# Evaluation of lesinurad-induced cardiotoxicity in cardiomyoblastic cells

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**ABSTRACT**: Lesinurad, a uric acid transporter-1 (URAT1) inhibitor, is a uricosuric medicine and administered in combination therapy with a xanthine oxidase inhibitör (XOI) when the targeted serum uric acid level with XOIs cannot be reached in the treatment of gout. The cardiovascular adverse effects related to the use of lesinurad have been reported by authorities, but the underlying causes have not been elucidated yet. Therefore, to investigate the cardiotoxic potential and its mechanisms, oxidative stress, apoptosis, and troponin I/T protein expressions that are important in cardiac functions were evaluated using the rat cardiomyoblast (H9c2) cell model after lesinurad treatment (0.625-0.5 M) for 24 h. The half-maximal inhibitory concentration ($IC_{50}$) was calculated to be 0.84 M. Necrotic cell death was induced at 0.125-0.5 M doses and glutathione (GSH) was depleted at all studied concentrations. The ROS levels did not show significant change after lesinurad treatment, which may be a consequence of reactive oxygen species (ROS) scavenging activity of GSH. The protein expressions of troponin T and troponin I slightly decreased at the highest concentration. According to the findings, lesinurad-induced cardiotoxicity might be associated with oxidative stress and necrotic cell death. However, further detailed mechanistic studies are needed to clarify the lesinurad-induced cardiotoxic effects.

**KEYWORDS**: Lesinurad-1; URAT-1 inhibitors-2; gout-3; cardiotoxicity-4.

## 1. INTRODUCTION

Gout is a metabolic disease characterized by recurrent acute arthritic attacks resulting from the accumulation of monosodium urate crystals in the joint and surrounding tissues due to hyperuricemia [1]. The most important risk factor for gout is serum urate level. Hyperuricemia results from increased uric acid production or decreased uric acid excretion or both mechanisms [2,3]. The drugs used in the treatment of hyperuricemia are divided into three categories: (i) decreasing uric acid production (xanthine oxidase inhibitors, XOIs), (ii) increasing uric acid excretion (uricosurics), and (iii) uricases, which allow uric acid to be excreted by enzymatic means [4].

Lesinurad, a uric acid transporter-1 inhibitor (URAT-1), is approved for the treatment of hyperuricemia in gout patients by the Food and Drug Administration (FDA)in 2015 and by the European Medicines Agency (EMA) in 2016. URAT-1 transporter plays important role in the reabsorption of uric acid from the proximal tubule thereby decreasing plasma uric acid levels. Lesinurad is indicated for gout patients, when the target serum uric acid values are not achieved with xanthine oxidase inhibitors, 200 mg combination therapy with a xanthine oxidase inhibitor once a day is administered [5,6]. Lesinurad treatment is associated with adverse effects such as increasing in creatinine level, reversible nephrolithiasis, urolithiasis. Renal failure cases have also been reported if the daily dose of lesinurad exceeds 400 mg. Lesinurad-induced cardiovascular adverse effects have been reported in Phase III studies [7,8,9]. Moreover, FDA warned the patients about major cardiovascular adverse effects defines as cardiovascular deaths, non-fatal myocardial infarctions, or non-fatal strokes [5]. The causal relationships between cardiotoxicity and lesinurad have not been known well. Oxidative stress and cardiomyocte death are known to result in cardiotoxicity. Therefore, it was evaluated the cardiotoxic potential of lesinurad on the rat cardiomyoblast (H9c2) cell line focusing on oxidative stress and apoptosis. Troponin T and I protein expression levels were investigated after lesinurad treatment. The H9c2 cell line, from embryonic rat heart tissue, is widely used in vitro studies due to its morphological, biochemical and electrophysiological properties similar to cardiac myocytes [10].

## 2. RESULTS AND DISCUSSION

Lesinurad, approved by the FDA in 2015 and by EMA in 2016, is a uricosuric used for the treatment of hyperuricemia in chronic gout patients. There are clinical studies showing cardiotoxic adverse effects after treatment with lesinurad [6-8]. However, the underlying mechanisms of cardiotoxic effects of lesinurad have not been fully elucidated. In the present study, $IC_{50}$ value was determined as 0.845 M following 24 h lesinurad treatment (Figure 1).
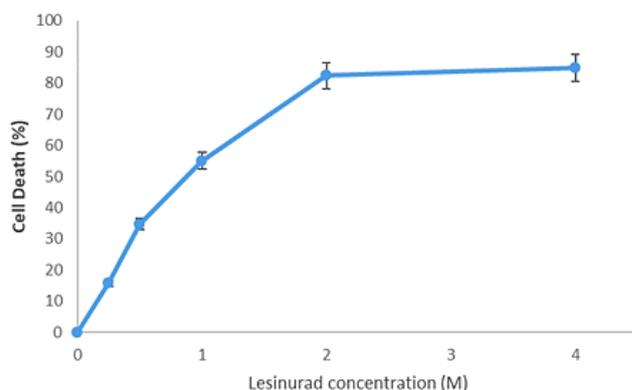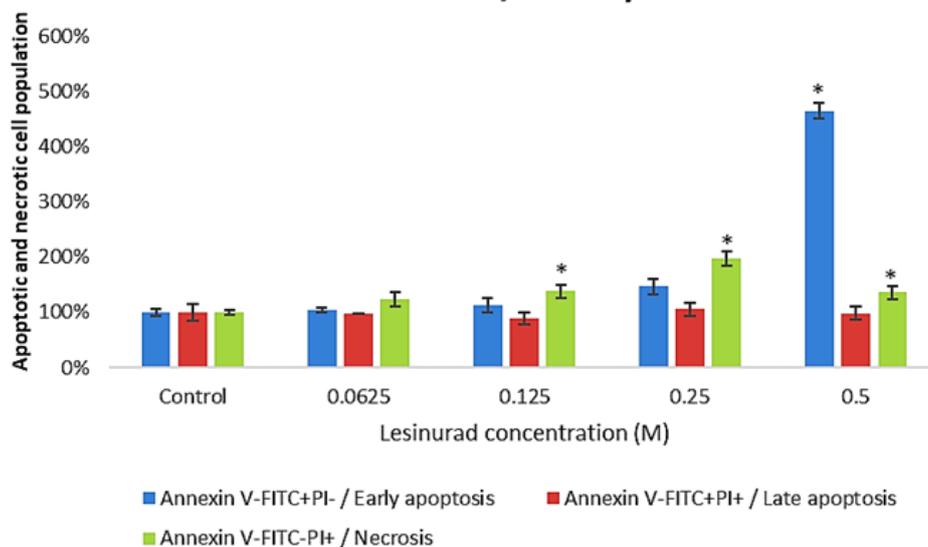


**Figure 1:** The effects of lesinurad on cell viability. Data were expressed as mean ±SD.

Apoptosis in cardiomyocytes occurs in various clinical-pathological conditions such as myocardial infarction, congestive heart failure and transplant rejection [11,12]. Also, apoptosis may play an important role in congestive heart failure, which is an important cause of cardiovascular-based hospitalizations [13]. Olivetti et al. reported that the condition accompanying congestive heart failure may be associated with apoptosis and necrosis [14]. In the present study, it was detected that early apoptosis was significantly induced following 0.5 M lesinurad treatment compared to the control ($p<0.05$) (Figure 2), whereas there was no significant difference between the treatment concentrations in late apoptosis. Necrotic cell death was significantly increased after ≥0.125 M lesinurad treatments comparing with the control group (Figure 2). It has been shown that necrotic cell death plays a role in drug-induced cardiotoxicity with in vitro and in vivo experimental models [15,16].
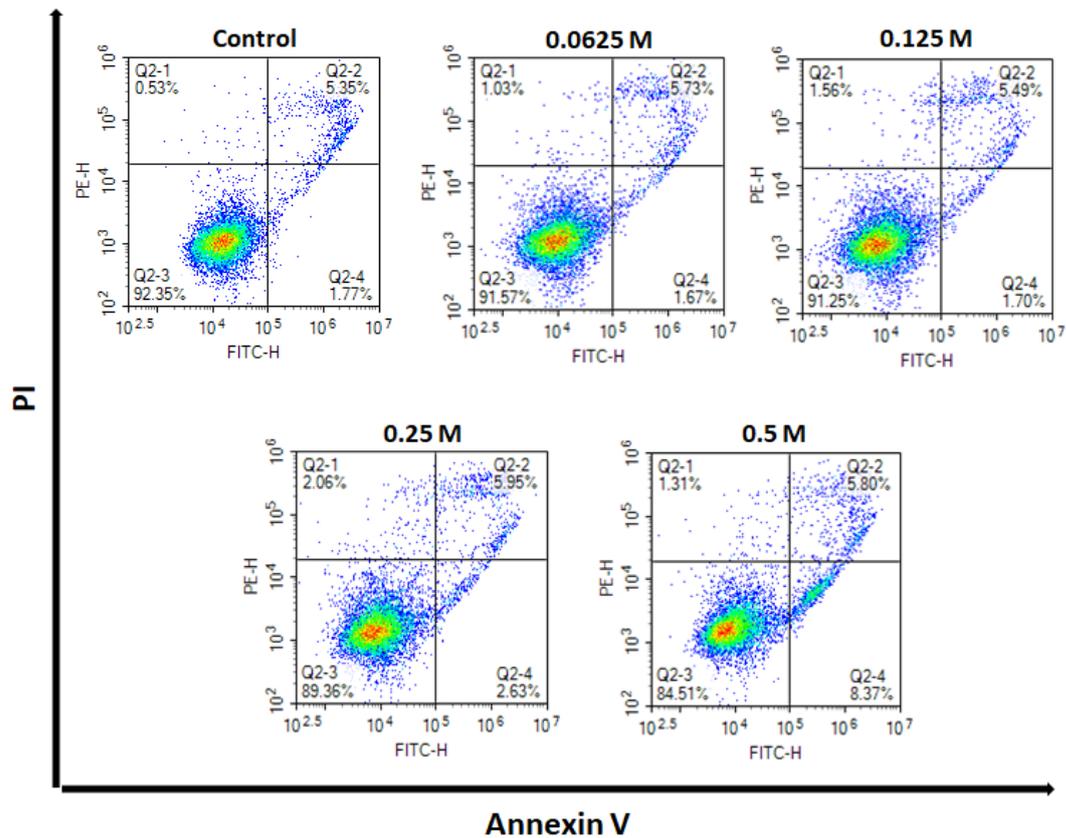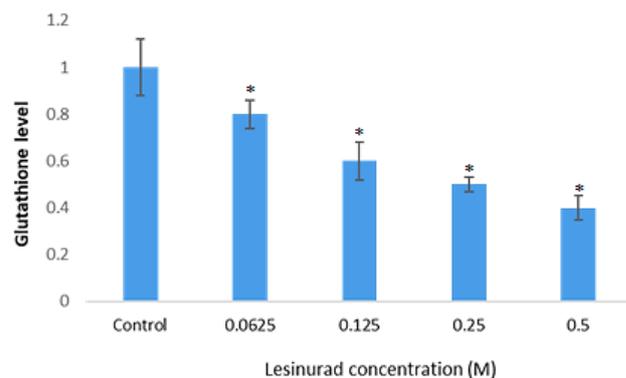
**Figure 2**: The effects of lesinurad on apoptosis and necrosis in H9c2 cell line. Data were expressed as mean ±SD. *$p<0.05$ vs the control group. Percentage of apoptotic/necrotic cells in treatment groups were normalized to the control group.

The relationship between cardiovascular disease and oxidative damage has been established in numerous in vivo and clinical studies. Oxidative damage is known to underlie in many cardiac disorders including myocardial hypertrophy, contractile dysfunction, development of cardiac fibrosis and endothelial dysfunction [17,18]. Indeed, oxidative damage plays an important role in drug-induced cardiotoxicity [19]. GSH is an important indicator of cellular redox potential and the activity of cell death pathways. GSH depletion is generally considered a marker of oxidative damage caused by reactive oxygen and nitrogen derivatives [20]. In our results, it was detected a dose-dependent significant reduction in the GSH levels compared to the control at the concentration range of 0.0625-0.5 M ($p<0.05$) (Figure 3). However, it was not

observed any significant difference compared to control in the ROS at same concentration range ($p<0.05$) (Figure 4) GSH depletion might be a consequence of ROS scavenging in the cardiomyoblastic cells.



**Figure 3:** The effects of lesinurad on the GSH level in H9c2 cell line. Data are expressed as mean ±SD.*$p<0.05$ vs control group. Amount of glutathione level in per mg protein in treatment groups were normalized to the control group.
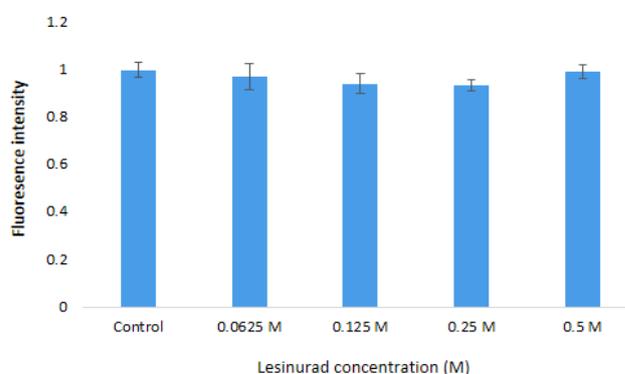


**Figure 4:** The Eevaluation of ROS production after lesinurad exposure in H9c2 cell line by H2DCFDA. Data are expressed as mean ±SD. The mean fluoresence intensity of treatment groups was normalized to the control group.

cTnI and cTnT are regulatory proteins that play important role for contractility of the heart [21]. The plasma cTnI and cTnT levels are important markers with high sensitivity and specificity used in clinical and pre-clinical studies to monitor potential drug-induced myocardial injury. cTnT plays structural role as it may bind to other troponin complements. In clinical studies, abnormally high serum concentrations of cTnT and cTnI have been reported to increase 4-5 fold the risk of acute myocardial infarction. Therefore, it has been suggested that cardiac troponins are very useful for stratification of patients at high risk for adverse effects and for selecting patients with acute coronary syndrome [22,23]. It has been shown that reduced cTnT expression in the heart might be associated with cardiac damage [24,25]. Similarly, the cTnI protein expression has been found to decrease following doxorubicin treatment in human-induced pluripotent stem cell-derived cardiomyocytes [26]. According to our results, at 0.5 M concentration, it was detected 1.39 and 1.14-fold reduction compared to the control in the cTnI and cTnT levels, respectively (**Figure 5**). When the data were evaluated, any statistically significant change was found between the control and treatment concentrations ($p<0.05$).
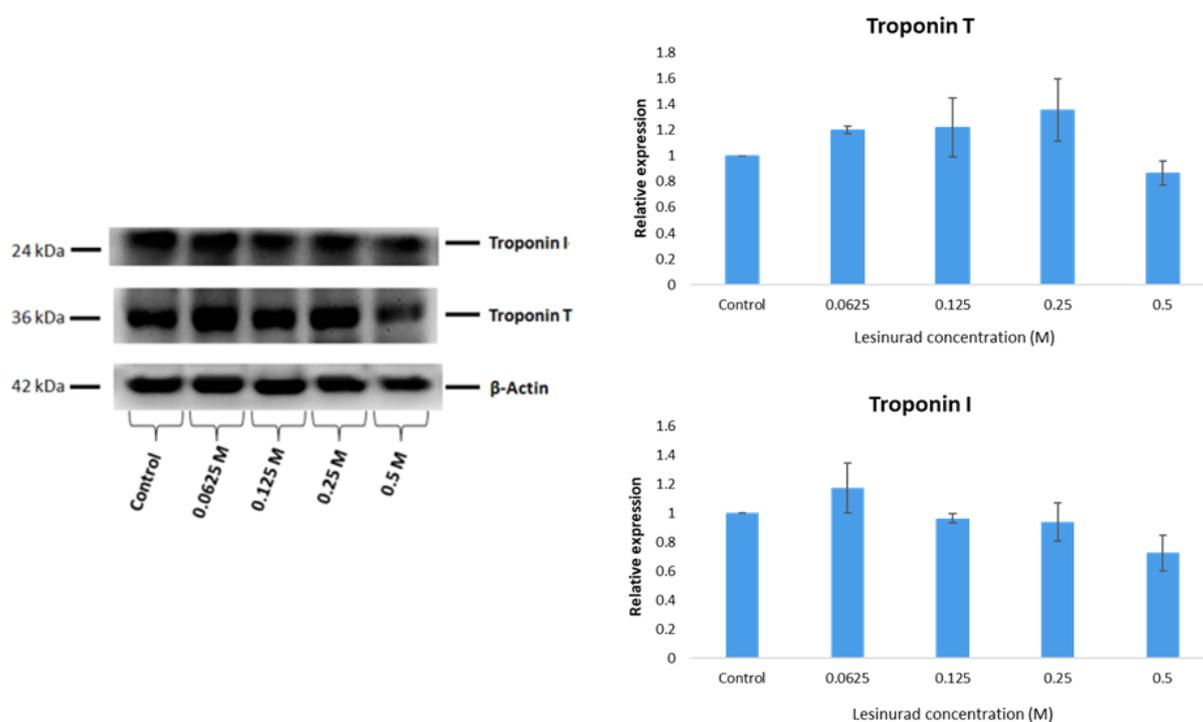
**Figure 5:** The expression levels of cTnI and cTnT after lesinurad exposure in H9c2 cell line by western blot technique. Data are expressed as mean ±SD. Protein expression of treatment groups were normalize to the control group.

## 3. CONCLUSION

In conclusion, the present study is a preliminary investigation of the relationship between lesinurad and cardiovascular toxicity. Lesinurad impaired cardiac cell viability, causing GSH loss and necrotic cell death. It also slightly decreased cTnT and cTnI protein expression on cardiac myocytes at the highest concentration. However, further studies should be conducted on the toxic effects of lesinurad in the cardiovascular system, such as effects on mechanical function of cardiac muscle, morphological damage, and changes in vascular system function.

## 4. MATERIALS AND METHODS

The material and chemicals were provided by Wisent Multicell (Quebec, Canada). Lesinurad, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and 2′,7′-dichloro-dihydro-fluorescein diacetate (H2DCFDA) were by Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kit for the detection of GSH was by Elabscience (Wuhan, China). FITC Annexin V Apoptosis Detection Kit with PI were from Biolegend (San Diego, CA) and the primer antibodies for detection of troponin I (cTnI, Cat. No STJ96727-100) and troponin T (cTnT, Cat. No STJ96114-100) were from St. John's Laboratory (London, UK), the primer antibody for β-actin (Cat. No SC-47778) was from Santa Cruz Biotechnology (Heidelberg, Germany), the secondary antibodies were from Abcam (Cambridge, UK) and protein ladder was from Thermo Fisher Scientific (Life Techonologies Ltd., UK).

The rat cardiomyoblast cell line (ATCC-CRL 1446, H9c2) provided by the American Type Culture Collection (ATCC, USA) were used. Dulbecco's Modified Eagle's Medium/Nutrient F-12 (DMEM/F12) (with 10% heat-inactivated fetal bovine serum and 1% antibiotics) were used for cell cultured and the cells were incubated at 37 °C. The treatment concentrations were in the range of 0.0625-0.5 M for 24 h. It was used 105 cells/mL for cytotoxicity assay, $2.5 \times 10^5$ cells/mL for GSH detection, $10^5$ cells/mL for flow cytometry assays and $2.5 \times 10^5$ cells/mL for protein isolation assays. The control group was exposed to DMSO (1%) containing cell culture medium.

MTT assay were used to evaluate the cytotoxic effect of lesinurad. The cell viability was examined with MTT dye after treatment with the range of 0-4 M concentrations. Following 24 h treatment, MTT dye was added to each well and incubated for 2 h in standard culture conditions. Purple-colored formazan crystals was dissolved in DMSO. A microplate spectrophotometer system (Epoch, Germany) was used to

measure the optical densities (ODs) at 590 nm. The concentration-cell death (%) curves were used to calculate the half-maximal inhibition concentration (IC50) responsible for the death of 50% of cells.H2DCFDA dye was used for the evaluation of ROS induction. . At the end of 24 h expoure time, cells were collected by trypsinization and washed with PBS. Then the cells were incubated with 20 μM H2DCFDA dye for 30 min. Following washing steps fluoresence intensity was measured with flow cytometry (Acea Novocyte, California, USA) at Ex/Em: 485/535 nm. The results were analysed using Novocyte soft-ware. Median Fluoresence Intensity (MFI) level of treatment groups were normalized to control group. FITC Annexin V Apoptosis Detection Kit with PI were used for the identification early and late apoptotic and necrotic cells PI following the manufacturer's guideline. After trypsinization and washing steps, the cells were incubated with annexin V and PI dyes at room temperature for 15 min. Then, fluoresence intensity was read using Acea flowcyotometry at Ex/Em: 494/519 nm for Annexin V-FITC and at Ex/Em: 535/617 nm. The GSH levels were assessed using ELISA kit (Elabscience E-EL-0026) according to the manufacturer's instructions. The OD values were measured at 450 nm using a microplate spectrophotometer. The results were calculated from the curves of the standard supplied by the kits. The GSH levels were normalized to protein amount of each sample, and then normalized to control group. The levels of cTnI and cTnT proteins were determined by western blot technique using same amount of protein. Protein isolation was performed with RIPA lysis buffer system and the protein levels were measured by Bradford method [27]. Equal amount of protein samples were separated with SDS-PAGE electrophoresis and then the proteins were blotted to the PVDF membrane. After blocking (5% non-fat dry milk) and washing (tris buffered saline with tween 20), membrane was incubated with cTnI and cTnT in 1:500 dilutions. β-actin (1:1000 dilution) was used as a reference protein in the evaluation. The results were quantified using Image J soft-ware programme. The results were shown after normalization to the control group.

All results were normalized to the control group. The statistical analysis was performed one-way analysis of variance (ANOVA) (Post Hoc- Tukey) using SPSS v.20. Data were expressed as mean ± standard deviation (SD). The values of $p<0.05$ were considered statistically significant.

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