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NLRP3 inflammasome as a therapeutic target in cardiovascular diseases: An update of preclinical and clinical evidence

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Abstract
The role of inflammation in the pathogenesis of various diseases is of paramount importance, with a significant impact on morbidity and mortality. This underscores the pivotal role of cardiovascular diseases as the leading cause of mortality globally, including in Chile. In this context, the NLRP3 inflammasome, a multiprotein complex of the innate immune system, has been the subject of intense research with regard to its expression and activation in cardiovascular pathologies. The present review addresses the preclinical and clinical scientific evidence, using databases and proceeded to classify the collected molecules according to their mechanism of inhibition of NLRP3 inflammasome activation, exploring their potential therapeutic effect in cardiovascular diseases. In conclusion, it is highlighted that the molecules currently under study show significant improvements in cardiovascular disease symptoms by influencing the priming or activation of the NLRP3 inflammasome.
Introduction: . Materials and Methods: . Results: . Discussion: .
Keywords: inflammation; NLRP3 inflammasome; cardiovascular diseases; NLRP3 inhibitor.

Resumen
La inflamación desempeña un papel crucial en diversas enfermedades de gran impacto en la morbi- mortalidad, destacando las enfermedades cardiovasculares como principal causa de fallecimiento a nivel mundial y en Chile. En este contexto, el inflamasoma NLRP3, un complejo multiproteico del sistema inmune innato, ha sido objeto de intensa investigación en relación con su expresión y activación en patologías cardiovasculares. La presente revisión aborda la evidencia científica preclínica y clínica, empleando bases de datos y se procedió a clasificar las moléculas recopiladas según su mecanismo de inhibición de la activación del inflamasoma NLRP3, explorando su potencial efecto terapéutico en enfermedades cardiovasculares. Como conclusión, se destaca que las moléculas actualmente en estudio muestran mejoras significativas en los síntomas de enfermedades cardiovasculares al influir en el *priming* o la activación del inflamasoma NLRP3.
Palabras Clave:inflamación; inflamasoma NLRP3; enfermedades cardiovasculares; inhibidor de NLRP3

Introduction

Inflammation represents a physiological process whereby the body mounts an immune response to combat damage, whether caused by an injury or a pathogenic agent (Caillon&Schiffrin, 2016). The inflammatory process commences with an acute phase, which is directed towards the eventual resolution of the inflammatory response through the action of pro-resolutive mediators. However, if this inflammatory process is not resolved, it can evolve into a chronic inflammatory state, which may result in tissue damage. The role of inflammation in the pathogenesis of various diseases is well documented. These include atherosclerosis (Wei et al., 2023), cardiomyopathy (P. Nordet et al., 1996), hypertension (Caillon&Schiffrin, 2016), heart failure (Murphy et al., 2020), asthma (Lambrecht & Hammad, 2015), arthritis (Aletaha et al., 2010), and even some types of cancer (Singh et al., 2019).

In this regard, the resolution of chronic inflammation represents an intriguing therapeutic objective, particularly in the context of diseases with elevated morbidity and mortality, such as cardiovascular diseases (Caillon&Schiffrin, 2016). Cardiovascular diseases represent the leading cause of death globally and in Chile,in the year 2020, the Department of Statistics and Health Information (DEIS) (Ministry of Health, 2023) reported that 29,035 deaths were attributed to diseases of the circulatory system, representing 27.9% of all deaths. Conversely, the leading cause of death worldwide since 2000 is ischemic heart disease, representing 16% of all deaths, according to the World Health Organization (World Health Organization, n.d.). Consequently, addressing the chronic inflammatory component of

cardiovascular pathologies represents a significant area of interest, primarily to identify new therapeutic targets and complement patient therapy.

Mediators involved in the inflammatory mechanism

The initiation of the inflammatory response is contingent upon the presence of a stimulus that corresponds to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Mangan et al., 2018). These patterns are recognized by pattern recognition receptors (PRRs) and are capable of initiating a local and systemic inflammatory response, releasing proinflammatory molecules such as prostaglandins, thromboxanes, and cytokines (Mangan et al., 2018). Two proinflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18), are of particular importance for this review as they participate in the mechanism of action of the protein complex, the inflammasome, which will be addressed later. These cytokines are regulated by the nuclear factor enhancing kappa light chains of activated B cells (NFκB: Nuclear Factor kappa-beta) through the activation of their gene transcriptional mechanism (Murphy et al., 2020). In the absence of an infectious agent, inflammation is collectively referred to as sterile inflammation, given that its mechanism is primarily mediated by DAMPs.

For example, in the context of atherosclerosis, endogenous elements and the formation of atheromatous plaques are identified as DAMPs, which then trigger a sustained inflammatory response in the vascular endothelium (Kong et al., 2022). This is interpreted as a sterile inflammatory process, given the absence of a pathogen. Furthermore, elevated levels of angiotensin II in patients with arterial hypertension and atherosclerosis have been demonstrated to induce vasoconstriction (Poznyak et al., 2021) and activate the transcriptional factor NFκB, thereby perpetuating the inflammatory state that ultimately leads to vascular damage (Barnabei et al., 2021).

The treatment of patients with vascular disease does not incorporate the use of anti-inflammatory drugs, although some clinical trials have addressed this therapeutic aspect. The results of these trials have not yielded conclusive efficacy (De Miguel et al., 2021; Wohlford et al., 2020). Consequently, research has been directed towards identifying inflammation targets in order to develop molecules that inhibit the initiation of inflammatory processes, such as NFκB activation and the NLRP3 inflammasome.

Role of the NLRP3 inflammasome in chronic inflammation in CVD

The NLRP3 inflammasome is a multiprotein complex comprising three distinct domains or regions. It represents a component of the innate immune system, as it is triggered by the detection of non-specific stimuli. Activation of the NLRP3 inflammasome involves the generation of a conformation signal and an activation signal, which in turn activates NFκB and results in the release of proinflammatory cytokines, primarily IL-1β and IL-18. In recent years, research has been conducted on the expression and activation of the inflammasome in cardiovascular pathologies. Activation of the inflammasome has been observed in cardiac muscle cells (Mangan et al., 2018), vascular endothelium (Tanase et al., 2023), and cardiac fibroblasts (Caillon&Schiffrin, 2016) during the course of cardiovascular diseases or after a cardiovascular event, such as ischemia/reperfusion damage, during the inflammatory process associated with cardiac remodeling, cardiomyopathy, and other conditions (Mangan et al., 2018). The most relevant of these are:

- Hypertension: The inflammasome, through pyroptosis, which corresponds to a type of programmed cell death, plays a role in this pathology that has yet to be fully elucidated. However, it has been possible to establish a correlation between it and certain polymorphisms and diverse mechanisms that impact cardiac function (De Miguel et al., 2021).
- Atherosclerosis: Electronegative low-density lipoprotein (LDL^-) A minor fraction of normal LDL, designated as electronegative low-density lipoprotein (LDL(-)), has the capacity to stimulate the release of IL-1β in monocytes through the activation of Toll-Like Receptor 4 (TLR4). This, in turn, activates the priming and activation pathways of the NLRP3 inflammasome (Estruch et al., 2015), a process that will be elucidated in the following sections.
- Heart failure: Since IL-1β and IL-18 are at high levels, therefore, the inflammasome can be a therapeutic target in this pathology (Wohlford et al., 2021).

In recent years, there has been a growing interest in the study of different natural and synthetic molecules, many of which have shown potential in inhibiting the formation or activation of the NLRP3 inflammasome. This could be a promising avenue for modulating the inflammatory response in pathological contexts where this exacerbation of the response has caused damage. Nevertheless, the current body of evidence, both preclinical and clinical, is insufficient to confirm whether this represents a promising therapeutic target for the development of new drugs and to assess the potential clinical outcomes. Accordingly, the present review assesses the scientific evidence pertaining to the therapeutic benefits of NLRP3 inflammasome inhibition in the context of cardiovascular disease.

Inflammasome activation

The various types of inflammasomes share a common structural composition, comprising three distinct regions: the Nucleotide Oligomerization Domain (NOD), the Caspase Activation and Recruitment Domains (CARD), and the Inhibitor of Apoptosis (IAP) (Montaño Estrada Luis Felipe et al., n.d.). However, the NLRP3 inflammasome, in addition to this common structure, is composed of the Nlrp3 protein, which contains: The NACHT domain (comprising NAIP, neuronal apoptosis inhibitory protein; CIITA, MHC class II transcription activator; HET-E, incompatibility locus protein from Podosporaanserin; and TP1, telomerase-associated protein) is accompanied by a leucine-rich repeat (LRR) and the pyrin domain (PYD) containing protein 3, as illustrated in Figure 2A. To execute its function, the inflammasome requires the ASC protein (apoptosis-associated speck-like protein containing a caspase recruitment domain), which facilitates the recruitment of pro-caspase 1 (Colunga Biancatelli et al., 2022; UrcuquiInchima Silvio, n.d.). Inactivated Nlrp3 proteins undergo a conformational change in response to the detection of a proinflammatory stimulus at the intracellular level, which serves as the initial trigger for the formation of the NLRP3 inflammasome complex. The proteins are coupled in the form of a disk, with their PYD domains arranged at the center and the ASC protein recruited to form a filament (Fig. 2C). Figure 2 provides an overview of the aforementioned structures, their conformations, and interactions.

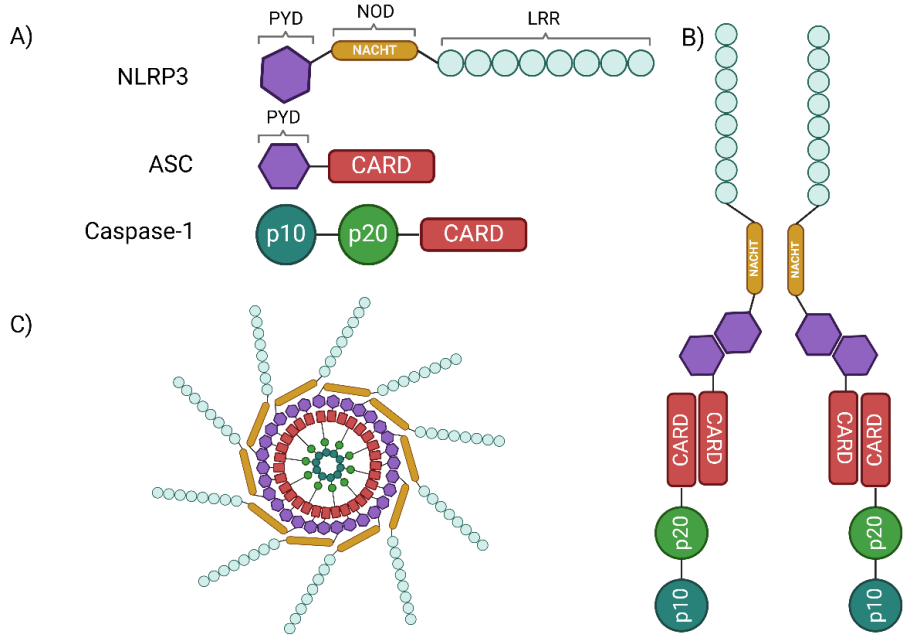

Figure 2. Structure and conformation of NLPR3 inflammasome. **A)** Structure and domains of the proteins that make up the NLPR3 inflammasome. **B)** Assembly mode of Nlpr3, ASC and Caspase-1 proteins. **C)** Oligomerization and formation of the NLRP3 inflammasome complex.

The activation of the NLRP3 inflammasome is initiated by two signals that culminate in pyroptosis. The initial signal, designated as signal 1 or priming, is mediated by PRRs (pattern recognition receptors), such as the tumor necrosis factor receptor (TNFr), which interacts with the tumor necrosis factor a (TNF). Tumor necrosis factor (TNF) activates intracellular signaling that involves the phosphorylation of NF-κB, thereby releasing the p65/ReIB complex to be translocated to the nucleus, which in turn promotes mRNA transcription and increases Nlrp3 protein expression (Coll et al., 2022).

The second signal, or activation signal, is as follows: The NLRP3 inflammasome is activated through various mechanisms, as outlined in Table 1. These mechanisms lead to the oligomerization or assembly of the inactive Nlrp3 proteins, forming the NLRP3 inflammasome. The NLRP3 inflammasome then cleaves procaspase-1 to caspase-1.

The latter is responsible for cleaving pro-IL-1β and pro-IL-18 to IL-1β and IL-18, thereby generating their release through the pores generated during pyroptosis (Coll et al., 2022).

Pyroptosis is a type of programmed cell death characterized by the cleavage of the gasdermin-D protein by caspase-1, resulting in the generation of GSDMD-N. The N-terminal domain of GSDMD-N plays a crucial role in the formation of a pore in the membrane, leading to the release of interleukins until cell death is induced (Coll et al., 2022). It is noteworthy that evidence of inflammasome activation or overexpression has been observed in numerous chronic inflammatory pathologies (Wang et al., 2018).

It is evident that the molecules involved in the inflammasome activation signal (Table 1) are not suitable as therapeutic targets since they are involved in the mediation of numerous processes throughout the body, including nerve transmission, muscle contraction (Stefania Manetti, 2022), bone development (Stefania Manetti, n.d.), blood coagulation, plasma tonicity contribution, pH regulation (Hume V., 2018), and others. It is for this reason that a review of the scientific literature on studies of molecules that generate direct inhibition of the inflammasome or its signaling cascade is a valuable exercise in identifying new approaches to therapy.

Table 1. Molecules that trigger signal 2 or inflammasome activation and the mechanism by which they do so.

MOLECULE	ACTIVATION MECHANISM	REFERENCE
Potassium	Potassium efflux is a trigger for formation	(Kelley et al.,
	of the inflammasome by allowing the binding of NEK7 (NEK7: *NIMA-related kinase 7)* a protein Nlrp3.	2019) (Yang et al., 2019)
	Efflux of chlorinePromotes activityATPase	
Chlorine	allowing the activation of caspase-1 and an increase in IL-1β production.	(Kelley et al., 2019) (Yang et al., 2019)
Sodium	Sodium influx induces activation of the NLRP3 inflammasome through potassium efflux.	(Kelley et al., 2019) (Yang et al., 2019)
Calcium	Its mechanism of action is not entirely clear, it is thought that an excessive release of calcium from the endoplasmic reticulum causes an overload in the mitochondria generating mitochondrial reactive oxygen species.	(Kelley et al., 2019) (Yang et al., 2019)
Cholesterol crystals	They generate the release of extracellular ATP that	
Uric acid **Asbestos Crystals**	Allows the activation of the NLRP3 inflammasome, amplifying its inflammatory response.	(Yang et al., 2019) (Mangan et al., 2018)
Imiquimod, CLO97	Production of reactive oxygen species.	(Kelley et al., 2019)

Preclinical evidence of direct NLRP3 inhibitory molecules

The following section will present a review of the molecules that are currently being studied as inhibitors of this machinery, along with an analysis of their potential therapeutic effect in the context of cardiovascular diseases. To this end, the molecules will be classified into two categories: direct inhibitory molecules of the NLRP3 inflammasome and indirect inhibitory molecules. The former category encompasses those whose mechanism of action involves acting on the components of the inflammasome structure, as illustrated in Figure 2. The aforementioned molecules will be synthesized in Table 2, along with those that act indirectly.

Dapansutrile: dapansutrile (OLT1177®, Olatec) is a molecule that acts on signal 2 by reducing the ATPase activity of the Nlrp3 protein by directly inhibiting recruitment and interaction with ASC, and consequently prevents the oligomerization of the inflammasome (Marchetti et al., 2018; Klück et al., 2020).

To assess the efficacy of this molecule in ischemic heart disease, a total of 60 male ICR mice (ICR: Institute of Cancer Research) were utilized. These mice are commonly employed in drug research due to their ease of handling and reproduction. Dapansutrile was administered at mealtimes for a period of nine weeks, and the mice were divided into four groups. The first three groups underwent the AMI procedure, with the first group receiving 3. The first group received 75 g/kg of OLT1177 (n=10), the second group received 7.5 g/kg (n=9), and the third group served as the control and received the same diet as the other groups without OLT1177 (n=10). A fourth group underwent a sham procedure and received the same diet as the third group (n=6). The preservation of contractile reserve and diastolic function of the heart was evaluated in all four groups throughout the course of the dietary intervention. During this period, two mice from the first group, two from the second, three from the third, and none from the Sham group died. The causes of death were not specified.

To ascertain whether OLT1177 induces an enhancement in the preservation of contractility, the "isoproterenol challenge" was conducted, yielding favorable outcomes for both groups to which the drug was administered in comparison to the control group (Group 1: +33 ± 11%; Group 2: +40 ± 6%; Group 3: +9 ± 7%). Additionally, the end-diastolic diameter of the left ventricle was measured to assess diastolic function. The results demonstrated an improvement in the first two groups (Group 1: 3.2 ± 0.5 mmHg and Group 2: 4.5 ± 0.5 mmHg vs. 9.6 ± 1.4 mmHg). In light of these preclinical findings, dapansutrile may be regarded as a potential therapeutic agent capable of preserving diastolic function and contractility in the context of ischemic heart disease. Subsequently, articles from clinical trials pertaining to this drug will be discussed (Aliaga et al., 2021).

ZYIL1: Despite the absence of published preclinical evidence, given the drug's recent development and ongoing study, it merits further investigation. Its efficacy, safety, and pharmacokinetics have been demonstrated in human trials, which provides a foundation for further analysis (*Drug Bank. ZYL-1*, 2023).

MCC950 is a molecule that has been shown to inhibit the Walker B motif of the NACHT domain, preventing the conformational change and oligomerization of the NLRP3 inflammasome by blocking the release of IL-1β (Coll et al., 2019; Coll MCC950 is a molecule that inhibits the Walker B motif of the NACHT domain, thereby preventing the conformational change and oligomerization of the NLRP3 inflammasome through the blocking of IL-1β release (Coll et al., 2019; Coll et al., 2015). The following section presents a review of two studies examining the effects of this drug on atherosclerosis and cardiotoxicity induced by doxorubicin (DOX). One study suggests a potential link between DOX-induced cardiotoxicity and the action of the NLRP3 inflammasome (TavakoliDargani& Singla, 2019).

MCC950 has been proposed as a molecule that may attenuate the cardiotoxicity of DOX, which in certain cases has been observed to lead to insufficiency. To test this hypothesis, an in vitro study was conducted using H9c2 cells, and an in vivo study was performed on male C57BL/6J mice (n=50) over the course of eight weeks. The subjects were randomly assigned to one of three groups: a control group (n=10), a group treated with DOX alone (n=20) to induce myocardial injury, and a third group treated with DOX and MCC950 (n=20). The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick-end labeling) test (Kyrylkova et al., 2012) was employed to evaluate the impact of DOX on NLRP3 inflammasome-mediated pyroptosis, a form of cell death that is increased by DOX in the myocardium. This test also assessed the expression of NLRP3 mRNA, ASC, and caspase-1 in the myocardium. In the group that received MCC950, there was a notable decrease in the expression of NLRP3, ASC, and caspase-1, which correlated with a reduction in pyroptosis in the myocardium. In vitro, doxorubicin (DOX) has been demonstrated to reduce cell viability and elevate levels of interleukin-1 beta (IL-1β) and IL-18, both of which have been linked to the generation of inflammation and cardiotoxicity. However, the administration of MCC950 has been shown to diminish the levels of both cytokines, thereby reducing pyroptosis and consequently, the associated cardiotoxicity.

The results of the study indicate that MCC950 has the potential to provide cardioprotective effects by reducing DOX-induced heart damage in mice, improving cardiac function through the inhibition of NLRP3 inflammasome activation and pyroptosis, and by decreasing the expression of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 in both mice and H9c2 cells (Zhang et al., 2021).

In a separate in vivo study, the impact of MCC950 was assessed in mice lacking NLRP3 or IL-1β and exhibiting reduced atherosclerosis. It was observed that ox-LDL can trigger macrophage pyroptosis through the activation of the NLRP3 inflammasome, a crucial process in the formation of atheromatous plaques (Zeng et al., In 2021). Truong et al. (2021) and Lo Sasso et al. (2016) conducted a study in which mice were fed a "Western" diet for 12 weeks and then separated into a model group (n=15) and a group that was given a 10 mg/kg intraperitoneal injection of MCC950 (n=15). A control group of C57BL/6J mice (n=15) was maintained on a normal diet. A total of

30 apoE−/− mice were utilized in this study, as the absence of apoprotein E predisposes these mice to the development of atherosclerosis. To facilitate a comparative analysis of the results obtained from the various experimental groups, the lesion formation in the aortic tissue was evaluated through the use of several methodologies, including western blotting. This approach involved the extraction of proteins from the aorta in an in vitro setting and macrophages from the aortic tissue in an in vivo context. Furthermore, the formation of membrane pores during pyroptosis was evaluated using the TUNEL assay (Kyrylkova et al., 2012). Additionally, a serum analysis was conducted to evaluate the expression levels of inflammatory factors.

The results obtained after twelve weeks of dietary intervention demonstrate that MCC950 was capable of significantly reducing the formation of atheromatous plaque in the aorta when compared to the model groups and the MCC950 group (4.84 ± 1.07 mm² vs 7.25 ± 0. The analysis of the serum revealed a reduction in IL1-β and IL-18 levels. However, this was not observed in the lipid profile, as triglyceride, ox-LDL, and C-LDL levels were similar in both the model group and the MCC950 group. This suggests that MCC950 does not affect the lipid profile. It was observed that MCC950 in the aorta decreased the levels of NLRP3 and caspase-1 proteins, which prevented the activation of the NLRP3 inflammasome and therefore decreased macrophage pyroptosis. The activation of the NLRP3 inflammasome generated by ox-LDL was then evaluated, resulting in the fact that, despite treatment with MCC950, the mRNA levels of NLRP3 and caspase-1 were elevated in both groups, but at the protein level, there was a significant reduction. In light of these findings, it can be concluded that MCC950 possesses the capacity to diminish the formation of atheromatous plaque by inhibiting the activation of the NLRP3 inflammasome and reducing the pyroptosis of macrophages in the aorta by lowering the levels of proteins involved in this inflammatory process. Consequently, it can be regarded as a molecule with potential for pharmacological applications (Zeng et al., 2021).

Oridonin is a bioactive component extracted from Rabdosiarubescent, a traditional Chinese medicinal plant with documented anti-cancer and anti-inflammatory properties. However, its application in cancer therapies has been limited, in part, due to the uncertainty regarding its mode of action and the relatively moderate potency observed until a few years ago. Moreover, the molecule has been demonstrated to inhibit the activation of NF-κB, the priming phase induced by an LPS (lipopolysaccharide)-mediated stimulus, and to reduce reactive oxygen species through the expression of the transcription factor Nrf2, which exhibits antioxidant activity (Lin et al., 2022; He et al., 2018).

A current study has demonstrated the mechanism of action through a series of assays, as described below: First, oridonin (Ori) was identified as a specific inhibitor of the NLRP3 inflammasome. The model employed was that of bone marrow-derived macrophages stimulated with monosodium urate, ATP, and LPS crystals. Furthermore, the cells are treated with nigericin in doses of 0.5. μM, 1 μM and 2 μM to stimulate the activation of caspase-1 and secretion of IL-1β, through the induction of potassium efflux and chloride.

The presence of pro-caspase-1, caspase-1, pro-IL-1β, and IL-1β was quantified through western blotting, while TNF-α expression was determined through enzyme-linked immunosorbent assay (ELISA). Consequently, a reduction in P20 and IL-1β was noted, indicating that caspase-1 activation and IL-1β release were suppressed. Moreover, no impact was observed on the expression of TNF-α, which is independent of the inflammasome. It is noteworthy that the same tests were also conducted on human peripheral blood mononuclear cells with LPS-induced caspase-1 activation, and the same results were obtained. This study demonstrates that Ori acts before ASC oligomerization. However, it was observed that Ori does not affect signaling events prior to oligomerization of Nlrp3 proteins, such as ROS production, mitochondrial damage, or potassium and chlorine efflux. Therefore, it can be postulated that Ori interferes with the NEK7 and Nlrp3 interaction, which is a fundamental step for the assembly of the inflammasome complex. To ascertain this, HEK-293T cells (immortalized human embryonic kidney cells) treated with nigericin were utilized. Through immunoprecipitation and western blotting, the following interactions were analyzed: The following experiments were conducted: NEK7-Nlrp3 in purified HEK293T cells, purified NEK7 and Nlrp3, oligomerization of Nlrp3 in HEK-293T cells, and ASC-Nlrp3 in HEK-293T cells.

Consequently, Ori was unable to impede the direct Nlrp3-Nlrp3 interaction in HEK-293T cells, thereby indicating that Ori does not inhibit NLRP3 oligomerization. Consequently, Ori does not influence the ATPase activity of Nlrp3, which is crucial for the oligomerization of Nlrp3. Furthermore, it was observed that the compound was unable to inhibit the interaction between NLRP3 and ASC in HEK-293T cells. Although NEK7 has been previously reported to interact with NEK9 during mitosis, the findings indicate that Ori is unable to block the NEK7-NEK9 interaction. Consequently, these results suggest that Ori inhibits the activation of the NLRP3 inflammasome by directly blocking the NEK7-Nlrp3 interaction.

To ascertain whether Ori binds directly to NLRP3, the Nlrp3 protein extracted from LPS-stimulated bone marrow-derived macrophage lysates utilized in the initial test was incubated and combined with a green, fluorescent marker, designated as GFP. The interaction between GFP-Nlrp3 and Ori was investigated by culturing the two

proteins together. The results demonstrated that GFP-NLRP3 was attracted to Ori, confirming that it interacts directly with NLRP3. Furthermore, a microscale thermophoresis (MST) assay was employed to quantify the direct interaction between Ori and GFP-Nlrp3. The equilibrium dissociation constant (KD) was determined to be approximately 52.5 nM. Furthermore, the expression of the PYD, NACHT, and LRR domains was evaluated via western blot analysis of macrophage lysates derived from bone marrow. The results demonstrated that Ori interacts directly with NACHT.

To ascertain the nature of the interaction between Ori and NACHT, LPS-stimulated bone marrow-derived macrophages were incubated with Ori and induced with nigericin. The objective was to ascertain whether IL-1β production could be inhibited after three washes for fifteen minutes. It was observed that IL-1β secretion remained blocked, which suggests that Ori binds irreversibly to Nlrp3 through a covalent bond. Additionally, Ori contains an active carbon-carbon double bond with the potential to react with the thiol groups of cysteine-containing Nlrp3, forming a covalent bond.

BLAST was employed to identify the cysteine residues present in NACHT and ascertain which one was involved in the interaction with Ori. Nlrp3 protein mutants were created in which the amino acid cysteine was replaced by alanine. As a result, it was determined that in the mutant where cysteine 279 was modified, Ori was unable to bind with Nlrp3, indicating that this may correspond to its binding site.

In conclusion, the mechanism by which direct inhibition of the NLRP3 inflammasome is achieved is through covalent binding to cysteine 279 of the NACHT domain of the Nlrp3 protein (H. He et al., 2018).
Following the clarification of Ori's mechanism of action, its potential therapeutic applications in cardiovascular diseases, particularly its impact on cardiac remodeling, were investigated (J. Lin et al., 2022; S. Lin et al., 2022; Gao et al., 2021). C57BL/6 male mice with ligated right coronary arteries were utilized to induce myocardial infarction, with treatment with Ori occurring thrice weekly for a period of four weeks. Cardiac function and myocardial fibrosis were evaluated through the expression of IL-1β and IL-18, as well as fibrotic markers, which were analyzed using western blotting, immunofluorescence, ELISA, and quantitative real-time polymerase chain reaction (RT-PCR). As a result, a notable reduction in cardiac fibrosis was observed in the Ori-treated groups, with a percentage of 16.71±2.38 compared to the control group, which exhibited 23.38±1.65. Furthermore, there was evidence of a decrease in the expression of NLRP3, IL-1β, and IL-18, as well as a reduction in the presence of cardiac macrophages and the entry of neutrophils in the Ori-treated group, in contrast to the untreated group. New Ori-derived compounds with increased potency and specificity have now been developed (Pang et al., 2023).

Ginsenoside RG3 (RG3) is a component of a traditional Chinese plant. This component has been described as having anti-inflammatory properties related to both the activation signals of the NLRP3 inflammasome (Liu et al., 2023; Shi et al., 2020).It was demonstrated that the compound affects signal 1, specifically by inhibiting the NF-κB signaling pathway and the expression of pro-IL-1β. Conversely, the mechanism by which it affects signal 2 is not fully elucidated; however, it has been postulated that this may be through the disruption of NEK7-NLRP3, which is crucial for the oligomerization of the inflammasome (Shi et al., 2020).

The objective of this study is to examine the in vivo effects of RG3 on myocardial hypertrophy and to relate these effects to in vitro observations of SIRT1 (SIRT1: Sirtuin 1), a protein that inhibits the NF-kβ signaling pathway and may have significant implications for the mechanism of action of RG3.

To evaluate the studies, a variety of techniques were employed. Levels of myocardial hypertrophy markers (ANP, BNP, and beta-MHC) were quantified through RT-PCR. Levels of proteins associated with myocardial fibrosis (TGF-B1: Transforming Growth Factor Beta-1) and proteins related to the inflammasome were determined through western blot analysis. Additionally, the ELISA assay (ELISA: Enzyme-linked immunosorbent assay) was utilized to assess markers of NLRP3 inflammasome, hypertrophy, and fibrosis.

In the in vivo study, Sprague-Dawley (SD) mice underwent a CT procedure (deAlmeida et al., 2010), which generates myocardial hypertrophy in this model. Subsequently, the mice were divided into two groups. The first group underwent CT scans and was administered RG3 at a dose of 30 mg/kg per day for a period of two weeks. The second group served as the control and only underwent the CT procedure, receiving a dose of saline solution equivalent to the RG3 dose administered to the first group.

Following a fourteen-day period, the expression of proteins associated with myocardial hypertrophy and the NLRP3 inflammasome was evaluated, resulting in a lower expression in the TAC+RG3 group compared to the CT group. Additionally, the evaluation of IL-1β and IL-18 levels via ELISA revealed a notable reduction in the TAC+RG3 group. Finally, the expression of SIRT1/NF-κB was evaluated, and elevated levels were observed in the first group. These findings indicate a reduction in the progression of myocardial hypertrophy. The expression of NLRP3, ASC, and caspase 1 induced by ANG-2 was found to be less pronounced in the presence of RG3.

With regard to SIRT1, it was observed that ANG II has the effect of decreasing its expression, thereby promoting the NF-κB signaling pathway. In the presence of RG3, this phenomenon is reversed, with an increase in

SIRT1 and a decrease in the NF-κB pathway. This suggests that RG3 exerts its effect through an increase in SIRT1, which in turn inhibits NF-κB and the expression of Nlrp3 proteins. To substantiate the aforementioned findings, AGK2, a SIRT1 inhibitor, was employed in a group that also received ANG II and RG3. An increase in proteins associated with myocardial fibrosis was observed in the group that received ANG II and RG3, compared to the group that only received ANG II. This suggests that RG3 has a weaker effect, and that SIRT1, which deacetylates lysine 310 of p65, may play an important role in the mechanism of action of RG3. This is supported by the findings of Yang et al. (2022), who demonstrated that SIRT1 has anti-inflammatory effects.

It can be concluded that RG3 has the potential to reduce myocardial hypertrophy by reducing the proteins associated with this pathology and by reducing levels of proteins associated with the NLRP3 inflammasome and IL-1β and IL-18. It is also noteworthy that the study illustrates that RG3 exerts a considerable degree of its action through the regulation of SIRT1. This suggests that RG3 may have a more pronounced impact on signal 1 than on signal 2.

Preclinical Evidence of NLRP3 Signaling Pathway Inhibitory Molecules

In the in vitro study, AC16 and HCM cells were treated with ANG II (ANG II: Angiotensin II) at 200 nM for 24 hours to induce myocardial hypertrophy.The levels of IL-1β and IL-18 were evaluated by ELISA, which revealed a decrease in both when RG3 was applied. To corroborate the aforementioned findings, western blotting was performed to ascertain the signaling cascade, specifically to determine whether the compound acts on any molecule involved in the priming and activation processes of NLRP3, such as cytokines, transcription factors, or second messengers.

Colchicine (COLCOT): Colchicine is an alkaloid compound derived from Colchicum autumnale, which has the ability to bind to tubulin, thereby inhibiting the polymerization of microtubules and preventing mitosis. With regard to the phenomenon of inflammation, it has been demonstrated that this process can be inhibited, and the level of various immune cells can be reduced (Deftereos et al., 2022). The anti-inflammatory effect of colchicine, as demonstrated in the COLCOT (Colchicine Cardiovascular Outcomes Trial), has been well-documented (Wohlford et al., 2020). However, the precise mechanism of action remains unclear. It has been postulated that colchicine exerts its effects by interfering with the assembly and activation of the NLRP3 inflammasome (Martinez et al., 2018). In the context of atherosclerosis, it is of interest to ascertain whether the activation of the NLRP3 inflammasome results in an increase in NLRP3 protein levels within extracellular vesicles. This could potentially lead to the activation of other NLRP3 inflammasomes, thereby establishing a vicious cycle of inflammation. To test this hypothesis, an in vitro study was conducted using THP-1 cell cultures. The NLRP3 inflammasome was activated, and different doses of colchicine were applied to assess the extent of stimulation. It is regrettable that this study yielded unfavorable results. The use of colchicine resulted in a notable reduction in cell viability, which precluded the possibility of conducting the experiment (Silvis et al., 2021).

In a separate study, the potential of colchicine to treat dilated cardiomyopathy was investigated using a mouse model induced with doxorubicin. The results of the comparison between the control group and the group that was given colchicine yielded disparate outcomes. The left ventricular ejection fraction and its shortening exhibited a notable improvement, while the levels of IL-1β, IL-18, and TNF-α demonstrated a reduction. Additionally, the activation of the NLRP3 inflammasome was found to be lower, and SIRT2, which inhibits the NLRP3 inflammasome, was induced (M. He et al., 2020). This study demonstrates, in vitro, that colchicine improves the development of dilated cardiomyopathy by inducing SIRT2, which suppresses the NLRP3 inflammasome. Further investigation is required to determine the potential therapeutic use of this approach (Sun et al., 2022).

Anakinra is a recombinant antagonist of IL-1β (Cvetkovic & Keating, 2002). However, despite extensive research, no published preclinical evidence could be found for this review. One possible reason for this is that it is a relatively new drug. Nevertheless, the identified clinical studies addressing the inhibitory effect of anakinra on IL-1β will be examined later.

Rilonacept (recombinant IL-1β blocker) is a recombinant fusion protein comprising a portion of the IL-1β receptor and an access protein of the IL-1β receptor fused with Fc belonging to IgG1. Its mechanism of action is linked to its binding to IL-1β, which results in the inhibition of chronic inflammatory processes (Rilonacept, 2012).

It has been proposed that this molecule may possess anti-inflammatory properties. However, there are currently no preclinical studies available on this subject. Nevertheless, the subject of clinical studies on this molecule will be addressed subsequently.

Clinical evidence of direct NLRP3 inhibitory molecules or signaling

Dapansutrilo: A study was conducted to evaluate the pharmacodynamic, safety, and tolerability parameters of dapansutrile in patients with heart failure. The study was a phase 1B, randomized, double-blind, dose-escalation

study conducted at a single health center (Wohlford et al., 2021, p. 1). A total of 30 patients were enrolled in the study, all of whom met the following inclusion criteria: age 18 or above, diagnosis of stable heart failure with reduced ejection fraction (HFrEF), and maximum aerobic exercise capacity of 40% or below. The exclusion criteria included pregnancy, autoimmune diseases, and infections. Once the recruitment phase was complete, the subjects were divided into four groups. The first received a dose of 500 mg of dapansutrile, the second 1,000 mg, the third 2,000 mg, and the fourth was the placebo group. This allocation was maintained for the duration of the twenty-eight-day research period. Throughout the course of the research, a variety of parameters were evaluated, including exercise time. The 2000 mg dose demonstrated a notable improvement in this area, while the other doses did not exhibit any discernible change.

Conversely, an evaluation of the left ventricular ejection fraction via echocardiography demonstrated a notable improvement from day 12±2 in all groups receiving dapansutrile, with the 200 mg dose group exhibiting the most pronounced improvement. With regard to adverse reactions, 60% of the subjects experienced at least one adverse reaction, but none of these reactions were serious or life-threatening. In light of these findings, it can be concluded that patients with HFrEF exhibit a favorable tolerance to dapansutrile. However, high doses are necessary to achieve a notable impact on patient outcomes. Consequently, further research involving high doses may be warranted (Wohlford et al., 2021).

ZYIL 1 is a molecule that acts as an inhibitor of the NLRP3 inflammasome and has undergone human clinical trials, including phase 1 and phase 2 studies. The objective of these studies was to evaluate the safety, tolerability, pharmacodynamics, and pharmacokinetics of the drug by administering single and multiple doses to healthy subjects and those suffering from cryopyrin-associated autoinflammatory syndromes (CAPS), respectively (Parmar et al., 2023; Hissaria et al., 2024).

Two separate studies were conducted as part of the Phase 1 study, one examining the safety, tolerability, pharmacodynamics, and pharmacokinetics of ZY-IL1 in healthy individuals aged 18 to 55 years, and the other evaluating the same parameters for single and multiple doses.

The safety and tolerability of the drug were evaluated based on the severity and incidence of adverse effects, as well as through the measurement of vital signs, electrocardiograms, and clinical laboratory parameters. In contrast, the pharmacokinetics were evaluated through the analysis of blood and urine samples. To assess the pharmacodynamic effects, the NLRP3 inflammasome was activated in whole blood following the administration of ZY-IL1, and the levels of IL-1β and IL-18 were quantified using an ELISA assay. Descriptive statistics were employed to determine the percentage of IL-1β inhibition.

The findings demonstrated that the medication was well-tolerated at doses of up to 400 mg as a single dose and up to 100 mg as a multiple dose (administered twice daily for fourteen days). No alterations in vital signs were noted, and a total of eleven adverse events were documented, comprising eight mild, one moderate, and two serious adverse effects. Furthermore, ZY-IL1 was demonstrated to be readily absorbed orally in both single and multiple doses, with excretion occurring primarily in the urine.

In both studies, ZY-IL1 demonstrated the capacity to inhibit over 90% of IL-1β following administration. A single dose of 25, 50, and 100 mg demonstrated inhibition of over 90% of IL-1β for up to twenty-four hours, while the 12.5 mg group exhibited inhibition for up to twelve hours and the 50 and 100 mg groups-maintained inhibition for up to thirty-six hours. It is noteworthy that no inhibition of other proinflammatory cytokines, such as IL-6 and TNF-α, was observed.

In Phase 2 of the study, the same methodology was employed, and the findings were consistent with those observed in the previous phase. Consequently, this molecule could have a substantial impact on cardiovascular therapies due to its ability to be administered orally, a characteristic that is not shared by the majority of the most studied molecules currently, as they are, for the most part, biological products (Parmar et al., 2023; Hissaria et al., 2024).

Anakinra, a recombinant human IL-1β receptor antagonist that has received FDA approval for the treatment of rheumatoid arthritis (Kron et al., 2021), is a promising option for modulating inflammation at both the systemic and cardiac levels in patients at cardiovascular risk, as suggested by the reviewed evidence. This prompts the question of whether this approach could be applied in the context of therapies aimed at this group of patients.

A single study indicates that the inhibition of IL-1β by anakinra results in a notable reduction in white blood cell count, thereby suggesting a decrease in inflammation. A total of 99 patients diagnosed with ST-elevation acute myocardial infarction were enrolled in the study and randomly assigned to either the anakinra treatment group or the placebo group.

The objective was to evaluate the impact of interleukin-1 beta (IL-1β) inhibition via anakinra on white blood cell count. Consequently, a notable reduction in white blood cell and neutrophil count was observed at seventy-two hours in the anakinra cohort in comparison to the placebo group. Furthermore, patients who received

anakinra and experienced heart failure-related clinical events exhibited a notable decline in white blood cell and neutrophil count at seventy-two hours, in comparison to those who received anakinra and did not experience heart failure-related clinical events at follow-up. Similarly, patients who received anakinra and experienced heart failure-related adverse events also exhibited a significant reduction in white blood cell percentage and absolute neutrophil count at fourteen days compared to those who received anakinra and had no heart failure-related adverse events at follow-up.

In conclusion, the observed benefit in patients evaluated in this study may be extended to those suffering from diseases characterized by chronic inflammation, which in turn contributes to the development of cardiovascular diseases (Del Buono et al., 2022).

Rilonacept: The fusion protein rilonacept has been evaluated as a potential therapeutic agent for pericarditis, a common inflammatory disorder of the heart (Rilonacept, 2012). A phase 2 study enrolled individuals diagnosed with recurrent pericarditis who were undergoing corticosteroid therapy. The participants were divided into five groups based on the underlying cause and C-reactive protein levels (Klein, Lin et al., 2021).

On the initial day of the study, patients were administered a loading dose of 320 mg of rilonacept subcutaneously, followed by maintenance doses of 160 mg for a period of five weeks. The concomitant use of nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids was permitted. The assessment of pain and inflammation demonstrated a notable reduction, with an average decrease of approximately four points on the eleven-point numerical pain scale. Moreover, the administration of prednisone was diminished in the majority of patients. The treatment was generally well tolerated, although there was one instance of a serious adverse reaction.

In a phase 3 study comprising 86 patients who experienced a second recurrence of pericarditis (Klein, Imazio et al., 2021, p. 3), rilonacept was administered for twelve weeks following a one-week loading phase. Thereafter, the stability of these patients was observed for nine weeks, and concomitant therapies were discontinued for a further two weeks. Subsequently, 61 patients who had exhibited a clinical response were randomly assigned to receive rilonacept as monotherapy or a placebo to assess its efficacy. To evaluate the efficacy of the treatment, the pain scale and C-reactive protein levels were measured. The results demonstrated a rapid and significant decrease in both the pain scale and C-reactive protein levels, as well as a notable reduction in the recurrence of pericarditis in the rilonacept group compared to the placebo group.

In conclusion, both studies demonstrated that rilonacept is an effective treatment for reducing pain and inflammation associated with recurrent pericarditis. These findings suggest that this pharmacological agent may have significant potential for the treatment of inflammation in this clinical context.

Canakinumab is a human monoclonal antibody that selectively binds to and inhibits the action of the proinflammatory cytokine interleukin-1 beta (IL-1β). The interleukin-1β inhibitor antibody was the subject of study in the study entitled "Results of Anti-inflammatory Thrombosis with Canakinumab" (De Benedetti et al., 2018). The clinical trial, which spanned approximately three years and eight months, enrolled 10,611 patients with a history of myocardial infarction and high-sensitivity C-reactive protein levels exceeding 2 mg/L. The patients were administered 50, 150, and 30 mg of canakinumab.

The effects of canakinumab on the incidence of serious cardiovascular events, including acute myocardial infarction, unstable angina, and coronary revascularization, were assessed. It is important to note that all events were accounted for, including those that occurred on the same day. The results demonstrate that therapy with canakinumab is associated with a notable reduction in the risk of experiencing the initial episode of a serious cardiovascular event. Furthermore, the therapy has been demonstrated to limit the overall incidence of cardiovascular events and, in general, to decrease the rates of serious cardiovascular events in both male and female populations, as a result of IL-1β inhibition (Everett et al., 2020). Additional research that adopted a similar experimental design has shown that the use of canakinumab leads to a significant decrease in the incidence of anemia, as reflected in the increase in hemoglobin levels. Nevertheless, an elevated risk of infections has been documented, and a correlation has been identified between cases of thrombocytopenia and neutropenia (Vallurupalli et al., 2020). One disadvantage of this therapy is that it has been demonstrated to be non-cost-effective(Sehested et al., 2019).

Table 2. Categorization of the molecules examined into their mode of action (direct or indirect), the type of evidence accumulated (pre-clinical or clinical), and the synthesis of the mechanism of action together with their biological effect.

EVIDENCE	DIRECT				INDIRECT			
	MOLECULE	**MECHANISMS OF ACTION**	**BIOLOGICAL EFFECT**	**REF**	**MOLECULE**	**MECHANISMS OF ACTION**	**BIOLOGICAL EFFECT**	**REF**
PRECLINICAL	Dapansutril	Reduces ATPase activity of Nlrp3 protein which inhibits ASC recruitment and prevents NLRP3 inflammasome oligomerization.	Improves preservation of cardiac contractility. Improves diastolic function of the heart	(33)	Colchicine	Regarding NLRP3, its mechanism has not been elucidated, it has been related to inhibit the assembly and inhibition of the inflammasome.	Induction of SIRT2 has been shown to improve the symptoms of dilated cardiomyopathy.	(58)
	MCCC950	Inhibits NLRP3 inflammasome formation by interacting with the Walker B motif of NACHT	Reduction of atheromatous plaque Cardioprotective effect in myocardium damaged by DOX.	(35)				
	Oridonin	Reversible inhibition of Nlrp3 through the formation of an x-covalent bond.	Reduction of myocardial fibrosis and preservation of cardiac function	(47)				
	RG3	Inhibits via NF-kB signaling and pro-c expression, Interruption of NEK7-NLRP3 preventing oligomerization of the inflammasome.	Reduces NLRP3 and interleukin inflammasome-associated proteins.	(51)				
CLINICAL	Dapansutril	Reduces ATPase activity of Nlrp3 protein which inhibits ASC recruitment and prevents NLRP3 inflammasome oligomerization.	Improves exercise time and left ventricular ejection fraction in heart failure.	(33)	Canakinumab	IL-1β inhibitory antibody	Decreased rates of severe cardiovascular events in both men and women, attributed to IL-1β inhibition.	(67)
					Anakinra	Recombinant IL-1β antagonist.	A marked reduction in the leukocyte Recount,	(64)

							indicative of a decrease in inflammation, and in clinical events associated with heart failure.	
	ZYIL 1	Not described	Inhibition of more than 90% of IL-1β after administration.	(61)	Rilonacept	Binds to IL-1, blocking it and preventing inflammation.	Reduction of the scale of pain and inflammation in recurrent pericarditis.	(66)

Projections

The objective of this review is to present an updated perspective on the involvement of the NLRP3 inflammasome in chronic inflammation associated with cardiovascular diseases, emphasizing its potential as a pharmacological target. Furthermore, it aims to demonstrate that molecules currently under investigation have therapeutic potential in the context of cardiovascular diseases, suggesting that, in the future, they may represent an innovative alternative to conventional pharmacological therapies.

Furthermore, the objective is to identify deficiencies in current knowledge and areas that require further investigation, particularly with regard to the inflammasome and its mechanism of action, as well as to new molecules, whether directly or indirectly acting. Furthermore, the objective is to underscore the clinical relevance of this mechanism in the pathogenesis of specific diseases, which could inform the approach and treatment of these diseases by health professionals. It is anticipated that the review will have a considerable impact on both research and clinical practice, contributing to the advancement of knowledge and enhancing the range of therapeutic options available for cardiovascular disease.

Conclusion

The molecules currently under investigation have demonstrated the capacity to significantly ameliorate symptoms associated with cardiovascular disease by influencing priming or activating the NLRP3 inflammasome. Nevertheless, further investigation of specific molecules is necessary to fully elucidate their potential therapeutic benefits.

The majority of investigational therapies approved for cardiovascular diseases are oriented towards the inhibition of IL-1β by antibodies, which are biological products susceptible to fragilities and various technological limitations. In light of these considerations, the development of molecules such as dapansutrile, MCC950, ZYIL1, and Ori represents a promising strategy.

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(The article must conclude with a list of the documents and books cited in the text that must not exceed 40 references and must use the format described in https://arsmedica.cl/index.php/MED/about/submissions#authorGuidelines)

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