Glyceraldehyde derivatives inspired by empagliflozin as potential anti-heart failure agents independent of glucose-lowering effects

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In brief
This study explores structural modifications of sodium-glucose cotransporter 2 inhibitors to separate their cardiovascular benefits from glucose-lowering effects. Compound 12, derived from JX22, demonstrated enhanced cardioprotective efficacy and safety in heart failure models without impacting glucose levels.

Highlights
• Compound 12, emerging from structural modifications of JX22, significantly enhances cardiomyocyte protection without the glucose-lowering effect of typical sodium-glucose cotransporter 2 inhibitors.
• Compound 12 effectively inhibits NHE1 on the myocardial membrane, maintaining intracellular ion homeostasis and improving cardiac function and exercise tolerance in heart failure models.
• Demonstrating a favorable safety profile and promising pharmacokinetic properties, compound 12 supports the efficacy of scaffold hopping strategy in developing focused therapeutic agents.
Glyceraldehyde derivatives inspired by empagliflozin as potential anti-heart failure agents independent of glucose-lowering effects

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ABSTRACT

Sodium-glucose cotransporter 2 inhibitors are a class of glucose-lowering drugs known for robust cardiovascular protective properties. However, the side effects induced by Sodium-glucose cotransporter 2 inhibition limit application in cardiovascular medicine. Our prior research showed that thoughtful structural modifications can dissociate the anti-heart failure activity from glucose-lowering effects. Moreover, we showed that the glyceraldehyde derivative, JX22, developed by scaffold hopping from empagliflozin, exhibits a superior cardiomyocyte protective effect, albeit with increased cytotoxicity compared to empagliflozin. In the current study systematic structural modifications of JX22 were performed to enhance anti-heart failure efficacy and safety, while reducing glucose-lowering activity. Twenty glyceraldehyde-based derivatives were synthesized and compound 12 emerged as an optimal candidate by exhibiting an improved cytoprotective effect compared to JX22. Compound 12 significantly inhibited the activity of NHE1 on the myocardial membrane, thereby maintaining intracellular ion homeostasis. In vivo efficacy results demonstrated that compound 12 at 10 mg/kg significantly ameliorated cardiac dysfunction, myocardial fibrosis, and exercise tolerance in isoproterenol-induced heart failure mice without a glucose-lowering effect. Furthermore, compound 12 exhibited favorable safety profiles in single-dose toxicity and hERG inhibition tests, along with promising pharmacokinetic properties in mice. The current study not only underscores the potential of compound 12 for further investigation but also highlights the effectiveness of the scaffold hopping strategy.

Keywords: anti-heart failure, empagliflozin, glyceraldehyde structure, glucose-lowering effect

1. INTRODUCTION

Heart failure (HF) is a complex clinical syndrome characterized by high mortality and frequent hospitalizations. HF significantly affected the quality of life, especially in individuals > 60 years of age [1]. According to epidemiologic statistics, HF patients number 80 million worldwide, with China reporting an HF prevalence of 13.7 million among adults in 2015 [2]. The prevalence of HF poses a growing health and economic burden on
individuals and society [3]. Despite notable progress in drug treatments for HF, especially in heart failure patients with a reduced ejection fraction (HFrEF), challenges in treatment persist [4]. The use of a standard HF drug treatment has significantly reduced mortality and hospitalization rates, yet the quality of life needs further improvement. Furthermore, the treatment landscape for patients with a preserved ejection fraction (HFpEF) remains largely empirical, relying on symptom and co-morbidity management and a lack of specific drugs [5, 6].

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a new class of glucose-lowering agents known for remarkable cardioprotective effects [7]. Empagliflozin (EMPA) and dapagliflozin (DAPA) have been approved for the treatment of patients with HFrEF and HFpEF, whether or not the patient has diabetes mellitus, and shown to be the 4th pillar of HF medical therapy [8]. While generally well-tolerated, concerns about urinary and genital tract infections, hypoglycemia, and euglycemic diabetic ketoacidosis [9], which are induced by SGLT2 inhibition, have partly restricted the widespread use of SGLT2 inhibitors as first-line drugs for HF [10]. Many studies have demonstrated that the cardioprotective mechanism underlying SGLT2 inhibition is independent of the glucose-lowering effect [11], but rather involves multiple mechanisms [12-16]. Our previous work has shown that the cardioprotective effects of EMPA partly stems from the inhibition of sodium hydrogen exchanger 1 (NHE1) on the myocardial membrane [17]. A corollary study showed that rational structural repurposing of EMPA dissociates anti-HF activity from glucose-lowering effects [18], and supports an SGLT2-independent molecular mechanism as the key role for the cardioprotective effects of SGLT2 inhibitors. The subtle modification of the glucose ring, an SGLT2 inhibitor pharmacophore for sustaining SGLT2 inhibitory activity, in EMPA generated the candidate JX01, which exhibits a superior cardioprotective effect with reduced glycosuria and fewer glucose-lowering side effects to EMPA. Despite the successful separation of anti-HF activity and glucose-lowering effects, the striking structural similarity between JX01 and EMPA has not led to innovation in its design.

Scaffold hopping is an effective strategy for obtaining novel lead compounds [19]. During modification of the EMPA glucose ring, we obtained a new compound, JX22, by replacing the glucose ring with glyceraldehyde [18]. JX22 exhibits superior cardiomyocyte protective effects compared to EMPA, albeit with a notable increase in cytotoxicity. Furthermore, whether this skeletal transition attenuates “old” glucose-lowering effects while enhancing the “new” anti-HF activity is unknown. Nevertheless, JX22, with its novel skeleton, has emerged as a promising lead compound. In the current study a systematic structural modification of JX22 was carried out to further enhance the cardioprotective effects and improve safety. The ultimate objective was to obtain an anti-HF candidate compound featuring a novel skeletal structure (Figure 1). Twenty glyceraldehyde derivatives were designed and synthesized, among which compound 12 exhibited superior cardiomyocyte protective effects and lower cytotoxicity compared to the lead compound, JX22. An acute glucose-lowering experiment in vivo revealed that compound 12 (10 mg/kg) had no effect on the blood glucose level of mice. Compound 12 (10 mg/kg) significantly improved cardiac dysfunction and myocardial fibrosis in an isoproterenol (ISO)-induced

![Figure 1](image-url)
HF mouse model, surpassing the EMPA positive control. Of note was the moderate impact of EMPA on improving exercise tolerance, whereas compound 12 demonstrated a significant enhancement in exercise tolerance. Mechanistic studies indicated that compound 12 inhibited the activity of NHE1 in a dose-dependent fashion, which is consistent with the findings of a previous study [18]. In addition, compound 12 exhibited good pharmacokinetic and safety profiles. In conclusion, the glyceraldehyde derivative, compound 12, is a promising candidate as an anti-HF agent, underscoring that scaffold hopping is an effective strategy for rejuvenating SGLT2 inhibitors, transitioning from the “old” glucose-lowering interactions to “new” anti-HF functionalities.

2. METHODS

2.1 Compound synthesis

The synthetic route for compound 01 is depicted in Scheme 1. Using 4-bromo-1-chloro-2-(4-ethoxybenzyl) benzene as the starting material, the distal phenyl ethyl ether moiety was initially removed by boron tribromide, yielding compound 1a. Then, the distal phenolic hydroxyl group was protected by tert-butylimethyl, which gave rise to compound 1b. Following this, compound 1b reacted with n-butylithium and (R)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde in tetrahydrofuran to obtain the intermediate compound, 1c. Subsequent oxidation with Dess-Martin periodinane resulted in compound 1d, which was then deprotected with tetrabutylammonium fluoride in tetrahydrofuran to yield compound 1e with an exposed hydroxyl group. Ultimately, compound 01 was obtained by 4M HCl in methanol.

The synthetic route for compounds 02-13 is depicted in Scheme 2. Using intermediate compound 1e as the starting material, various substituents were introduced through nucleophile substitution or the Mitsunobu reaction, leading to the formation of intermediate compounds 2a-13a. The subsequent reaction with 4 M HCl in methanol facilitated the removal of the isopropylidene group, yielding compounds 02-13.

The synthetic route for compounds 14-20 is depicted in Scheme 3. Using different starting materials, such as heteraryl, bromo-benzo-cycloalkyl, and bromo-benzo-epoxide, as the starting materials, reactions with 2-chloro-5-bromo-benzaldehyde in tetrahydrofuran using n-butylithium resulted in compounds 14a-20a. Subsequently, the hydroxyl groups were removed by utilizing boron trifluoride ether and triethylsilane, yielding compounds 14b-20b. In a subsequent step, the intermediate compounds, 14b-20b, underwent reactions with n-butylithium and (R)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde to yield compounds 14c-20c, followed by Dess-Martin oxidation to yield compounds 14d-20d. Finally, compounds 14-20 were obtained with 4 M HCl in methanol.

The detailed synthesis and characterization of compounds 01-20 are described in the Supporting Information.

2.2 Cell culture and isolation of neonatal rat cardiomyocytes

H9c2 cells were obtained from the laboratory of Professor Yaozu Xiang (School of Life Sciences and Technology, Tongji University, China), and cultured in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and a 1% penicillin/streptomycin solution. H9c2 cells were maintained in a humidified cell incubator at 37°C with an atmosphere of 5% CO₂. Primary cardiomyocytes were isolated from neonatal Wistar rats (1–2 days old). The neonatal rats were dissected and the hearts were promptly removed

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**Scheme 1 | Synthesis of compound 01**

**Reagents and conditions:** (a) BBr₃, DCM, N₂, 0°C, 4 h, 95%; (b) Et₂N, DMAP, TBDMSCl, DCM, rt, 4 h, 92%; (c) n-BuLi, (R)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde, N₂, -78°C—rt, 13 h, 50%; (d) Dess-Martin Periodinane, DCM, rt, 2 h, 90%; (e) TBAF, THF, rt, 1 h, 65%; (f) 4M HCl, MeOH, rt, overnight, 50%.
and placed in a prechilled phosphate-buffered saline solution (PBS). After removing the connective tissues and expressing the remaining blood, tissue fragments were digested in 0.05% trypsin at 37°C. After discarding the supernatant, the tissue fragments were digested in 0.5 mg/mL type II collagenase diluted with Dulbecco’s modified Eagle’s medium/F-12 containing 5% heat-activated horse serum, 0.1 mmol/L ascorbate, insulin-transferring-sodium selenite media supplement, 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.1 mmol/L bromodeoxyuridine. Then, the cell suspension was centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended and cultured by differential sedimentation for 90 min. After removing the cardiac fibroblasts by blowing off the upper cells, the cardiomyocytes were diluted to 1×10^6 cells/mL and plated in 10 mg/mL-culture dishes.

2.3 Cell protective effects test of derivatives

The protective effects of all derivatives were evaluated in a glucose deprivation (GD)-induced H9c2 cell injury model. Cultured cardiomyocytes were seeded at a density of 8000 cells per well in a 96-well plate. After 24 h of seeding, the cells were exposed to the following concentrations of compounds: 1; 10; 50; and 100 μM. After incubation, the medium was removed and the cells were washed once with PBS. DMEM without glucose and fetal bovine serum was then added to induce cell injury. After 36–48 h of GD treatment, 10% CCK-8 solution was added to the cells for a 2-h incubation. The OD value was determined at 450 nm. Additionally, the protective effect of the optimal compound against GD-induced H9c2 cell injury was investigated by crystal violet staining. Cultured cardiomyocytes were seeded at 25,000 cells per well in 24-well plates. The cells were washed twice with prechilled PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After removing 4% paraformaldehyde, 0.5% crystal violet solution was added to the cells for incubation for 10 min at room temperature. After staining, the 0.5% crystal violet solution was recycled and the plates were washed several times with water. Images were collected using a Nikon Eclipse Ti2 microscope. The efficacy evaluation in primary cardiomyocytes followed the method described above.

2.4 Glucose-lowering test in mice

Male C57BL/6J mice were purchased from a subsidiary corporation of Shanghai SLAC Laboratory (Shanghai, China) and divided into 4 groups (n = 8 for each group), as follows: vehicle (saline-treated group); EMPA-treated group (10 mg/kg intragastric [i.g.]); JX22-treated group (10 mg/kg i.g.); and compound 12-treated (10 mg/kg i.g.). The mice were fasted for 12 h before the test. Blood samples were obtained from the tail tip at 0, 1, 2, 4, and 6 h; the tail tip was excised by 1–2 mm for each sample collection. The first drop of blood was discarded and the glucose levels were estimated using a glucometer (Sanicare; city, state, country). Food was withheld throughout the study.
2.5 Animal study
Male C57BL/6J mice were purchased from a subsidiary corporation of Shanghai SLAC Laboratory. The animals were raised under specific pathogen-free conditions with a 12 h light-dark cycle at 25°C and 55% humidity. The mice were given free access to water and food. All mouse studies were approved by the Hainan University Animal Research Committee (SYXK2023-0031). We established HF mice by injecting isoproterenol. Mice (7–8 weeks old) were randomly divided into 5 groups (n = 10 for each group), including sham, vehicle, EMPA-treated (10 and 30 mg/kg), and compound 12-treated groups (10 mg/kg). Compounds were administrated orally by i.g. administration per day. Except for the sham group, mice were injected subcutaneously with isoproterenol twice a day at 10-h intervals for a 2-week period. For days 1–2, 3–7, and 8–14 the dosage was set at 40, 20, and 10 mg/kg, respectively; mice in the sham group were durably injected with saline.

2.6 Echocardiography
This study assessed cardiac function by transthoracic echocardiography (TTE) using a Vevo 3100 high-resolution imaging system (VisualSonics, city, state, country) under light anesthesia. Rodents were anesthetized with 1.5% isoflurane inhalation before undergoing echocardiography monitoring. The images were obtained with the rat at rest and lying in the lateral decubitus position. The parameters were obtained by > 3 beats and the average was calculated. The cardiac structure was imaged in the two-dimensional parasternal short-axis view and an M-mode echocardiogram of the mid-ventricle was recorded at the level of the papillary muscles. Diastolic-LVIDd and systolic-LV diameters (LVIDs) were measured. The left ventricular fractional shortening and left ventricular ejection fraction were calculated.

2.7 Masson staining
The isolated heart issue was immobilized in 4% paraformaldehyde, dehydrated with gradient alcohol, and paraffin-embedded into 5 μm-thick sections. The sections were stained separately with Masson’s trichrome. The collagen deposition was observed under a light microscope and the blue area in the heart was quantified with Image-Pro Plus 6.0.
2.8 Exercise tolerance test
Gripping force test: The gripping force tester was placed horizontally and the mice were plated on the gripping force plate. After the mouse grasped the gripping force plate, the mouse was pulled back and able to release its claw. Then, the maximum gripping force was recorded. Each sample was measured three times, and the average value was taken as the result to evaluate the muscle strength of the mouse.

Rotating rod test: The mice were trained 2 days in advance to adapt to the rotating rod movement at 20 rpm for 10 min each day, and the time the mouse stayed on the rotating rod was recorded. Qualified training was defined by a stay time > 300 s more than 3 times. In the formal experiment, the mice were gently placed on a runner rotating at a speed of 20 rpm, observed for 5 min, and the duration of persistence on the rod was recorded.

2.9 Measuring pH changes with BCECF-AM
Cultured cardiomyocytes were seeded at 10,000 cells per well in 96-well plates. After incubation for 24 h, the medium was removed and the cells were washed with Hank's balanced salt solution (SH30268.01; Hyclone, city, state, country). HBSS containing 5 mmol/L of BCECFAM, 20 mmol/L of NH4Cl, and the tested compounds were added to the cells and incubated in the dark for 30 min at 37°C in 5% CO2. At the end of the incubation period, the buffer was removed and cells were washed twice for 5 min with HBSS and 100 μL of HBSS was added to each well. The fluorescence value of BCECF-AM (excitation, 488 nm; emission, 535 nm) was detected using a microplate reader (BioTek, city, state, country).

2.10 Single-dose toxicity study in mice
Male C57BL/6J mice were purchased from a subsidiary corporation of Shanghai SLAC Laboratory. The mice were divided into 3 groups (n = 5 for each group), including vehicle (saline-treated), JX22-treated (1000 mg/kg i.g.), and 12-treated groups (1000 mg/kg i.g.). After administration of a single-dose, observations focused on mortality as well as changes in behavior, skin, eyes, fur, and somatomotor activity. Individual body weight was measured daily throughout the observation period (7 days). At the end of the observation period, all mice were sacrificed and dissected. Macroscopic evaluation was performed and hearts, livers, spleens, lungs, and kidneys were removed for relative weight calculation.

2.11 Pharmacokinetic studies
Pharmacokinetic studies were commissioned by WuXi AppTec Co., Ltd. (Shanghai, China). Briefly, C57BL/6J mice were fasted for 10–14 h before administration of the compound and allowed free access to food and water 4 h after administration. Blood was obtained (0.20 mL/time point) via the jugular vein at appropriate time points (0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h for the intravenous administration; and 0.25, 0.5, 1, 2, 4, 8, 16, and 24 h for the oral administration group). Samples were placed in tubes containing K2-EDTA and stored on ice until centrifuged. The blood samples were centrifuged at 3200 x g for 10 min at 2–8°C within 1 h of collection and stored at approximately −80°C. Plasma concentration-time data were analyzed and the pharmacokinetic parameters were calculated.

2.12 Liver microsomal stability test
Liver microsomal stability studies were commissioned by WuXi AppTec Co., Ltd. The details of the protocols are described in the Supporting Information.

2.13 Statistical analyses
Data are presented as the means ± standard deviations (SDs). Statistical significance was determined using an unpaired two-tailed Student's t-test between the two groups of data. When more than two groups were compared, statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using Prism 8 software (GraphPad Software, city, state, country). A P<0.05 was considered statistically significant.

3. RESULTS

3.1 Cardiomyocyte protective effects of compounds 01-20
In our previous work we primarily modified the substituents on the distal aromatic ring of EMPA and found that hydrophilic substitutions were beneficial for maintaining or even improving cardioprotective activity. Hydrophilic substitutions are generally thought to reduce the toxicity of compounds. In the present work the substituents on the distal benzene ring of JX22 were replaced with hydrophilic groups, such as an aromatic heterocyclic ring or heterocycles containing N, O, and S, as well as some non-hydrophilic groups, which generated compounds 01-13. In addition, to diversify the structural types, the distal benzene ring was replaced with five-membered heterocyclic rings, naphthalene rings, benzo-aliphatic rings, and benzo oxygen-containing heterocycles, which generated compounds 14-20. The cell protective effects of all compounds were tested on a GD-induced H9c2 cell injury model at concentrations of 1, 10, 50, and 100 μM; the results are presented in Figure 2 and Supporting Information Table S2.

In general, replacing the tetrahydrofuran ring with hydrophobic groups at the distal benzene ring is not conducive to cardioprotective activity, as indicated by the lower maximum cell viability of compounds 02-04, 08, and 09 compared to JX22 and EMPA. It is regrettable that hydrophilic substitutions, such as an oxygen heterocyclic ring, did not improve the cell protection effect or reduce the cell toxicity (compounds 05-07 vs. JX22).

Compound 01, which replaced the tetrahydrofuran ring with a strong hydrophilic hydroxyl group, exhibited a...
cellular protective effect comparable to JX22. Among the derivatives substituted with aromatic heterocycles (compounds 10-13), compound 12, which was substituted with pyridazine, demonstrated a higher maximum cell protective effect and lower cytotoxicity than JX22 and achieved the intended objective. For compounds 14-20, produced by modification at region B, changes in substituents at the proximal benzene ring did not enhance cell protective effects. However, the type of substituent affected the cell toxicity of compounds. Specifically, compounds 14-17, which were modified with thiophene, fused aromatic hydrocarbons, and a benzo aliphatic ring, did not have cardiomyocyte protective effects or safety. Only hydrophilic substituents, such as 2,3-dihydrobenzofuran and 1,3-benzodioxole, decreased the cell toxicity of compounds 18-20 compared to JX22.

Figure 2 | Chemical structures and cardiomyocyte protective effects of derivatives. (A) Structures of all derivatives. (B) Cardioprotective effects of all derivatives in the GD-induced H9c2 cell line. The data are presented as the percentage of surviving cells relative to control cells and as the mean ± SD, n = 3, *P<0.05, **P<0.01, ***P<0.001.
Overall, it is challenging to enhance the cell protective effect and reduce cytotoxicity through structural modification based on the glyceraldehyde skeleton. Among the derivatives, only compound 12 met the criteria, whereas compound 01 maintained a cell protective effect comparable to JX22, yet the cytotoxicity remained unchanged. Therefore, compounds 01 and 12 were selected as preferred compounds for further evaluation.

3.2 Compound 12 exhibited superior cardiomyocyte protective effect compared to JX22 in vitro

To further verify the protective effect of compounds 01 and 12 on GD-induced cardiomyocyte injury, crystal violet staining was introduced to evaluate cell viability in 24-well plates. Approximately 80% of cardiomyocytes were dead after GD treatment, as indicated by a decrease in crystal violet staining (Figure 3A, B). Compound JX22 exhibited a moderate cell protective effect at 10 μM, but apparent cell toxicity at a concentration of 50 μM. Compound 01 significantly increased the cell survival at 10 μM, while compound 12 exhibited significant, dose-dependent improvement of cell viability at 10 μM and 50 μM, indicating that compound 12 has a superior cardiomyocyte protective effect and higher safety compared to JX22. The cytotoxicity of compounds 12 and JX22 was evaluated in two human cell lines (HL7702 and MRC5). The cytotoxicity of compound 12 was lower than JX22 (Supporting Information Figure S1). In addition, primary cardiomyocytes were isolated from neonatal rats to further evaluate the efficacy of compound 12. As shown
in Figure 3C, compound 12 prevented GD-induced cellular injury with a lower effective concentration (1 μM) and was comparable to EMPA at 50 μM. Taken together, compound 12 demonstrated a superior protective effect on both primary cardiomyocytes and the H9c2 cell line compared to EMPA and JX22.

3.3 Acute glucose-lowering test of compound 12 in vivo
Given that glucose-lowering effects are a primary obstacle in the clinical application of SGLT2 inhibitors for cardiovascular disease, we determined whether glyceraldehyde compound 12 attenuated the glucose-lowering effects. The acute glucose-lowering test was introduced to investigate the effect of compound 12 on the blood glucose in mice. Male C57BL/6J mice were fasted for 12 h in advance and the blood glucose levels of the mice were monitored at 0, 1, 2, 4, and 6 h after oral administration of compound JX22, compound 12, and EMPA at dose of 10 mg/kg. EMPA showed a significant glucose-lowering effect in mice. The glucose-lowering effect of the lead compound, JX22, was noticeably weaker than EMPA, although JX22 retained some efficacy. Compound 12 had no impact on blood glucose levels during the 0–6 h window (Figure 4), indicating that replacement of the glucose ring with glyceraldehyde significantly weakened the glucose-lowering effect.

3.4 Compound 12 exhibited superior anti-HF activity compared to EMPA in vivo
Based on previous work, EMPA exhibited cardioprotective and glucose-lowering effects on MI- and ISO-induced HF mice at a dose of 30 mg/kg, while treatment with 10 mg/kg failed to improve cardiac dysfunction. To determine whether the anti-HF activity of compound 12 is superior to EMPA, the efficacy in ISO-induced HF mice at a dose of 10 mg/kg was evaluated, but the blood glucose was not affected. As shown in Figure 5, administration of compound 12 (10 mg/kg) significantly alleviated hypertrophy induced by an ISO infusion, as illustrated by a decrease in the heart-to-body mass ratio (Figure 5C). In addition, compound 12 ameliorated left ventricular systolic dysfunction and ventricular remodeling, as evidenced by the increase in the ejection fraction and left ventricular fractional shorting (Figure 5D, E), accompanied by a decrease in left ventricular end-systolic and end-diastolic diameter (Figure 5F, G) and a reduction in the area of fibrosis (Figure 5J, K). In contrast, the EMPA-treated group failed to ameliorate hypertrophy and left ventricular systolic dysfunction at 10 mg/kg, indicating that compound 12 exhibited a significant advantage in terms of cardioprotective effect compared to EMPA at the same dose. Considering that low exercise tolerance contributes to the poor prognosis of end-stage HF patients, we delved into the improvement provided by compound 12. As expected, HF mice exhibited significantly lower exercise tolerance than mice in the sham group, as illustrated by the decrease in the gripping force and drop time of rotating rod ability (Figure 5H, I). Treatment with compound 12 prolonged the athletic ability in the rotating rod test and strengthened the grip ability, surpassing that of the EMPA-treated (10 mg/kg) group. Collectively, the results mentioned above demonstrated that compound 12 exhibited a lower effective dose and a more robust improvement in exercise tolerance compared to EMPA.

3.5 Compound 12 inhibited NHE1 activity
In our previous work we showed that EMPA inhibited NHE1 on the myocardial cell membrane, thereby preventing autophagy-induced cell death. To determine

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**Figure 4 | Acute glucose-lowering test in vivo.**
(A) Blood glucose changes. (B) AUC of blood glucose levels between 0 and 6 h in 12 h-fasted mice after oral administration of compounds 12 and JX22, and EMPA. The data are presented as the mean ± SD, n = 8, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 5 | Effects of compound 12 on cardiac function in ISO-induced HF mice.
(A) Schematic of the experimental design. (B) Representative echocardiographic tracings. (C) Heart weight-body weight ratio. (D) Left ventricular ejection fraction. (E) Left ventricular fractional shortening. (F) Left ventricular systolic diameter. (G) Left ventricular diastolic diameter. (H) Grip strength test. (I) Rotating rod test. Effect of compound 12 on exercise endurance in mice. (J) Representative left ventricular sections stained with Masson's trichrome. (K) Quantification of Masson's trichrome staining. The data are shown as the mean ± SD, n = 10. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
whether glyceraldehyde derivatives mediate cytoprotective effects through a similar mechanism, we indirectly evaluated the inhibitory activity of compounds JX22, 01, and 12 against NHE1 using a pH fluorescence probe. Following a previously described method, an NH₄Cl solution was used to induce an intracellular acidic environment that mimicked an ischemia-reperfusion injury. The cytoplasmic pH in H9c2 cells, after removing the NH₄Cl solution from the medium, was used to indicate NHE1 activity. The fluorescence value decreases with the reduction in pH value, indicating a stronger inhibitory activity against NHE1. Without affecting the cell survival (Figure 6A), treatment with compound JX22, 01, and 12 inhibited intracellular pH recovery, suggesting their ability to inhibit NHE1. Among these compounds, JX22 and 12 exhibited comparable NHE1 inhibitory activity (Figure 6B). These results suggested that glyceraldehyde derivatives also have a myocardial protective role by inhibiting NHE1.

3.6 Pharmacokinetics and safety profiles of compound 12
We further evaluated the pharmaceutical and safety profiles of compound 12. The pharmacokinetics of an oral single dose of compound 12 in blood was performed in mice. As shown in Table 1, compound 12 exhibited an excellent oral bioavailability (F = 116%), acceptable terminal half-life (t₁/₂ = 3.19 h), and low total plasma clearance (6.09 mL/min/kg). In addition, compound 12 displayed good liver microsomal stability (> 80% of the parent remained after a 60-min incubation in both human and mouse liver microsomes; Table 2). These results indicated that compound 12 possesses favorable pharmacokinetic properties.

The hERG inhibition and single-dose toxicity tests were performed. Compound 12 displayed low hERG inhibition activity (IC₅₀ > 30 μM; Table 2). The safety of compound 12 and lead compound JX22 were compared in the single-dose toxicity test. There were no instances of mortality or signs of morbidity in mice from either group within 7 days following a single administration (1000 mg/kg i.g.). However, a significant increase in the spleen-to-body mass ratio and a nearly significant increase in the heart-to-body mass ratio were noted in

Table 2 | Liver microsomal stability and hERG inhibition of compound 12.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver microsomal stability</td>
<td>Mouse: 94.4% of parent remained after a 60 min incubation</td>
</tr>
<tr>
<td></td>
<td>Human: 82.9% of parent remained after a 60 min incubation</td>
</tr>
<tr>
<td>hERG inhibition</td>
<td>20.82% at 30 μM (IC₅₀ &gt; 30 μM)</td>
</tr>
</tbody>
</table>

Table 1 | Pharmacokinetic parameters of compound 12 in mice.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dosage (mg/kg)</th>
<th>Tₘₚ (h)</th>
<th>Cₘₚ (ng/mL)</th>
<th>t₁/₂ (h)</th>
<th>AUC₀₋ₜ (ng·h/mL)</th>
<th>CL (mL/min/kg)</th>
<th>F%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>10 (p.o)</td>
<td>0.33</td>
<td>16767</td>
<td>3.19</td>
<td>28130</td>
<td>/</td>
<td>116%</td>
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<td></td>
<td>5 (i.v)</td>
<td>/</td>
<td>20908</td>
<td>1.34</td>
<td>13600</td>
<td>6.09</td>
<td>/</td>
</tr>
</tbody>
</table>

Tₘₚ: peak time; Cₘₚ: peak concentration; t₁/₂: terminal half-life; CL: total plasma clearance; AUC₀₋ₜ: area under the plasma concentration-time curve; F: bioavailability.
the JX22-treated group compared to the vehicle-treated group. No differences were observed between the compound 12-treated and vehicle groups, indicating that compound 12 has a superior safety profile compared to JX22 in vivo (Figure 7). In summary, the preliminary data suggest that compound 12 has good pharmaceutical and safety properties.

4. DISCUSSION

Cardiovascular diseases have become major health problems worldwide. With persistent drug discovery and treatment, the symptoms of HF patients have shown improvement. Nevertheless, elevated mortality rates and frequent hospitalizations persist, posing a significant threat to modern society. Despite the considerable efficacy of SGLT2 inhibitors, the widespread use of SGLT2 inhibitors in cardiovascular medicine is hindered by glucose-lowering side effects. In our prior work EMPA was structurally modified to dissociate the anti-HF activity from the glucose-lowering effects. Compound JX22, featuring a novel skeleton, demonstrated a remarkable cardioprotective effect.

In the current study we conducted a comprehensive modification of compound JX22 to enhance the cardiomyocyte protective effects and reduce cell toxicity. Among 20 derivatives, compound 12, incorporating a pyridazine modification in the distal benzene, exhibited notable results. Cell viability reached up to 90% (Figure 2) and the safety range on cell lines and primary cardiomyocytes was increased as well (Figure 3B, C). Given the limitations of EMPA due to its glucose-lowering side effects, we assessed the glucose-lowering effect of compound 12, which showed no influence on blood glucose (Figure 4). Additionally, at a dose of 10 mg/kg, compound 12 demonstrated a remarkable protective effect on ISO-induced HF mice, surpassing the efficacy of EMPA at the same dose, especially in improvement of exercise tolerance (Figure 5). In addition, we verified that the protective efficiency was mediated by NHE1,
with compound 12 exhibiting a potent inhibition of NHE1 (Figure 6). Compound 12 also exerted good pharmacokinetic and safety profiles.

In summary, our study introduced a novel structural skeleton by incorporating glyceraldehyde, which successfully separated the efficacy against HF from glucose-lowering side effects. This finding presents a promising strategy for the modification of SGLT2 inhibitors. Moreover, NHE1 was shown, to some extent, to serve as a therapeutic target for HF. This work provides an essential candidate compound and lays the groundwork for effective treatments in patients with HF.

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CONFLICTS OF 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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