New non-sulfonylurea-based NLRP3 inhibitors: discovery and selection of INF200, a 1,3,4-oxadiazol-2-one-based NLRP3 inhibitor

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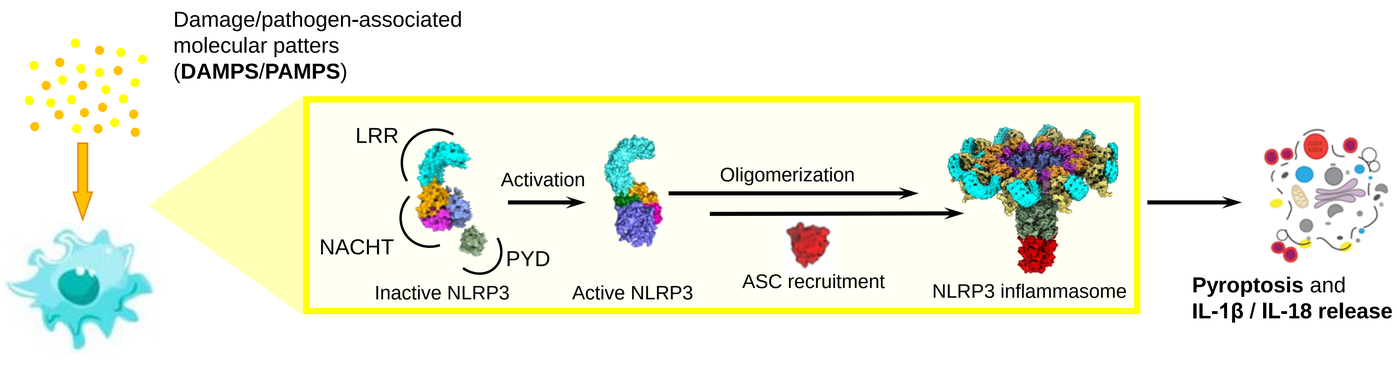
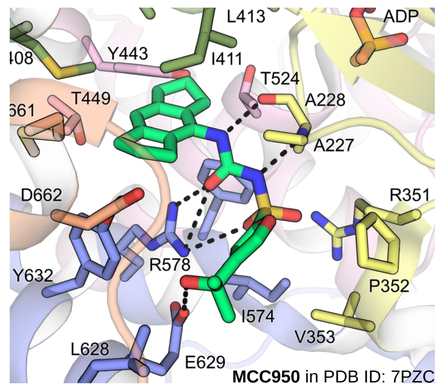
Focus on NLRP3

The cytosolic multiprotein complex nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3 (**NLRP3**) inflammasome, plays an important role in the **initiation and maintenance of the inflammatory state**. Once activated and assembled, the NLRP3 inflammasome triggers the auto-proteolytic cleavage of pro-caspase-1 into the active caspase-1, converting the pro-inflammatory cytokines pro-interleukin (IL)-1β and pro-IL-18 into their active forms and causing pyroptotic cell death.





An **aberrant activation** of NLRP3 inflammasome has been detected in **chronic inflammatory diseases** such as autoinflammatory, autoimmune, neurodegenerative and cardiovascular diseases.

The **inhibition of the NLRP3 inflammasome activation** represents an interesting **new approach** for the development of a new class of anti-inflammatory drugs. To date, **the most studied NLRP3 inhibitor is MCC950**, a disubstituted sulfonylurea derivative, binding to the NACHT domain of NLRP3.

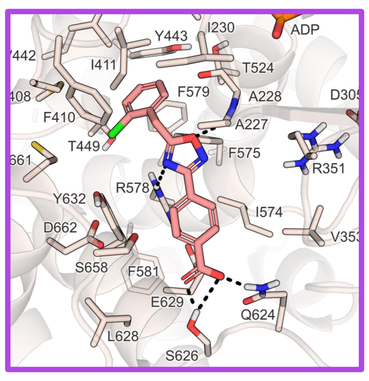
Can we obtain non-sulfonylurea NLRP3 inhibitors?

Main Results

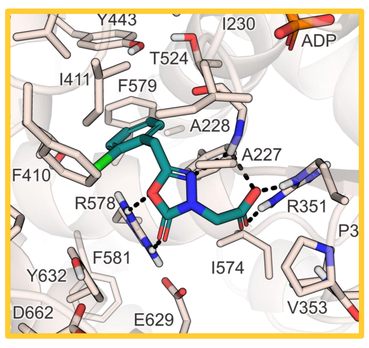
1. Structure-based design

Induced-fit docking was performed on a set of core heterocycles, which were chosen to mimic the hydrogen bond pattern established by the sulfonylurea core with key residues **Ala228**, **Arg351** and **Arg578**.

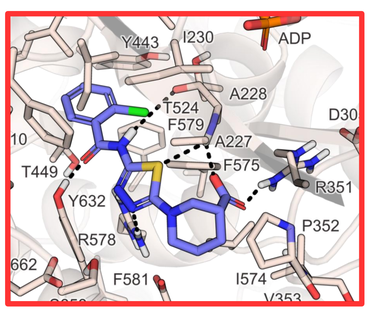
1,2,4 - oxadiazoles



1,3,4 – oxadiazol-2-ones

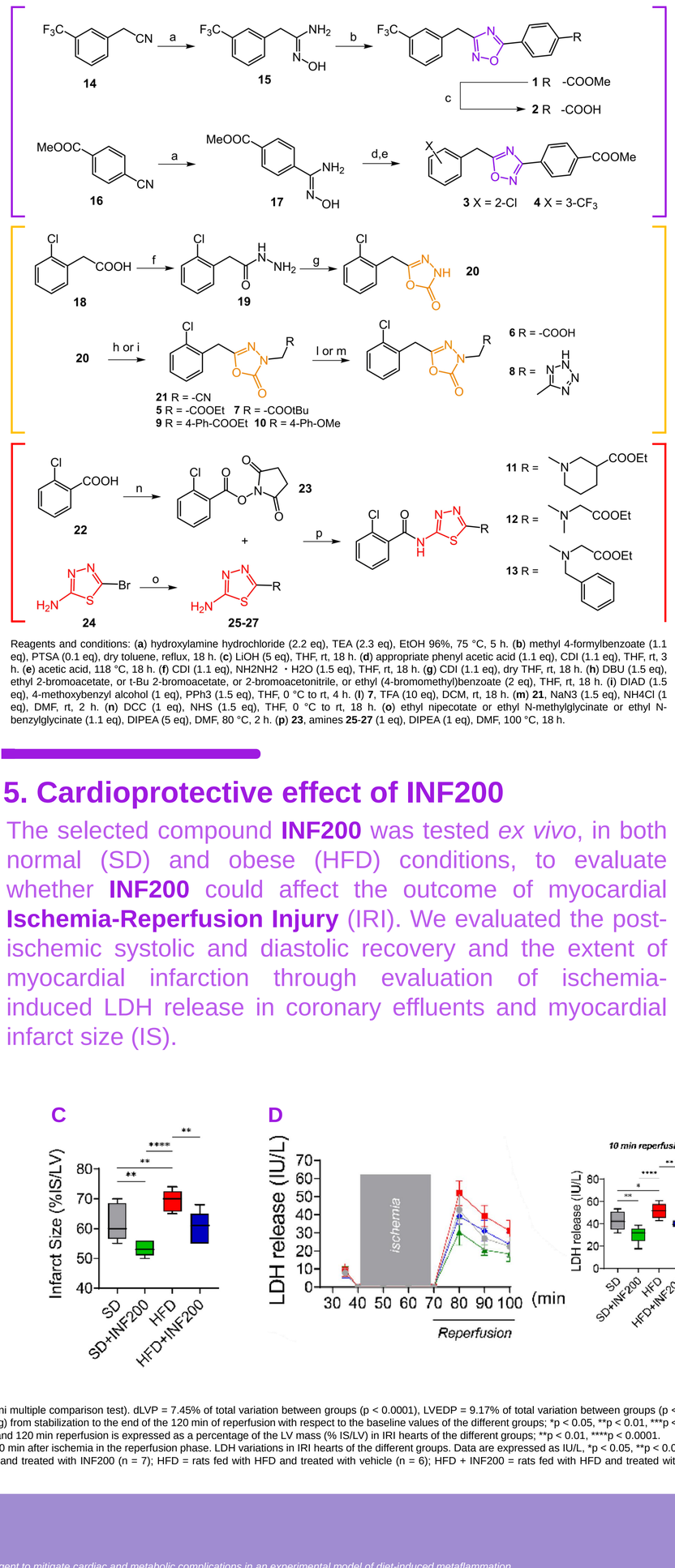


1,3,4 - thiadiazoles



2. Synthesis

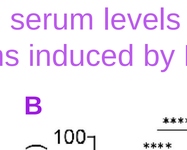
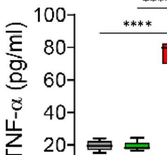
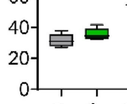
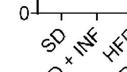
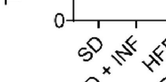
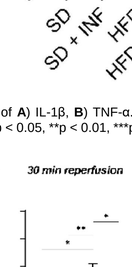
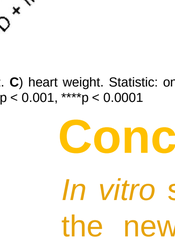
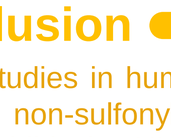
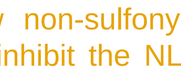
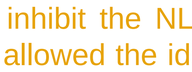
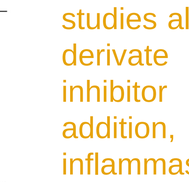
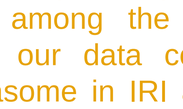
The 1,2,4-oxadiazole (**1** - **4**), 1,3,4-oxadiazol-2-one (**5** - **10**) and 1,3,4-thiadiazole series (**11** – **13**) were synthesized with limited modulations of the lipophilc and polar terminal portions.



Reagents and conditions: (**a**) hydroxylamine hydrochloride (2.2 eq), TEA (2.3 eq), EtOH 96%, 75 °C, 5 h. (**b**) methyl 4-formylbenzoate (1.1 eq), PTSA (0.1 eq), dry toluene, reflux, 18 h. (**c**) LiOH (5 eq), THF, rt, 18 h. (**d**) appropriate phenyl acetic acid (1.1 eq), CDI (1.1 eq), THF, rt, 3 h. (**e**) acetic acid, 118 °C, 18 h. (**f**) CDI (1.1 eq), NH2NH2 · H2O (1.5 eq), THF, rt, 18 h. (**g**) CDI (1.1 eq), dry THF, rt, 18 h. (**h**) DBU (1.5 eq), ethyl 2-bromoacetate, or t-Bu 2-bromoacetate, or 2-bromoacetonitrile, or ethyl (4-bromomethyl)benzoate (2 eq), THF, rt, 18 h. (**i**) DIAD (1.5 eq), 4-methoxybenzyl alcohol (1 eq), PPh3 (1.5 eq), THF, 0 °C to rt, 4 h. (**l**) **7**, TFA (10 eq), DCM, rt, 18 h. (**m**) **21**, NaN3 (1.5 eq), NH4Cl (1 eq), DMF, rt, 2 h. (**n**) DCC (1 eq), NHS (1.5 eq), THF, 0 °C to rt, 18 h. (**o**) ethyl nipecotate or ethyl N-methylglycinate or ethyl N-benzylglycinate (1.1 eq), DIPEA (5 eq), DMF, 80 °C, 2 h. (**p**) **23**, amines **25**-**27** (1 eq), DIPEA (1 eq), DMF, 100 °C, 18 h.

3. *In vitro* analysis

We evaluated the **% inhibition of pyroptosis and IL-1β release** of the obtained compounds.

Cpd	R_1	Heterocycle	R_2	Pyroptosis decrease[a] % inhibition ± SEM at 10 μM	IL-1β release[b] % inhibition ± SEM at 10 μM	Cytotoxicity[c] TC50 (μM)
1				46.3 ± 17.3[d]	< 10	>100
2				14.2 ± 2.1	NT	27.3 ± 1.3
3				< 10	NT	>100
4				< 10	NT	94.5 ± 1.1
5 (INF200)				66.3 ± 6.6[e]	35.5 ± 8.1[d]	76.5 ± 1.2
6				45.9 ± 8.4[d]	30.3 ± 14.6[d]	94.6 ± 1.2
7				< 10	NT	98.9 ± 15.0
8				27.6 ± 9.5	< 10	>100
9				17.7 ± 6.4	NT	>100
10				11.4 ± 3.8	NT	64.8 ± 5.6
11				39.7 ± 13.3	22.4 ± 9.5	79.7 ± 7.0
12				25.7 ± 5.8	20.7 ± 12.6	96.1 ± 4.0
13				60.0 ± 13.2[e]	49.0 ± 12.4[e]	91.6 ± 14.5
NP3-146				56.1 ± 2.5[e]	54.7 ± 7.2[e]	92.5 ± 4.1

NT = not tested. [a] Pyroptosis of differentiated THP-1 cells was triggered using LPS/ATP. Data are reported as the % inhibition of pyroptosis of cells treated with test compound (10 μM) *vs* vehicle-treated cells. [b] IL-1β inhibition was measured in the cell supernatants form the same experiments. Data are reported as % inhibition ± SEM of three to five experiments run in triplicate. [c] Cytotoxicity was determined after 72 h treatment of THP-1 cells with increasing conc. (0.1–100 μM) of test compounds. Data are reported as TC_{50} ± SEM of three experiments. [d] $p < 0.05$ *vs* vehicle treated cells. [e] $p < 0.01$ *vs* vehicle treated cells.

4. NLRP3 and metaflammation

We evaluated the *in vivo* effects of **INF200** on the HFD-dependent systemic inflammation, monitoring the serum levels of IL-1β and TNFα. We evaluated the anthropometric alterations induced by HFD regimen.

Plasma levels of **A**) IL-1β, **B**) TNF-α. **C**) heart weight. Statistic: one-way ANOVA and Newman–Keuls multiple comparison test. Data are expressed as means ± SEM statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

5. Cardioprotective effect of INF200

The selected compound **INF200** was tested *ex vivo*, in both normal (SD) and obese (HFD) conditions, to evaluate whether **INF200** could affect the outcome of myocardial **Ischemia-Reperfusion Injury** (IRI). We evaluated the post-ischemic systolic and diastolic recovery and the extent of myocardial infarction through evaluation of ischemia-induced LDH release in coronary effluents and myocardial infarct size (IS).

A) dLVP and **B**) LVEDP variations. Grey boxes indicate ischemic administration (Bonferroni multiple comparison test). dLVP = 7.45% of total variation between groups ($p < 0.0001$), LVEDP = 9.17% of total variation between groups ($p < 0.0001$). Inset graph shows dLVP and LVEDP at the end of reperfusion. Data are expressed as changes of dLVP and LVEDP values (mmHg) from stabilization to the end of the 120 min of reperfusion with respect to the baseline values of the different groups; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
C) Infarct size (IS). The amount of necrotic tissue measured after 30 min global ischemia and 120 min reperfusion is expressed as a percentage of the LV mass (% IS/LV) in IRI hearts of the different groups; **p < 0.01, ****p < 0.0001.
D) LDH activity was assessed in coronary effluent 5 min before ischemia and 10, 20 and 30 min after ischemia in the reperfusion phase. LDH variations in IRI hearts of the different groups. Data are expressed as IU/L, *p < 0.05, **p < 0.01, ****p < 0.0001z.
SD = rats fed with SD and treated with vehicle (n = 6); SD + INF200 = rats fed with SD and treated with INF200 (n = 7); HFD = rats fed with HFD and treated with vehicle (n = 6); HFD + INF200 = rats fed with HFD and treated with INF200 (n = 7). Statistic: one-way ANOVA and Newman–Keuls multiple comparison test.

Conclusion

In vitro studies in human macrophages have shown that the new non-sulfonylurea-based NLRP3 inhibitors are able to inhibit the NLRP3 activation. In particular, these studies allowed the identification of 1,3,4-oxadiazol-2-one derivate **5** (**INF200**) as the most promising NLRP3 inhibitor among the three series of compounds. In addition, our data confirm an important role of this inflammasome in IRI and they pave the way for further improvements in the field of these inhibitors for future application in the clinical field.

Simone Gastaldi et al,
Discovery of a novel 1,3,4-oxadiazol-2-one-based NLRP3 inhibitor as a pharmacological agent to mitigate cardiac and metabolic complications in an experimental model of diet-induced metaflammation,
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, **257**(2023), 115542