ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

DOI: 10.5336/cardiosci.2017-55134

Cardioprotection Potential of Some Hydroxypyridine Iron Chelators Against H_2O_2-Induced H9C2 Cell Injury

H9C2 Hücrelerinde H_2O_2'nin Oluşturduğu Hasarlara Karşı Bazı Hidroksipiridin Demir Bağlayıcıların Potansiyel Kardiyoprotektif Etkileri

Mohaddeseh BEHJATI,[a]
Afshin FASSIHI,[b]
Mehrdad Mohammadpour DEHKORDI,[b]
Mahtab KESHVARI[a]

[a]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, [b]Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. IRAN

Geliş Tarihi/*Received:* 07.02.2017
Kabul Tarihi/*Accepted:* 09.05.2017

Yazışma Adresi/*Correspondence:*
Mohaddeseh BEHJATI
Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, IRAN
behjati@med.mui.ac.ir

ABSTRACT Objective: Iron-induced cardiotoxicity serves as the main cause of heart failure in young adults and is formidable in many patients. Intracellular iron chelation either by restoring the disturbed cellular iron homeostasis or by removing redox-active iron is the only well-established strategy for cardioprotection. We aimed to evaluate the cardioprotective role of some hydroxypyridine derivative iron chelators on rat myoblast cell line. **Material and Methods:** Five different derivatives of the 3-hydroxypyridine-4-one scaffold were prepared according a previously reported study. Compounds (10-100 μM) or L1 as standard iron chelator (30-100 μM) dissolved in dimethyl sulfoxide and applied to dulbecco's minimal essential medium (DMEM) culture medium 1 h prior and during H_2O_2 (100 μM) exposure. H9C2 cells were exposed with FeSO4 (3–30 μM). **Results:** Cell survival studies were evaluated through lactate dehydrogenase (LDH) leakage and Annexin-V/ propidium iodide counter staining. Application of iron chelators in the culture media of injured H9C2 cells resulted in non-significant decrease in the amounts of released LDH. Indeed, these compounds were not associated with significant induction of cell death through apoptosis, necrosis or both. **Conclusion:** The present study demonstrates that these hydroxypyridine derivatives have no effect on nullifying H_2O_2-induced oxidative stress on in vitro model of cardiac injury.

Keywords: Iron chelating agents; myoblasts, cardiac; cells, cultured

ÖZET Amaç: Genç bireylerde kalp yetmezliğinin ana nedeni olan demir-nedenli kardiyotoksite, birçok hastada yönetilebilir bir durumdur. Kalbin korunması için, hasarlı hücrelerde demir homeostazını sağlamak üzere hem intraselüler demir şelasyonu düzenlenmeli hem de redoks-aktif demirin uzaklaştırılması en iyi stratejidir. Biz, rat miyoblast hücre hattında bazı hidroksipiridin türevi demir bağlayıcıların kardiyoprotektif etkilerini inceledik. **Gereç ve Yöntemler:** Bir önceki çalışmamızda bildirdiğimiz şekilde hazırladığımız, 3-hidroksipiridin-4-one çatı molekülünün 5 farklı türevlerini kullandık. 10-100 μM bileşikleri veya L1 standart demir bağlayıcılar (30-100 μM), dimetil sülfoksit içinde çözündürülüp, dulbecco minimum esansiyel vasat (DMEM) kültür kabına, H_2O_2 (100 μM) ile maruziyet sırasında ve maruziyetten 1 saat önce eklendi. H9C2 hücreleri $FeSO_4$ (3-30 μM) ile muamele edildi. **Bulgular:** Hücre sağkalım çalışmaları laktat dehidrogenaz (LDH) salınımı ve Anneksin-V/propidium iyot boyanma sayımı üzerinden değerlendirildi. Hasarlı H9C2 hücreleri içeren kültür ortamına demir bağlayıcıların eklenmesi LDH salınım miktarlarında önemli olmayan azalma sağladı. Gerçekte bu bileşikler apoptozis, nekrozis veya her ikisi üzerinden hücre ölümünün önemli şekilde indüklenmesi ile bağlantılı değildi. **Sonuç:** Çalışmamızın sonuçlarına göre, bu hidroksipiridin türevleri, H_2O_2'nin hasar verdiği, oksidatif strese maruz kalmış; kardiyak hasarın in vitro modelinde etkili değildirler.

Anahtar Kelimeler: Demir bağlayıcı edici ajanlar; miyoblastlar, kardiyak; hücreler, kültüre edilmiş

Iron is an essential transition metal for normal cellular function, general health, cellular metabolism and enzyme function.[1] In this sense, the quantity of iron in the body is tightly regulated; to avoid the toxic effects of excess total body iron. Hence, humans have no mechanisms for elimination of the excess iron; its cumulative overload in diverse disease conditions inevitably results in progressive damage to many organs, like the heart.[2.] Deregulation of localized iron homeostasis is implicated in the existence of labile form of iron which is so called Non-Transferrin bound iron (NTBI).[3] Represented NTBI comprise labile plasma iron pool (LIP) and labile cellular iron pool.[4] Today's a merit candidate iron chelator should potently target the labile cellular iron pool as well as LPI.

Accumulated NTBI over time compromises organ functions and patient survival. These catalytically active components overwhelm the normal regulatory cellular capacity for iron and are capable of initiating free radical reactions. In this way, it accelerates damage to proteins, DNA (especially mitochondrial DNA); with subsequent loss of normal cellular function which manifest as abnormal myocyte contractility.[5] In partial support of this idea earlier studies have demonstrated the beneficial effects in term of cardioprotection by reducing iron-evoked formation of reactive oxygen species (ROS) and restoration of cardiomyocyte contractility via application of iron chelators.[6]

In hand, the general toxic effects of catalytic iron are linked to the oxidative stress through Fenton reaction, in which the oxidized less harmful H2O2 and superoxides in the presence of iron promotes their auto-oxidation and generation of extremely toxic hydroxyl radicals.[7.]These Fenton products, in turn, initiate myocardial membrane lipid peroxidation which progress toward cell death via a cascade of signal transduction pathways.[8] The significant vulnerability of cardiomyocytes to ROS is explainable by the presence of a relatively poor antioxidant defense system and a limited regenerative capacity as a post replicative cell type.[9,10] Indeed, these limited well-tuned scavenger mecha nisms in order to perturb oxygen radical mediated damages, are surpassed by many disease conditions. Therefore, the main venue of cardiop rotection interventions was focused on the ROS scavengers.[11-13] But their limited efficacy lend scientist to speculate that prevention of ROS formation is superior to scavenging the already formed ROS or bolstering of the cellular antioxidant effects.

Iron-induced cardiotoxicity serves as the main cause of heart failure in young adults and is formidable in many patients by intensive iron chelating therapies, either by restoring the disturbed cellular iron homeostasis or by removing redox-active iron.[14,15] Therefore, the research focus in appealed in the design and promotion of candidate iron chelators. By now there are many compounds evaluated both *in vitro* and *in vivo* in order to determine their cardioprotective effects but due to the failure of such achievement the search line is ongoing.[16,17] In our previous work we have determined the cardioprotective role of two novel hydroxypyridine derivative iron chelators on rat myoblast cell line. These two novel iron chelators were effective cardioprotective agents. Thus, we have decided to evaluate the efficacy of further hydroxypyridine derivative iron chelators. Therefore, we have synthesized further hydroxypyridine derivative iron chelators based on the concept of previous efficacy. Thus, the aim of this study is to evaluate the cardioprotective role of some hydroxypyridine derivative iron chelators on rat myoblast cell line.

MATERIAL AND METHODS

CHEMISTRY

All chemicals used for the synthesis were purchased from Merck (Germany) or Fluka. Melting points were determined on a Mettler capillary melting point apparatus and were uncorrected. The FTIR spectra were recorded with a WQF-510 Ratio Recording FTIR spectrometer as a KBr disc (n, cm-1) (t). The 1H-NMR spectra (dimethyl sulfoxide (DMSO)-d6) were recorded on a Bruker 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). The purity of the compounds

was checked by thin layer chromatography (TLC) on silica gel plate using chloroform and methanol. The synthesis pathway followed for the preparation method for the compounds are reported previously.[18] Chemistry, antioxidant evaluation, hydrogen peroxide scavenging assay, iron chelating ability and DPPH free radical scavenging assay of these compounds are resented in the above mentioned article.[18,19]

CELL LINE AND CELL CULTURE

H9C2 cells derived from fetal rat hearts were purchased from National Cell bank of Iran (NCBI). This immortalized cell line at a density of 105/cm^2 were cultured as monolayers in dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% streptomycin and penicillin (Invitrogen). Culture medium was replaced every 2 days by fresh medium. Cells were counted by invert microscopy using improved hemo-cytometer neobar. For each evaluation, all experiments were run in triplicate.

PHARMACOLOGY

H9C2 cells (cell line) at 70% confluency were used in this study. Serum containing medium was replaced with freshly prepared ADS buffer (5.3 mM KCl, 1 mM $CaCl_2$, 116 mM NaCl, 1.13 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose and 25 mM HEPES, pH 7.40). Synthetized iron chelator compounds (10-100 μM) or L1 as standard iron chelator (30-100 μM) were dissolved in DMSO (0.01% v/v) and applied to DMEM culture medium 1 h prior and during H_2O_2 (100 μM) exposure. H9C2 cells (cell line) were exposed with fresh $FeSO_4$ (3-30 μM). Lactate dehydrogenase (LDH) leakage into the culture media was measured after 24 h. Prevention of LDH leakage has been used as a method for demonstration of cardioprotection.[10]

CELL SURVİVAL ASSAYS

Cell survival studies were evaluated in cells treated for 24 h with 100 μM of compounds or L1 together with H2O2 (200 μM) and challenged with FeSO4 (30 μM). For this purpose, Annexin-V/ propidium iodide (PI) counter staining was used. Cell survival assay is used as a method for demonstration of cardioprotection.[20] This technique provides a convenient and rapid assay for detection of apoptosis using recombinant Annexin V conjugated fluorescein (FITC-Annexin) with ready-to-use red-florescent PI nucleic binding dye. PI stains dead cells by binding tightly to cellular nucleic acid. Following counter staining, cells in binding buffer green florescent indicates apoptotic cells while red and green florescent implies to dead cells. Little or no florescence is detected from live cells.

RESULTS

CHEMİSTRY

(E)-5-(benzyloxy)-2-((pyridine-4-ylimi nomethyl) pyridin-4 (1H)-one (a), (E)-5-(benzyloxy) 4-oxo-1, 4-dihydropyridin-2- carbaldeh ydeoxime(b), (E)-5-hydroxy-2- ((4methoxyphen ylmino) methyl)-1-methylpyridin-4(1H)-one(c),(E)-1-hydroxy-((5-hydroxy-4-oxo-1,4-dihydropyridin-2-yl) methylene) thiosemicarbazide (d), (E)-5-hydrox yl-1-methyl-4-oxo-1,4- dihydropyridine-2-carbal dehyde oxime (e) compounds have been used in this study (Figure 1). Spectral data elucidating the structural detailsof these novel synthesized compounds were all in accordance with the previously reported data.[18]

PHARMACOLOGY

LDH leakage

Application of iron chelators in the culture media of injured H9C2 cells (cell line) resulted in non-significant decrease in the amounts of released LDH ($P>0.05$). The data of LDH release amounts are presented in Figure 2.

Cell survival

Cell survey assay using flow cytometry analysis demonstrates that there was a non-significant difference in induction of death through apoptosis or necrosis in cells by application of these agents ($P>0.05$). The data of cell survival assays are depicted in Figure 3.

DISCUSSION

This study compared the efficacy of some of the Hydroxypyridine derivative iron chelators on the

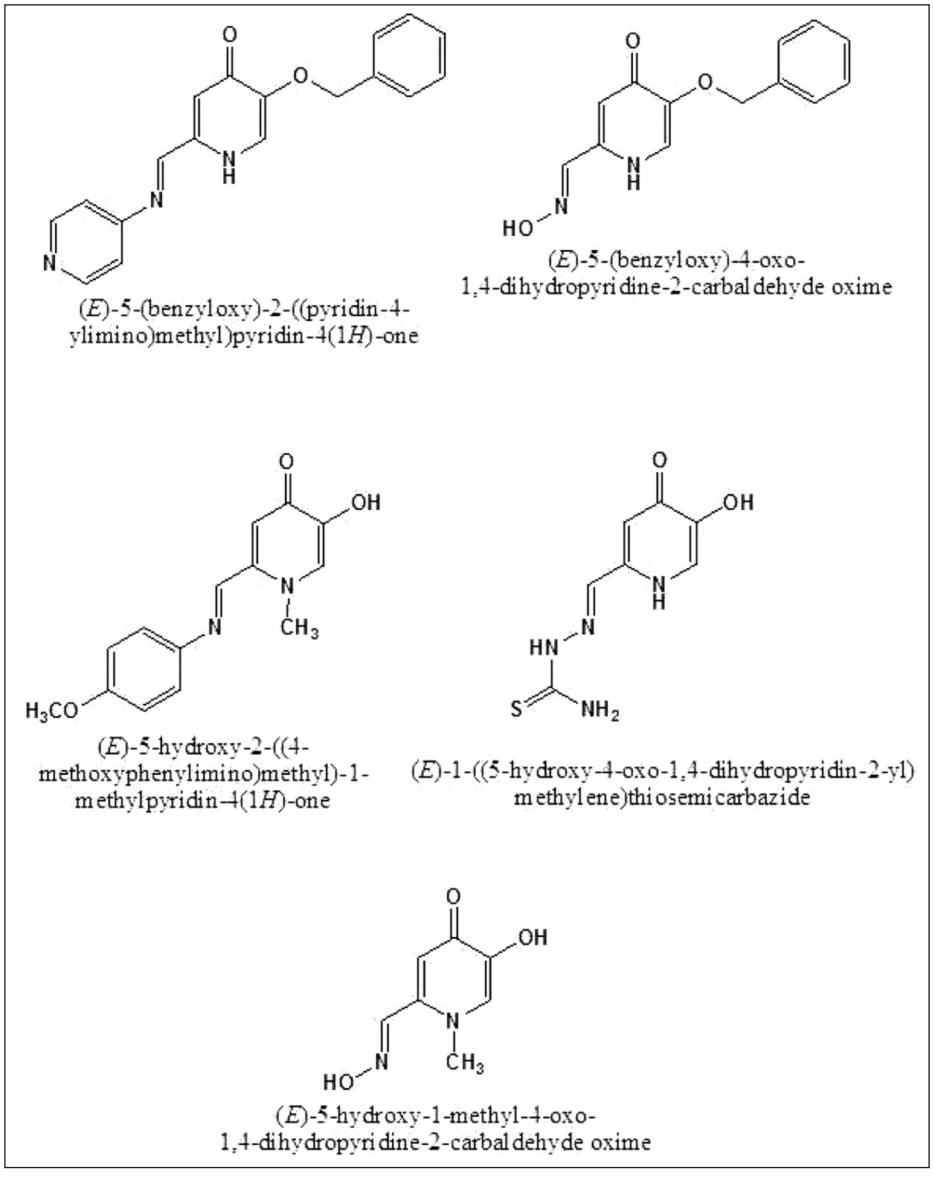

FIGURE 1: Chemistry of compounds from **a)** left top, **b)** right top, **c)** left second column, **d)** right second column and e; finally compounds are demonstrated.

protection of cardiac cells, against oxidative stress induced by Fenton reagents (H2O2 and Fe+3). In this study, LDH release assay demonstrated that induction of oxidative stress by iron was established successfully H9C2 cells. But applied compounds that we have applied were non-cardioprotective.

Heart, a near terminally differentiated organ, is particularly vulnerable to the toxic damages induced by ROS. Iron-induced cardiotoxicity occurs both in the setting of iron-overload and or in the conditions as consumption of anti-cancer drugs.[21,22] Although the precise underlying mechanisms are not truly clarified, but accumulated iron-catalyzed formation of free radicals seems to be an obvious explanation.[23] There is a general agreement that extracting labile intracellular iron pool is a very powerful toll against oxidant-induced myocardial damage.[24] The ensued cardiotoxicity is conferred by reduction of iron-redox activity.[18] So far, iron chelation is the only pharmacological intervention against either iron-induced cardiac failure or anthracycline-induced cardiotoxicity (mediated by the imbalance iron homeostasis).[25] But the progress in this area is lagged by the lack of effective agents.[26]

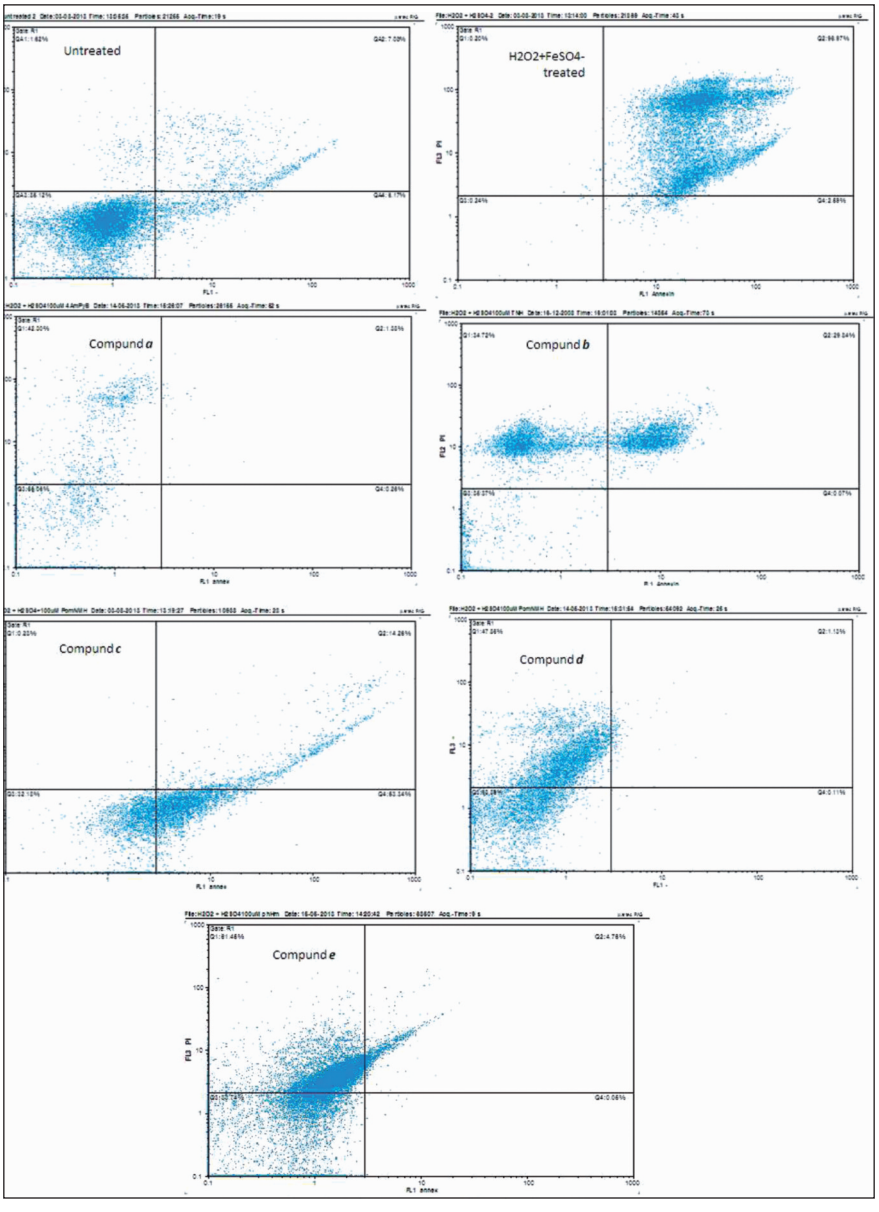

FIGURE 2: LDH leakage data from cells with and without treatment.

Numerous agents tested so far, only a few are currently in further development. Ultimately, interrupted attempts to shed more lights to the field of cardioprotective iron chelators will significantly advance the rational design of safe and more effective cardioprotective agents. Hydroxypyridine derivativeiron chelators have been applied for various medicinal issues as treatment of malaria but cardioprotection efficacy of these agents has not been widely studied. Antioxidant properties of hydroxypyridine derivative has been demonstrated previously. Hydroxypyridine derivative have been showed to reduce oxidative stress injury.[18] Indeed, previously, we have shown the cardioprotective efficacy of two hydroxypyridine derivative-iron chelators against H_2O_2-induced H9C2 cell injury.[20.] Since our novel agents were effective in reduction of iron-induced cardiac injury, we have decided to extend synthesis of this kind of iron chelator derivatives. Various kinds of hydroxypyridine derivative-iron chelators should be assessed to find out effective chelators

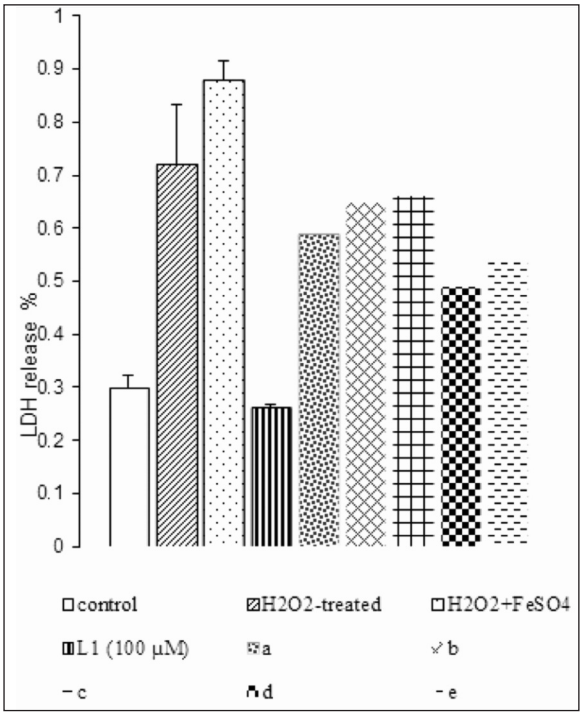

FIGURE 3: Flow cytometry results of H9C2 cells treated with synthetized iron chelators. **a)** untreated cells, **b)** cells treated with H2O2 plus FeSO4, **c)** cells treated with compound a, **d)** cells treated with compound b, e; cells treated with compound **e.**

among them. Thus, reporting negative results derived from our investigation might help other scientist and chemists for designation of effective hydroxypyridine derivative-iron chelators. Indeed, this report prevents repetitive synthesis of these non-effective agents.

Yielding better results and clearly significant cardioprotection obtained by our new compounds encourage us to screen more powerful iron chelators both in vitro and in vivo. In this investigation, we have tried the cardioprotective efficacy of our synthetized hydroxypyridine derivative-iron chela tors. But we have found no cardioprotective efficacy from these ligands. This negative data prevents other investigators from testing these ligands in future.

CONCLUSION

The present study demonstrates that these hydroxypyridine derivatives have no effect on nullifying H_2O_2-induced oxidative stress on the in vitro model of cardiac injury.

Acknowledgements

The authors gratefully acknowledge authorities at Isfahan University of Medical Sciences for financial support of this study.

Conflict of Interest

Authors declared no conflict of interest or financial support.

Authorship Contributions

All contributed to the study. Acquired data and prepared the drafting: Mohaddeseh Behjati, Afshin Fassihi, Mehrdad Mohammadpour Dehkordi, Mahtab Keshvari; Revised it critically for important intellectual content and submitted it: Mahtab Keshvari; Prepared the main drafting: Mohaddeseh Behjati.

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