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Celastrol-type HSP90 modulators allow for potent cardioprotective effects☆

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ABSTRACT

Aims: Cardiac ischemic conditioning has been shown to decrease ischemic injury in experimental models and clinically. Activation of survival pathways leading to heat shock proteins (HSP) modulation is an important contributor to this effect. We have previously shown that celastrol, an HSP90 modulator, achieves cardioprotection through activation of cytoprotective HSP's and heme-oxygenase-1 (HO-1). This is the first comparative evaluation of several modulators of HSP90 activity for cardioprotection. Furthermore, basic celastrol structure-activity relationship was characterized in order to develop novel potent infarct sparing agents suitable for clinical development.

Main methods: Combining *in vitro* cell culture using rat myocardial cell line exposed to ischemic and ischemia/reperfusion (I/R) stresses, and *ex vivo* Langendorff rat heart perfusion I/R model, we evaluated cardioprotective effects of various compounds. Selected signalling pathways were evaluated by western blot and reporter gene activation.

Key findings: From a variety of HSP90 modulator chemotypes, the celastrol family was most efficient in inducing cytoprotective HSP70 and HO-1 protein overexpression and cell survival *in vitro*. Celastrol and two synthetic analogs were protective against ischemia and prevented ischemia/reperfusion (I/R) injury when given as pre-treatment or at time of reperfusion, increasing viability and reducing mitochondrial permeability transition pore opening. *Ex vivo* experiments demonstrated that the two synthetic analogs show cardioprotective activity at lower concentrations compared to celastrol, with activation of multiple survival pathways.

Significance: Celastrol backbone is essential for cardioprotection through HSP90 activity modulation. These compounds hold promise as novel adjunct treatment to improve outcome in the clinical management of I/R injury.

1. Introduction

Despite advances in reperfusion protocols to reduce ischemic injury, irreversible loss of cardiomyocytes still occurs during ischemia, in response to reperfusion and afterwards [1]. It is estimated that up to 50% of the final infarct size may be due to reperfusion injury [2,3]. Despite promising results from clinical trials, myocardial reperfusion injury remains an unmet clinical need [4].

Ischemic conditioning, the activation of endogenous protective mechanisms through repeat short episodes of ischemia followed by reperfusion, decreases infarct size in preclinical models [1]. Similarly, ischemic postconditioning, (repeated brief episodes of ischemia at the time of reperfusion) [1] has been extensively studied in several clinical trials. Compelling data in humans report the cardioprotective effects of ischemic postconditioning with reduction of infarct size by 35% on average [1,5] or with some improvement of cardiac function [6].

Many pharmacological treatments aimed at known cardioprotective targets, including adenosine receptors, beta1-adrenoceptors and the mitochondrial permeability transition pore (mPTP), have been proposed for treatment of ischemia/reperfusion (I/R) injury, but these have shown neutral results in clinical trials [4].

Celastrol, a pentacyclic triterpenoid $C_{29}H_{38}O_4$ compound isolated from the root bark of the "thunder god vine" (*Tripterygium wilfordii* Hook. F.), interacts with the heat shock protein (HSP) 90 complex. By

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disrupting HSP90-CDC37 interaction [7,8] and stimulating the heat shock response (HSR) through Heat shock transcription factor 1 (HSF1) activation and the antioxidant response through Nuclear factor (erythroid-derived 2)-like 2 (NRF2) activation [9], celastrol triggers powerful survival signals including heme-oxygenase-1 (HO-1). Our group was first to show that celastrol activates pro-survival signalling pathways and upregulate cytoprotective HSF1 and HO-1 responsible for improved cardiac cell survival under hypoxic conditions. In the rat ischemic myocardium with permanent coronary ligation, continuous celastrol treatment for two weeks improves cardiac function and abrogates adverse left ventricular remodelling [10].

Based on the infarct sparing effects of the HSP90 modulator celastrol, we set out to evaluate other known HSP90 modulators and chemically-related synthetic analogs of celastrol, in order to identify the efficient compound family, target and minimal structural characteristics essential for efficient cardioprotection.

Herein we demonstrate that celastrol and two analogs have the best protective effects among the *in vitro* screened compounds. The two screened synthetic analogs demonstrate *ex vivo* cardioprotective characteristics at lower concentrations, differentially activating multiple target cardioprotective pathways when compared to the parent naturally occurring celastrol.

2. Methods

2.1. Materials

The known HSP90 modulators SNX-5422 (Adooq Bioscience, Irvine, CA), Radicicol, Withanolide A, Celastrol (all from Cayman Chemical, Ann Arbor, MI), Conglobatin (Santa Cruz Biotechnology, Mississauga, ON), Ganetespib (Medkoo Biosciences, Morrisville, NC) were commercially available.

Celastrol analogs were synthetized by Piramal Healthcare Canada Ltd. (Aurora, ON) according to our specifications.

All other products were purchased from Sigma-Aldrich Canada (Oakville, ON) unless specified otherwise.

2.2. Cell culture, signalling and viability assays

Rat H9c2 cardiomyoblasts (ATCC Cat# CRL-1446, Manassas, VA) were seeded in 96 well plates (Eppendorf, Mississauga, ON) at a density of 5,000 cells/well for reporter gene, mitochondrial permeability pore opening assay or viability analyses, or in 6 cm plates (Sarstedt, Montreal, Qc) at a density of 400,000 cell/plate for protein analyses. Cells were cultured overnight in DMEM high glucose (Life technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Life technologies) and 1% penicillin/streptomycin (Life technologies).

Reporter gene analyses were performed using the Cignal Antioxidant Response Reporter (luc) Kit or the Cignal Heat Shock Response Reporter (luc) Kit (Quiagen Cat# CCS-5020L and CCS-4023L, Toronto, ON). Transfection of H9c2 cells was performed at the moment of plating using lipofectamine 2000 (Thermo-Fisher scientific, Waltham, MA) following manufacturer's recommendations. Stimulations were performed after 24 h transfection. Cells were stimulated with the different compounds for 5 h in DMEM high glucose 1% FBS, then allowed to recuperate for 2 h in DMEM high glucose 10% FBS. Firefly and Renilla luciferase were measured using Dual-Glo Luciferase assay System (Promega, Madison, WI). Luminescence was measured using a Victor3 1420 Multilabel counter running Wallac 1420 Workstation software V3.0 (Perkin Elmer, Waltham, MA). Values were normalized to fold induction compared to corresponding dimethyl sulfoxide (vehicle, normalized to 1).

For selected signalling analyses (ERK and Akt activation, and HSP70 and HO-1 expression), cells were stimulated with the different compounds for 4 h in DMEM high glucose 1% FBS, then allowed to recuperate for 3 h in DMEM high glucose 10% FBS, then processed for Western blot (See protein expression).

For viability screening tests, cells were pre-treated with different compounds or vehicle (Dimethyl sulfoxide (DMSO), Sigma-Aldrich Canada, Oakville, ON; final concentration < 1%v/v) in DMEM high glucose 1% FBS for 1 h, then cells were cultured in DMEM low glucose (Life technologies), serum starved and placed in hypoxic conditions (< 1% O_2) for 48 h in an hypoxia chamber (Billups-Rothenberg, Del Mar, CA) (Ischemic stress) or cultured in DMEM no glucose (Life technologies), serum starved and placed in hypoxic conditions (< 1% O_2) for 18 h, then reoxygenated in DMEM high glucose for 6 h (I/R stress). Stress conditions were selected following preliminary tests (data not shown).

Follow-up concentration-response tests were performed similarly, but stimulation was performed at the moment of reoxygenation for 1 h, and then reoxygenation was continued in DMEM high glucose for an additional 5 h. Cell viability was measured using the Live/dead kit (Life technologies) according to the manufacturer's recommendations as previously reported [10]. Nuclei were stained with Hoechst 33342 (Life technologies). Images were captured and analysed using a High content screening system Operetta, running Harmony High-Content Imaging and Analysis software ver. 4.1 (Perkin Elmer, Waltham, MA). Viability was calculated as Alive cells in stressed conditions/Alive cells in non-stressed condition*100 to account for the loss of observable dead cells with prolonged stress.

2.3. Mitochondrial permeability transition pore (mPTP) opening assay

mPTP opening assessment was performed at reoxygenation using the Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit (Invitrogen Eugene, OR) following manufacturer recommendations. Cells were loaded with calcein and $CoCl_2$ at the moment of reoxygenation. After 15 min, images were taken and calcein fluorescence was measured using a High content screening system Operetta, running Harmony High-Content Imaging and Analysis software ver. 4.1 (Perkin Elmer). If calcein escapes the mitochondria following mPTP opening, fluorescence is quenched by $CoCl_2$ [11]. The higher the detected fluorescence, the lower the mPTP opening. Fluorescence data was normalized to DMSO (vehicle, 100%).

2.4. Animals

Male Lewis rats (250–300 g, Charles River, St Constant, Qc) were used in *ex vivo* experiments. Animals were housed in a temperature controlled facility, with 12 h day/night cycle. All animals were handled according to the Guide for the Care and Use of Laboratory Animals [12]. All experiments were approved by the institutional animal care and use committee of the CHUM Research Center, protocol number: CM14044NNr.

2.5. Ex vivo studies

Thirty-two rats were randomly assigned to the following experimental groups: Vehicle (N = 7), Celastrol 1, 0.1 or 0.01 μmol/L, Analog 1 0.01 μmol/L or Analog 3 0.01 μmol/L (N = 5 each). Under isoflurane anesthesia, rats were injected with Heparin (i.p., 1000 I.U./kg, Novartis, Dorval, Qc), then medial thoracotomy with rapid heart excision was performed. Hearts were immediately submerged in ice cold Krebs buffer (in mmol/L: NaCl 113, KCl 4.5, NaH_2PO_4 1.6, $CaCl_2$ 1.25, $MgCl_2 + 6H_2O$ 1, D-Glucose 5.5, $NaHCO_3$ 25) for transport to the *ex vivo* system. The hearts were rapidly fastened *via* the aorta to a metallic cannula using a suture loop and perfused in a Langendorff system (Radnoti, Monrovia, CA) with a constant aortic pressure of 55–60 mm Hg, using Krebs buffer at 37 °C, bubbled with 5% CO_2 balanced O_2. A latex balloon connected to a pressure transducer was inserted into the left ventricle (LV) *via* left atria and adjusted to 15 mm Hg (LV preload). Hearts were paced at a physiological rate of 300 bpm [13]

and allowed 20 min of stabilization before starting experiments.

Intraventricular pressures were continuously monitored (Sampling: 1000/s) using a PowerLab 8/30 data acquisition system (ADinstruments, Colorado Springs, CO), and data was recorded and analysed off-line using LabChart pro v.7.3.7 software (ADinstruments) for calculation of average end diastolic pressure (EDP), delta pressure (maximum systolic pressure-minimal pressure), maximum rate of pressure increase (+dP/dt) and decrease (−dP/dt), and contractility index (+dP/dt divided by pressure at +dP/dt).

To ensure that Celastrol, analogs or vehicle (DMSO) were in contact with the heart from the very start of the reperfusion period, the system was primed at the moment of inducing warm global ischemia, achieved by stopping cardiac pace and perfusion for 30 min. Reperfusion was started using Krebs buffer with Celastrol (1, 0.1 or 0.01 μmol/L), Analog 1 0.01 μmol/L, Analog 3 0.01 μmol/L or vehicle (DMSO) for 10 min, then continued (Krebs buffer) for a total reperfusion time of 120 min. Pacing was reengaged once stable rhythmic contractions were observed.

Coronary effluent was collected for 5 min and measured (coronary flow) at the end of the stabilization period, at 5 min reperfusion and then every 15 min for 60 min, and samples kept at −80 °C for later analyses. For detection of Troponin T, eluent samples were evaluated at the Clinical biochemistry department of the CHUM Hospital using high sensitive Troponin T assay by electrochemiluminescence method (ECLIA; COBAS analyser, Roche Diagnostics Canada, Laval, Qc).

At the end of reperfusion, hearts were weighed and sliced transversally (6 slices, 1–2 mm thick). One slice per heart was immediately snap frozen for measuring protein expressions. Other slices were colored with 5% 2,3,5-Triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich Canada) in phosphate buffer saline pH 7.4 (PBS, Life technologies) for 20 min at 37 °C, then washed twice with PBS and fixed with 10% formalin overnight. Infarcted areas appeared white and viable tissue was stained brick red. Slices were weighted, then images of both sides were taken using a Stemi 508 Stereo microscope coupled to an AxioCam ERc 5s camera and processed with Zen 2.3 imaging software (Carl Zeiss Canada, Toronto, ON). Planimetric image analyses were performed using ImageJ 1.51h shareware (NIH, Bethesda, MD). Infarct area was normalized to slice weight and averaged for total hearth weight.

2.6. Protein expression

Western blot analyses in cells or tissues were performed as described [10]. In short, cell or tissues were homogenized in NP-40 buffer (in mmol/L: HEPES: 50, EDTA: 4, Na_3VO_4: 1, NaF: 10, PMSF: 1, sodium pyrophosphate 1, and 1% NP-40). Protein content was determined using the Bradford method (Bio-Rad, Saint-Laurent, Qc). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and electroblotted to a polyvinylidene fluoride membrane (Millipore Canada, Etobicoke, ON). After blocking with 5% skimmed milk, membranes were incubated with anti-HSP70 (1:1000, Cat# ADI-SPA-810-D), anti-HO-1 (1:2000, Cat# ADI-SPA-895) (both from Enzo Life Sciences Farmingdale, NY), anti-phospho-Akt (ser473) (1:1000, Cat# 9271), anti-phospho-p44/p42 (Thr202/Tyr204) (1:1000, Cat# 9106), anti-Akt (1:2000, Cat# 9272), anti-p44/p42 (1:2000, Cat# 9102, all from Cell Signaling Technology, Danvers, MA) and GAPDH (1:10,000, Cat# G9545, Sigma-Aldrich, Oakville, ON, loading control), then membranes were incubated with corresponding horseradish peroxidase-conjugated anti-mouse or anti-rabbit (1:4000, Cat# sc-2005 and Cat# sc-2004, Santa Cruz Biotechnology, Dallas, TX). Signals were visualized by Western Lightning ECL Pro (PerkinElmer). Images were analysed using ImageJ 1.51h shareware (NIH, Bethesda, MD).

2.7. Statistical analyses

Data are expressed as mean ± standard error or median with 95% confidence interval. ANOVA test was used for group comparison of non-repeated measurements. For repeated measurements, linear mixed-effect models were used to compare groups (MIXED procedures in SAS software, version 9.3) (SAS Institute, Cary, NC). Between-group differences were assessed. For non-normally distributed measurements, such as indexes and ratios, a log transformation of the measurements was used. For hemodynamic measurements, as much as 200 measurements per rat per time point were used in the model, with each single measurement weighted accordingly (*i.e.* 1/200). For concentration-response data, concentration was transformed to Log concentration and plotted on the X axis and fold change *vs.* DMSO was plotted on Y axis. Non-linear variable slope regression curve fit was performed Using Prism 5 software (GraphPad Software, La Jolla, CA). For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Celastrol molecular structure allows for heat shock response element (HSE) and antioxidant response element (ARE) activation, downstream expression of the cardioprotective proteins HSP70 and HO-1, and activation of the ERK1/2 and Akt pathways

To efficiently screen several compounds and conditions for specific pathway activation underpinning cardioprotective effects related to HSP90 activity modulation, we used selected reporter gene assays in H9c2 cells. From the commercially available classical HSP90 modulators tested, celastrol demonstrates to be the most effective inducer of HSE and ARE responses (all 1 μmol/L, Fig. 1).

Furthermore, synthetic analogs of celastrol tested at the same (1 μmol/L) concentration (Fig. 2), confirm the effectiveness of the celastrol family of compounds to generate a HSE and AR response. Chemical modifications in the celastrol backbone 'E' ring provided the highest activation of both HSE and ARE (Analog 1, Analog 3) (Fig. 2).

Other concentrations were also studied, with similar results demonstrating maximal activation in the micromolar range (Data not shown). Detailed concentration/response analyses of celastrol, Analog 1 and Analog 3 show that, compared to celastrol, Analog 1 and Analog 3 provided a higher maximum HSE fold change, and Analog 3 show lower ARE effective concentration 50% (EC50) (Fig. 3).

Downstream expressions of the cardioprotective, HSE- and ARE-dependant HSP70 and HO-1 proteins are increased an average of 8.5 and 9.5 times respectively upon stimulation with celastrol (Fig. 4). Celastrol and its two synthetic Analogs 1 and 3 activate both ERK1/2 and Akt survival kinase pathways as demonstrated by increased protein phosphorylation (Fig. 4).

3.2. Only celastrol-type HSP90 inhibitors protect against hypoxic and I/R stresses

To evaluate if the observed pathway signalling changes functionally correlate with viability, H9c2 cardiomyoblasts were subjected to ischemic and ischemia/reoxygenation (I/R) stresses. Only compounds with a celastrol-like structure and known to modulate HSP90 activity through hindrance of its association with its essential co-factor CDC37, show cytoprotective effects. Cardioprotection is achieved when treatment is initiated prior to hypoxia (Fig. 5A). Since none of the classical HSP90 inhibitors tested show protective effects, only celastrol and its analogs were further studied for I/R *in vitro* assays. The best protective effects were observed with Analog 1, Analog 3 and Celastrol (Fig. 5B). Of note, these same analogs demonstrated higher induction of both HSE and ARE responses (Figs. 2 and 3) with a resulting increase in HSP70 and HO-1 protein expression and activation of ERK1/2 and Akt kinases (Fig. 4).

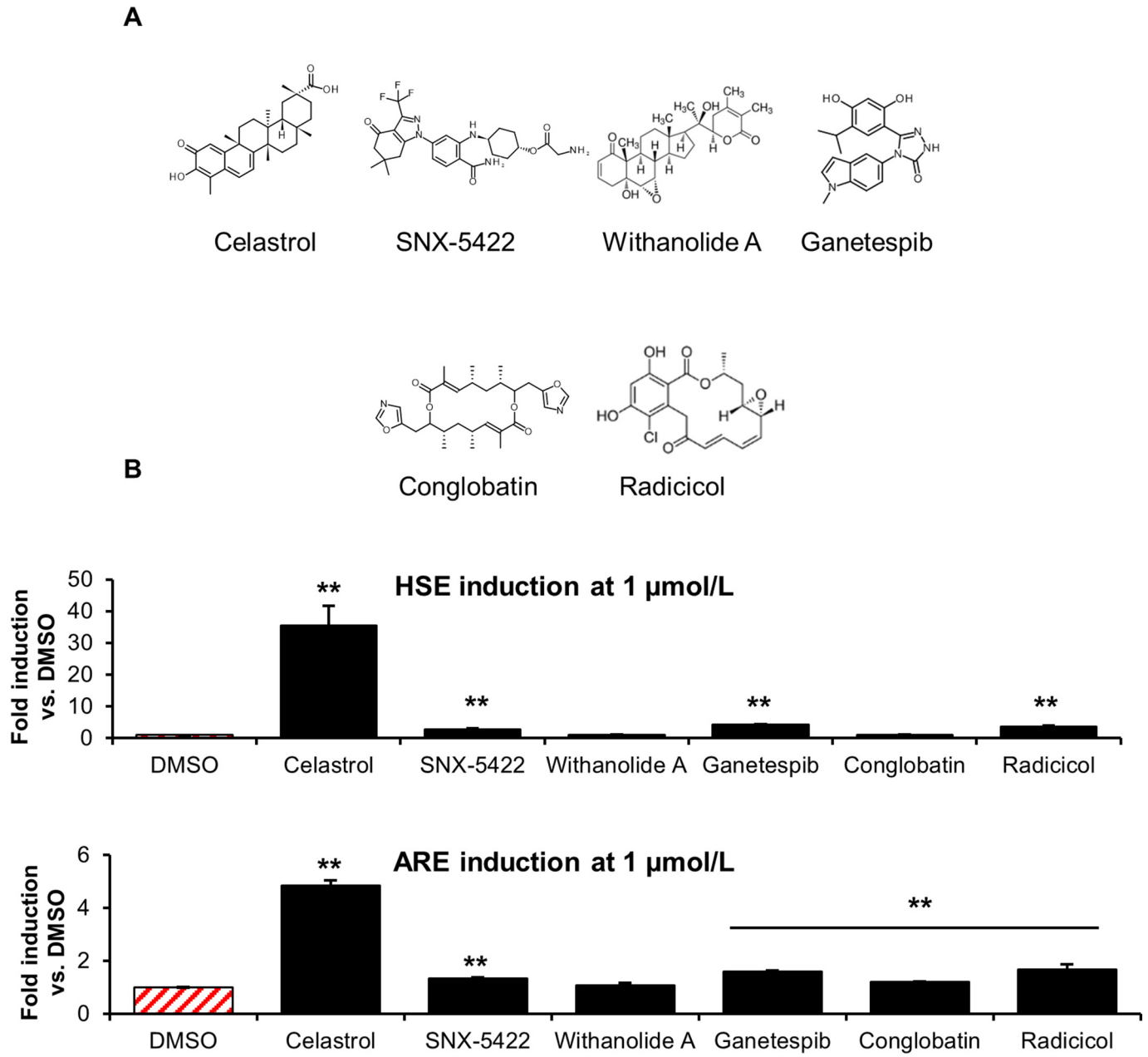

Fig. 1. (A) Chemical structure of the HSP90 modulators tested. (B) Normalized to DMSO/vehicle induction of the transcription factors heat shock response element (HSE) or antioxidant response element (ARE) following 4 h treatment and 3 h recuperation. N = 6 experiments, each compound 3 repeats per experiment. **P < 0.01 *vs.* DMSO.

3.3. Celastrol and its structurally-related Analogs 1 and 3 protect from I/R injury and prevent the opening of the mitochondrial permeability transition pore (mPTP)

In the I/R *in vitro* model, celastrol, Analog 1 and Analog 3 are protective despite being introduced at the moment of reoxygenation, with a peak effect at 1 µmol/L. At this concentration, Analog 1 demonstrates better protective effectiveness when compared to celastrol (Fig. 6A). Mitochondria are vitally important in cell fate in the context of I/R injury. During early reoxygenation, prolonged opening of the mPTP leads to the equilibration of ions across membranes, collapse of the membrane potential, ATP depletion, matrix swelling, and the release of pro-apoptotic proteins, all together leading to cell death. Using the same I/R *in vitro* model, treatments with celastrol, Analog 1 and Analog 3 at the moment of reoxygenation significantly reduced early (15 min) mPTP opening (Fig. 6B).

3.4. Celastrol and Analogs 1 and 3 improve cardiac functional recovery and reduce tissue damage in an ex vivo Langendorff I/R heart injury model

Cytoprotective effects of celastrol and synthetic analogs were demonstrated *in vitro* with underlying survival pathways activation. To thoroughly evaluate integrated contractile and relaxation functions at the whole organ level with complete control of confounding variables such as pre- and after-load and heart rate, the retained compounds from *in vitro* screens were tested using an *ex vivo* Langendorff heart perfusion model with constant pressure, pacing and pre-determined pre-load. Compared to vehicle-treated control hearts, celastrol 0.1 µmol/L significantly improves cardiac functional indexes, with improvement of systolic function (+dP/dt), contractility index, generated delta pressure, diastolic function (−dP/dt) and relaxation (lower end-diastolic pressure) (Fig. 7, Supplementary Table S1). Similarly, Analog 1 improves cardiac functional recovery at 1 log lower concentration

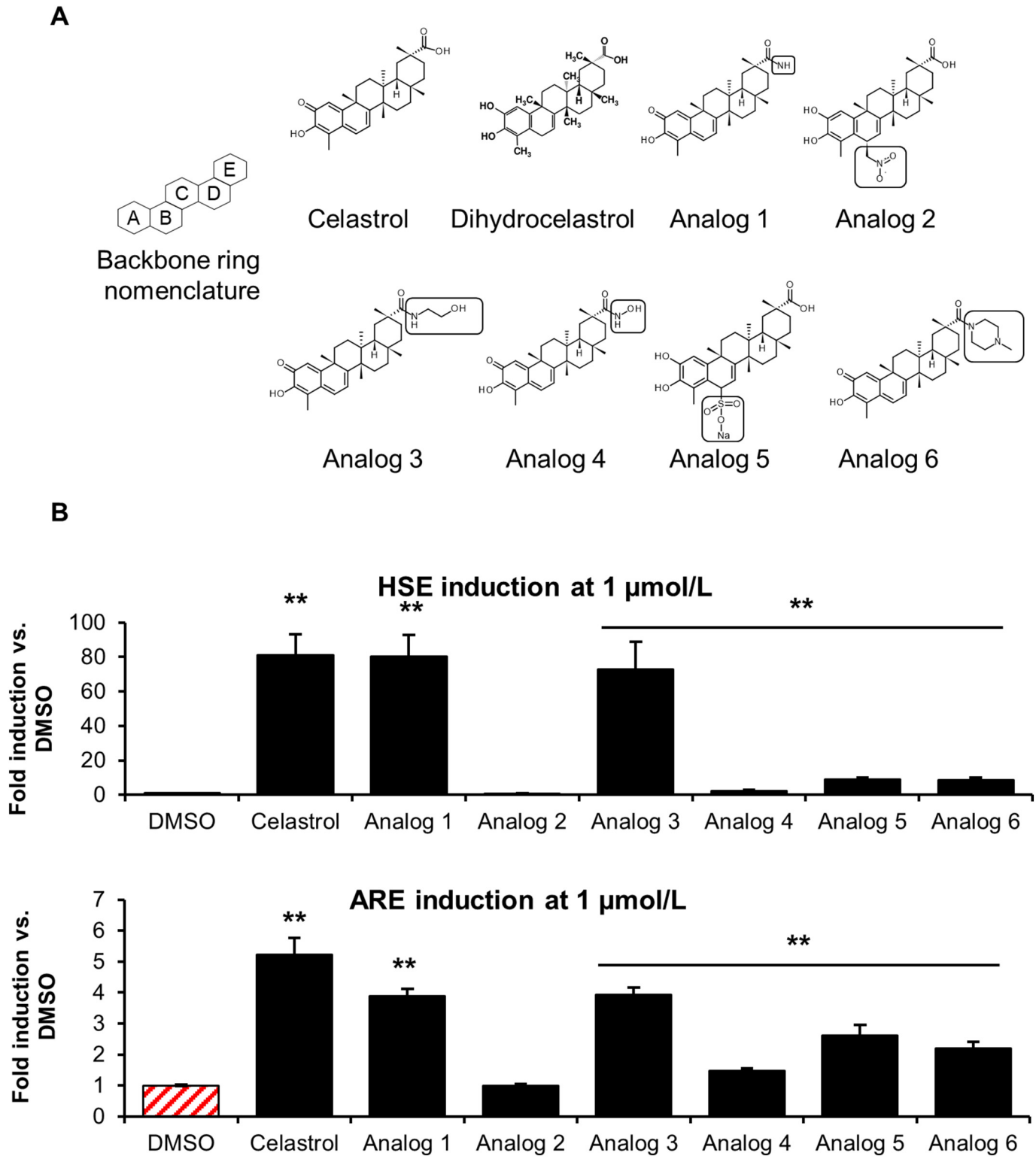

Fig. 2. (A) Chemical structure of synthetized triterpenoid analogs based on Celastrol or Dihydrocelastrol. (B) Normalized to DMSO/vehicle induction of the HSP90 downstream transcription factors heat shock element (HSE) or antioxidant response element (ARE) following 4 h treatment and 3 h recuperation. N = 6 experiments, each compound and condition 3 repeats per experiment. **P < 0.01 *vs.* DMSO.

(0.01 µmol/L). Celastrol and Analog 3 at 0.01 µmol/L had no significant effect compared to vehicle. Interestingly, Analog 3 0.01 µmol/L demonstrates improved contractility (+dP/dt) and relaxation (−dP/dt) when compared to the same concentration of celastrol, suggesting a superior protective potential (Supplementary Table S1).

Celastrol 0.1 µmol/L significantly abrogates ischemic damages as demonstrated by reduced infarct volume (measured by TTC staining) and cardiac high sensitive Troponin T (hs-cTnT) release in coronary effluent compared to vehicle-treated hearts (Fig. 8). Other concentrations tested had no effect (data not shown). Analog 1 at 0.01 µmol/L reduces both infarct volume and hs-cTnT release. Analog 3 at 0.01 µmol/L reduces infarct volume and has tendency to reduce hs-

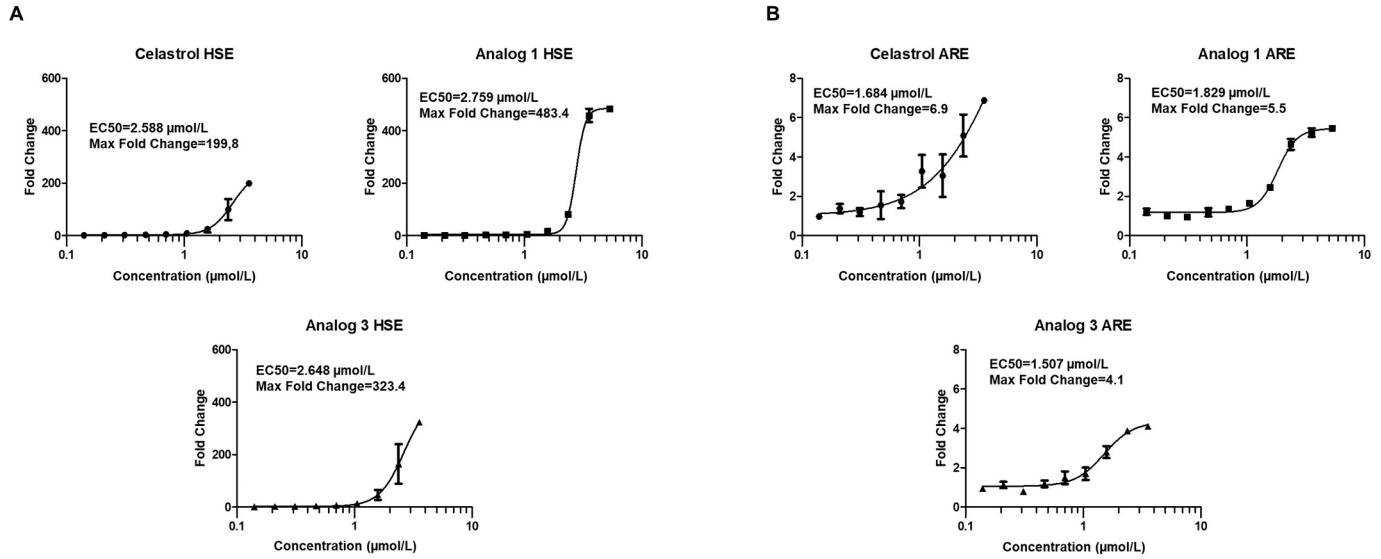

Fig. 3. Concentration-response curves for (A) HSE and (B) ARE activation from selected compounds showing *in vitro* differences effective concentration 50% (EC50) and maximum fold change after 4 h treatment and 3 h recuperation. N = 4 experiments, each point 3 repeats per experiment.

cTnT release (Fig. 8).

3.5. Celastrol and Analogs 1 and 3 differentially modulate cardioprotective pathways in an ex vivo Langendorff I/R heart injury model

Signalling pathway analyses from tissues taken at the end of

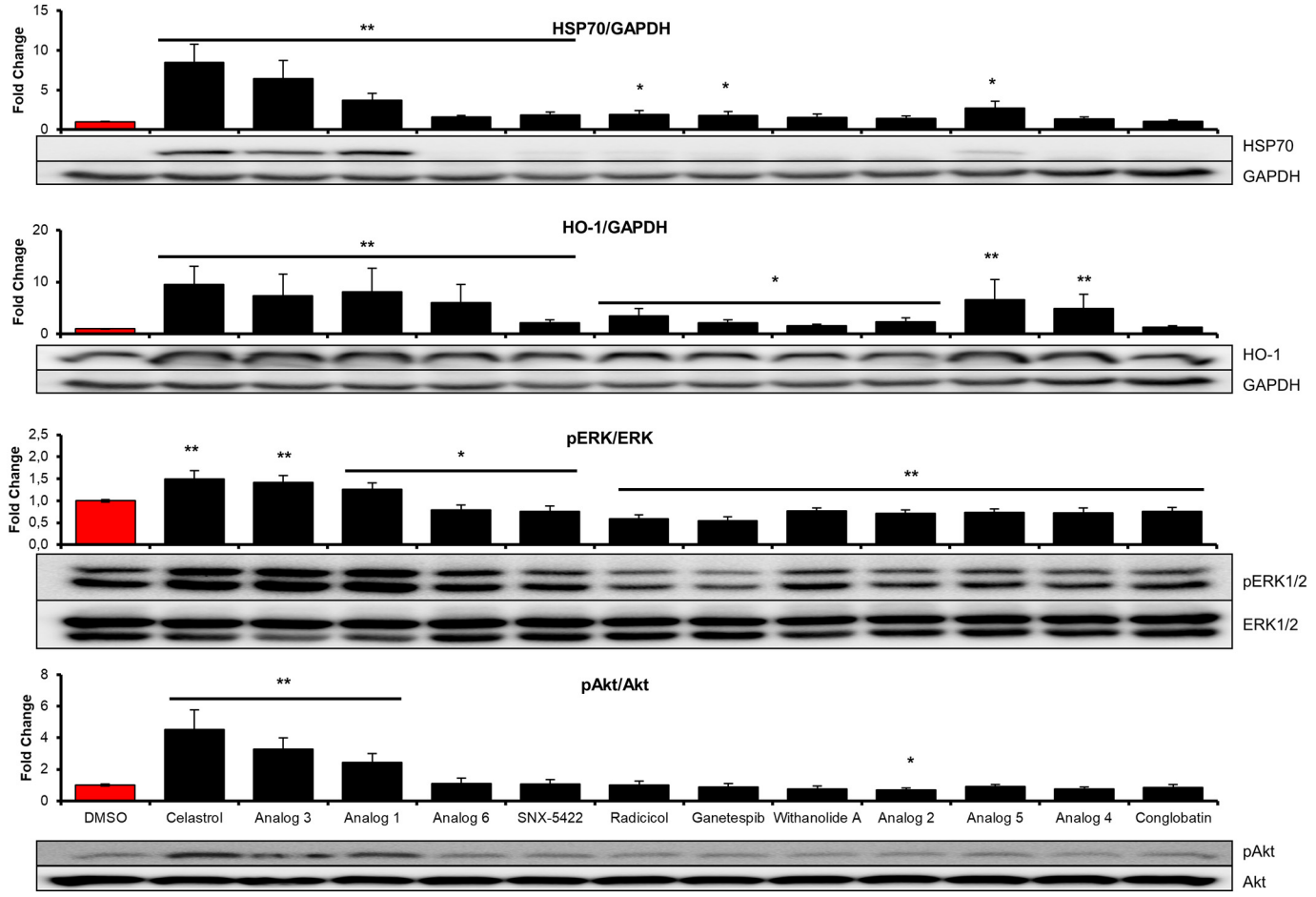

Fig. 4. Bar graphs showing the relative expression or phosphorylation of the cardioprotective proteins HSP70 and HO-1 and the survival kinases ERK1/2 and Akt following 4 h stimulation and 3 h recuperation in H9c2 cardiomyoblasts. All stimulations at 1 µmol/L. N = 6 experiments for each compound and condition. *P < 0.05, **P < 0.01 *vs.* DMSO.

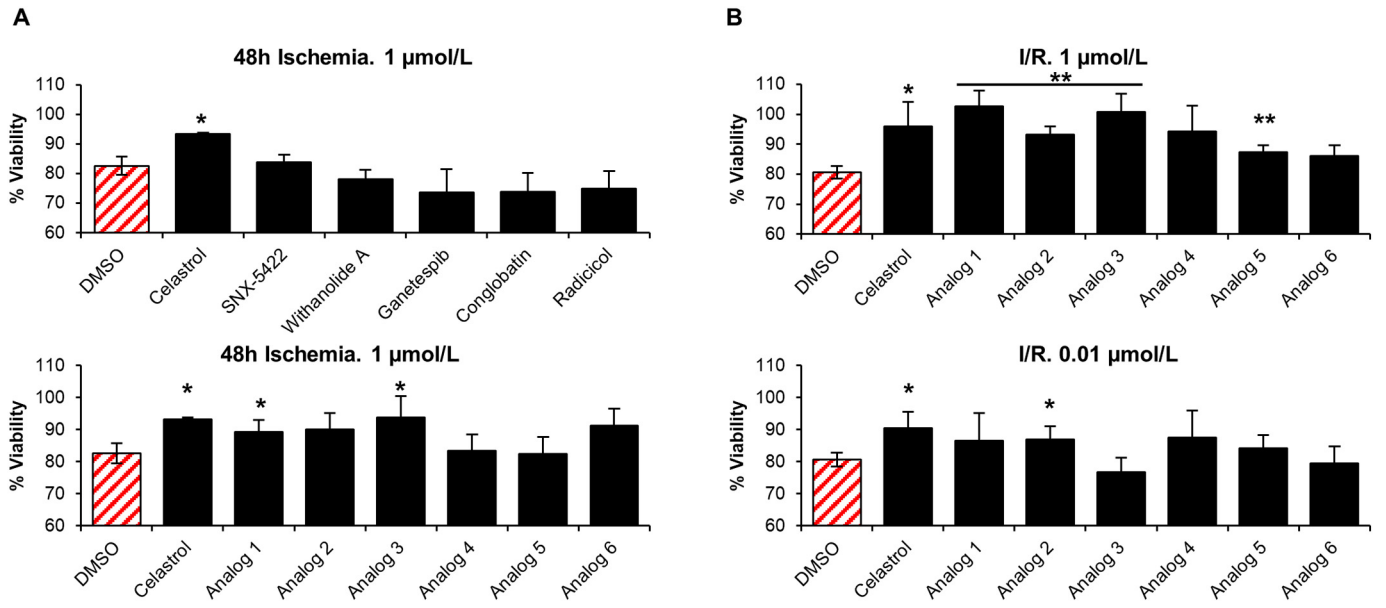

Fig. 5. Effect of 1 h treatment prior to (A) 48 h ischemia or (B) 18 h ischemia followed by 6 h reperfusion, with the different compounds in H9c2 cells. N = 5 experiments. 3 repeats per experiment. *P < 0.05, **P < 0.01 *vs.* DMSO.

experiments (2 h reperfusion time) demonstrate that celastrol 0.1 µmol/L induces the expression of HSP70, and activates both ERK1/2 and Akt phosphorylation (Fig. 9). Analog 3 (0.01 µmol/L) induces both HSP70 and HO-1 expression, and activates both ERK1/2 and Akt phosphorylation. Analog 1 (0.01 µmol/L) induces both HSP70 and HO-1, and activates Akt phosphorylation but not ERK1/2 at this time point.

4. Discussion

This study compares for the first time structurally different HSP90 modulators for cardioprotective effects in a model of myocardial ischemia/reperfusion injury. Results show that, from various classes of HSP90 modulators tested, only the natural compound celastrol and two of its synthetized analogs, reduce cellular death *in vitro* in conditions mimicking myocardial infarct. Furthermore, the infarct sparing effect was demonstrated in the whole organ using *ex vivo* heart perfusion system, with improved cardiac function and reduced tissue injury. Importantly, these compounds demonstrate cardioprotective properties when introduced at the clinically relevant moment of reperfusion to prevent I/R-related injury. Finally, the *ex vivo* cardioprotective effect of Analog 1 is observed at 10 times lower concentration compared to Celastrol. In addition, Analogs 1 and 3 show different cardioprotective kinase activation patterns, suggesting different target kinetics or the activation of additional cardioprotective pathways.

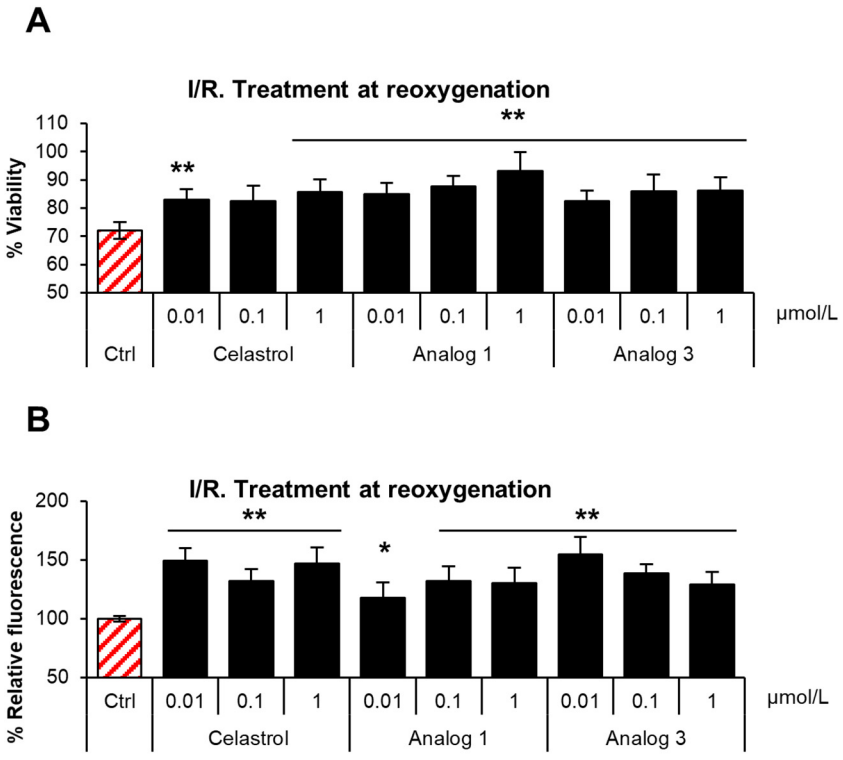

Fig. 6. Concentration/response effect of 1 h treatment of H9c2 cells with selected compounds at the moment of reoxygenation. (A) Viability assay. N = 6 experiments, 4 repeats per experiment. (B) Mitochondrial permeability transition pore activity normalized to control (100%). Mitochondrial calcein fluorescence is inversely related to pore opening. N = 5 experiments, 3 repeats per experiment. *P < 0.05, *P < 0.01 *vs.* DMSO.

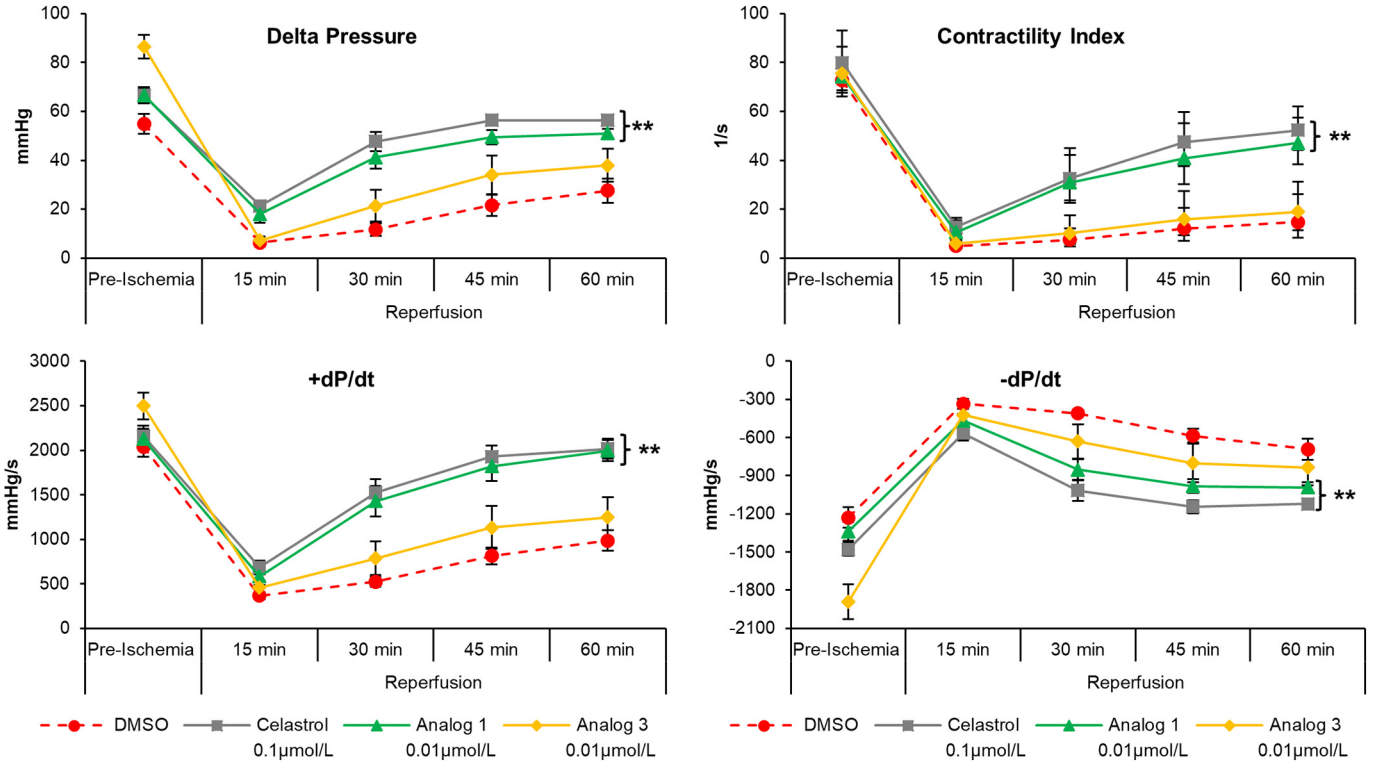

Fig. 7. Functional changes observed following treatment at reperfusion in Langendorff hearts subjected to ischemia/reperfusion stress. Delta pressure: maximum pressure minus end diastolic pressure. N = 5 hearts/group (7 for DMSO (Vehicle)). **P < 0.01 *vs.* DMSO (Vehicle).

Celastrol modulates HSP90 activity by disrupting binding of the CDC37 co-factor to HSP90 [7,8]. Our group has previously shown celastrol's infarct-sparing effect with daily administrations of the compound in a model of permanent coronary ligation. At 2 weeks post myocardial infarction, celastrol reduced infarct size, peri-infarct infiltration of inflammatory macrophages and myofibroblasts, reduced fibrotic scarring, and preserved cardiac function [10]. Celastrol activates Akt and ERK1/2 survival kinases, the transcription factor HSF1 targeting HSE, which represents the main regulator of HSP expression [14], and cardioprotective HO-1 protein [10]. These pathways are similar to those being activated by ischemic conditioning. Conditioning induces several cardioprotective pathways, notably the reperfusion injury salvage kinase (RISK) pathway (which includes ERK1/2 and Akt) [15], and activates several transcription factors including the Nuclear factor (erythroid-derived 2)-like 2 (NRF2) and HSF1 [1], with downstream induction of the antioxidant response and the heat shock response, exemplified by an increase in HO-1 and HSP70 [16].

The HSP90 chaperone has an important role in cardiac ischemic conditioning. Inhibiting HSP90 expression impedes ischemic preconditioning in H9c2 cardiomyoblasts [17], and overexpression of HSP90 protects from I/R stress in pig hearts [18]. HSP90 interacts with over a thousand client proteins with diverse functions including cell survival and proliferation [19,20]. HSP90 modulators can modify its activity by interacting with the N- or C-terminal domains, and/or disrupting the co-chaperone or client protein binding [21]. The cellular effect of each type of interaction is different. C- or N-terminal ATP binding pocket modulators induce widespread degradation of client proteins, N-terminal induce a robust heat shock response, while the C-terminal induce little to none [20,21]. On the other hand, disruptors of co-chaperone binding modify client protein activation without client degradation [22].

Modulation of HSP90 activity by celastrol is cardioprotective [10], nevertheless, other HSP90 modulators have conflicting results regarding cardioprotection. For instance the N-terminal modulator geldanamycin protects neonatal rat cardiomyocytes from ischemic stress following 4 h pretreatment [23], while inhibits ischemic preconditioning *in vitro* [17], cardiac ischemic postconditioning *in vivo* [24], and increases cellular damage when given at reperfusion in an *ex vivo* I/R model [25]. Similarly, 4 h pretreatment with radicicol protects neonatal rat cardiomyocytes from ischemia [26], yet short term (10 min) pre-treatment does not offer cardioprotection on an *in vivo* I/R model [27], demonstrating divergent responses of different HSP90 targeting molecules in the context of ischemia and ischemia/reperfusion injury.

We therefore assessed HSP90 modulators that interact with the N-terminal (SNX-5422, Radicicol and Ganetespib) [21,28] and inhibitors of the CDC37-HSP90 binding (Conglobatin, Withanolide A and Celastrol) [7,29,30] for potential cardioprotective effects *in vitro*. The most efficient inductor of both HSE and ARE was celastrol. In parallel, celastrol shows marked activation of both ERK1/2 and Akt kinases. These signalling changes result in cellular protection from ischemic stress, an effect not observed with the non-celastrol-like HSP90 modulators tested, suggesting that the celastrol family of compounds are prime candidates for cardioprotection.

Chemical modifications of the celastrol backbone affected HSE and ARE activation. Modifications on 'B' ring (Analogs 2 and 5) induce a reduction of HSE activity, HSP70 expression and Akt phosphorylation, while maintaining some of the ARE activity and HO-1 expression, an effect similar to the one observed with the addition of big chemical structures on the 'E' ring (Analog 6). These signalling changes translate into a reduced cellular protection compared to celastrol. On the other hand, small structures added to the 'E' ring, like those in Analog 1 and 3, induce an increase in HSE maximum fold change, while maintaining other pathways activities, which translates in enhanced cardioprotection. These results show that celastrol has two molecular targets for activity modulation, ring 'B' and ring 'E', and that the synergic effects of various pathways activated by celastrol, Analog 1 and 3 are crucial for an efficient cardioprotection.

Protective effects against I/R stress of celastrol, Analog 1 and Analog 3 are also present when added at the clinically relevant moment

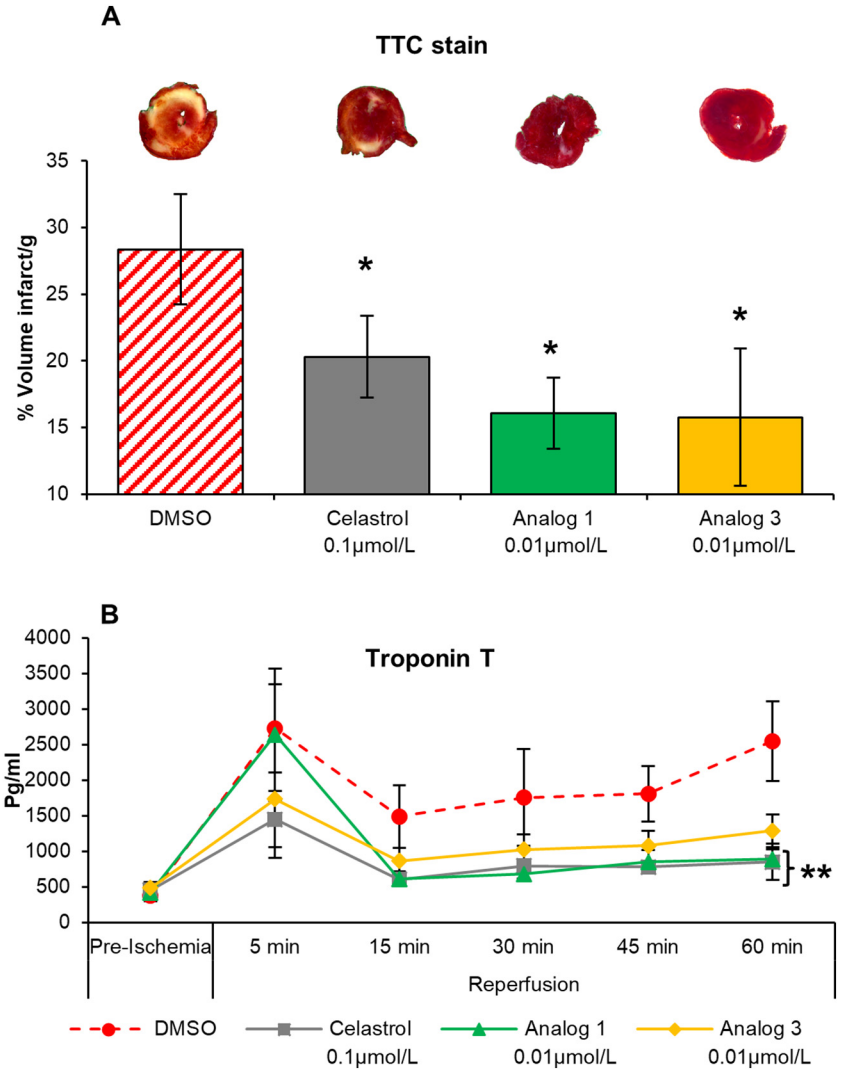

Fig. 8. (A) Infarct volume (measured by TTC staining, viable tissue stains brick red) and (B) biochemical (troponin T release) effects of treatment at reperfusion in Langendorff hearts subjected to ischemia/reperfusion stress. N = 5 hearts/group (7 for DMSO (Vehicle)). *P < 0.05, **P < 0.01 *vs.* DMSO (Vehicle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of reperfusion *in vitro*. During reperfusion opening of the mPTP plays a significant role in cell fate. mPTP channels are important in the regulation of mitochondrial cell death pathways. Prolonged opening has been linked to mitochondrial swelling and release of pro-apoptotic cytochrome C [31]. The abrupt normalisation of intracellular pH and the increase in free radical production observed in the first minutes of cardiac reperfusion opens the mPTP, leading to cell death [32,33]. mPTP opening was significantly reduced by the use of any of the three selected molecules at the moment of reperfusion, an effect related to cardiac cell protection [34]. Cardioprotective mechanisms reducing mPTP opening include reduction of cellular oxidative stress and activation of RISK kinases [32]. The observed activation of Akt following treatment might explain this protective effect, nevertheless, the exact mechanisms activated by each analog and concentration might be different and need further characterization.

Post-ischemic cardioprotection was confirmed in the whole organ, with reduction of infarct size in Langendorff heart I/R injury model. Structural protection was corroborated by a decrease in the acute hs-cTnT release, a specific marker of cardiac cell damage [35], in heart eluent fractions during reperfusion.

Infarct size has a direct correlation with late ventricular dilatation, and an inverse correlation with ejection fraction [36]. Thorough functional measurements using the Langendorff system showed that celastrol 0.1 µmol/L and Analog 1 at 0.01 µmol/L significantly improve systolic (+dP/dt, LV maximum pressure, LV contractility index) and diastolic (−dP/dt, LV end diastolic pressure) cardiac function immediately after I/R. While celastrol failed to produce a statistically significant effect on cardiac functional parameters at 0.01 µmol/L, it is interesting that Analog 3 0.01 µmol/L improves +dP/dt and −dP/dt compared to equivalent concentration of celastrol, showing that Analog 3 is more potent. *Ex vivo* Analog 1 has the highest cardioprotective potency at 0.01 µmol/L compared to other compounds. In parallel, an increase in cardioprotective HO-1 and HSP70 expression was observed both *in vitro* (all 3 selected molecules) and *ex vivo* (Analog 1 or 3).

HO-1 is a potent and highly inducible antioxidant enzyme, whose function limits I/R-induced structural and functional cardiac damage [37]. In *ex vivo* I/R rat models, HO-1 expression is induced at the moment of reperfusion, with mRNA beginning to increase significantly at 15 min, and its activity remaining elevated after 2 h reperfusion [38,39]. On the other hand, HSP70 is a chaperone protein that impedes aggregation of misfolded proteins and facilitates protein folding [40]. HSP70 overexpression reduces I/R damage *in vitro* [41], similarly, adenoviral transfection of HSP70 reduces functional and infarct size *ex vivo* [42] confirming its cardioprotective effects. The increased HO-1 expression of Analog 1 and Analog 3 compared to celastrol may explain, at least in part, its increased protective effects. Nevertheless, oxidative

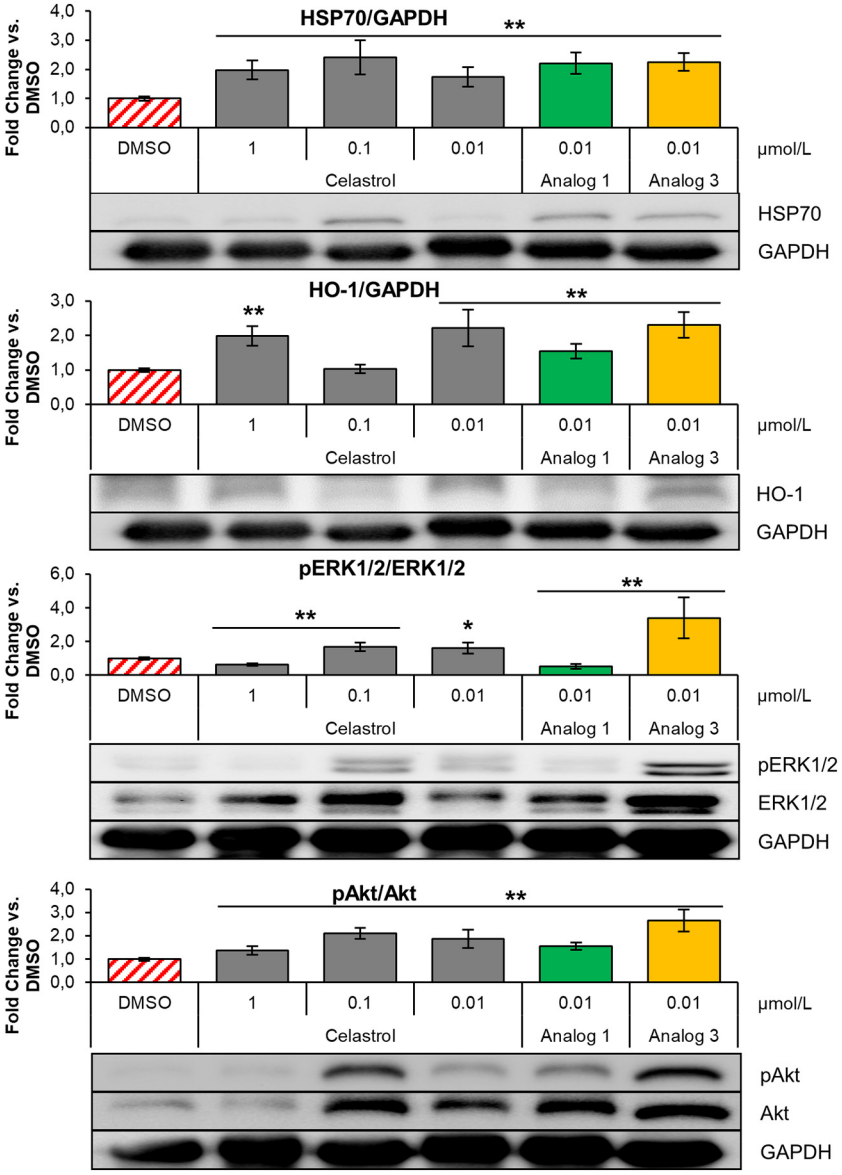

Fig. 9. Bar graphs and representative western blots showing the relative expression or phosphorylation of the cardioprotective proteins HSP70 and HO-1 and the survival kinases ERK1/2 and Akt *ex vivo*. N = 5 hearts/group (7 for DMSO (Vehicle)). *P < 0.05, **P < 0.01 *vs.* DMSO (Vehicle).

stress and mPTP opening is detectable almost immediately after initiation of cardiac reperfusion [43], thus the activation of pre-synthetized fast acting cardioprotective mechanisms, including kinases, is needed for efficient cardioprotection.

The *ex vivo* modulation of fast-acting cardioprotective kinases differ between celastrol and Analog 1 and 3. All of them activate Akt, an effect that has been related to decrease mPTP activation and cardioprotection [15,32] but modulate ERK1/2 differently. Celastrol and Analog 3 increases ERK1/2 activity, an effect generally considered cardioprotective [1]. On the other hand, Analog 1 treated hearts show reduced activity observed at the end of reperfusion period compared to control. Treatment with the antioxidant tyrosol was demonstrated to reduce I/R-induced ERK1/2 activation in H9c2 cardiomyoblasts by reducing oxidative stress at reperfusion, while increasing cell survival [44]. Fast activation of antioxidant mechanisms by Analog 1 compared to other compounds might in part explain the observed reduced ERK1/2 activation coupled to potent cardioprotective effects *ex vivo*. On the other hand, ERK1/2 activation kinetics might be different in Analog 1, requiring detailed dose and time/response experiments to fully characterize its signalling. Those differences imply that Analog 1 may have different pharmacokinetics or might activate additional protective mechanisms compared to its parent compound, allowing for more efficient cardioprotection when used at the moment of reperfusion.

The two described analogs represent a novel multi-targeting approach able to activate multiple (fast and long term) cardioprotective pathways, thereby conveying a more holistic molecular approach to cardiac protection.

Screening was performed using H9c2 cardiomyoblasts, which for some may be viewed as a limitation. However, the H9c2 cell line is a widely used in cardiovascular research, particularly in the screening for cardioprotective compounds [45,46]. H9c2 cells behave similarly to primary cardiomyocytes under ischemia/reperfusion stress *in vitro* [47], thus facilitating translation of results to more clinically relevant models as confirmed in the present study. The use of a short term *ex vivo* Langendorff model might also be seen as a limitation. The Langendorff preparation allows for a thorough structural and functional evaluation, with tightly controlled ischemic and reperfusion times. Nevertheless, it only evaluates the acute changes, due to a decrease of 5–10% per hour in contractile function [48], without observing the long-term effects on cicatrisation, development of ventricular hypertrophy and cardiac

function, among other relevant parameters, that need to be addressed before translation is considered [49]. Finally, in this screening protocol, a fixed molecule concentration and treatment time were used, limiting the mechanistic and concentration/response information gathered. In order to fully define the molecular characteristics and kinetics of the selected compounds, studies involving a range of treatment times with different follow up times, treatment concentrations and durations are required.

5. Conclusion

In this study we show that celastrol, Analog 1 and Analog 3 procure cardioprotective effects observed both in experimental reperfused and non-reperfused myocardial infarction *in vitro* and *ex vivo* models. Of particular interest these compounds protect efficiently when used at the clinically relevant moment of reperfusion. Mechanistic screening shows the need of simultaneous activation of multiple pathways in order to obtain cardioprotection. The most efficient analog, Analog 1, behaves differently to its parent compound, potentiating its cardioprotective effects at lower concentration. These promising compounds should be further investigated with the objective of developing an adjunct therapy to current reperfusion protocols in the clinical setting.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.025.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

CRediT author statement

Henry Aceros: Investigation, visualization, data curation, writing-Original draft. **Shant Der Sarkissian**: Conceptualization, investigation, data curation, writing-review and editing, funding acquisition. **Mélanie Borie**: Investigation, data curation, resources. **Louis-Mathieu Stevens**: Conceptualization, formal analysis, writing-review and editing. **Samer Mansour**: Conceptualization, writing-review and editing. **Nicolas Noiseux**: Conceptualization, investigation, writing-review and editing, supervision, funding acquisition.

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