Brief Report

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Synthesis and biological evaluation of novel anthranilamide derivatives as anticancer agents

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ABSTRACT: A new series of anthranilamide derivatives were synthesized and evaluated for their antiproliferative activities against human colon carcinoma cell lines (HCT 116) and human breast adenocarcinoma cell lines (MDA-MB-231) *in vitro*. The bioassay results indicated that compounds 7a-7d, 11a, and 11b with flexible linkers showed promising antiproliferative activity against both cell lines. Among the compounds synthesized, 7c showed the most significant antiproliferative activity. Flow cytometric analysis indicated that 7c inhibited HCT 116 and MDA-MB-231 cell growth by inducing apoptosis in a dose-dependent manner and suppressed HCT 116 cell proliferation by G1 and S phase arrest. Compound 7c may serve as a lead candidate in the development of novel anticancer agents.

Keywords: Anthranilamide, anticancer agent, antiproliferative activity

1. Introduction

Cancer is a main public health problem and a leading cause of death worldwide (*1*). During past decades, extensive efforts have been carried out to develop more specific and efficacious anticancer agents. With an increasing mechanistic understanding of biological pathways regulating human cancers and normal cells in recent years, these efforts have shifted from older, cytotoxic therapeutic options toward chemical and biological therapies that are precisely designed to target a critical gene or pathway (*2*). In recent years, many antitumor agents targeting one or more critical targets of the tumor signal pathway network have entered clinical trials or been approved for clinical use.

PTK787 (*N*-(4-chlorophenyl)-4-(pyridin-4-yl-methyl)phthalazin-1-amine, Figure 1), a potent and selective tyrosine kinase inhibitor of vascular endothelial growth factor receptor-1, 2, 3 (VEGFR-1, 2, 3), platelet-derived growth factor receptor (PDGFR) and stem cell growth factor receptor (SCFR, also known as C-Kit) with anti-tumor activity, has undergone clinical trials for the treatment of breast, colorectal and other cancers (*3-5*). P. Furet *et al*. reasoned that an anthranilamide moiety (Figure 1) presenting a strong intramolecular hydrogen bond between the amine and keto functionalities to form a pseudo six membered ring could mimic the phthalazine ring of PTK787 (*6*). Thus, a series of anthranilamide derivatives with promising *in vivo* anti-tumor effects were investigated. Among these anthranilamide derivatives, AAL993 (2-((4-pyridyl) methyl) amino-*N*-(3-(trifluoromethyl) phenyl) benzamide, Figure 1) is a highly potent and selective inhibitor of VEGFRs with potent anti-tumor properties, good pharmacological properties and excellent oral bioavailability (*7,8*). CI-1040 (2-((2-chloro-4-iodophenyl)amino)-N-(cyclopropylmethoxy)-3,4-difluorobenzamide, Figure 1), the first mitogen activated protein kinase kinase 1/2 (MEK1/2) inhibitor in clinical development, has shown antitumor activity in a variety of *in vitro* and *in vivo* tumor models (*9,10*). The anthranilamide moieties presented in these structures are considered responsible for the potent antitumor properties (*7,11*). The anthranilamide has been widely used as a privileged scaffold to generate various therapeutic molecules (*12-14*). Furthermore, many anthranilamide containing derivatives were previously reported as antitumor agents (*12,13,15*).

In these experiments, we introduced some aromatic heterocycles which were widely presented in antitumor drugs with the anthranilamide scaffold through a linker and synthesized seventeen novel anthranilamide derivatives (Figure 1). The antiproliferative activity of these compounds was evaluated on human colon carcinoma cell lines (HCT 116) and human breast adenocarcinoma cell lines (MDA-MB-231).

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2. Materials and Methods

2.1. *Chemistry*

The synthesis of the target anthranilamide derivatives was performed in a manner described in Scheme I. All the target compounds were synthesized with commercially available 2-nitrobenzoic acid **1** (Energy Chemical, Shanghai, China) as starting material. 2-Nitrobenzoic acid **1** was treated with thionyl chloride to generate acyl chloride **2**. Subsequent treatment of acyl chloride **2** with different substituted anilines yielded the intermediates 2-nitrobenzamides **3a-3e**. Reduction of 2-nitrobenzamides **3a-3e** with reduced

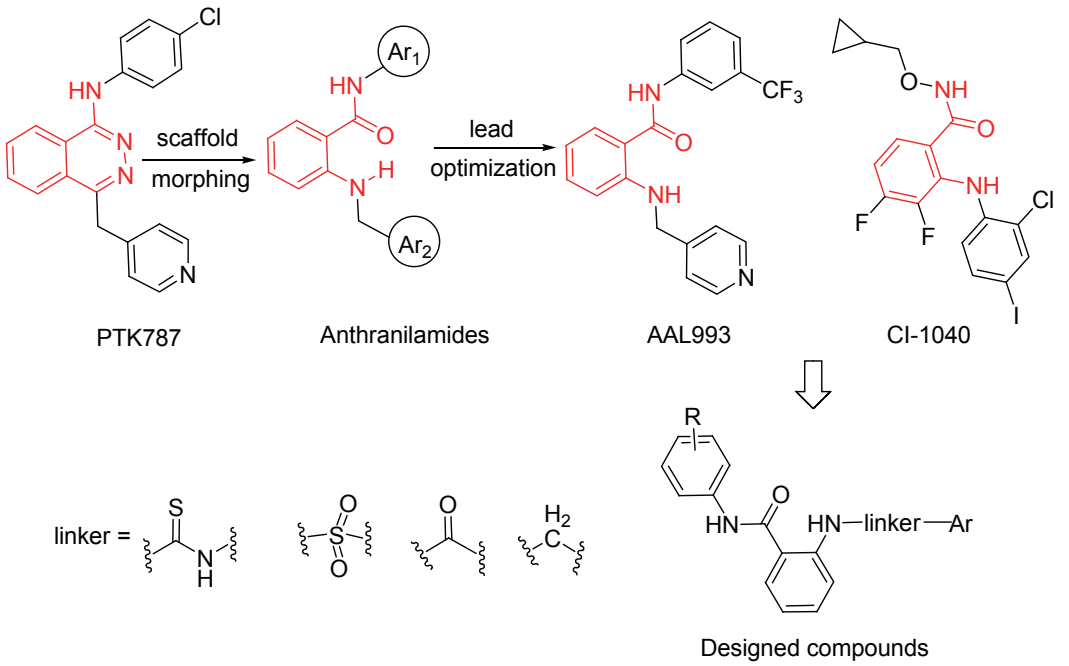
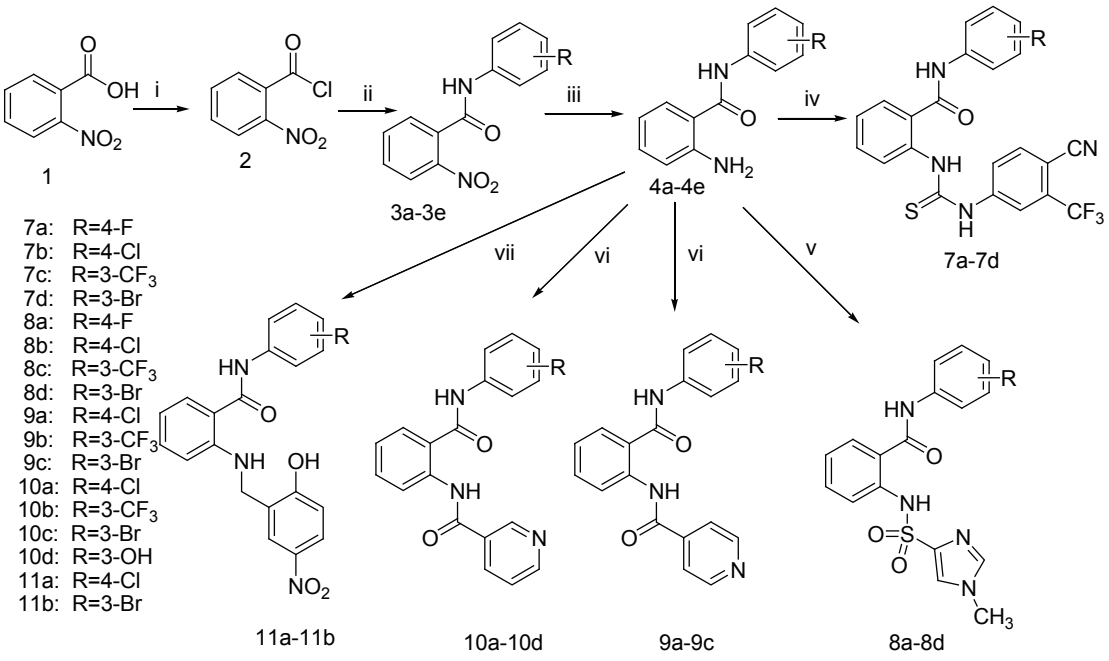


Figure 1. Structures of lead compounds (PTK787, AAL993, and CI-1040) and designed compounds.



Scheme I. Synthesis of target compounds. Reagents and conditions: (*i*) thionyl chloride, dichloromethane, 60°C, 4 h; (*ii*) aromatic amine, triethylamine, dichloromethane, 0°C, 1 h; (*iii*) Fe, acetic acid, ethyl acetate, 80°C, 4 h; (*iv*) 4-isothiocyanato-2-(trifluoromethyl)benzonitrile, dichloromethane, r.t., 5 h; (*v*) 1-methyl-1*H*-imidazole-4-sulfonyl chloride, pyridine, 0°C, 10 h; (*vi*) isonicotinoyl chloride or nicotinoyl chloride, dichloromethane, triethylamine, 0°C, 0.5 h, then r.t., 2 h; (*vii*) 5-nitrosalicylaldehyde, acetic acid, r.t., 8h, then $NaCNBH_3$, methyl alcohol, r.t., 24 h.

iron powder in ethyl acetate generated the key intermediate anthranilamides **4a-4e**. 4-Isothiocyanato-2-(trifluoromethyl)benzonitrile was added to **4a-4e** to give target compounds **7a-7d**. Treatment **4a-4e** with 1-methyl-1*H*-imidazole-4-sulfonyl chloride, isonicotinoyl chloride or nicotinoyl chloride respectively obtained target compounds **8a-8d**, **9a-9c**, and **10a-10d**. Anthranilamides **4a-4e** reacted with 2-hydroxy-5-nitrobenzaldehyde in the presence of $NaCNBH_3$ to yield target compounds **11a-11b**.

2.2. *Cell lines and culture*

Human cancer cell lines HCT 116 and MDA-MB-231 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured separately in RPMI-1640 and DMEM medium (HyClone®, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco®, Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

2.3. *Cell growth inhibition assay*

The cell growth inhibitory activity of anthranilamide derivatives was assessed by means of a standard MTT colorimetric assay as previously reported (*16*). All compounds were dissolved in dimethylsulfoxide (DMSO) to form the stock solutions with concentrations of 1-16 mM. Serial dilutions were prepared from these stocks to yield final concentrations of 5-80 μmol/L in medium with a DMSO content less than 0.5%. HCT 116 and MDA-MB-231 cells were plated in 96-well microplates at a density of 3,000 cells per well for 12 h, after which all the prepared compounds and positive controls were added. After 72 h incubation at 37°C, 10 μL MTT (5 μg/μL) was added followed by another 4 h incubation, then the supernatant was removed and 200 μL DMSO was added to dissolve the formazan formed. Optical density was measured at 570 nm on a microplate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA). Each experiment was performed three times.

2.4. *Flow cytometric analysis*

For cell cycle assays, cells treated with 10 μmol/L compounds for 24 h were harvested and washed in PBS. These cells were then fixed in 75% ethanol (ice cold) for 4 h at 4°C, washed twice with PBS, resuspended in 100 μL RNase (Sigma, St. Louis, MO, USA) solution (100 μg/mL) and incubated for 30 min at 37°C. Next, 100 μL of propidium iodide (100 μg/mL) was added followed by 30 min incubation. Fluorescence was quantified by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA), and the percentage of cells in each phase was calculated using ModFit software (BD Biosciences, San Jose, CA, USA).

A BU-Annexin V-FITC apoptosis detection kit (BioUniquer Technology CO., LTD, Nanjing, Jiangsu, China) was used to determine the apoptosis induced by the target compounds at a dosage of 1 and 10 μM. After treatment for 24 h, cells were harvested and washed with ice cold PBS, cells were then resuspended in 200 μL Annexin V binding buffer containing 2 μL Annexin V-FITC and 2 μL propidium iodide. After 10 min reaction in the dark at room temperature, stained cells were analyzed by flow cytometry. Unstained cells, cells stained with Annexin V-FITC alone and cells stained with PI alone were used to set up compensation and quadrants. The data were analyzed using Win MDI 2.9 software (The Scripps Research Institute, Jupiter, FL, USA).

2.5. *VEGFR-2 inhibitory activity assay*

VEGFR-2 inhibitory activity was determined by detecting phosphorylated substrate with a LANCE® *Ultra* TR-TRET biochemical platform (PerkinElmer, Waltham, MA, USA) using a *ULight*-TK peptide substrate with a phospho-specific Europium-labeled anti-phosphotyrosine (PT66). VEGFR-2 used in a TR-FRET assay was purchased from Carna Bioscience (Chuo-ku, Kobe, Japan). Complete assay optimization was initially conducted. VEGFR-2 kinase (0.0337 ng/μL) was added to 50 μM ATP, 50 nM peptide substrate and tested compounds (9 points, ranging from 0.45 nM to 3μM) in a kinase buffer composed of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA; 2 mM DTT, 0.01% Tween 20 in 10 μL total volume. Reactions were terminated by the addition of 10 mM EDTA after incubation for 1 h at room temperature, 2 nM Eu-labeled antibody in 1× detction buffer was then added in 20 μL total volume. After incubation for 1 h, samples were excited at 340 nM, and emission was read at 665 nM using an EnSpire® Multimode Plate Reader (PerkinElmer). All reactions were performed in a 384 well plate.

3. Results and Discussion

The target compounds were initially evaluated for their antiproliferative activity *in vitro* against human cancer cell lines HCT 116 and MDA-MB-231 with AAL 993 as a positive control by a standard MTT-based colorimetric assay. The bioassay results listed in Table 1 indicated that compounds **7a-d**, **9a**, **11a**, and **11b** exhibited potential antiproliferative activities against HCT 116 cells compared to AAL993. By comparison with AAL993, compounds **7a-7d**, **11a**, and **11b** showed improved antiproliferative activities on MDA-MB-231 cells. Among them, compound **7c** showed the most significant cytotoxicity on both cell lines with IC_{50} values of 14.60 and 13.86 μmol/L respectively.

It was also noticed that the compounds with a more flexible linker between the amino of anthranilamide moiety and the aromatic ring (**7a-7d**, **11a**, and **11b**) showed a greater potential antiproliferative activity than these compounds with carbonyl (**10a-10d**) as linker. Compounds **8a-8d** exhibited no antiproliferative activity against either HCT 116 or MDA-MB-231. From the view point of chemistry, it might be explained that the linker sulfonamide moiety in the compound **8** series could ionize a proton under physiological pH conditions, resulting in a compound unable to pass the cell membrane. Compared to compound **9b** that showed no antiproliferative activity with AAL 993, it was found that the linker between the amino of the anthranilamide moiety and the pyridine ring was carbonyl in compound **9b** and methylene in compound AAL 993. From this point, it was inferred that the linker between the amino of the anthranilamide moiety and the aromatic ring influenced the antiproliferative activity significantly.

Table 1. Antiproliferative activities and VEGFR-2 inhibitory activities of target compounds

Compound	IC_{50}/μM		
	HCT-116[a]	MDA-MB-231[a]	VEGFR-2[b]
7a	34.06 ± 4.52	22.90 ± 3.84	> 3
7b	23.37 ± 1.39	18.96 ± 5.76	> 3
7c	14.60 ± 3.54	13.86 ± 1.44	> 3
7d	19.12 ± 1.73	17.16 ± 1.06	> 3
8a	> 80	> 80	–
8b	> 80	> 80	–
8c	> 80	> 80	–
8d	> 80	> 80	–
9a	38.39 ± 3.92	> 80	–
9b	> 80	> 80	–
9c	> 80	> 80	–
10a	> 80	> 80	–
10b	> 80	> 80	–
10c	> 80	> 80	–
10d	> 80	> 80	–
11a	18.75 ± 2.01	20.34 ± 5.16	> 3
11b	15.88 ± 2.14	18.39 ± 7.44	> 3
AAL-993	19.11 ± 0.86	> 80	1.89 ± 0.47 nM

[a] IC_{50} indicates the concentration of each compound required for a 50% decrease in cell viability; [b] IC_{50} represents the concentration of compound that inhibits 50% of phosphorylation of the substrate compared to substrate of untreated VEGFR-2. The data shown are the means ± S.D. of three independent experiments.

The synthesized target compounds share the same anthranilamide scaffold with compound AAL 993 which is a good VEGFR-2 inhibitor. To determine whether the VEGFR-2 inhibitory activity plays a role in the observed cell growth inhibitory activities, the representative compounds **7a-7d**, **11a**, and **11b** with potent anticancer activities were selected for further analysis and the results are shown in Table 1. It was disappointing that all of the tested compounds showed no inhibitory potency against VEGFR-2.

To gain additional insight into the possible mechanism involved in the cell growth inhibitory activities, HCT 116 and MDA-MB-231 cells treated with compound **7c** were chosen for further biological evaluation. The effects of **7c** on apoptosis and cell cycle distribution were investigated by flow cytometry analysis. The results suggested that compound **7c** could significantly induce apoptosis in human cancer cell lines HCT 116 and MDA-MB-231 in a dose-dependent manner (Figure 2). After treatment with compound **7c** at the concentration of 0, 1, and 10 μM for 24 h, the cell apoptotic population of HCT 116 increased from 17.95% of the vehicle control to 25.25% and 50.45%. For MDA-MB-231, the cell apoptotic population increased from 17.76% to 24.67% and 56.79%.

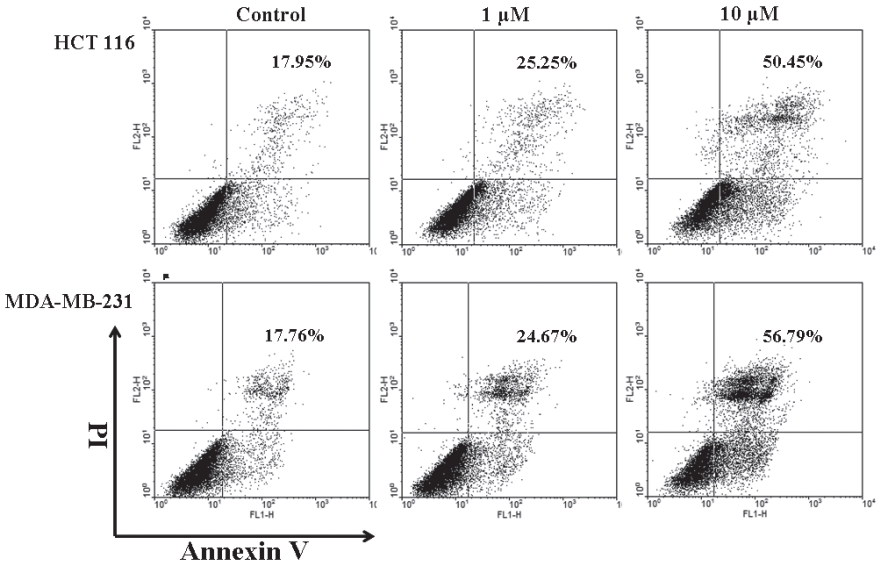


Figure 2. Apoptosis induced by 7c on HCT 116 and MDA-MB-231. HCT 116 and MDA-MB-231 cells were treated for 24 h with **7c** (1 and 10 μM) or DMSO (control). The treated cells were harvested and stained with Annexin V-FITC and propidium iodide, and the stained cells were analyzed by flow cytometry to determine the percentage of cells that were undergoing apoptosis.

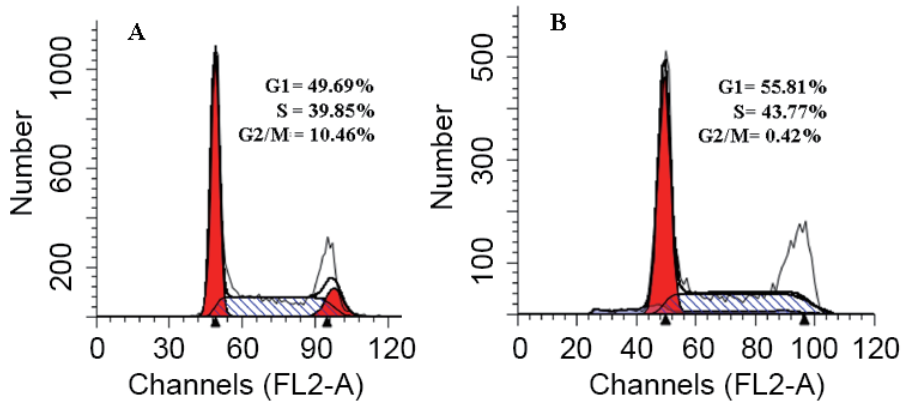


Figure 3. Cell cycle progression involved in compound 7c cytotoxicity on HCT 116. (A) Cell cycle distribution of HCT 116 cells treated with DMSO (control). **(B)** Cell cycle distribution of HCT 116 cells treated with 10 μM of **7c**.

Compound **7c** could also induce the accumulation of G1 and S phases in HCT 116 cells at a concentration of 10 μM by preventing the cell cycle progressing from S phase to the G2/M phase (Figure 3). These results implied that compound 7c might disturb duplication of the DNA.

In conclusion, a series of novel anthranilamide derivatives with potential antiproliferative activities were synthesized. Compounds with flexible linkers between the amino of the anthranilamide moiety and the aromatic ring (**7a-7d**, **11a**, and **11b**) showed promising antiproliferative activity against the selected cell lines. The anthranilamide derivative **7c** displayed the most significant antiproliferative activity in the series of compounds. Further bioassay results showed that compound **7c** plays a role in suppressing HCT 116 and MDA-MB-231 cell proliferation by inducing apoptosis in a dose-dependent manner and inhibits HCT 116 cell growth by influencing normal cell cycle progression. Compound **7c** may serve as a lead candidate in the development of novel anticancer agents, however, the exact target is still unknown, whether it has an exact target or is just a cytotoxic agent, the detailed mechanisms of the inhibitory effects need to be further investigated.

Acknowledgements

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Appendix

Chemistry: general procedures

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Melting points (Mp) were determined using a Büchi capillary melting point apparatus and were uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker Avance DRX600 instrument using tetramethylsilane (TMS) as an internal standard in DMSO-d_6 solutions. The chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS and the coupling constants (J) are expressed in Hertz (Hz). Multiplicities are designated as singlet (s), doublet (d), triplet (t), or multiplet (m) and broad peaks indicated as "br". ESI-MS spectra were recorded using an API 4000 and the high-resolution mass spectral data were determined using an Accela UPLC-LTQ Orbitrap Mass Spectrometer. The purity of the target compounds was determined using SHIMADZU LC-20AT High-performance Liquid Chromatography. Analytical thin-layer chromatography (TLC) was carried out on silica gel GF254 plates (layer thickness, 0.2 mm) to monitor reactions and the compounds were visualized using UV light. All column chromatography was carried out using silica gel (60 Å, 200-300 mesh).

General procedures for the preparation of 2-nitrobenzoyl chloride 2

To a 40 mL dichloromethane solution of 2-nitrobenzoic acid (3.34 g, 20 mmol), thionyl chloride (4 mL) was added. The mixture was heated to 60°C and stirred for 4 h. The contents were then cooled to room temperature and concentrated in vacuo to remove the excessive thionyl chloride. The products were dissolved in 25 mL anhydrous dichloromethane without further purification.

General procedures for the preparation of *N*-phenyl-2-nitrobenzamide 3

Substituted aniline (20 mmol) was dissolved in anhydrous dichloromethane (15 mL) and 5 mL triethylamine was added to the solution. 2-Nitrobenzoyl chloride dichloromethane solution was then slowly added to the substituted aniline solution at 0°C. The contents were stirred for 1h after dropping off. The mixture was filtered and filtrate was evaporated under reduced pressure. The products were purified by chromatography on silica gel to afford **3**.

*N-(4-fluorophenyl)-2-nitrobenzamide (**3a**)*. White solid. Yield 89.7%. Mp: 167-171°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.21, 7.68 (A′ABB′, J_{AB} = 9.0 Hz, $J_{AA'}$ = $J_{BB'}$ = 3.0 Hz, 4H), 7.77 (td, J = 8.4Hz, 1.2Hz, 1H), 7.78 (dd, J =7.8Hz, 1.2Hz, 1H), 7.88 (td, J = 7.8Hz, 1.2Hz, 1H), 8.16 (dd, J=8.4Hz, 1.2Hz, 1H), 10.04 (s, 1H). MS (calcd/found) $[M+H]^+$: 261.06/261.2.

*N-(4-chlorophenyl)-2-nitrobenzamide (**3b**)*. White solid. Yield 73.8%. Mp: 185-187°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.44 (dd, J = 8.7 Hz, 2.4 Hz, 2H), 7.69 (dd, J = 8.7 Hz, 2.4 Hz, 2H), 7.77 (td, J = 8.1 Hz, 1.2 Hz, 1H), 7.79 (dd, J = 8.1 Hz, 1.2 Hz, 1H), 7.88 (td, J = 8.1 Hz, 1.2 Hz, 1H), 8.17(dd, J = 8.1 Hz, 1.2 Hz, 1H), 10.81 (s, 1H). MS (calcd/found) $[M + H]^+$: 277.03/277.3.

*N-(3-(trifluoromethyl)phenyl)-2-nitrobenzamide (**3c**)*. White solid. Yield 75.9%. Mp: 135-137°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.50 (d, J = 7.5 Hz, 1H), 7.62 (dd, J = 8.4 Hz, 7.5 Hz, 1H), 7.79 (td, J = 8.4 Hz, 1.2 Hz, 1H), 7.82 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.85 (d, J = 8.4 Hz, 1H), 7.91 (td, J = 7.8 Hz, 1.2 Hz, 1H), 8.16 (s, 1H), 8.19 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 11.02 (s, 1H). MS (calcd/found) $[M + H]^+$: 311.06/311.3.

*N-(3-bromophenyl)-2-nitrobenzamide (**3d**)*. White solid. Yield 89.4%. Mp: 168-170°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.32-7.34 (m, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.78 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.89 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 8.01 (s, 1H), 8.17 (d, J = 7.8 Hz, 1H), 10.85(s, 1H). MS (calcd/found) $[M + H]^+$: 320.98/321.2.

*N-(3-hydroxyphenyl)-2-nitrobenzamide (**3e**)*. White solid. Yield 52.9%. Mp: 176-179°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.52 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 7.02 (d, J = 7.8 Hz, 1H), 7.12 (dd, J = 8.4 Hz, 7.8

Hz, 1H), 7.26 (d, J = 1.8 Hz, 1H), 7.73-7.76 (m, 2H), 7.86 (t, J = 7.2 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 9.49 (br s, 1H),10.53 (s, 1H). MS (calcd/found) $[M + H]^+$: 259.06/259.1.

General procedures for the preparation of *N*-phenyl-2-amino-benzamide 4

To a three-necked flask, compound **3** (15 mmol), iron (10 eqv.), acetic acid (20 mL) and ethyl acetate (30 mL) were added in order and the mixture was refluxed for 4 h and then cooled to room temperature. The contents were filtered and the filtrate was washed with saturated NaCl solution (3 × 20 mL). The solution was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by chromatography on silica gel to afford **4**.

*N-(4-fluorophenyl)-2-amino-benzamide (**4a**)*. White solid. Yield 87.8%. Mp: 130-132°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.32 (s, 2H), 6.59 (td, J = 7.8 Hz, 1.2 Hz, 1H), 6.75 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.15-7.21 (m, 3H), 7.61(dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.72 (AA′ of AA′BB′, 2H), 10.04 (s, 1H). MS (calcd/found) $[M + H]^+$: 231.09/231.3.

*N-(4-chlorophenyl)-2-amino-benzamide (**4b**)*. White solid. Yield 83.7%. Mp: 148-150°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.33 (s, 2H), 6.59 (td, J = 8.1 Hz, 0.6 Hz, 1H), 6.75 (dd, J = 8.1 Hz, 0.6 Hz, 1H), 7.21 (td, J = 8.1 Hz, 1.2 Hz, 1H), 7.38 (dd, J = 7.8 Hz, 1.5 Hz, 2H), 7.61 (dd, J = 8.1 Hz, 1.2 Hz, 1H), 7.75 (dd, J = 7.8 Hz, 1.5 Hz, 2H), 10.11 (s, 1H). MS (calcd/found) $[M + H]^+$: 247.06/247.3.

*N-(3-(trifluoromethyl)phenyl)-2-amino-benzamide (**4c**)*. White solid. Yield 85.5%. Mp: 136-138°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.38 (s, 2H), 6.60 (td, J = 8.1 Hz, 0.6 Hz, 1H), 6.77 (dd, J = 8.1 Hz, 0.6 Hz, 1H), 7.22 (td, J = 8.1 Hz, 1.5 Hz, 1H), 7.42 (d, J = 7.8 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.65 (dd, J = 8.1 Hz, 1.5Hz, 1H), 7.97 (d, J = 7.8 Hz, 1H), 8.21 (s, 1H), 10.28 (s, 1H). MS (calcd/found) $[M + H]^+$: 281.08/281.3.

*N-(3-bromophenyl)-2-amino-benzamide (**4d**)*. White solid. Yield 79.4%. Mp: 137-141°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.35 (s, 2H), 6.59 (td, J = 8.4 Hz, 1.2 Hz, 1H), 6.76 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 7.21 (td, J = 8.4 Hz, 1.2 Hz, 1H), 7.26 (dt, J = 7.8 Hz, 1.8 Hz, 1 H), 7.30 (t, J = 7.8 Hz, 1H), 7.61 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 7.68 (dt, J = 7.8 Hz, 1.8 Hz, 1H), 8.06(t, J = 1.8 Hz, 1H), 10.12 (s, 1H). MS (calcd/found) $[M + H]^+$: 291.01/291.2.

*N-(3-hydroxyphenyl)-2-amino-benzamide (**4e**)*. Light brown solid. Yield 63.1%. Mp: 127-131°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.27 (s, 2H), 6.47 (dt, J = 6.6 Hz, 2.4 Hz, 1H), 6.58 (td, J = 7.8 Hz, 1.2 Hz, 1H), 6.74 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.08-7.09 (m, 2H), 7.19 (td, J = 7.8 Hz, 1.5 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.58 (dd, J = 7.8 Hz, 1.5 Hz, 1H), 9.35 (s, 1H), 9.86 (s, 1H). MS (calcd/found) $[M + H]^+$: 229.09/229.2.

General procedures for the preparation of target compound *N*-substituted phenyl-2-(3-(4-cyano-3-(trifluoromethyl)phenyl)thioureido)benzamide 7

To a 6 mL dichloromethane solution of compound **4** (1 mmol), 4-isothiocyanato-2-trifluoromethyl-benzonitrile (1 mmol) dissolved in 6mL dichloromethane was added slowly at 0°C. The mixture was stirred for another 5 h at room temperature. The granulated solid crystallized from the mixture and the mixture was then filtered. The filter cake was washed with ether (2 × 5 mL) to afford **7**.

*N-(4-fluorophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7a**)*. White solid. Yield 79.0%. Mp: 182-185°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.17,7.70 (AA′BB′, J_{AB} = $J_{A'B'}$ = 9.0 Hz, $J_{AA'}$ = $J_{BB'}$ =2.1 Hz, 4H),7.37 (dd, J = 8.1 Hz, 7.2 Hz, 1H), 7.56 (dd, J = 8.1 Hz, 7.2 Hz, 1H), 7.71 (d, J = 8.1 Hz, 1H), 7.79 (d, J = 8.1 Hz, 1H), 8.04 (dd, J = 8.7 Hz, 1.8 Hz, 1H), 8.06 (d, J = 8.7 Hz, 1H), 8.38 (d, J = 1.8 Hz, 1H), 10.50(s, 1H), 10.51 (s, 1H), 10.94 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_4N_4OS$ $[M + H]^+$: calculated 459.0897 found 459.0899. HPLC purity = 95.5%.

*N-(4-chlorophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7b**)*. White solid. Yield 76.3%. Mp: 191-193°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.37 (t, J = 7.5Hz, 1H), 7.39 (d, J = 8.4 Hz, 2H), 7.56 (dd, J = 8.4 Hz, 7.5 Hz, 1H), 7.69-7.75 (m, 4H), 8.03 (dd, J = 8.4 Hz, 1.8 Hz, 1H), 8.05 (d, J = 8.4 Hz, 1H), 8.37 (d, J = 1.8 Hz, 1H), 10.46 (s, 1H), 10.57 (s, 1H), 10.91 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_3ClN_4OS$ $[M + H]^+$: calculated 475.0602 found 475.0602. HPLC purity = 98.0%.

*N-(3-(trifluoromethyl)phenyl)-2-(3-(4-cyano-3-(trifluoromethyl)phenyl)thioureido)benzamide (**7c**)*. White solid. Yield 80.1%. Mp: 188-191°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.39 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.44(d, J = 7.2 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.58 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.73 (d, J = 7.2 Hz, 1H), 7.74 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 8.01 (dd, J = 8.4 Hz, 1.5 Hz, 1H), 8.03 (d, J = 8.4 Hz, 1H), 8.17 (s, 1H), 8.36 (d, J = 1.5 Hz, 1H), 10.47 (s, 1H), 10.75 (s, 1H), 10.89 (s, 1H). HRMS (ESI) m/z for $C_{23}H_{15}F_6N_4OS$ $[M + H]^+$: calculated 509.0865 found 509.0869. HPLC purity = 95.7%.

*N-(3-bromophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7d**)*. White solid. Yield 81.9%. Mp: 178-180°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.35-7.39 (m, 3H), 7.55 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.69-7.21 (m, 3H), 7.74 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 8.1 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 8.37 (s, 1H), 10.46 (s, 1H), 10.57 (s, 1H), 10.91 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_3BrN_4OS$ $[M + H]_+$: calculated 519.0097 found 519.0099. HPLC purity = 96.1%.

General procedures for the preparation of target compound *N*-substitutedphenyl-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide 8

To a 10 mL pyridine solution of compound **4** (1 mmol), 1-methyl-1*H*-imidazole-4-sulfonyl chloride (1 mmol) was added at 0°C. The mixture was stirred for 10 h at room temperature and then water (5 ml) was added with stirring for another 0.5 h. 2 mol/L hydrogen chloride solution was added to the contents to pH 6-7. The mixture was extracted with dichloromethane (4 × 15 mL) and the combined organic layers were washed with a saturated NaCl aqueous solution. The dichloromethane layers were dried in situ using $MgSO_4$ and concentrated in vacuo. The crude product was purified by chromatography on silica gel to afford **8**.

*N-(4-fluorophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8a**)*. White solid. Yield 81.3%. Mp: 171-173°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.20 (dd, J = 9.0 Hz, 6.6 Hz, 1H), 7.23,7.72 (A′ABB′, JAB = $J_{A'B'}$ = 9.0 Hz, $J_{A'A}$ = $J_{BB'}$ = 2.4 Hz, 4H), 7.50 (dd, J = 9.0 Hz, 8.1 Hz, 1H), 7.59 (d, J = 8.1 Hz, 1H), 7.70 (s, 1H), 7.80 (d, J = 6.6Hz, 1H), 7.90 (d, J = 1.2 Hz, 1H), 10.50 (s, 1H), 10.67 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}FN_4O_3S$ $[M + H]^+$: calculated 375.0922 found 375.0925. HPLC purity = 99.2%.

*N-(4-chlorophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8b**)*. White solid. Yield 85.1%. Mp: 178-180°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.21 (dd, J = 7.8 Hz, 7.2Hz, 1H), 7.45 (d, J = 8.7 Hz, 2H), 7.50 (dd, J = 8.4 Hz, 7.2 Hz, 1H), 7.59 (d, J = 8.4 Hz, 1H), 7.70 (s, 1H), 7.75 (d, J = 8.7 Hz, 2H), 7.80 (d, J = 7.8 Hz, 1H), 7.90 (s, 1H), 10.57 (s, 1H), 10.58 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}ClN_4O_3S$ $[M + H]^+$: calculated 391.0626 found 391.0621. HPLC purity = 98.8%.

*N-(3-(trifluoromethyl)phenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8c**)*. White solid. Yield 82.1%. Mp: 168-170°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.22 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 7.50-7.53 (m, 2H), 7.59 (d, J = 7.8 Hz, 1H), 7.63 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.70 (s, 1H), 7.81 (d, J = 6.9 Hz, 1H), 7.90 (s, 1H), 7.97 (d, J = 7.8 Hz, 1H), 8.18 (s, 1H), 10.52 (s, 1H), 10.74 (s, 1H). HRMS (ESI) m/z for $C_{18}H_{16}F_3N_4O_3S$ $[M + H]^+$: calculated 425.0890 found 425.0893. HPLC purity = 98.4%.

*N-(3-bromophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8d**)*. White solid. Yield 79.4%. Mp: 157-160°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.21 (dd, J = 8.7 Hz, 7.8 Hz, 1H), 7.35 (d, J = 6.6 Hz, 1H), 7.45 (d, J = 7.5 Hz, 1H), 7.50 (dd, J = 8.1 Hz, 7.8 Hz, 1H), 7.58 (d, J = 8.1 Hz, 1H), 7.70 (s, 1H), 7.75 (d, J = 8.7 Hz, 1H), 7.79 (dd, J = 7.5Hz, 6.6 Hz, 1H), 7.90 (s, 1H), 8.03 (s, 1H), 10.51(s, 1H), 10.58 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}BrN_4O_3S$ $[M + H]^+$: calculated 435.0121 found 435.0124. HPLC purity = 98.1%.

General procedures for the preparation of *N*-(2-(substituted phenylcarbamoyl)phenyl)isonicotinamide 9 and *N*-(2-(substituted phenylcarbamoyl)phenyl) nicotinamide 10

Compound **4** (1 mmol) was dissolved in 10 mL dichloromethane, and 1 mL triethylamine was added to the solution. Nicotinoyl chloride or isonicotinoyl chloride (1 mmol) dichloromethane solution was then slowly added at 0°C. The mixture was stirred for 2 h at room temperature and saturated $NaHCO_3$ aqueous solution was then added with stirring until no bubbles were formed. The mixture was extracted with dichloromethane (4 × 15 mL) and the combined organic layers were washed with a saturated NaCl aqueous solution. The methylene chloride layers were dried in situ using Na_2SO_4 and concentrated in vacuo. The crude product was purified by chromatography on silica gel to afford **9** and **10**.

*N-(2-((4-chlorophenyl)carbamoyl)phenyl) isonicotinamide (**9a**)*. White solid. Yield 65.3%. Mp: 231-233°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.35 (td, J = 7.8 Hz, 1.2 Hz, 1H), 7.42 (d, J = 9.0 Hz, 2H), 7.64 (td, J = 7.8 Hz, 1.8 Hz, 1H), 7.75 (d, J = 9.0 Hz, 2H), 7.80 (dd, J = 4.8 Hz, 1.8 Hz, 2H), 7.90 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 8.29 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 8.81 (dd, J = 4.8 Hz, 1.8 Hz, 2H), 10.65 (s, 1H), 11.56 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}Cl\ N_3O_2$ $[M + H]_+$: calculated 352.0847 found 352.0850. HPLC purity = 98.9%.

*N-(2-((3-(trifluoromethyl)phenyl)carbamoyl)phenyl) isonicotinamide (**9b**)*. White solid. Yield 53.5%. Mp: 229-231°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.37 (t, J = 7.8 Hz, 1H), 7.47 (d, J = 7.8 Hz, 1H), 7.60 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.80 (dd, J = 6.0 Hz, 1.2 Hz, 2H), 7.89 (d, J = 7.8 Hz, 1H), 8.00 (d, J = 8.4 Hz, 1H), 8.11(s, 1H), 8.20 (d, J = 7.8 Hz, 1H), 8.80 (dd, J = 6.0 Hz, 1.2 Hz, 2H), 10.78 (s, 1H), 11.38 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{15}F_3N_3O_2$ $[M + H]^+$: calculated 386.1111 found 386.1113. HPLC purity = 99.3%.

*N-(2-((3-bromophenyl)carbamoyl)phenyl) isonicotinamide (**9c**)*. White solid. Yield 51.7%. Mp: 231-233°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.30-7.33 (m, 2H), 7.36 (t, J = 7.8 Hz, 1H), 7.64 (t, J = 7.8Hz, 1H), 7.69 (d, J = 7.2 Hz, 1H), 7.80(d, J = 6.0 Hz, 2H), 7.87 (d, J = 7.8 Hz, 1H), 8.00 (s, 1H), 8.23 (d, J = 7.8 Hz, 1H), 8.82 (d, J = 6.0 Hz, 2H), 10.65 (s, 1H), 11.43 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}BrN_3O_2$ $[M + H]^+$: calculated 396.0342 found 396.0346. HPLC purity = 96.5%.

*N-(2-((4-chlorophenyl)carbamoyl)phenyl) nicotinamide (**10a**)*. White solid. Yield 55.4%. Mp: 207-210°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.34 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.60 (dd, J = 7.8 Hz, 5.1 Hz, 1H), 7.63 (dd, J = 8.4 Hz, 7.2 Hz, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.8 Hz, 1H), 8.24 (dt, J = 7.8 Hz, 1.5 Hz, 1H), 8.27 (d, J = 8.4 Hz, 1H), 8.78 (dd, J = 5.1 Hz, 1.5 Hz, 1H), 9.07 (d, J = 1.5Hz, 1H), 10.63 (s, 1H), 11.47 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}ClN_3O_2$ $[M + H]^+$: calculated 352.0847 found 352.0849. HPLC purity = 99.3%.

*N-(2-((3-(trifluoromethyl)phenyl)carbamoyl)phenyl) nicotinamide (**10b**)*. White solid. Yield 63.9%. Mp: 218-220°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.36 (dd,

J = 7.8 Hz, 7.2 Hz, 1H), 7.47 (d, *J* = 7.8 Hz, 1H), 7.58-7.65 (m, 3H), 7.88 (d, *J* = 7.8 Hz, 1H), 8.00 (d, *J* = 8.4 Hz, 1H), 8.14 (s, 1H), 8.19 (d, *J* = 8.4 Hz, 1H), 8.24 (dt, *J* = 8.4 Hz, 1.8 Hz, 1H), 8.77 (dd, *J* = 5.4 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 10.78 (s, 1H), 11.30 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{15}F_3N_3O_2$ $[M + H]^+$: calculated 386.1111 found 386.1115. HPLC purity = 99.3%.

*N-(2-((3-bromophenyl)carbamoyl)phenyl) nicotinamide (**10c**)*. White solid. Yield 51.3%. Mp: 199-201°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.30-7.36 (m, 3H), 7.60 (dd, *J* = 7.8 Hz, 4.8 Hz, 1H), 7.64 (td, *J* = 8.1 Hz, 1.8 Hz, 1H), 7.69 (dd, *J* = 7.2 Hz, 1.8 Hz, 1H), 7.86 (d, *J* = 8.1Hz, 1H), 8.02 (s, 1H), 8.21 (d, *J* = 8.1 Hz, 1H), 8.25 (dt, *J* = 7.8 Hz, 1.8 Hz, 1H), 8.78 (dd, *J* = 4.8 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 10.64 (s, 1H), 11.53 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}BrN_3O_2$ $[M + H]^+$: calculated 396.0342 found 396.0348. HPLC purity = 95.1%.

*N-(2-((3-hydroxyphenyl)carbamoyl)phenyl) nicotinamide (**10d**)*. White solid. Yield 52.2%. Mp: 207-210°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.53 (dt, *J* = 7.2 Hz, 1.8 Hz, 1H), 7.09-7.14 (m, 2H), 7.30-7.34 (m, 2H), 7.60-7.64 (m, 2H), 7.88 (d, *J* = 7.8 Hz, 1H), 8.25 (dt, *J* = 7.8 Hz, 1.8 Hz, 1H), 8.33 (d, *J* = 7.8 Hz, 1H), 8.79 (dd, *J* = 4.8 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 9.45 (s, 1H), 10.41(s, 1H), 11.61 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{16}N_3O_3$ $[M + H]^+$: calculated 334.1186 found 334.1189. HPLC purity = 96.2%.

General procedures for the preparation of target compound *N*-substituted phenyl-2-((2-hydroxy-5-nitrobenzyl)amino)benzamide 11

To a solution of compound **4** (0.75 mmol) and acetic acid (50 µL, 2.9 mmol) in methanol (10 mL), 2-hydroxy-5-nitrobenzaldehyde (0.17 g, 1 mmol) was added and stirred at ambient temperature for 8 h. The resulting reaction mixtures were treated with 1.0 mL of a solution of $NaCNBH_3$ in methyl alcohol (10 mL) and stirred for a further 24 h at ambient temperature. The solvent was evaporated under reduced pressure and the products were diluted with dichloromethane (15 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 10 mL) followed by saturated NaCl (2 × 10 mL). The organic layer was dried over Na_2SO_4 and filtered. The crude product was purified by column chromatography to afford **11**.

*N-(4-chlorophenyl)-2-((2-hydroxy-5-nitrobenzyl) amino)benzamide (**11a**)*. Yellow solid. Yield 52.7%. Mp: 168-169°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 4.40 (d, *J* = 5.4 Hz, 2H), 6.63-6.67 (m, 2H), 7.01 (d, *J* = 8.7 Hz, 1H), 7.29 (dd, *J* = 8.7 Hz, 7.2 Hz, 1H), 7.40 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.76(d, *J* = 8.7 Hz, 2H), 7.86 (br t, d = 5.4 Hz, 1H), 8.04 (dd, *J* = 9.0 Hz, 3.0 Hz, 1H), 8.08 (d, *J* = 3.0Hz, 1H), 10.28 (s, 1H), 11.41 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{17}ClN_3O_4$ $[M + H]^+$: calculated 398.0902 found 398.0904. HPLC purity = 98.0%.

*N-(3-bromophenyl)-2-((2-hydroxy-5-nitrobenzyl) amino)benzamide (**11b**)*. Yellow solid. Yield 55.6%. Mp: 146-149°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 4.41 (d, *J* = 6.0 Hz, 2H), 6.63 (d, *J* = 8.7 Hz, H), 6.66 (dd, *J* = 7.8 Hz, 7.2 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 7.27-7.33 (m, 3H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.69 (d, *J* = 7.8 Hz, 1H), 7.87 (br t, *J* = 6.0 Hz, 1H), 8.05 (dd, *J* = 8.7 Hz, 2.4 Hz, 1H), 8.08 (d, *J* = 2.4 Hz, 1H), 8.10 (s, 1H), 10.30 (s, 1H), 11.41 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{17}BrN_3O_4$ $[M + H]^+$: calculated 442.0397 found 442.0399. HPLC purity = 96.8%.