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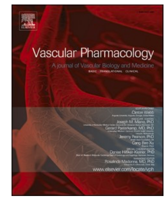

Macrophage and cardiomyocyte roles in cardioprotection: Exploiting the NLRP3 Inflammasome inhibitor INF150

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ABSTRACT

Background: Cardiovascular diseases remain the leading cause of disability and death in the Western world. Effective cardioprotection involves limiting ischemia/reperfusion injury (IRI), including cell death (pyroptosis) driven by the NLRP3 inflammasome. While various cardiac resident cellular populations contribute to cardioprotection, it remains unclear whether targeting resident macrophages is inherently cardioprotective. Given that INF150, an NLRP3 inhibitor, exhibits varying abilities to penetrate cardiomyocytes and macrophages, we sought to address this question.
Methods: We studied the cardioprotective potential of INF150, the potent metabolite of the NLRP3 inhibitor INF195, in isolated hearts or cells. In isolated hearts, we measured infarct size, caspase-1 cleavage, and interleukins (IL) release, while in macrophages, naïve H9c2 and differentiated H9c2 cells, we analyzed cell viability, and pyroptosis markers, including IL-1β release and Gasdermin D cleavage, following hypoxia/reoxygenation (H/R).
Results and conclusion: While INF150 effectively shielded macrophages from LPS/ATP challenges, it failed to penetrate H9c2 and differentiated H9c2, even at high concentrations (no changes in pyroptosis markers induced by H/R). In the isolated mice heart model, INF150 did not demonstrate cardioprotective effects: infarct size, IL-1β, cleaved caspase-1 levels did not change significantly across tested concentrations of INF150. These findings suggest that while INF150 shows promise in macrophage/phagocytic models, its inability to penetrate cardiomyocytes limits its effectiveness in the whole cardiac tissue. Our results underscore the importance of cardiomyocyte uptake for effective cardioprotection, highlighting the need for NLRP3 inhibitors capable of targeting these cells directly. Future research should focus on enhancing the delivery and cardiomyocyte uptake of NLRP3 inhibitors to achieve cardioprotection. Unlike its precursor, INF195, which penetrates H9c2 cells, INF150 does not appear to offer cardioprotection in the whole organ.

1. Introduction

Cardiovascular diseases, notably acute myocardial infarction, are the leading cause of death and disability in the Western world. The main treatment for preserving cardiac function and reducing the risk of post-ischemic heart failure (HF) is the primary percutaneous coronary intervention [1,2]. However, the reperfusion process can exacerbate myocardial damage, impairing mitochondrial integrity, contractility, and leading to cell death, collectively known as ischemia/reperfusion injury (IRI) [3]. During ischemia and early reperfusion, cardiomyocytes trigger several death mechanisms, including a death mechanism prominently involving the nucleotide-binding oligomerization domain-like receptors with a pyrin-domain 3 (NLRP3) inflammasome, leading to pyroptosis through the activation of Gasdermin D (GSDMD) [1]. Indeed,

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NLRP3 interacts with apoptosis-associated speck-like (ASC) and pro-caspase-1, activating caspase-1, thus regulating the cleavage of GSDMD and the maturation and secretion of proinflammatory cytokines, interleukins (ILs), namely IL-1β and IL-18, which amplify the innate immune response [2,4–7]. Then, IRI involves oxidative stress and inflammation, which expand the infarct area and further damage the heart [8,9]. Therefore, the NLRP3 inflammasome is involved in IRI-induced early cardiomyocyte death, *via* the GSDMD mediated pyroptosis [10], and in the amplification of the inflammatory response, *via* IL-1β and IL-18, both leading to HF [5].

Inhibiting the NLRP3 inflammasome can offer cardioprotection by reducing IRI and preventing HF. Various NLRP3 inhibitors aiming at different components of this multiprotein complex have been tested so far, with discordant results [4,7,10–12]. Importantly, the effectiveness of these inhibitors may depend on their ability to penetrate specific cell types, such as cardiomyocytes, where NLRP3 plays a critical role in the cell death and inflammatory responses, and/or macrophages where NLRP3 inflammasome is abundantly expressed [5,8,13,14].

Recently, our group identified a novel NLRP3 inhibitor, INF195, a small molecule that demonstrated cardioprotective effects by reducing infarct size and the release of IL-1β and caspase-1 in isolated perfused hearts. The active metabolite of INF195, named INF150 (or Compound 5), similarly inhibits NLRP3-driven pyroptosis and IL-1β release in a macrophage cellular model. However, INF150 did not penetrate H9c2 cells [15]. Given the proposed crucial role of NLRP3 and macrophages in cardioprotection [13,14], we sought to determine whether INF150 could provide cardioprotective benefits despite its inability to penetrate cardiomyocyte-like cells. First, we assessed whether INF150 was ineffective in reducing cell pyroptosis in both naïve (myoblasts) [15] and differentiated H9c2 cells (cardiac-like phenotype) [16], in a hypoxia/reoxygenation model [17]. Then, to investigate its potential cardioprotective effects, we studied INF150 in an isolated rodent heart model, where macrophages and cardiomyocytes coexist. The assessment included measuring infarct size, IL-1β levels, and active caspase-1 levels at the end of reperfusion.

2. Materials and methods

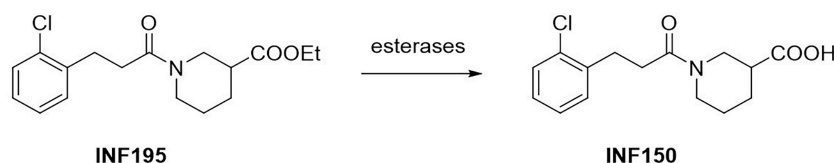
Synthesis of INF150 (also called compound 5) has been previously described [15]. It is the active metabolite of INF 195 (Fig. 1A).

2.1. In vitro models

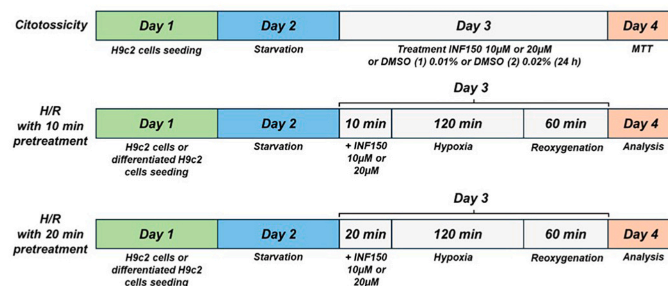
H9c2 cells and macrophages, were treated and maintained as previously reported [15,18]. Briefly, naïve H9c2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % (*v*/v) streptomycin/penicillin (Wisent Inc., Quebec, QC, Canada), at 37 °C and 5 % CO_2. H9c2 cells were differentiated towards a more cardiac phenotype, by pretreatment with Retinoic Acid (RA). Briefly, H9c2 cells were treated with DMEM supplemented with 1 % FBS and 1 μg/mL RA (RA was dissolved in DMSO and stored at −20 °C in the dark; Sigma, St. Louis, MO). The medium was refreshed every 24 h for five days. On day 5, differentiation was complete, and the cells were ready for further analysis [16].

Human monocytic cells THP-1 were cultured in RPMI 1640 medium with 10 % fetal bovine serum (Aurogene, Rome, Italy), 2 mM L-glutamine (Aurogene, Rome, Italy), 100 IU/mL penicillin (Aurogene, Rome, Italy), and 100 mg/mL streptomycin (Aurogene, Rome, Italy) at 37 °C and 5 % CO_2, and differentiated into macrophages by treatment with phorbol myristate acetate (PMA; 50 nM; 24 h; Sigma-Aldrich). In previous experiments macrophages challenged with LPS/ATP were exposed to increasing concentrations of INF195 and INF150 (0.1–100 μM) to effectively inhibit pyroptosis without cytotoxicity [15]. Here, we performed experiments with a single concentration of INF150 (10 μM) to confirm that macrophage pyroptosis is blocked by INF150. To this aim, the Cytotox 96 nonradioactive cytotoxicity assay (Promega Corporation, Madison, MI, USA) was used to evaluate LDH activity in order to quantify pyroptotic cell death. The Victor X4 (PerkinElmer, Waltham, MA, USA) was used to detect absorbance at λ = 490 nm. As directed by the manufacturer, the Human IL-1β Uncoated ELISA kit and the Human TNF-α Uncoated ELISA kit (Invitrogen, Waltham, MA, USA) were used to measure the release of IL-1β and TNF-α in differentiated THP-1

A) Structure of NLRP3 inhibitors INF195 and INF150



B) *In vitro* protocol



C) *Ex vivo* protocol

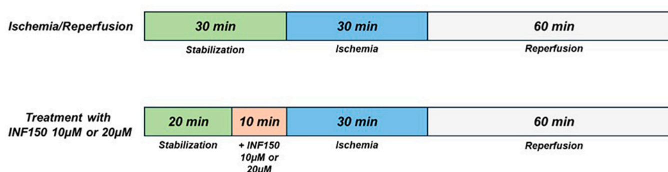


Fig. 1. Structure of NLRP3 inhibitors INF195 and INF150 (**A**), *in vitro* (**B**) and *ex vivo* (**C**) protocols used for the treatment with INF150 or not of H9c2 cells and mice hearts, respectively.

supernatant [15].

Given that INF150 does not penetrate naïve H9c2 cells [15], here we exposed these cells to increasing concentrations of INF150 (0.1–50 μM) for 24 h to assess cytotoxicity. To evaluate the cytotoxicity, we tested INF150 (10–20 μM), as done for INF195 in [15] and then an MTT assay was performed [18]. Since DMSO (Sigma, St. Louis, MO; final concentration 0.02 %) was used to dissolve the INF150 inhibitor to prepare a 10 mM stock solution, H9c2 cells were exposed to a final concentration of 0.01 % and 0.02 % DMSO [DMSO (1) and DMSO (2), respectively] to assess solvent cytotoxicity (Fig. 1B).

2.1.1. Hypoxia/Reoxygenation

Under hypoxia/reoxygenation (H/R) conditions, we also evaluated IL-1β release following pretreatment with INF150 at concentrations of 10 and 20 μM in either naïve or differentiated H9c2 cells. The H/R protocol was obtained with a reagent mixture that can create an anaerobic environment using the manufacturer's instructions (Millipore, Germany) [17]. The protocol consisted of pretreatment with the NLRP3 inhibitor for 10 or 20 min, followed by 120 min of hypoxia/anoxia (oxygen levels near 0 %) and then 60 min of reoxygenation (21 % O_2 and 5 % CO_2). Subsequently, IL-1β release was measured in supernatants using ELISA kit according to the manufacturer's instructions (FineTest, Wuhan, Hubei, China). The differentiated H9c2 cells were used to evaluate also GSDMD expression and cleavage [4,10].

2.2. Ex vivo cardiac model studies

Experiments were conducted on hearts isolated from male FVB mice (Harlan Laboratories, Udine, Italy; $n = 21$; age 10–12 weeks; weight 25–35 g). The animals were handled in accordance with the European Directive 2010/63/EU on the protection of animals used for research purposes (n 285/2019 – PRE 669C-44).

2.3. Ex vivo protocol

Mice were anesthetized with zoletyl (40 mg/kg) and xylazine (5 mg/kg), and heparinized with 500 U of heparin [15,19]. Subsequently, their hearts were rapidly removed, placed in ice-cold buffer solution, and weighed. The isolated hearts were then connected to a perfusion apparatus and retrogradely perfused with Krebs-Henseleit solution containing (mM): NaCl (127), KCl (5.1), $NaHCO_3$ (17.7), $MgCl_2$ (1.26), $CaCl_2$ (1.5), D-glucose (11), pH 7.4 and maintained at 37 °C and oxygenated (95 % O_2 and 5 % CO_2) [20].

2.4. Ex vivo ischemia-reperfusion protocol

The hearts were used for experimental purposes only if the perfusion pressure reached and maintained a value of 80 mmHg. Four hearts were discarded at the end of stabilization because they did not meet the required perfusion standards for experimental purposes [21]. DMSO stock solution was diluted in Krebs-Henseleit buffer to achieve final concentrations of 10 μM and 20 μM for INF150. The quantity of DMSO used does not exhibit cardiotoxicity, as previously reported [20,22].

After the stabilization period (30 min), hearts were randomly assigned to one of the experimental groups described below (Fig. 1 C). All hearts underwent 30 min of normothermic global ischemia followed by 60 min of reperfusion (I/R), regardless of the experimental group [21].

Group A, I/R ($n = 8$): 30 min of global ischemia followed by 60 min of reperfusion only;

Group B, INF150–10 μM ($n = 5$): 10 min of pretreatment with INF150 at the end of the stabilization period, followed by the I/R protocol;

Group C, INF150–20 μM ($n = 4$): 10 min of pretreatment with INF150 at the end of stabilization and subsequent I/R protocol.

2.5. Measurement of the infarct size

The evaluation of the infarct area was performed using ImageJ software on heart slices stained with 2,3,5-triphenyltetrazolium chloride (TTC). Infarct size is expressed as a percentage of the area at risk, which in global ischemia is represented by the whole heart, as reported in the literature [23]. Briefly, at the end of the reperfusion, the apex of each heart was removed and quickly frozen in liquid nitrogen for subsequent analysis. The remaining ventricles were frozen, then sectioned into slices and incubated with TTC (10 mg/mL in phosphate buffer, at 37 °C for 5 min) [20].

2.6. Determination of IL-1β and cleaved caspase-1 in hearts homogenates

To quantify the release of IL-1β and cleaved caspase-1, homogenized cardiac apices were used according to the indications reported by the manufacturers of the ELISA kits used (FineTest, Wuhan, Hubei, China). Briefly, apices were lysed by RIPA in the presence of protease and phosphatase inhibitors. Proteins were subsequently quantified using the Bradford method. Total protein assayed was employed to quantify IL-1β and cleaved caspase-1 according to the manufacturer's instructions [24].

2.7. Western blots

To obtain total cellular extracts, differentiated H9c2 cells were harvested and homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors (1:1000, Merck, Darmstadt, Germany). Protein concentrations were determined using the Bradford method [25]. The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5 % non-fat milk at room temperature for 1 h, followed by overnight incubation with primary antibodies: Gasdermin D (Cell Signaling, Danvers, MA) and β-actin (Santa Cruz, Dallas, TX) [10]. After incubation with anti-rabbit or anti-mouse secondary antibodies (Cell Signaling Technology, Danvers, MA), the chemiluminescence signal was detected using the chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA). Images were captured with the ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, USA), and Bio-Rad Image Lab Software 6.0.1 was used to analyze the immunoreactive bands.

2.8. Data analysis

Data are expressed as mean ± SEM (standard error of the mean). Statistical analysis using ANOVA and Dunnett and Tukey's multiple comparison test. P value ≤ 0.05 was considered significant. Statistical analyses were conducted using GraphPad Prism 8.0 software.

3. Results and discussion

3.1. INF150: Variable efficacy in in vitro models

In a previous study, we demonstrated the ability of INF150 to reduce pyroptosis in macrophages exposed to increasing concentrations of the compound [15]. To further validate the effectiveness of the inhibitor, macrophages were treated with INF150 (10 μM) and subsequently challenged with LPS/ATP. This challenge induces pyroptosis [15] as evidenced by increased LDH and IL-1β release. INF150 treatment led to a significant reduction in pyroptosis, decreasing LDH levels by approximately 30 % and IL-1β release by about 50 % in LPS/ATP-challenged macrophage.

Cytotoxicity was evaluated in H9c2 cells exposed to increasing concentrations of INF150 (0.1–50 μM) for 24 h. No effect on the viability of H9c2 cells was detected, even at the highest concentration (Fig. 2).

To further corroborate previous findings indicating that INF150 does

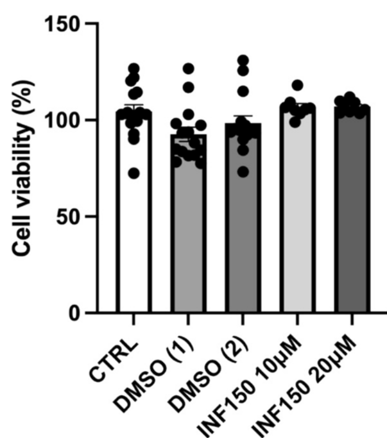


Fig. 2. Cell viability on H9c2 cells treated or not (CTRL) with 10 or 20 μM INF150 or DMSO (1) and (2) doses (vehicle) for 24 h. Both INF150 and DMSO at tested concentrations were not cytotoxic. Data are expressed as mean ± SEM.

not penetrate cardiomyocytes, we pretreated naïve H9c2 and differentiated H9c2 cells with INF150 at 10 and 20 μM for 10 and 20 min under H/R conditions.

In naïve H9c2 cells, H/R conditions led to an increase in IL-1β release with respect to normoxia, regardless of INF150 pretreatment (Fig. 3).

To validate these findings, we repeated the experiments in differentiated H9c2 cells, assessing IL-1β release and GSDMD expression and cleavage — key events in pyroptosis — under H/R conditions with or without INF150 pretreatment (Fig. 4).

Data show the presence of the GSDMD precursor in differentiated H9c2 cells. Under H/R conditions, IL-1β release was increased compared to normoxia, regardless of INF150 pretreatment, and this was accompanied by GSDMD cleavage.

3.2. Lack of cardioprotective effects of INF150 in isolated mouse hearts

To assess the protective effects of INF150 against myocardial IRI, we measured infarct size (IS) after pretreatment with two concentrations (10 or 20 μM) of the inhibitor in isolated hearts. The IS in the I/R group was 65 ± 2 of the risk area. Treatment with INF150 at 10 μM resulted in an infarct size (IS) of 57 ± 2 %, while 20 μM yielded an IS of 63 ± 5.6 %, with no significant differences compared to the ischemia/reperfusion (I/R) group (Fig. 5).

3.3. Cleaved caspase-1 and IL-1β in heart tissue: Lack of effect of INF150

We measured cleaved caspase-1 and IL-1β levels by ELISA to assess NLRP3 activation, and the results showed no significant changes in either cytokine at the tested concentrations of INF150 compared to the I/R group (Fig. 6).

4. Conclusion

We developed a series of NLRP3 inhibitors, specifically focusing on two distinct compounds: INF195 and INF150. In biological systems, INF195 is cardioprotective acting as the precursor to the active agent INF150 [15]. In this study, we explored the potential cardioprotective benefits of INF150 *per se*. INF150 does not penetrate H9c2 cell line, but penetrates macrophages [15], and given the crucial role of NLRP3 within macrophages in cardioprotection [13,14], we wondered whether INF150 could be protective in whole heart were macrophages and cardiomyocytes coexist. To address this, we evaluated the effects of INF150 using an isolated rodent heart model, where both macrophages and cardiomyocytes are present. Our assessment focused on measuring infarct size, IL-1β levels, and active caspase-1 levels at the end of reperfusion.

Our present findings confirm that INF150 effectively protects macrophages from LPS/ATP challenges but is ineffective on H9c2 cells. Notably, INF150 does not exhibit cytotoxic effects in either cell type, even at high concentrations. Moreover, INF150 is ineffective in differentiated H9c2 cells. Importantly, when differentiated with retinoic acid, H9c2 cells acquire adult cardiomyocyte-like features and exhibit a more 'physiological' response to hypoxia [16]. Despite the INF150's ability to protect isolated macrophages, in our mouse heart model, INF150 does not provide any cardioprotective benefits, as indicated by unchanged infarct size and no significant changes in IL-1β and caspase-1 levels across the inhibitor tested concentrations. This outcome contrasts sharply with the cardioprotective effects observed with its precursor, namely INF195 [15].

The present study suggests that while INF150 is promising in macrophage models, its inability to enter cardiomyocytes (both naïve H9c2 and differentiated H9c2) limits its effectiveness in cardiac tissue. This highlights the crucial role of cardiomyocyte uptake in the effectiveness of NLRP3 inhibitors as cardioprotective agents; indeed, merely being effective in macrophages, as demonstrated by INF150, is not sufficient for inducing cardioprotection. In contrast, INF195 is cardioprotective because it penetrates both macrophages and

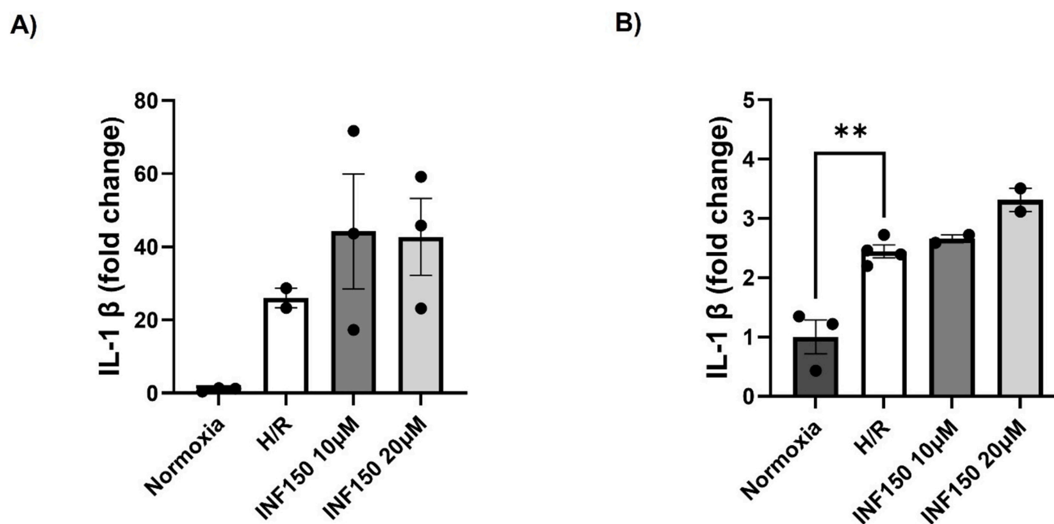


Fig. 3. Fold change of IL-1β assessed by ELISA in supernatants of H9c2 cells under normoxic or H/R conditions, with and without INF150 pretreatment. In (A) INF150 pretreatment lasted 10 min and in (B) 20 min. Data are presented as mean ± SEM. Statistical significance: **($p < 0.01$) compared to Normoxia.

Fig. 4. (A, C) Representative Western blot images showing cleaved GSDMD and its precursor in cell lysates from differentiated H9c2 cells pretreated with INF150 (10 μM and 20 μM). In (A), INF150 pretreatment lasted 10 min before H/R exposure, whereas in (C), it lasted 20 min. β-actin was used as a loading control. Graphs show band density expressed as fold change relative to H/R.
(B, D) IL-1β release, expressed as fold change, assessed by ELISA in the supernatants of differentiated H9c2 cells under normoxic or H/R conditions, with or without INF150 pretreatment. In (B), INF150 pretreatment lasted 10 min before H/R exposure, while in (D), it lasted 20 min. Data are presented as mean ± SEM. Statistical significance: *($p < 0.05$), ($p < 0.01$) *vs.* Normoxia.

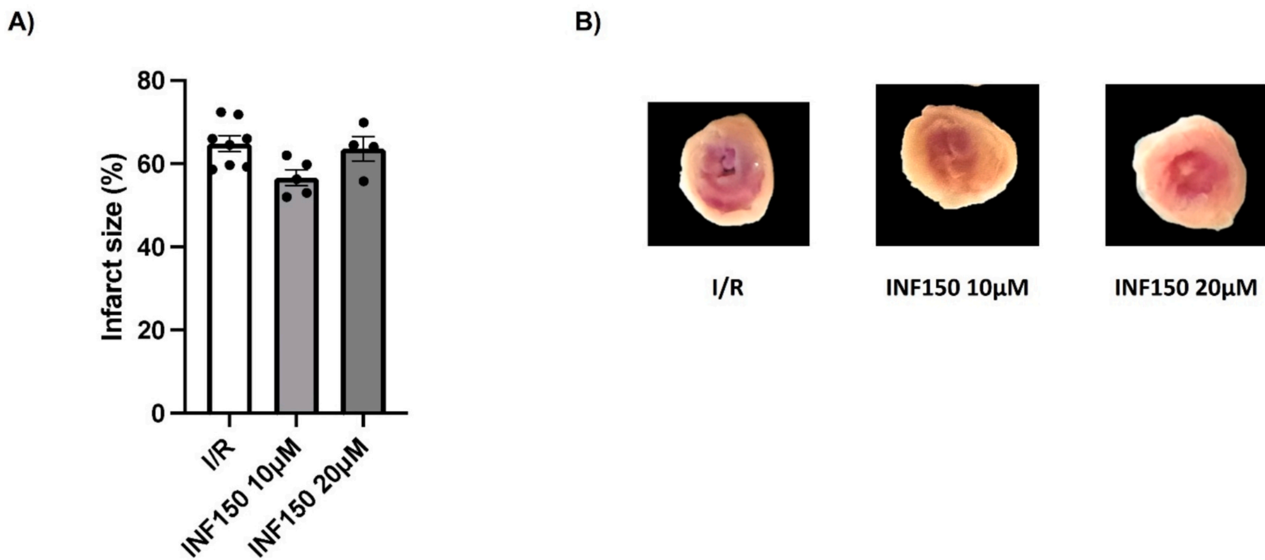


Fig. 5. Infarct size in mouse hearts subjected to an ischemia/reperfusion (I/R) protocol, with or without pretreatment with 10 or 20 μM INF150 in the perfusate 10 min prior to ischemia. A) Infarct size is expressed as a percentage of the area at risk in mouse hearts. B) Representative images of left ventricle slices from each experimental group, showing the infarcted area in white and the remaining area at risk in red. Data are presented as mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

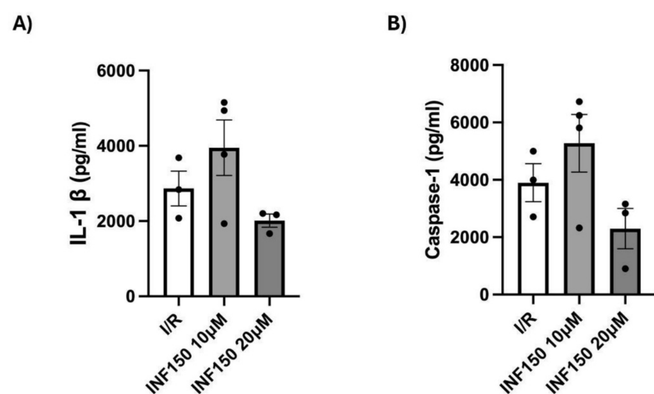


Fig. 6. Concentrations of IL-1β (A) and cleaved caspase-1 (B) measured by ELISA in extracts from the left ventricular apex of mice subjected to ischemia/reperfusion (I/R), with or without pretreatment with INF150. Data are presented as mean ± SEM.

cardiomyocytes, where it is metabolized to INF150 [15].

Ischemia and reperfusion serve as priming and triggering stimuli for NLRP3 activation in cardiomyocytes. Indeed, adult murine cardiomyocytes have exhibited inducible inflammasome formation associated with increased cell death by pyroptosis [26]. Here, we show for the first time that GSDMD, which is required for IL-1β secretion and pyroptosis, is present in differentiated H9c2 cells and is cleaved upon exposure to H/R. Yet, INF150 is unable to limit this cell death, as evidenced by unchanged GSDMD cleavage and IL-1β levels in the supernatant.

Previous studies suggested that the main role of NLRP3 in cardioprotection is played by cells of the immune system, including macrophages [13,14]. Here, we show that effective cardioprotection requires inhibitors that can readily penetrate cardiomyocytes, where the NLRP3 inflammasome plays a direct role in exacerbating IRI.

Many cellular populations have been proposed to contribute to the cardioprotective potential of various cardioprotective approaches, and some authors have suggested that NLRP3 inhibition exerts its cardioprotective effects by acting mainly on inflammatory cellular components rather than directly on cardiomyocytes [14,27–30]. As a matter of fact, NLRP3 is active in various cardiac cell types, including fibroblasts, leukocytes, and cardiomyocytes, each contributing to the inflammatory response and subsequent cardiac damage [31]. However, it remains unclear whether targeting cells other than cardiomyocytes is inherently cardioprotective.

INF150 enters macrophages, likely through a phagocytic process [15], and inhibits NLRP3 activation. However, it does not protect the heart against IRI. Therefore, our results challenge the notion that macrophages are central to the cardioprotective effects of NLRP3 inhibitors, at least in the *ex vivo* model. Indeed, the isolated heart model provides precise control over experimental variables and ensures high reproducibility by eliminating systemic influences, such as neural, neurohumoral and immune responses [21].

5. Limitations of the study

The present study has some limitations. The sample size of the heart groups is limited; however, the small SEM in each group suggests that the data are robust, reducing the need for additional animals. Moreover, the use of different cell models supports our conclusions.

While recruited macrophages influence infarct size after ischemia/reperfusion injury, resident macrophages coordinate inflammatory responses to promote cardiac remodeling [32]. Since NLRP3 is primarily expressed in myeloid cells, we cannot rule out that the cardioprotective effect of NLRP3 inhibitors *in vivo* may also be due to reduced macrophage infiltration [33]. Nevertheless, recruited macrophages contribute to the healing phase [32], while in the present study we focused on acute cell death, where cardiac cell death by pyroptosis may play a pivotal role [1,5,10].

Future research should focus on quantifying INF195 and INF150 within the cells they penetrate and/or developing alternative delivery methods for INF150 to achieve the cardioprotective effects observed with INF195, *in situ* [15]. Additionally, exploring other molecular targets within the NLRP3 inflammasome pathway may offer new avenues for therapeutic intervention in IRI.

In summary, our results suggest that for NLRP3 inflammasome inhibitors to be effective as cardioprotective agents, they must specifically target and penetrate cardiomyocytes. Successful NLRP3 inhibition in isolated macrophages alone does not predict cardioprotective effects in whole organs. Our study highlights the complexity of translating *in vitro* findings to *ex vivo* models and eventually to *in vivo* settings. It also emphasizes the need for thorough evaluations of potential therapeutic agents across various biological systems before considering clinical applications.

Ethics approval and consent to participate

The animals were maintained following the European Directive 2010/63/EU about the protection of animals being used for research reasons.

Consent for publication

All the authors read and approved the final version of the manuscript.

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CRediT authorship contribution statement

Magalì Giordano: Writing – original draft, Investigation, Data curation. **Saveria Femminò:** Writing – original draft, Investigation, Data curation. **Federica Blua:** Methodology, Investigation. **Francesca Boccato:** Methodology, Investigation. **Chiara Rubeo:** Methodology, Investigation. **Beatrice Mantuano:** Methodology, Investigation. **Francesca Cioffi:** Methodology, Investigation. **Stefano Comità:** Investigation. **Arianna Brovero:** Methodology, Investigation. **Rosa Ciullo:** Investigation, Methodology. **Massimo Bertinaria:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Claudia Penna:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Pasquale Pagliaro:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

Request for data used in this manuscript should be addressed to the corresponding author.

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