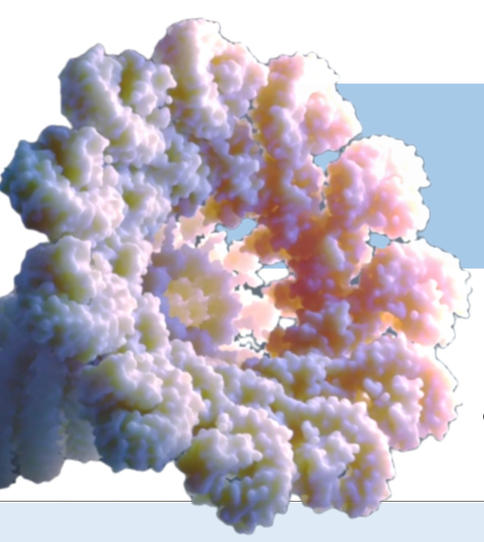

Design and synthesis of INF195, a new NLRP3 Inflammasome Inhibitor with ex vivo cardioprotective effect

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Introduction

The NLRP3 inflammasome is a cytosolic multiprotein complex involved in the release of pro-inflammatory cytokines (such as IL-1β and IL-18). NLRP3 expression and over-activation is found in acute damage in several cardiovascular diseases, including ischemia-reperfusion injury (IRI) which occurs after myocardial infarction. The discovery of new NLRP3 inhibitors may provide cardioprotective effects in heart diseases.

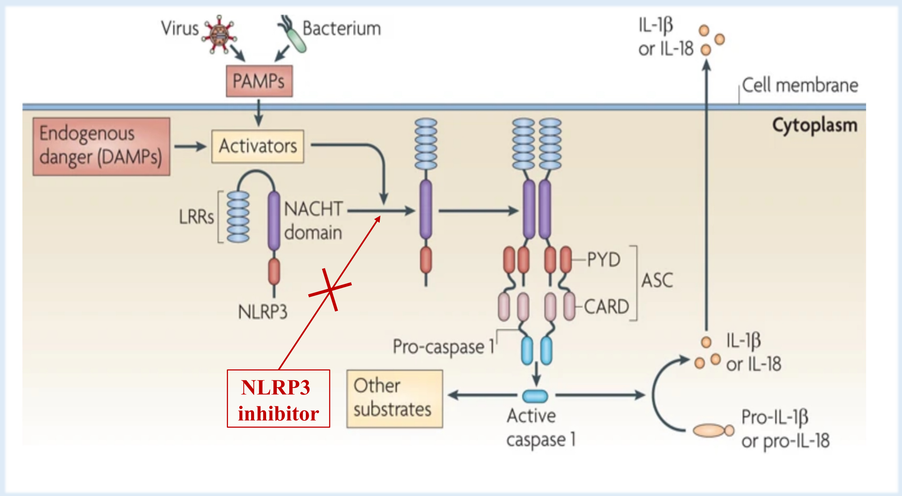


Figure 1: The designed non-covalent NLRP3 inhibitors act by binding to the NACHT domain of the protein, thereby blocking conformational changes which trigger the oligomerization of the protein and its assembly. The assembled NLRP3 is able to activate the caspase 1, causing the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18.

In silico design of novel NLRP3 inhibitors

In molecular docking studies of the previously developed inhibitor **INF120,** the benzimidazole-2-one moiety interacts with Arg578 and Glu629 (*Figure 2A*), but not with Arg351, a key residue for the binding to the NACHT domain. In order to establish an interaction with this residue, we replaced the benzimidazole portion with a piperidine ring bearing a carboxyl group in position 3 or 4 (*Figure 3*). As shown in *Figure 2B-C*, **compound 5** maintains the 2-chlorophenyl orientations sandwiched between Ile411 and Phe579 and the polar part of the molecule engages residues Ala228, Arg578 and Arg351.

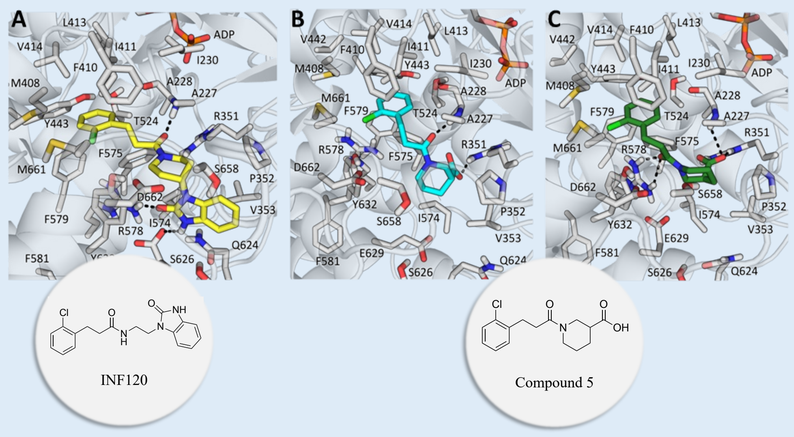


Figure 2: Molecular docking of (A) INF120 (yellow), (B) **5** (cyan, R-stereoisomer) and (C) **5** (green, S-stereoisomer). Protein is depicted as grey cartoon, residues lining the inhibitor binding site are shown as sticks and labelled with one-letter code. Distances and angles compatible with hydrogen bonds are highlighted with black dashes.

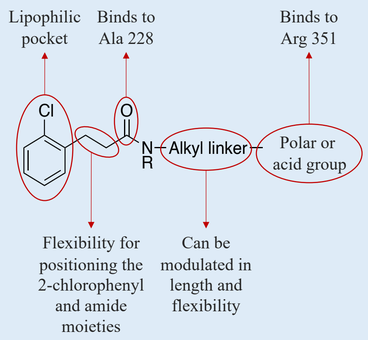
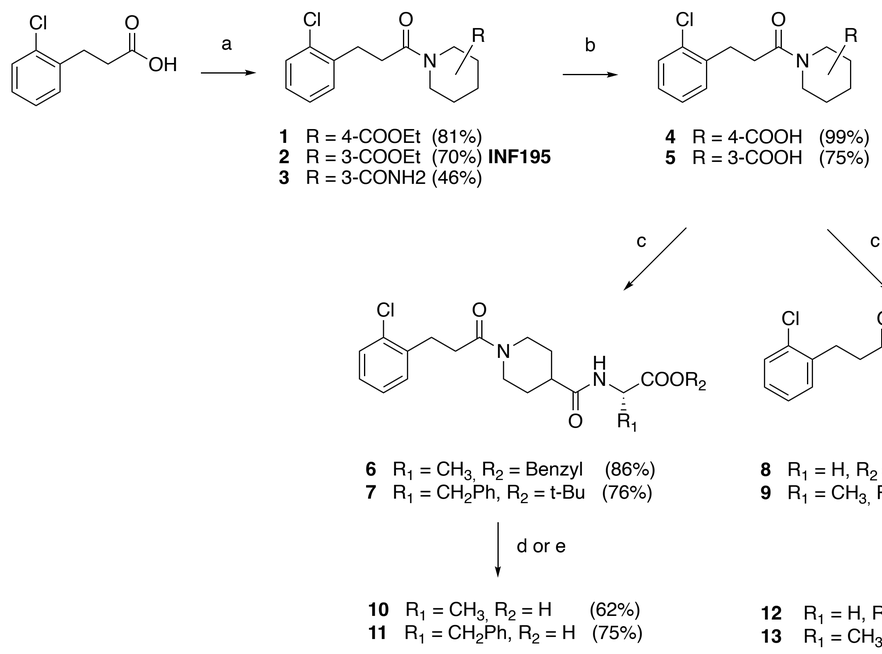


Figure 3: General structure of the designed compounds and the predicted interactions to receptor NACHT domain pocket.

Synthetic route to the designed compounds



Scheme 1. Reagents and conditions: (**a**) (i) CDI, CH_2Cl_2, rt, 30 min; (ii) substituted piperidine, rt, 18 h. (**b**) NaOH 2.5 M, EtOH, rt, 18 h. (**c**) aminoacid, HBTU, HOBt, DIPEA, DMF, 18 h. (**d**) TFA , CH_2Cl_2, rt, 18 h. (**e**) Pd/C 10 %, H_2, MeOH, rt, 18 h.

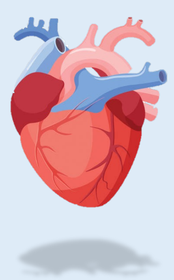
Selection of compound INF195

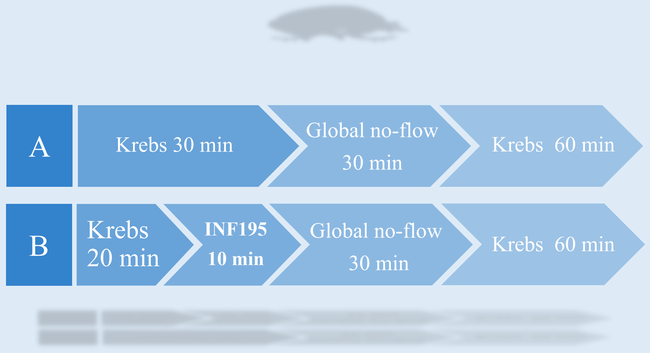
In vitro inhibitory effect of synthesised compounds on NLRP3-dependent pyroptotic cell death and IL-1β release in differentiated THP-1 cells as well as cytotoxicity in THP-1 cells was measured. Only compounds with polar group in position 3 of the piperidine ring (compounds **2**, **3**, **5**) showed significant inhibition of NLRP3 and among them, compound **2** (**INF 195**), the ethyl ester prodrug of the most active compound **5**, was selected for *ex vivo* studies.

Compound	Pyroptosis decrease[a] *% inhibition at 10 μM*	IL-1β inhibition[b] *% inhibition at 10 μM*	Cytotoxicity[c] *TC_{50} (μM)*
1	11.0 ± 9.0	NT	> 100
2 (INF 195)	32.9 ± 3.4[d]	38.0 ± 7.5[d]	> 100
3	22.7 ± 10.3	13.2 ± 1.1	> 100
4	< 10	NT	> 100
5	40.4 ± 11.9[e]	40.5 ± 5.5[d]	> 100
10	11.4 ± 6.2	< 10	74.3 ± 1.5
11	< 10	NT	> 100
12	22.8 ± 7.09	24.4 ± 8.2	> 100
13	< 10	NT	> 100

Table 1: [a] Pyroptosis of differentiated THP-1 cells was triggered using LPS/ATP. Data are reported as the % inhibition of pyroptosis of cells treated with 10 μM of test compounds vs vehicle-treated cells. Data are the mean ± SEM of three to five experiments run in triplicate. [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 concentration (0.1–100 μM) of test compounds. Data are reported as TC50 ± SEM of three experiments. [d] $p < 0.05$ vs vehicle-treated cells; e $p < 0.01$ vs vehicle-treated cells; NT = not tested.

Ex-vivo cardiac ischemia/reperfusion protocol





A (IR): 30 minutes of stabilization with Krebs solution, and 30 minutes of ischemia, followed by 60 minutes of reperfusion; **B**: 30 minutes of stabilization with the infusion of **INF195** at concentrations of 5, 10 or 20 μM in the last 10 minutes; then 30 minutes of ischemia followed by 60 minutes of reperfusion.

Infarct size evaluation

At the end of reperfusion, hearts were freezed and sliced. Slices were immersed in a nitro-blue tetrazolium solution with phosphate buffer. *ImageJ* processing tool was used to quantify the infarct size.

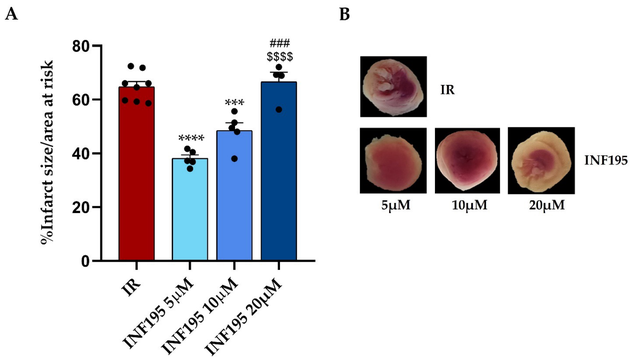


Figure 5. A) Infarct size after IR exposition is expressed as a percentage of area at risk of mice hearts. Black circles depict individual hearts, whereas coloured bars show group means and SEM. **B)** Representative pictures of left ventricle slices of mice of the different experimental groups. The area at risk is indicated in red, while the infarct area in white. Data are mean ± SEM. *** $p < 0.001$ *vs* IR; **** $p < 0.0001$ *vs* IR; $^{\$\$\$\$}$ $p < 0.0001$ *vs* INF195 5μM; $^{\#\#\#}$ $p < 0.001$ *vs* INF195 10μM.

IL-1β and Caspase-1 levels evaluation

At the end of reperfusion, hearts were freezed and the apices were lysed in a RIPA buffer with protease and phosphatase inhibitors. The concentration of IL-1β and caspase-1 in tissue homogenates were then quantified with specific ELISA kits.

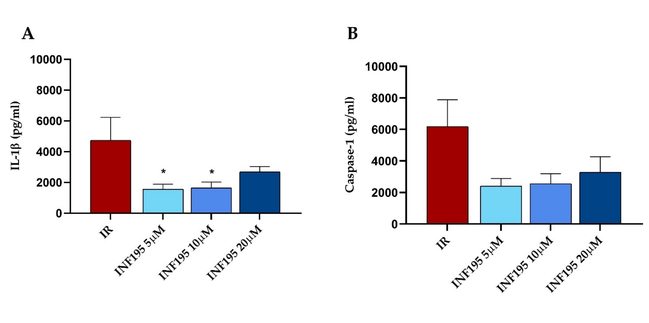


Figure 4. IL-1β (**A**) and caspase-1 (**B**) concentrations evaluated by ELISA in extracts of left ventricular apex from mice exposed to IR pre-treated or not with INF195. Data are presented as the means ± SEM. Statistical significance: *$p < 0.05$ *vs* IR.

Conclusions

NLRP3 activation contributes to infarct size in an *ex vivo* model by inducing the production of active caspase-1 and IL-1β. NLRP3 inhibition by treatment with low doses of the novel inhibitor INF195 showed significantly reduction in IL-1β levels and therefore decreased infarct size after IR. Overall, INF195 has highlighted cardioprotective properties in *in vitro* and *ex vivo* models.

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