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Structural optimization of a CXCR2-directed antagonist that indirectly inhibits γ-secretase and reduces Aβ

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ABSTRACT

Amyloid β (Aβ), a key molecule in the pathogenesis of Alzheimer's disease (AD), is derived from the amyloid precursor protein (APP) by sequential proteolysis via β- and γ-secretases. Because of their role in generation of Aβ, these enzymes have emerged as important therapeutic targets for AD. In the case of γ-secretase, progress has been made towards designing potent inhibitors with suitable pharmacological profiles. Direct γ-secretase inhibitors are being evaluated in clinical trials and new strategies are being explored to block γ-secretase activity indirectly as well. In this regard, we have previously reported an indirect regulation of γ-secretase through antagonism of CXCR2, a G-protein coupled receptor (GPCR). We demonstrated that *N*-(2-hydroxy-4-nitrophenyl)-*N*′-(2-bromophenyl)urea (**SB225002**), a selective inhibitor of CXCR2 also plays a role in an indirect inhibition of γ-secretase. Furthermore, we reported a ~5-fold difference in the selective inhibition of APP versus Notch processing via γ-secretase following treatment with **SB225002**. Herein we describe the synthesis and optimization of **SB225002**. By determination of the structure–activity relationship (SAR), we derived small molecules that inhibit Aβ40 production with IC_{50} values in the sub-micromolar range in a cell-based assay and also validated the potential of CXCR2 as a new target for therapeutic intervention in AD.

1. Introduction

Alzheimer's disease (AD) is a progressive dementia believed to be caused by accumulation of Aβ in the brain.[1] Aβ peptides are generated by the sequential action of β- and γ-secretases on the amyloid precursor protein (APP), a type 1 integral membrane protein.[2] In this amyloidogenic cleavage pathway, β-secretase (also referred to as beta-site APP cleaving enzyme, or BACE) generates the NH_2-terminus of Aβ, cleaving APP at the cell-surface to produce a soluble version of APP (sAPPβ) and a 99-residue COOH-terminal fragment (C99) that remains membrane bound.[2] C99 is then cleaved by γ-secretase in the intramembrane region to produce the 4-kD Aβ and a cytoplasmic tail, APP-intracellular domain (AICD).[2] Proteolysis of C99 by γ-secretase is heterogeneous and produces Aβ peptides that vary in length from 37 to 43 amino acids. The most common Aβ species are a 40-residue peptide (Aβ40), and a 42-residue peptide (Aβ42).[3] In an alternative non-amyloidogenic pathway, α-secretase cuts within the Aβ region to produce soluble version of APP (sAPPα) and an 83-residue membrane bound COOH-terminal fragment (C83).[3] C83 subsequently also serves as a substrate for γ-secretase, producing an N-terminally truncated form of Aβ called p3 (a non-amyloidogenic fragment) and AICD.[3]

Because of the evident involvement of Aβ in the pathogenesis of AD, several drug discovery efforts are aimed toward inhibition of Aβ production, prevention of Aβ aggregation, and enhancing Aβ clearance. Preventing Aβ production has focused on targeting the enzymes involved in APP processing.[4,5] Detailed structural characterization of β-secretase[6–9] led to the discovery of many transition-state based inhibitors with activity in the low nanomolar range,[10] though their in vivo efficacy was found to be limited.[10,11] The large active site of β-secretase has made it difficult to develop small molecules with suitable pharmacological profiles.[12] This problem has recently been overcome, and these well-designed inhibitors await further investigation of their potential as drug candidates.[12–14] Despite significant progress towards the biochemical characterization of γ-secretase, its mechanism of action is still not fully understood.[15] Biochemical evidence demonstrates that the full spectrum of γ-secretase activity can be reconstituted by the co-expression of four integral membrane proteins, Presenilin (PS), Nicastrin (NCT), Aph-1, and Pen-2, which are the minimally required constituents of physiologically active γ-secretase.[16–18] This membrane associated γ-secretase complex has resisted purification efforts that would allow crystallization and detailed characterization.[15] Recently, cryoelectron microscopy images provided the first available structure of the γ-secretase complex at 12 Å resolution.[19,20]

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Additionally, several structurally diverse low molecular-weight γ-secretase inhibitors have been reported that have helped to understand the nature and role of γ-secretase with regard to its structure and regulation.[21] Based on the mechanism of inhibition of γ-secretase, these inhibitors have been classified as direct and indirect inhibitors.[21] These inhibitors have enhanced our understanding of the binding pocket of γ-secretase, as well as the biomolecular pathways that interact with and regulate γ-secretase and Aβ production.[22–26] Recently, numerous γ-secretase substrates (>30) have been identified that raise concerns about targeting γ-secretase for therapeutic intervention of AD.[5,27] Although one γ-secretase inhibitor (LY450139) has advanced into late-phase clinical trials,[28,29] the in vivo toxicity observed with use of γ-secretase inhibitors that block Aβ production and apparently proteolysis of the Notch receptor (an important substrate of γ-secretase) is a cause of concern.[5,21,27] Therefore it is desirable to develop γ-secretase inhibitors that have the ability to selectively inhibit Aβ production without hindering Notch proteolysis.[27] In this regard, evidence is emerging that many biochemical pathways can impact γ-secretase activity.[30–34]

Towards finding such pathways that can alter γ-secretase activity, we showed that CXCR2, a GPCR which mediates inflammatory signaling through binding with its ligands, blocks Aβ production through inhibition of γ-secretase.[31] We also demonstrated that **SB225002**, which is a selective antagonist for the CXCR2 receptor,[35] is a potent inhibitor of Aβ production.[31] On exploring the inhibitory mechanism of **SB225002** on Aβ production, we observed and reported a dose-dependent indirect inhibition of γ-secretase activity with no effect on α- and β-secretase activity.[31] Thus, our initial study suggested an important role of CXCR2 as a novel target in regulation of γ-secretase activity. At the time of our report, two other labs independently reported the effect of other GPCRs on the regulation of γ-secretase activity, although the proposed mechanisms of action of these GPCRs differ from one another.[32,33]

Herein we describe the synthesis and structural optimization of **SB225002** with the aims of improving activity and determining the steric and electronic requirements for binding to CXCR2, which then results in indirect inhibition of γ-secretase activity. The present study also elucidates the importance of the phenolic 2-hydroxy moiety necessary for γ-secretase inhibition. The acidic nature of phenolic 2-hydroxy has been suggested as an important structural feature in CXCR2 antagonism and inhibition of its associated downstream pathways.[36,37]

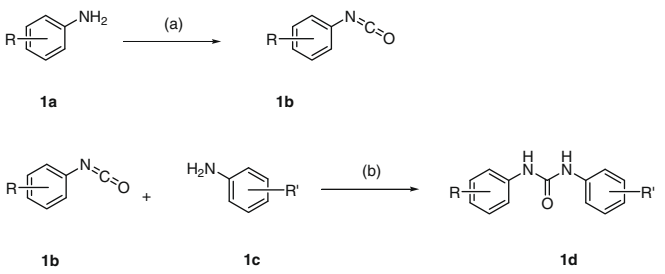
2. Results and discussion

2.1. Chemistry

We divided **SB225002** (**2**) into two domains (A and B) for SAR optimization.

For the synthesis of methoxy substituted *N*,*N*′-diphenyl urea analogues (**11–16**), we methylated the phenolic hydroxyl group of commercially available substituted anilines. Briefly, the hydroxyl substituted aniline was dissolved in acetone with K_2CO_3 (1.2 equiv), and was subsequently alkylated by MeI (5 equiv). The reaction was stirred at 50 °C for 15 h, then quenched with H_2O, and extracted with CH_2Cl_2. The methoxy-containing substituted anilines was dried, concentrated, purified and subsequently coupled with phenylisocyanate as described below to obtain compounds **11–16**.

The final compounds **2–46** were synthesized using a previously described route.[26] Briefly, to access the compounds **2–46**, substituted phenylisocyanate **1b** (Scheme 1), was made by stirring substituted aniline **1a** (1 equiv) in a mixture of CH_2Cl_2 and aqueous saturated $NaHCO_3$, followed by the addition of phosgene solution (20% in toluene; 2 equiv) into the settled CH_2Cl_2 layer of the mixture at 0 °C. After 30 min of vigorous stirring at room temperature, the organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The *N*,*N*′-diarylurea **1d** (**2–46**) was then made by coupling another substituted aniline **1c** (1 equiv) with the phenylisocyanate **1b** in a minimal amount of THF at 50 °C for 12 h (Scheme 1). The final compound **1d** was purified using flash chromatography and crystallization to >95% purity which was determined using HPLC and elemental analysis. Compounds that met these purity criteria were then tested in the cell-based assay described below.



Scheme 1. Reaction conditions (a) DCM, aqueous saturated $NaHCO_3$, phosgene solution (20% in toluene), 0 °C, 30 min; (b) THF, 50 °C, 12 h.

3. Biological evaluation

Recent studies performed by our group identified **SB225002** (**2**) as an inhibitor of Aβ production through the indirect blocking of γ-secretase activity.[31] The cellular and molecular biology data revealed a pivotal role of an inflammatory receptor, CXCR2, in regulation of γ-secretase.[31] **SB225002** (**2**), reported to be a selective inhibitor of the CXCR2 receptor,[35] was also found to indirectly inhibit γ-secretase activity without any effect on α- and β-secretase activities.[31] In this study we focused our effort to (1) improve activity (2) determine the steric and electronic requirement of **SB225002** (**2**) pharmacophore needed for interaction with the CXCR2 binding site required for blocking γ-secretase activity, and (3) elucidate the importance of the phenolic 2-hydroxy moiety of the **SB225002** (**2**) pharmacophore for γ-secretase inhibition. The acidic nature of the phenolic 2-hydroxy of **SB225002** (**2**) has been shown to be an important structural feature in antagonizing CXCR2 and its association with G-proteins.[36–38]

Our previous work demonstrated that the CXCR2 antagonists, **SB225002** (**2**) and Repertaxin (chemically dissimilar from **SB225002** (**2**)), inhibit Aβ40 and 42 production with tight correlation between their IC_{50} values, suggesting a similar γ-secretase is responsible for Aβ40 and 42 generation.[31] Thus in the present study, the SAR of synthesized compounds was derived by evaluating their effect on Aβ40 production in Chinese Hamster Ovary (CHO) cells stably transfected with human APP 751 (7w CHO cell line (7w))[26,39] as described previously. Briefly, stock concentrations of analogues were made in DMSO and added to the media to a final concentration of 1% DMSO; positive controls contained 1% DMSO only. The compounds were incubated with cells for 18 h, media was collected, centrifuged, and protein-normalized supernatant was checked for secreted Aβ40 by ELISA.

The optimized compounds derived through the SAR were further validated for their inhibitory effect on agonist-induced calcium mobilization assay using a previously described procedure[35] with slight modifications. Briefly, 7w cells in a 96-well plate were loaded with Fura-4 NW (no wash required) using manufacturer's instructions. The stock concentrations of analogues (in 1% DMSO, final concentration) were added at the indicated concentrations (negative controls contained 1% DMSO only), followed 15 s later by addition

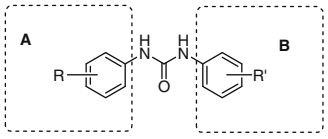
of Groα (33 nM final concentration). The maximal calcium concentration attained after Groα stimulation was quantitated as described previously.[35]

We initially focused on hydrogen and different halogen substitutions at position 2 on the phenyl ring of domain **A**, keeping the same position of $-NO_2$ and –OH groups on the phenyl ring of domain **B** in **SB225002** (**2**). Substituting the 2-Br from **SB225002** (**2**) with –H (**3**), –F (**4**) and –Cl (**5**) revealed that both –Br (**2**) and –Cl (**5**) at position 2 (Table 1) are well-tolerated. However, both H (**3**) and F (**4**) substitutions in position 2 reduced the potency by approximately 20-fold compared to the lead (Table 1).

A significant reduction in activity was noticed with both removal of phenolic –OH (**6**) (Table 1, compare **4** vs **6**) or with exchange of positions between –OH and $-NO_2$ group (**7**) (Table 1, compare **4** vs **7**) from domain **B**, suggesting the requirement for the –OH group at position 2′ of the phenyl ring for optimum activity. Removal of either the 4′-NO_2 group alone (Table 1, compare **8** vs **5** and **9** vs **2**) or both 2′-OH and 4′-NO_2 (**10**) from the phenyl ring of domain B was found to be detrimental for inhibition of Aβ40 production. Methylation of 2′-OH on the phenyl ring of domain **B** to 2′-OMe (Table 1), with the domain **A** phenyl ring substituted at position 2, either with –H or –Br or –Cl or –F resulted in compounds **11**, **12**, **13** and **14**, respectively. Compounds **11**, **12** and **13** lacked activity even at 100 μM (Table 1, compare **11** vs **3**; **12** vs **2**; **13** vs **5**), implicating the 2′-OH group on the phenyl ring of domain **B** as an important chemical feature necessary for blocking γ-secretase through CXCR2. However, compound **14** (Table 1) was found to be more active than analogue **4** (Table 1), indicating the requirement of a H-bonding group at position 2 of one phenyl ring and a bulky group like –Br, –Cl or even –OMe on the second phenyl ring. Together, these data indicate a preference for (1) a phenolic –OH (or at least a substituent that can be involved in H-bonding (both –OH and –F containing molecules are active)) on one ring (preferably at position 2) and (2) a bulky group substitution on the second ring (although **4** contains both –OH and –F, it is less active than **14** that contains a bulky group and –F). Changing the position of –F on the phenyl ring of domain **A** from position 2 to 3 was found to be detrimental for activity (Table 1, compare **14** vs **15**), which is consistent with our earlier results. Also, exchanging the position of $-NO_2$ and –OMe on the phenyl ring of domain **B** while keeping –F at position 2 on the ring of domain **A** (**16**) reduced activity (Table 1, compare **14** vs **16**). This further confirms the requirement of a substituent on one ring that can be either involved in H-bonding or have an acidic proton and a bulky group on the second ring.

After demonstrating the importance of phenolic –OH group in domain **B**, we next focused on optimizing domain **A** keeping domain **B** unchanged. To optimize steric effects and further map the binding domain of CXCR2, we substituted domain **A** with multiple halogens while leaving domain **B** unchanged as in **SB225002** (**2**). We synthesised several di-halosubstituted compounds, and their inhibitory effect on Aβ40 production is illustrated in Table 2. Substitution at positions 2 and 4 of domain **A** with –Br (**17**), –Cl (**18**) and –F (**19**) exhibited inhibitory activity in order of –Cl (**18**) > –Br (**17**) > –F (**19**) (Table 2). Because of the difficulty encountered in synthesis of dibrominated analogues, we chose to synthesize dichloro-substituted analogues instead. On exploring the optimal position of the –Cl groups on the phenyl ring **A**, the order of activity was found to be 2,4-dichloro (**18**) > 2,5-dichloro (**20**) > 3,4-dichloro (**21**) (Table 2). However, the order of activity with difluoro substitution was found to be 3,4-difluoro (**22**) > 2,4-difluoro (**19**) (Table 2). For comparison, we made several tri-substituted derivatives (**23–28**) using commercially available –Cl and –F tri-substituted anilines. However, the syntheses were sluggish and resulted in poor yield of the final compounds. With Cl substitution, the order of activity was found to be in order of 2,3,4-trichloro (**23**) = 2,4,5-trichloro (**24**) (Table 2). Interestingly, for trifluoro-substituted analogues , the order of activity was found to be 3,4,5-trifluoro (**25**) = 2,3,4-trifluoro (**27**) > 2,4,5-trifluoro (**28**) > 2,4,6-trifluoro (**26**) (Table 2). Importantly, trichloro-substituted analogues were found to be less active than di-substituted and even mono-substituted analogues. In contrast, the trifluoro analogues inhibited

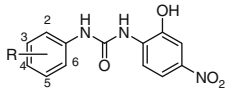
Table 1
Inhibitory effect of compounds on Aβ40 production in 7w cells



Compound no.	Domain A R	Domain B R′	Aβ40/IC_{50}[a] (μM)
SB225002 (**2**)	R^2 = Br	$R^{2'}$ = OH, $R^{4'}$ = NO_2	0.5 ± 0.03
3	$R^2 = R^3 = R^4 = R^5 = R^6$ = H	$R^{2'}$ = OH, $R^{4'}$ = NO_2	12 ± 0.1
4	R^2 = F	$R^{2'}$ = OH, $R^{4'}$ = NO_2	10 ± 0.08
5	R^2 = Cl	$R^{2'}$ = OH, $R^{4'}$ = NO_2	0.6 ± 0.05
6	R^2 = F	$R^{4'}$ = NO_2	30 ± 0.2
7	R^2 = F	$R^{4'}$ = OH, $R^{2'}$ = NO_2	NA
8	R^2 = Cl	$R^{2'}$ = OH	NA
9	R^2 = Br	$R^{2'}$ = OH	NA
10[b]	R^2 = Br		NA
11	$R^2 = R^3 = R^4 = R^5 = R^6$ = H	$R^{2'}$ = OMe, $R^{4'}$ = NO_2	NA
12	R^2 = Br	$R^{2'}$ = OMe, $R^{4'}$ = NO_2	NA
13	R^2 = Cl	$R^{2'}$ = OMe, $R^{4'}$ = NO_2	NA
14[b]	R^2 = F	$R^{2'}$ = OMe, $R^{4'}$ = NO_2	5 ± 0.1
15[b]	R^3 = F	$R^{2'}$ = OMe, $R^{4'}$ = NO_2	12 ± 0.2
16	R^2 = F	$R^{4'}$ = OMe, $R^{2'}$ = NO_2	10 ± 0.1

NA: not active at 100 μM.
[a] Values are the mean of three separate experiments ± SEM.
[b] Purchased from Sigma–Aldrich.

Table 2
Inhibitory effect of compounds on Aβ40 production in 7w cells



Compound no.	R	Aβ40/IC_{50}[a] (μM)
17	$R^2 = R^4$ = Br	0.8 ± 0.08
18	$R^2 = R^4$ = Cl	0.3 ± 0.02
19	$R^2 = R^4$ = F	15 ± 0.1
20[b]	$R^2 = R^5$ = Cl	0.5 ± 0.1
21	$R^3 = R^4$ = Cl	0.7 ± 0.2
22	$R^3 = R^4$ = F	8 ± 0.06
23	$R^2 = R^3 = R^4$ = Cl	3 ± 0.02
24	$R^2 = R^4 = R^5$ = Cl	3 ± 0.01
25	$R^3 = R^4 = R^5$ = F	8 ± 0.1
26	$R^2 = R^4 = R^6$ = F	15 ± 0.1
27	$R^2 = R^3 = R^4$ = F	8 ± 0.1
28	$R^2 = R^4 = R^5$ = F	10 ± 0.3

NA: not active at 100 μM.
[a] Values are the mean of three separate experiments ± SEM.
[b] Purchased from Sigma–Aldrich.

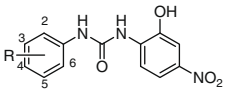
Aβ40 production with almost the same potency as mono and difluoro analogues, suggesting stringent steric and spatial requirements of the CXCR2 interacting site.

To more precisely define the electronic versus steric requirements on ring **A** for inhibition of Aβ production, we made a third series of compounds (Table 3) using a more diverse set of substituents. For this we purchased all commercially available mono-alkyl (methyl to butyl) substituted anilines. The syntheses became more difficult with increase in bulk, and so we restricted the series to no more than a butyl group. Substitution of position 2 on the phenyl ring of domain **A** with methyl, ethyl and *n*-propyl groups (2-*n*-butyl aniline is not commercially available) resulted in compounds (**29**), (**30**) and (**31**), respectively (Table 3). The order of activity was *n*-propyl (**31**) = *n*-ethyl (**30**) > *n*-methyl (**29**) (Table 3), suggesting that an increase in steric bulk at position 2 of the phenyl ring of domain **A** correlates with an improved inhibitory effect on Aβ production. The ability to lower Aβ40 production was significantly reduced by changing the position of similar substituents on the phenyl ring (Table 3, compare **30** vs **33** and **34** and **31** vs **35** (3*n*-propyl aniline is not commercially available)). Also, branching at position 2 of the phenyl ring of domain **A** and groups larger than 3-carbon containing alkyl chains reduced inhibitory activity (Table 3, compare **31** vs **36**; **31** vs **32** and **37** and **32** vs **37**). Next, in order to see if additional bulk at other positions of domain **A** would improve activity, we used dialkyl-substituted anilines to make compounds **38–46** (Table 4). All dialkyl derivatives showed less activity compared to monoalkyl-substituted analogues with only 2,4-dimethyl and 3,5-dimethyl analogues compared to **29**. This is consistent with our earlier findings with dihalosubstituted analogues, and suggests stringent steric requirements at position 2 of the phenyl ring of domain **A**.

Using the SAR, we were able to derive two compounds, **30** and **31**, that reduced Aβ40 production via inhibition of γ-secretase activity beyond starting molecules **2** and **5** (Tables 1 and 3; Fig. 1).

Finally, to determine if compounds **30** and **31** (optimized for Aβ inhibition) were also functional CXCR2 antagonists, we monitored their effects on intracellular calcium mobilization stimulated by GROα. For a positive control, we used **SB225002** (**2**). In these cells, compounds **30**, 31 and **SB225002** (**2**) produced a concentration-dependent inhibition of GROα-mediated calcium mobilization with ~IC_{50} values of 100 nM, 300 nM and 1 μM, respectively (Fig. 2). The order of potency of these compounds towards inhibition of Aβ production and inhibition of GROα-mediated calcium mobilization is the same. Further, we found a tight correlation ((regression) $r^2 = 0.989$) between the IC_{50} for Aβ inhibition with

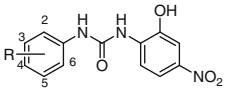
Table 3
Inhibitory effect of compounds on Aβ40 production in 7w cells



Compound no.	R	Aβ40/IC_{50}[a] (μM)
29	R^2 = Me	2 ± 0.03
30	R^2 = Et	0.09 ± 0.05
31	R^2 = *n*-Pr	0.1 ± 0.08
32	R^2 = *sec*-Bu	1 ± 0.01
33	R^3 = Et	4 ± 0.02
34	R^4 = Et	3 ± 0.06
35	R^4 = *n*-Pr	4 ± 0.02
36	R^2 = *i*-Pr	0.3 ± 0.01
37	R^2 = *t*-Bu	15 ± 0.05

NA: not active at 100 μM.
[a] Values are the mean of three separate experiments ± SEM.

Table 4
Inhibitory effect of compounds on Aβ40 production in 7w cells



Compound no.	R	Aβ40/IC_{50}[a] (μM)
38	R^2 = R^4 = Me	3 ± 0.02
39	R^2 = R^5 = Me	10 ± 0.02
40	R^3 = R^4 = Me	15 ± 0.1
41	R^3 = R^5 = Me	2 ± 0.1
42	R^2 = Et, R^6 = Me	7 ± 0.2
43	R^2 = Et, R^6 = *i*-Pr	3 ± 0.06
44	R^2 = *i*Pr, R^6 = Me	5 ± 0.02
45	R^2 = R^6 = *i*-Pr	8 ± 0.01
46	R^2 = *sec*-Bu, R^6 = Et	15 ± 0.1

NA: not active at 100 μM.
[a] Values are the mean of three separate experiments ± SEM.

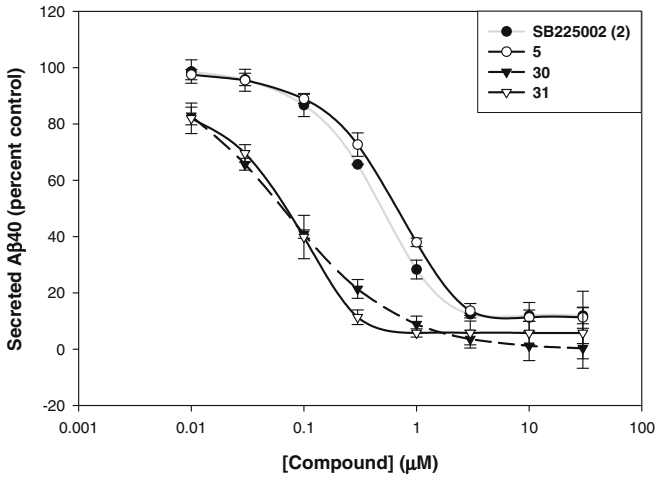


Figure 1. Effects of designed inhibitors **SB225002** (**2**), **5**, **30** and **31** on Aβ40 production. 7w cells stably transfected with human APP751 were treated with various concentrations of the indicated compounds for 18 h, whereupon the media was collected for Aβ sandwich ELISA analysis. Values are means ± s.d. from three separate experiments and are expressed relative to control values.

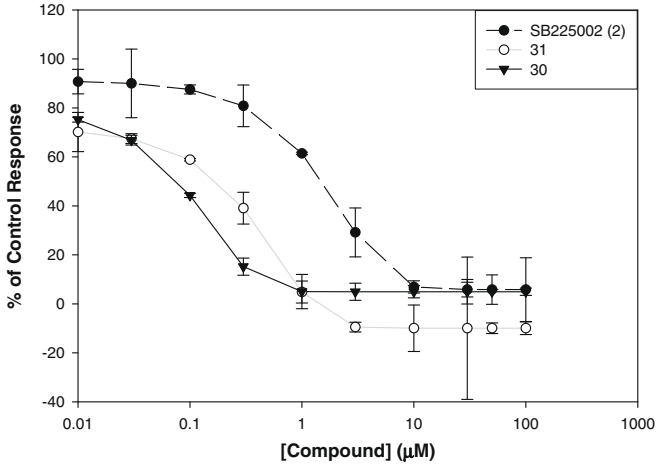


Figure 2. Effects of designed inhibitors **SB225002** (**2**), **30** and **31** on Groα-induced calcium mobilization. 7w cells were pretreated for 15 s with the indicated concentrations of **SB225002** (**2**), **30**, **31** (10–100,000 nM) before the addition of the agonist (Groα). Each data point represents means ± s.d ($n = 8$) and the result is a representative from three separate experiments.

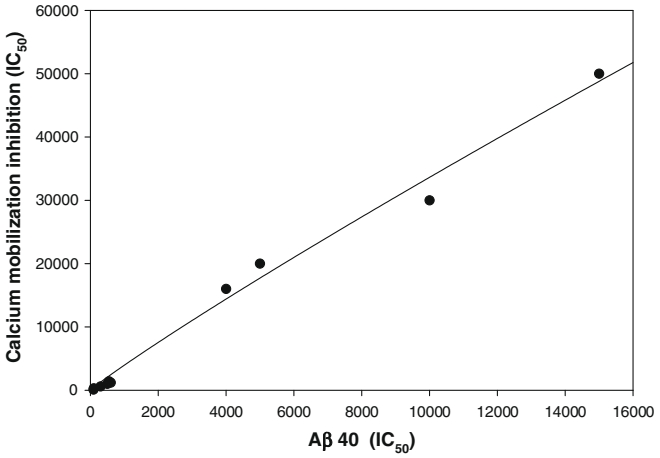


Figure 3. Correlation between the IC_{50} for Aβ inhibition with IC_{50} for inhibition of Groα-induced intracellular calcium mobilization. Nonlinear regression was used to derive the correlation between the IC_{50} values for Aβ inhibition (*x*-axis) with IC_{50} values for Groα-induced intracellular calcium mobilization inhibition (*y*-axis) for compounds **SB225002** (**2**), **4**, **5**, **14**, **20**, **30**, **31** and **36** ($r = 0.9949$; $r^2 = 0.9898$).

IC_{50} for inhibition of Groα-induced intracellular calcium mobilization for compounds **SB225002** (**2**), **4**, **5**, **14**, **20**, **30**, **31** and **36** (Fig. 3). This observation and our previously published studies lend further support to the hypothesis that CXCR2 regulates γ-secretase activity and Aβ production.

4. Conclusions

Alzheimer's disease (AD) is a progressive chronic disorder that leads to cognitive decline.[1] Recent advances in therapeutic intervention in AD through blocking γ-secretase activity have identified direct and indirect inhibitors of γ-secretase.[21,24] Because of the loose substrate specificity of γ-secretase, in vivo studies suggest that inhibition of γ-secretase with compounds that equally prevent processing of APP and apparently Notch can result in toxicity.[5,21,24,27] It has been demonstrated that γ-secretase-mediated final processing of APP can be regulated through different biochemical pathways.[30,32,33,21,24,40,41] For instance, γ-secretase has been shown to be modulated through another GPCR, GPR3, that affects cell-surface localization of the mature γ-secretase and thus impacts γ-secretase specificity towards APP and Notch processing.[33] In this regard, through studying the association of chemokine receptors with altered APP processing and increased production of Aβ, we have demonstrated an important role of CXCR2 in regulation of γ-secretase activity and in inhibition of Aβ40/42 production.[31] Further, we reported a small molecule, **SB225002** (**2**), known to selectively antagonize CXCR2,[35] which blocked Aβ40/42 production in a cell-based assay without affecting α- and β-secretases.[31] **SB225002** (**2**) was found to alter APP processing through indirect regulation of γ-secretase activity via CXCR2 antagonism.[31] Specifically, we found that antagonism of CXCR2 leads to reduction in PS1-CTF (a critical component of γ-secretase, required for its activity) at the protein level that in turn leads to degradation, and thus reduction of other γ-secretase components.[31] This finding is consistent with recent findings that demonstrate that inhibition of basal activity of c-jun-NH_2-terminal kinase (JNK) (JNK, downstream of CXCR2) represses the expression of FLPS[42] and reduces the stabilization of PS1-CTF.[34] Similarly, it is plausible that CXCR2 might mediate stabilization of PS1-CTF (half life ~ 24 h),[43] an endoproteolysed product of FLPS[43] via JNK.

In the present study, we sought to define the topological requirements around **SB225002** (**2**) by iterative modification of substituents around the pharmacophore. This systematic SAR study led to the conclusion that the phenolic –OH, a prerequisite for antagonism of the CXCR2 receptor,[44] is also required to inhibit Aβ production through indirect inhibition of γ-secretase activity. Further, we found that in addition to the acidic proton of the phenolic-OH at position 2 of ring **A**, the ability of the substituents to undergo H-bonding, has a positive effect on the interaction with the CXCR2 binding domain. Thus, effects of systematic chemical manipulation in the parent molecule on Aβ production suggest that there are stringent steric and electronic requirements at position 2. Specifically, we found a preference for (1) a bulky group at position 2 of one of the phenyl rings and (2) a substituent at position 2′ on the other phenyl ring that can be involved in H-bonding or have acidic proton. Using this SAR, we were able to derive two compounds, **30** and **31**, that reduced Aβ40 production via inhibition of γ-secretase activity beyond starting molecules **2** and **5** (Tables 1 and 3; Fig. 1). Finally, we confirmed that compounds **30** and **31**, which were optimized for Aβ production inhibition, are also functional CXCR2 antagonists, as both of them inhibited Groα-induced intracellular calcium mobilization in a dose-dependent manner (Fig. 2). Importantly, as demonstrated in Figure 3, we found a tight correlation ((regression) $r^2 = 0.989$) between the IC_{50} for Aβ and the IC_{50} for Groα-induced intracellular calcium mobilization for compounds **SB225002** (**2**), **4**, **5**, **14**, **20**, **30**, **31** and **36**. In conclusion, we have identified compounds that maximize the inhibition of CXCR2-mediated γ-secretase activity, which in addition to their Aβ-lowering properties are likely to mitigate the CXCR2-mediated inflammation which accompanies and exacerbates the AD.

The data in this manuscript and in recently published work demonstrates that antagonism of CXCR2 with structurally unrelated ligands[31] reduces γ-secretase activity, suggesting an impact on the pathways from the former to the latter. Furthermore, we have also validated CXCR2 as a potential target in gamma-secretase regulation in vivo (Bakshi et al., unpublished result). The differential manipulation of downstream pathways (permissive antagonism) of CXCR2 needs further exploration with these compounds and may lead to inhibition of γ-secretase-mediated APP processing and without inhibition of other substrates.

5. Experimental procedures

5.1. General techniques

All reactions requiring anhydrous conditions were conducted in oven-dried glassware under an atmosphere of nitrogen. Preparative chromatographic separations were performed using a Combiflash Companion (Isco Inc., Lincoln, NE); reactions were followed by TLC analysis using silica plates with a fluorescent indicator (254 nm) and visualized with UV phosphomolybdic acid or 4-hydroxy-3-methoxybenzaldehyde. All commercially available reagents were purchased from Aldrich and Acros and were typically used as supplied unless stated otherwise.

HPLC analyses were carried out on an Agilent 1100 series system using an analytical HPLC column (Eclipse XDB-C18, 5 μm, 4.6 mm i.d.) and a diode array as the detector. Compounds were separated over a period of 40 min gradient of water and acetonitrile (0–80%) at a flow rate of 1 ml/min. ^1H and ^{13}C NMR spectra were recorded in Fourier transform mode at field strengths specified on a Varian AS500 spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm, TMS) as an internal standard for ^1H resonances. Multiplicities in the ^1H NMR spectra are described as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad; coupling constants (*J*) are reported in hertz (Hz). High resolution mass spectra (HRMS) were measured on a LTQ-Orbitrap mass spectrometer (calibrated immediately prior to analysis) utilizing an Advion Bioscience Nanomate electrospray source. Full scan MS spectra

were acquired in Fourier Transform (FTMS) mode at a resolution of 60,000 FWHM in centroid mode. Samples were dissolved in 50% methanol, 0.1% formic acid. Reserpine was added to each sample at 2 ng/μl as an internal lockmass standard ([M+H$^+$] = 609.2812). Ion mass/charge (*m/z*) ratios are reported as values in atomic mass units.

5.2. General method of synthesis

5.2.1. Method A: 1-(2-bromophenyl)-3-(2-hydroxy-4-nitrophenyl)urea SB225002 (2)

2-Bromophenylisocyanate was made by stirring 2-bromoaniline (0.57 mL; 5 mmol) in a mixture of CH_2Cl_2 and aqueous saturated $NaHCO_3$, followed by the addition of phosgene solution (20% in toluene; 5.18 mL, 10 mmol) into the settled CH_2Cl_2 layer of the mixture. After 30 min of vigorous stirring, the organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The 2-hydroxy-4-nitroaniline (154.1 mg, 1 mmol) was then coupled with bromophenylisocyanate in a minimal amount of THF at 50 °C for 12 h. The reaction was concentrated and purified by flash chromatography over silica gel (EtOAc/hexanes 1:1). The solid obtained after evaporation was crystallized from acetone/hexane to >98% purity. Yellow solid (34% yld); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.06 (1H, td, J = 1.3, 7.9 Hz, CH_{Ar}), 7.41 (1H, td, J = 1.3, 7.9 Hz, CH_{Ar}), 7.63 (1H, dd, J = 1.3, 7.9 Hz, CH_{Ar}), 7.77 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.84 (1H, dd, J = 2.5, 9.0 Hz, CH_{Ar}), 8.19 (1H, dd, J = 1.3, 7.9 Hz, CH_{Ar}), 8.47 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.57 (1H, br s, NH), 8.95 (1H, br s, NH), 9.85 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.5 (CH_{Ar}), 114.1 (C_{Ar}), 116.5 (CH_{Ar}), 118.2 (CH_{Ar}), 123.7 (CH_{Ar}), 125.0 (CH_{Ar}), 128.2 (CH_{Ar}), 132.8 (CH_{Ar}), 135.2 (C_{Ar}), 137.2 (C_{Ar}), 142.2 (C_{Ar}), 145.6 (C_{Ar}), 152.4 (CO). HRMS (ES) *m/z* 352 (M+H)$^+$; *m/z* 351.9858 (calcd for $C_{13}H_{11}BrN_3O_4$ (M+H)$^+$: 351.9855).

5.2.2. 1-(2-Hydroxy-4-nitrophenyl)-3-phenylurea (3)

Method A was utilized for synthesis. Yellow solid (49% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.06 (1H, t, J = 7.4 Hz, CH_{Ar}), 7.33 (2H, t, J = 7.4 Hz, CH_{Ar}), 7.59 (2H, d, J = 8.0 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.0 Hz, CH_{Ar}), 7.83 (1H, dd, J = 2, 9.0 Hz, CH_{Ar}), 8.33 (1H, br s, NH), 8.49 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.92 (1H, br s, NH), 9.89 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.3 (C_{Ar}), 116.6 (C_{Ar}), 117.5 (C_{Ar}), 118.8 (C_{Ar}), 118.9 (C_{Ar}), 122.9 (C_{Ar}), 129.0 (C_{Ar}), 135.5 (C_{Ar}), 139.5 (C_{Ar}), 139.7 (C_{Ar}), 141.8 (C_{Ar}), 146.1 (C_{Ar}), 152.2 (CO). HRMS MS (ES) *m/z* 274 (M+H)$^+$; *m/z* 274.0841 (calcd for $C_{13}H_{12}N_3O_4$ (M+H)$^+$: 274.084).

5.2.3. 1-(2-Fluorophenyl)-3-(2-hydroxy-4-nitrophenyl)urea (4)

Method A was utilized for synthesis. Yellow solid (82% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.07–7.10 (1H, m, H_{Ar}), 7.16–7.21 (2H, m, H_{Ar}), 7.77 (1H, d, J = 2.6 Hz, H_{Ar}), 7.84 (1H, d, J = 2.6, 9.0 Hz, H_{Ar}), 8.34 (1H, t, J = 9.0 Hz, H_{Ar}), 8.51 (1H, d, J = 9.0 Hz, H_{Ar}), 8.78 (1H, br s, NH), 8.90 (1H, br s, NH), 9.90 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.3 (C_{Ar}), 115.0 (C_{Ar}), 116.5 (C_{Ar}), 117.8 (C_{Ar}), 121.5 (C_{Ar}), 123.4 (C_{Ar}), 124.7 (C_{Ar}), 127.6 (C_{Ar}), 135.3 (C_{Ar}), 142.0 (C_{Ar}), 145.4 (C_{Ar}), 152.2 (C_{Ar}), 153.8 (CO). HRMS (ES) *m/z* 292 (M+H)$^+$; *m/z* 292.0659 (calcd for $C_{13}H_{11}FN_3O_4$ (M+H)$^+$: 292.0655).

5.2.4. 1-(2-Chlorophenyl)-3-(2-hydroxy-4-nitrophenyl)urea (5)

Method A was utilized for synthesis. Yellow solid (26% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.11 (1H, td, J = 1.4, 7.8 Hz, CH_{Ar}), 7.38 (1H, td, J = 1.4, 8.3 Hz, CH_{Ar}), 7.45 (1H, dd, J = 1.4, 8.0 Hz, CH_{Ar}), 7.77 (1H, d, J = 2.6 Hz, CH_{Ar}), 7.84 (1H, dd, J = 2.6, 9.1 Hz, CH_{Ar}), 8.30 (1H, dd, J = 1.4, 8.3 Hz, CH_{Ar}), 8.49 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.72 (1H, br s, NH), 8.93 (1H, br s, NH), 9.85 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.4 (C_{Ar}), 116.5 (C_{Ar}), 122.5 (C_{Ar}), 122.6 (C_{Ar}), 123.3 (C_{Ar}), 123.9 (C_{Ar}), 124.2 (C_{Ar}), 127.7 (C_{Ar}), 129.5 (C_{Ar}), 135.2 (C_{Ar}), 136.1 (C_{Ar}), 145.5 (C_{Ar}), 152.3 (CO). HRMS (ES) *m/z* 308 (M+H)$^+$, *m/z* 308.0329 (calcd for $C_{13}H_{11}ClN_3O_4$ (M+H)$^+$: 308.036).

5.2.5. 1-(2-Fluorophenyl)-3-(4-nitrophenyl)urea (6)

Method A was utilized for synthesis. Brown solid (22% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.06–7.11 (1H, m, CH_{Ar}), 7.16–7.22 (2H, m, CH_{Ar}), 8.03 (4H, A_2B_2, J_{AB} = 9.2 Hz CH_{Ar}), 8.29 (1H, br t, J = 8.0 Hz, CH_{Ar}), 9.12 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 99.6 (C_{Ar}), 119.7 (C_{Ar}), 122.5 (C_{Ar}), 122.6 (C_{Ar}), 126.0 (C_{Ar}), 128.2 (C_{Ar}), 129.4 (C_{Ar}), 129.8 (C_{Ar}), 132.2 (C_{Ar}), 147.0 (C_{Ar}), 150.9 (C_{Ar}), 156.5 (C_{Ar}), 158.5 (CO). HRMS (ES) *m/z* 276 (M+H)$^+$; *m/z* 276.0707 (calcd for $C_{13}H_{11}FN_3O_3$ (M+H)$^+$: 276.0706).

5.2.6. 1-(2-Fluorophenyl)-3-(4-hydroxy-2-nitrophenyl)urea (7)

Method A was utilized for synthesis. Brown solid (69% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.04–7.09 (1H, m, CH_{Ar}), 7.13–7.19 (2H, m, CH_{Ar}), 7.26 (1H, dd, J = 2.6, 9.1 Hz, CH_{Ar}), 7.57 (1H, dd, J = 2.6 Hz, CH_{Ar}), 8.22 (1H, t, J = 7.9 Hz, CH_{Ar}), 8.30 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.94 (2H, br s, NH), 9.47 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 115.0 (C_{Ar}), 119.7 (C_{Ar}), 126.7 (C_{Ar}), 127.6 (C_{Ar}), 128.1 (C_{Ar}), 129.1 (C_{Ar}), 130.0 (C_{Ar}), 123.2 (C_{Ar}), 132.3 (C_{Ar}), 143.5 (C_{Ar}), 156.9 (C_{Ar}), 157.2 (C_{Ar}), 158.6 (CO). HRMS (ES) *m/z* 292 (M+H)$^+$; *m/z* 292.0658 (calcd for $C_{13}H_{11}FN_3O_4$ (M+H)$^+$: 292.0655).

5.2.7. 1-(2-Chlorophenyl)-3-(2-hydroxyphenyl)urea (8)

Method A was utilized for synthesis. White solid (80% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.76 (t, J = 7.5 Hz, 1H, CH_{Ar}), 6.87–6.81 (m, 2H, CH_{Ar}), 7.03 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.29 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.45 (d, J = 7.5 Hz, 1H, CH_{Ar}), 8.00 (d, J = 8.0 Hz, 1H, CH_{Ar}), 8.10 (d, J = 7.5 Hz, 1H, CH_{Ar}), 8.95 (s, 1H, NH), 8.97 (br s, 1H, NH), 9.89 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 115.3 (CH_{Ar}), 119.8 (CH_{Ar}), 120.2 (CH_{Ar}), 122.9 (CH_{Ar}), 123.0 (CH_{Ar}), 123.2 (CH_{Ar}), 124.0 (CH_{Ar}), 128.1 (CH_{Ar}), 128.2 (C_{Ar}), 130.0 (C_{Ar}), 136.9 (C_{Ar}), 146.9 (C_{Ar}), 153.2 (CO). HRMS (ES) *m/z* 263 (M+H)$^+$; *m/z* 263.0585 (calcd for $C_{13}H_{12}ClN_2O_2$ (M+H)$^+$: 263.0587).

5.2.8. 1-(2-Bromophenyl)-3-(2-hydroxyphenyl)urea (9)

Method A was utilized for synthesis. White solid (62% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.75 (t, J = 7.5 Hz, 1H, CH_{Ar}), 6.87–6.81 (m, 2H, CH_{Ar}), 6.98 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.33 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.61 (d, J = 7.5 Hz, 1H, CH_{Ar}), 7.96 (d, J = 8.5 Hz, 1H, CH_{Ar}), 7.99 (d, J = 8.0 Hz, 1H, CH_{Ar}), 8.80 (s, 1H, NH), 8.95 (s, 1H,NH), 9.89 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ114.5 (CH_{Ar}), 115.3 (CH_{Ar}), 119.8 (CH_{Ar}), 120.2 (CH_{Ar}), 122.9 (CH_{Ar}), 124.2 (CH_{Ar}), 124.9 (CH_{Ar}), 128.2 (CH_{Ar}), 128.6 (C_{Ar}), 133.2 (C_{Ar}), 138.1 (C_{Ar}), 146.9 (C_{Ar}), 153.3 (CO). HRMS (ES) *m/z* 307 (M+H)$^+$; *m/z* 307.0081 (calcd for $C_{13}H_{12}BrN_2O_2$ (M+H)$^+$: 307.0082).

5.2.9. Method B: 1-(2-methoxy-4-nitrophenyl)-3-phenylurea (11)

To a solution of 2-amino-5-nitrophenol (52.4 mg, 0.34 mmol, 1 equiv) in acetone (3 mL) was added K_2CO_3 (97 mg, 0.41 mmol, 1.2 equiv) followed by MeI (0.11 mL, 1.71 mmol, 5 equiv). The reaction was stirred at 50 °C for 15 h, quenched with H_2O and extracted with DCM. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by chromatography over silica gel. The 2-methoxy-4-nitroaniline (168.15 mg, 1 mmol) was then coupled with the 1-phenylisocyanate (synthesized as described in method A) in a minimal amount of THF at 50 °C for 12 h. The reaction was concentrated and purified by chromatography over silica gel. Yellow

solid (46% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 4.05 (3H, s, CH_3), 7.05 (1H, br t, J = 8.3 Hz, CH_{Ar}), 7.32 (2H, td, J = 0.9, 8.3 Hz, CH_{Ar}), 7.57 (2H, dd, J = 0.9, 8.3 Hz, CH_{Ar}), 7.79 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.91 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 8.38 (1H, br s, NH), 8.57 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.86 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 56.3 (CH_3), 105.5 (CH_{Ar}), 116.7 (CH_{Ar}), 117.6 (CH_{Ar}), 118.9 (CH_{Ar}), 122.9 (CH_{Ar}), 129.0 (C_{Ar}), 136.2 (C_{Ar}), 139.6 (C_{Ar}), 147.5 (C_{Ar}), 152.0 (CO). HRMS (ES) m/z 288 $(M+H)^+$; m/z 288.0909 (calcd for $C_{14}H_{14}N_3O_4$ $(M+H)^+$: 288.0906).

5.2.10. 1-(2-Bromophenyl)-3-(2-methoxy-4-nitrophenyl)urea (12)

Method B was utilized for synthesis. Yellow solid (14% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 4.06 (3H, s, CH_3), 7.05 (1H, td, J = 7.9, 1.4 Hz, CH_{Ar}), 7.40 (1H, t, J = 8.2 Hz, CH_{Ar}), 7.63 (1H, d, J = 7.0 Hz, CH_{Ar}), 7.82 (1H, d, J = 2.3 Hz, CH_{Ar}), 7.93 (1H, dd, J = 2.3, 9.1 Hz, CH_{Ar}), 8.19 (1H, d, J = 8.3 Hz, CH_{Ar}), 8.46 (1H, br s, NH), 9.03 (1H, d, J = 8.2 Hz, CH_{Ar}), 9.05 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 56.3 (CH_3), 105.6 (CH_{Ar}), 114.0 (C_{Ar}), 117.4 (CH_{Ar}), 117.4 (CH_{Ar}), 123.6 (CH_{Ar}), 125.0 (CH_{Ar}), 128.2 (CH_{Ar}), 132.8 (CH_{Ar}), 136.0 (C_{Ar}), 137.2 (C_{Ar}), 142.2 (C_{Ar}), 147.9 (C_{Ar}), 152.1 (CO). HRMS (ES) m/z 366 $(M+H)^+$; m/z 366.0031 (calcd for $C_{14}H_{13}BrN_3O_4$ $(M+H)^+$: 366.0011).

5.2.11. 1-(2-Chlorophenyl)-3-(2-methoxy-4-nitrophenyl)urea (13)

Method B was utilized for synthesis. Yellow solid (26% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 4.06 (3H, s, CH_3), 7.10 (1H, td, J = 1.4, 8.0 Hz, CH_{Ar}), 7.35 (1H, td, J = 1.4, 8.0 Hz, CH_{Ar}), 7.44 (1H, dd, J = 1.4, 8.0 Hz, CH_{Ar}), 7.81 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.93 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 8.29 (1H, dd, J = 1.4, 8.0 Hz, CH_{Ar}), 8.58 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.60 (1H, br s, NH), 9.03 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 26.3 (CH_3), 105.6 (CH_{Ar}), 117.3 (CH_{Ar}), 117.4 (CH_{Ar}), 122.5 (CH_{Ar}), 123.2 (C_{Ar}), 124.2 (CH_{Ar}), 127.7 (CH_{Ar}), 129.5 (CH_{Ar}), 136.0 (C_{Ar}), 136.0 (C_{Ar}), 142.2 (C_{Ar}), 147.9 (C_{Ar}), 152.0 (CO). HRMS (ES) m/z 322 $(M+H)^+$; m/z 322.0519 (calcd for $C_{14}H_{13}ClN_3O_4$ $(M+H)^+$: 322.0516).

5.2.12. 1-(2-Fluorophenyl)-3-(4-methoxy-2-nitrophenyl)urea (16)

Method B was utilized for synthesis. Yellow solid (40% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 3.92 (3H, s, CH_{Ar}), 7.05–7.11 (1H, m, CH_{Ar}), 7.15–7.21 (2H, m, CH_{Ar}), 7.36 (1H, dd, J = 3.1, 9.3 Hz, CH_{Ar}), 7.64 (1H, d, J = 3.1 Hz, CH_{Ar}), 8.23 (1H, td, J = 1.5, 8.1 Hz, CH_{Ar}), 8.40 (1H, d, J = 9.3 Hz, CH_{Ar}), 9.00 (1H, br s, NH), 9.56 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 55.7 (CH_3), 108.4 (CH_{Ar}), 115.0 (CH_{Ar}), 115.2 (CH_{Ar}), 122.2 (CH_{Ar}), 122.5 (C_{Ar}), 123.6 (CH_{Ar}), 123.7 (C_{Ar}), 124.6 (C_{Ar}), 124.6 (CH_{Ar}), 125.2 (CH_{Ar}), 129.0 (C_{Ar}), 152.3 (C_{Ar}), 154.8 (CO). HRMS (ES) m/z 306 $(M+H)^+$; m/z 306.0814 (calcd for $C_{14}H_{13}FN_3O_4$ $(M+H)^+$: 306.0812).

5.2.13. 1-(2,4-Dibromophenyl)-3-(2-hydroxy-4-nitrophenyl) urea (17)

Method A was utilized for synthesis. Yellow solid (27% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.58 (1H, dd, J = 2.2, 8.9 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.81 (1H, d, J = 2.2 Hz, CH_{Ar}), 7.84 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 8.19 (1H, d, J = 8.9 Hz, CH_{Ar}), 8.48 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.65 (1H, br s, NH), 8.99 (1H, br s, NH), 9.91 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.4 (CH_{Ar}), 114.4 (C_{Ar}), 115.6 (CH_{Ar}), 116.4 (C_{Ar}), 118.2 (CH_{Ar}), 124.5 (CH_{Ar}), 131.2 (CH_{Ar}), 134.7 (CH_{Ar}), 135.0 (C_{Ar}), 136.9 (C_{Ar}), 142.3 (C_{Ar}), 145.6 (C_{Ar}), 152.2 (CO). HRMS (ES) m/z 431 $(M+H)^+$, m/z 430.9191 (calcd for $C_{13}H_{10}Br_2N_3O_4$ $(M+H)^+$: 430.9186).

5.2.14. 1-(2,4-Dichlorophenyl)-3-(2-hydroxy-4-nitrophenyl) urea (18)

Method A was utilized for synthesis. Yellow solid (35% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.40 (1H, dd, J = 2.4, 8.9 Hz, CH_{Ar}), 7.52 (1H, dd, J = 2.4 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.84 (1H, dd, J = 9, 11.5 Hz, CH_{Ar}), 8.34 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.48 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.80 (1H, br s, NH), 8.94 (1H, br s, NH), 9.94 (1H, br s, OH). HRMS (ES) m/z 342 $(M+H)^+$, m/z 342.0031 (calcd for $C_{13}H_{10}Cl_2N_3O_4$ $(M+H)^+$: 342.0040).

5.2.15. 1-(2,4-Difluorophenyl)-3-(2-hydroxy-4-nitrophenyl) urea (19)

Method A was utilized for synthesis. Yellow solid (43% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.01–7.06 (1H, m, CH_{Ar}), 7.11 (1H, ddd, J = 2.9, 8.7, 11.4 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.6 Hz, CH_{Ar}), 7.83 (1H, dd, J = 2.6, 9.1 Hz, CH_{Ar}), 8.29 (1H, td, J = 6.1, 9.1 Hz, CH_{Ar}), 8.49 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.73 (1H, br s, NH), 8.86 (1H, br s, NH), 9.93 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 103.6 (CH_{Ar}), 109.3 (CH_{Ar}), 111.0 (C_{Ar}), 111.2 (C_{Ar}), 116.5 (CH_{Ar}), 117.7 (CH_{Ar}), 122.8 (C_{Ar}), 122.8 (C_{Ar}), 135.2 (CH_{Ar}), 142.1 (C_{Ar}), 145.3 (CH_{Ar}), 152.2 (C_{Ar}), 159.0 (CO). HRMS (ES) m/z 310 $(M+H)^+$, m/z 310.0565 (calcd for $C_{13}H_{10}F_2N_3O_4$ $(M+H)^+$: 310.0561).

5.2.16. 1-(3,4-Dichlorophenyl)-3-(2-hydroxy-4-nitrophenyl) urea (21)

Method A was utilized for synthesis. Yellow solid (31% yld; 95% purity); ^{1}H NMR (DMSO-d_6, 500 MHz,) δ 7.30 (1H, dd, J = 2.5, 9.0 Hz, CH_{Ar}), 7.56 (1H, d, J = 9.0 Hz, CH_{Ar}), 7.68 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.77 (1H, dd, J = 2.5, 9.0 Hz, CH_{Ar}), 7.91 (1H, d, J = 2.5 Hz, CH_{Ar}), 8.35 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.79 (1H, s, NH), 9.87 (1H, s, NH), 11.20 (1H, br s, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 109.2 (C_{Ar}), 116.6 (C_{Ar}), 117.4 (C_{Ar}), 119.0 (C_{Ar}), 120.0 (C_{Ar}), 124.4 (C_{Ar}), 131.5 (C_{Ar}), 131.9 (C_{Ar}), 135.5 (C_{Ar}), 140.1(C_{Ar}), 141.7 (C_{Ar}), 146.1 (C_{Ar}), 152.4 (CO); HRMS (ES) 342 $(M+H)^+$; m/z 342.1 (calcd for $C_{13}H_{10}Cl_2N_3O_4$ $(M+H)^+$: 341.997).

5.2.17. 1-(3,4-Difluorophenyl)-3-(2-hydroxy-4-nitrophenyl) urea (22)

Method A was utilized for synthesis. Yellow solid (52% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.19–7.21 (1H, m, CH_{Ar}), 7.27 (1H, q, J = 9.4 Hz, CH_{Ar}), 7.75–7.87 (3H, m, CH_{Ar}), 8.33 (1H, br s, CH_{Ar}), 8.47 (1H, d, J = 9.0 Hz, NH), 9.09 (1H, br s, NH), 9.92 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 113.1 (C_{Ar}), 114.4 (CH_{Ar}), 119.9 (CH_{Ar}), 121.8 (CH_{Ar}), 122.7 (CH_{Ar}), 140.3 (CH_{Ar}), 141.9 (C_{Ar}), 147.2 (C_{Ar}), 150.1 (C_{Ar}), 150.4 (CH_{Ar}), 152.1 (C_{Ar}), 154.4 (C_{Ar}), 156.3 (CO). HRMS (ES) m/z 310 $(M+H)^+$; m/z 310.0568 (calcd for $C_{13}H_{10}F_2N_3O_4$ $(M+H)^+$: 310.0561).

5.2.18. 1-(2-Hydroxy-4-nitrophenyl)-3-(2,3,4-trichlorophenyl) urea (23)

Method A was utilized for synthesis. Yellow solid (28% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.56 (1H, d, J = 9.2 Hz, CH_{Ar}), 7.75 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.82 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 8.30 (1H, d, J = 9.2 Hz, CH_{Ar}), 8.47 (1H, d, J = 8.1 Hz, CH_{Ar}), 8.89 (1H, br s, NH), 8.98 (1H, br s, NH), 9.89 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.3 (CH_{Ar}), 116.4 (CH_{Ar}), 118.2 (C_{Ar}), 120.8 (C_{Ar}), 123.0 (CH_{Ar}), 126.8 (C_{Ar}), 128.6 (C_{Ar}), 130.9 (CH_{Ar}), 134.8 (CH_{Ar}), 137.0 (C_{Ar}), 142.3 (C_{Ar}), 145.6 (C_{Ar}), 152.0 (CO). HRMS (ES) m/z 376 $(M+H)^+$; m/z 375.9561 (calcd for $C_{13}H_9Cl_3N_3O_4$ $(M+H)^+$: 375.958).

5.2.19. 1-(2-Hydroxy-4-nitrophenyl)-3-(2,4,5-trichlorophenyl) urea (24)

Method A was utilized for synthesis. Yellow solid (44% yld; 165 mg; 0.44 mmol; >98% purity); ^{1}H NMR (acetone-d_6,

500 MHz) δ 7.68 (1H, s, CH_{Ar}), 7.76 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.84 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 8.50 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.64 (1H, s, CH_{Ar}), 8.89 (1H, br s, NH), 9.00 (1H, br s, NH), 9.92 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.3 (CH_{Ar}), 116.4 (CH_{Ar}), 118.2 (CH_{Ar}), 121.8 (C_{Ar}), 122.4 (CH_{Ar}), 125.5 (C_{Ar}), 130.3 (CH_{Ar}), 131.0 (C_{Ar}), 134.7 (C_{Ar}), 136.3 (C_{Ar}), 142.4 (C_{Ar}), 145.7 (C_{Ar}), 151.9 (CO). HRMS (ES) m/z 376 $(M+H)^+$; m/z 375.954 (calcd for $C_{13}H_9Cl_3N_3O_4$ $(M+H)^+$: 375.958).

5.2.20. 1-(2-Hydroxy-4-nitrophenyl)-3-(3,4,5-trifluorophenyl) urea (25)

Method A was utilized for synthesis. Yellow solid (36% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.42 (2H, m, CH_{Ar}), 7.76 (1H, d, J = 2.6 Hz, CH_{Ar}), 7.85 (1H, dd, J = 2.6, 9.1 Hz, CH_{Ar}), 8.36 (1H, br s, NH), 8.46 (1H, d, J = 9.1 Hz, CH_{Ar}), 9.22 (1H, br s, NH), 10.04 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 104.2 (CH_{Ar}), 109 (CH_{Ar}), 113.5(CH_{Ar}), 118.7 (CH_{Ar}), 132.7 (C_{Ar}), 134.6 (C_{Ar}), 145.3 (C_{Ar}), 146 (C_{Ar}), 147.2 (C_{Ar}), 148.9 (C_{Ar}), 152 (CO). HRMS (ES) m/z 328 $(M+H)^+$; m/z 328.0465 (calcd for $C_{13}H_9F_3N_3O_4$ $(M+H)^+$: 328.0467).

5.2.21. 1-(2-Hydroxy-4-nitrophenyl)-3-(2,4,6-trifluorophenyl) urea (26)

Method A was utilized for synthesis. Yellow solid (21% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.07 (1H, dd, J = 8.9, 7.7 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.79 (1H, dd, J = 2.5, 9.0 Hz, CH_{Ar}), 8.39 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.47 (1H, br s, NH), 8.61 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 100.4 (C_{Ar}), 100.4 (C_{Ar}), 100.6 (CH_{Ar}), 100.8 (C_{Ar}), 100.2 (C_{Ar}), 116.3 (CH_{Ar}), 117.4 (CH_{Ar}), 135.2 (C_{Ar}), 142.1 (C_{Ar}), 145.5 (C_{Ar}), 152.5 (CO). HRMS (ES) m/z 328 $(M+H)^+$, m/z 328.0468 (calcd for $C_{13}H_9F_3N_3O_4$ $(M+H)^+$: 328.0467).

5.2.22. 1-(2-Hydroxy-4-nitrophenyl)-3-(2,3,4-trifluorophenyl) urea (27)

Method A was utilized for synthesis. Yellow solid (41% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.21 (1H, dd, J = 2.4, 10.2, 18.3 Hz, CH_{Ar}), 7.77 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.84 (1H, dd, J = 2.5, 9.0 Hz, CH_{Ar}), 8.06–8.12 (1H, m, CH_{Ar}), 8.49 (1H, d, J = 9.0 Hz, CH_{Ar}), 8.74 (1H, br s, NH), 8.98 (1H, br s, NH), 9.92 (1H, br s, OH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 109.3 (CH_{Ar}), 111.6 (CH_{Ar}), 111.6 (C_{Ar}), 111.8 (CH_{Ar}), 111.8 (C_{Ar}), 115.8 (C_{Ar}), 115.9 (C_{Ar}), 116.5 (CH_{Ar}), 117.8 (CH_{Ar}), 136.0 (C_{Ar}), 142.2 (C_{Ar}), 145.4 (C_{Ar}), 152.1 (CO). HRMS (ES) m/z 328 $(M+H)^+$; m/z 328.0474 (calcd for $C_{13}H_9F_3N_3O_4$ $(M+H)^+$: 328.0467).

5.2.23. 1-(2-Hydroxy-4-nitrophenyl)-3-(2,4,5-trifluorophenyl) urea (28)

Method A was utilized for synthesis. Yellow solid (59% yld; >98% purity); ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.35 (1H, td, J = 7.35, 10.6 Hz, CH_{Ar}), 7.76 (1H, d, J = 2.5 Hz, CH_{Ar}), 7.85 (1H, dd, J = 2.5, 9.1 Hz, CH_{Ar}), 7.36 (1H, m, CH_{Ar}), 8.46 (1H, d, J = 9.1 Hz, CH_{Ar}), 8.79 (1H, br s, NH), 9.05 (1H, br s, NH); ^{13}C NMR (acetone-d_6, 125 MHz) δ 105.0 (CH_{Ar}), 105.2 (CH_{Ar}), 105.2 (C_{Ar}), 105.4 (C_{Ar}), 109.2 (CH_{Ar}), 109.4 (C_{Ar}), 109.4 (C_{Ar}), 116.4 (CH_{Ar}), 117.8 (CH_{Ar}), 134.9 (C_{Ar}), 142.2 (C_{Ar}), 145.4 (C_{Ar}), 152.0 (CO). HRMS (ES) m/z 328 $(M+H)^+$, m/z 328.0463 (calcd for $C_{13}H_9F_3N_3O_4$ $(M+H)^+$: 328.0467).

5.2.24. 1-(2-Hydroxy-4-nitrophenyl)-3-o-tolylurea (29)

Method A was utilized for synthesis. Yellow solid (48% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.26 (s, 3H, CH_3), 6.99 (s, 1H, CH_{Ar}), 7.16 (d, J = 7.0 Hz, 1H, CH_{Ar}), 7.19 (d, J = 7.0 Hz, 1H, CH_{Ar}), 7.67 (s, 1H, CH_{Ar}), 7.75 (d, J = 9.0 Hz, 1H, CH_{Ar}), 7.78 (d, J = 7.5 Hz, 1H, CH_{Ar}), 8.38 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.82 (s, 1H, NH), 9.16 (s, 1H, NH), 11.07 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 18.7 (CH), 109.2 (CH_{Ar}), 116.6 (CH_{Ar}), 117.3 (CH_{Ar}), 122.5 (CH_{Ar}), 124.0 (CH_{Ar}), 126.8 (CH_{Ar}), 129.1 (CH_{Ar}), 130.9 (C_{Ar}), 136.3 (C_{Ar}), 137.5 (C_{Ar}), 141.2 (C_{Ar}), 146.0 (C_{Ar}), 153.0 (CO). HRMS (ES) m/z 288 $(M+H)^+$; m/z 288.0981 (calcd for $C_{14}H_{14}N_3O_4$ $(M+H)^+$: 288.0984).

5.2.25. 1-(2-Hydroxy-4-nitrophenyl)-3-(2-ethylphenyl)urea (30)

Method A was utilized for synthesis. Yellow solid (53% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.26 (t, J = 7.0 Hz, 3H, CH_3), 2.54 (t, J = 7.5 Hz, 2H, CH_2), 6.94 (s, 1H, CH_{Ar}), 7.08 (d, J = 7.0 Hz, 1H, CH_{Ar}), 7.14 (d, J = 7.0 Hz, 1H, CH_{Ar}), 7.62 (s, 1H, CH_{Ar}), 7.75 (d, J = 9.0 Hz, 1H, CH_{Ar}), 7.80 (d, J = 7.5 Hz, 1H, CH_{Ar}), 8.41 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.84 (s, 1H, NH), 9.18 (s, 1H, NH), 11.10 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 15.6 (CH), 21.5 (CH), 109.2 (CH_{Ar}), 116.6 (CH_{Ar}), 117.2 (CH_{Ar}), 123.6 (CH_{Ar}), 124.0 (CH_{Ar}), 126.8 (CH_{Ar}), 129.4 (CH_{Ar}), 130.6 (C_{Ar}), 136.3 (C_{Ar}), 136.9 (C_{Ar}), 141.2 (C_{Ar}), 146.0 (C_{Ar}), 153.0 (CO). HRMS (ES) m/z 301 $(M+H)^+$; m/z 301.1065 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 301.1063).

5.2.26.1-(2-Hydroxy-4-nitrophenyl)-3-(2-propylphenyl)urea (31)

Method A was utilized for synthesis. Yellow solid (36% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.93 (t, J = 7.0 Hz, 3H, CH_3), 1.56 (m, 2H, CH_2), 2.59 (t, J = 7.5 Hz, 2H, CH_2), 7.03 (td, J = 7.5, 1.5 Hz, 1H, CH_{Ar}), 7.14–7.19 (m, 2H, CH_{Ar}), 7.66–7.69 (m, 2H, CH_{Ar}), 7.73 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.36 (d, J = 9.0, 1H, CH_{Ar}), 8.76 (s, 1H, NH), 9.11 (s, 1H, NH), 11.06 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 14.5 (CH), 23.3 (CH), 33.3 (CH), 109.2 (CH_{Ar}), 116.6 (CH_{Ar}), 117.4 (CH_{Ar}), 124.1 (CH_{Ar}) , 124.5 (CH_{Ar}), 126.7 (CH_{Ar}), 130.1 (CH_{Ar}), 134.1 (C_{Ar}), 136.3 (C_{Ar}), 136.7 (C_{Ar}), 141.2 (C_{Ar}), 146.0 (C_{Ar}), 153.3 (CO). HRMS (ES) m/z 316 $(M+H)^+$; m/z 316.1296 (calcd for $C_{16}H_{18}N_3O_4$ $(M+H)^+$: 316.1297).

5.2.27. 1-(2-*sec*-Butylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (32)

Method A was utilized for synthesis. Yellow solid (30% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.78 (t, J = 7.5 Hz, 3H, CH_3), 1.16 (d, J = 7.0 Hz, 3H, CH_3), 1.56 (m, 2H, CH_2), 2.97 (m, 1H, CH), 7.09–7.16 (m, 2H, CH_{Ar}), 7.24 (dd, J = 7.5, 2.0 Hz, 1H, CH_{Ar}), 7.56 (dd, J = 7.5, 2.0 Hz, 1H, CH_{Ar}), 7.66 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.73 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.35 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.78 (s, 1H, NH), 9.05 (s, 1H, NH), 11.06 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 12.6 (CH), 21.9 (CH), 30.4 (CH), 34.2 (CH), 109.1 (CH_{Ar}), 116.6 (CH_{Ar}), 117.2 (CH_{Ar}), 125.3 (CH_{Ar}), 125.4 (CH_{Ar}), 126.3 (CH_{Ar}), 126.5 (CH_{Ar}), 136.0 (C_{Ar}), 136.4 (C_{Ar}), 140.1 (C_{Ar}), 141.1 (C_{Ar}), 145.9 (C_{Ar}), 153.3 (CO). HRMS (ES) m/z 330 $(M+H)^+$; m/z 330.1453 (calcd for $C_{17}H_{20}N_3O_4$ $(M+H)^+$: 330.1454).

5.2.28. 1-(3-Ethylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (33)

Method A was utilized for synthesis. Yellow solid (42% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.18 (t, J = 7.5 Hz, 3H, CH_3), 2.58 (q, J = 7.5 Hz, 2H, CH_2), 6.85 (d, J = 7.5 Hz, 1H, CH_{Ar}), 7.19 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.25 (d, J = 8.5 Hz, 1H, CH_{Ar}), 7.33 (s, 1H, CH_{Ar}), 7.65 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.74 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.36 (d, J = 9.5 Hz, 1H, CH_{Ar}), 8.71 (s, 1H, NH), 9.53 (s, 1H, NH), 11.09 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 16.2 (CH), 28.9 (CH), 109.1 (CH_{Ar}), 116.3 (CH_{Ar}), 116.7 (CH_{Ar}), 117.1 (CH_{Ar}), 118.2 (CH_{Ar}), 122.5 (CH_{Ar}), 129.5 (CH_{Ar}), 136.1 (C_{Ar}), 139.9 (C_{Ar}), 141.2 (C_{Ar}), 145.1(C_{Ar}), 145.8 (C_{Ar}), 152.6 (CO). HRMS (ES) m/z 302 $(M+H)^+$; m/z 302.1138 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1141).

5.2.29. 1-(4-Ethylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (34)

Method A was utilized for synthesis. Yellow solid (53% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.18 (t, J = 8.0 Hz, 3H, CH_3), 2.57 (q, J = 7.5 Hz, 2H, CH_2), 7.15 (d, J = 8.5 Hz, 1H, CH_{Ar}), 7.38(d, J = 8.5 Hz, 1H, CH_{Ar}), 7.67 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.78 (dd, J = 9.0,

2.5 Hz, 1H, CH_{Ar}), 8.37 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.71 (s, 1H, NH), 9.51 (s, 1H, NH), 11.13 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 16.5 (CH), 28.2 (CH), 109.1 (CH_{Ar}), 116.7 (CH_{Ar}), 117.0 (CH_{Ar}), 119.1 (CH_{Ar}), 128.8 (CH_{Ar}), 136.2 (C_{Ar}), 137.6 (C_{Ar}), 138.4 (C_{Ar}), 141.2 (C_{Ar}), 145.9 (C_{Ar}), 152.6 (CO). HRMS (ES) *m/z 302* $(M+H)^+$; *m/z* 302.1138 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1144).

5.2.30.1-(2-Hydroxy-4-nitrophenyl)-3-(4-propylphenyl)urea (35)

Method A was utilized for synthesis. Yellow solid (48% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.90 (t, J = 7.5 Hz, 3H, CH_3), 1.58 (sextet, J = 7.5 Hz, 2H, CH_2), 2.52 (t, J = 7.5 Hz, 2H, CH_2), 7.13 (d, J = 8.0 Hz, 1H, CH_{Ar}), 7.38(d, J = 8.5 Hz, 1H, CH_{Ar}), 7.67 (d, J = 2.0 Hz, 1H, CH_{Ar}), 7.77 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.38 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.72 (s, 1H, NH), 9.51 (s, 1H, NH), 11.11 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 14.3 (CH), 24.9 (CH), 37.3 (CH), 109.1 (CH_{Ar}), 116.8 (CH_{Ar}), 117.1 (CH_{Ar}), 119.0 (CH_{Ar}), 129.4 (CH_{Ar}), 136.2 (C_{Ar}), 136.8 (C_{Ar}), 137.6 (C_{Ar}), 141.2 (C_{Ar}), 145.9 (C_{Ar}), 152.6 (CO). HRMS (ES) *m/z* 316 $(M+H)^+$; *m/z* 316.1294 (calcd for $C_{16}H_{18}N_3O_4$ $(M+H)^+$: 316.1297).

5.2.31. 1-(2-Hydroxy-4-nitrophenyl)-3-(2-isopropylphenyl)urea (36)

Method A was utilized for synthesis. Yellow solid (46% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.19 (d, J = 7.0 Hz, 6H, CH_3), 3.20 (m, 1H, CH), 7.09–7.17 (m, 2H, CH_{Ar}), 7.29 (dd, J = 7.5, 1.5 Hz, 1H, CH_{Ar}), 7.59 (dd, J = 8.0, 1.5 Hz, 1H, CH_{Ar}), 7.66 (d, J = 3.0 Hz, 1H, CH_{Ar}), 7.73 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.36 (d, J = 9.5, 1H, CH_{Ar}), 8.81 (s, 1H, NH), 9.08 (s, 1H, NH), 11.06 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 23.9 (CH), 27.4 (CH), 109.1 (CH_{Ar}), 116.6 (CH_{Ar}), 117.2 (CH_{Ar}), 125.0 (CH_{Ar}), 125.2 (CH_{Ar}), 126.1 (CH_{Ar}), 126.4 (CH_{Ar}), 135.5 (C_{Ar}), 136.4 (C_{Ar}), 141.0 (C_{Ar}), 141.1(C_{Ar}), 145.9 (C_{Ar}), 153.5 (CO). HRMS (ES) *m/z* 316 $(M+H)^+$; *m/z* 316.129 (calcd for $C_{16}H_{18}N_3O_4$ $(M+H)^+$: 316.1297).

5.2.32. 1-(2-*tert*-Butylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (37)

Method A was utilized for synthesis. Yellow solid (25% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.38 (s, 9H, CH_3), 7.22–7.19 (m, 3H, CH_{Ar}), 7.40 (m, 1H, CH_{Ar}), 7.68 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.73 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.35 (d, J = 9.0 Hz, 1H, NH), 11.04 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 31.3 (CH), 35.4 (C), 109.2 (CH_{Ar}), 116.7 (CH_{Ar}), 117.2 (CH_{Ar}), 127.0 (CH_{Ar}), 127.1 (CH_{Ar}), 131.9 (CH_{Ar}), 136.1 (C_{Ar}), 136.7 (C_{Ar}), 141.0 (C_{Ar}), 145.9 (C_{Ar}), 146.2 (C_{Ar}), 154.1 (CO). HRMS (ES) *m/z* 330 $(M+H)^+$; *m/z* 330.1453 (calcd for $C_{17}H_{20}N_3O_4$ $(M+H)^+$: 330.1454).

5.2.33. 1-(2,4-Dimethylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (38)

Method A was utilized for synthesis. Yellow solid (39% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.23 (s, 3H, CH_3), 2.25 (s, 3H, CH_3), 6.98 (d, J = 8.0 Hz, 1H, CH_{Ar}), 7.02 (s, 1H, CH_{Ar}), 7.62 (d, J = 8.0 Hz, 1H, CH_{Ar}), 7.68 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.75 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.38 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.76 (s, 1H, NH), 9.09 (s, 1H, NH), 11.07 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 18.7 (CH), 21.1 (CH), 109.2 (CH_{Ar}), 116.7 (CH_{Ar}), 117.3 (CH_{Ar}), 122.9 (CH_{Ar}), 127.3 (CH_{Ar}), 129.3 (CH_{Ar}), 131.5 (C_{Ar}), 133.1 (C_{Ar}), 134.9 (C_{Ar}), 136.4 (C_{Ar}), 141.1 (C_{Ar}), 145.9 (C_{Ar}), 153.1 (CO). HRMS (ES) *m/z 302* $(M+H)^+$; *m/z* 302.1139 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1141).

5.2.34. 1-(2,5-Dimethylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (39)

Method A was utilized for synthesis. Yellow solid (47% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.22 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 6.82 (d, J = 8.0 Hz, 1H, CH_{Ar}), 7.07 (d, J = 7.5 Hz, 1H, CH_{Ar}), 7.64(s, 1H, CH_{Ar}), 7.68 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.75 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.39 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.77 (s, 1H, NH), 9.17 (s, 1H, NH), 11.07 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 18.3 (CH), 21.6 (CH), 109.2 (CH_{Ar}), 116.7 (CH_{Ar}), 117.4 (CH_{Ar}), 123.1 (CH_{Ar}), 124.7 (CH_{Ar}), 125.9 (CH_{Ar}), 130.8 (C_{Ar}), 135.8 (C_{Ar}), 136.4 (C_{Ar}), 137.3 (C_{Ar}), 141.2 (C_{Ar}), 146.1 (C_{Ar}), 153.0 (CO). HRMS (ES) *m/z* 302 $(M+H)^+$; *m/z* 302.1138 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1141).

5.2.35. 1-(3,4-Dimethylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (40)

Method A was utilized for synthesis. Yellow solid (53% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.18 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 7.06 (d, J = 8.5 Hz, 1H, CH_{Ar}), 7.19 (d, J = 8.0 Hz, 1H, CH_{Ar}), 7.25(s, 1H, CH_{Ar}), 7.67 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.76 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.37 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.70 (s, 1H, NH), 9.43 (s, 1H, NH), 11.07 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 19.4 (CH), 20.4 (CH), 109.1 (CH_{Ar}), 116.5 (CH_{Ar}), 116.8 (CH_{Ar}), 117.0 (CH_{Ar}), 120.3 (CH_{Ar}), 130.5 (CH_{Ar}), 130.8 (C_{Ar}), 136.2 (C_{Ar}), 137.3 (C_{Ar}), 137.6 (C_{Ar}), 141.2 (C_{Ar}), 145.9 (C_{Ar}), 152.6 (CO). HRMS (ES) *m/z* 302 $(M+H)^+$; *m/z* 302.1139 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1141).

5.2.36. 1-(3,5-Dimethylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (41)

Method A was utilized for synthesis. Yellow solid (36% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.26 (s, 6H, CH_3), 6.67 (s, 1H, CH_{Ar}), 7.10 (s, 1H, CH_{Ar}), 7.67(d, J = 2.0 Hz, 1H, CH_{Ar}), 7.76 (dd, J = 9.0, 2.0 Hz, 1H, CH_{Ar}), 8.37 (d, J = 8.0 Hz, 1H, CH_{Ar}), 8.73 (s, 1H, NH), 9.45 (s, 1H, NH), 11.08 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 21.8 (CH), 109.1 (CH_{Ar}), 116.6 (CH_{Ar}), 117.1 (CH_{Ar}), 124.7 (CH_{Ar}), 136.2 (C_{Ar}), 138.6 (C_{Ar}), 139.8 (C_{Ar}), 141.2 (C_{Ar}), 146.0 (C_{Ar}), 152.6 (CO). HRMS (ES) *m/z* 302 $(M+H)^+$; *m/z* 302.1138 (calcd for $C_{15}H_{16}N_3O_4$ $(M+H)^+$: 302.1141).

5.2.37. 1-(2-Ethyl-6-methylphenyl)-3-(2-hydroxy-4-nitrophenyl) urea (42)

Method A was utilized for synthesis. Yellow solid (42% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.14 (t, J = 7.5 Hz, 3H, CH_3), 2.21 (s, 3H, CH_3), 2.59 (q, J = 7.5 Hz, 2H, CH_2), 7.20–7.10 (m, 3H, CH_{Ar}), 7.68 (s, 1H, CH_{Ar}), 7.74 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.65 (s, 1H, NH), 8.87 (s, 1H, NH), 11.09 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 15.5 (CH), 18.9 (CH), 25.2 (CH), 109.2 (CH_{Ar}), 116.7 (CH_{Ar}), 116.8 (CH_{Ar}), 126.9 (CH_{Ar}), 127.2 (CH_{Ar}), 128.5 (CH_{Ar}), 134.8 (C_{Ar}), 136.6 (C_{Ar}), 141.0 (C_{Ar}), 141.9 (C_{Ar}), 145.7 (C_{Ar}), 153.5 (CO). HRMS (ES) *m/z* 316 $(M+H)^+$; *m/z* 316.1295 (calcd for $C_{16}H_{18}N_3O_4$ $(M+H)^+$: 316.1297).

5.2.38. 1-(2-Ethyl-6-isopropylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (43)

Method A was utilized for synthesis. Yellow solid (23% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.22–1.12 (m, 9H, CH_3), 2.56 (q, J = 7.5 Hz, 2H, CH_2), 3.15 (septet, J = 7.0 Hz, 1H, CH), 7.12 (d, J = 7.0 Hz, 1H, CH_{Ar}), 7.25–7.18 (m, 2H, CH_{Ar}), 7.68 (d, J = 2.5 Hz, 2H, CH_{Ar}), 7.74 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.32 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.60 (s, 1H, NH), 8.87 (s, 1H, NH), 11.08 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 15.4 (CH), 24.7 (CH), 25.3 (CH), 28.6 (CH), 109.2 (CH_{Ar}), 116.8 (CH_{Ar}), 123.9 (CH_{Ar}), 126.6 (CH_{Ar}), 127.9 (CH_{Ar}), 133.3 (C_{Ar}), 136.7 (C_{Ar}), 140.9 (C_{Ar}), 142.5 (C_{Ar}), 145.6 (C_{Ar}), 147.1 (C_{Ar}), 154.2 (CO). HRMS (ES) *m/z* 344 $(M+H)^+$; *m/z* 344.16108 (calcd for $C_{18}H_{22}N_3O_4$ $(M+H)^+$: 344.161).

5.2.39. 1-(2-Hydroxy-4-nitrophenyl)-3-(2-isopropyl-6-methylphenyl)urea (44)

Method A was utilized for synthesis. Yellow solid (31% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.18 (d, J = 6.5 Hz, 6H, CH_3), 2.22 (s, 3H, CH_3), 3.19 (m, 1H, CH), 7.18–7.11 (m, 3H, CH_{Ar}), 7.69 (s,

1H, CH_{Ar}), 7.74 (d, J = 8.5 Hz, 1H, CH_{Ar}), 8.34 (d, J = 8.5 Hz, 1H, CH_{Ar}), 8.65 (s, 1H, NH), 8.89 (s, 1H, NH), 11.06 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 19.1 (CH), 24.4 (CH), 28.6 (CH), 109.2 (CH_{Ar}), 116.9 (CH_{Ar}), 123.9 (CH_{Ar}), 127.6 (CH_{Ar}), 128.3 (CH_{Ar}), 134.0 (C_{Ar}), 136.8 (C_{Ar}), 141.0 (C_{Ar}), 145.7 (C_{Ar}), 146.5 (C_{Ar}), 153.8 (CO). HRMS (ES) m/z 330 $(M+H)^+$; m/z 330.1453 (calcd for $C_{17}H_{20}N_3O_4$ $(M+H)^+$: 330.1454).

5.2.40. 1-(2,6-Diisopropylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (45)

Method A was utilized for synthesis. Yellow solid (28% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.76 (d, J = 6.5 Hz, 12H, CH_3), 3.14 (septet, 7.0 Hz, 2H, CH), 7.17 (s, 1H, CH_{Ar}), 7.18 (s, 1H, CH_{Ar}), 7.28(t, J = 7.5 Hz, 1H, CH_{Ar}), 7.68 (d, J = 2.5 Hz, 1H, CH_{Ar}), 7.74 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.32 (d, J = 9.5 Hz, 1H, CH_{Ar}), 8.59 (s, 1H, NH), 8.86 (s, 1H, NH), 11.08 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 23.7 (CH), 24.7 (CH), 28.8 (CH), 109.2 (CH_{Ar}), 116.8 (CH_{Ar}), 116.9 (CH_{Ar}), 123.7 (CH_{Ar}), 128.2 (CH_{Ar}), 132.5 (C_{Ar}), 136.7 (C_{Ar}), 140.9 (C_{Ar}), 145.6 (C_{Ar}), 147.1 (C_{Ar}), 154.4 (CO); HRMS (ES) m/z 358 $(M+H)^+$; m/z 358.1765 (calcd for $C_{19}H_{24}N_3O_4$ $(M+H)^+$: 358.1767).

5.2.41. 1-(2-*sec*-Butyl-6-ethylphenyl)-3-(2-hydroxy-4-nitrophenyl)urea (46)

Method A was utilized for synthesis. Yellow solid (25% yld; >98% purity); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.76 (t, J = 7.5 Hz, 3H, CH_3), 1.16–1.13 (m, 6H, CH_3), 1.52 (br s, 2H, CH_2), 2.52 (q, J = 7.0 Hz, 2H, CH2), 2.90 (m, 1H, CH), 7.14–7.10 (m, 2H, CH_{Ar}), 7.23 (t, J = 7.5 Hz, 1H, CH_{Ar}), 7.68 (d, J = 2.0 Hz, 1H, CH_{Ar}), 7.74 (dd, J = 9.0, 2.5 Hz, 1H, CH_{Ar}), 8.32 (d, J = 9.0 Hz, 1H, CH_{Ar}), 8.57 (s, 1H, NH), 8.87 (s, 1H, NH), 11.08 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 12.9 (CH), 15.3 (CH), 21.8 (CH), 25.4 (CH), 31.1 (CH), 35.6 (CH), 109.1 (CH_{Ar}), 116.8 (CH_{Ar}), 124.3 (CH_{Ar}), 126.5 (CH_{Ar}), 127.9 (CH_{Ar}), 133.9 (CH_{Ar}), 136.8 (C_{Ar}), 141.0 (C_{Ar}), 142.6 (C_{Ar}), 145.6 (C_{Ar}), 146.0 (C_{Ar}), 154.1 (CO); HRMS (ES) m/z 358 $(M+H)^+$, m/z 358.1765 (calcd for $C_{19}H_{24}N_3O_4$ $(M+H)^+$: 358.1767).

5.3. Reagents for in vitro assays

All cell culture reagents were from Invitrogen, unless otherwise noted. The BCA protein assay reagent kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL). The human Aβ40 colorimetric ELISA kit were purchased from BioSource International Inc (Carlsbad, California). The Fluo-4 NW calcium assay kit was purchased from Molecular Probes, Invirogen (Carlsbad, California). All reagents used for synthesis were purchased from Aldrich and Acros and were typically used as supplied unless otherwise stated.

5.4. Cell lines and cultures

All cells were cultured at 37 °C in the presence of 5% CO_2. The 7w CHO cell line (expressing APP751), were cultured as described previously.[26,39]

5.5. Measurement of Aβ40

Compounds were tested in CHO cells stably transfected with the human 751 amino acid splice variant of APP (7w cells). Cells were grown to 60% confluency in 24-well plates. Stock concentrations of the compound in DMSO were added to DMEM to reach a final concentration of 1% DMSO. A minimum of eight different concentrations of each compound were done in triplicate and the experiment was repeated in triplicate as well. Positive controls contained 1% DMSO alone. After 18 h, the medium was removed and centrifuged at 10,000g for 5 min, and the supernatant was stored at −80 °C until the ELISA assays were carried out. Aβ40 levels secreted in conditioned media were determined using a quantitative human Aβ40 sandwich ELISA kit as described in manufacturer's instructions.

5.6. Calcium mobilization assay

Compounds were tested in 7w cells. Cells were grown to ~100% confluency (30,000–40,000 cells) in 96-well plates. After removing the growth medium from the adherent 7w cell culture, 100 μl of the dye loading solution (made according to manufacturer's instructions) was carefully added to each well and the plate was incubated at 37 °C for an hour. Following incubation, the dye solution was replaced with 100 μl of 1X HBSS buffer with component C from the calcium assay kit. Stock concentrations of the compounds in DMSO were added to 1X Hank's balanced salt solution (HBSS) to reach a final concentration of 1% DMSO. A minimum of nine different concentrations of each compound were tested (n = 8 for each concentration), and the experiment was repeated in triplicate. Negative controls contained 1% DMSO alone. The plate was read at Ex/Em: 494/516 in a Biotek Synergy (with automated injector) plate reader, followed 15 s later by addition 50 μl of 100 nM of Groα (final [Groα] = 33 nM). The maximal calcium concentration attained after Groα stimulation was quantitated as described previously.[35]

5.7. Statistical analysis

All dose–response graphs were plotted using Sigma plot 8.0 and the standard sigmoid 4-parameter equation $y = y_o + \frac{a}{1+e^{-\frac{x-x_0}{b}}}$ was used to fit the dose–response data. The scaling of x-axis is in log.

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Supplementary data

Supplementary data (HPLC data and elemental analysis of the discussed compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.051.

References and notes

1. Hardy, J.; Selkoe, D. J. *Science* **2002**, *297*, 353.
2. Wilquet, V.; De Strooper, B. *Curr. Opin. Neurobiol.* **2004**, *14*, 582.
3. Esler, W. P.; Wolfe, M. S. *Science* **2001**, *293*, 1449.
4. Dominguez, D. I.; De Strooper, B. *Trends Pharmacol. Sci.* **2002**, *23*, 324.
5. Jakob-Roetne, R.; Jacobsen, H. *Angew. Chem., Int. Ed.* **2009**, *48*, 3030.
6. Yan, R.; Bienkowski, M. J.; Shuck, M. E.; Miao, H.; Tory, M. C.; Pauley, A. M.; Brashier, J. R.; Stratman, N. C.; Mathews, W. R.; Buhl, A. E.; Carter, D. B.; Tomasselli, A. G.; Parodi, L. A.; Heinrikson, R. L.; Gurney, M. E. *Nature* **1999**, *402*, 533.
7. Hussain, I.; Powell, D.; Howlett, D. R.; Tew, D. G.; Meek, T. D.; Chapman, C.; Gloger, I. S.; Murphy, K. E.; Southan, C. D.; Ryan, D. M.; Smith, T. S.; Simmons, D. L.; Walsh, F. S.; Dingwall, C.; Christie, G. *Mol. Cell Neurosci.* **1999**, *14*, 419.
8. Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. *Science* **1999**, *286*, 735.
9. Sinha, S.; Anderson, J. P.; Barbour, R.; Basi, G. S.; Caccavello, R.; Davis, D.; Doan, M.; Dovey, H. F.; Frigon, N.; Hong, J.; Jacobson-Croak, K.; Jewett, N.; Keim, P.; Knops, J.; Lieberburg, I.; Power, M.; Tan, H.; Tatsuno, G.; Tung, J.; Schenk, D.; Seubert, P.; Suomensaari, S. M.; Wang, S.; Walker, D.; John, V. *Nature* **1999**, *402*, 537.
10. Guo, T.; Hobbs, D. W. *Curr. Med. Chem.* **2006**, *13*, 1811.

11. Dominguez, D.; Tournoy, J.; Hartmann, D.; Huth, T.; Cryns, K.; Deforce, S.; Serneels, L.; Camacho, I. E.; Marjaux, E.; Craessaerts, K.; Roebroek, A. J.; Schwake, M.; D'Hooge, R.; Bach, P.; Kalinke, U.; Moechars, D.; Alzheimer, C.; Reiss, K.; Saftig, P.; De Strooper, B. *J. Biol. Chem.* **2005**, *280*, 30797.
12. Ghosh, A. K.; Kumaragurubaran, N.; Hong, L.; Koelsh, G.; Tang, J. *Curr. Alzheimer Res.* **2008**, *5*, 121.
13. Evin, G.; Kenche, V. B. *Recent Pat. CNS Drug Disc.* **2007**, *2*, 188.
14. Hills, I. D.; Vacca, J. P. *Curr. Opin. Drug Discovery Dev.* **2007**, *10*, 383.
15. Wolfe, M. S. *Biochemistry* **2006**, *45*, 7931.
16. Edbauer, D.; Winkler, E.; Regula, J. T.; Pesold, B.; Steiner, H.; Haass, C. *Nat. Cell Biol.* **2003**, *5*, 486.
17. Kimberly, W. T.; LaVoie, M. J.; Ostaszewski, B. L.; Ye, W.; Wolfe, M. S.; Selkoe, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6382.
18. Takasugi, N.; Tomita, T.; Hayashi, I.; Tsuruoka, M.; Niimura, M.; Takahashi, Y.; Thinakaran, G.; Iwatsubo, T. *Nature* **2003**, *422*, 438.
19. Li, H.; Wolfe, M. S.; Selkoe, D. J. *Structure* **2009**, *17*, 326.
20. Osenkowski, P.; Li, H.; Ye, W.; Li, D.; Aeschbach, L.; Fraering, P. C.; Wolfe, M. S.; Selkoe, D. J.; Li, H. *J. Mol. Biol.* **2009**, *385*, 642.
21. Wolfe, M. S. *Neurotherapeutics* **2008**, *5*, 391.
22. Esler, W. P.; Das, C.; Wolfe, M. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1935.
23. Esler, W. P.; Kimberly, W. T.; Ostaszewski, B. L.; Ye, W.; Diehl, T. S.; Selkoe, D. J.; Wolfe, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2720.
24. Wolfe, M. S. *Curr. Alzheimer Res.* **2008**, *5*, 158.
25. Kornilova, A. Y.; Bihel, F.; Das, C.; Wolfe, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3230.
26. Bakshi, P.; Wolfe, M. S. *J. Med. Chem.* **2004**, *47*, 6485.
27. Wolfe, M. S. B. M. C. *Neuroscience* **2008**, *9*, S4.
28. Bateman, R. J.; Siemers, E. R.; Mawuenyega, K. G.; Wen, G.; Browning, K. R.; Sigurdson, W. C.; Yarasheski, K. E.; Friedrich, S. W.; Demattos, R. B.; May, P. C.; Paul, S. M.; Holtzman, D. M. *Ann. Neurol.* **2009**.
29. Fleisher, A. S.; Raman, R.; Siemers, E. R.; Becerra, L.; Clark, C. M.; Dean, R. A.; Farlow, M. R.; Galvin, J. E.; Peskind, E. R.; Quinn, J. F.; Sherzai, A.; Sowell, B. B.; Aisen, P. S.; Thal, L. J. *Arch. Neurol.* **2008**, *65*, 1031.
30. Kukar, T. L.; Ladd, T. B.; Bann, M. A.; Fraering, P. C.; Narlawar, R.; Maharvi, G. M.; Healy, B.; Chapman, R.; Welzel, A. T.; Price, R. W.; Moore, B.; Rangachari, V.; Cusack, B.; Eriksen, J.; Jansen-West, K.; Verbeeck, C.; Yager, D.; Eckman, C.; Ye, W.; Sagi, S.; Cottrell, B. A.; Torpey, J.; Rosenberry, T. L.; Fauq, A.; Wolfe, M. S.; Schmidt, B.; Walsh, D. M.; Koo, E. H.; Golde, T. E. *Nature* **2008**, *453*, 925.
31. Bakshi, P.; Margenthaler, E.; Laporte, V.; Crawford, F.; Mullan, M. *ACS Chem. Biol.* **2008**, *3*, 777.
32. Ni, Y.; Zhao, X.; Bao, G.; Zou, L.; Teng, L.; Wang, Z.; Song, M.; Xiong, J.; Bai, Y.; Pei, G. *Nat. Med.* **2006**, *12*, 1390.
33. Thathiah, A.; Spittaels, K.; Hoffmann, M.; Staes, M.; Cohen, A.; Horre, K.; Vanbrabant, M.; Coun, F.; Baekelandt, V.; Delacourte, A.; Fischer, D. F.; Pollet, D.; De Strooper, B.; Merchiers, P. *Science* **2009**, *323*, 946.
34. Kuo, L. H.; Hu, M. K.; Hsu, W. M.; Tung, Y. T.; Wang, B. J.; Tsai, W. W.; Yen, C. T.; Liao, Y. F. *Mol. Biol. Cell* **2008**.
35. White, J. R.; Lee, J. M.; Young, P. R.; Hertzberg, R. P.; Jurewicz, A. J.; Chaikin, M. A.; Widdowson, K.; Foley, J. J.; Martin, L. D.; Griswold, D. E.; Sarau, H. M. *J. Biol. Chem.* **1998**, *273*, 10095.
36. Jin, Q.; Nie, H.; McCleland, B. W.; Widdowson, K. L.; Palovich, M. R.; Elliott, J. D.; Goodman, R. M.; Burman, M.; Sarau, H. M.; Ward, K. W.; Nord, M.; Orr, B. M.; Gorycki, P. D.; Busch-Petersen, J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4375.
37. Widdowson, K. L.; Elliott, J. D.; Veber, D. F.; Nie, H.; Rutledge, M. C.; McCleland, B. W.; Xiang, J. N.; Jurewicz, A. J.; Hertzberg, R. P.; Foley, J. J.; Griswold, D. E.; Martin, L.; Lee, J. M.; White, J. R.; Sarau, H. M. *J. Med. Chem.* **2004**, *47*, 1319.
38. McCleland, B. W.; Davis, R. S.; Palovich, M. R.; Widdowson, K. L.; Werner, M. L.; Burman, M.; Foley, J. J.; Schmidt, D. B.; Sarau, H. M.; Rogers, M.; Salyers, K. L.; Gorycki, P. D.; Roethke, T. J.; Stelman, G. J.; Azzarano, L. M.; Ward, K. W.; Busch-Petersen, J. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1713.
39. Koo, E. H.; Squazzo, S. L. *J. Biol. Chem.* **1994**, *269*, 17386.
40. Liao, Y. F.; Wang, B. J.; Cheng, H. T.; Kuo, L. H.; Wolfe, M. S. *J. Biol. Chem.* **2004**, *279*, 49523.
41. Kuo, L. H.; Hu, M. K.; Hsu, W. M.; Tung, Y. T.; Wang, B. J.; Tsai, W. W.; Yen, C. T.; Liao, Y. F. *Mol. Biol. Cell* **2008**, *19*, 4201.
42. Lee, S.; Das, H. K. *Brain Res.* **2008**, *1207*, 19.
43. Campbell, W. A.; Iskandar, M. K.; Reed, M. L.; Xia, W. *Biochemistry* **2002**, *41*, 3372.
44. Baxter, A.; Bennion, C.; Bent, J.; Boden, K.; Brough, S.; Cooper, A.; Kinchin, E.; Kindon, N.; McInally, T.; Mortimore, M.; Roberts, B.; Unitt, J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2625.