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Structural Insights of Benzenesulfonamide Analogues as NLRP3 Inflammasome Inhibitors: Design, Synthesis, and Biological Characterization

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Abstract

NLRP3 inflammasome plays critical roles in a variety of human diseases and represents a promising drug target. In this study, we established the in vivo functional activities of **JC124**, a previously identified NLRP3 inflamma-some inhibitor from our group, in mouse models of Alzheimer's disease and acute myocardial infarction. To understand the chemical space of this lead structure, a series of analogues were designed, synthesized, and biologically characterized. The results revealed the critical roles of the two substituents on the benzamide moiety of **JC124**. On the other hand, modifications on the sulfonamide moiety of **JC124** are well tolerated. Two new lead compounds, **14** and **17**, were identified with improved inhibitory potency (IC₅₀ values of 0.55 ± 0.091 and 0.42 ± 0.080 μ M, respectively). Further characterization confirmed their selectivity and in vivo target engagement. Collectively, the results strongly encourage further development of more potent analogues based on this chemical scaffold.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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Molecular formula strings (CSV)

Notes

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the A β pathology of AD.²⁰ NLRP3 inflammasome also plays critical roles in the inflammatory responses to myocardial injury during AMI.²³ In the early phases of AMI, the acute ischemic injury induces the expression of NLRP3 inflammasome components (priming), which concomitantly provides the stimuli leading to NLRP3 activation and formation of the macromolecular aggregate (trigger), leading to an active inflammasome.^{23,24} Caspase-1 is detected in the heart starting 3–6 h after ischemia and its activity peaks between 24 and 72 h, while low grade activation persists for weeks after the initial insult.^{23,25} Reperfusion, while it effectively reduces infarct size, does not prevent activation of the NLRP3 inflammasome and leads to further injury through caspase-1-dependent inflammatory cell death.²⁶ To support this notion, studies by Abbate and others demonstrated that mice with genetic deletion of NLRP3 or ASC exhibited smaller infarct size in the experimental AMI model, and reduced tendency toward adverse remodeling and heart failure, consistent with the previously reported central role of caspase-1 in AMI.^{25,27,28} Transgenic mice expressing constitutively active caspase-1, on the other hand, developed adverse cardiac remodeling and heart failure.²⁹ Collectively, these studies strongly suggest that this pathway could be targeted for the treatment of a variety of diseases. Indeed, several biological agents have been successfully developed and approved by FDA by targeting this pathway as treatments for CAPS, and this includes IL-1 receptor antagonist anakinra, IL-1 β antibody canakinumab, and decoys of IL-1 receptor rilonacept.^{18,30}

Although the pathogenic roles of the NLRP3 inflammasome in a variety of human disorders are quickly emerging, the basis of NLRP3 inflammasome activation and its contribution to disease progression remain not fully understood. It is therefore of importance to develop novel and specific NLRP3 inflammasome inhibitors (NLRP3Is) as pharmacological tools, which will complement ongoing molecular and genetic studies to precisely define the role of NLRP3 inflammasome in the pathogenesis of related human diseases, and as potential therapeutics. To this end, several small molecules have recently been reported to inhibit the NLRP3 inflammasome signaling pathway with different or unknown mechanisms of action (MOA) (Figure 1).³¹ Among these inhibitors, glyburide is an antidiabetic drug promoting insulin release and has shown inhibitory activity on NLRP3 inflammasome in myeloid cells in vitro.³² In contrast, glipizide, another sulfonylurea antidiabetic agent, lacks this inhibitory effect on the NLRP3 inflammasome.¹¹ This suggests that the observed inhibitory effects on NLRP3 inflammasome by glyburide are independent from its actions on the K_{ATP} channels, which are involved in insulin release. Further studies suggested that the sulfonyl and benzamide moieties within this structure are necessary for the observed inhibitory activity.³² Although the observed anti-inflammatory properties of glyburide suggest beneficial effects, further development of this compound is limited by the need of high doses that potentially induce lethal hypoglycemia.

Our laboratories have recently designed and developed a sulfonamide analogue **JC121** based on the structure of glyburide (Figure 1).^{33–35} Biological characterization established that **JC121** is a selective NLRP3I and exhibits promising in vivo protective activities in mouse AMI models. Furthermore, as summarized in Figure 2, our studies suggested that **JC121** may directly interfere with the formation of the NLRP3 inflammasome complex,³⁴ based on the facts that (1) it blocks ASC aggregation; (2) it does not directly inhibit caspase-1

activation as NLRC4 and AIM2 pathways are not affected by **JC121**; and (3) it blocks the release of IL-1 β and the activation of caspase-1 in macrophages expressing constitutively active NLRP3 from mutant mice. Our ongoing studies also demonstrated the direct binding interactions of our inhibitor with the NLRP3 protein (unpublished data). On the basis of the structure of **JC121**, we further developed a methylated analogue, **JC124**, to balance the hydrophilicity and hydrophobicity (Figure 1). Our initial studies established that **JC124** is an active and selective NLRP3I.³⁶ Our studies also demonstrated the in vivo efficacy of this compound in reducing AD pathology in a transgenic mouse AD model.³⁶ Herein, we report further characterization of **JC124** in a transgenic AD mouse model and a mouse AMI model to confirm its therapeutic effects. Furthermore, structure activity relationship (SAR) studies were conducted to help understand and explore the chemical space of **JC124**, providing guidance for further structural optimization/refinement of this chemical scaffold.

RESULTS AND DISCUSSION

Therapeutic Effects of JC124 on Cognitive Functions in APP/PS1 Mice.

Our previous studies in TgCRND8 mice, a widely used mouse AD model,^{37,38} demonstrated that treatment with **JC124** (50 mg/kg) modified multiple AD pathologies, including beta-amyloid (A β) deposit, oxidative stress, inflammatory responses, and synaptic degeneration.³⁶ With the promising activity on AD pathologies, we decided to examine whether such modifications by **JC124** can lead to improved cognitive functions. We then employed APP/PS1 mice, another widely used mouse AD model,³⁹ to test **JC124** treatment with a battery of behavioral tests including Novel Object Recognition (NOR) and Morris Water Maze (MWM). In our previous studies, moderate improvement on AD pathology was observed for **JC124** at a dose of 50 mg/kg;³⁶ we therefore decided to use a higher dose, 100 mg/kg, in APP/PS1 mice for the behavioral studies. Treatment started at the age of 7 months old and continued for 8 weeks by oral administration (5 times/week). This protocol mimics the therapeutic settings because significant AD pathologies have developed by 7 months old in APP/PS1 mice.³⁹ As shown in Figure 3A, as compared to the wild-type (WT) mice, transgenic APP/PS1 (Tg) mice in both vehicle or **JC124** treated groups showed a discrimination index (DI) lower than 50%, which suggested cognitive deficits of Tg mice. **JC124** treatment group showed a trend to improve cognitive function with higher DI in **JC124**-treated group as compared to the vehicle-treated group. However, the difference did not reach a statistical significance. In MWM fixed-platform test, mice in all groups showed improved learning with time during the 4 days latency trials; however, at the last day of trial, the WT vehicle group had shorter latency to find goal platform than did the Tg groups, indicating better performance in the WT vehicle group in this test (Figure 3B). Notably, in the MWM fixed platform probe trial test, Tg animals treated with **JC124** spent significantly longer time in the goal quadrant than did the vehicle-treated Tg group, suggesting improved memory function by **JC124** treatment (Figure 3C). As noted in the MWM probe trial test of WT animals, **JC124** treated mice spent shorter time in the goal quadrant as compared to the vehicle treated group. Although the difference did not reach statistical significance, the observed difference may indicate some degree of immune suppressive effects by **JC124** treatment in normal animals that could affect cognitive function. This speculation needs to be confirmed in future study by examining the systemic immune responses. Collectively,

under therapeutic trial settings, we found that **JC124** exhibits beneficial effects on cognition as evidenced by the improved memory function (probe trial).

Therapeutic Effects of **JC124** in an Experimental Model of AMI.

To further confirm the *in vivo* efficacy of **JC124**, we examined its effects to protect cardiac functions in an experimental AMI model induced by transient myocardial ischemia followed by reperfusion (I/R model), in which the NLRP3 inflammasome has critical roles in postischemia inflammatory responses and heart failure.^{25,40} To explore the effective doses of **JC124** and based on the preliminary pharmacokinetic data of **JC124** (unpublished data), we selected a lower dose of 30 mg/kg in this experiment. Two different I/R models were used to evaluate the protective effects of **JC124**: (1) coronary ligation was continued for 30 min; and (2) a more severe model in which coronary ligation was continued for 75 min. As shown in Figure 4, treatment with **JC124** significantly limited the infarct size as measured with triphenyltetrazolium chloride (TTC) staining or troponin I levels in both models when compared to the vehicle treatment group, thus further confirming the *in vivo* efficacy of **JC124** at this dose.

Design and Synthesis of **JC124** Analogues.

With the confirmation of *in vivo* efficacy, we decided to conduct SAR studies of **JC124** to understand the chemical space around this lead structure. In addition, the results of SAR studies may help shed light on whether this chemical scaffold functions by a single mechanism of action, thus providing valuable information on target interaction. The design of **JC124** congeners was mainly focused on the phenyl domain of the benzamide moiety and the sulfonamide domain to understand the influence of substituents on their biological activities (Figure 5). Compounds **1–4** were designed to examine the importance of the 2-CH₃O and 5-Cl to their biological activities using the deconstruction strategy. Analogues **5–9** were designed to investigate how the positional change of the CH₃O- and Cl- substituents will influence their inhibitory activities. The results of **JC124** suggested that the sulfonamide moiety could be optimized to improve biological activity. Therefore, analogues **10–21** were designed to understand the space at this domain for further optimization. To evaluate whether a cyclized version of sulfonamide analogues will provide improved inhibitory activity, compounds **22–26** were designed. We also designed analogues **27** and **28** to investigate how the linkage length between the two phenyl rings will impact the biological activity.

The chemical syntheses of the designed analogues were successfully achieved by employing the conditions detailed in Schemes 1 and 2. Briefly, condensation of 2-phenylethylamine **29** with phthalic anhydride gave **30**, which on sulfonation with chlorosulfonic acid gave intermediate **1**. Reaction of **31** with methylamine afforded intermediate **32**. Refluxing of **32** with NH₂NH₂ in ethanol followed by coupling reactions with corresponding substituted benzoic acids under standard peptide coupling conditions yielded analogues **1–9** (Scheme 1). Compounds **10–26** were synthesized following the conditions listed in Scheme 2. Coupling of **29** with 5-chloro-2-methoxybenzoic acid **34** followed by sulfonation afforded sulfonyl chloride intermediate **36**. Reaction of intermediate **36** with various amines afforded compounds **10–26**. Similarly, compounds **27** and **28** were obtained by following the same

strategies with 3-phenylpropylamine **37** or 4-phenylbutylamine **38** as starting material, respectively.

Evaluation of the Inhibitory Potency for the Designed Analogues on IL-1 β Release in J774A.1 Cells.

Our previous studies have established a cellular model using mouse macrophage J774A.1 cells in which the release of IL-1 β is mediated through the activation of NLRP3 inflammasome upon stimulation with lipopolysaccharide (LPS) and adenosine triphosphate (ATP).³³ The level of secreted IL-1 β was monitored by an enzyme-linked immunosorbent assay (ELISA). As shown in Table 1, lead compound **JC124** showed an inhibitory potency of 3.25 μ M under the current experimental conditions. Removal of 2-OCH₃ or 5-Cl resulted in a significant decrease of inhibitory potency (>4-fold) as demonstrated by analogues **1** and **2**. The importance of the 2-OCH₃ and 5-Cl was further demonstrated by analogue **3** in which both substituents were removed with a complete loss of inhibitory activity on IL-1 β release. When the 2-OCH₃ was demethylated to a 2-OH moiety, a reduced inhibitory potency (~3-fold) was observed in analogue **4**. The results of **JC124** and **4** suggest that modification of the 4-OH moiety to other alkoxy groups at this position could lead to improved inhibitory potency. Moving of the 5-Cl to different positions on the phenyl ring all led to a significant decrease of inhibitory potency on IL-1 β release in J774A.1 cells as evidenced by analogues **5–7**. Interestingly, a positional change of the 2-OCH₃ to the meta-position led to analogue **8** with potency comparable to that of **JC124**, while a change to the para-position, as demonstrated by **9**, led to significant loss of the inhibitory potency. This may indicate that the electronic effect by the CH₃O substituent is not an essential factor for the observed biological activity. Taken together, the results of **5–9** strongly suggest the important roles of the 2,5-disubstitution on the observed inhibition of IL-1 β release under the experimental conditions.

Structural modifications at the sulfonamide moiety, on the other hand, were well tolerated. As shown in Table 1, with the increase of the size of the substituents on the sulfonamide N, the inhibitory potency improves on IL-1 β release as reflected by analogues **10–14**. Compound **14** with a dibutyl substitution on the sulfonamide N is the most potent one within this series of analogues with an IC₅₀ of 0.55 μ M. With this observation, we then turned our attention to more bulky substituents at this position. As shown by **15**, aniline substitution on the sulfonamide moiety resulted in an inhibitory potency comparable to that of **JC124**. Notably, when the benzene ring was replaced with a benzyl moiety as in **16**, the inhibitory potency was increased by 2.5-fold as compared to that of **JC124**. Substitution on the benzyl moiety with a 4-OCH₃ as illustrated by **17** further improved the inhibitory potency to 0.42 μ M, a >3-fold increase as compared to that of **16**. Analogue **18** with a 4-OH at this position exhibited potency comparable to that of **17**. This may suggest that an electron-donating group at this specific position favors the inhibitory activity on IL-1 β release under the LPS/ATP challenge conditions in J774A.1 cells. This notion is further supported by the results of **19** and **20** as a 4-Cl or 4-CF₃ substitution, both electron-withdrawing, led to a comparable or decreased inhibitory potency as compared to that of **16**. Further structural extension at this position by a phenylethyl moiety, as shown in **21**, resulted in a slight improvement when compared to **16**. Analogues with a heterocyclic moiety on the

sulfonamide moiety, as demonstrated by compounds **22–26**, exhibited inhibitory potency comparable to that of **JC124**. The results of the mono- or disubstituted analogues suggested that bulky substituents with rotational flexibility are preferred to providing analogues with improved inhibitory potency on IL-1 β release. Structural extension slightly improved the inhibitory potency under the current experimental conditions as evidenced by **27** and **28** with IC₅₀ values of 2.03 and 1.74 μ M, respectively. This is in agreement with the results of compounds **17** and **21**, thus suggesting that hydrophobic interactions at this domain may be important for the observed inhibition on IL-1 β release.

Compounds **14** and **17** Are Selective NLRP3Is.

After the establishment of inhibitory potency on IL-1 β release in J774A.1 cells for the designed analogs, we selected **14** and **17**, the two most potent analogues, for further characterization in mouse bone marrow derived macrophages (BMDMs) and in mice. As shown in Figures 6A, both **14** and **17** dose dependently suppressed the release of IL-1 β from BMDMs upon LPS/ATP challenge with IC₅₀ values of 0.12 ± 0.067 and 0.36 ± 0.043 μ M, respectively. Analogue **14** is ~4 times more potent in BMDMs than in J774A.1 cells to inhibit the release of IL-1 β , while the potency of **17** is comparable in these two cell populations. This may suggest that BMDMs are more sensitive to **14** under the current experimental conditions, consistent with our previous studies.⁴¹ We next examined the selectivity of **14** and **17** to NLRP3 inflammasome. To this end, J774A.1 cells were stimulated with LPS/poly(dA:dT) or LPS/flagellin to activate the NLRC4 and AIM2 inflammasome, respectively. As shown in Figure 6B and C, treatment of J774A.1 cells with **14** or **17** under these experimental conditions did not significantly interfere with the production of IL-1 β (by ANOVA one-way analysis), thus confirming the relatively specific inhibition of NLRP3 inflammasome by **14** and **17**. Consistent with the reported results,^{36,42} no inhibitory activity was observed for **JC124** and **MCC950**, a recently reported and potent inhibitor that targets the NLRP3 inflammasome pathway,⁴² under these conditions as well. The results from the selectivity studies also serve as indirect evidence to support the MOA that analogues derived from this chemical scaffold interfere with the NLRP3 inflammasome complex, instead of the upstream priming step by the LPS. This is consistent with our previously reported results.³⁴ Finally, we tested the in vivo engagement of the NLRP3 inflammasome by **14** and **17**. Mice were pretreated with **14** or **17** before intraperitoneal injection of LPS, which has been shown to trigger IL-1 β production in a NLRP3-dependent manner.⁴³ **MCC950** was tested as a positive control. As shown in Figure 6D, the serum level of IL-1 β was sharply reduced by the treatment of all three compounds at the tested dose, thus strongly suggesting the engagement of the NLRP3 inflammasome pathway in the observed effects by **14** and **17**. Although compounds **14** and **17** exhibit significantly improved potency when compared to **JC124**, they are still less potent than **MCC950**. However, compounds **14** and **17** exhibited activity comparable to that of **MCC950** in this in vivo LPS-challenge experiment. This could be caused by different pharmacokinetic properties of these analogues as well as the unique MOA of our inhibitors. Further studies are warranted to investigate and confirm these speculations.

CONCLUSION

In our efforts to develop small molecule inhibitors by targeting the NLRP3 inflammasome, a sulfonamide-based chemical scaffold was identified to provide analogues as active NLRP3Is. In this study, we further confirmed the *in vivo* activity of **JC124**, a recently identified lead inhibitor from our group, in a transgenic AD mouse model and a mouse AMI model. The results clearly provided confidence for further development of new analogues based on this chemical scaffold. Further SAR studies revealed the positions on the structure of **JC124** for further optimization. Our results demonstrated that the 2-OCH₃ and 5-Cl are essential to the inhibitory activity on the NLRP3 inflammasome as positional change or removal of them led to a significant decrease of inhibitory activity. On the other hand, structural modifications on the N-substituents of the sulfonamide moiety are tolerated, and bulky groups tend to provide analogues with improved potency. As a result of this SAR study, two new lead compounds, **14** and **17**, were identified with improved inhibitory potency. Further biological characterization in BMDMs and J774A.1 cells confirmed the inhibitory potency and selectivity of these two lead compounds to the NLRP3 inflammasome. More importantly, studies in LPS-challenged mice, a mouse model in which the release of IL-1 β is NLRP3 inflammasome dependent, confirmed that both **14** and **17** significantly suppressed the production of IL-1 β , thus confirming the *in vivo* engagement of this inflammasome complex by these two compounds. Collectively, the results strongly encourage further studies and the development of new analogues based on this chemical scaffold as NLRP3Is and explore their potential therapeutic applications.

EXPERIMENTAL SECTION

Chemistry.

Reagents and solvents were obtained from commercial suppliers and used as received unless otherwise indicated. All reactions were carried out under inert atmosphere (N₂) unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) (precoated silica gel 60 F₂₅₄ plates, EMD Chemicals) and visualized with UV light or by treatment with phosphomolybdic acid (PMA). Flash chromatography was performed on silica gel (200–300 mesh, Fisher Scientific) using solvents as indicated. ¹H NMR and ¹³C NMR spectra were routinely recorded on a Bruker ARX 400 spectrometer. The NMR solvent used was CDCl₃ or DMSO-*d*₆ as indicated. Tetramethylsilane (TMS) was used as internal standard. The purity of target NLRPIs was determined by HPLC using a Varian 100–5 C18 250 × 4.6 mm column with UV detection (288 nm) (50% acetonitrile/50% H₂O/0.1% trifluoroacetic acid (TFA) and 80% methanol/19.9% H₂O/0.1% TFA two solvent systems) to be 95%.

3-Chloro-N-(4-(N-methylsulfamoyl)phenethyl)benzamide (1).—Compound **1** was prepared from 3-chlorobenzoic acid (0.47 mmol) following method C in 91% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.69 (t, *J* = 5.44 Hz, 1H), 7.83 (t, *J* = 1.76 Hz, 1H), 7.76 (dt, *J* = 7.80, 1.35 Hz, 1H), 7.70 (d, *J* = 8.28 Hz, 2H), 7.61–7.59 (m, 1H), 7.52–7.46 (m, 4H), 3.53 (q, *J* = 6.64 Hz, 2H), 2.95 (t, *J* = 7.16 Hz, 2H), 2.39 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.8, 144.3, 137.2, 136.5, 133.1, 130.9, 130.8, 129.4, 126.9, 126.7, 125.8, 40.4, 34.6, 28.6.

2-Methoxy-N-(4-(N-methylsulfamoyl)phenethyl)benzamide (2).—Compound **2** was prepared from 2-methoxybenzoic acid (0.47 mmol) following method C in 65% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.18 (t, *J* = 5.58 Hz, 1H), 7.70–7.74 (m, 3H), 7.50 (d, *J* = 8.38 Hz, 2H), 7.45 (dt, *J* = 2.01, 7.91 Hz, 1H), 7.38 (q, *J* = 5.03 Hz, 1H), 7.11 (d, *J* = 8.20 Hz, 1H), 7.02 (dt, *J* = 7.57, 0.78 Hz, 1H), 3.81 (s, 3H), 3.57 (q, *J* = 6.60 Hz, 2H), 2.94 (t, *J* = 7.02 Hz, 2H), 2.41 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.9, 156.9, 144.4, 137.2, 132.1, 130.3, 129.5, 126.7, 123.0, 120.0, 111.9, 55.8, 40.1, 34.7, 28.6.

N-(4-(N-Methylsulfamoyl)phenethyl)benzamide (3).—Compound **3** was prepared starting from benzoic acid (0.47 mmol) following method C in 56% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.61 (t, *J* = 5.46 Hz, 1H), 7.85 (d, *J* = 8.00 Hz, 2H), 7.75 (d, *J* = 8.00 Hz, 2H), 7.59–7.48 (m, 5H), 7.41 (q, *J* = 5.01 Hz, 1H), 3.59 (q, *J* = 6.68 Hz, 2H), 3.00 (t, *J* = 7.20 Hz, 2H), 2.44 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.2, 144.5, 137.1, 134.5, 131.0, 129.4, 128.2, 127.0, 126.7, 40.3, 34.8, 28.6.

5-Chloro-2-hydroxy-N-(4-(N-methylsulfamoyl)phenethyl)-benzamide (4).—Compound **4** was prepared from 5-chlorosalicylic acid (0.47 mmol) following method C in 60% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.94 (t, *J* = 5.46 Hz, 1H), 7.71 (d, *J* = 8.28 Hz, 2H), 7.48 (d, *J* = 8.28 Hz, 2H), 7.42 (dd, *J* = 9.03, 2.69 Hz, 1H), 7.37 (q, *J* = 5.03 Hz, 1H), 6.93 (d, *J* = 8.84 Hz, 1H), 3.58 (q, *J* = 6.64 Hz, 2H), 2.96 (t, *J* = 7.14 Hz, 2H), 2.39 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.4, 158.4, 144.1, 137.2, 133.2, 129.4, 127.3, 126.8, 122.9, 119.3, 116.9, 40.1, 34.5, 28.6.

3-Chloro-2-methoxy-N-(4-(N-methylsulfamoyl)phenethyl)-benzamide (5).—Compound **5** was prepared from 3-chloro-2-methoxybenzoic acid (0.27 mmol) following method C in 40% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.46 (t, *J* = 5.48 Hz, 1H), 7.77 (d, *J* = 8.24 Hz, 2H), 7.62 (dd, *J* = 8.16, 1.53 Hz, 1H), 7.56 (d, *J* = 8.24 Hz, 2H), 7.44–7.40 (m, 2H), 7.24 (t, *J* = 7.84 Hz, 1H), 3.73 (s, 3H), 3.61 (q, *J* = 6.59 Hz, 2H), 3.01 (t, *J* = 7.04 Hz, 2H), 2.46 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 165.1, 152.8, 144.3, 137.2, 132.1, 131.7, 129.4, 128.2, 127.1, 126.7, 125.0, 61.5, 34.5, 28.6.

4-Chloro-2-methoxy-N-(4-((methylamino)sulfinyl)phenethyl)-benzamide (6).—Compound **6** was prepared from 4-chloro-2-methoxybenzoic acid (0.54 mmol) following method C in 60% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.22 (t, *J* = 5.58 Hz, 1H), 7.77 (d, *J* = 8.33 Hz, 2H), 7.74 (d, *J* = 8.32 Hz, 1H), 7.53 (d, *J* = 8.32 Hz, 2H), 7.42 (q, *J* = 4.84 Hz, 1H), 7.25 (d, *J* = 1.88 Hz, 1H), 7.13 (dd, *J* = 8.34, 1.95 Hz, 1H), 3.88 (s, 3H), 3.60 (q, *J* = 6.95 Hz, 2H), 2.98 (t, *J* = 6.97 Hz, 2H), 2.45 (d, *J* = 4.88 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.0, 157.6, 144.4, 137.2, 136.3, 131.7, 129.4, 126.7, 122.1, 120.5, 112.5, 56.3, 40.1, 34.7, 28.6.

2-Chloro-6-methoxy-N-(4-((methylamino)sulfinyl)phenethyl)-benzamide (7).—Compound **7** was prepared from 2-chloro-6-methoxybenzoic acid (0.54 mmol) following method C in 62% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.49 (t, *J* = 5.05 Hz, 1H), 7.70 (d, *J* = 8.32 Hz, 2H), 7.50 (d, *J* = 8.32 Hz, 2H), 7.39–7.31 (m, 2H), 7.04–7.01 (m, 2H), 3.75 (s, 3H), 3.49 (q, *J* = 7.01 Hz, 2H), 2.91 (t, *J* = 7.01 Hz, 2H), 2.41 (d, *J* = 5.04 Hz, 3H); ¹³C NMR

(100 MHz, DMSO- d_6) δ 163.9, 156.9, 144.3, 137.1, 130.4, 129.5, 127.2, 126.6, 120.9, 110.3, 56.0, 34.5, 28.6.

3-Chloro-5-methoxy-N-(4-(N-methylsulfamoyl)phenethyl)-benzamide (8).—

Compound **8** was prepared from 3-chloro-5-methoxybenzoic acid (0.36 mmol) following method C in 70% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.66 (t, J = 5.48 Hz, 1H), 7.70 (d, J = 8.33 Hz, 2H), 7.46 (d, J = 8.33 Hz, 2H), 7.42–7.41 (m, 1H), 7.36 (q, J = 5.03 Hz, 1H), 7.34–7.32 (m, 1H), 7.19–7.18 (m, 1H), 3.82 (s, 3H), 3.53 (q, J = 6.64 Hz, 2H), 2.94 (t, J = 7.14 Hz, 2H), 2.40 (d, J = 5.04 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.6, 160.1, 144.3, 137.3, 137.2, 133.8, 129.4, 126.7, 119.1, 111.9, 55.81, 40.4, 34.6, 28.6.

3-Chloro-4-methoxy-N-(4-(N-methylsulfamoyl)phenethyl)-benzamide (9).—

Compound **9** was prepared from 3-chloro-4-methoxybenzoic acid (0.36 mmol) following method C in 70% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.59 (t, J = 5.46 Hz, 1H), 7.94 (d, J = 2.24 Hz, 1H), 7.85 (dd, J = 2.24, 8.71 Hz, 1H), 7.75 (d, J = 8.29 Hz, 2H), 7.51 (d, J = 8.28 Hz, 2H), 7.40 (q, J = 5.06 Hz, 1H), 7.27 (d, J = 8.72 Hz, 1H), 3.96 (s, 3H), 3.56 (q, J = 7.06 Hz, 2H), 2.98 (t, J = 7.06 Hz, 2H), 2.44 (d, J = 5.06 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.5, 156.7, 144.4, 137.2, 129.4, 128.7, 127.7, 127.5, 126.7, 120.8, 112.3, 56.4, 40.3, 34.8, 28.6.

5-Chloro-N-(4-(N,N-diethylsulfamoyl)phenethyl)-2-methoxybenzamide (10).—

Compound **10** was prepared from **36** (0.39 mmol) and diethylamine (1.95 mmol) following method B in 87% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.26 (t, J = 5.77 Hz, 1H), 7.73 (d, J = 8.38 Hz, 1H), 7.60 (d, J = 2.70 Hz, 1H), 7.51–7.46 (m, 3H), 7.16 (d, J = 9.54 Hz, 1H), 3.81 (s, 3H), 3.57 (q, J = 6.93 Hz, 2H), 3.96 (q, J = 7.60 Hz, 4H), 2.95 (t, J = 7.60 Hz, 2H), 1.04 (t, J = 7.25 Hz, 6H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.5, 137.7, 137.4, 129.6, 129.5, 129.7, 124.8, 124.3, 114.2, 56.2, 41.7, 40.1, 34.6, 14.0.

5-Chloro-2-methoxy-N-(4-(N-propylsulfamoyl)phenethyl)-benzamide (11).—

Compound **11** was prepared from **36** (0.39 mmol) and propylamine (1.95 mmol) following method B in 61% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.24 (t, J = 3.50 Hz, 1H), 7.72 (d, J = 8.20 Hz, 2H), 7.61 (d, J = 2.08 Hz, 1H), 7.51–7.46 (m, 3H), 7.15 (d, J = 8.88 Hz, 1H), 3.81 (s, 3H), 3.55 (q, J = 6.60 Hz, 2H), 2.92 (t, J = 7.02 Hz, 2H), 2.67 (q, J = 6.68 Hz, 2H), 1.36 (sex, J = 7.16 Hz, 2H), 0.77 (t, J = 7.38 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.1, 138.6, 131.5, 129.6, 129.4, 126.5, 124.9, 124.3, 114.2, 56.2, 44.3, 40.1, 34.6, 22.3, 11.1.

5-Chloro-N-(4-(N,N-dipropylsulfamoyl)phenethyl)-2-methoxy benzamide (12).—

Compound **12** was prepared from **36** (0.39 mmol) and dipropylamine (1.95 mmol) following method B in 75% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.24 (t, J = 5.50 Hz, 1H), 7.72 (d, J = 8.28 Hz, 2H), 7.61 (d, J = 2.80 Hz, 1H), 7.51–7.47 (m, 3H), 7.15 (d, J = 8.88 Hz, 1H), 3.83 (s, 3H), 3.56 (q, J = 6.58 Hz, 2H), 3.00 (t, J = 7.54 Hz, 4H), 2.94 (t, J = 6.96 Hz, 2H), 1.46 (sex, J = 7.47 Hz, 4H), 0.80 (t, J = 7.38 Hz, 6H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.5, 137.4, 131.4, 129.6, 129.5, 126.8, 124.8, 124.3, 114.2, 56.3, 49.7, 40.1, 34.6, 21.6, 10.9.

N-(4-(N-Butylsulfamoyl)phenethyl)-5-chloro-2-methoxybenzamide (13).—

Compound **13** was prepared from **36** (0.39 mmol) and butylamine (1.95 mmol) following method B in 56% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.25 (t, J = 5.58 Hz, 1H), 7.73 (d, J = 8.32 Hz, 2H), 7.62 (d, J = 2.80 Hz, 1H), 7.51–7.47 (m, 3H), 7.15 (d, J = 8.92 Hz, 1H), 3.82 (s, 3H), 3.55 (q, J = 6.60 Hz, 2H), 2.93 (t, J = 7.00 Hz, 2H), 2.71 (q, J = 6.60 Hz, 2H), 1.37–1.17 (m, 4H), 0.78 (t, J = 7.28 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.1, 138.5, 131.4, 129.5, 129.4, 126.5, 124.9, 124.3, 114.2, 56.2, 42.2, 40.2, 34.6, 31.0, 19.4, 13.4.

5-Chloro-N-(4-(N,N-dibutylsulfamoyl)phenethyl)-2-methoxybenzamide (14).—

Compound **14** was prepared from compound **36** (0.39 mmol) and dibutylamine (1.95 mmol) following method B in 86% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.26 (t, J = 5.42 Hz, 1H), 7.71 (d, J = 8.32 Hz, 2H), 7.63 (d, J = 2.80 Hz, 1H), 7.51–7.47 (m, 3H), 7.15 (d, J = 8.92 Hz, 1H), 3.82 (s, 3H), 3.54 (q, J = 6.53 Hz, 2H), 3.02 (t, J = 7.52 Hz, 4H), 2.93 (t, J = 7.00 Hz, 2H), 1.41 (qun, J = 7.52 Hz, 4H), 1.22 (sex, J = 7.40 Hz, 4H), 0.837 (t, J = 7.34 Hz, 6H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.1, 138.5, 131.4, 129.5, 129.4, 126.5, 124.9, 124.3, 114.2, 56.2, 42.2, 40.2, 34.6, 31.0, 19.2, 13.5.

5-Chloro-2-methoxy-N-(4-(N-phenylsulfamoyl)phenethyl)-benzamide (15).—

Compound **15** was prepared from compound **36** (0.26 mmol) and aniline (1.30 mmol) following method B in 80% yield. Purity was confirmed by HPLC: ^1H NMR (400 MHz, DMSO- d_6) δ 8.25 (t, J = 5.56 Hz, 1H), 7.70 (d, J = 8.36 Hz, 2H), 7.61 (d, J = 2.80 Hz, 1H), 7.49 (dd, J = 8.88, 2.89 Hz, 1H), 7.42 (d, J = 8.36 Hz, 2H), 7.19 (d, J = 2.78 Hz, 2H), 7.13 (d, J = 8.92 Hz, 1H), 7.09 (d, J = 4.34 Hz, 2H), 7.00 (t, J = 7.64 Hz, 1H), 3.73 (s, 3H), 3.50 (q, J = 6.56 Hz, 2H), 2.87 (t, J = 7.02 Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.6, 144.8, 137.7, 137.5, 131.4, 129.5, 129.4, 129.1, 126.7, 124.8, 124.3, 123.9, 119.9, 114.1, 56.1, 34.5, 30.6.

N-(4-(N-Benzylsulfamoyl)phenethyl)-5-chloro-2-methoxybenzamide (16).—

Compound **16** was prepared from compound **36** (1.28 mmol) and benzylamine (6.40 mmol) following method B in 72% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.27 (t, J = 5.56 Hz, 1H), 8.09 (t, J = 5.90 Hz, 1H), 7.76 (d, J = 8.32 Hz, 2H), 7.65 (d, J = 2.08 Hz, 1H), 7.50 (dd, J = 9.05, 3.15 Hz, 1H), 7.47 (d, J = 8.32 Hz, 2H), 7.29–7.19 (m, 3H), 7.16 (d, J = 8.92 Hz, 1H), 3.97 (d, J = 5.60 Hz, 2H), 3.83 (s, 3H), 3.56 (q, J = 6.62 Hz, 2H), 2.93 (t, J = 7.06 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.2, 138.7, 137.6, 131.5, 129.5, 129.4, 128.2, 127.5, 127.1, 126.6, 124.9, 124.4, 114.2, 56.3, 46.1, 40.2, 34.6.

5-Chloro-2-methoxy-N-(4-(N-(4-methoxybenzyl)sulfamoyl)phenethyl)benzamide (17).—

Compound **17** was prepared from compound **36** (0.26 mmol) and 4-methoxybenzylamine (1.30 mmol) following method B in 69% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.28 (t, J = 5.46 Hz, 1H), 8.00 (t, J = 6.22 Hz, 1H), 7.75 (d, J = 8.12 Hz, 2H), 7.64 (d, J = 2.68 Hz, 1H), 7.51 (dd, J = 8.99, 2.39 Hz, 1H), 7.46 (d, J = 8.20 Hz, 2H), 7.17 (d, J = 8.92 Hz, 1H), 7.13 (d, J = 8.56 Hz, 2H), 6.83 (d, J = 8.60 Hz, 2H), 3.90 (d, J = 6.20 Hz, 2H), 3.83 (s, 3H), 3.72 (s, 3H), 3.56 (q, J = 6.55 Hz, 2H), 2.94 (t, J = 7.04

Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 158.4, 155.7, 144.2, 138.7, 131.5, 129.5, 129.4, 128.9, 126.6, 126.7, 124.9, 124.3, 114.2, 113.6, 56.3, 55.03, 45.7, 40.2, 34.7.

5-Chloro-N-(4-(N-(4-hydroxybenzyl)sulfamoyl)phenethyl)-2-methoxybenzamide (18).—Compound **18** was prepared from compound **36** (0.39 mmol) and 4-

(aminomethyl)phenol (1.95 mmol) following method B in 51% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 9.30 (br s, 1H), 8.28 (t, J = 5.46 Hz, 1H), 7.92 (t, J = 6.20 Hz, 1H), 7.74 (d, J = 8.20 Hz, 2H), 7.64 (d, J = 2.80 Hz, 1H), 7.50 (dd, J = 8.96, 2.89 Hz, 1H), 7.46 (d, J = 8.24 Hz, 2H), 7.16 (d, J = 8.92 Hz, 1H), 7.00 (d, J = 8.04 Hz, 2H), 6.65 (d, J = 8.44 Hz, 2H), 3.83–3.82 (m, 5H), 3.55 (q, J = 6.60 Hz, 2H), 2.93 (t, J = 7.02 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 156.5, 155.7, 144.2, 138.7, 131.5, 129.5, 129.4, 128.4, 127.6, 124.9, 124.3, 114.9, 114.2, 56.4, 56.3, 45.8, 40.3, 34.7.

5-Chloro-N-(4-(N-(4-chlorobenzyl)sulfamoyl)phenethyl)-2-methoxybenzamide (19).—Compound **19** was prepared from compound **36** (0.39 mmol) and 4-

chlorobenzylamine (1.95 mmol) following method B in 45% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.28 (t, J = 5.58 Hz, 1H), 8.14 (t, J = 6.26 Hz, 1H), 7.74 (d, J = 8.32 Hz, 2H), 7.64 (d, J = 2.80 Hz, 1H), 7.50 (dd, J = 8.79, 2.77 Hz, 1H), 7.46 (d, J = 8.28 Hz, 2H), 7.32 (d, J = 8.56 Hz, 2H), 7.24 (d, J = 8.56 Hz, 2H), 7.16 (d, J = 8.92 Hz, 1H), 3.97 (d, J = 6.12 Hz, 2H), 3.82 (s, 3H), 3.55 (q, J = 6.63 Hz, 2H), 2.93 (t, J = 7.08 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.3, 138.6, 136.8, 131.7, 131.5, 129.6, 129.5, 129.4, 128.1, 126.5, 124.9, 124.3, 114.2, 56.2, 45.3, 40.2, 34.7.

5-Chloro-N-(4-(N-(4-(trifluoromethyl)benzyl)sulfamoyl)-phenethyl)-2-methoxybenzamide (20).—Compound **20** was prepared from compound **36** (0.39 mmol)

and 4-(trifluoromethyl)benzylamine (1.95 mmol) following method B in 48% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.23–8.27 (m, 2H), 7.74 (d, J = 7.88 Hz, 2H), 7.62–7.64 (m, 3H), 7.51 (d, J = 8.88 Hz, 1H), 7.44–7.46 (m, 4H), 7.17 (d, J = 8.84 Hz, 1H), 4.09 (d, J = 5.92 Hz, 2H), 3.82 (s, 3H), 3.56 (q, J = 6.24 Hz, 2H), 2.93 (t, J = 6.80 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.3, 142.6, 138.5, 131.4, 129.4, 128.1, 126.5, 125.0, 124.9, 124.8, 124.3, 114.1, 56.2, 45.5, 40.2, 34.6.

5-Chloro-2-methoxy-N-(4-(N-phenethylsulfamoyl)phenethyl)-benzamide (21).—

Compound **21** was prepared from compound **36** (0.32 mmol) and phenylethylamine (1.60 mmol) following method B in 69% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.25 (t, J = 5.58 Hz, 1H), 7.74 (d, J = 8.26 Hz, 2H), 7.66 (t, J = 5.78 Hz, 1H), 7.63 (d, J = 2.76 Hz, 1H), 7.51–7.46 (m, 3H), 7.25 (d, J = 7.52 Hz, 2H), 7.20 (d, J = 7.24 Hz, 1H), 7.14 (d, J = 8.44 Hz, 2H), 3.80 (s, 3H), 3.55 (q, J = 6.58 Hz, 2H), 2.98–2.91 (m, 4H), 2.68 (t, J = 7.50 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.3, 138.7, 138.3, 131.4, 129.5, 128.6, 128.3, 126.6, 126.2, 124.9, 124.3, 114.1, 56.2, 40.2, 44.0, 35.3, 34.6, 18.6.

N-(4-(Azetidin-1-ylsulfonyl)phenethyl)-5-chloro-2-methoxybenzamide (22).—

Compound **22** was prepared from compound **36** (0.39 mmol) azetidine hydrochloride (1.95 mmol) following method B in 64% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.29 (t, J = 5.52 Hz, 1H), 7.74 (d, J = 8.24 Hz, 2H), 7.60–7.56 (m, 3H), 7.50 (dd, J = 8.92, 2.80 Hz, 1H), 7.15 (d, J = 8.88 Hz, 1H), 3.83 (s, 3H), 3.64 (t, J = 7.66 Hz, 4H), 3.58 (q, J = 6.63 Hz, 2H), 2.98

(t, J = 7.00 Hz, 2H) 1.99–1.92 (m, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 145.5, 131.6, 131.4, 129.8, 129.4, 128.2, 124.9, 124.3, 114.2, 56.2, 50.7, 40.1, 34.8, 14.7.

5-Chloro-2-methoxy-N-(4-(pyrrolidin-1-ylsulfonyl)phenethyl)-benzamide (23).—

Compound **23** was prepared from compound **36** (0.26 mmol) and pyrrolidine (1.30 mmol) following method B in 73% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.25 (t, J = 5.68 Hz, 1H), 7.73 (d, J = 8.28 Hz, 2H), 7.59 (q, J = 2.80 Hz, 1H), 7.52–7.48 (m, 3H), 7.15 (d, J = 8.88 Hz, 1H), 3.82 (s, 3H), 3.56 (q, J = 6.60 Hz, 2H), 3.12 (t, J = 6.74 Hz, 4H), 2.94 (t, J = 6.98 Hz, 2H), 1.62–1.54 (m, 4H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 144.9, 134.2, 131.4, 129.6, 129.4, 127.3, 124.9, 124.3, 114.2, 56.2, 47.7, 40.1, 34.7, 24.6.

5-Chloro-2-methoxy-N-(4-(piperidin-1-ylsulfonyl)phenethyl)-benzamide (24).—

Compound **24** was prepared from compound **36** (0.26 mmol) and piperidine (1.30 mmol) following method B in 70% yield. ^1H NMR (400 MHz, CDCl_3) δ 8.16 (d, J = 2.76 Hz, 1H), 7.78 (br s, 1H), 7.71 (d, J = 8.28 Hz, 2H), 7.40 (d, J = 8.30 Hz, 1H), 7.38 (dd, J = 2.80, 8.80 Hz, 2H), 6.88 (d, J = 8.78 Hz, 1H), 3.79 (s, 3H), 3.76 (q, J = 6.78 Hz, 2H), 2.99 (t, J = 5.50 Hz, 2H), 3.01 (t, J = 6.80 Hz, 4H), 1.64 (quin, J = 5.71 Hz, 4H), 1.42 (quin, J = 5.96 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.6, 145.0, 133.3, 131.4, 129.6, 129.4, 127.4, 124.9, 124.3, 114.1, 56.2, 46.5, 40.1, 34.7, 24.6, 22.8.

5-Chloro-2-methoxy-N-(4-(piperazin-1-ylsulfonyl)phenethyl)-benzamide (25).—

Compound **25** was prepared from compound **36** (0.39 mmol) and piperazine (1.95 mmol) following method B in 60% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.29 (t, J = 5.52 Hz, 1H), 7.66 (d, J = 8.24 Hz, 2H), 7.62 (d, J = 2.76 Hz, 1H), 7.54–7.49 (m, 3H), 7.16 (d, J = 8.92 Hz, 1H), 3.83 (s, 3H), 3.55 (q, J = 7.06 Hz, 2H), 2.95 (t, J = 7.06 Hz, 2H), 2.77–2.70 (m, 8H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 156.7, 145.2, 132.8, 131.4, 129.6, 129.6, 129.5, 127.6, 124.9, 124.3, 114.2, 56.3, 46.6, 44.6, 34.7.

5-Chloro-2-methoxy-N-(4-(morpholin-1-ylsulfonyl)phenethyl)-benzamide (26).—

Compound **26** was prepared from compound **36** (0.39 mmol) and morpholine (1.95 mmol) following method B in 91% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.27 (t, J = 5.56 Hz, 1H), 7.68 (d, J = 8.24 Hz, 2H), 7.60 (d, J = 2.80 Hz, 1H), 7.55 (d, J = 8.24 Hz, 2H), 7.49 (dd, J = 9.01, 2.83 Hz, 1H), 7.15 (d, J = 8.92 Hz, 1H), 3.82 (s, 3H), 3.62 (t, J = 4.66 Hz, 4H), 3.57 (q, J = 6.56 Hz, 2H), 2.96 (t, J = 6.98 Hz, 2H), 2.84 (t, J = 4.62 Hz, 4H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.6, 155.7, 145.5, 132.3, 131.4, 129.7, 129.4, 127.7, 124.9, 124.3, 114.1, 65.2, 56.2, 45.9, 40.1, 34.7.

5-Chloro-2-methoxy-N-(3-(4-(N-methylsulfamoyl)phenyl)-propyl)benzamide (27).—

Compound **27** was prepared from **41** (0.37 mmol) and methylamine HCl (0.19 mmol) following method B in 72% yield. ^1H NMR (400 MHz, DMSO- d_6) δ 8.29 (t, J = 5.30 Hz, 1H), 7.70 (d, J = 8.28 Hz, 2H), 7.64 (d, J = 2.76 Hz, 1H), 7.51 (dd, J = 9.91, 2.91 Hz, 1H), 7.47 (d, J = 8.24 Hz, 2H), 7.37 (q, J = 5.01 Hz, 1H), 7.17 (d, J = 8.88 Hz, 1H), 3.88 (s, 3H), 3.28 (q, J = 6.18 Hz, 2H), 2.73 (t, J = 7.66 Hz, 2H), 2.40 (t, J = 5.04 Hz, 3H), 1.85 (quin, J = 7.32 Hz, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.8, 155.6, 146.7, 136.8, 131.2, 129.3, 129.0, 125.7, 125.5, 124.2, 114.1, 56.3, 38.7, 32.3, 30.3, 28.6.

5-Chloro-2-methoxy-N-(4-(4-(N-methylsulfamoyl)phenyl)butyl)-benzamide (28).

—Compound **28** was prepared from **42** (0.37 mmol) and methylamine HCl (0.19 mmol) following method B in 84% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.24 (t, *J* = 5.54 Hz, 1H), 7.70 (d, *J* = 8.24 Hz, 2H), 7.62 (d, *J* = 2.76 Hz, 1H), 7.50 (dd, *J* = 8.66, 2.62 Hz, 1H), 7.45 (d, *J* = 8.20 Hz, 2H), 7.35 (q, *J* = 5.05 Hz, 1H), 7.16 (d, *J* = 8.88 Hz, 1H), 3.85 (s, 3H), 3.29 (q, *J* = 6.67 Hz, 2H), 2.71 (t, *J* = 7.50 Hz, 2H), 2.40 (d, *J* = 5.04 Hz, 3H), 1.65 (quin, *J* = 8.15 Hz, 2H), 1.54 (quin, *J* = 7.06 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.7, 155.6, 147.2, 136.7, 131.2, 129.3, 129.0, 126.7, 125.5, 124.3, 114.1, 56.3, 38.8, 34.5, 28.7, 28.6, 27.9.

2-Phenethylisoindoline-1,3-dione (30).—Phthalic anhydride (103.20 mmol) and 2-phenylethylamine **29** (82.50 mmol) were added to acetic acid (30 mL), and refluxed for 3 h. The solution was cooled to room temperature and was poured into ice–water. The precipitate was filtered and purified by recrystallization in ethanol to give **30** as a white solid (17.25 g, 33%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.84–7.81 (m, 4H), 7.27–7.16 (m, 5H), 3.82 (t, *J* = 7.36 Hz, 2H), 2.93 (t, *J* = 7.34 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.6, 138.2, 134.4, 131.5, 128.6, 128.4, 126.4, 122.3, 38.8, 33.6.

Method A: 4-(2-(1,3-Dioxoisindolin-2-yl)ethyl)benzenesulfonyl chloride (31).

To the solution of **30** (59.74 mmol) in dichloromethane (DCM) was added chlorosulfonic acid (15 mL) at –20 °C. The solution then was heated to 60 °C for 1 h. The solution was cooled to room temperature and poured onto crushed ice. The product was extracted into DCM, concentrated, and purified by column chromatography to give **31** as a white solid (17.05 g, 82%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.83–7.82 (m, 4H), 7.49 (d, *J* = 7.55 Hz, 2H), 7.16 (d, *J* = 7.56 Hz, 2H), 3.81 (t, *J* = 7.16 Hz, 2H), 2.92 (t, *J* = 7.16 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.6, 146.1, 138.7, 134.4, 131.4, 127.9, 125.6, 123.0, 38.6, 33.4.

Method B: 4-(2-(1,3-Dioxoisindolin-2-yl)ethyl)-N-methylbenzenesulfonamide (32).

—To the solution of compound **31** (10.40 mmol) in DCM (200 mL) were added methylamine (52.00 mmol) and trimethylamine (Et₃N) (52.00 mmol). The solution was stirred overnight at room temperature. The reaction was diluted with H₂O and the product was extracted into DCM, concentrated, and purified by column chromatography to give **32** (3.36 g, 84%) as a white solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.83–7.82 (m, 4H), 7.65 (d, *J* = 8.28 Hz, 2H), 7.44 (d, *J* = 8.24 Hz, 2H), 7.35 (q, *J* = 5.01 Hz, 1H), 3.86 (t, *J* = 7.12 Hz, 2H), 3.02 (t, *J* = 7.06 Hz, 2H), 2.35 (d, *J* = 5.04 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.6, 143.2, 137.3, 134.4, 131.4, 129.5, 126.8, 123.0, 38.4, 33.5, 28.5.

4-(2-Aminoethyl)-N-methylbenzenesulfonamide (33).

—Hydrazine (30.00 mmol) was added dropwise to a stirring solution of **32** (6.01 mmol) in EtOH (10 mL). After addition, the solution was heated to 60 °C for 12 h. The precipitate was then washed with hexane. The filtrate was collected and concentrated under reduced pressure. The crude product was then purified by column chromatography to give **33** as a yellow liquid (1.01 g, 79%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.69 (d, *J* = 8.32 Hz, 2H), 7.43 (d, *J* = 8.32 Hz, 2H), 2.80 (t, *J* = 6.54 Hz, 2H), 2.72 (t, *J* = 6.60 Hz, 2H), 2.40 (s, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 145.5, 136.8, 129.3, 126.6, 43.1, 39.4, 28.6.

Method C: 5-Chloro-2-methoxy-N-phenethylbenzamide (35).—2-Methoxy-5-chloro-benzoic acid **34** (0.47 mmol) and Et₃N (0.93 mmol) were dissolved in DCM (4 mL), and the mixture was cooled to 0 °C. To this solution were added 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC, 0.70 mmol) and hydroxybenzotriazole (HOBt, 0.70 mmol). After 1 h, **29** (0.47 mmol) was added and the reaction was allowed to room temperature overnight. The solution was diluted with DCM and H₂O, and was extracted into DCM. The combined organic phase was concentrated and purified by column chromatography to give **35** as a white powder (37% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.22 (d, *J* = 3.04 Hz, 1H), 7.66 (br s, 1H), 7.50 (dd, *J* = 8.80, 3.00 Hz, 1H), 7.32 (d, *J* = 7.22 Hz, 2H), 7.28 (d, *J* = 8.03 Hz, 2H), 7.24–7.20 (m, 1H), 7.16 (d, *J* = 9.46 Hz, 1H), 3.81 (s, 3H), 3.54 (q, *J* = 7.06 Hz, 2H), 2.85 (t, *J* = 6.20 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.4, 155.7, 139.4, 131.5, 129.6, 128.7, 128.3, 126.1, 124.7, 124.3, 114.1, 56.2, 40.7, 34.9.

4-(2-[(5-Chloro-2-methoxyphenyl)formamido]ethyl)benzene-1-sulfonyl Chloride (36).—Compound **36** was prepared from compound **35** (9.50 mmol) following method A in 62% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.20 (br s, 1H), 7.66 (s, 1H), 7.56 (d, *J* = 8.28 Hz, 2H), 7.50 (dd, *J* = 8.78, 2.76 Hz, 1H), 7.22 (d, *J* = 8.53 Hz, 2H), 7.16 (d, *J* = 9.04 Hz, 1H), 3.80 (s, 3H), 3.51 (q, *J* = 7.30 Hz, 2H), 2.84 (t, *J* = 6.80 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.5, 155.7, 146.1, 139.8, 131.5, 129.5, 128.0, 125.6, 124.7, 124.3, 114.2, 56.2, 40.6, 34.6.

5-Chloro-2-methoxy-N-phenpropylbenzamide (39).—Compound **39** was prepared from **34** (5.36 mmol) and **37** (5.36 mmol) following method C in 59% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.26 (t, *J* = 5.28 Hz, 1H), 7.63 (d, *J* = 2.80 Hz, 1H), 7.51 (dd, *J* = 8.92, 2.91 Hz, 1H), 7.32–7.16 (m, 6H), 3.88 (s, 3H), 3.28 (q, *J* = 6.62 Hz, 2H), 2.64 (t, *J* = 7.68 Hz, 2H), 1.82 (quin, *J* = 7.37 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.4, 155.7, 139.4, 131.5, 129.6, 128.7, 128.3, 126.1, 124.7, 124.3, 114.1, 56.2, 40.7, 34.9.

5-Chloro-2-methoxy-N-(4-(4-(N-methylsulfamoyl)phenyl)butyl)-benzamide (40).—Compound **40** was prepared from **34** (5.36 mmol) and **38** (5.36 mmol) following method C in 73% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.07 (d, *J* = 5.73 Hz, 1H), 7.46 (d, *J* = 2.82 Hz, 1H), 7.33 (dd, *J* = 9.29, 3.00 Hz, 1H), 7.13–6.97 (m, 6H), 3.68 (s, 3H), 3.12 (q, *J* = 6.67 Hz, 2H), 2.46 (t, *J* = 7.73 Hz, 2H), 1.53–1.33 (m, 4H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 164.1, 156.1, 142.7, 131.7, 129.8, 128.8, 128.7, 126.2, 126.1, 126.0, 124.7, 114.5, 56.7, 39.3, 35.3, 29.2, 28.9.

4-(3-(5-Chloro-2-methoxybenzamido)propyl)benzenesulfonyl Chloride (41).—Compound **41** was prepared from **39** (3.18 mmol) following method A in 53% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.26 (t, *J* = 5.46 Hz, 1H), 7.63 (d, *J* = 2.80 Hz, 1H), 7.54 (d, *J* = 8.12 Hz, 2H), 7.48 (dd, *J* = 8.91, 2.92 Hz, 1H), 7.19 (d, *J* = 8.16 Hz, 2H), 7.16 (d, *J* = 8.92 Hz, 1H), 3.87 (s, 3H), 3.27 (q, *J* = 6.56 Hz, 2H), 2.63 (t, *J* = 7.66 Hz, 2H), 1.80 (quin, *J* = 7.34 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.8, 155.6, 145.4, 142.3, 131.2, 129.3, 127.6, 125.6, 125.5, 124.24, 114.1, 56.3, 38.7, 32.6, 30.7.

4-(4-(5-Chloro-2-methoxybenzamido)butyl)benzenesulfonyl Chloride (42).—Compound **42** was prepared from **40** (3.10 mmol) following method A in 38% yield. ¹H

NMR (400 MHz, DMSO-*d*₆) δ 8.21 (t, *J* = 5.54 Hz, 1H), 7.61 (d, *J* = 2.80 Hz, 1H), 7.52 (d, *J* = 8.08 Hz, 2H), 7.48 (dd, *J* = 8.90, 2.80 Hz, 1H), 7.17–7.13 (m, 3H), 3.84 (s, 3H), 3.27 (q, *J* = 6.47 Hz, 2H), 2.61 (t, *J* = 7.40 Hz, 2H), 1.64–1.48 (m, 4H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.7, 155.7, 145.4, 142.7, 131.2, 129.3, 127.5, 125.5, 124.4, 114.1, 56.3, 38.8, 34.4, 28.5, 28.2.

Biological Assays.

Cells.—J774A.1 murine macrophage cells were purchased from American Type Cell Culture (ATCC, Manassas, VA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.

IL-1 β Release Assays.—J774A.1 cells were plated into a 96-well plate (1×10^5 cells/well) for 24 h in growth medium. Cells were primed with *Escherichia coli* 0111:B4 LPS (Sigma-Aldrich) (final concentration: 1 μ g/mL) for 4.5 h. Next, test compounds were added for 30 min. ATP (5 mM) was added at the same time when compounds were added to induce NLRP3 inflammasome activation. After 30 min, the supernatants were collected and the level of IL-1 β was measured with a mouse IL-1 β ELISA kit following the manufacturer's instructions. BMDMs were similarly treated as described with J774A.1 cell line followed by ELISA analysis for IL- β production.

Inhibition on the NLRP4 and AIM2 Inflammasomes.—J774A.1 cells were plated into a 96-well plate (1×10^5 cells/well) for 24 h in growth medium. Cells were treated with LPS (1 μ g/mL) and test compounds for 1 h. Flagellin or poly deoxyadenylic-deoxythymidylic acid sodium salt (poly(dA:dT)) was used to induce the formation of the NLRP4 and the AIM2 inflammasomes. Flagellin (Enzo Life Sciences, Farmingdale, NY), isolated from *Salmonella typhimurium* strain 14028, was added in DMEM (Invitrogen) without fetal bovine serum (FBS) to the plate (1 μ g/mL) and allowed to incubate for 6 h. Flagellin cell-transfection was accomplished utilizing the Polyplus transfection kit (PULSin, New York, NY). For AIM2 activation, cells were incubated with poly(dA:dT) (4 μ g/mL) (InvivoGen, San Diego, CA) for 8 h. The supernatants were collected, and levels of IL-1 β were measured with a mouse IL-1 β ELISA kit following the manufacturer's instructions.

Animals.—All animal experiments were conducted under the guidelines of the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (revised 2011). ICR mice (8–12 weeks old) were purchased from Harlan Laboratories (Charles River, MA). C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). APP/PS1 female transgenic mice (B6C3-Tg (APP^{swe}, PSEN 1dE9)85Dbo/Mmjax) and matching wild-type female mice were purchased from the Jackson Laboratory.

Experimental Model of AMI.—Briefly, ICR mice were anesthetized using pentobarbital (50–70 mg/kg, Sigma-Aldrich, St. Louis, MO) followed by orotracheal intubation. After being placed in the right lateral decubitus position, the mice were subjected to left thoracotomy, pericardiectomy, and the proximal left coronary artery was ligated for 30 or 75 min and then released (I/R model). Different groups of mice were treated with the compound or a matching volume of vehicle (0.1 mL) (*N* = 6 in each group). Myocardial

damage was determined by measuring the serum troponin I level at 24 h after surgery and with pathology assessment of viability. Briefly, mice were anesthetized and the blood was drawn from the inferior vena cava and collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) for serum isolation. Mouse troponin I levels were determined by ELISA (Life Diagnostic Inc., West Chester, PA). Infarct size was measured at 24 h after induction of ischemia using TTC (Sigma-Aldrich) to stain viable myocardium.

LPS Challenge in Vivo and Compound Treatments.—C57BL/6 mice were injected intraperitoneally (i.p.) with 20 mg/kg LPS (Sigma-Aldrich) or PBS 1 h after compound or vehicle treatment. 2.5 h after LPS injection, serum levels of IL-1 β were measured by ELISA.

Studies in APP/PS1 Mice.—At the age of 7 months old, animals were randomly divided into the following experimental groups: WT, vehicle group ($n = 4$), JC124 treatment group ($n = 6$); APP/PS1 mice, vehicle group ($n = 5$), JC124 treatment group ($n = 7$). For i.p., JC124 was dissolved in 10% DMSO with PEG-100. For oral dosing, JC124 was dissolved in 2% DMSO in corn oil. Starting at the age of 7 months old, animals first received i.p. injection of JC124 or vehicle single daily for 5 days (Monday through Friday), then changed to oral dosing single daily for 5 days during weekdays for 7 weeks. After completing the 8 weeks treatment, animals were tested on cognitive functions using NOR and MWM tests.

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ABBREVIATIONS USED

AD	Alzheimer's disease
AMI	acute myocardial infarction
A β	amyloid- β
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
A β	beta-amyloid
BMDMs	mouse bone marrow derived macrophages
CAPS	cryopyrin-associated periodic syndrome
DCM	di chloromethane
DI	discrimination index
DMEM	Dulbecco's Modified Eagle Medium

EDCI	N-(3-(dimethylamino)-propyl)-N-ethyl-carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HOBt	hydroxybenzotriazole
IL-1β	interleukin-1 β
LPS	lipopolysaccharide
MOA	mechanisms of action
MS	multiple sclerosis
MWM	Morris Water Maze
NLRP3	NOD-like receptor family pyrin-domain-containing 3
NLRP3Is	NLRP3 inflammasome inhibitors
NOR	Novel Object Recognition
PBS	phosphate-buffered saline
poly(dAdT)	dT)poly deoxyadenylic-deoxythymidylic acid sodium salt
PMA	phosphomolybdic acid
RIG-I	retinoic acid-inducible gene I
SAR	structure activity relationship
TBI	traumatic brain injury
TFA	trifluoroacetic acid
TMS	tetramethylsilane
TTC	triphenyltetrazolium chloride
Et₃N	trimethylamine

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Figure 1.
Chemical structures of small molecule inhibitors targeting the NLRP3 inflammasome pathway.

***Including constitutively active NLRP3**

Figure 2.
Proposed mechanism of action for the benzenesulfonamide analogues as NLRP3Is.

Figure 3.

Effects of **JC124** treatment on cognitive functions in APP/PS1 mice. At the age of 7 months old, animals were randomly divided into the following experimental groups: WT mice, vehicle group ($n = 4$), JC124 treatment group ($n = 6$); Tg mice, vehicle group ($n = 5$), JC124 treatment group ($n = 7$). Starting at the age of 7 months old, animals were treated for 8 weeks (5 times/week). After completing treatment, animals were tested on cognitive functions using NOR (A) and MWM (B and C) methods.

Figure 4.

Effects of **JC124** in an experimental model of AMI. Mice ($n = 6$ for each group) were subjected to I/R for 30 or 75 min. The infarct size (A) then was measured by TTC staining, and serum troponin I level (B) was determined by ELISA.

Figure 5.
Chemical structures of the designed analogues of **JC124**.

Figure 6.

Compounds **14** and **17** block NLRP3 inflammasome activation and IL-1 β production in primary macrophages and LPS-challenged mice. (A) BMDMs were primed with LPS (1 μ g/mL) for 4.5 h and then treated with indicated doses of **14** or **17** when adding ATP (5 mM) stimulation for 30 min. IL- β in the culture media was assayed by ELISA. (B and C) J774A.1 cells were treated with LPS (1 μ g/mL) and test compounds (10 μ M) for 1 h. Flagellin (1 μ g/mL) was added and allowed to incubate for 6 h or (poly(dA:dT)) (4 μ g/mL) for 8 h. The supernatants were collected, and levels of IL-1 β were measured by ELISA. (D) Serum levels of IL-1 β from C57BL/6 mice pretreated with **14** and **17** (0.5 mg/kg) or vehicle control were measured by ELISA 2.5 h after i.p. injection of LPS (20 mg/kg).

Scheme 1. Chemical Synthesis of the Designed Analogues^a

^aReagents and conditions: (a) phthalic anhydride, AcOH, reflux; (b) ClSO₃H, 70 °C; (c) methylamine, Et₃N, DCM; (d) NH₂NH₂, EtOH, 60 °C; (e) various benzoic acids, EDCI, HOBt, Et₃N, DCM.

Scheme 2. Chemical Synthesis of the Designed Analogues^a^aReagents and conditions: (a) EDCI, HOBT, Et₃N, DCM; (b) ClSO₃H, 70 °C; (c) various amines, Et₃N, DCM.

Table 1.

Inhibitory Potency of the Designed Analogues on the Production of IL-1 β by J774A.1 Cells upon Stimulation with LPS/ATP

Compound	R ₁	R ₂	n=	IC ₅₀ (μ M)
JC124			1	3.25 $\pm$ 1.34
1.			1	13.36 $\pm$ 2.26
2.			1	17.10.25 $\pm$ 4.32
3.			1	146.56 $\pm$ 26.31
4.			1	10.48 $\pm$ 0.091

Compound	R ₁	R ₂	n=	IC ₅₀ (μM)
5.			1	31.78 ± 3.19
6.			1	18.031 ± 0.27
7.			1	44.42 ± 6.32
8.			1	5.83 ± 1.36
9.			1	32.75 ± 3.78
10.			1	2.01 ± 0.43

Compound	R ₁	R ₂	n=	IC ₅₀ (μM)
11.			1	0.64 ± 0.20
12.			1	1.40 ± 0.62
13.			1	0.97 ± 0.053
14.			1	0.55 ± 0.091
15.			1	2.08 ± 0.59
16.			1	1.31 ± 0.16
17.			1	0.42 ± 0.080
18.			1	0.63 ± 0.22

Compound	R ₁	R ₂	n=	IC ₅₀ (μM)
19.			1	1.50 ± 0.78
20.			1	1.90 ± 0.20
21.			1	0.81 ± 0.12
22.			1	1.66 ± 1.21
23.			1	1.53 ± 1.68

Compound	R ₁	R ₂	n=	IC ₅₀ (μM)
24.			1	5.01 ± 1.33
25.			1	1.40 ± 0.62
26.			1	3.44 ± 0.97
27.			2	2.03 ± 0.61

Compound	R ₁	R ₂	n=	IC ₅₀ (μM)
28.			3	1.74 ± 0.61