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Research Article

Synthesis, Activity, and Docking Study of Novel Phenylthiazole-Carboxamido Acid Derivatives as FFA2 Agonists

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Free fatty acid receptor 2 (FFA2), also known as GPR43, is activated by short-chain fatty acids (SCFAs) that are mainly produced by the gut microbiota through the fermentation of undigested carbohydrates and dietary fibers. FFA2 currently appears to be a potential target in the management of obesity, diabetes, inflammatory diseases, and cancer. In the study, a series of novel phenylthiazole-carboxamido acid derivatives has been synthesized and evaluated as potential orthosteric FFA2 ligands for the study of structure–activity relationships. Compound 6e was found to exhibit the twofold potent agonistic activity in the stable hFFA2-transfected CHO-K1 cells (EC_{50} = 23.1 μM) as that of positive control propionate (EC_{50} = 43.3 μM). We also reported the results of mutagenesis studies based on the crystal structure of hFFA1 bound to TAK-875 at 2.3 Å resolution to identify important residues for orthosteric agonist 6e inducing FFA2 activation.

Key words: agonist activity, FFA2, flexible docking, phenylthiazolecarboxamido acids

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Free fatty acid receptor 2 (FFA2), also known as GPR43, is a member of G protein-coupled receptor (GPCR) superfamily (1,2). FFA2 is activated by short-chain fatty acids (SCFAs, carbon number ≤6) that are mainly produced by the gut microbiota through the fermentation of undigested carbohydrates and dietary fibers (3,4). Acetate, propionate, and butyrate are the most potent natural ligands for FFA2 that could induce coupling to both Gαi and Gαq (5). FFA2 is highly expressed in adipose tissue and gastrointestinal tract and mediates SCFA-promoted GLP-1 release. FFA2 agonists promote GLP-1 release, and they furthermore increase glucose uptake in adipocytes, thus providing support for the notion that FFA2 agonists could be of interest for the treatment of obesity and diabetes. Additionally, FFA2 is also expressed in immune cells especially in neutrophils and mediates the chemotactic effects of SCFAs on neutrophils. Therefore, FFA2 currently appears to be a potential target in the management of several pathological conditions such as obesity, diabetes, and inflammatory diseases (6,7).

The activated potencies of SCFAs for FFA2 are low, in the high micromolar to millimolar concentrations (4). Previously, Amgen Inc. has discovered phenylacetamide derivative AMG7703 as the first class of non-SCFA allosteric agonist of FFA2 which induced positive cooperativity with natural SCFAs (8). Euroscreen S.A. has patented a compound series of agonists including compounds **1** and **2** with potent FFA2-activated potency and claimed their uses in treating metabolic disorders (9). Additionally, compound **3** as a constrained lactam analog has been explored to increased GLP-1 secretion from a rat lower intestinal cell preparation (10). These reported agonists all possessed the moiety of thiazol-2-amine group, which gave us a guideline to the structural design of FFA2 modulators.

Here, a series of novel phenylthiazole-carboxamido acid derivatives as potential orthosteric FFA2 ligands has been synthesized and evaluated for the study of structure–activity relationship (SAR). In the study, based on the crystal structure of hFFA1 bound to TAK-875 at 2.3 Å resolution, we also reported the results of mutagenesis studies to identify important residues for novel orthosteric FFA2 agonist **6e**-induced receptor activation (11) (Figure 1).

Methods and Materials

FFA2 assay in stably transfected CHO-K1 cells

CHO-K1 cells expressing human recombinant FFA2 grown prior to the test in media without antibiotic are detached by gentle flushing with PBS–EDTA (5 mM EDTA), recovered by centrifugation, and resuspended in assay buffer

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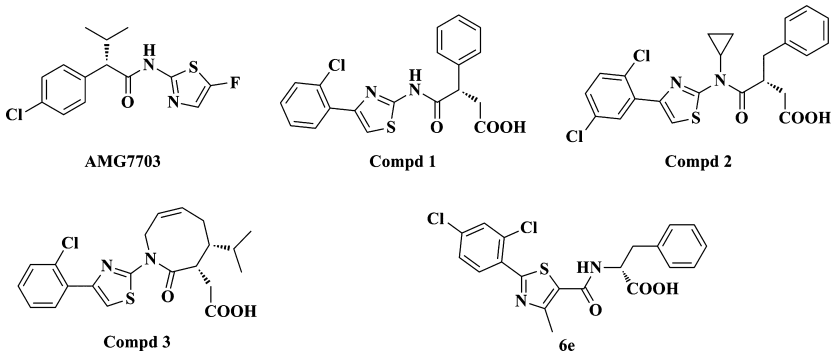


Figure 1: Selected small-molecule FFA2 agonists and compound **6e** in this study.

(KRH: 5 mM KCl, 1.25 mM $MgSO_4$, 124 mM NaCl, 25 mM HEPES, 13.3 mM glucose, 1.25 mM KH_2PO_4, 1.45 mM $CaCl_2$, 0.5 g/L BSA). Dose–response curves are performed in parallel with the reference compounds. For agonist test (96-well): 12 μL of cells was mixed with 6 μL of the test compound at increasing concentrations and 6 μL of forskolin and then incubated for 30 min at room temperature. After the addition of the lysis buffer and incubation for 1 h, cAMP concentrations are estimated according to the manufacturer specification with the HTRF kit (Cisbio International, Paris, France). Agonist activity of test compound will be expressed as a percentage of the activity of the reference agonist at its EC_{100} concentration.

Chemical synthesis

Chemical reagents of analytical grade were purchased from Chengdu Changzheng Chemical Factory (Sichuan, P. R. China). TLC was performed on 0.20 mm silica gel 60 F_{254} plates (Qingdao Ocean Chemical Factory, Shandong, China). NMR was recorded at 400 MHz on a Waters spectrometer and reported in parts per million. Chemical shifts (δ) are quoted in ppm relative to TMS as an internal standard, where (δ) TMS = 0.00 ppm. The multiplicity of the signal is indicated as s, singlet; brs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet, defined as all multipeak signals where overlap or complex coupling of signals makes definitive descriptions of peaks difficult. Mass spectra were measured by Q-TOF Premier mass spectrometer utilizing electrospray ionization (ESI) (Micromass, Manchester, UK). Room temperature (RT) was within the range 20–25 °C. The purity was analyzed by HPLC system (Waters 2695, Separations Module) with a photodiode array detector (Waters 2996, Milford, MA, USA), and the chromatographic column was a reversed-phase C18 column (Waters, 150 mm × 4.6 mm, i.d. 5 μm). All compounds were supplied in HPLC-degree methanol with 10 μL, which were injected on a partial loop fill with isocratic elution from 90% methanol and 10% water to 10% methanol and 90% water (containing 0.1% formic acid) within 30 min at a flow rate of 1 mL/min. The purity of all tested compounds was ≥95% according to our analytical HPLC method.

Synthesis of benzothioamides

A Schlenk tube was charged under N_2 atmosphere with the corresponding benzamide (0.2 mmol, 2.0 eq), Lawesson's reagent (0.1 mmol, 1.0 eq), and toluene (4 mL) and stirred at 60 °C. After 6 h, when the reaction was completed as determined by TLC, neutral aluminum oxide (2.0 g) was added and the solvent was evaporated. The resulting solid was placed on a short column packed with silica. The column was eluted with EtOAc (20–50 mL) to give benzothioamides without further purification.

Synthesis of 2-phenylthiazole-4-carboxylic acids (1–3)

To a solution of the corresponding benzothioamides (5.0 mmol) in MeOH (~10 mL) was added ethyl 3-bromo-2-oxopropanoate (0.77 mL, 5.5 mmol) at room temperature. The reaction mixture was stirred for 4 h at 80 °C and then cooled to room temperature. The resulting precipitate was isolated by filtration and washed with Et_2O to give a solid product. The solid was dissolved in EtOH (~15 mL), and aqueous lithium hydroxide (2 M, 10 mL) was added. The mixture was stirred overnight at room temperature and neutralized by 1 N HCl to pH 4–5, resulting in the formation of a solid precipitate. The solid product was washed with water and dried *in vacuo* to give a pure solid product.

2-(2-Chlorophenyl)thiazole-4-carboxylic acid (1). Yield: 78.9% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.23 (s, 1H), 8.66 (s, 1H), 8.21–7.19 (m, 1H), 7.70–7.68 (m, 1H), 7.58–7.53 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.73, 162.03, 146.95, 131.66, 130.90, 130.71, 130.09, 127.84. MS (ESI), m/z: 238.2 $[M-H]^-$.

2-(4-Chlorophenyl)thiazole-4-carboxylic acid (2). Yield: 61.2% for two steps; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.32 (s, 1H), 8.32 (s, 1H), 7.95 (d, J = 8.5 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.07, 161.91, 148.15, 135.31, 131.30, 129.36, 129.18, 128.10. MS (ESI), m/z: 238.1 $[M-H]^-$.

2-(2,4-Dichlorophenyl)thiazole-4-carboxylic acid (3). Yield: 74.1% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ

13.26 (s, 1H), 8.67 (s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 7.88 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.95, 161.66, 147.00, 135.38, 132.02, 131.76, 130.29, 130.12, 129.69, 128.14. MS (ESI), m/z: 272.1 [M−H]$^-$.

Synthesis of 2-phenylthiazole-5-carboxylic acids (4–6)

To a suspension of the corresponding benzothioamides (5.0 mmol) in EtOH (~10 mL) was added ethyl 2-chloro-3-oxobutanoate (0.69 mL, 5.0 mmol). The solution was stirred and refluxed for 4 h, and then, the solvent was removed under reduced pressure. The solid was stirred in cooled hexane (15 mL) for 30 min, filtered, and washed with hexane (2 × 10 mL) to give a crude product without any further purification. To a cooled solution of crude solid (10 mmol) in EtOH (~40 mL) was added a solution of lithium hydroxide (2 M, 20 mL). The mixture was stirred overnight at room temperature, and after the evaporation of half of EtOH solvent, the solution was neutralized by 1 N HCl to pH 4–5, resulting in the formation of a solid precipitate. The solid product was washed with water and dried *in vacuo* to give a pure solid product.

2-(2-Chlorophenyl)-4-methylthiazole-5-carboxylic acid (4). Yield: 89.3% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.49 (s, 1H), 8.00 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 2.68 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.93, 162.80, 159.51, 135.80, 131.10, 129.37, 129.21, 128.15, 127.60, 123.45, 17.01. MS (ESI), m/z: 252.2 [M−H]$^-$.

2-(4-Chlorophenyl)-4-methylthiazole-5-carboxylic acid (5). Yield: 86.6% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.53 (brs, 1H), 8.28 (d, J = 8.0 Hz, 1H), 7.83 (s, 1H), 7.59 (d, J = 8.0 Hz, 1H), 2.69 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.79, 162.08, 158.00, 135.64, 131.91, 131.68, 130.23, 129.34, 128.03, 124.64, 16.83. MS (ESI), m/z: 252.3 [M−H]$^-$.

2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxylic acid (6). Yield: 92.9% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 1H), 8.68 (t, J = 7.2 Hz, 1H), 8.26 (dd, J = 7.2, 2.0 Hz, 1H), 7.69–7.67 (m, 1H), 7.57–7.51 (m, 1H), 3.92 (d, J = 5.2 Hz, 1H), 2.67 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.44, 161.75, 161.55, 154.72, 132.19, 131.35, 131.11, 130.99, 128.33, 127.68, 41.78, 17.37. MS (ESI), m/z: 286.1 [M−H]$^-$.

Synthesis of phenylthiazole-carboxamido acids (*1a-6f*)

To a solution of the corresponding 2-phenylthiazole-4(5)-carboxylic acids (1.00 mmol) and amino acid ester (1.05 mmol) in DMF (~5 mL) were added EDCI (287.6 mg, 1.50 mmol) and DMAP (244.3 mg, 2.00 mmol). The reaction mixture was stirred overnight at room temperature. When the reaction was completed as determined by TLC, the mixture was poured into the ice water and then the precipitate was formed. The resulting precipitate was isolated by filtration and washed with water to give a crude product. The solid (0.3 mmol) was dissolved in EtOH (~5 mL), and aqueous lithium hydroxide (2 M, 0.6 mL) was added. The mixture was stirred overnight at room temperature and neutralized by 1 N HCl to pH 4–5, resulting in the formation of a solid precipitate. The solid product was washed by water and EtOH and dried *in vacuo* to give a pure solid product.

2-(2-(2-Chlorophenyl)thiazole-4-carboxamido)acetic acid (1a). Yield: 31.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.69 (s, 1H), 8.87 (s, 1H), 8.49 (s, 1H), 8.43 (s, 1H), 7.69–7.56 (s, 3H), 3.98 (d, J = 4.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.09, 162.47, 160.59, 149.00, 131.68, 130.97, 130.92, 130.74, 130.56, 127.70, 125.92, 40.87. MS (ESI), m/z: 295.1 [M−H]$^-$.

2-(2-(4-Chlorophenyl)thiazole-4-carboxamido)acetic acid (2a). Yield: 44.0% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.72 (s, 1H), 8.82 (t, J = 6.0 Hz, 1H), 8.37 (s, 1H), 8.09 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H), 3.97 (d, J = 8.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.07, 165.88, 160.50, 150.20, 135.36, 131.23, 129.33, 128.11, 124.86, 40.88. MS (ESI), m/z: 295.1 [M−H]$^-$.

2-(2-(2,4-Dichlorophenyl)thiazole-4-carboxamido) acetic acid (3a). Yield: 34.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.78 (s, 1H), 8.90 (s, 1H), 8.51–8.47 (m, 2H), 7.89 (s, 1H), 7.67 (d, J = 8.0 Hz, 1H), 3.97 (d, J = 4.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.09, 161.39, 160.48, 149.04, 135.40, 132.07, 131.76, 130.16, 129.56, 128.00, 126.15, 40.94. MS (ESI), m/z: 329.2 [M−H]$^-$.

2-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)acetic acid (4a). Yield: 36.3% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 1H), 8.68 (t, J = 7.2 Hz, 1H), 8.26 (dd, J = 7.2, 2.0 Hz, 1H), 7.69–7.67 (m, 1H), 7.57–7.51 (m, 1H), 3.92 (d, J = 5.2 Hz, 1H), 2.67 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.44, 161.75, 161.55, 154.72, 132.19, 131.35, 131.11, 130.99, 128.33, 127.68, 41.78, 17.37. MS (ESI), m/z: 309.1 [M−H]$^-$.

2-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)acetic acid (5a). Yield: 29.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 1H), 8.59 (t, J = 8.0 Hz, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.59 (d,

J = 8.0 Hz, 2H), 3.91 (d, J = 8.0 Hz, 1H), 2.64 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.91, 161.14, 159.92, 154.23, 135.39, 131.67, 130.21, 129.45, 128.08, 127.48, 41.30, 16.83. MS (ESI), m/z: 309.1 [M−H]$^-$.

2-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)acetic acid (6a). Yield: 48.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.86 (brs, 1H), 8.68 (s, 1H), 8.28 (s, 1H), 7.85 (s, 1H), 7.61 (s, 1H), 3.93 (m, 2H), 2.67 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.93, 164.76, 161.16, 155.44, 135.48, 131.17, 129.41, 127.98, 126.23, 41.31, 17.02. MS (ESI), m/z: 343.5 [M−H]$^-$.

3-(2-(2-Chlorophenyl)thiazole-4-carboxamido) propanoic acid (1b). Yield: 41.2% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.29 (s, 1H), 8.63 (t, J = 8.0 Hz, 1H), 8.45 (s, 1H), 8.41–8.38 (m, 1H), 7.69–7.67 (m, 1H), 7.56–7.54 (m, 2H), 3.52 (q, J = 8.0 Hz, 2H), 2.56 (t, J = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.98, 162.37, 160.29, 149.39, 131.63, 131.02, 130.90, 130.71, 130.59, 127.68, 125.52, 34.98, 33.81. MS (ESI), m/z: 310.0 [M−H]$^-$.

3-(2-(4-Chlorophenyl)thiazole-4-carboxamido) propanoic acid (2b). Yield: 22.6% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.30 (s, 1H), 8.63 (t, J = 8.0 Hz, 1H), 8.45 (s, 1H), 8.40 (s, 1H), 7.68–7.67 (m, 1H), 7.55 (m, 2H), 3.52 (q, J = 8.0 Hz, 2H), 2.56 (t, J = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.98, 162.37, 160.29, 149.39, 131.63, 131.02, 130.90, 130.71, 130.59, 127.68, 125.52, 34.98, 33.81. MS (ESI), m/z: 309.8 [M−H]$^-$.

3-(2-(2,4-Dichlorophenyl)thiazole-4-carboxamido) propanoic acid (3b). Yield: 29.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.66 (s, 1H), 8.44 (s, 1H), 7.85 (s, 1H), 7.63 (s, 1H), 3.51 (m, 2H), 2.55 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.01, 161.36, 160.30, 149.32, 135.38, 132.11, 131.74, 130.11, 129.54, 127.96, 125.81, 35.00, 33.79. MS (ESI), m/z: 343.2 [M−H]$^-$.

3-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)propanoic acid (4b). Yield: 46.5% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.30 (s, 1H), 8.41 (s, 1H), 8.24 (m, 1H), 7.66 (m, 1H), 7.52 (m, 2H), 3.44 (m, 2H), 2.63 (s, 3H), 2.51 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.80, 160.90, 160.72, 153.64, 131.61, 130.83, 130.79, 130.57, 127.81, 127.74, 35.67, 33.61, 16.81. MS (ESI), m/z: 323.6 [M−H]$^-$.

3-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)propanoic acid (5b). Yield: 25.5% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.30 (s, 1H), 8.35 (t, J = 4.0 Hz, 1H), 7.95 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 3.44 (q, J = 8.0 Hz, 2H), 2.60 (s, 3H), 2.54–2.52 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.80, 164.41, 160.82, 154.83, 135.39, 131.22, 129.39, 127.90, 126.80, 35.67, 33.62, 16.96. MS (ESI), m/z: 323.4 [M−H]$^-$.

3-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)propanoic acid (6b). Yield: 33.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.59 (s, 1H), 8.42 (s, 1H), 8.29–8.25 (m, 1H), 7.84 (s, 1H), 7.59 (d, J = 8.0 Hz, 1H), 3.45 (d, J = 8.0 Hz, 2H), 2.62 (s, 3H), 2.53 -2.51 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.80, 160.79, 159.57, 153.69, 135.30, 131.63, 131.59, 130.19, 129.49, 128.07, 35.68, 33.59, 16.83, 16.77. MS (ESI), m/z: 357.2 [M−H]$^-$.

(R)-2-(2-(2-Chlorophenyl)thiazole-4-carboxamido) butanoic acid (1c). Yield: 23.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.83 (s, 1H), 8.55 (s, 1H), 8.44 (s, 1H), 7.68 (s, 1H), 7.57 (s, 1H), 4.41 (s, 1H), 1.90 (d, J = 2.4 Hz, 2H), 0.94 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.18, 162.55, 160.29, 149.00, 131.66, 131.13, 130.90, 130.71, 130.56, 127.75, 126.00, 53.38, 24.11, 10.41. MS (ESI), m/z: 323.5 [M−H]$^-$.

(R)-2-(2-(4-Chlorophenyl)thiazole-4-carboxamido) butanoic acid (2c). Yield: 35.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.82 (s, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.38 (s, 1H), 8.11 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.0 Hz, 2H), 4.40 (q, J = 5.6 Hz, 1H), 1.90 (dq, J = 12.0, 4.0 Hz, 2H), 0.94 (t, J = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.17, 165.95, 160.22, 150.16, 135.37, 131.21, 129.29, 128.21, 124.94, 53.36, 24.13, 10.41. MS (ESI), m/z: 323.2 [M−H]$^-$.

(R)-2-(2-(2,4-Dichlorophenyl)thiazole-4-carboxamido) butanoic acid (3c). Yield: 37.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.83 (s, 1H), 8.52 (m, 3H), 7.88 (s, 1H), 7.66 (d, J = 8.0 Hz, 1H), 4.40 (s, 1H), 1.90 (d, J = 2.4 Hz, 2H), 0.94 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.16, 161.47, 160.23, 149.03, 135.39, 132.27, 131.75, 130.13, 129.57, 128.02, 126.28, 53.41, 24.10, 10.46. MS (ESI), m/z: 357.2 [M−H]$^-$.

(R)-2-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)butanoic acid (4c). Yield: 28.3% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.69 (s, 1H), 8.58 (d, J = 8.0 Hz, 1H), 8.25 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 4.0 Hz, 1H), 7.54 (m, 2H), 4.27 (d, J = 4.0 Hz, 1H), 2.71 (s, 3H), 1.87–1.77 (m, 2H), 0.97 (t, J = 6.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.20, 161.31,

160.97, 153.82, 131.61, 130.83, 130.60, 130.57, 127.81, 127.54, 54.27, 23.79, 16.82, 10.77. MS (ESI), m/z: 337.1 $[M-H]^-$.

(R)-2-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)butanoic acid (5c). Yield: 45.2% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.75 (s, 1H), 8.51 (d, J = 4.0 Hz, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 4.26 (d, J = 4.0 Hz, 1H), 2.62 (s, 3H), 1.87–1.76 (m, 2H), 0.96 (t, J = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.20, 164.63, 161.20, 155.03, 135.41, 131.23, 129.39, 127.93, 126.54, 54.31, 23.81, 16.97, 10.71. MS (ESI), m/z: 337.3 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)butanoic acid (6c). Yield: 33.5% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.91 (brs, 1H), 8.59 (m, 1H), 8.29 (m, 1H), 7.86 (m, 1H), 7.61 (m, 1H), 4.28 (s, 1H), 2.65 (s, 3H), 1.86–1.77 (m, 2H), 0.97 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.20, 164.63, 161.20, 155.03, 135.41, 131.23, 129.39, 127.93, 126.54, 54.31, 23.81, 16.97, 10.71. MS (ESI), m/z: 371.2 $[M-H]^-$.

(R)-2-(2-(2-(2-Chlorophenyl)thiazole-4-carboxamido)-2-phenylacetic acid (1d). Yield: 13.8% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.26 (s, 1H), 8.85 (d, J = 8.0 Hz, 1H), 8.53 (s, 1H), 8.38 (m, 1H), 7.70 (m, 1H), 7.57 (m, 2H), 7.50 (d, J = 8.0 Hz, 2H), 7.41–7.34 (m, 3H), 5.61 (d, J = 8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.53, 162.78, 159.65, 148.63, 137.40, 131.74, 131.13, 130.90, 130.75, 130.49, 128.53, 127.94, 127.85, 127.67, 126.43, 56.15. MS (ESI), m/z: 371.1 $[M-H]^-$.

(R)-2-(2-(4-Chlorophenyl)thiazole-4-carboxamido)-2-phenylacetic acid (2d). Yield: 56.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.29 (s, 1H), 8.80 (d, J = 8.0 Hz, 1H), 8.41 (s, 1H), 8.09 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H), 7.50 (d, J = 8.0 Hz, 2H), 7.41–7.34 (m, 3H), 5.60 (d, J = 8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.52, 166.17, 159.57, 149.78, 137.40, 135.42, 131.11, 129.35, 128.53, 128.24, 127.94, 127.66, 125.38, 56.15. MS (ESI), m/z: 371.1 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)thiazole-4-carboxamido)-2-phenylacetic acid (3d). Yield: 51.3% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.26 (s, 1H), 8.89 (d, J = 8.0 Hz, 1H), 8.55 (s, 1H), 8.45 (d, J = 8.0 Hz, 2H), 7.90 (d, J = 4.0 Hz, 1H), 7.67 (dd, J = 8.0, 4.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.41–7.34 (m, 3H), 5.62 (d, J = 8.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.52, 161.69, 159.62, 148.67, 137.34, 135.44, 132.29, 131.77, 130.16, 129.51, 128.51, 128.11, 127.94, 127.71, 126.70, 56.16. MS (ESI), m/z: 405.2 $[M-H]^-$.

(R)-2-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)-2-phenyl-acetic acid (4d). Yield: 42.5% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.05 (s, 1H), 9.09 (d, J = 8.0 Hz, 1H), 8.25 (dd, J = 8.0, 4.0 Hz, 1H), 7.67–7.66 (m, 1H), 7.56–7.49 (m, 4H), 7.42–7.33 (m, 3H), 5.54 (d, J = 8.0 Hz, 1H), 2.65 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.54, 161.14, 160.99, 154.14, 136.67, 131.63, 130.85, 130.58, 130.53, 128.46, 128.09, 128.00, 127.82, 127.30, 57.07, 16.91. MS (ESI), m/z: 385.2 $[M-H]^-$.

(R)-2-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)-2-phenyl-acetic acid (5d). Yield: 21.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.06 (s, 1H), 9.00 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.58 (d, J = 8.0 Hz, 2H), 7.50 (d, J = 8.0 Hz, 1H), 7.42–7.33 (m, 3H), 5.52 (d, J = 8.0 Hz, 1H), 2.62 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d6) δ 171.52, 164.83, 160.90, 155.37, 136.66, 135.44, 131.20, 129.40, 128.47, 128.02, 127.94, 126.32, 57.07, 17.05. MS (ESI), m/z: 385.2 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)-2-phenyl-acetic acid (6d). Yield: 18.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.16 (s, 1H), 9.11 (d, J = 8.0 Hz, 1H), 8.28 (d, J = 8.0 Hz, 1H), 7.87 (s, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.42–7.35 (m, 3H), 5.54 (d, J = 4.0 Hz, 1H), 2.64 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.51, 160.89, 160.01, 154.22, 136.62, 135.37, 131.77, 131.69, 130.25, 129.51, 128.47, 128.12, 128.09, 128.01, 127.56, 57.08, 16.87. MS (ESI), m/z: 419.2 $[M-H]^-$.

(R)-2-(2-(2-Chlorophenyl)thiazole-4-carboxamido)-3-phenylpropanoic acid (1e). Yield: 43.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.30 (brs, 1H), 8.48 (d, J = 8.0 Hz, 1H), 8.44 (s, 1H), 8.34 (m, 1H), 7.68 (m, 1H), 7.56 (m, 2H), 7.27–7.18 (m, 5H), 4.68–4.67 (m, 1H), 3.23 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.62, 162.48, 159.90, 149.00, 137.80, 131.67, 130.93, 130.91, 130.76, 130.49, 129.19, 128.14, 127.77, 126.35, 125.86, 53.56, 36.38. MS (ESI), m/z: 385.2 $[M-H]^-$.

(R)-2-(2-(4-Chlorophenyl)thiazole-4-carboxamido)-3-phenylpropanoic acid (2e). Yield: 23.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.02 (brs, 1H), 8.49 (d, J = 8.0 Hz, 1H), 8.32 (s, 1H), 8.06 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H), 7.28–7.20 (m, 5H), 4.71–4.70 (m, 1H), 3.23 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.63, 165.90, 159.99, 150.01, 137.63, 135.39, 131.17,

129.34, 129.10, 128.24, 128.13, 126.47, 124.95, 53.44, 36.25. MS (ESI), m/z: 385.1 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)thiazole-4-carboxamido)-3-phenylpropanoic acid (3e). Yield: 18.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.47 (brs, 1H), 8.50 (d, J = 8.0 Hz, 1H), 8.46 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.87 (s, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.26–7.18 (m, 5H), 4.65 (m, 1H), 3.22 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.56, 161.41, 159.78, 149.10, 137.88, 135.39, 132.03, 131.76, 130.18, 129.51, 129.19, 128.11, 128.05, 126.31, 126.09, 53.69, 36.43. MS (ESI), m/z: 419.2 $[M-H]^-$.

(R)-2-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)-3-phenyl propanoic acid (4e). Yield: 40.0% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.90 (s, 1H), 8.65 (d, J = 8.0 Hz, 1H), 8.22 (d, J = 4.0 Hz, 1H), 7.66 (m, 1H), 7.52 (m, 2H), 7.31–7.22 (m, 5H), 4.61 (m, 1H), 3.23–3.02 (m, 2H), 2.48 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.70, 161.05, 153.71, 137.88, 131.63, 130.82, 130.61, 130.53, 129.08, 128.19, 127.80, 127.48, 126.41, 54.32, 36.05, 16.66. MS (ESI), m/z: 435.9 $[M + Cl]^-$.

(R)-2-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)-3-phenyl propanoic acid (5e). Yield: 35.0% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.90 (s, 1H), 8.57 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.0 Hz, 2H), 7.58 (d, J = 8.0 Hz, 2H), 7.31–7.22 (m, 5H), 4.58 (m, 1H), 3.23–3.00 (m, 2H), 2.44 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.52, 164.83, 160.90, 155.37, 136.66, 135.44, 131.20, 129.40, 128.47, 128.02, 127.94, 126.32, 57.07, 17.05. MS (ESI), m/z: 399.3 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)-3-phenylpropanoic acid (6e). Yield: 41.0% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.85 (s, 1H), 9.47 (d, J = 8.0 Hz, 1H), 9.05 (d, J = 8.0 Hz, 1H). 8.67 (s, 1H), 8.41 (d, J = 8.0 Hz, 1H), 8.12–8.02 (m, 5H), 5.42 (m, 1H), 4.04–3.82 (m, 2H), 3.28 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.98, 162.24, 161.24, 155.06, 139.17, 136.67, 133.02, 132.96, 131.53, 130.81, 130.38, 129.49, 129.41, 129.07, 127.72, 55.64, 37.36, 17.93. MS (ESI), m/z: 433.3 $[M-H]^-$.

(R)-2-(2-(2-Chlorophenyl)thiazole-4-carboxamido)-3-(1H-indol-3-yl) propanoic acid (1f). Yield: 28.5% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 1H), 8.42 (s, 1H), 8.31 (d, J = 4.0 Hz, 1H), 8.13 (dd, J = 8.0, 4.0 Hz, 1H), 7.65 (dd, J = 8.0, 4.0 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.53–7.48 (m, 2H), 7.29 (d, J = 8.0 Hz, 1H), 7.14 (m, 1H), 7.00 (t, J = 8.0 Hz, 1H), 6.87 (t, J = 8.0 Hz, 1H), 4.53 (d, J = 8.0 Hz, 1H), 3.40–3.27 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.92, 162.30, 159.43, 149.65, 135.90, 131.57, 130.84, 130.80, 130.71, 130.46, 127.87, 127.75, 125.30, 123.50, 120.56, 118.51, 118.04, 111.10, 110.48, 54.15, 27.07. MS (ESI), m/z: 424.2 $[M-H]^-$.

(R)-2-(2-(4-chlorophenyl)thiazole-4-carboxamido)-3-(1H-indol-3-yl) propanoic acid (2f). Yield: 18.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H), 10.92 (s, 1H), 8.33 (s, 1H), 8.30 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.61 (m, 3H), 7.35 (d, J = 8.0 Hz, 1H), 7.21 (m, 1H), 7.09 (t, J = 8.0 Hz, 1H), 6.97 (t, J = 8.0 Hz, 1H), 4.73 (dd, J = 16.0, 8.0 Hz, 1H), 3.36–3.35 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.91, 165.86, 159.94, 149.94, 136.09, 135.38, 131.05, 129.29, 128.11, 127.30, 124.89, 123.72, 121.00, 118.51, 118.22, 111.44, 109.43, 52.90, 26.53. MS (ESI), m/z: 424.3 $[M-H]^-$.

(R)-2-(2-(2,4-Ddichlorophenyl)thiazole-4-carboxamido)-3-(1H-indol-3-yl)propanoic acid (3f). Yield: 25.1% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 8.44 (s, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 8.0 Hz, 1H), 7.84 (s, 1H), 7.57 (t, J = 8.0 Hz, 2H), 7.29 (s, J = 8.0 Hz, 2H), 7.14 (s, 1H), 7.01 (t, J = 8.0 Hz, 1H), 6.87 (t, J = 8.0 Hz, 1H), 4.53 (d, J = 4.0 Hz, 1H), 3.40–3.27 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.83, 161.23, 159.37, 149.65, 135.93, 135.27, 131.88, 131.71, 130.13, 129.46, 128.01, 127.82, 125.62, 123.51, 120.59, 118.50, 118.06, 111.14, 110.43, 54.11, 27.05. MS (ESI), m/z: 458.3 $[M-H]^-$.

(R)-2-(2-(2-Chlorophenyl)-4-methylthiazole-5-carboxamido)-3-(1H-indol-3-yl)propanoic acid (4f). Yield: 33.9% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.29 (d, J = 8.0 Hz, 1H), 8.21 (dd, J = 8.0, 2.0 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.80 (d, J = 8.0 Hz, 1H), 7.54–7.48 (m, 2H), 7.32 (d, J = 8.0 Hz, 1H), 7.18 (s, 1H), 7.04 (t, J = 8.0 Hz, 1H), 6.95 (t, J = 8.0 Hz, 1H), 4.55 (d, J = 4.0 Hz, 1H), 3.37–3.18 (m, 2H), 2.49 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.99, 160.85, 160.62, 153.35, 136.03, 131.57, 130.81, 130.56, 128.03, 127.76, 127.45, 123.56, 120.76, 118.30, 118.22, 111.26, 110.44, 54.45, 26.60, 16.66. MS (ESI), m/z: 438.3 $[M-H]^-$.

(R)-2-(2-(4-Chlorophenyl)-4-methylthiazole-5-carboxamido)-3-(1H-indol-3-yl)propanoic acid (5f). Yield: 39.0% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.88 (s, 1H), 10.87 (s, 1H), 8.46 (d, J = 4.0 Hz, 1H), 7.94 (d, J = 8.0 Hz, 2H), 7.58–7.57 (m, 3H), 7.35 (d, J = 8.0 Hz, 1H), 7.22 (s, 1H), 7.07 (t, J = 8.0 Hz, 1H), 6.99 (t, J = 8.0 Hz, 1H), 4.63 (m, 1H), 3.34–3.18 (m, 2H), 2.47 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.05, 164.65, 160.97, 155.05, 136.13, 135.43, 131.20, 129.38,

127.92, 127.11, 126.47, 123.67, 120.95, 118.38, 118.13, 111.41, 110.05, 53.86, 26.48, 16.85. MS (ESI), m/z: 438.2 $[M-H]^-$.

(R)-2-(2-(2,4-Dichlorophenyl)-4-methylthiazole-5-carboxamido)-3-(1H-indol-3-yl)propanoic acid (6f). Yield: 33.2% for five steps from the starting chloro-substituted benzamide; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.02 (s, 1H), 10.87 (s, 1H), 8.55 (d, J = 8.0 Hz, 1H), 8.25 (d, J = 8.0 Hz, 1H), 7.84 (s, 1H), 7.61–7.60 (m, 2H), 7.35 (d, J = 8.0 Hz, 1H), 7.22 (s, 1H), 7.07 (t, J = 8.0 Hz, 1H), 6.99 (t, J = 8.0 Hz, 1H), 4.66 (m, 1H), 3.35–3.19 (m, 2H), 2.50 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.02, 160.96, 159.86, 153.86, 136.12, 135.34, 131.67, 130.27, 130.20, 129.50, 128.07, 127.74, 127.13, 123.63, 120.93, 118.38, 118.15, 111.40, 110.09, 53.87, 26.46, 16.66. MS (ESI), m/z: 472.2 $[M-H]^-$.

Modeling simulation

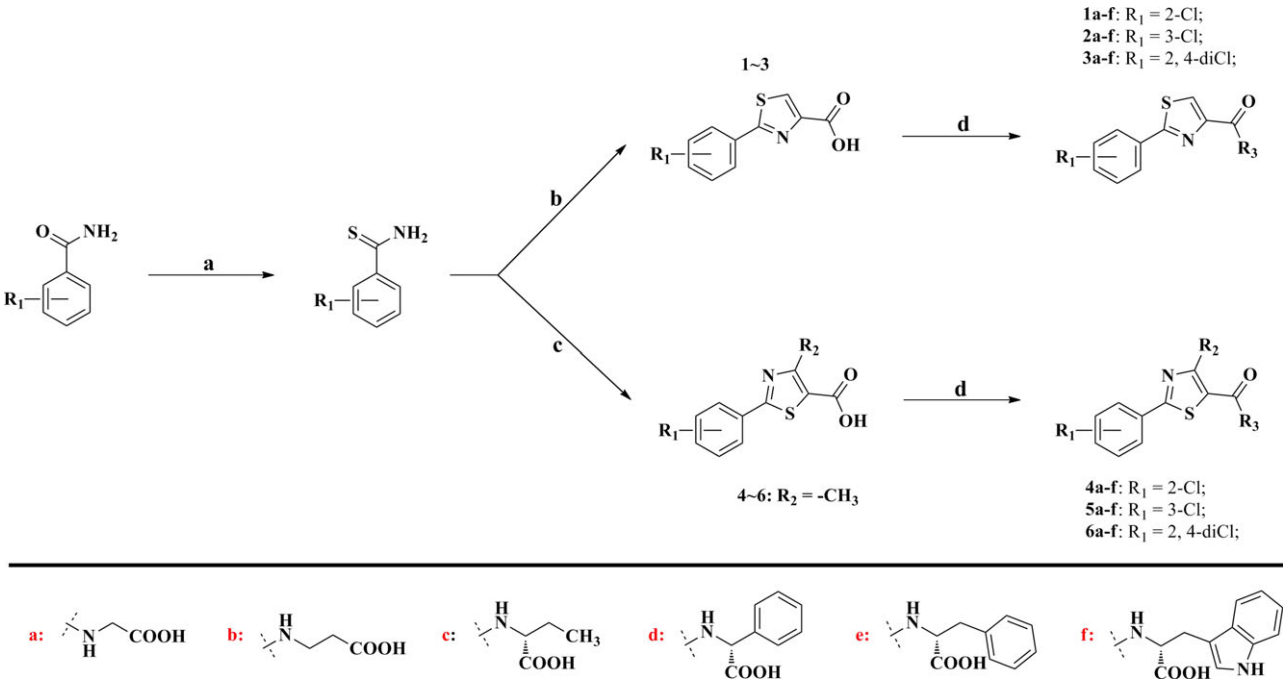
To further investigate the bound mode of our compound, FFA2 model was built by using multithreading alignments. To search the similar sequence of human FFA2, the PSI-BLAST program was employed directed from its Web site at http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi (12). EASYMODELLER 4.0 was used manually built homology models of human FFA2 from human FFA1 (PDB code: 4PHU) and other GPCR protein structures (13). In addition, the following web server also were implemented from Protein Model Portal (http://www.proteinmodelportal.org/) (14): M4T (15), I-TASSER (16), Phyre2 (17), IntFOLD2 (18), RaptorX (19). The quality of models was assessed by Verify Models implemented by DISCOVERY STUDIO 3.1 and PROCHECK (20). Finally, based on the reported mutational analysis and the model evolution results, the most rational structure was recruited for molecular docking (21–23).

Flexible docking was performed using the Flexible Docking protocol implemented in DISCOVERY STUDIO 3.1. In the process of docking, the following residues were selected as flexible residues: Tyr90, Arg180, Tyr238, His242, and Arg255. Residue Arg180 was used to define the binding site, and the sphere radius was set as 12 Å. The best docked conformations of small agonists were selected according to docking score and the binding action of short-chain fatty acids and other small carboxylic modulators of FFA2.

Results and Discussion

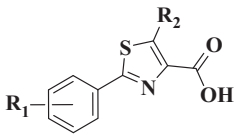
Chemistry

The preparation of a library of 2-phenylthiazole-carboxylic acids (1–6) and their amido acid derivatives (**1a**–**6f**) has been carried out in a tandem five-step sequence from the commercially available corresponding benzamides as described in Scheme 1. Treatment of benzamides with Lawesson's reagent in the solvent of toluene afforded benzothioamides in a good yield without any further purification for the next step (24–26). 2-Phenylthiazole-4-car-

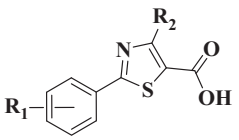


Scheme 1: Synthesis of phenylthiazole-carboxamido acid derivatives [a]. [a]Reagent and conditions: (a) Lawesson's reagent, toluene, 60 °C, 6 h; (b) ethyl 3-bromo-2-oxopropanoate, MeOH, reflux, 4 h, then 2 M LiOH, EtOH, rt; (c) ethyl 2-chloro-3-oxobutanoate, EtOH, reflux, 4 h; (d) amino acid ester, EDCI, DMAP, DMF, rt, then 2 M LiOH, EtOH, rt;

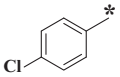
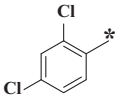
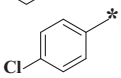
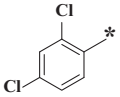
Table 1: In vitro agonistic activity of test compounds **1**–**6** for hFFA2[a]



1~3



4~6

Compounds	R_1	R_2	% Activation (at 100 μM)
1		H	10.56 ± 5.93
2		H	8.17 ± 1.05
3		H	13.73 ± 2.41
4		$-CH_3$	31.25 ± 0.90
5		$-CH_3$	10.93 ± 2.72
6		$-CH_3$	41.49 ± 4.54

[a]In vitro agonist activity of test compounds at 100 μM was performed in a cAMP assay for human G_i-coupled FFA2. Agonistic activity of the test compound was expressed as a percentage of the activity of the reference agonist at the EC_{100} concentration. The activity of propionate is 85.35 ± 5.89% at a concentration of 100 μM.

boxylic acids (**1**–**3**) were synthesized via a condensation of ethyl 3-bromo-2-oxopropanoate with respective benzothioamides in refluxed methanol and then the hydrolysis using 2 M LiOH in ethanol at room temperature (27–29). The 2-phenylthiazole-5-carboxylic acids (**4**–**6**) were prepared via a similar chemical condensation of ethyl 2-chloro-3-oxobutanoate with appropriate benzothioamides in refluxed ethanol and then hydrolysis by the solution of 2 M LiOH at room temperature (28, 30). The desired phenylthiazole-carboxamido acid derivatives **1a**–**6f** were obtained by the condensation of **1**–**6** with appropriate amino acid ester (**a**–**f**) using EDCI and DMAP as efficient condensing agents, followed by the hydrolysis in the LiOH/EtOH solution (2 M), which was further neutralized by 1 N HCl to pH 4–5 (28, 31). At this stage, all final products were fully analyzed and characterized by NMR, MS and HPLC before submitted to the biological screening.

Agonistic activity in vitro

In this study, we evaluated the FFA2 agonistic activity of test compounds by the assay of cAMP concentrations with the homogeneous time-resolved fluorescence (HTRF) kits in stable human recombinant FFA2-transfected CHO-K1 cells. Natural potent ligand propionate was selected as positive control in the assay, and agonistic activity of the test compounds was expressed as a percentage of the activity of the reference agonist at the EC_{100} concentration.

As shown in Table 1, we initially accessed the agonistic potency of six chloro-substituted phenylthiazole-5-carboxylic acid and chloro-substituted phenyl-4-methylthiazole-5-carboxylic acid derivatives, which have been designed on the basis of previously reported thiazol-2-amine agonists. By the inspection of their structural features and agonistic potency, compounds of 2,4-dichloro-

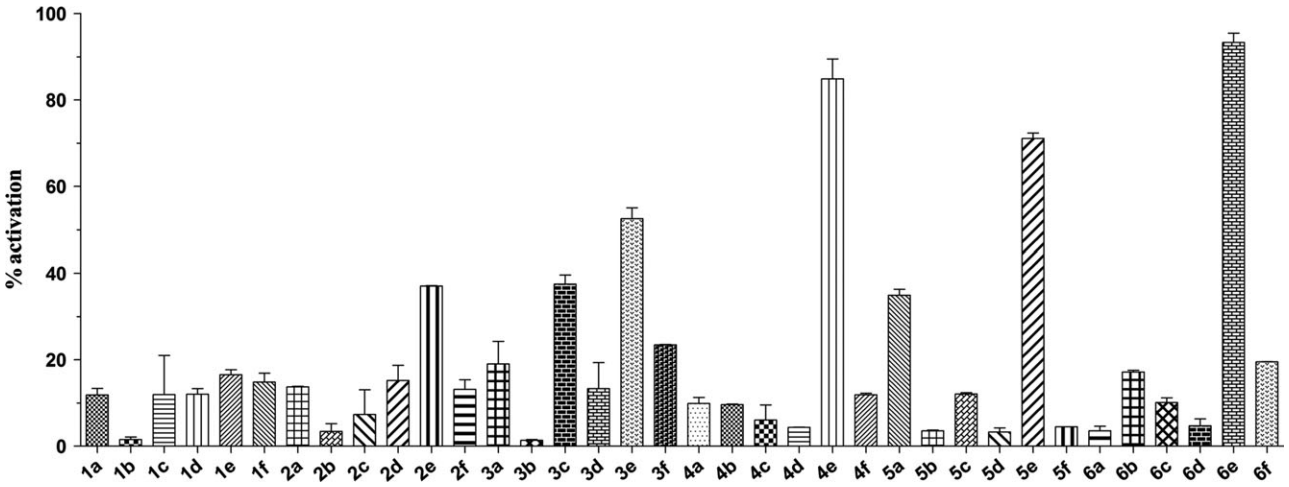
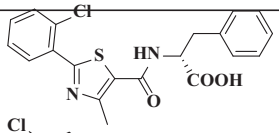
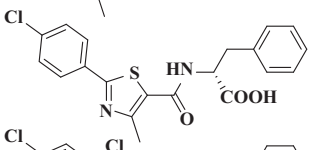
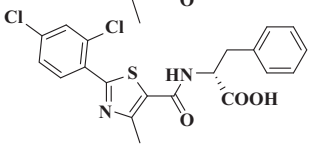
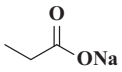


Figure 2: In vitro agonistic activity of test compounds **1a**–**6f** for hFFA2. The agonist activity of **1a**–**6f** at 100 μM was performed in a cAMP assay for human G_i-coupled FFA2. Agonistic activity of test compound was expressed as a percentage of the activity of the reference agonist at the EC_{100} concentration.

Table 2: EC_{50} values of selected compounds for hFFA2.

Compounds	Chemical structures	EC_{50} (μM)
4e		60.6 ± 1.98
5e		89.6 ± 10.4
6e		23.1 ± 2.46
Propionate		43.3 ± 1.71

phenyl group exhibited more potent activity than those of 2-chlorophenyl group and compounds of 4-chlorophenyl group were weak (compound **3** > **1** > **2**). By comparison with thiazole (**1**–**3**) and 4-methylthiazole derivatives (**4**–**6**), the introduction of methyl group to the phenylthiazole moiety contributed to agonistic activity (compound **4** > **1** or **5** > **2** or **6** > **3**). Herein, we concluded that the number and substituent site of chloro atom and 4-methylthiazole group seemed to be crucial for *in vitro* hFFA2 activation (Figure 2).

As shown in Table 2, three selected compounds were further investigated for the hFFA2 agonistic activity. We found that compounds (**4e**, **5e** and **6e**) exhibited EC_{50} values of 60.6, 89.6 and 23.1 μM, respectively, compared to 43.3 μM of the positive control propionate. These results were in keeping with the aforementioned screening data and SAR (compound **6e** > **4e** > **5e**) and were also profitable to structural design and exploration for orthosteric FFA2 agonists.

Mutagenesis analysis and molecular simulation

As for the orthosteric FFA2 agonist **6e**, we further investigated the mechanism of action using Flexible Docking protocol implemented in DISCOVERY STUDIO 3.1. In the process of docking, the following residues were selected as flexible residues: Tyr90, Arg180, Tyr238, His242, Arg255 (11). Residue Arg180 was used to define the binding site, and the sphere radius was set as 12 Å. The best docked conformations of small agonists were selected according to the docking score and the binding action of short-chain fatty acids and other small carboxylic modulators of

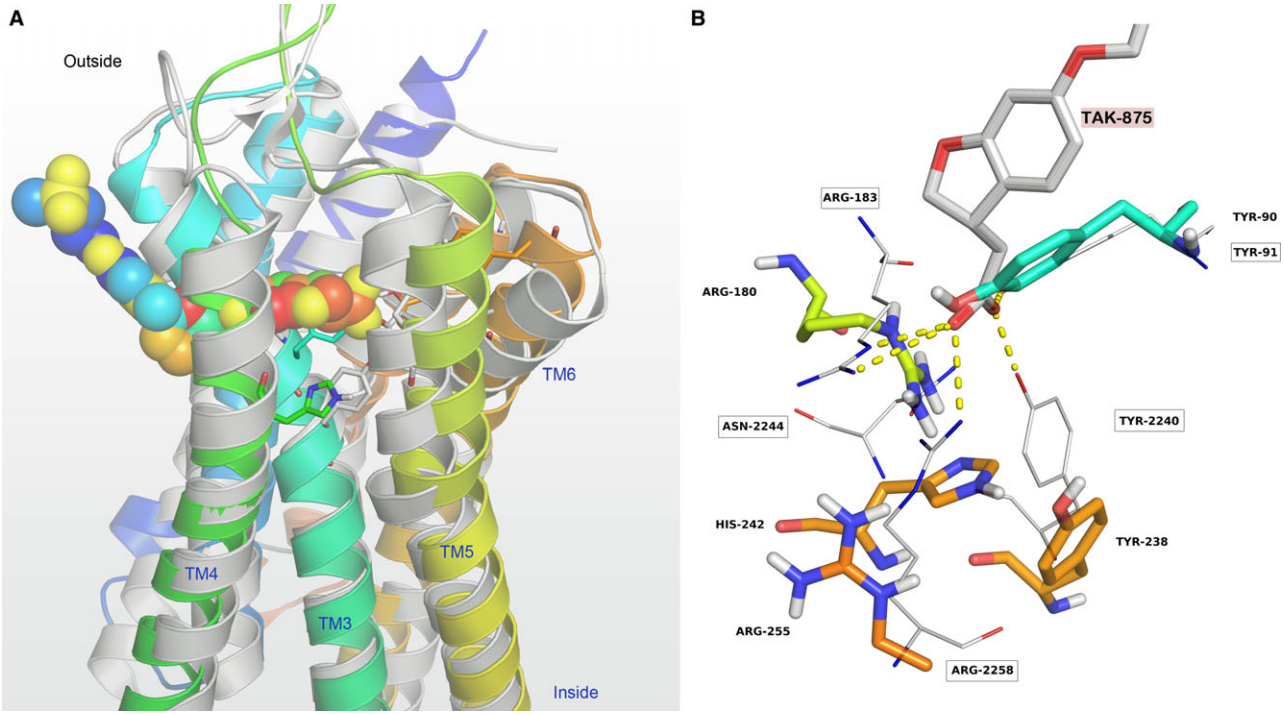


Figure 3: Structure alignment of the crystal structure of FFA1 (4PHU) and FFA2. (A) The conserved seven-transmembrane helical bundle fold of I-FFA2 was represented as rainbow, while seven-transmembrane helical bundle fold of FFA1 was painted gray. TAK-875, bound between TM3 and TM4 in FFA1 crystal structure, was shown as sphere; (B) Arg90, Arg255, His242, Tyr90 and Tyr238 were shown as rainbow stick, and the corresponding residues in the FFA1 were represented as gray lines. The part of TAK-875 was displayed as gray stick, and its carboxylate moiety was highly co-ordinated by these critical residues. Hydrogen bonds are represented by yellow dashes.

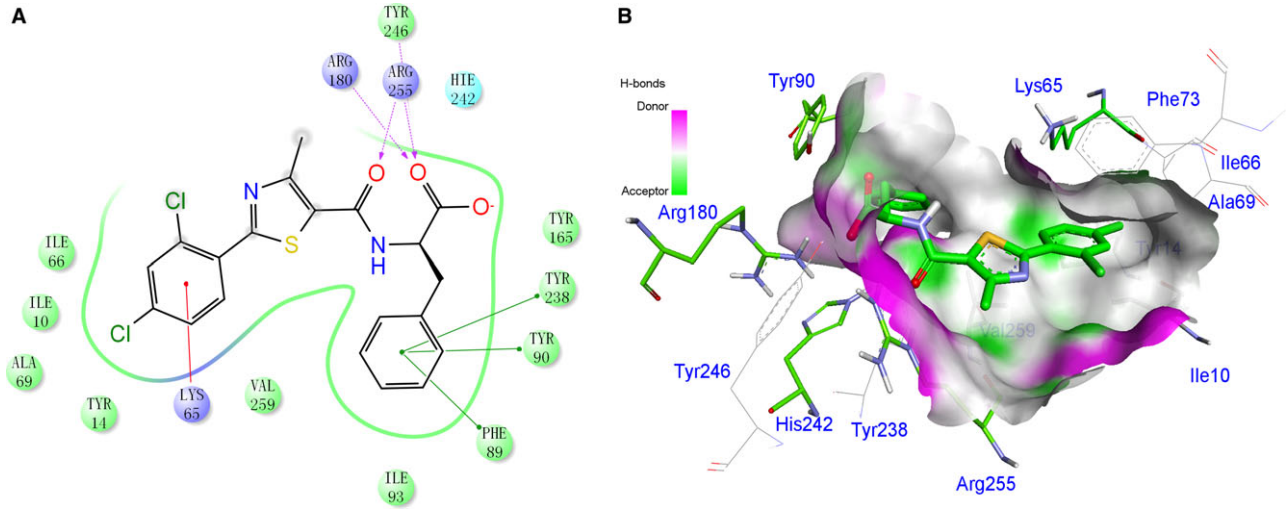


Figure 4: The binding mode of **6e** in orthosteric binding sites of FFA2. (A) The interaction between I-FFA2 and compound **6e** on 2D diagram; (B) The binding site of **6e** in the surface of I-FFA2.

FFA2. By site-directed mutagenesis, Arg180 and Arg255 in FFA2 (the two corresponding residues in FFA1: Arg183 and Arg258) were found to be critical for the recognition of SCFAs. Thus, the two arginine residues functioned as conserved anchoring residues of the fatty acid carboxylate group in FFA1 and FFA2. Moreover, site-directed mutagenesis of Tyr90 and His242 exhibited detrimental effects on the binding affinity of SCFAs (21–23). Therefore, rational conformation of these residues was the first thing to be assessed after the FFA2 models were constructed. There are five FFA2 models that generated by above-mentioned methods. Among these methods, I-FFA2 model, generated by I-TASSER web server, showed the lowest DOPE score with the value of −42259.32. *Ramachandran* plot of phi and psi angles revealed the stereochemical quality of the models, and the model generated by I-TASSER showed that 80% of the residues are in core regions and 15.1% in allowed regions; 95.1% of allowed regions were sufficient for GPCR to conform the reliability of the model.

The FFA1 crystal structure shared 32% sequence identify and 73% query coverage with query sequence of FFA2. Superimposing I-FFA2 and FFA1 (4PHU) yielded an RMSD of 2.98 Å, indicating that FFA2 approximately adopted the same conformation. The seven-transmembrane helices bundle (TM), typical of GPCR structures, remained conserved in the I-FFA2 model. Notably, I-FFA2 model had a conserved hairpin loop between the transmembrane helix 3 (Cys82) and the C-terminal portion of the ECL2 loop (Cys164). The gap between TM3 and TM4 in the FFA1 was wider than in the I-FFA2 model. So the modulators of FFA1 accessed into the binding pocket through lipid interface from the side between TM3 and TM4. However, FFA2 possessed canonical solvent-accessible binding pocket and the modulator could enter into the binding pocket from extracellular surface (Figure 3A). In the I-FFA2 model, Arg180, Arg255 and His242 formed charge center and hydrogen bond network. In addition to the arginine residues, Tyr90 and Tyr238 may be involved in the stabilization of the carboxylate moiety (Figure 3B).

Mutagenesis analysis and molecular simulation revealed that our carboxylic modulators functioned as agonists by binding in the orthosteric site. So compound **6e** was docked into the canonical binding site, and the rational conformation was singled out based on docking scores and mutagenesis experiments. Three hydrophilic residues Arg180, Arg255 and His242 acted as anchors for the carboxylate group, while phenyl rings of Phe89, Tyr90 and Tyr238 were involved in the formation of π-π stacking interactions. 2, 4-Dichlorophenyl moiety formed a cation–π interaction with the side chain of Lys65 (Figure 4A). As shown in Figure 4B, the conformation of 6e was exhibited in a crescent shape and reached into the binding pocket. These results may be a right guideline for our further structural modification and exploration for potent agonists.

Conclusion

In the study, a series of novel phenylthiazole-carboxamido acid derivatives has been synthesized and evaluated as potential orthosteric FFA2. Compound **6e** was found to exhibit more potent agonistic activity in the stable hFFA2-transfected CHO-K1 cells (EC_{50} = 23.1 μM) in contrast to positive control propionate (EC_{50} = 43.3 μM). As for the study of structure–activity relationships, we concluded that the number and substituent site of chloro atom and 4-methylthiazole group were crucial for *in vitro* hFFA2 activation. Based on the crystal structure of hFFA1 bound to TAK-875 at 2.3 Å resolution, we also reported the results of mutagenesis studies to identify important residues for

orthosteric agonist **6e** inducing the activation of FFA2. These results may be a guideline for next structural modification and exploration for potent agonists.

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Conflict of Interest

The authors have declared no conflict of interest.

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