.96 Hz, 2H), 3.63 (t, J = 6.78 Hz, 2H), 2.63 (s, 3H), 2.23 (d, J = 0.9 Hz, 3H), 2.11 (quintet, J = 7.14 Hz, 2H). MS (ESI) m/z: 331 $[M+H]^+$.

4.1.7.52. 1-(Benzo[*d*]oxazol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (69). Prepared from commercially available 6-aminobenzoxazole by following the general thiourea coupling procedure: 40% yield, pink solid, mp = 59–60 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.46 (s, 1H), 7.83 (d, J = 2.01 Hz, 1H), 7.72 (d, J = 8.4 Hz, 1H), 7.60 (d, J = 1.08 Hz, 1H), 7.30 (dd, J_1 = 2.01 Hz, J_2 = 8.58 Hz, 1H), 6.67 (s, 1H), 4.03 (t, J = 7.14 Hz, 2H), 3.65 (t, J = 7.14 Hz, 2H), 2.25 (d, J = 1.08 Hz, 3H), 2.14 (quintet, J = 7.32 Hz, 2H). MS (ESI) m/z: 316 $[M+H]^+$.

4.1.7.53. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(2-methylbenzo[*d*]oxazol-6-yl)thiourea (70). Prepared from commercially available 2-methylbenzo[*d*]oxazol-6-amine by following the general thiourea coupling procedure: 51% yield, white solid, mp = 75–76 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.68 (d, J = 1.83 Hz, 1H), 7.60 (d, J = 1.08 Hz, 1H), 7.58 (d, J = 8.43 Hz, 1H), 7.22 (dd, J_1 = 1.83 Hz, J_2 = 8.43 Hz, 1H), 6.67 (s, 1H), 4.02 (t, J = 7.32 Hz, 2H), 3.64 (t, J = 7.14 Hz, 2H), 2.62 (s, 3H), 2.23 (d, J = 0.93 Hz, 3H), 2.11 (quintet, J = 6.96 Hz, 2H). MS (ESI) m/z: 331 $[M+H]^+$.

4.1.7.54. 1-(Benzo[*d*]thiazol-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (71). Prepared from commercially available 5-aminobenzothiazole by following the general thiourea coupling procedure: 29% yield, white solid, mp = 78–79 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 9.26 (s, 1H), 9.00 (s, 1H), 8.22 (d, J = 1.83 Hz, 1H), 8.04 (d, J = 8.61 Hz, 1H), 7.50 (dd, J_1 = 1.65 Hz, J_2 = 8.22 Hz, 1H), 7.30 (s, 1H), 4.30 (t, J = 6.96 Hz, 2H), 3.72 (t, J = 6.18 Hz, 2H), 2.38 (d, J = 1.08 Hz, 3H), 2.24–2.20 (m, 2H). MS (ESI) m/z: 333 $[M+H]^+$.

4.1.7.55. 1-(Benzo[*d*]thiazol-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (72). Prepared from commercially available 6-aminobenzothiazole by following the general thiourea coupling procedure: 44% yield, white solid, mp = 72–73 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 9.20 (s, 1H), 8.13 (d, J = 2.19 Hz, 1H), 8.04 (d, J = 8.61 Hz, 1H), 7.60 (s, 1H), 7.46 (dd, J_1 = 2.04 Hz, J_2 = 8.79 Hz, 1H), 6.67 (s, 1H), 4.03 (t, J = 7.14 Hz, 2H), 3.65 (t, J = 6.78 Hz, 2H), 2.23 (d, J = 1.08 Hz, 3H), 2.14 (quintet, J = 7.14 Hz, 2H). MS (ESI) m/z: 333 $[M+H]^+$.

4.1.7.56. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(pyridin-2-yl)thiourea (73). Prepared from commercially available 2-aminopyridine by following the general thiourea coupling procedure: 53% yield, white solid, mp = 146–147 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.23–8.20 (m, 1H), 7.75–7.69 (m, 1H), 7.62 (d, J = 0.93 Hz, 1H), 7.03–6.99 (m, 1H), 6.97 (d, J = 8.43 Hz, 1H), 6.65 (s, 1H), 4.13 (t, J = 7.14 Hz, 2H), 3.76 (t, J = 6.96 Hz, 2H), 2.23 (d, J = 0.93 Hz, 3H), 2.19 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 276 $[M+H]^+$.

4.1.7.57. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(pyridin-3-yl)thiourea (74). Prepared from commercially available 3-aminopyridine by following the general thiourea coupling procedure: 55% yield, white solid, mp = 51–52 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.55 (d, J = 2.55 Hz, 1H), 8.30 (dd, J_1 = 1.29 Hz, J_2 = 4.77 Hz, 1H), 8.00–7.96 (m, 1H), 7.61 (s, 1H), 7.42–7.37 (m, 1H), 6.69 (s, 1H), 4.04 (t, J = 7.14 Hz, 2H), 3.64 (t, J = 6.96 Hz, 2H), 2.24 (d, J = 0.72 Hz, 3H), 2.08 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 276 $[M+H]^+$.

4.1.7.58. 1-(Isoquinolin-3-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (75). Prepared from commercially available 3-aminoisoquinoline by following the general thiourea coupling procedure: 21% yield, white solid, mp = 184–185 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 9.04 (s, 1H), 8.01 (d, J = 8.22 Hz, 1H), 7.79 (d, J = 8.04 Hz, 1H), 7.70 (d, J = 7.71 Hz, 1H), 7.64 (s, 1H), 7.52–7.47 (m, 1H), 7.31 (s, 1H), 6.64 (s, 1H), 4.11 (t, J = 7.14 Hz, 2H), 3.81 (t, J = 6.78 Hz, 2H), 2.25 (s, 3H), 2.23–2.10 (m, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.59. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-3-yl)thiourea (76). Prepared from commercially available 3-aminoquinoline by following the general thiourea coupling procedure: 62% yield, white solid, mp = 85–86 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.88 (d, J = 2.37 Hz, 1H), 8.38 (s, 1H), 7.97 (d, J = 8.4 Hz, 1H), 7.90 (d, J = 8.07 Hz, 1H), 7.74–7.68 (m, 1H), 7.62 (s, 1H), 7.59–7.57 (m, 1H), 6.69 (s, 1H), 4.06 (t, J = 7.14 Hz, 2H), 3.65 (t, J = 7.14 Hz, 2H), 2.25 (d, J = 1.08 Hz, 3H), 2.13 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.60. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-2-yl)thiourea (77). Prepared from commercially available 2-aminoquinoline by following the general thiourea coupling procedure: 64% yield, white solid, mp = 221–222 °C. ^{1}H NMR (500 MHz, CD_3OD): δ 8.19 (d, J = 8.9 Hz, 1H), 7.82 (t, J = 9.25 Hz, 2H), 7.70 (t, J = 7.25 Hz, 1H), 7.62 (s, 1H), 7.47 (t, J = 7.45 Hz, 1H), 7.12 (d, J = 8.85 Hz, 1H), 6.63 (s, 1H), 4.13 (t, J = 7.00 Hz, 2H), 3.84 (t, J = 6.7 Hz, 2H), 2.29 (quintet, J = 6.9 Hz, 2H), 2.21 (s, 3H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.61. 1-(Isoquinolin-1-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (78). Prepared from commercially available 1-aminoisoquinoline by following the general thiourea coupling procedure: 45% yield, white solid, mp = 136–137 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.34 (d, J = 8.61 Hz, 1H), 8.10 (d, J = 5.85 Hz, 1H), 7.91 (d, J = 8.22 Hz, 1H), 7.81 (t, J = 6.96 Hz, 1H), 7.71 (t, J = 7.14 Hz, 1H), 7.63 (s, 1H), 7.46 (d, J = 6.21 Hz, 1H), 6.64 (s, 1H), 4.12 (t, J = 7.14 Hz, 2H), 3.83 (t, J = 6.78 Hz, 2H), 2.25 (d, J = 0.9 Hz, 3H), 2.25–2.21 (m, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.62. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-6-yl)thiourea (79). Prepared from commercially available 6-aminoquinoline by following the general thiourea coupling procedure: 46% yield, white solid, mp = 57–58 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.79 (dd, J_1 = 1.65 Hz, J_2 = 4.38 Hz, 1H), 8.32 (d, J = 7.68 Hz, 1H), 8.01 (s, 1H), 7.97 (d, J = 8.97 Hz, 1H), 7.77 (dd, J_1 = 2.37 Hz, J_2 = 8.97 Hz, 1H), 7.64 (s, 1H), 7.54 (dd, J_1 = 4.38 Hz, J_2 = 8.22 Hz, 1H), 6.69 (s, 1H), 4.06 (t, J = 7.14 Hz, 2H), 3.67 (t, J = 6.96 Hz, 2H), 2.25 (d, J = 0.93 Hz, 3H), 2.10 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.63. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-7-yl)thiourea (80). Prepared from commercially available 7-aminoquinoline by following the general thiourea coupling procedure: 30% yield, white solid, mp = 100–101 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.80 (dd, J_1 = 4.38 Hz, J_2 = 1.65 Hz, 1H), 8.33 (d, J = 7.86 Hz, 1H), 8.13 (s, 1H), 7.92 (d, J = 8.97 Hz, 1H), 7.69–7.63 (m, 2H), 7.49–7.44 (m, 1H), 6.68 (s, 1H), 4.07 (t, J = 7.14 Hz, 2H), 3.68 (t, J = 6.57 Hz, 2H), 2.25 (d, J = 0.9 Hz, 3H), 2.14 (quintet, J = 7.14 Hz, 2H). MS (ESI) m/z: 326 $[M+H]^+$.

4.1.7.64. 1-(Isoquinolin-6-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (81). Prepared from commercially available 6-aminoisoquinoline by following the general thiourea coupling procedure: 68% yield, white solid, mp = 95–96 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 9.10 (s, 1H), 8.35 (d, J = 5.85 Hz, 1H), 8.12 (s, 1H), 8.04 (d, J = 8.79 Hz, 1H), 7.72–7.69 (m, 2H), 7.63 (s, 1H), 6.68 (s, 1H), 4.06 (t, J = 7.14 Hz, 2H), 3.68 (t, J = 6.78 Hz, 2H), 2.24 (s, 3H), 2.17 (quintet, J = 6.96 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.65. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-8-yl)thiourea (82). Prepared from commercially available 8-aminoquinoline by following the general thiourea coupling procedure: 64% yield, white solid, mp = 142–143 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.98 (d, J = 7.53 Hz, 1H), 8.85 (dd, J_1 = 1.83 Hz, J_2 = 4.2 Hz, 1H), 7.63 (d, J = 1.17 Hz, 1H), 7.60 (d, J = 1.26 Hz, 1H), 7.56 (s, 1H), 7.54–7.51 (m, 1H), 6.68 (s, 1H), 4.08 (t, J = 7.14 Hz, 2H), 3.69 (t, J = 6.96 Hz, 2H), 2.25 (d, J = 1.11 Hz, 3H), 2.17 (quintet, J = 7.14 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.66. 1-(Isoquinolin-5-yl)-3-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)thiourea (83). Prepared from commercially available 5-aminoisoquinoline by following the general thiourea coupling procedure: 49% yield, white solid, mp = 117–118 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 9.29 (s, 1H), 8.49 (d, J = 6.06 Hz, 1H), 8.11 (dd, J_1 = 3.12 Hz, J_2 = 6.03 Hz, 1H), 7.85 (d, J = 6.03 Hz, 1H), 7.76 (s, 1H), 7.74–7.70 (m, 1H), 7.57 (s, 1H), 6.65 (s, 1H), 3.97 (t, J = 6.96 Hz, 2H), 3.61 (t, J = 6.96 Hz, 2H), 2.19 (s, 3H), 2.04 (quintet, J = 6.93 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.67. 1-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)-3-(quinolin-5-yl)thiourea (84). Prepared from commercially available 5-aminoquinoline by following the general thiourea coupling procedure: 47% yield, white solid, mp = 187–188 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.89 (dd, J_1 = 1.65 Hz, J_2 = 4.2 Hz, 1H), 8.42 (d, J = 7.89 Hz, 1H), 8.05 (d, J = 8.61 Hz, 1H), 7.81 (t, J = 7.32 Hz, 1H), 7.61–7.50 (m, 3H), 6.65 (s, 1H), 3.95 (t, J = 7.14 Hz, 2H), 3.59 (t, J = 7.14 Hz, 2H), 2.19 (s, 3H), 2.02 (quintet, J = 7.32 Hz, 2H). MS (FAB) m/z: 326 $[M+H]^+$.

4.1.7.68. 1-(3-(5-Methyl-1H-imidazol-1-yl)propyl)-3-(quinoxalin-6-yl)thiourea (85). Prepared from commercially available 6-aminoquinoxaline by following the general thiourea coupling procedure: 34% yield, yellow solid, mp = 74–75 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.80 (d, J = 1.83 Hz, 1H), 8.79 (d, J = 2.01 Hz, 1H), 7.62 (s, 1H), 6.68 (s, 1H), 4.07 (t, J = 7.32 Hz, 2H), 3.68 (t, J = 6.93 Hz, 2H), 2.26 (d, J = 0.93 Hz, 3H), 2.17 (quintet, J = 7.14 Hz, 2H). MS (ESI) m/z: 327 $[M+H]^+$.

4.1.7.69. 2-Amino-*N*-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)benzamide (86). A mixture of isatoic anhydride (0.553 mmol) and amine **40** (0.052 mmol) in THF (3 mL) was stirred overnight at room temperature and concentrated in vacuo. The residue was shaken with dichloromethane and 3 mL of 1 N NaOH, and the layers were separated. The organic layer was washed twice with H_2O, dried over $MgSO_4$, and concentrated. The residue was triturated with ethyl acetate, and the product was recovered by filtration.

4.1.7.70. 3-(3-(5-Methyl-1*H*-imidazol-1-yl)propyl)quinazoline-2,4(1*H*,3*H*)-dione (88). Ethylchloroformate (1.4 mL) was added to benzamide **86** (0.35 mmol) at 0 °C. The reaction mixture was heated at reflux temperature for 3 h to give crude ethyl 2-(3-(5-methyl-1*H*-imidazol-1-yl)propylcarbamoyl)phenylcarbamate **(87)**. The crude **87** was dissolved in EtOH and then concentrated, after which EtOH (4 mL) and KOH (72 mg) were added. The mixture was heated at reflux temperature for 3 h and again concentrated. The residue was diluted with H_2O, HOAc was added to a pH of 6–7, dichloromethane was added, and the layers were separated. The organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The residue was triturated with ethyl acetate, and the product **88** was isolated by column chromatography with a MeOH:MC (1:9) system in 74% yield, mp = 192–193 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 8.04 (dd, J_1 = 1.47 Hz, J_2 = 8.07 Hz, 1H), 7.66–7.60 (m, 2H), 7.25–7.13 (m, 2H), 6.66 (s, 1H), 4.09–4.01 (m, 4H), 2.22 (d, J = 0.9 Hz, 3H), 2.17 (quintet, J = 7.32 Hz, 2H). MS (ESI) m/z: 285 $[M+H]^+$.

4.2. In vitro QC activity assay

QC activity was evaluated by fluorometric analysis using H-Gln-βNA (L-glutaminyl-β-naphthylamine, BACHEM, Switzerland). The samples were prepared in a total volume of 200 μl of assay buffer (20 mM Tris-Cl (pH 8.0), 200 mM potassium chloride) containing the fluogenic substrate (0.05 mM), 0.025U pGAPase (pyroglutaminyl aminopeptidase, QUIAZEN, Germany), 50 μg of HEK293 cell lysate, and variable amounts of the test inhibitory compounds. The assay reaction was initiated by addition of the cell lysate and incubated for 1 h at 37 °C. Excitation/emission wavelengths were 320/415 nm. QC activity was determined from the standard curves of β-naphthlyamine under assay conditions (Sigma). IC_{50} values of compounds were collected from 3 independent experiments.

Acknowledgements

This work was supported by the Global Frontier Project Grant (NRF-M3A6A4–2012M3A6A4054253) of National Research Foundation funded by the Ministry of Education, Science and Technology of Korea and by SNU Hospital Grant 03–2012–0120 for I.M.-J.

References and notes

1. Bockers, T. M.; Kreutz, M. R.; Pohl, T. *J. Neuroendocrinol.* **1995**, *7*, 445.
2. Fischer, W. H.; Spiess, J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 3628.
3. Busby, W. H., Jr.; Quackenbush, G. E.; Humm, J.; Youngblood, W. W.; Kizer, J. S. *J. Biol. Chem.* **1987**, *262*, 8532.
4. Sykes, P. A.; Watson, S. J.; Temple, J. S.; Bateman, R. C., Jr. *FEBS Lett.* **1999**, *455*, 159.
5. Schilling, S.; Manhart, S.; Hoffmann, T.; Ludwig, H.-H.; Wasternack, C.; Demuth, H.-U. *Biol. Chem.* **2003**, *384*, 1583–1592.
6. Schilling, S.; Zeitschel, U.; Hoffmann, T.; Heiser, U.; Francke, M.; Kehlen, A.; Holzer, M.; Hutter-Paier, B.; Prokesch, M.; Windisch, M.; Jagla, W.; Schlenzig, D.; Lindner, C.; Rudolph, T.; Reuter, G.; Cynis, H.; Montag, D.; Demuth, H. U.; Rossner, S. *Nat. Med.* **2008**, *14*, 1106.
7. Ezura, Y.; Kajita, M.; Ishida, R.; Yoshida, S.; Yoshida, H.; Suzuki, T.; Hosoi, T.; Inoue, S.; Shiraki, M.; Orimo, H.; Emi, M. *J. Bone Miner. Res.* **2004**, *19*, 1296.
8. Batliwalla, F. M.; Baechler, E. C.; Xiao, X.; Li, W.; Balasubramanian, S.; Khalili, H.; Damle, A.; Ortmann, W. A.; Perrone, A.; Kantor, A. B.; Gulko, P. S.; Kern, M.; Furie, R.; Behrens, T. W.; Gregersen, P. K. *Genes Immun.* **2005**, *6*, 388.
9. Muthusamy, V.; Duraisamy, S.; Bradbury, C. M.; Hobbs, C.; Curley, D. P.; Nelson, B.; Bosenberg, M. *Cancer Res.* **2006**, *66*, 11187.
10. Cynis, H.; Scheel, E.; Saido, T. C.; Schilling, S.; Demuth, H. U. *Biochemistry* **2008**, *47*, 7405.
11. Russo, C.; Violani, E.; Salis, S.; Venezia, V.; Dolcini, V.; Damonte, G.; Benatti, U.; D'Arrigo, C.; Patrone, E.; Carlo, P.; Schettini, G. *J. Neurochem.* **2002**, *82*, 1480.
12. Harigaya, Y.; Saido, T. C.; Eckman, C. B.; Prada, C. M.; Shoji, M.; Younkin, S. G. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 422.
13. Nussbaum, J. M.; Schilling, S.; Cynis, H.; Silva, A.; Swanson, E.; Wangsanut, T.; Tayler, K.; Wiltgen, B.; Hatami, A.; Ronicke, R.; Reymann, K.; Hutter-Paier, B.; Alexandru, A.; Jagla, W.; Graubner, S.; Glabe, C. G.; Demuth, H. U.; Bloom, G. S. *Nature* **2012**, *485*, 651.
14. Schilling, S.; Appl, T.; Hoffmann, T.; Cynis, H.; Schulz, K.; Jagla, W.; Friedrich, D.; Wermann, M.; Buchholz, M.; Heiser, U.; von Hörsten, S.; Demuth, H. U. *J. Neurochem.* **2008**, *106*, 1225.
15. Buchholz, M.; Hamann, A.; Aust, S.; Brandt, W.; Böhme, L.; Hoffmann, T.; Schilling, S.; Demuth, H.-U.; Heiser, U. *J. Med. Chem.* **2009**, *52*, 7069.
16. Buchholz, M.; Heiser, U.; Schilling, S.; Niestroj, A. J.; Zunkel, K.; Demuth, H.-U. *J. Med. Chem.* **2006**, *49*, 664.
17. Huang, K. F.; Liu, Y. L.; Cheng, W. J.; Ko, T. P.; Wang, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13117.
18. Cynis, H.; Schilling, S.; Bodnar, M.; Hoffmann, T.; Heiser, U.; Saido, T. C.; Demuth, H. U. *Biochim. Biophys. Acta* **2006**, *1764*, 1618.
19. Schilling, S.; Hoffmann, T.; Wermann, M.; Heiser, U.; Wasternack, C.; Demuth, H. U. *Anal. Biochem.* **2002**, *303*, 49.