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Discovery of a series of ester-substituted NLRP3 inflammasome inhibitors

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ABSTRACT

The NLRP3 inflammasome is a component of the innate immune system involved in the production of proinflammatory cytokines. Aberrant activation by a wide range of exogenous and endogenous signals can lead to chronic, low-grade inflammation. It has attracted a great deal of interest as a drug target due to the association with diseases of large unmet medical need such as Alzheimer's disease, Parkinson's disease, arthritis, and cancer. To date, no drugs specifically targeting inhibition of the NLRP3 inflammasome have been approved. In this work, we used the known NLRP3 inflammasome inhibitor CP-456,773 (aka CRID3 or MCC 950) as our starting point and undertook a Structure-Activity Relationship (SAR) analysis and subsequent scaffold-hopping exercise. This resulted in the rational design of a series of novel ester-substituted urea compounds that are highly potent and selective NLRP3 inflammasome inhibitors, as exemplified by compounds **44** and **45**. It is hypothesized that the ester moiety acts as a highly permeable delivery vehicle and is subsequently hydrolyzed to the carboxylic acid active species by carboxylesterase enzymes. These molecules are greatly differentiated from the state-of-the-art and offer potential in the treatment of NLRP3-driven diseases, particularly where tissue penetration is required.

Letter

Inflammasomes are multiprotein complexes first identified and named by Jürg Tschopp as the molecular machinery by which caspases are activated.[1] Upon activation these complexes are formed in the cytosol and consist of an upstream sensor protein of the NOD-like (nucleotide-binding oligomerization domain-containing protein) receptor (NLR) family, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and the effector procaspase. Active caspases in turn process proinflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) from their inactive to active forms. Most immune cells of the monocyte-macrophage lineage, such as peripheral blood monocytes, bone marrow-derived macrophages (BMDMs), Kupffer cells, peritoneal macrophages and alveolar macrophages, can form inflammasomes.

The NLRP3 (NOD-like receptor, Leucine-rich Repeat and Pyrin domain-containing protein 3) inflammasome is of particular therapeutic interest because of its links to human disease. NLRP3 activation is a two-step signalling process. The first step, known as priming, occurs when endogenous cytokines or microbial-derived molecules (e.g. lipopolysaccharide (LPS)) bind to extracellular receptors such as toll-like receptors, IL-1 receptors and tumor necrosis factor (TNF) receptors.[2] This upregulates the transcription and production of intracellular NLRP3, proIL-1β and proIL-18 through activation of transcription factor NF-κB. The second step, termed activation, requires a second stimulus. This leads to NLRP3 inflammasome assembly, the cleavage of

Abbreviations: NLRP3, NOD-like receptor, Leucine-rich Repeat and Pyrin domain-containing protein 3; NOD-like, nucleotide-binding oligomerization domain-containing protein-like; ASC, apoptosis-associated speck-like protein containing a CARD; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; NSAIDs, non-steroidal anti-inflammatory drugs; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; BMDMs, bone marrow-derived macrophages; PBMC, peripheral blood mononuclear cells; ATP, adenosine triphosphate; PAMPA, parallel artificial membrane permeability assay; CES, carboxylesterase

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procaspase-1 to the active form caspase-1, and the release of mature IL-1β and IL-18. Caspase-1 also cleaves and activates Gasdermin-D (GSDMD),[3] a protein that forms pores in the cell membrane, leading to pyroptosis (programmed inflammatory cell death) which facilitates release of inflammatory cytokines into extracellular space.

The NLRP3 inflammasome is formed upon activation by a wide range of second stimuli, categorized as either pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). PAMPs can be peptidoglycan, and both viral or bacterial RNA and DNA. DAMPs include adenosine triphosphate (ATP), beta-amyloid, ceramides, monosodium urate crystals, cholesterol crystals, calcium pyrophosphate dihydrate crystals, fatty acids, silica, alum and asbestos. This broad range of activating 'sterile' danger signals is unique to the NLRP3 inflammasome and it therefore has great potential as a drug target in a wide range of human diseases. Therapeutic intervention to inhibit NLRP3 inflammasome activation could have clinical benefits for conditions where chronic, low-grade inflammation caused by inappropriate activation of the NLRP3 inflammasome is implicated. These include Alzheimer's disease,[4] Parkinson's disease,[5] gout,[6] type II diabetes[7,8] and atherosclerosis.[9]

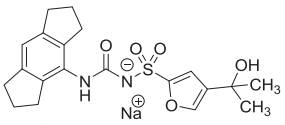
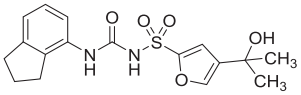
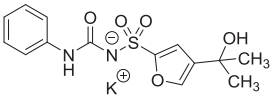
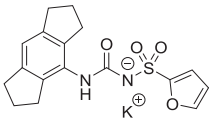
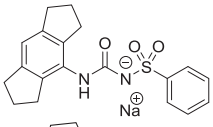
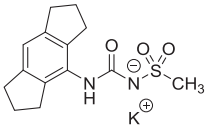
Various small molecules have been reported to inhibit NLRP3 in both *in vitro* and *in vivo* studies, the structures of some of these are shown in Fig. 1.

In research conducted prior to the discovery of the NLRP3 inflammasome, Perregaux, *et. al.* observed that the anti-diabetic sulfonylurea glyburide (also known as glibenclamide) inhibited IL-1β post-translational processing.[10] Further optimization of glyburide led to the discovery of the clinical candidate CP-456,773, which advanced into a Phase II trial.[11,12] A subsequent study by Coll, *et. al.* established that CP-456,773 inhibited IL-1β release via selective inhibition of NLRP3 inflammasome activation.[13] A number of other scaffolds have been associated with inhibition of NLRP3 activation. For example, Baldwin, *et. al.* reported that BC23, amongst a series of oxazaborine compounds, was an effective NLRP3 inflammasome inhibitor both *in vitro* and *in vivo*.[14] Some fenamate NSAIDs, exemplified by mefenamic acid, were shown by Daniels, *et. al.*, to inhibit the NLRP3 inflammasome and showed *in vivo* effects in a rodent Alzheimer's disease model.[15] Other small molecules such as 2-(4-nitrophenyl)-3H-quinazolin-4-one,[16] JC-171,[17] the natural product isoliquiritigenin,[18] CY-09,[19] Bay 11-7082[20] and Fc11a-2[21] have also been reported to show NLRP3-inhibitory effects in various models. Various extensive reviews of small molecule NLRP3 inflammasome inhibitors have been published.[22–25]

Our efforts to discover novel NLRP3 inhibitors began with the selective NLRP3 inhibitor CP-456,773 as a chemical starting point. This represented an attractive lead as the molecule had been through pre-clinical safety studies and advanced into the clinic. We took a *de novo* approach to systematically truncate the molecule and assess the SAR of the resultant fragment molecules. *In vitro* potency was assessed in a human peripheral blood mononuclear cell (PBMC) assay. This assay measures the ability of a compound to inhibit IL-1β release caused by LPS and ATP stimulation. As a compound is required to permeate the cell in order to disrupt the NLRP3 inflammasome, the potency is affected by both functional activity and cell permeability.

The initial results of the truncation approach are shown in Table 1. Removal of one or both fused cyclopentyl rings on the

Table 1
Truncation of CP-456773.

Compound	Structure	PBMC IL-1β IC_{50} (μM)[a]
CP-456,773		0.030
1		> 20
2		> 20
3		0.45
4		1.8
5		2.8

[a] Values represent the numerical average of at least two experiments.

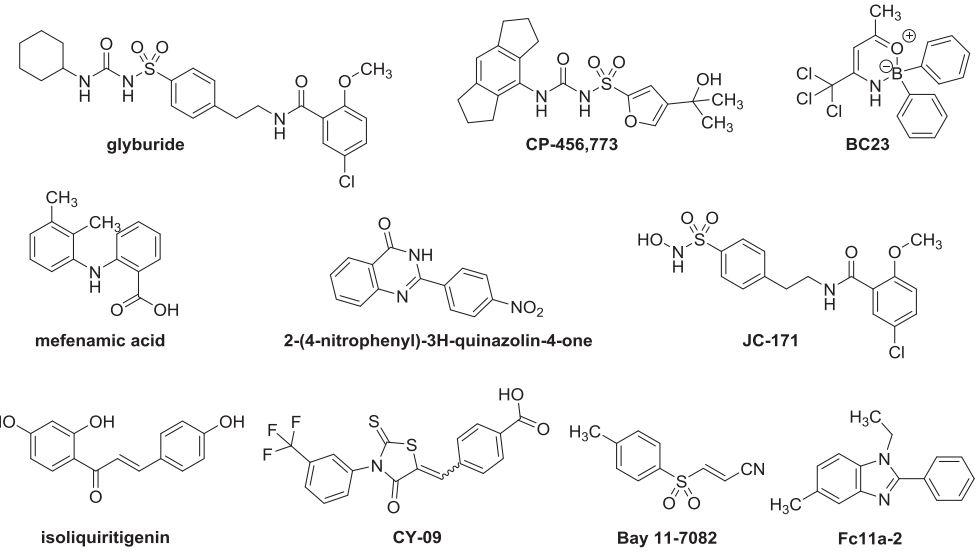


Fig. 1. Small molecule NLRP3 inhibitors.

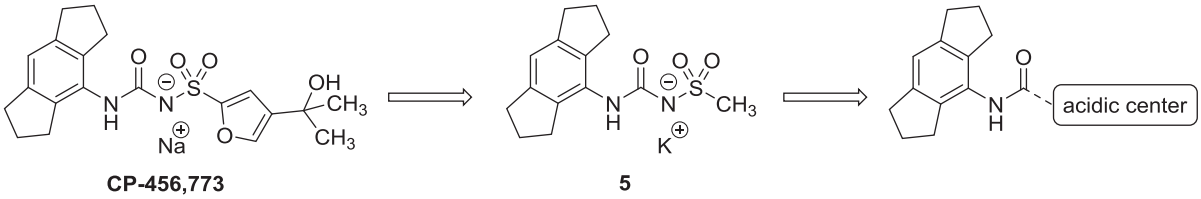
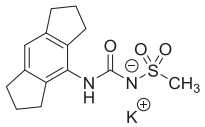
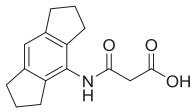
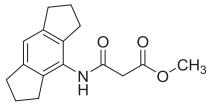
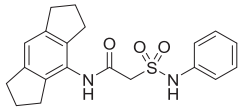
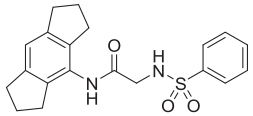
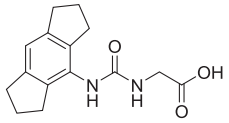
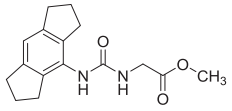


Fig. 2. Design rationale for acid-containing compounds.

hexahydroindacene, resulting in indane (compound **1**) and phenyl (**2**), respectively, led to a dramatic loss of activity in the PBMC assay. From this we concluded that the hexahydroindacene ring made an important contribution to the potency of CP-456,773. The furan portion of the molecule proved to be far more amenable to simplification, with the unsubstituted furan (**3**), phenyl (**4**) and even methyl sulfone (**5**) all retaining some activity in the cellular assay.

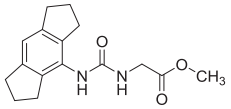
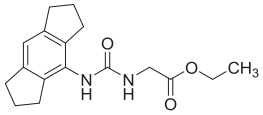
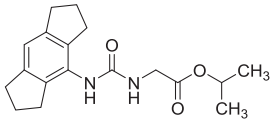
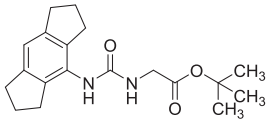
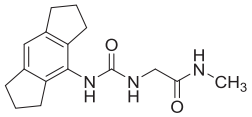
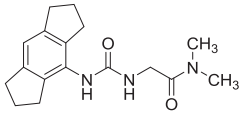
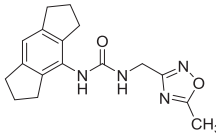
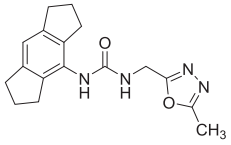
Encouraged by the result for **5** we sought to assess the importance of the acidic center of the sulfonylurea and to explore alternatives for it, as shown in Fig. 2. We designed a small set of compounds that maintained the features of hexahydroindacene ring connected to an amide moiety and featured an acidic center. The compounds synthesized and their corresponding cellular PBMC assay IC_{50} values are shown in Table 2. The carboxylate compound **6** and corresponding methyl ester intermediate **7** were both inactive. The sulfonamide compounds **8** and **9**, chosen as they both maintained the sulfone moiety along with a weak acidic center, were also devoid of activity. The carboxylic acid substituted urea (**10**) also did not show any activity. Surprisingly the corresponding methyl ester (**11**), synthesized as a precursor of the acid,

Table 2
Results for acid replacement compounds.

Compound	Structure	Calculated pK_a (most acidic)	PBMC IL-1β IC_{50} (μM)[a]
5		3.4	2.8
6		4.3	> 20
7		n/a	> 20
8		7.8	> 20
9		10.2	> 20
10		4.0	> 20
11		n/a	6.8

[a] Values represent the numerical average of at least two experiments.

Table 3
Exploring the ester substituent.

Compound	Structure	PBMC IL-1β IC_{50} (μM)[a]
11		6.8
12		1.6
13		1.8
14		> 20
15		> 20
16		> 20
17		> 20
18		> 20

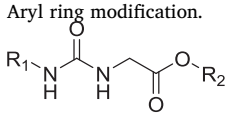
[a] Values represent the numerical average of at least two experiments

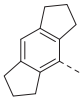
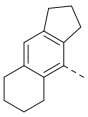
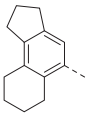
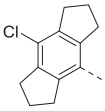
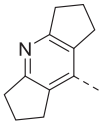
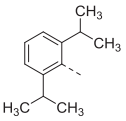
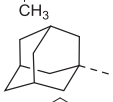
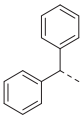
showed a modest level of inhibition.

Encouraged that a relatively low molecular weight and neutral molecule had shown an inhibitory effect in the PBMC IL-1β assay, we next focused on compound **11** as a new lead. An exploration of the methyl ester commenced with a view to identifying its role. The results of the methyl ester SAR exploration are shown in Table 3.

Along with the methyl ester (**11**), the ethyl (**12**) and isopropyl (**13**) esters showed single digit micromolar activity in the primary assay. The *tert*-butyl ester (**14**), however, was devoid of activity, despite being structurally similar to the isopropyl ester. Both the secondary (**15**) and tertiary amide (**16**) compounds were inactive (defined as having an IC_{50} greater than the top concentration of the assay). Two oxadiazole-containing compounds designed as nonclassical methyl ester bioisosteres (**17** and **18**) also failed to exhibit any activity. The similar level of

Table 4
Aryl ring modification.



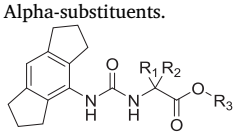
Compound	R1	R2	PBMC IL-1β IC_{50} (μM)[a]
12		Et	1.6
19		Et	> 20
20		Et	3.1
21		Et	> 20
22		Et	> 20
23		Me	> 20
24		Et	> 20
25		Et	> 20
26		Et	> 20

[a] Values represent the numerical average of at least two experiments.

potency of the methyl, ethyl and isopropyl esters and the inactivity of the chemically and enzymatically more stable *tert*-butyl ester led to a working hypothesis that the ester acts as a delivery vehicle for the carboxylic acid. Given that the primary assay was a cellular rather than isolated protein assay, cell permeability was required in order to measure activity. It is likely that the ester moiety greatly improves cellular permeation relative to the carboxylic acid, then subsequent intracellular enzymatic cleavage delivers the active species.

The next round of modification focused on the aryl substituent adjacent to the urea, the results of which are shown in Table 4. Expansion of the 5-membered to a 6-membered alkyl ring to give compound **19** resulted in ablation of activity. Interestingly, activity could be mostly restored by translocating the 5-membered ring to give the 5-substituted heptahydro-cyclopenta[*a*]naphthalene (**20**). Variation of the *para* position of the aryl ring included chloro substitution (**21**) and conversion to the 4-aza ring (**22**) yielded only inactive compounds. 2,6-*Bis*-alkyl substituted phenyls (**23** and **24**), which were reasoned to impart an orthogonal twist of the aryl group relative to the urea carbonyl and thus mimic the hexahydroindacene, did not give promising results. Finally, replacement of the hexahydroindacene group with other large lipophilic groups such as 1-adamantyl (**25**) or benzhydryl (**26**) resulted in

Table 5
Alpha-substituents.



Compound	R1	R2	R3	PBMC IL-1β IC_{50} (μM)[a]
12	H	H	Et	1.6
27	Me	Me	Me	> 20
28	*rac*-benzyl	H	Me	14
29	(S)-benzyl	H	Me	> 20
30	(R)-benzyl	H	Me	6.1
31	CH_2-CH_2-phenyl	H	Me	> 20

[a] Values represent the numerical average of at least two experiments.

complete loss of potency. The hexahydroindacene was therefore selected as the optimal substituent for the urea.

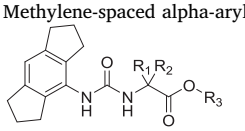
We next turned our attention toward the position alpha- to the carboxylate. A small number of derivatives were tested (Table 5). *Gem*-dimethyl substitution (**27**) resulted in a complete loss of activity. The racemic benzyl substituent (**28**), although weakly potent, encouraged the synthesis of both enantiomers (**29** and **30**) to probe for a stereochemical preference. Indeed the (R)-benzyl example, compound **30**, was found to be the eutomer and the (S)-benzyl example (**29**) was devoid of activity. The phenethyl substituted compound (**31**) was inactive. These findings warranted further investigation of the alpha substituent, with a focus on aryl and heteroaryl groups linked via a methylene spacer. Where possible, the (R)- enantiomer was profiled based on the previously observed stereochemical preference. These results are shown in Table 6.

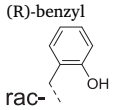
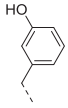
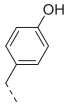
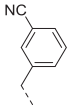
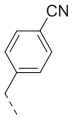
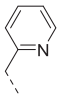
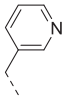
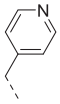
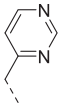
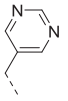
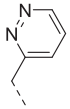
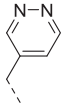
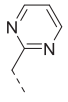
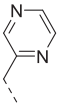
A scan of *ortho*, *meta* and *para* hydroxy around the phenyl ring (**32**, **33** and **34**) showed that all these substituents slightly improved potency. Similarly, *meta* and *para* nitrile substitution (**35** and **36**) brought about slight improvements to the potency compared with the unsubstituted phenyl. There was more of a contrast when comparing the three pyridyl isomers; whilst the *ortho* pyridyl (**37**) offered no potency advantage, the *meta* pyridyl (**38**) was more potent than the corresponding phenyl ring and the *para* pyridyl (**39**) was highly disfavoured. Other azines were tolerated, with the 4- and 5-pyrimidine (**40** and **41**) and 3- and 4-pyridazine (**42** and **43**) boasting similar to or slightly improved potency than the phenyl analogue. Large potency gains were seen with both the 2-pyrimidine (**44**) and the pyrazine group (**45**).

The two most potent compounds, **44** and **45**, were tested in the human whole blood (WB) assay along with the reference compound CP-456,773. This assay quantifies NLRP3 inflammasome disruption by measuring the inhibition of IL-1β release stimulated by LPS and ATP in human whole blood. The binding of a compound to proteins within the blood, principally human serum albumin and α-1 glycoprotein, causes a reduction in available concentration of compound and consequently reduces the measured half-maximal inhibitory concentration compared with the PBMC assay. It is well known that acidic compounds are generally more highly protein bound than neutral compounds and that protein binding also increases with lipophilicity.

As shown in Table 7, in the PBMC assay the two leading compounds from this series were equipotent to the NLRP3 inflammasome inhibitor CP-456,773. By contrast, in the whole blood assay there was a significant improvement for compounds **44** and **45** compared with CP-456,773, with IC_{50} values approximately 20-fold and 8-fold more potent, respectively. The whole blood attenuation (Δ = WB IC_{50} ÷ PBMC IC_{50}) for CP-456,773 is 97-fold, whereas for compound **44** it is only 4-fold. CP-456,773 is an acidic sulfonylurea (estimated $pK_a \approx 5$) and as such is negatively charged at physiological *p*H. This acidic center is likely to contribute to the binding to plasma proteins as implied by the high whole blood attenuation. By contrast, compounds **44** and **45** are uncharged at physiological *p*H and this may explain why the

Table 6
Methylene-spaced alpha-aryl substituents.



Compound	R1	R2	*R3*	PBMC IL-1β IC_{50} (μM)[a]
30	(R)-benzyl	H	Me	6.1
32	 rac-	H	Me	2.5
33	 rac-	H	Me	2.0
34	 (R)-	H	Me	2.7
35	 (R)-	H	Me	1.5
36	 (R)-	H	Me	1.5
37	 (R)-	H	Me	4.2
38	 (R)-	H	Me	1.5
39	 (R)-	H	Me	> 20
40	 rac-	H	Et	6.8
41	 (R)-	H	Me	4.5
42	 (R)-	H	Me	3.3
43	 rac-	H	Et	6.0
44	 (R)-	H	Et	0.036
45	 (R)	H	Et	0.030

[a] Values represent the numerical average of at least two experiments.

Table 7
Comparison and further profiling of CP-456,773, **44** and **45.**

Compound	CP-456,773	44	45
PBMC IL-1β IC_{50} (μM)[a]	0.030	0.036	0.030
WB IL-1β IC_{50} (μM)[a]	2.9	0.16	0.37
Δ WB/PBMC	97	4	12
Speck formation inh IC_{50} (μM)	0.028	0.033	0.20
TNFα inh IC_{50} (μM)	> 40	2.7	7.6
IL-6 inh IC_{50} (μM)	> 40	4.3	6.1
PAMPA Pe (nm/s)[b]	< 0.003	82	139
Hu blood stability (% at 1 h)[c]	NT	92.4	79.2
Mu blood stability (% at 1 h)[c]	NT	0	0

[a] Values represent the numerical average of at least two experiments. [b]Parallel Artificial Membrane Permeability Assay. [c]Defined as percentage remaining after incubation in whole blood at 37° C for one hour. NT = not tested.

attenuation to whole blood for both compounds is significantly reduced compared to CP-456,773.

Compounds **44** and **45** were profiled further to ensure they retained the profile of a selective NLRP3 inflammasome inhibitor, as displayed by CP-456,773. The ASC speck assay measures the aggregation of green fluorescent protein (GFP) tagged ASC in THP-1 cells using confocal microscopy. This assay uses nigericin as signal 2 (activation), rather than ATP as used in the PBMC assay. This provides additional mechanistic information that the compound does not have signal 2-specific activity, for example P2X7 antagonism. Combined with the fact that the ASC speck assay reads out ASC speck formation rather than downstream events (for example caspase activation, IL-1β release), inhibition in both the PBMC and speck assays is a strong indication that a compound inhibits a common component of NLRP3 activation or inflammasome assembly. Both **44** and **45** show potent inhibition of ASC speck formation in this assay, with the former having a comparable half maximal inhibitory concentration to CP-456,773. This demonstrates that these compounds inhibit the NLRP3 inflammasome upstream of inflammasome formation. The TNFα and IL-6 selectivity assays measure the inhibition of these cytokines from human PBMCs stimulated with LPS. NLRP3 inflammasome activation results in IL-1 and IL-18 release and therefore in the timeframe of the assay TNFα and IL-6 release should not be inhibited by a specific NLRP3 inflammasome inhibitor. CP-456,773 shows no effect in the TNFα and IL-6 assays up to 40 μM concentration. Compounds **44** and **45** show a modest inhibition in both selectivity assays when tested at higher concentrations, but with a selectivity window of two orders of magnitude compared to the PBMC assay half maximal inhibitory concentrations.

With favourable potency and selectivity profiles determined, the membrane permeability of **44** and **45** were assessed in a PAMPA assay. CP-456,773 demonstrated an unmeasurably low rate of membrane permeability (< 0.003 nm/s). By contrast, **44** and **45** both had a high rate of membrane permeation (82 and 139 nm/s, respectively).

The stability of **44** and **45** were assessed in both human and mouse blood. It was found that both showed reasonable stability in human whole blood, with 92 and 79 percent of the **44** and **45** remaining, respectively, after one hour of incubation. This was not the case in mouse whole blood. Here, complete metabolism of the parent molecule had taken place after one hour of incubation. We hypothesized that the rapid turnover of **44** and **45** in mouse blood was due to circulating carboxylesterases (CES), which are capable of hydrolyzing ethyl esters to carboxylic acids.[25] Due to the rapid turnover of these ester compounds in mouse blood, the pharmacokinetic profiles of **44** and **45** were not assessed.

In conclusion, a deconstruction of CP-456,773 and subsequent rational design of acid-containing compounds led to the discovery of ester **11**. The corresponding carboxylic acid, **10**, did not inhibit at the top concentration of the PBMC assay. Subsequent optimization of **11** led to the discovery of a novel series of substituted 2-[(phenylcarbamoyl) amino]acetate based NLRP3 inflammasome inhibitors. Two leading

exemplars from this series, **44** and **45**, were further profiled to confirm excellent potency in PBMC, whole blood and ASC speck formation inhibition assays, with selectivity over TNFα and IL-6 release, high rates of membrane permeability and good stability in human whole blood. Our working hypothesis is that the ester moiety acts as a delivery vehicle (prodrug), enabling higher rates of membrane permeability compared to the carboxylic acid. The ester is converted to the active species by carboxylesterase enzymes. The SAR of the ester substituent and the large difference in blood stability between human and mouse of compounds **44** and **45** support this prodrug hypothesis. These molecules offer a differentiated profile to the widely studied inhibitor CP-456,773 and have potential utility in the treatment of diseases where NLRP3 inflammasome inhibition is required along with high tissue and membrane permeability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127560.

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