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Molecular Cardiotoxic Effects of Proteasome Inhibitors Carfilzomib and Ixazomib and Their Combination with Dexamethasone Involve Mitochondrial Dysregulation

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Abstract

With the development and approval of new proteasome inhibitors, proteasome inhibition is increasingly recognized in cancer therapy. Besides successful anti-cancer effects in hematological cancers, side effects such as cardiotoxicity are limiting effective treatment. In this study, we used a cardiomyocyte model to investigate the molecular cardiotoxic mechanisms of carfilzomib (CFZ) and ixazomib (IXZ) alone or in combination with the immunomodulatory drug dexamethasone (DEX) which is frequently used in combination therapies in the clinic. According to our findings, CFZ showed a higher cytotoxic effect at lower concentrations than IXZ. DEX combination attenuated the cytotoxicity for both proteasome inhibitors. All drug treatments caused a marked increase in K48 ubiquitination. Both CFZ and IXZ caused an upregulation in cellular and endoplasmic reticulum stress protein (HSP90, HSP70, GRP94, and GRP78) levels and DEX combination attenuated the increased stress protein levels. Importantly, IXZ and IXZ-DEX treatments caused upregulation of mitochondria fission and fusion gene expression levels higher than caused by CFZ and CFZ-DEX combination. The IXZ-DEX combination reduced the levels of OXPHOS proteins (Complex II–V) more than the CFZ-DEX combination. Reduced mitochondrial membrane potential and ATP production were detected with all drug treatments in cardiomyocytes. Our findings suggest that the cardiotoxic effect of proteasome inhibitors may be due to their class effect and stress response and mitochondrial dysfunction may be involved in the cardiotoxicity process.

Keywords Proteasome inhibitors · Cardiotoxicity · Stress response · Mitochondrial toxicity

Introduction

Drug-induced cardiotoxicity is an increasing concern as therapeutic side effect to "targeted" anti-cancer therapies become first-line treatments. The usage of proteasome inhibitors as chemotherapeutic drugs has considerably increased survival of the patients with hematologic malignancies. However, their use has also resulted in serious cardiovascular problems [1]. Carfilzomib (CFZ), a second-generation proteasome inhibitor, is being successfully used in the treatment of multiple myeloma (MM) alone or in combination with immunomodulatory drugs. CFZ therapy has been associated with cardiotoxicity in several clinical settings [2]. Although it has been suggested that CFZ causes cardiotoxicity due to irreversible proteasome inhibition, data on cardiotoxic side effects of other reversible proteasome inhibitors suggest that proteasome inhibitors-induced cardiotoxicity may be a class effect [3].

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Ixazomib (IXZ) is a reversible proteasome inhibitor and IXZ is the first FDA-approved orally bioavailable proteasome inhibitor for the treatment of MM in combination with immunomodulatory drugs. Phase I/II study of IXZ therapy did not note cardiotoxic effects and IXZ is thought to have a low incidence of cardiotoxicity [4]. However, Jouni et al. [3] presented the first case report of cardiotoxicity after IXZ treatment. Also, a recent systematic review and meta-analysis of twenty studies showed that IXZ was associated with increased high-grade cardiovascular adverse effects [5].

Cardiotoxicity risks of immunomodulatory drugs with proteasome inhibitors are not well documented. A recent systematic review indicated that an immunomodulatory drug combination with CFZ does not cause a different response in cardiotoxicity [6]. Additionally, the recent phase III study of relapsed or refractory AL amyloidosis (amyloid light chain or primary amyloidosis) patients results showed that IXZ in combination with dexamethasone (DEX) resulted in a cardiac deterioration compared to the DEX-based non-proteasome inhibitors containing regimen patients [7]. A meta-analysis of randomized clinical trials on the cardiotoxicity of combination therapy of immunomodulatory drugs and proteasome inhibitors showed that immunomodulatory drugs and proteasome inhibitors are independently associated with cardiotoxicity and CFZ was associated with a maximum risk of cardiotoxicity among proteasome inhibitors [8].

Mitochondrial dysfunction and impaired ubiquitin–proteasome system are two important hallmarks of cardiotoxicity [9, 10]. Given that cardiomyocytes are non-proliferative and have greater proteasome activity than other tissues, they tend to be particularly vulnerable to proteasome inhibition [11]. However, the data on the molecular mechanisms of cardiotoxicity induced by proteasome inhibitors are limited. Understanding the mechanisms of proteasome inhibitors' cardiotoxicity may facilitate the development of strategies for its prevention or management.

Mitochondrial health is very important for many cell types, especially for the ones that are post-mitotic and have high energy demands, such as cardiomyocytes. Thus, in this study, we explored the molecular cardiotoxicity mechanisms of two second-generation proteasome inhibitors, CFZ and IXZ, alone or in combination with DEX which is an immunomodulatory drug and has been used in combination with proteasome inhibitors in anti-cancer therapy in a cardiomyocyte model. We focused on stress-associated proteins and parameters related to mitochondrial dysregulation and dysfunction.

Materials and Methods

Materials

CFZ (Cat#S2853) and IXZ (Cat#S2180) were bought from Selleck Chemicals. DEX (Cat#D4902) was from Sigma. H9c2 cardiomyocytes (Cat#CRL-1446) were from American Type Culture Collection (ATCC). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat#A3338) was purchased from Biomatik. ATP determination kit (Cat#A22066) was bought from Invitrogen. JC-1 Mitochondrial Membrane Potential Assay Kit (Cat#ab113850) and Total OXPHOS Rodent WB Antibody Cocktail (Cat#ab110413) were purchased from Abcam. GRP78 (Cat#3177S), GRP94 (Cat#2104S), HSP70 (Cat#4872S), HSP90 (Cat#4877S) K48-linked ubiquitin (Cat#8081S), and GAPDH (Cat#51332S) antibodies were from Cell Signaling. Anti-mouse- and anti-rabbit HRP (Cat#sc-2005, Cat#sc-2357)-conjugated secondary antibodies were from SCBT. Fetal bovine serum (FBS, Cat#10,091), DMEM:F12 with GlutaMAX medium (Cat#10,565,018), and antibiotic antimicotic solution (Cat#15,240,062) were bought from Gibco. RNA isolation kit was from Roche (Cat#11,828,665,001) and the cDNA synthesis kit (Cat#1,708,890) was from Bio-Rad. SYBR green (Cat#BIO-96005) was from Bioline.

Cell Culture and Treatments

H9c2 cardiomyocytes were grown in DMEM:F12 medium supplemented with 10% FBS and 1% antibiotic antimicotic solution in a humidified atmosphere at 37 °C. In order to maintain myoblastic cell population, the cells were passaged before reaching confluency and passage number between 5 and 15 were used for this study.

For evaluation of cytotoxicity, 5×10^3 cells per well were seeded into 96-well plates and incubated overnight. The next day, the cells were treated with 10, 25, 50, 100, 250, 500 nM, and 1 µM CFZ or 100, 250, 500 nM, 1, 2.5, 5, and 10 µm IXZ, both in combination with 100 nm, 1 µM, and 10 µM DEX for 24 h. Control groups were treated with 0.1% DMSO. Then MTT assay was carried out. Briefly, 5 mg/mL MTT dye solution was added into wells and incubated for 3 h for formazan formation. The medium was discarded and the formed formazan crystals were dissolved in DMSO and absorbance were read with a microplate reader (BioTek) at 590 nm. The cell viability was calculated as the percentage of the control groups. The cytotoxicity experiments were performed at least in triplicate.

Table 1 Primer sequences for determination of gene expression levels

Gene	Forward primer	Reverse primer	Melting temperature (°C)
MFN1	AGAATTTCCTTGGGCTCCAT	AGAAAGGAGGTGGACGGTTT	60
OPA1	GCATCTGGTTTCCCGAAGTA	GAGCCTTGCAGCTAACCTTG	60
DRP1	GTAGGTGATCAGCCCAAGGA	CATCTGGATCTACCTCTCTGGAA	60
FIS1	AAATGATGCTACGCAGGCTT	CCTGGACCATGACCAAGTTT	60
Beta Actin	ATGGTGGGTATGGGTCAGAA	CTTTTCACGGTTGGCCTTAG	60

Detection of Mitochondrial Membrane Potential

Mitochondria membrane potential was evaluated with JC-1 Mitochondrial Membrane Potential Kit (Abcam) according to the manufacturer's protocol. Briefly, 5×10^3 cells per well were seeded into 96-well clear-bottomed black plates and incubated overnight. Then, the cells were treated with 100 nM CFZ and 100 nM IXZ and both in combination with 1 µM DEX for 24 h. Then, the cells were stained with 20 µM JC-1 dye in dilution buffer for 10 min at dark at 37 °C. After that, the wells were washed twice with dilution buffer. The assay was performed at least in triplicate. The plates were read at excitation/emission wavelengths at 490/540 nm with a multimode reader (PerkinElmer).

Total ATP Content

Total ATP content was measured with the ATP Bioluminescence Kit (Invitrogen) according to the manufacturer's instructions. 5×10^4 cells were seeded into 24 well plates and incubated overnight. Following drug treatments as aforementioned, the cells were lysed in a lysis buffer (2 M NaCl, 200 mM Tris, 20 mM EDTA, 1% DTT, 0.2% Triton X-100; pH 7.5). The cell lysates were mixed with reaction solution and the luminescence intensity was measured with a multimode plate reader (PerkinElmer). The assay was performed in triplicate. The protein concentration of the lysates was measured with the Bradford Assay (Sigma) and the results are given as the percentage of the control group.

Gene Expression Levels

The mitochondrial dynamic-related gene expressions of Mitofusin 1 (*MFN1*), Optic atrophy 1 (*OPA1*), Dynamine-related protein (*DRP1*), and Fission 1 (*FIS1*) were analyzed with RT-qPCR. Beta actin was used as the house-keeping gene. Total RNA extraction and cDNA synthesis were done according to the manufacturers' instructions. Gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [12]. The studied primer sequences are given in Table 1.

Protein Expression Levels

For protein expression analysis, western blot was performed. Briefly, after drug treatments, protein extracts were prepared in RIPA buffer according to the manufacturer's instruction (SCBT). Protein concentrations of the lysates were measured with the Bradford assay (Sigma) and equal amounts of protein extract were separated in 10–12% SDS-PAGE gels. Separated proteins were transferred onto PVDF or nitrocellulose membranes and membranes were blocked with 5% non-fat milk. The membranes were then incubated with the primary antibodies against K-48 ubiquitin, OXPHOS complexes (CV-ATP5A, CIV-MTCO1, CIII-UQCRC2, and CII-SDHB), HSP90, HSP70, GRP78, GRP94, and GAPDH overnight at 4 °C and followed by the appropriate secondary antibodies for 2 h at room temperature. Finally, the bands were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo) and the band intensities were analyzed using Chemidoc MP imaging system, Image Lab, and Image J softwares. GAPDH was used as an internal control.

Statistical Analysis

Statistical differences were analyzed using One-way analysis of variance (ANOVA) followed by Tukey's test as post-ANOVA, employing Graphpad Prism 7.0 software. The data has been presented as the mean ± standard deviation (S.D.). Experiments were conducted in three independent biological replicates. $P < 0.05$ was considered statistically significant.

Results

According to the cytotoxicity assay results, CFZ was more cytotoxic to the cardiomyocytes compared to IXZ. In the preliminary study, 10 nM to 1 µM concentration range was used for both of the proteasome inhibitors. There was no significant cytotoxicity with low concentrations of IXZ (Supplementary Material Fig. 1) thus, tenfold higher (100 nM–10 µM) concentrations were used for IXZ. As can be seen in Fig. 1A and B both of the proteasome inhibitors

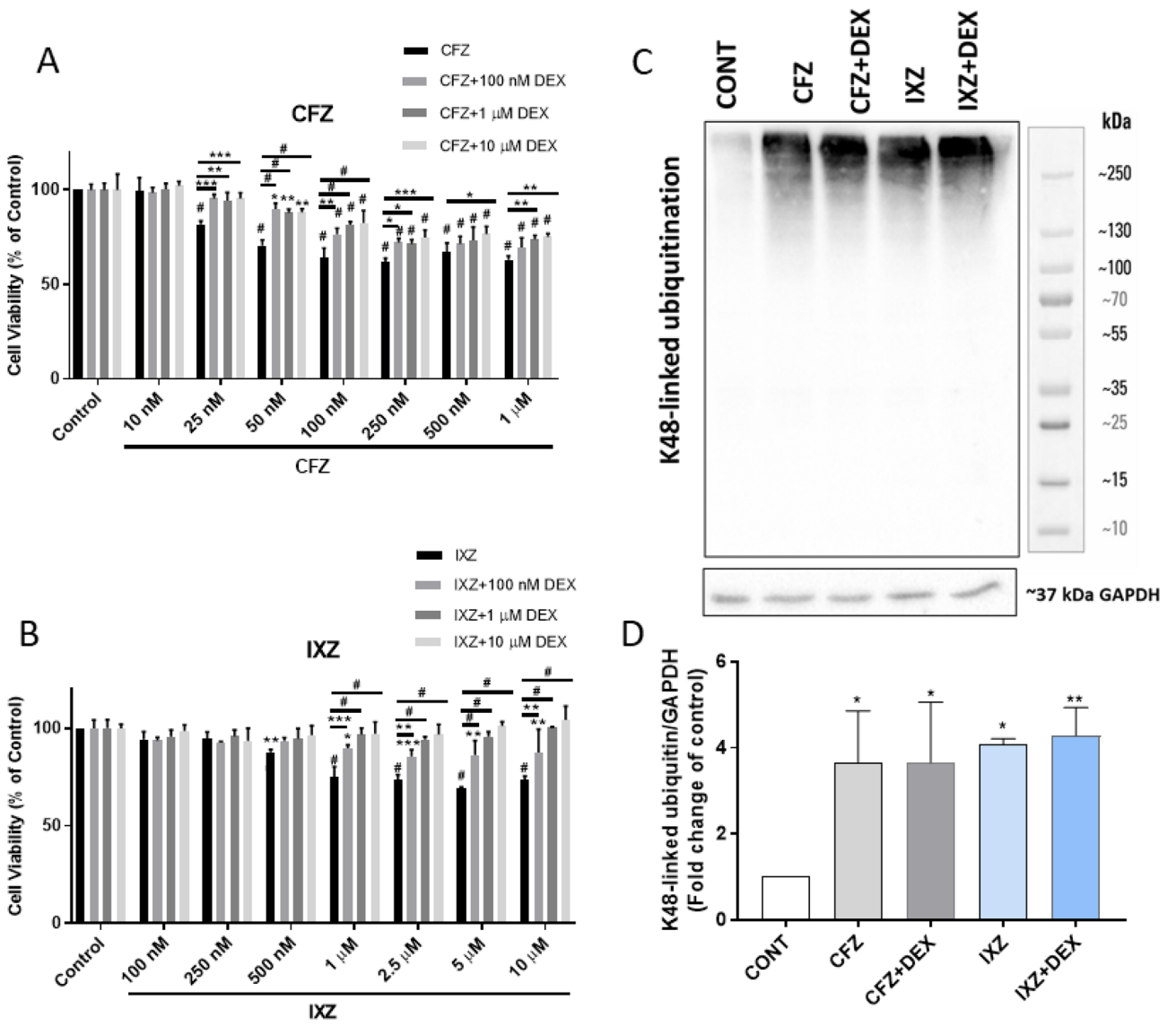

Fig. 1 H9c2 cardiomyocytes were treated with different concentrations of CFZ and IXZ alone and in combination with 100 nM, 1 μM, and 10 μM DEX for 24 h. The cytotoxic effects of CFZ alone and in combination with DEX (**A**) and IXZ alone and in combination with DEX (**B**) were evaluated with MTT assay. Effects of 100 nM proteasome inhibitors alone and in combination with 1 μM DEX on K48-linked ubiquitination by western blot. K48: lysine-48 specific, $*p<0.05$, $**p<0.01$, $***p<0.001$, and $\#p<0.0001$, $n=3$

caused cytotoxicity in a concentration-dependent manner and CFZ showed more cytotoxic activity on H9c2 cardiomyocytes than IXZ at lower concentrations. CFZ started to show cytotoxic activity on H9c2 cardiomyocytes at 25 nM concentration, while IXZ showed cytotoxicity from 500 nM. Also, CFZ was more cytotoxic than IXZ at the same concentrations (100 nM–1 μM). Combinational use of DEX with proteasome inhibitors significantly reduced the cytotoxic effect of CFZ and IXZ. We continued with further studies using 100 nM proteasome inhibitors, the CFZ dose we used in our previous studies, which results in > 80% viability with 1 μM and 10 μM DEX concentrations. There was no significant difference in viability between the DEX concentrations used in the study, thus, 1 μM DEX concentration was used for further studies.

Proteasome inhibition was also validated by measuring the K48 protein ubiquitination level. The selected concentrations of proteasome inhibitors caused accumulation of K48-linked protein pool similarly and DEX did not have an effect on K48 protein ubiquitination level (Fig. 1C).

Further, to understand the cellular stress effect on cardiomyocyte toxicity, heat shock proteins HSP90 and HSP70 levels were measured by western blot. According to the data, both proteasome inhibitors significantly increased the HSP90 and HSP70 levels, and DEX combination with proteasome inhibitors was observed to attenuate HSP90

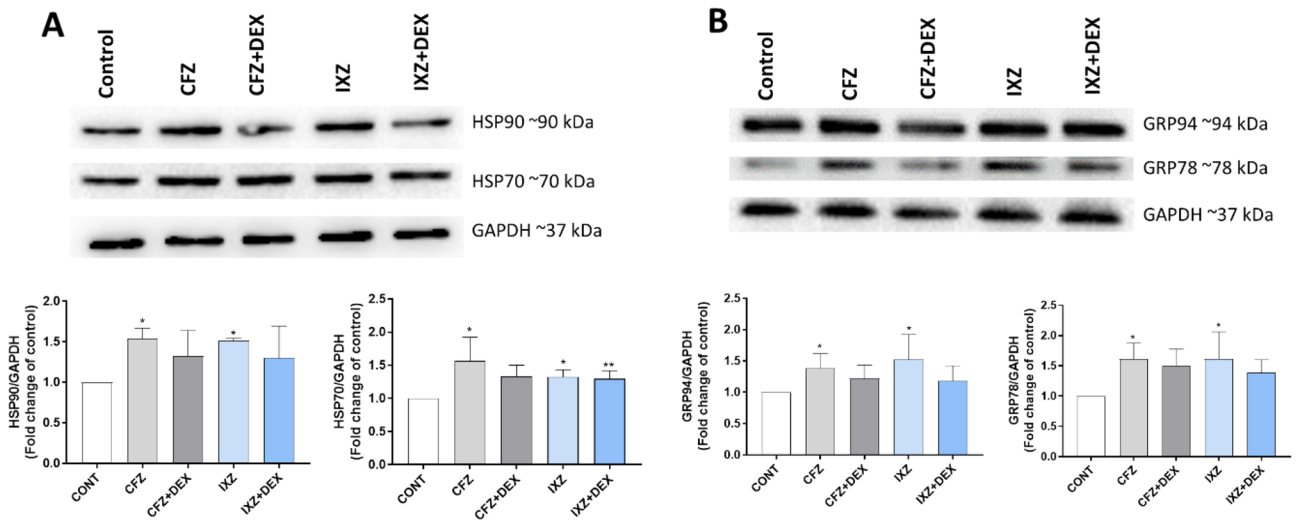

Fig. 2 H9c2 cardiomyocytes were treated with 100 nM CFZ and 100 nM IXZ alone and in combination with 1 μM DEX for 24 h. Effects of proteasome inhibitors alone and in combination with DEX on the protein levels of cellular stress markers (**A**) and ER stress markers (**B**) by western blot. *$p < 0.05$ and **$p < 0.01$, $n = 3$

elevation, whereas HSP70 induction was prominent with DEX combination (Fig. 2A).

Endoplasmic reticulum resided chaperones, the glucose-regulated proteins GRP94 and GRP78 levels were measured to evaluate the unfolded protein response in cardiomyocytes after drug treatments. Both GRP94 and GRP78 protein levels were significantly upregulated with CFZ and IXZ treatments and DEX combination with proteasome inhibitors attenuated the proteasome inhibitors-induced-ER stress (Fig. 2B).

Mitochondrial health is very important for many cell types, especially for the ones that are post-mitotic and have high energy demand, such as cardiomyocytes. To elucidate the cardiotoxicity mechanism we further focused on mitochondrial dynamics, dysfunction, and energy metabolism. Mitochondrial dynamics are mainly regulated by the fusion (mitofusins and *OPA1*) and fission genes (*DRP1* and *FIS1*). RT-qPCR results showed that expression levels of *MFN1* and *OPA1* were significantly increased with all drug treatments relative to the control group (Fig. 3 A, B). IXZ caused significantly higher expression levels of both fusion genes than CFZ alone or in combination with DEX. Interestingly, the combination of DEX with proteasome inhibitors caused a slight decrease in the expression levels of both *MFN1* and *OPA1*. Similarly fission-related genes *DRP1* and *FIS1* were significantly upregulated with all drug treatments compared to the control group. Similar to MFN1 and OPA1, DEX combination with CFZ resulted in a slight decrease. Contrarily, IXZ combination with DEX caused a slight increase in both expression levels of *DRP1* and *FIS1* genes (Fig. 3C, D).

Then, we measured the protein levels of mitochondrial electron transport chain complexes (CV-ATP5A, CIV-MTCO1, CIII-UQCRC2, and CII-SDHB). Our results showed that both proteasome inhibitors did not alter the CV–CIV–CIII and CII complex protein levels alone (Fig. 4). Importantly, IXZ combination with DEX caused a significant decrease in CIV, CIII and CII protein levels.

Further, we investigated whether the cardiotoxic effects of proteasome inhibitors and DEX were caused by the loss of mitochondria membrane potential in cardiomyocytes. The analysis exhibited that both of the proteasome inhibitors alone or in combination with DEX caused significantly compromised mitochondrial membrane potential in cardiomyocytes (Fig. 5A). Reduction in the electron transfer chain complex proteins can cause the inhibition of ATP synthesis. Thus, we measured the cellular ATP levels. As can be seen in Fig. 5B, cellular ATP concentrations were reduced significantly with proteasome inhibitors alone and in combination with DEX.

Discussion

The human proteasome has three different catalytic subunits (β1, β2, and β5) and clinically approved proteasome inhibitors, bortezomib (BTZ), CFZ, and IXZ show their anti-cancer activity through the inhibition of the main catalytic β5 subunit of the proteasome [13]. Clinically used proteasome inhibitors can cause cardiotoxicity, with varying degrees of severity suggesting a potential class effect and the incidence of cardiotoxicity was found to be higher with CFZ treatment compared to other proteasome inhibitors [14]. CFZ irreversibly inhibits proteasome activity and it is thought to be one of the reasons for the higher incidence of cardiotoxic effects

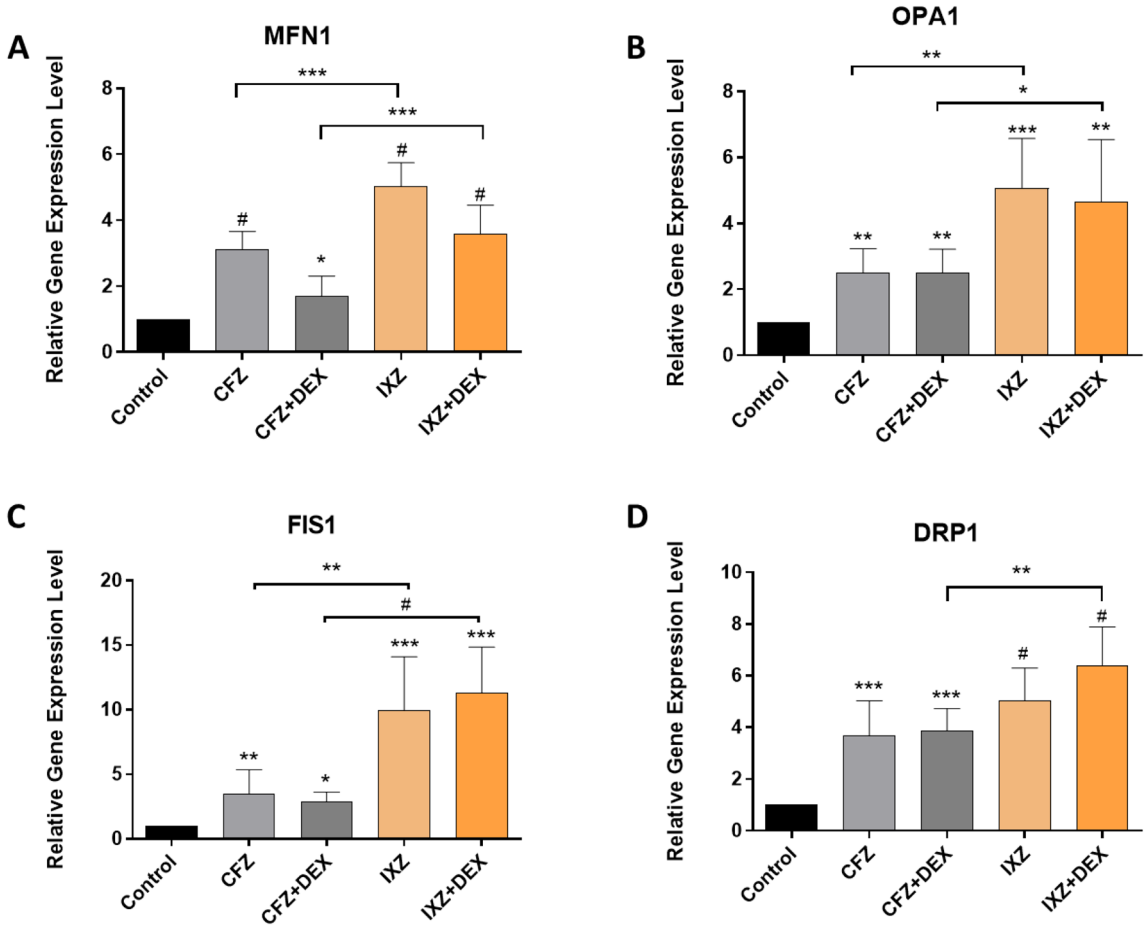

Fig. 3 H9c2 cardiomyocytes were treated with 100 nM CFZ and 100 nM IXZ alone and in combination with 1 μM DEX for 24 h. Gene expression levels related to mitochondrial fusion (*MFN1* and *OPA1*), **A** and fission (*FIS1* and *DRP1*), **B** were measured by RT-qPCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and #$p < 0.0001$

[15]. Additionally, as with BTZ, IXZ also co-inhibits the β1 and β5 subunits of the proteasome at nM range. However, it is known that BTZ, CFZ, and IXZ can inhibit all three proteasome catalytic subunits (β1, β2, and β5) at higher concentrations [16–18]. It has been reported in a study with a primary murine cardiomyocyte model that while the CFZ co-inhibition of β2 and β5 provides more effective proteasome inhibition, it may be especially responsible for cardiac toxicity [19]. Particularly, lower basal total proteasome activity in heart tissues may be a cause of cardiac vulnerability to CFZ at high concentrations and CFZ treatment (intravenous 4 mg/kg) in mice showed a significant reduction of both β5 and β2 proteasome activity after 2 h of injection in heart tissue [20]. It is worth noting that earlier studies with CFZ reported that β2 inhibition by CFZ is weaker than β1 inhibition in RPMI 8226 or HT-29 cells [17].

The results of a phase III trial of CFZ and immunomodulatory drugs (lenalidomide and/or dexamethasone) showed that the combination increased the cases of cardiovascular adverse events [21]. Also, there is a MM patient case report suggesting that CFZ use in combination with an immune modulatory drug synergistically results in cardiotoxic events [22]. The recently approved proteasome inhibitor, IXZ, has not undergone as extensive research as BTZ or CFZ. It is believed that IXZ has a low incidence of cardiotoxicity [23]. However, recent clinical studies reported that the novel orally available proteasome inhibitor IXZ also shows cardiotoxic effects in myeloma patients [7]. Jouni et al. [3] reported that a patient developed acute heart failure after four cycles of IXZ and DEX treatment. Additionally, a recent systematic review of the literature also underlined adverse cardiovascular events in IXZ-treated MM patients and suggested that cardiotoxic events are a potential adverse class effect of proteasome inhibitors [5].

Cellular protein synthesis and turnover are tightly regulated by the ubiquitin–proteasome system and the autophagic pathway. Elevated proteasomal activity has been shown in cardiac tissues. Thus, inhibition of the proteasomal activity in cancer treatment results in a compromised protein quality control system in cardiac tissues [15]. On the contrary,

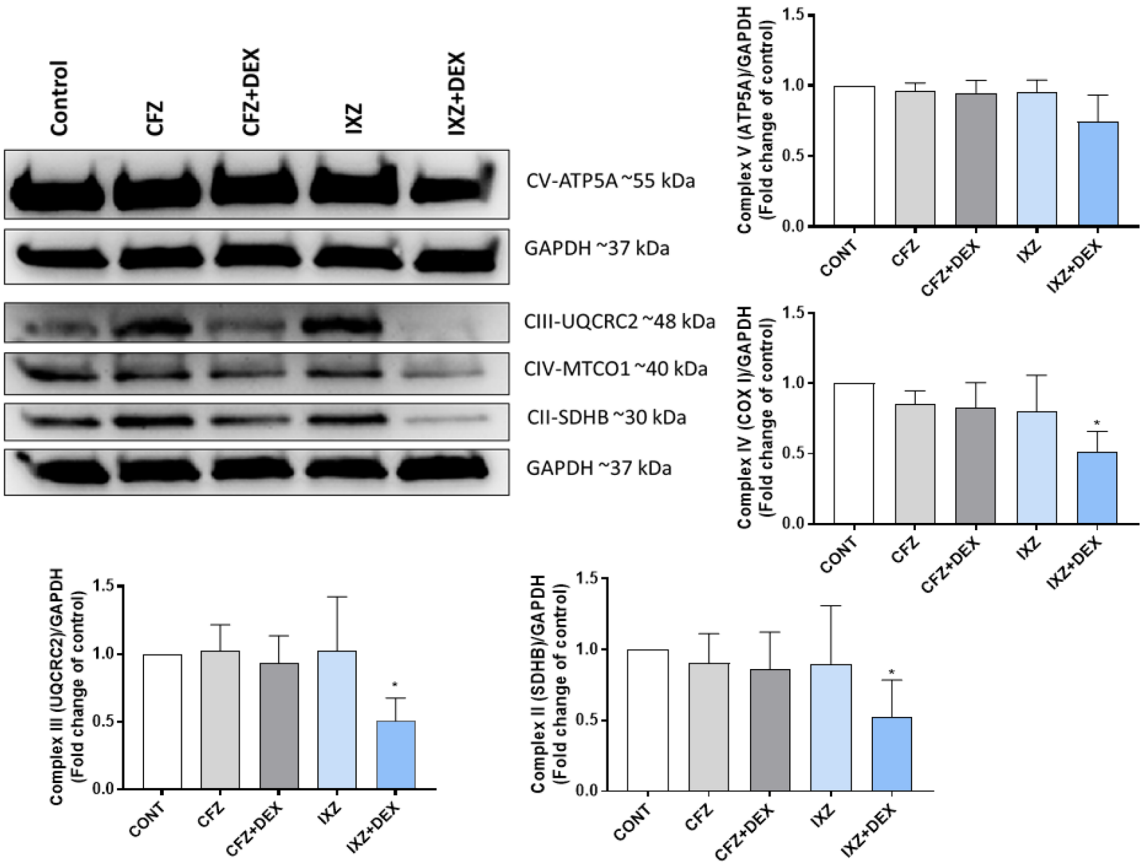

Fig. 4 H9c2 cardiomyocytes were treated with 100 nM CFZ and 100 nM IXZ alone and in combination with 1 µM DEX for 24 h. Effects of proteasome inhibitors alone and in combination with DEX on the protein levels of mitochondrial respiratory chain complex proteins by western blot. *$p < 0.05$, **$p < 0.01$, $n = 3$

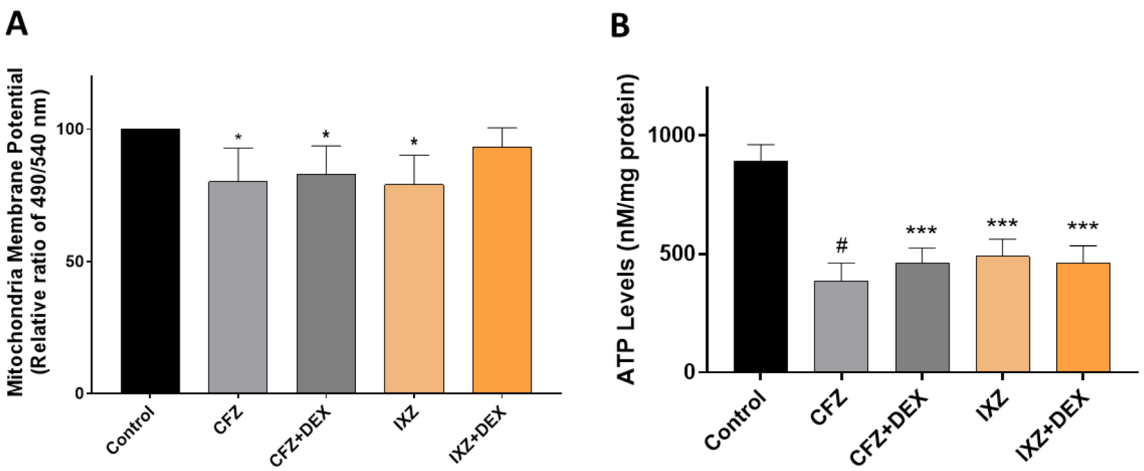

Fig. 5 H9c2 cardiomyocytes were treated with 100 nM CFZ and 100 nM IXZ alone and in combination with 1 µM DEX for 24 h. **A** The effect on the mitochondrial membrane potential with JC-10 dye. **B** The effect on the cellular ATP levels by luminometric ATP detection. *$p < 0.05$, ***$p < 0.001$, and #$p < 0.0001$

in a recent study, the heart tissue was found to have a lower level of proteasome activity compared to other organs and it is thought that this may contribute to the toxicity of proteasome inhibitors [19]. Accordingly, molecular mechanisms involved in proteasome inhibitors-induced cardiotoxicity remains to be fully elucidated. To better understand

the molecular differential cardiotoxic effects of CFZ and IXZ and their combination with DEX, we analyzed several parameters related to the stress response, mitochondrial dysregulation, and dysfunction in a cardiomyocyte model.

Proteasome inhibitors are known to have co-inhibitory effects on all three proteasomal subunits at higher doses. BTZ and IXZ begin to inhibit β1 activity at lower doses than the doses necessary to inhibit β2, whereas CFZ inhibits β2 activity at lower doses compared to the doses needed to inhibit β1 activity [16]. This is thought to be a contributing factor to the higher cytotoxicity and proteotoxicity of CFZ [19, 20]. Our findings also showed that CFZ was more cytotoxic to cardiomyocytes than IXZ. However, as a marker of proteasome inhibition, K48 ubiquitinated protein accumulation, which leads to proteotoxic stress was similar for both of the drugs. Even though DEX combination attenuated the cytotoxic effects of the proteasome inhibitors, K48 protein ubiquitination was similar for all groups. A study with rat neonatal cardiac myoblasts showed that IXZ showed more myocyte damage than CFZ which was measured with LDH release and these effects slightly correlated with their binding kinetics [24]. Another study with primary murine cardiomyocytes using all approved proteasome inhibitors (BTZ, CFZ, and IXZ) with the exception of marizomib showed that CFZ affected the contractility of the cardiomyocytes and this was due to β5 and β2 co-inhibition which resulted in higher proteotoxic stress [19]. Interestingly, Hasinoff and Patel (2018) treated neonatal cardiac myocytes with different proteasome inhibitors and found that BTZ was the most toxic proteasome inhibitor, followed by IXZ and the least toxic was CFZ. They speculated that the greater myocyte damage with boronic acid inhibitors, BTZ, IXZ, and delanzomib might be due to their faster proteasome inhibitory activity than the epoxyketone inhibitors CFZ and oprozomib [24]. As we showed in this study, the cellular and ER stress protein (HSP90, HSP70, GRP94, and GRP78) levels similarly increased with both CFZ and IXZ treatments in cardiomyocytes. DEX combination reduced the stress level, despite HSP70, and this can be interpreted as cellular and ER stress increment is related to the cytotoxic effects of both proteasome inhibitors.

As cardiomyocytes are heavy on energy expenditure, for evaluating the molecular mechanisms of CFZ- and IXZ-induced cardiotoxicity, we focused on some molecular pathways of mitochondrial dysfunction, such as changes in mitochondrial dynamics and mitochondrial complex protein levels, mitochondrial membrane potential, and cellular ATP levels.

The interconnected processes involving mitochondrial fission and fusion regulate the size, number, shape, and metabolic activity of mitochondria, as well as the response to stress [25]. Since the mitochondrial fission and fusion proteins are recycled by the ubiquitin–proteasome system, regulation of the mitochondrial dynamics may be affected by proteasome inhibition [26]. In this regard, it was worth analyzing the mitochondrial dynamics after proteasome inhibition. Both proteasome inhibitors caused significant upregulation in fission and fusion genes and we observed higher mitochondrial dynamics dysregulation with IXZ alone or in combination with DEX compared to CFZ and CFZ-DEX combination. With different experimental models, proteasome inhibition was proven to increase the mitofusion protein levels and it is thought to be having a protective effect in cardiomyocytes and myocytes [27, 28]. DEX combination with the proteasome inhibitors expressed lower fusion genes compared to proteasome inhibitors alone, and this can be due to the less cellular stress since increased mitochondrial dynamics is a sign for coping with stress. Even BTZ-resistant myeloma cells were reported to have higher mitochondrial dynamics with stronger defense [29]. Related to that, upregulated fusion and fission genes might be related to a defense mechanism in the cardiomyocytes to protect them from proteasome inhibitor toxicity. Notably, IXZ, which is less cytotoxic than CFZ in cardiomyocytes, has been attributed to higher gene expression of mitochondrial dynamics. To our knowledge, this is the first report showing that the new oral proteasome inhibitor IXZ is showing stronger alterations than CFZ in the mitochondrial markers of cardiomyocytes.

Under normal conditions, cardiomyocytes mainly generate ATP via oxidative phosphorylation [30]. Cells with disturbed mitochondrial networks have low mitochondrial membrane potential due to inefficient electron transport and proton pumping [31]. Also, electron transport and ATP synthesis are affected by mitochondrial fusion and fission disruption [32]. Supportively, we determined a decrease in the mitochondrial membrane potential and reduced ATP levels in all treatment groups. Similarly, in a recent study, cardiotoxic effects of CFZ were investigated with human-induced pluripotent stem cell-derived cardiomyocytes and according to the study results CFZ treatment reduced mitochondrial membrane potential, ATP level, and mitochondrial oxidative respiration. Also, they were able to show reduced contractility and upregulated stress-responsive proteins with transcriptomic and proteomic analyses. Contrarily, with their cardiomyocyte model *MFN1* and *OPA1* gene expression levels were downregulated [33].

Considering that IXZ causes reversible proteasome inhibition and co-inhibits the same subunits similar to BTZ, our results demonstrate that at 100 nM concentration, reversibility of proteasome inhibition and co-inhibition pattern of proteasome subunits did not make a difference as suggested by previous studies, at least on the molecular toxicity mechanisms that we focused on. Contrarily, IXZ resulted in higher alteration in the mitochondrial dynamic gene expression and OXPHOS protein levels than CFZ. Also, we determined

for the first time IXZ and DEX combination exacerbated OXPHOS protein levels in cardiomyocytes. This supports studies indicating that DEX may cause cardiotoxicity synergistically, either by itself or in combination [6–8].

The mechanism of action of glucocorticoids involves the direct regulation of glucocorticoid receptors in target tissues. Due to their anti-inflammatory and immunosuppressive effects, glucocorticoids are successfully in therapeutic use for various conditions [34]. Nevertheless, the information in the literature indicates that glucocorticoids affect energy metabolism, mitochondrial respiration, and oxidative phosphorylation in different tissues [35]. Additionally, DEX is shown to enhance protein turnover and cause mitochondrial fragmentation and mitophagy in myotubes [36]. DEX can promote cardiomyocyte maturation through activation of Parkin-mediated mitophagy induction [37]. DEX administration has been shown to reduce antioxidant enzyme levels and alter electrocardiographic parameters in the heart [38]. Also here we showed that DEX combination with proteasome inhibitors, especially IXZ, resulted in reduced mitochondrial complex protein levels. IXZ and DEX reduced mitochondrial complex V, IV, III, and II levels more than CFZ and DEX. Reduced levels of ATP production that we observed in this study can be a predicted consequence of decreased oxidative phosphorylation complex proteins and mitochondrial membrane potential.

We have previously established BTZ and CFZ neurotoxicity with mouse and human neuronal cell models and found that BTZ causes more skeletal damage and proteotoxicity than CFZ. Besides, the human neuronal cell model showed that both drugs can cause mitochondrial toxicity and this can be one of the underlying mechanisms of proteasome inhibitors-induced neurotoxicity [39, 40]. With the contribution of the results we obtained from these studies, it is better understood that mitochondrial dysregulation and dysfunction may contribute to the neurotoxic and cardiotoxic events due to proteasome inhibitors. The absence of a prominent difference in mitochondrial toxicities of different proteasome inhibitors supports the *class effect hypothesis* for cardiotoxicity side effects of proteasome inhibitors.

Conclusion

In conclusion, we determined a few molecular pathways that are involved in the cardiotoxic effects of CFZ, IXZ, and their combination with DEX in this study. As data suggest, CFZ and IXZ similarly induced cellular and ER stress responses in cardiomyocytes. DEX combination helped to reduce stress response related to proteasome inhibition. However, IXZ caused higher alteration in the genes related to mitochondrial dynamics than CFZ. Especially, the combination of IXZ with DEX more than CFZ resulted in a decrease in mitochondrial OXPHOS complex protein levels. The results from this study may have important implications toward the discovery of new proteasome inhibitors to overcome the cardiotoxic side effects of existing proteasome inhibitors. In addition, identified molecular changes may lead to a new path for the creation of biomarkers for the early detection of proteasome inhibitors-induced cardiotoxicity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12012-023-09785-7.

Author Contributions ATJ, BKY, and BA designed the experiments. ATJ, NSK, AGA, and SAE performed the experiments and analyzed the data. ATJ wrote the manuscript. All of the authors reviewed and edited the manuscript.

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Declarations

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

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