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Discovery and Optimization of 4-Oxo-2-thioxo-thiazolidinones as NOD-like receptor (NLR) family, pyrin domain–containing protein 3 (NLRP3) Inhibitors

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Graphical abstract

Abstract

Aberrant activation of NLRP3 inflammasome is present in a subset of acute and chronic inflammatory diseases. The NLRP3 inflammasome has been recognized as an attractive therapeutic target for developing novel and specific anti-inflammatory inhibitors. Cellular structure-activity relationship-guided optimization resulted in the identification of 4-oxo-2-thioxo-thiazolidinone derivative **9** as a selective and direct small-molecule inhibitor of NLRP3 with IC_{50} of 2.4 μM, possessing favorable ex vivo and in vivo pharmacokinetic properties. Compound **9** may represent a lead for the development of anti-inflammatory therapeutics for treating NLRP3-driven diseases.

Keywords:NLRP3, thiazolidinones, Structure-activity relationship (SAR)

Introduction

Inflammasomes are large, cytosolic, multimeric protein complexes and respond to a diverse set of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs).[1-3] Once activated, assembly of the NLRP3 inflammasome promote the maturation and secretion of important pro-inflammatory cytokines such as IL-1β and IL-18.[4-5] Whereas numerous inflammasomes have been identified,[6] NLRP3 is the best characterized and the most elusive one.[7] Aberrant NLRP3 activation has previously been implicated in several auto-inflammatory and auto-immune diseases such as Alzheimer's disease, type 2 diabetes mellitus, atherogenesis and gout.[8-11] Among the NLRP3 inhibitors identified so far, including benzenesulfonamide-based compounds,[12-13] novel boron compound series,[14] acrylate and acrylamide derivatives,[15-16] OLT1177,[17] BAY 11-7082[18] and MNS,[19] there is no evidence showing that these inhibitors can specifically and directly inhibit NLRP3 itself.[20] Therefore, the development of novel and specific NLRP3 inhibitors for the treatment of NLRP3-driven diseases is urgently needed. In this work, we reported the identification, characterization, and structure-activity relationship (SAR) for a novel chemotype of inhibitors of NLRP3. Iterative rounds of optimization efforts led to the discovery of 4-oxo-2-thioxo-thiazolidinone derivative, **9**, as a potent and specific NLRP3 inhibitor.

fied as a potent 'hit' for blocking NLRP3 inflammasome activation (Figure 1A), which is a reported inhibitor of the cystic fibrosis transmembrane conductance regulator (CFTR) channel.[21] $CFTR_{inh}$-172 exhibited a dose-dependent inhibitory activity on monosodium urate crystals (MSU)-induced caspase-1 activation and IL-1β secretion in LPS-primed bone marrow–derived macrophages (BMDMs) (Figure 1B and 1C). Having identified **1** as a promising 'hit', a library (**2−21**) bearing thiazolidinone core was designed and synthesized using a concise three-step synthetic route (Schemes 1−2). Briefly, reaction of commercially available amines with an excess of thiophosgene under basic conditions followed by cyclization in the presence of methyl 2-sulfanylacetate afforded **24a−k** in moderate yield. Connection between the thiazolidinones and aromatic aldehydes under microwave-assisted conditions afforded the desired 4-oxo-2-thioxo-3-substituted-thiazolidin-5-ylidene-aromatic derivatives **1−15**, and **18−21** (Scheme 1). 2,4-dioxo-3-substituted-thiazolidin-5-ylidene-aromatic derivatives **16−17** were prepared as described in Scheme 2. The geometry of the exocyclic double bond is assumed to be (*Z*) configuration due to steric effects.[22]

Using **1** as a lead, the SAR for 2-thioxothiazolidin-4-one was explored, utilizing inhibition of IL-1β secretion in BMDMs and intracellular chloride efflux in HT29 cells as readout to measure the inhibitory activity against NLRP3 inflammasome and CFTR, respectively. We first investigated the effects of modification to the aryl ring (R^2) by introducing a hydrophobic group or a hydrophilic group resulted in compounds **2−8**. As shown in Table 1, introduction of 4-methylpiperazin-1-yl, morpholino and 4-hydroxyl-piperidin-1-yl groups to **1** led to **2−4**, respectively, which all showed slightly improved NLRP3 inhibitory activity. When the trifluoromethyl group was replaced with trifluoromethoxy and nitro groups (**2** *vs* **5**, **3** *vs* **6**, **2** *vs* **7**), respectively, the anti-inflammatory activity was maintained. However, compound **8** containing substituent with anilino group dramatically abolished the inhibitory activity (32%) against NLRP3 at 10 μM. These modifications all yielded compounds possessing less potent activities against CFTR, which provided the improved NLRP3 selectivity.

We next explored the effects of introducing additional carbon linker between phenyl ring and thiazolidinone core, resulting compounds **9−11** with improved NLRP3 inhibitory activities, having IC_{50}s of 2.40, 5.91 and 4.32 μM, respectively (Table 2). Surprisingly, compounds **9−11** didn't show CFTR inhibitory activities at 20 μM. These results indicated that proper length of the linker appears to be critical for achieving both activity and selectivity against NLRP3. Replacement the carboxyl group with tetrazolyl bioisostere yielded compounds **12−14**, which exhibited a slight decrease of inhibitory potency against NLRP3 compared with their counterparts (**9−11**). Compound **15** containing a substituent with 4-methylpiperazin-1-yl group showed a sharp loss of inhibitory activity against NLRP3, suggesting a limited tolerance for 4-amide derivatization under the condition of one carbon linker (n = 1). The distinct SAR profiles were observed with different carbon linker (**1** *vs* **9**, and **2** *vs* **15**).

Subsequently, 2,4-dioxo-thiazolidinedione derivatives (**16−17**) were synthesized and evaluated, which exhibited decrease activities against NLRP3. This result suggested the positive impact of the thioxo group (X = S) in the thiazolidinone core. Finally, the substitutents of R^4 ranging from 3-trifluoromethoxylphenyl (**18**), 3-fluorophenyl (**19**), 3-pyridyl (**20**) to biphenyl (**21**), were investigated resulting in decreased NLRP3 inhibitory activities.

The SAR exploration of 2-thioxothiazolidin-4-one scaffold revealed that the one carbon spacer between phenyl ring and thiazolidinone core (n = 1), the thioxo group (X = S), 4-carboxyl group (R^3) and 3-trifluoromethylphenyl group (R^4) were key structural features for achieving both potent inhibitory activity and selectivity against NLRP3. The most active compound **9** inhibited IL-1β secretion in LPS-primed BMDMs with micromolar cellular IC_{50} value of 2.40 μM, but not blocked CFTR activity at a concentration of 20 μM.

With these new NLRP3 inhibitors in hand that demonstrated excellent inhibitory potency, the most potent compounds (**2**−**7** and **9**), as well as **1**, were tested against human liver microsomes (HLM) and mouse liver microsomes (MLM) in the presence of an NADPH regenerating system (Table 3). Of these, the stability of **1** was limited in mouse liver microsomes ($t_{1/2}$ = 12.6 min) but was better in human microsomes ($t_{1/2}$ = 277.2 min). The morpholino-amide derivatives **3** and **6** exhibited much better metabolic stability ($t_{1/2}$ >145 and 187.3 min, respectively), than 4-methylpiperazin-1-yl-amide derivatives (**2**, **5** and **7**) and 4-hydroxypiperidin-1-yl-amide derivative (**4**) in human microsomes. Compound **9** exhibited the best *in vitro* microsomal stability in MLM with lowest clearance values (CL^{hep} < 20.7 mL/min/kg) and longest half-life ($t_{1/2}$ > 145.0 min) among these analogs.

The effects of compound **9** on NLRP3 inflammasome activation were also further characterized in BMDMs. Compound **9** exhibited time-dependent inhibitory activities on IL-1β secretion at the dose of 5 μM in LPS-primed BMDMs when stimulated with different NLRP3 agonists, including nigericin, ATP and MSU (Figure 2A–C). Similarly, when LPS-primed BMDMs were treat ed with an increasing concentration of NLRP3 agonists along with **9** (5 μM), secreted IL-1β was also inhibited in a dose-dependent manner (Figure 2D–F). In contrast, **9** had no effects on tumor necrosis factor-α (TNF-α) production (Figure 2G) and could not inhibit NLRC4 or AIM2 inflammasome activation induced by *Salmonella* infection or dsDNA analog Poly(dA:dT) transfection, respectively (Figure 2H and 2I), which demonstrates that **9** is a specific inhibitor for NLRP3 inflammasome. Compound **9** also blocked nigericin or ATP-induced BMDMs death (Fig. S1).

Then, the pharmacokinetic properties of compound **9** were further evaluated in Sprague-Dawley rats administered a single intravenous or oral dose. Compound **9** exhibited moderate pharmacokinetics with a half-life of 1.8 hours, an area under the curve of 1928 (h·ng)/mL, and a bioavailability of 27% (Table S1).

Compound **9** has been proven to be a potent, selective, and direct inhibitor of NLRP3.[23] The *in vivo* experimental results showed that inhibition of NLRP3 by compound **9** can significantly prevent NLRP3-dependent acute inflammation in mice model of cryopyrin-associated autoinflammatory syndrome (CAPS) and reverse NLRP3-dependent metabolic disorders in diabetic mice. Furthermore, compound **9** is active *ex vivo* for monocytes from healthy individuals or synovial fluid cells from patients with gout (for a more comprehensive *in vivo* efficacy study, please see ref 23).

In conclusion, cell-based structure activity relationship (SAR) studies guided the discovery of compound **9**, which represents a new chemotype exhibiting potent and highly selective NLRP3 activities. Having excellent selectivity and favorable pharmacokinetic profile, compound **9** may serve as a good starting point for the development of new therapeutics against NLRP3 inflammasome related diseases.

Notes

The authors declare no competing financial interest.

Acknowledgments

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Figure 1. $CFTR_{inh}$-172 inhibits NLRP3 inflammasome activation. A) Chemical structure of $CFTR_{inh}$-172 (**1**). B) Immunoblot analysis of IL-1β and cleaved caspase-1 (p20) in culture supernatants (SN) of LPS-primed BMDMs treated with various doses of $CFTR_{inh}$-172 as indicated and then stimulated with MSU. C) ELISA of IL-1β from LPS-primed BMDMs treated with $CFTR_{inh}$-172 (0–20 μM) and then stimulated with MSU. Cytokine level is normalized to that of DMSO-treated control cells.

Figure 2. Compound 9 specifically inhibits NLRP3 inflammasome activation. (A-C) ELISA of IL-1β in supernatants from LPS-primed BMDMs treated with **9** (5 μM) and then stimulated with nigericin (A), ATP (B), MSU (C) for indicated time. (D–F) ELISA of IL-1β in supernatants from LPS-primed BMDMs treated with **9** (5 μM) and then stimulated with various doses of nigericin (10 μM) (D), ATP (2.5 mM) (E) or MSU (150 μg/mL) (F). (G) Production of TNF-α (as measured by ELISA) from LPS-primed BMDMs treated with **9** (5 μM) and stimulated with nigericin (10 μM), ATP (2.5 mM) or MSU (150 μg/mL). (H-I) ELISA of IL-1β in supernatants from LPS-primed BMDMs treated with **9** (5 μM) and then transfected with poly(dA:dT) (0.5 μg/ml) for various time points by using of Lipofectamine 2000 (Invitrogen) (H), or infected by Salmonella typhimurium (multiplicity of infection) (I) with different time.

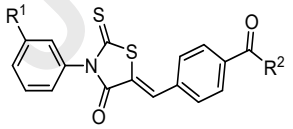
Scheme 1. Synthesis of Compounds 1-15 and 18-21[a]

[a]Reagents and conditions: (a) $NaHCO_3$, CH_2Cl_2, H_2O, 0 °C to room temperature (RT), overnight; (b) Methyl 2-sulfanylacetate, TEA, CH_2Cl_2, 0 °C to RT, overnight; (c) Substituted aromatic aldehydes, NaOAc, HOAc, microwave, 130 °C, 0.5 h.

Scheme 2. Synthesis of Compounds 16-17[a]

[a]Reagents and conditions: (a) K_2CO_3, DMF, 0 °C to RT, 12 h; (b) Substituted aromatic aldehydes, NaOAc, HOAc, microwave, 130 °C, 0.5 h.

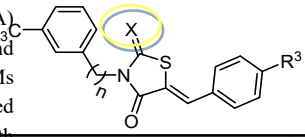
Table 1. SAR of Compounds 1-8 for NLRP3 and CFTR

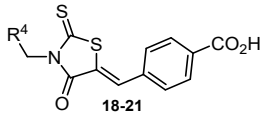


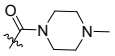
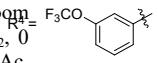
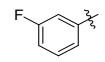
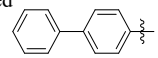
Cmpd	R^2	Inhibition of NLRP3 activity(%)[a]	Inhibition of CFTR activity(%)[b]	SI[c] NLRP3/CFTR	Inhibition of NLRP3 activity (IC_{50} μM)[d]
1		71	86	0.83	6.39±0.33
2		81	77	1.05	2.69±0.08
3		81	74	1.09	2.74±0.31
4		80	72	1.11	2.44±0.24
5		75	68	1.10	3.18±0.57
6		81	66	1.23	2.98±0.41
7		32	27	1.19	>10
8					

[a] BMDMs were treated with tested compounds at a concentration of 10 μM. [b] HT29 cells were treated with tested compounds at a concentration of 20 μM. [c] SI (Selectivity index) was determined by the ratio of % NLRP3 Inhibition at 10 μM and % CFTR Inhibition at 20 μM. [d] BMDMs were pretreated with varied doses of tested compounds and then primed with LPS and stimulated with nigericin. Production of IL-1β was measured by ELISA and then the cytokine level was normalized to that of DMSO-treated control cells. IC_{50} values were reported as the mean ± SD.

Table 2. SAR of Compounds 9-21 for NLRP3 and CFTR





Cmpd	Structure	Inhibition of NLRP3 activity(%)[a]	Inhibition of CFTR activity(%)[b]	Inhibition of NLRP3 activity (IC_{50} μM)[d]
9	R^3 = 	83	NI[d]	2.40±0.10
10		74	NI	5.91±0.24
11		68	NI	4.32±0.09
12		67	NI	5.58±0.13
13		67	NI	6.50±0.12
14		72	NI	4.85±0.35
15		35	10	>10
16		67	23	5.96±0.08
17		63	NI	5.61±0.14
18	R^4 = 	29	12	>10
19		63	NI	3.52±0.03
20		63	NI	6.27±0.16
21		73	11	7.63±0.36

[a] BMDMs were treated with tested compounds at a concentration of 10 μM. [b] HT29 cells were treated with tested compounds at a concentration of 20 μM. [c] BMDMs were pretreated with varied doses of tested compounds and then primed with LPS and stimulated with nigericin. Production of IL-1β was measured by ELISA and then the cytokine level was normalized to that of DMSO-treated control cells. IC_{50} values where reported as the mean ± SD. [d] NI means no inhibition (% inhibition @ 20 μM < 10).

Table 3. *In vitro* Metabolic Stability of Compounds 1-7 and 9 in HLM and MLM[a]

Cmpd	HLM		MLM	
	$t_{1/2}$ (min)	CL^{hep} (mL/min/kg)	$t_{1/2}$ (min)	CL^{hep} (mL/min/kg)
1	277.2	2.8	12.6	69.8
2	15.6	15.3	1.7	86.6
3	>145	<4.7	>145	<20.7

5	27.1	12.8	3.7	83
6	187.3	3.9	23.9	58.1
7	13.8	15.8	1.8	86.5
9	>145	<4.7	>145	<20.7
Testosterone	277.2	2.8	12.6	69.8
Propranolol	15.6	15.3	1.7	86.6
Clozapine	44.1	10.3	4	82.4

[a] $t_{1/2}$, half-life. CL^{hep}, hepatic clearance. Testosterone, propranolol, and clozapine were used as control.