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Reperfusion-induced injury and the effects of the dithioacetate type hydrogen sulfide donor ibuprofen derivative, BM-88, in isolated rat hearts

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ABSTRACT

Hydrogen sulfide (H_2S) plays an important role in cardiac protection by regulating various redox signalings associated with myocardial ischemia/reperfusion (I/R) induced injury. The goal of the present investigations is the synthesis of a newly designed H_2S-releasing ibuprofen derivative, BM-88, and its pharmacological characterization regarding the cardioprotective effects in isolated rat hearts. Cytotoxicity of BM-88 was also estimated in H9c2 cells. H_2S-release was measured by an H_2S sensor from the coronary perfusate. Increasing concentrations of BM-88 (1.0 to 20.0 μM) were tested in vitro studies. Preadministration of 10 μM BM-88 significantly reduced the incidence of reperfusion-induced ventricular fibrillation (VF) from its drug-free control value of 92% to 12%. However, no clear dose dependent reduction in the incidence of reperfusion-induced VF was observed while different concentrations of BM-88 were used. It was also found that 10 μM BM-88 provided a substantial protection and significantly reduced the infarct size in the ischemic/reperfused myocardium. However, this cardiac protection was not reflected in any significant changes in coronary flow and heart rates. The results support the fact that H_2S release plays an important role mitigating reperfusion-induced cardiac damage.

1. Introduction

Progressive degenerative processes are among the most important challenges in cardiovascular pharmacology. In particular, oxidative stress and chronic inflammation often lead to endothelial and smooth muscle dysfunction (Sinnenberg and Givertz, 2020). Therefore, preventive strategies are highly desirable in this regard, and it seems to be particularly important to identify and develop new and effective cardioprotective agents.

Ibuprofen as a nonsteroidal anti-inflammatory drug (NSAID) (McGettigan and Henry, 2013) is a widely used medication in heart injuries. Ibuprofen acts as an inhibitor of cyclooxygenases (COX-1 and COX-2) thereby blocking the synthesis of prostaglandins. At lower doses (below 1200 mg/day), ibuprofen possesses analgesic and antipyretic effects, while at higher doses (above 2400 mg/day) exerts anti-inflammatory activities (Bushra and Aslam, 2010; McGettigan and Henry, 2013). Although ibuprofen has fewer side effects compared to other NSAIDs (e.g. gastrointestinal bleeding), long term administration of higher doses (600–800 mg three times a day) may increase the risk of a heart attack, stroke, and also causes kidney and liver damage (Bushra and Aslam, 2010; McGettigan and Henry, 2013; Pawlosky, 2013).

Hydrogen sulfide (H_2S) was initially regarded as an environmental hazard and toxic gas, later, it was reported to possess cytoprotective properties under pathological conditions. The endogenous generation of H_2S was initially observed in the central nervous system as a key component that potentiates neuronal circuitry due to its cystathionine beta-synthase activity (Abe and Kimura, 1996; Kimura, 2000). This small gaseous molecule can freely diffuse through cell membranes to

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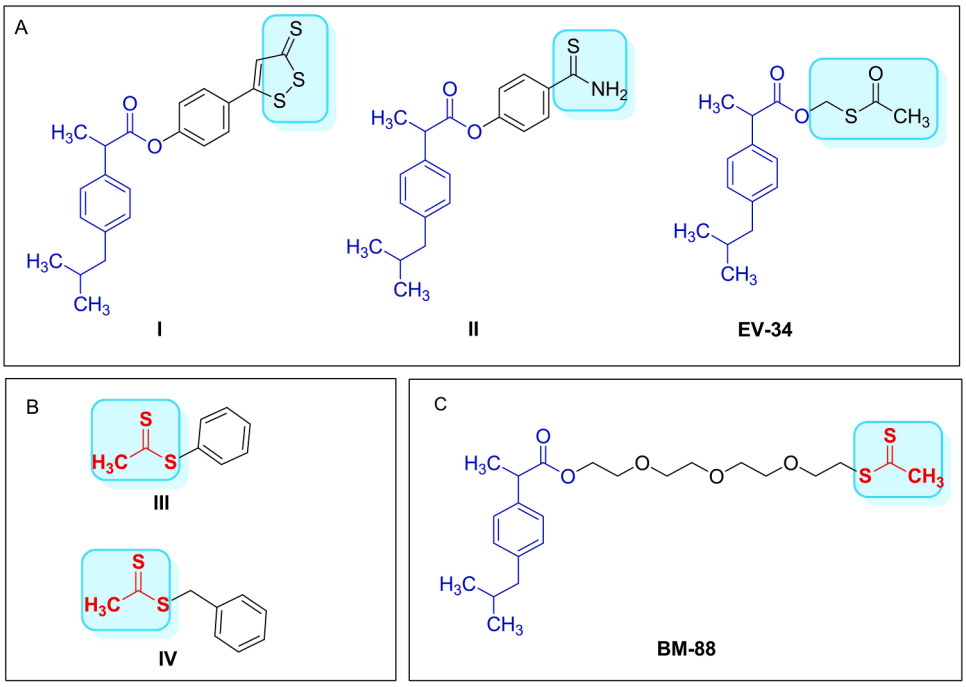


Fig. 1. Examples of H_2S donor ibuprofen (A) (Li et al., 2017; Hassan et al., 2019; Gyöngyösi et al., 2021) and dithioester derivatives (B) (Cerda et al., 2019); this work: structure of a new H_2S donor ibuprofen derivative containing a dithioester moiety (C). The H_2S-releasing group is highlighted with a blue box.

mediate cellular signaling processes, thus modifying several physiological and pathological functions in various cells and tissues (Calvert et al., 2010; Szabó, 2007). It was also reported that one of the cellular targets associated with H_2S is the group of ATP-sensitive potassium channels, because H_2S-induced vasodilatation has been shown to be mediated by the opening of these channels in smooth muscle cells of the vasculature (Calvert et al., 2010; Citi et al., 2020; Martelli et al., 2021; Zhao et al., 2001).

It is not a new idea that H_2S may play an important role in cardiac protection by regulating various redox signalings associated with myocardial ischemia/reperfusion (I/R) induced injury (Citi et al., 2020). For instance, H_2S shows an interaction with reactive oxygen species (Sikura et al.) and nitric oxide (NO) generated during reperfusion, thus contributes to the prevention of I/R-induced mitochondrial damage (Salloum et al., 2012). It was also reported that H_2S is produced in vascular tissues where it mediates smooth muscle relaxation, vasodilation and subsequent cytoprotection via a cGMP independent pathway (Andreadou et al., 2020; Salloum et al., 2012). Furthermore, H_2S is a well-documented signaling molecule and implicated in several pathological processes, including the attenuation of the severity of oxidative stress (Fukuto, 2022; Hosoki et al., 1997; Zhao and Wang, 2002). Additionally, H_2S has an anti-calcification effect in human cardiac valves, providing a novel therapeutic approach to prevent the malfunction of valves in humans (Murphy et al., 2019).

Several pathological conditions are associated with H_2S deficiency, since its endogenous level dramatically drops down in many disorders, such as cardiac ischemia and diabetes (Ansari et al., 2022; Sun et al., 2021a). Therefore, restoring the physiological levels of H_2S by exogenous H_2S-donors might contribute to prevention and/or treatment of vascular inflammatory conditions (Mendiola et al., 2021; Mitidieri et al., 2022). Some natural and synthetic H_2S donor compounds function as prodrugs (Kimura, 2014; Sikura et al., 2020; Sun et al., 2021b; Testai et al., 2016; Zhu et al., 2019) that are able to generate H_2S in biological systems. We and others have reported the synthesis and biological testing of various H_2S donor derivatives of the anti-inflammatory drug ibuprofen (Fig. 1, panel A). Li et al. (2017) and Hassan et al. (2019) prepared ibuprofen derivatives containing dithiolene-thione (**I**) or thiobenzamide (**II**) motifs, and these compunds proved to have strong anti-inflammatory effects (Zhang et al., 2019). Recently, we have reported on the synthesis and pharmacological characterization of a H_2S delivering ibuprofen derivative EV-34 (Fig. 1, A) containing a new type of hydrogen sulfide donor functional group: a bis-acylal bearing a thiolacetate ester moiety (Gyöngyösi et al., 2021). We showed that the hydrolysis of this group in biological milieu provided thiolacetic acid, which resulted in the release of hydrogen sulfide through a reaction cascade described by Liu and Orgel (Liu and Orgel, 1997). On the other hand, Pluth and co-workers (Cerda et al., 2019) synthesized some dithioester derivatives, e.g. **III** and **IV** (Fig. 1, panel B), as new H_2S donors, and demonstrated by in vitro experiments that the release of H_2S from the dithiocarboxylate moiety is triggered by cysteine.

Based on the above results, we designed a new H_2S donor ibuprofen derivative, which, similarly to the dithioesters published by Cerda et al. (2019), contains a dithioacetate functional group as the H_2S-releasing motif (BM-88, Fig. 1, panel C). We hypothesized that both the anti-inflammatory ibuprofen unit and the hydrogen sulfide released under physiological conditions may have protective effects against myocardial-ischemia reperfusion injury. Although ibuprofen is known for its association with incidental atrial fibrillation, on the other hand, it is also known that H_2S inhibits I_{to} potassium channels in cardiomyocytes and regulizes fatal arrythmia in myocardial infarction (Ma et al., 2015b). Therefore, we postulated that evolution of H_2S from our new ibuprofen derivative, BM-88, would compensate the arrythmogenic side effect of the parent ibuprofen. It is important to note that none of the previously prepared H_2S donor ibuprofen derivatives have been studied in a myocardial infarction model.

In the current study, we report on the synthesis of a promising new cardioprotective ibuprofen derivative, BM-88, containing a dithioacetate ester moiety, that evokes protective activities and reduces I/R-induced damage in isolated rat hearts. The newly synthesized compound, BM-88, could reduce the postischemic cardiac damage by its dual activity by regulating the H_2S signaling mechanism and exerting an anti-inflammatory effect.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. General information

Ibuprofen **1** and tetraethylene glycol **3** were purchased from Merck (Germany). Compound **2** (Yan et al., 2016), compound **4** (Caianiello et al., 2021) and dithioacetic acid (Alwaaly et al., 2015) were synthesized according to the literature.

TLC was carried out on Kieselgel 60 F254 (Merck, Darmstadt, Germany) with detection by immersing into ammonium molybdate-sulfuric acid solution followed by heating. Flash column chromatography was done using Silica gel 60 (Merck, Darmstadt, Germany, 0.040–0.063 mm). The ^{1H}NMR (360 MHz, 400 MHz) and ^{13C}NMR (90 MHz, 100 MHz) spectra were registered by a Bruker DRX-360 and DRX-400 spectrometers. Chemical shifts are referenced to Me_4Si (0.00 ppm for ^{1}H) and to the solvent residual signals. MALDI-TOF MS studies were carried out by a Bruker Autoflex Speed mass spectrometer equipped with a time-of-flight (TOF) mass analyzer. In all cases 19 kV (ion source voltage 1) and 16.65 kV (ion source voltage 2) were used. For reflectron mode, 21 kV and 9.55 kV were applied as reflector voltage 1 and reflector voltage 2, respectively. A solid phase laser (355 nm, ≥100 μJ/pulse) operating at 500 Hz was applied to produce laser desorption and 3000 shots were summed. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix and $F_3CCOONa$ as cationising agent in dimethylformamide. NMR and MS spectra can be found in the Supplementary Information (Figures S3-S7).

2.1.2. Synthesis of BM-88

2.1.2.1. Compound 5 (BM-86). Compound **4** (1.74 g, 5 mmol) was dissolved in acetone (30 ml) and tetrabutylammonium bromide (2.0 g, 6.2 mmol) was added. The reaction mixture was boiled for 3 h, and then the solvent was evaporated. The residue was dissolved in dichloromethane (50 ml) and washed with saturated aqueous NaCl solution (20 ml), the organic phase was dried over Na_2SO_4 then filtered and the solvent was evaporated. The residue was purified by flash column chromatography (hexane/acetone 6:4) to yield **5** (560 mg, 44%) as a colorless syrup. MALDI-TOF MS: *m/z* calculated for $C_8H_{17}BrO_4Na^+$: 279.0202 $[M+Na]^+$; found: 279.0208. It was used for the next step without NMR characterization.

2.1.2.2. Compound 6. Compound **5** (512 mg, 2 mmol) was dissolved in acetone (10 ml) and K_2CO_3 (557 mg, 4 mmol) was added under argon gas and was bubbling in the reaction mixture for 15 min. After addition of dithioacetic acid (200 mg, 2.2 mmol) the mixture was stirred for 3 h. The K_2CO_3 was filtered off, washed with acetone and the solvent was evaporated in vacuum. The residue was dissolved in dichloromethane (300 ml) and washed with saturated aqueous $NaHCO_3$ solution and saturated aqueous NaCl solution, the organic phase was dried over Na_2SO_4, then it was filtered and evaporated in vacuum. The product was purified by flash column chromatography (hexane/acetone 7:3) to produce compound **6** (400 mg, yield 75%) as a yellowish syrup. MALDI-TOF MS: *m/z* calculated for $C_{10}H_{20}O_4S_2Na^+$: 291.0695 $[M+Na]^+$; found: 291.0698. ^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 3.81–3.58 (m, 16H, CH_2), 3.48 (t, 2H, $J = 6.3$ Hz, CH_2), 2.84 (s, 3H, CH_3), 2.70 (bs, 1H, OH); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 72.6, 70.7, 70.5, 70.4, 68.1, 61.8, 36.8 (8C, CH_2), 39.3 (1C, CH_3).

2.1.2.3. BM-88. Compound **6** (250 mg, 0.93 mmol) was dissolved in anhydrous dichloromethane (7 ml), and then anhydrous pyridine (0.200 ml) was added, and the reaction mixture was cooled in an ice bath. Following the addition of compound **2** (225 mg, 1 mmol) in anhydrous dichloromethane (4 ml), the mixture was stirred for 3 h, and then 2 ml of water was added. The reaction mixture was diluted with dichloromethane (20 ml), washed with 10% aqueous $NaHSO_4$ and saturated $NaHCO_3$ solutions. The organic phase was dried on Na_2SO_4, filtered and evaporated in vacuum. Finally, the product was purified by flash column chromatography (hexane/acetone 8:2), yielding **BM-88** (220 mg, 52%) as a yellow syrup.

MALDI-TOF MS: *m/z* calculated for $C_{23}H_{36}O_5S_2Na^+$: 479.1896 $[M+Na]^+$; found: 479.1853.

^{1}H NMR (360 MHz, $CDCl_3$): δ (ppm) 7.21 (d, 2H, $J = 8.2$ Hz, aromatic C*H*), 7.08 (d, 2H, $J = 8.1$ Hz, aromatic C*H*), 4.30–4.14 (m, 2H, TEG-CH_2), 3.77–3.51 (m, 13H, 1 C*H*, 6 TEG-CH_2), 3.47 (t, 2H, $J = 6.3$ Hz, TEG-CH_2), 2.83 (s, 3H, CSCH_3), 2.44 (d, 2H, $J = 7.1$ Hz, ibuprofen-CH_2), 1.90–1.77 (m, 1H, C*H*), 1.49 (d, 3H, $J = 6.9$ Hz, CH_3), 0.89 (d, 6H, $J = 6.4$ Hz, 2 CH_3); ^{13}C NMR (90 MHz, $CDCl_3$): δ (ppm) 174.8 (1C, *C* = O), 140.6, 137.8 (2C, *C*q), 129.4, 127.3 (4C, aromatic *C*H), 70.8, 70.7, 70.5, 69.2, 68.1, 64.0 (7C, TEG-CH_2), 45.2 (1C, ibuprofen-CH_2), 45.1 (1C, *C*H), 39.4 (1C, *C*H), 37.0 (1C, TEG-CH_2), 30.3 (1C, CS*C*H3), 22.5 (2C, CH_3), 18.7 (1C, CH_3).

2.2. Detection of H_2S release and calibration

H_2S measurements were carried out by an amperometric H_2S selective sensor (ISO—H_2S-100, World Precision Instruments, Sarasota, FL, USA) connected to a WPI TBR 1025 One-Channel Free Radical Analyzer. The sensor was set to 10 nA range and the poise voltage to +150 mV and polarized before usage in phosphate buffered saline (PBS) for 12 h. The sensor was calibrated with freshly prepared Na_2S x 9 H_2O (reagent plus, 99.99+% from Sigma) stock solution prepared in water (HPLC grade) before each measurement (concentrations of Na_2S x 9 H_2O solution were 0, 1, 2, 4 and 6 μM, respectively) according to the Instruction Manual of the hydrogen sulfide sensor for use with WPI Analyzers.

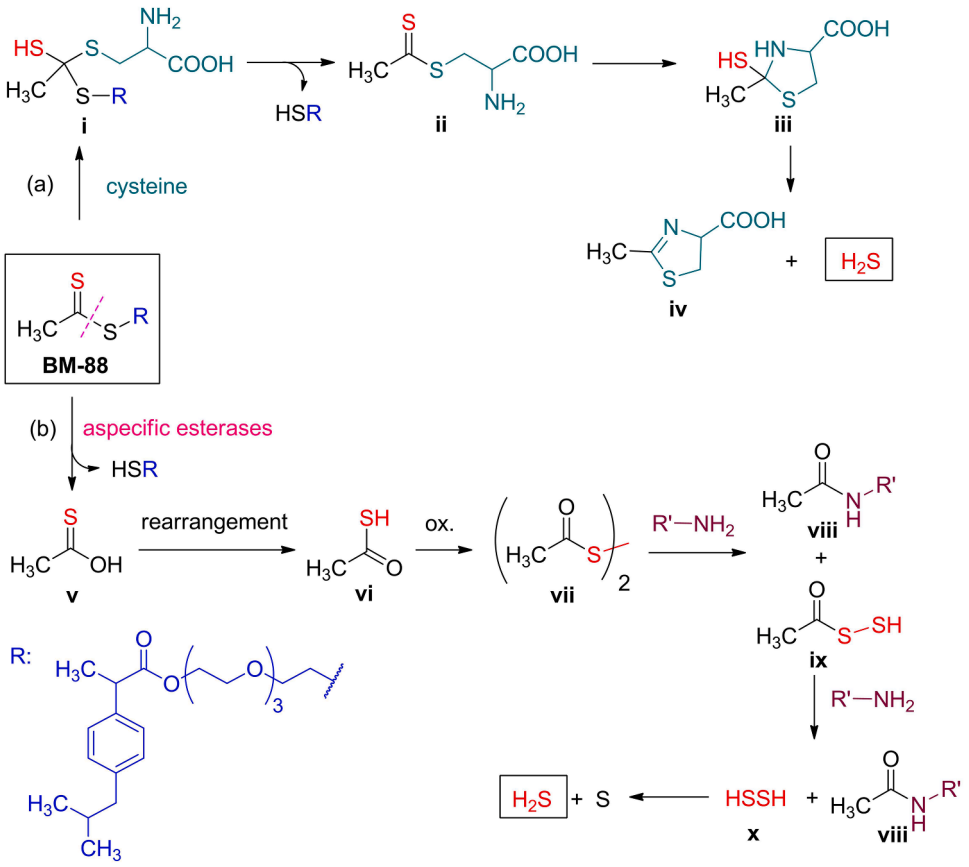
The H_2S donating property of the ibuprofen derivative BM-88 was tested in Dulbecco's modified Eagle's medium collected from H9c2 cell culture (derived from embryonic rat cardiomyocytes) as it was described by Gyöngyösi et al., 2021.

2.3. Cell culture and treatments for the determination of cellular toxicity

The H9c2 cells were obtained from ATCC, CRL-1446, LGC Standards GmbH Wesel, Germany. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified incubator consisting of 5% CO_2 and 95% air. Cells were cultured for one day to establish adhesion of the wells. Confluent cells (60–70% confluence) were employed to the experiments. Ibuprofen or BM-88 was dissolved in the medium contained 1% of DMSO, which resulted in a final concentration of DMSO less than 0.01%, and biocompatibility was measured by MTT [3-(4,5-dimethylthiazol 2-yl)−2,5-(diphenyltetrazolium bromide)] assay. Cells were seeded into 96-well culture plates with 3000 cells/well and treated with ibuprofen or BM-88 at the concentrations of 10 μM, 30 μM, 100 μM, respectively, for 24 h. Following the treatments, MTT solution (final concentration of 0.5 mg/ml) was added to each well and incubated for 3 h at 37 °C, and then, the medium was replaced by isopropyl alcohol to dissolve formazan product. Absorbance was measured by Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Ratastie, Finland) at 570 and 690 nm. The resulting colored solution was quantified by measuring the absorbance at 570 nm and the subtraction of background absorbance at 690 nm. The values were expressed relative to the positive control value, which was represented as 100% of viability of cells. 10% of DMSO was used as positive control (Pos. control), which significantly reduced the cell viability in the cell culture. The absorbance values were averaged across 4 replicate wells, and repeated 11 times ($n = 11$) in each group.

2.4. Animals

Male CFY rats with a body weight range of 220–290 g were used for all the experiments. All animals received humane care in compliance



Scheme 1. Two plausible pathways of H_2S release from ibuprofene derivative BM-88 containing a dithioacetate ester under physiological conditions; (a) H_2S release triggered by reaction with cysteine, (b) H_2S release induced by aspecific esterase-mediated hydrolysis.

with the "Principles of Laboratory Animal Care" (formulated by the U.S. National Society for Medical Research, as described in U.S. National Institutes of Health publication No. 86–23, revised 1996) and the "Guide for the Care and Use of Laboratory Animals". Maintenance and treatment of animals were additionally approved by the Institutional animal care and use committee of the University of Debrecen, (No. 6/2019/DEMAB, Debrecen, Hungary).

2.5. Isolated heart preparation and cardiac function assessment

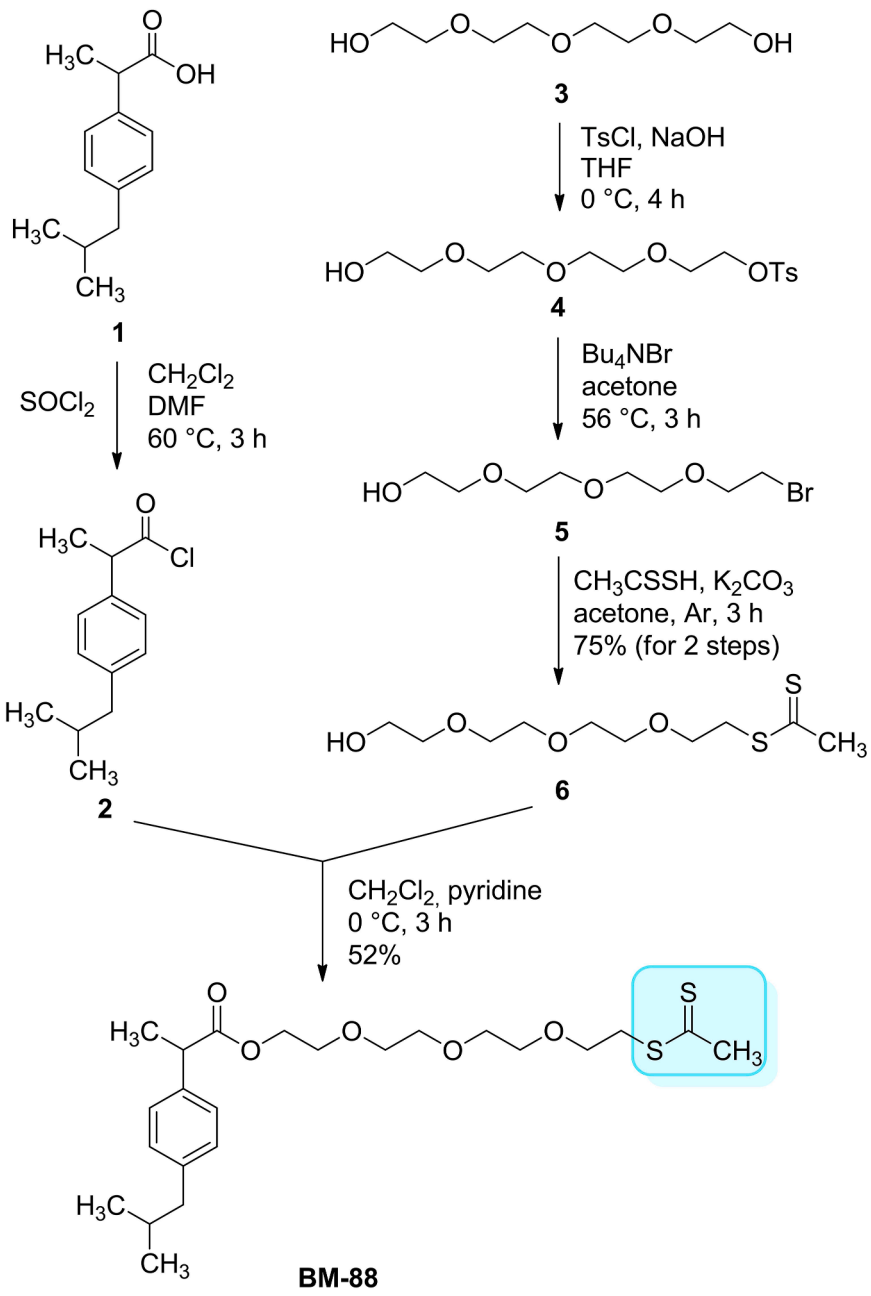
Rats were intraperitoneally anesthetized with ketamine/xylazine (50 mg/kg / 10 mg/kg), and heparin (400 U) was injected intravenously. After 5 min, the chest was opened, the hearts were excised and placed in modified Krebs-Henseleit bicarbonate buffer, and then the heart was cannulated via the aorta and perfused by the Langendorff technique (Langendorff, 1898) at a constant pressure equivalent to 71 mmHg (10.0 kPa). The perfusion buffer consists of the following modified Krebs-Henseleit perfusion medium (Krebs and Henseleit, 1932) in milimolar (Mitidieri et al.) concentrations of sodium chloride (118 mM), potassium chloride (4.7 mM), calcium chloride (1.7 mM), sodium bicarbonate (25 mM), potassium biphosphate (0.36 mM), magnesium sulfate (1.2 mM), and glucose (10.0 mM). Coronary flow (CF) rates were measured by the time collection of perfusates that dripped directly from the myocardium. Under these conditions, coronary flow rates were about 13 to 19 ml/min in the drug-free non-ischemic control hearts.

2.6. H_2S detection in the perfusion effluent of the isolated rat heart

H_2S detections were carried out by a WPI TBR 1025 One-Channel Free Radical Analyzer equipped with an amperometric H_2S sensor (ISO—H_2S-100) as described above. The tips of H_2S and temperature sensors were immersed about 10 mm into the coronary effluent and the current outputs were recorded. Five mililiters of each sample were collected from the coronary effluent of the isolated heart after 10 min of aerobic equilibration perfusion (drug-free perfusion), following 10 min perfusion of the hearts with the H_2S-donor, BM-88. Thirty min of global ISA was followed by 120 min of RE, and effluent samples were collected at 1 min, 5 min, 10 min, 60 min, 90 min and 120 min of RE, respectively. H_2S detections were carried out by immersing the sensor into the collected CF effluents, and values were registered after a few seconds, when the signal was stable and readable, and then, the baseline values (measured in modified Krebs-Henseleit bicarbonate buffer) were subtracted from the measured values.

2.7. Infarct size assessment

To estimate the infarcted areas of hearts after ischemia/reperfusion, triphenyl tetrazolium-chloride (TTC) staining method was used. After 30 min of ischemia followed by 120 min of reperfusion, the hearts were perfused with 50 ml 1% (wt/vol) solution of TTC in phosphate buffer for 5 min and stored at −80 °C for subsequent analysis. The frozen samples were sliced into equal sections, and excess moisture was drained off. The sections were weighted and scanned on a flat-bed scanner (Epson Perfection V33/330; Epson America, Inc.). Infarcted area (remains in white compared to the red coloration of viable heart tissues) and risk area (whole section) were measured using ImageJ software (ImageJ 1.53i; National Institutes of Health, USA). Infarcted zone magnitude was estimated by multiplying infarcted areas by weight of each section. Infarct size was expressed as a ratio of the weight of infarcted zone and risk zone (whole heart) (Czompa et al., 2014).



Scheme 2. Synthesis of BM-88. The H_2S releasing compound was prepared in the reaction of the acyl chloride derivative of ibuprofen (compound 2) and the monodithioacetic acid ester of tetraethylene glycol (compound 6). The H_2S-releasing unit of the molecule is marked in blue.

2.8. Histology and immunohistochemistry

Isolated hearts were subjected to 30 min of ISA followed by 120 min of RE. At the end of RE, all hearts (BM-88-free and BM-88 treated) were immersed in a fixative solution (4% buffered paraformaldehyde (PFA) solution). After 24 h of fixation, the hearts were dehydrated and embedded in paraffin. Heart tissue sections of 4–6 micrometers were cut and stained with hematoxylin-eosin (HE) and Giemsa/Picrosirius stainings for morphological assessment of the myocardial tissue.

For the detection of autophagy several immunohistochemistry reactions were carried out with specific primary antibodies, including LC3I/II (ab48394, Abcam, Cambridge, UK), Beclin-1 (ab62557), p62 (ab91526). Antigen retrieval was done in 0.1 M citrate buffer (pH 6.0), after blocking the nonspecific reactions, using 3% bovine serum albumin (BSA), the sections were incubated with primary antibodies overnight at 4 °C, respectively. All primary antibodies were used at 1:100 dilution (Abcam, Cambridge, UK). Secondary antibody system (ACUITYAdvenced Biotin Free Polymer Detection System 931201, Biolegend, MA, USA) was applied to the sections. Finally, the visualization procedure was carried out with a VIP kit (SK-4600, Vector Laboratories, Burlingame, CA, USA) followed by hematoxylin counterstain.

2.9. Statistical analysis

One-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If differences were established, the values (coronary flow, infarct size, H_2S release, cell toxicity) were compared with those of the drug-free control group by *t*-test (mean ± standard error of the mean). Because the incidence (%) of reperfusion-induced ventricular fibrillation (VF) followed the nonparametric (none Gaussian) distribution, therefore, chi-square test was used for the statistical analysis of the incidence of ventricular fibrillation.

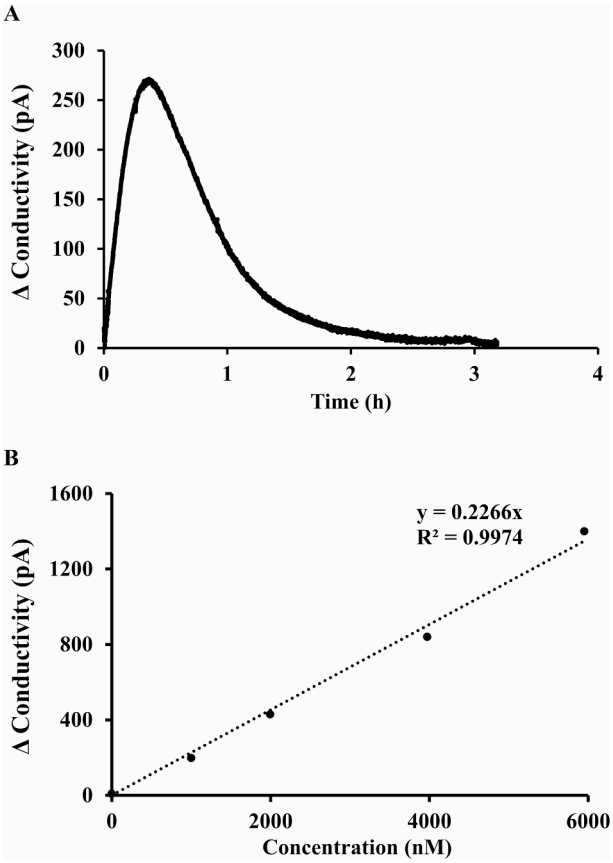


Fig. 2. (A): H_2S release of BM-88 in medium derived from H9c2 cardiomyoblast cells. (B): Calibration of H_2S sensor (dotted line: linear regression model, R^2: goodness-of-fit).

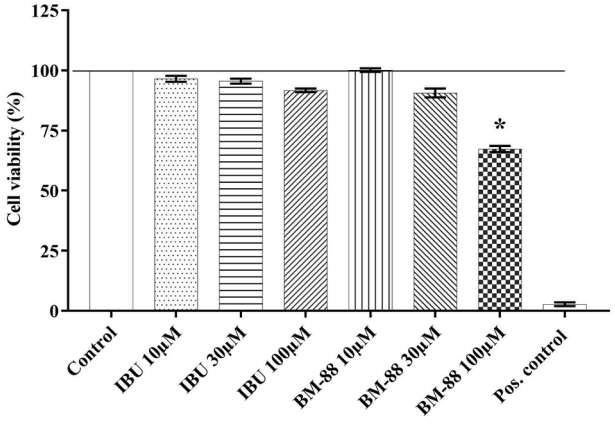


Fig. 3. Evaluation of BM-88 cytotoxicity. H9c2 cells were treated with ibuprofen (IBU) of 10 μM, 30 μM and 100 μM and the same concentrations of BM-88, respectively, for 24 h. The cell viability was detected using MTT assay and calculated as the percentage of cell surviving ibuprofen or BM-88 exposure. Data were expressed as the mean ± SEM, $n = 11$ in each group. *$p < 0.05$ in comparison with the Positive control (Pos. control) value. Control: untreated cells (100%); Pos. control: cytotoxicity of 10% DMSO.

Results were considered to be significant if *$p<0.05$.

3. Results

3.1. Design and synthesis of BM-88

According to literature results, a dithioester derivative can deliver hydrogen sulfide under physiological conditions by two mechanisms. On the one hand, it can react with cysteine via a native chemical ligation-like mechanism (Scheme 1, route a) to afford an equivalent of thiol (RSH), a cysteine-derived dihydrothiazole (**iv**) and H_2S (Cerda et al., 2019). On the other hand, hydrolysis of dithioesters catalyzed by native aspecific esterases (Scheme 1, route b) yields thiolcarboxylic acid (**v** → **vi**), which is spontaneously oxidized to disulfide (**vii**); reaction of the latter disulfide with native amines yields one equivalent of amide (**viii**); and half an equivalent of hydrogen sulfide (Liu and Orgel, 1997; Gyöngyösi et al., 2021).

In addition to the dual hydrogen sulfide release mechanism, the dithioester motif is also synthetically attractive because it is easy to create. The water solubility of the planned prodrug molecule was an important goal in the synthetic design, so we intended to connect the dithioacetate functional group and the ibuprofen unit via an amphiphilic tetraethylene glycol linker through an ester bond that is hydrolysable in a biological milieu releasing ibuprofen. (BM-88, Scheme 2). Thus, H_2S donating molecule, BM-88, was produced by the reaction of the acyl chloride derivative of ibuprofen (compound **2**) (Yan et al., 2016) and the monodithioacetic acid monoester of tetraethylene glycol (compound **6**). The latter was synthesized from monobromo derivative of tetraethylene glycol, compound **5** (Caianiello et al., 2021; Gugliotti et al., 2005) (Scheme 2). The prepared compound (BM-88) is stable in water solution (stability was tested by UV–Vis spectroscopy within an 8-hour time interval; Figure S1 in Supplementary Information), and it could deliver H_2S via either a cysteine-triggered mechanism or by enzymatic reactions in tissues.

3.2. Investigation of H_2S donating property of BM-88

The H_2S donating property of BM-88 was tested by a direct H_2S sensor in Dulbecco's modified Eagle's medium collected from H9c2 cell culture on the third day. Thus 0.1 mg of the ibuprofen derivative, BM-88, was dissolved in 5 ml of medium (concentration of the H_2S donating molecule was 43.8 μM). The concentration of H_2S started to raise and reached a maximum value at about 1.2 μM after 18 min (Fig. 2A). In control experiments, the stability of BM-88 in aqueous solution was also examined, and there was no H_2S release detected (Figure S2 in Supplementary Information). These results indicate that esterases and other enzymes present in biological milieu are essential for the degradation of the dithioacetic moiety of BM-88 to result hydrogen sulfide. Fig. 2B shows the calibration of the H_2S sensor, as the process described in the Materials and Methods.

3.3. Cytotoxicity

Cytotoxicity of BM-88 was evaluated in H9c2 cells in comparison with ibuprofen (IBU) at three different concentrations, respectively. The results showed that 10 μM and 30 μM of BM-88 did not cause any toxic effect, however 100 μM of BM-88 resulted in some cytotoxicity. Therefore, the use of BM-88 in 10 μM concentration for ischemia/reperfusion studies is safe (Fig. 3).

3.4. Incidence of reperfusion-induced ventricular fibrillation, coronary flow and heart rate

Isolated hearts were subjected to 30 min of global (zero flow) ischemia followed by 120 min of reperfusion and treated with various concentrations of BM-88. A dose-response reduction in the incidence of

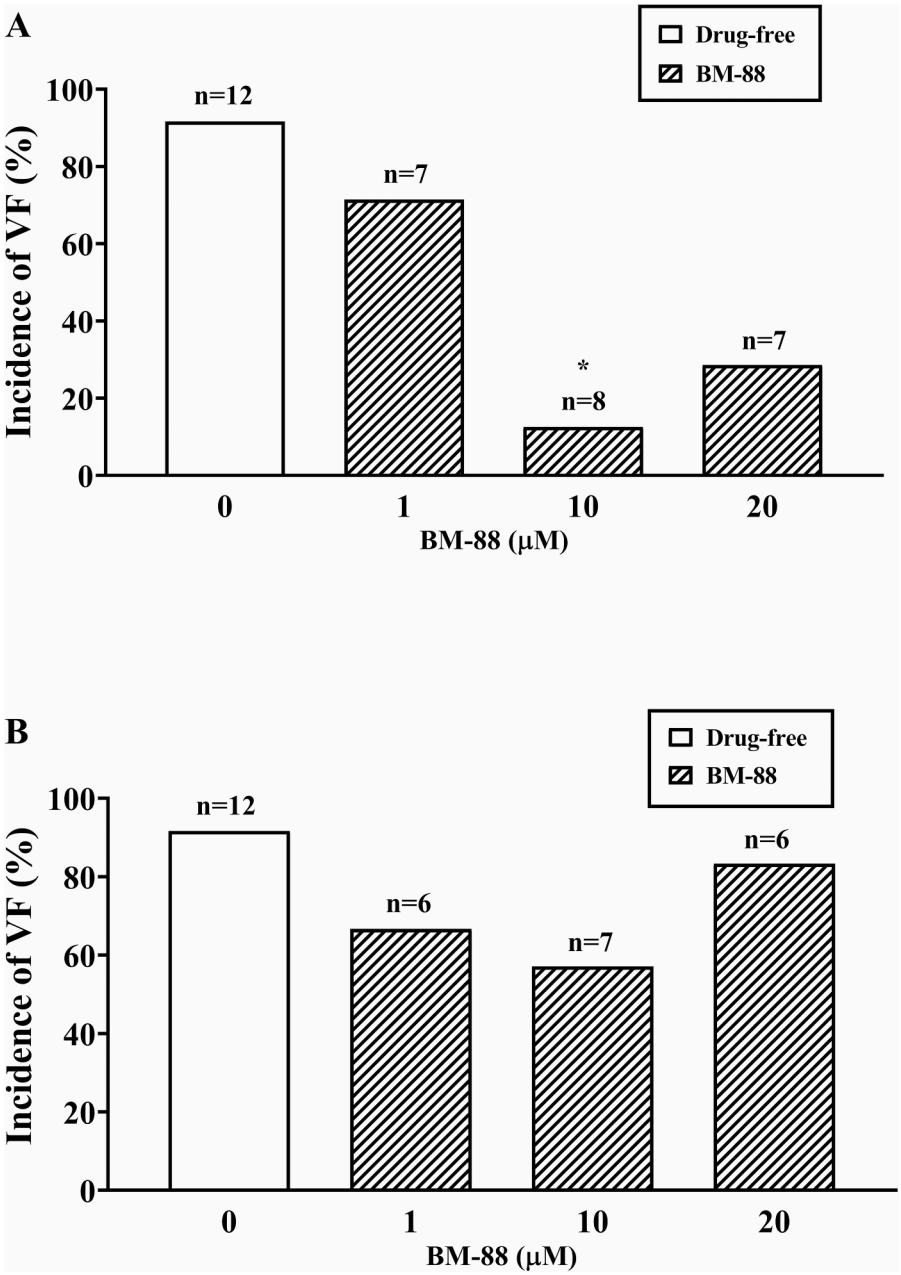


Fig. 4. The incidence (%) of reperfusion-induced ventricular fibrillation (VF). (A): BM-88 was administered 10 min before the induction of ischemia (preischemic treatment) in various concentrations. *$p<0.05$ in comparison with the drug-free control value (open bar). (B): BM-88 was given during the first 10 min of the reperfusion period (postischemic treatment). No significant changes were observed compared to the drug-free control value (open bar), if BM-88 was administered at the onset of reperfusion (postischemic treatment). n: numbers of hearts in each group.

reperfusion-induced VF was not observed (Fig. 4A), however, in hearts perfused with 10.0 μM BM-88 for 10 min before the induction of global ischemia (Fig. 4A), a significant reduction was detected in the incidence of reperfusion-induced VF from its control value of 92% to 12% (*$p<0.05$). When hearts were perfused with various concentrations of BM-88 at the onset and during the first 10 min of reperfusion (postischemic treatment), a reduction in the incidence of reperfusion-induced VF was not observed at a statistical level (Fig. 4B). Therefore, for additional studies, 10.0 μM of BM-88 was selected and administered before the induction of global ischemia (preischemic administration).

Fig. 5 shows the coronary flow (Fig. 5A) and heart rate (Fig. 5B) values at various time points in the 10.0 μM BM-88 treated group before the induction of ischemia and during the 2 h of the reperfusion period. Some reductions in CF values were measured at each time point during reperfusion in the BM-88 treated group (solid circles) in comparison with the drug-free control values (open circles), however, these decreases in CF were statistically not significant.

Heart rate was somewhat also reduced (Fig. 5B) during the 2 h of the reperfusion period in the BM-88 treated group in comparison with the drug-free control values, but these changes were not at a significant level at each time point. Indeed, several studies showed that slowing the heart rate is a protective intervention against ischemia/reperfusion-induced myocardial damage under experimental conditions. For instance, papers published some decades ago emphasized that reducing the heart rate affords cellular protection against the development of reperfusion-induced arrhythmias (Bernier et al., 1989; Tosaki et al., 1988, 1987) probably as the result of the effects on cellular energy metabolism and the rate of the degree of ischemic injury. Possible mechanisms between the reduction in heart beats and the incidence of arrhythmias have been recently reported in papers both under experimental and clinical conditions (Ikeda et al., 2022; Mugnai et al., 2022). It is of interest also to note that a reduction in the heart rate is arrhythmogenic in human beings, which clinical appearance could be genetically determined, e.g., long QT syndromes and 'torsades de pointes' arrhythmias (Naksuk et al., 2019; Wilde et al., 2022).

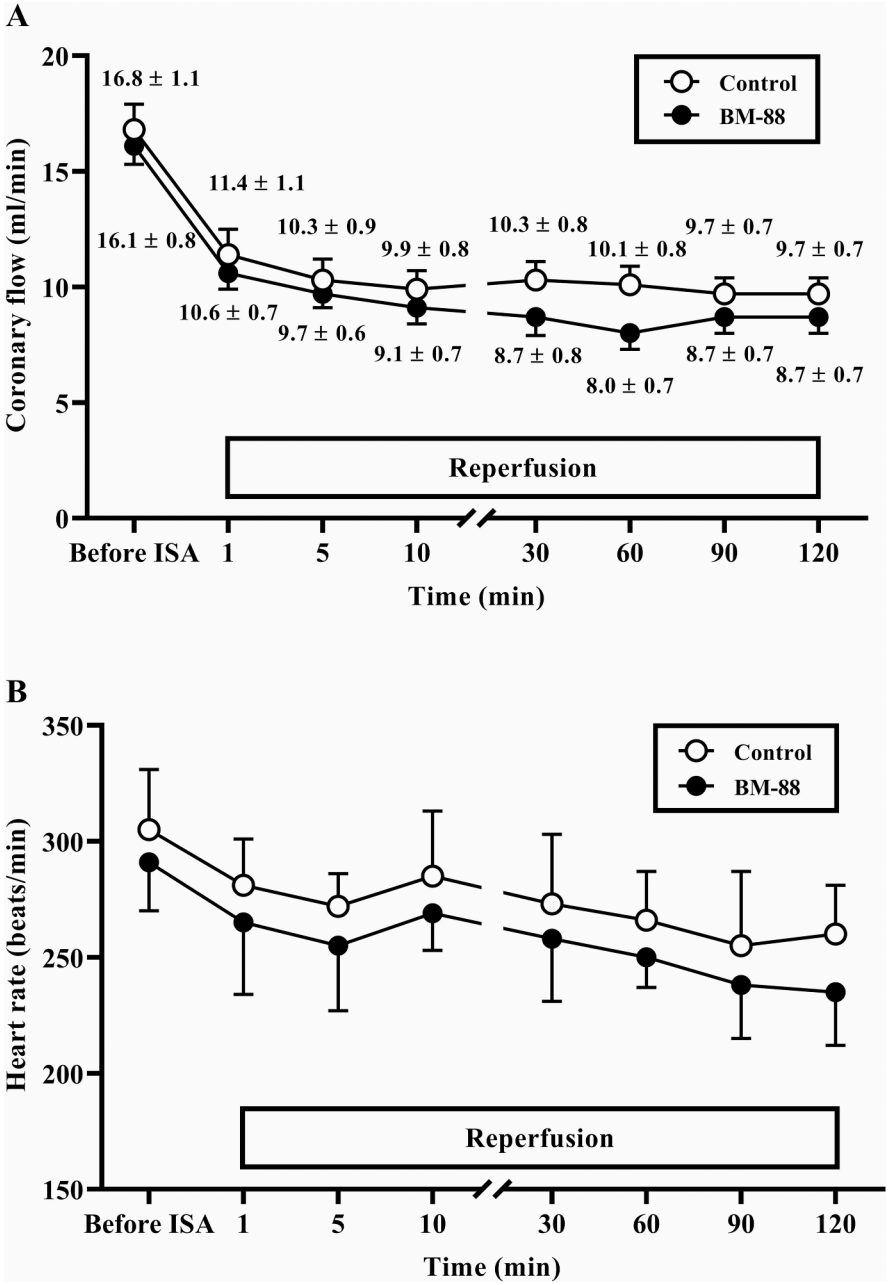


Fig. 5. Coronary flow and heart rate values in the BM-88 pre-treated group. Time course coronary flow (A) and heart rate (B) values in the control (open circles) and BM-88 (10.0 μM) pre-treated group (solid circles). $n = 12$ in the control (drug-free) group; $n = 8$ in the BM-88 treated group (mean ± standard error of the mean). Significant changes were not registered at any time point between the drug-free control and BM-88 treated values. ISA: ischemia.

3.5. Detection of H_2S release in the perfusion effluent

H_2S release was directly detected by a hydrogen sulfide sensor in the perfusion effluent (coronary flow) of hearts as described in the Methods. Release of a substantial amount of H_2S was not observed in hearts perfused with BM-88 before the induction of ischemia. After 30 min of global ischemia followed by reperfusion, a substantial amount of H_2S release was detected. H_2S release was increased during the first ten minutes of the reperfusion period and then suddenly returned to the preischemic values in all hearts treated with 10 μM BM-88 (Fig. 6). The levels of H_2S were raised after 1 min, 5 min and 10 min to 1.06 μM, 0.77 μM and 0.53 μM, respectively, following the initial phase of reperfusion (Fig. 6B), and then returned to the preischemic drug-free value of 0.4 μM, which were calculated from the calibrated curve (Fig. 2). However, a regular dose-response curve in H_2S release was not observed, using different concentrations of the H_2S-donor, BM-88.

3.6. Detection and measurement of infarct size area

Although BM-88 at a concentration of 10 μM did not increase coronary flow during the reperfusion period, the infarct size was significantly reduced from its control value of 38%±5% to 19%±7% (Fig. 7). Thus, our observations suggest that coronary flow rates do not show a significant and close correlation to either the extent of infarct size or the incidence of reperfusion-induced VF.

3.7. Histology and immunohistochemistry

Hematoxylin-Eosin (HE) and Giemsa/Picrosirius staining were used for morphological assessment of the heart tissues (Fig. 8). Both types of

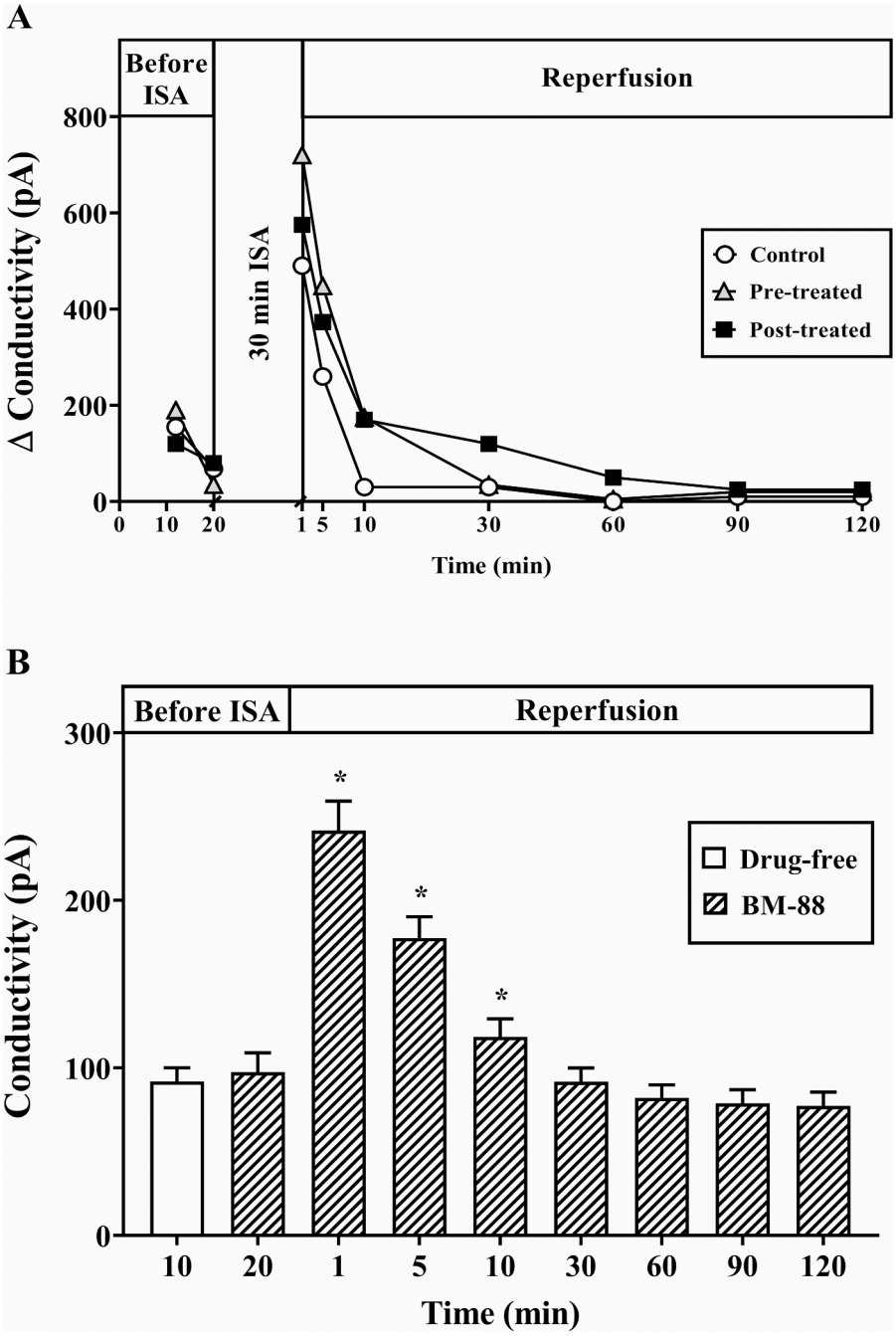


Fig. 6. Time-course H_2S release in hearts treated with BM-88 of 10.0 μM. (A): Each representative curve shows the time-course of H_2S release at each time point in a single heart, including the drug-free control (open circles), preischemic (solid triangles) and postischemic (solid squares) treated myocardium with 10.0 μM BM-88, respectively. (B): The columns show the quantitative values (mean ± standard error of the mean) of H_2S release in the BM-88 pre-treated (before the induction of ischemia) hearts. $n = 7$ at each time point. BM-88 was administered at 10 μM concentration for 10 min (hatched column) just before the induction of 30 min of global ischemia. *$p<0.05$ in comparison with the pre-ischemic (Before ISA) BM-88 treated value.

staining methods are pathologically suitable for assessing the extent of ischemic damage in the myocardium, which based on the degree of fragmentation of the fibers and the staining intensity. Fig. 8 shows the results obtained from 30 min of ISA followed by 120 min of RE, in hearts subjected to 10 μM of BM-88 treatment. Fragmentation of fibers was slightly reduced in BM-88 treated samples compared to drug-free ischemic control hearts.

Whether BM-88 treatment promoted autophagy was assessed by determining the conversion of LC3 I / II, an autophagosomal membrane protein used as a marker of autophagy, and the protein levels of p62 and Beclin-1. Beclin-1 is known to signal the onset of autophagy and p62 is a well-known substrate of autophagic processes. The results show that the baseline levels of autophagy-related proteins did not change in the hearts treated with BM-88 compared to the ischemic drug-free control. Using immunohistochemistry methods, no difference in the levels of p62, LC3I/II and Beclin-1 was detected (Fig. 9). The pictures were taken by an Olympus digital camera at 400x magnification under light microscopy (Leica Microsystems™ DM2000 LED Microscope, Germany).

4. Discussion

H_2S is produced enzymatically from cysteine in various mammalian tissues (Kimura, 2014; Murphy et al., 2019) and its physiological and pharmacological importance has been significantly increased during the past two decades (Bibli et al., 2015; Sodha et al., 2008; Wang et al., 2020). One of the beneficial effects of H_2S is the reaction with several reactive species, including hypochlorite, hydrogen peroxide, and superoxide radical leading to their neutralization and reduction of the degree of oxidative stress (Kabil and Banerjee, 2010) in various cells and tissues. The majority of investigations have reported extensive cellular

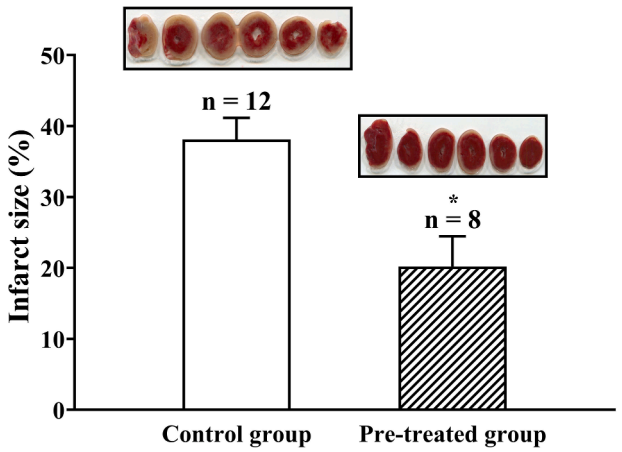


Fig. 7. Infarct size (%) in the drug-free control and BM-88 pre-treated groups. BM-88 was administered for 10 min before the induction of ischemia. The infarct size (%) was significantly reduced in the BM-88 pre-treated group (hatched bar) in comparison with the drug-free control (open bar) value $*p<0,05$. n = numbers of hearts in each group. Representative pictures of infarcted areas are shown on the top of each bar.

and tissue protection in various models of different diseases after administration of low levels of H_2S (Sun et al., 2021b). For the treatment of cardiovascular, diabetes and other diseases numerous H_2S-releasing compounds have been elaborated and studied as promising therapeutic tools. However, their therapeutical applications (Zhou et al., 2022) require a more detailed understanding of the pharmacokinetics, distribution, and mechanisms of actions (Li et al., 2018). In this context, it is of also interest to note that in an elegant review written by Wu et al. (2018) discussed and summarized the most important H_2S-mediated metabolisms and signaling pathways, including SR-A (scavenger receptor class A), PI3K(phosphatidylinositol 3-kinase)/SGK1(Serum- and glucocorticoid-responsive kinase-1)/GSK3beta(glycogen synthase kinase-3beta), PI3K/AKT(Akt: PKB: protein kinase B)/mTOR(mammalian target of rapamycin), Nrf2(NF-E2-related factor 2)-ROS(reactive oxygen species)-AMPK (adenosine monophosphate-activated protein kinase), AMPK/mTOR, and JNK1 (c-Jun N-terminal kinase 1) in autophagic processes.

Several H_2S donors have been published to show significant cellular protection against oxidative stress-induced pathological processes, including experimental models of ischemia/reperfusion and inflammation (Coavoy-Sánchez et al., 2020; Ellmers et al., 2020; Gyöngyösi et al., 2021; Peleli et al., 2022; Zhao et al., 2015), suggesting that the family of H_2S-releasing molecules could serve as a promising pharmacological group for the prevention of stress-induced cellular damage.

A publication has been recently reported from our group on the importance of H_2S release, showing a substantial protection against inflammatory processes induced by carrageenan in rat paws (Gyöngyösi et al., 2021). In the current study, a new H_2S-realeasing molecule, BM-88, was synthetized and characterized by NMR and mass spectrometry. BM-88 is a derivative of ibuprofen which is widely used as an anti-inflammatory drug. The cytotoxicity in H9c2 cells and the cardiovascular effects of BM-88 were assessed on the ischemic/reperfused myocardium, including the incidence of reperfusion-induced ventricular fibrillation, coronary resistance, and infarct size in connection with H_2S release directly measured from the perfusion effluent of isolated hearts.

In the in vitro experimental model of myocardial ischemia/reperfusion, the pre-administration of 10 μM BM-88 has led to a significant reduction in the incidence of reperfusion-induced VF from its drug-free control value of 92% to 12%. However, other concentrations of BM-88 were also studied, but no clear significant reduction and/or dose-dependency in the incidence of reperfusion-induced VF was observed by the application of various concentrations (e.g., 1.0 μM and 20.0 μM) of BM-88. Notably, lower or higher concentrations of BM-88 than 10 μM failed to significantly reduce the incidence of reperfusion-induced VF. It is valuable to note that at 10 μM concentration of BM-88 did not show any cytotoxic effect.

The results of the present study demonstrate that BM-88, the ibuprofen based organic H_2S donor designed specifically to release H_2S, can reduce the vulnerability of the myocardium to reperfusion-induced injury following a 30 min period of global ischemia. BM-88 exerted this cardioprotective effect without any major and significant effects on coronary flow rates. Although our observation is limited to the isolated rat heart, these results provide additional support for the fact that H_2S could play an important role in the reduction of reperfusion-induced VF and this inhibition may provide an effective means of controlling this potentially life threatening lethal ventricular arrhythmia.

The results also show if BM-88 was administered before the induction of ischemia (preischemic administration, Fig. 4A), the decrease in vulnerability of the hearts is attributed to BM-88 effects operating

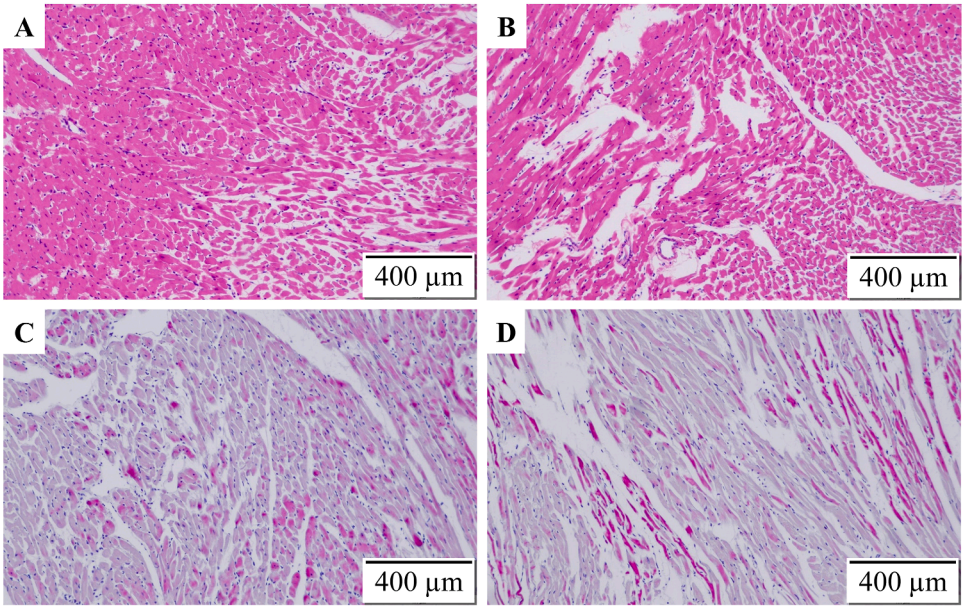


Fig. 8. Morphological analysis of heart tissues. Hematoxylin-Eosin (HE) (A and B) and Giemsa/Picrosirius (C and D) staining were used for morphological assessment of myocardial tissues. Figures A and C show samples obtained from ischemic control of drug-free hearts, while B and D represent the samples of BM-88 treated hearts obtained from hearts 30 min of ISA followed by 120 min of RE (reperfusion). The degree of fragmentation of fibers was slightly reduced in the BM-88-treated myocardium. The pictures were taken under light microscopy, original magnification is 400x, scale bar: 400 μm.

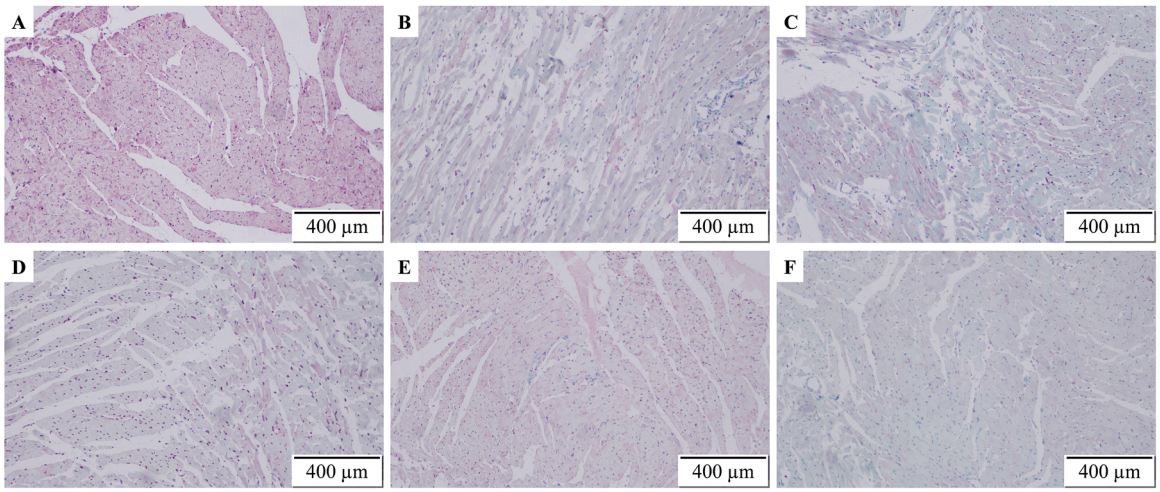


Fig. 9. Immunohistochemistry. Expression levels of LC3I/II, Beclin-1 and p62 proteins in representative heart tissue sections. The top row (A-C) represents the samples derived from the drug-free control myocardium, and the bottom row (D-E) represents the samples obtained from a BM-88 treated heart. After immunostaining with specific primary antibodies visualization was carried out with a VIP kit (SK-4600, Vector Laboratories, Burlingame, CA, USA) followed by hematoxylin counterstaining. Heart sections of all samples showed no immunohistochemically detectable level of LC3I/II (A, D), Beclin-1 (B, E) and p62 (C, F), although diffuse weak positivity can be recognized in some places, light brown staining, most likely reflects the background. No substantial immunohistochemical difference was observed in the levels of p62, LC3I/II and Beclin-1 between drug-free and BM-88 treated hearts. The pictures were taken under light microscopy, original magnification is 400x, scale bar: 400 μm.

during the ischemic period rather than to reperfusion itself as this drug was ineffective on the incidence of VF, when it was administered at the onset of reperfusion (postischemic treatment, Fig. 4B). The rate of H_2S based catabolism that takes place in the mitochondria, which is an oxygen dependent process; thus, the absence of oxygen affects the catabolism of H_2S leading to its accumulation (Li et al., 2022; Nesci et al., 2021). Although a non-significant reduction in the incidence of VF was still observed in the postischemic treatment of BM-88 (Fig. 4B), therefore it might be argued that in the case of the postischemic treatment of BM-88, the compound still exerted some protection during the reperfusion period. This means that BM-88, as a H_2S donor, somehow may alter this preischemic state during the subsequent reperfusion period. In this context, it is of considerable interest to ascertain whether other H_2S donors achieve a true antifibrillatory effect, or whether H_2S release at least reduces the vulnerability of the heart to reperfusion-induced VF. With regard to the mechanisms by which H_2S may participate VF could include changes in membrane integrity as a potent antioxidant (Kimura et al., 2006; Kimura and Kimura, 2004; Whiteman et al., 2004) through the modulation of the transcriptor factor Nrf2, which was found in the promoter region of genes such as HO-1 and other various antioxidant enzymes (Bak et al., 2010; Enayati et al., 2018; Martin et al., 2021; Sakurai et al., 2005; Tanito et al., 2007; Yeh et al., 2015; Zhu et al., 2005; Tosaki, 2020). The aforementioned findings and publications show that H_2S therapy may provide an environment in the myocardium that is resistant to oxidative stress related to the incidence of reperfusion-induced ventricular arrhythmias. However, it is of interest to note that Nrf2 may not have an immediate pathological relevance in arrhythmogenesis at the early minutes of reperfusion (1 min to 10 min), since the reperfusion-induced VF develops during the first few minutes of reperfusion. Thus, after 90 min to 120 min of reperfusion Nrf2 may have a substantial role in the development of the infarcted area because some genes could be present in inactive forms in the myocardium, which may be immediately activated during the reperfusion period, and after 2 h of reperfusion they could have some significant impact on necrosis-, apoptosis- and autophagia-induced cell deaths.

Various interventions, which modify the extent and rate of cellular death during both myocardial ischemia/reperfusion is a major therapeutic aim. Thus, a drug which could be used as an adjunct to thrombolysis to reduce myocardial ischemia/reperfusion-induced injury and would thereby give further benefit than reperfusion therapy alone, may substantially influence the final outcome from cardiac ischemia and/or infarction. In the present investigation we found that a concentration of BM-88, a H_2S donor, provided a substantial protection and significantly reduced the infarct size in an experimental model of myocardial ischemia/reperfusion. Several lines of evidence exist that cardiac tissue cell deaths during ischemia/reperfusion could take place via the processes of necrosis, apoptosis, and autophagy (de Freitas et al., 2021; Del *Re* et al., 2019; Haines et al., 2013; Heusch, 2020; Lekli et al., 2017) but the contributions of these three phenomena and at what point they contribute to cellular death is not completely clear. Since the activation of apoptotic and autophagic cell deaths via death ligands and mitochondrial damage are crucial processes and these events appear to be accelerated during ischemia/reperfusion, we studied the effect of BM-88 given at the onset of reperfusion (postischemic administration). Although several studies provide evidence that apoptosis and autophagy occur during the ischemic period, work from other laboratories suggests that these processes also substantially contribute to myocardial and vascular endothelial cell deaths, however, necrosis itself as a major line of cell death could mask the damage induced by apoptosis and autophagy (Gyongyosi et al., 2019). Herein we demonstrate that under our experimental conditions BM-88 significantly reduced the infarct size, if the drug was only administered before the induction of myocardial ischemia (preischemic treatment), however, BM-88 was ineffective on the infarcted area, if the drug was administered at the onset of the reperfusion period (postischemic treatment, data are not depicted).

It needs to be emphasized that using immunohistochemistry methods, no differences in the levels of p62, LC3I/II and Beclin-1 were detected in hearts treated with BM-88 compared to the ischemic drug-free controls. Several areas of data support the up-regulation of autophagy following I/R in cardiomyocytes, however, some revealed distinctive induction of autophagy in cultured H9c2 cells was reported. It was proved that the extent of the ischemic injury substantially affected the degree of autophagy processes, and studies revealed that metabolic inhibition used to induce ‘moderate’ or ‘severe’ ischemia resulted in apoptotic and necrotic cell deaths without any evidence of autophagy. Based on these results, we can also support the unchanged levels in the expression of autophagy proteins in the BM-88 treated hearts compared to the ischemic control values detected by immunohistochemistry (Ma et al., 2015a). The role of autophagy in myocardial I/R-induced injury has been investigated in vitro, *ex vivo* and in vivo experimental models in

various species. The controversy of autophagic processes during myocardial I/R injury may originate from several factors. These include the variability in the experimental conditions, and methodology used to study autophagic pathways, therefore, it does not seem contradictory that autophagy induction in our experimental model with the applied methods was not detectable (Dai et al., 2017; Lin et al., 2018; Ma et al., 2012). It is not clear, at the moment, to what extent autophagy and apoptosis individually cause myocardial cell deaths. However, our previous observations suggest that necrosis is the major type of cell death and autophagic/apoptotic pathways may govern and share several common molecular signaling mechanisms, contributing to the ischemia/reperfusion-induced necrotic cell death (Gyongyosi et al., 2019; Haines et al., 2013). Thus, the precise mechanism(s) by which BM-88, an H_2S donor, leads to the limitation of an infarcted area is not completely clear.

5. Conclusions and limitations of the study

The mechanism by which BM-88, an H_2S donor, leads to the reduction of infarct size and the attenuation in the incidence of reperfusion-induced VF at the present is not clear. Despite the fact that this H_2S donor exerts a cellular protection against myocardial cell deaths at a concentration of 10 μM should be treated with some cautions. Such caution includes the isolated nature of the heart preparation and the release and/or selectivity of H_2S on myocardial cells, because vasodilatation activity was not detected in coronary arteries, which reflected in the lack of the rate of coronary effluents (coronary flow) in our studies. Therefore, the authors cannot exclude several signal transduction mechanisms on myocardial tissues, which could control and govern various pathological processes of ischemia/reperfusion-induced injury, including apoptotic, autophagic and necrotic cell deaths. Indeed, H_2S release was detected in the perfusion effluent during reperfusion in our isolated experimental model, however, it is uncertain at the moment whether how much of H_2S was released and contributed to the myocardial protection during the previous ischemic episode. The obtained results show that BM-88 did not provide a significant and substantial protection against reperfusion-induced injury and cell death, if this compound was administered at the onset of the reperfusion period (postischemic treatment). Although it was not specifically investigated in our study what the relationship is between the H_2S catabolism and mitochondrial function, the results by Elrod et al. (Elrod et al., 2007) demonstrated that exogenous and/or endogenous elevation in H_2S levels at the time of reperfusion limits the extent of myocardial ischemia/reperfusion-induced infarct size in transgenic mice. Thus, the precise signal mechanism underlying cardiac tissue protection remains to be clarified, however our observations show that the release of H_2S could be a promising intervention for the development of pharmacological therapies to attenuate ischemia/reperfusion-induced injury in the myocardium.

Author contributions

P.H., A.B. and Á.T. designed the study; E.SZ., I.B., V.V., N.D. and Á.T. performed the experiments; I.B. and P.H. carried out the synthesis of the molecule, BM-88; V.V., E.SZ. performed the analysis of the data and visualization; Á.T., P.H. and A.B. wrote the manuscript. E.SZ., V.V., I.B. partially contributed to the writing; Á.T. supervised the experiments, conceptualized and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

All the authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. Approval number: 6/2019/DEMÁB.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106449.

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