European Journal of Pharmaceutical Sciences 195 (2024) 106721

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Effects of H_2S-donor ascorbic acid derivative and ischemia/reperfusion-induced injury in isolated rat hearts

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ARTICLE INFO

Keywords:
Hydrogen-sulfide
H_2S-donor
Ascorbic acid
Heart
Ischemia/reperfusion
Antioxidant activity
Apoptotic and autophagic proteins

ABSTRACT

Hydrogen sulfide (H_2S), a gasotransmitter, plays a crucial role in vasorelaxation, anti-inflammatory processes and mitigating myocardial ischemia/reperfusion-induced injury by regulating various signaling processes. We designed a water soluble H_2S-releasing ascorbic acid derivative, BM-164, to combine the beneficial cardiovascular and anti-inflammatory effects of H_2S with the excellent water solubility and antioxidant properties of ascorbic acid. DPPH antioxidant assay revealed that the antioxidant activity of BM-164 in the presence of a myocardial tissue homogenate (extract) increased continuously over the 120 min test interval due to the continuous release of H_2S from BM-164. The cytotoxicity of BM-164 was tested by MTT assay on H9c2 cells, which resulted in no cytotoxic effect at concentrations of 10 to 30 μM. The possible beneficial effects of BM-164 (30 μM) was examined in isolated 'Langendorff' rat hearts. The incidence of ventricular fibrillation (VF) was significantly reduced from its control value of 79 % to 31 % in the BM-164 treated group, and the infarct size was also diminished from the control value of 28 % to 14 % in the BM-164 treated group. However, coronary flow (CF) and heart rate (HR) values in the BM-164 treated group did not show significantly different levels in comparison with the drug-free control, although a non-significant recovery in both CF and HR was observed at each time point. We attempted to reveal the mechanism of action of BM-164, focusing on the processes of autophagy and apoptosis. The expression of key autophagic and apoptotic markers in isolated rat hearts were detected by Western blot analysis. All the examined autophagy-related proteins showed increased expression levels in the BM-164 treated group in comparison to the drug-free control and/or ascorbic acid treated groups, while the changes in the expression of apoptotic markers were not obvious. In conclusion, the designed water soluble H_2S releasing ascorbic acid derivative, BM-164, showed better cardiac protection against ischemia/reperfusion-induced injury compared to the untreated and ascorbic acid treated hearts, respectively.

1. Introduction

Cardiovascular diseases (CVDs) are among the leading causes of death according to the WHO, with an estimated 17.9 million deaths globally in each year. The prevalence, health effects and potential mortality rates of different CVDs has been thoroughly investigated in recent decades. With the improvements in medical technologies, pharmaceutical medications (pre- and post-treatment) and palliative care, in both developed and developing countries (Finegold et al., 2013; Roth et al., 2017), the demand of finding new potentially effective cardioprotective drugs is increasing. The consequence of the ischemia/reperfusion-caused injury in the myocardial tissue has been extensively investigated by the research community starting from the 1980s and continued nowadays both under experimental and clinical conditions (Dennis et al., 1983; Fox et al., 1985; Hearse and Tosaki, 1987; Heusch and Schulz, 2012; Kolluru et al., 2023; Manning and Hearse, 1984; Rathore et al., 2001; Sharma et al., 1983; Takata et al., 1996). The occlusion of the coronary arteries will lead to nutrient and energy starvation of the heart, can cause apoptotic, autophagic and necrotic changes in the myocardium, leading to arrhythmias,

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https://doi.org/10.1016/j.ejps.2024.106721
Received 27 November 2023; Received in revised form 30 January 2024; Accepted 4 February 2024
Available online 6 February 2024

bradycardia, tachycardia, fibrillation, heart failure and cardiac death in many patients. These outcomes can be mitigated somewhat with immediate medical intervention and post event care (Marin et al., 2021).

Ascorbic acid, also known as vitamin C is an essential vitamin, since in contrast with other mammalian species, humans cannot synthesize it, despite being an important molecule in numerous biochemical synthesis pathways. It is a water soluble, well characterized molecule whose importance is well known since the early 20th century and is on the World Health Organization model list of essential medicines: 22nd list (2021) (World Health Organization, 2021). Since ascorbic acid has antioxidant properties and vitamin functions according to several studies, it can prevent or mitigate the outcomes and complications of cardiovascular diseases (Al-Khudairy et al., 2017; Chen et al., 2013; Cheng et al., 2021; Ye et al., 2013).

The role and function of hydrogen-sulfide (H2S) has been reevaluated in recent years from a noxious and irritating gas to an essential signal molecule (gasotransmitter) in mammalian biology. It has been named the 3rd important gasotransmitter (along with nitric-oxide and carbon-monoxide) having numerous physiological effects (e.g. anti-inflammatory, cardioprotective) (Abe and Kimura, 1996; Murphy et al., 2019; Vandiver and Snyder, 2012). Because of these aforementioned reasons, the importance of H2S releasing drugs are a well-studied subject in numerous publications (Citi et al., 2020; Fukuto, 2022; Szabó, 2007).

We recently published the synthesis and pharmacological characterization of a new H2S-donor ibuprofen derivative BM-88 (Vass et al., 2023), which showed promising results in mitigating postischemic cardiac damage. The water solubility of the BM-88 prodrug molecule was ensured by incorporating an amphiphilic tetraethylene glycol linker between the H2S- releasing dithioacetate functional group and the anti-inflammatory ibuprofen unit. We found that preadministration of BM-88 significantly reduced the incidence of reperfusion-induced ventricular fibrillation (VF) and infarct size in ischemic/reperfused myocardium compared to drug-free controls, presumably through regulation of the H2S signaling pathway.

In the present study, we aimed to synthesize a new H2S-releasing ascorbic acid derivative and investigate its cardiovascular effects in a well-known isolated heart model as well on H9c2 cardiomyocyte cells derived from embryonic rat heart tissue (Kimes and Brandt, 1976). Our idea was to combine the beneficial cardiovascular and anti-inflammatory effects of H2S with the water solubility and well-known antioxidant effect of ascorbic acid (vitamin C) and investigate whether it has any beneficial effect on the basic cardiac functions. Our study included the determination of the antioxidant capacity of BM-164 under experimental conditions. Furthermore, in order to reveal the mechanism of cardiovascular effects, we also investigated the activity of BM-164 on autophagy and apoptotic processes.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. General information

Thin layer chromatography (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 performed using Silica gel 60 (Merck, Darmstadt, Germany, 0.040–0.063 mm). The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded by a Bruker DRX-400 spectrometer. 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 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 spectra of BM-164 can be found in the Supplementary Information (Fig. S1).

2.1.2. 6-S-Thioacetyl-6-thioascorbic acid [(R)-2-((R)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl)-2-hydroxyethyl ethanedithioate] (BM-164)

6-Bromo-6-deoxy-ascorbic acid ((*R*)-5-((*R*)-2-bromo-1-hydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one) (Bock et al., 1981) (474 mg, 1.98 mmol) was dissolved in acetone (50 mL), freshly prepared potassium dithioacetate (500 mg, 3.84 mmol) was added and the mixture stirred in argon atmosphere for 2 h in the presence of another 240 mg (1.84 mmol) of potassium dithioacetate. After 12 h stirring it was evaporated to dryness. The solid residue was dissolved in water (10 mL) and the solution was acidified with acetic acid. The product was extracted with 2 × 100 mL of ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The product was purified by flash column chromatography (CH_2Cl_2–MeOH 9:1 containing 0.1 % acetic acid) to produce compound **BM-164** (400 mg, yield 75 %) as a yellowish solid. R_f = 0.75 (CH_2Cl_2/MeOH 8:2 containing 0.1 % acetic acid); $[\alpha]_D$ = - 93.7 (c = 0.16, DMSO); ^{1}H NMR (400 MHz, CD_3OD): δ 4.72 (d, J = 1.6 Hz, 1H, H-4), 4.08 (d, J = 5.5 Hz, 1H, H-5), 3.65 (dd, J = 13.8, 5.1 Hz, 1H, H-6a), 3.51 – 3.41 (m, 1H, H-6b), 2.85 (s, 3H, C*H*$_3$S). (ppm); ^{13}C NMR (100 MHz, CD_3OD): δ 234.7 (1C, C═S), 173.3 (1C, C═O), 154.4 (1C, C-3), 120.2 (1C, C-2), 79.1 (1C, C-5), 67.9 (1C, C-4), 41.4 (1C, C6), 39.5 (1C, *C*H$_3$S) (ppm). MALDI-TOF MS: m/z calculated for $C_8H_{10}O_5S_2Na^+$: 272.9970 $[M+Na]^+$; found: 272.9900.

2.2. Detection of H_2S release of BM-164 in the medium

The release of H_2S was measured by an amperometric, H_2S specific sensor (ISO-H_2S-100, World Precision Instruments, Saratosa, FL, USA) connected to a WPI TBR 1025 One-Channel Free Radical Analyzer. The sensor was set to 10 nA range and poise voltage to +150 mV. Before each experiment, the sensor was polarized in phosphate buffered saline (PBS) for 12 h and calibrated with freshly prepared Na_2S x 9 H_2O solution (concentrations of Na_2S x 9 H_2O solutions were 0, 2, 4, 8 and 10 μM) (Vass et al., 2023).

The H_2S-donating ability of BM-164 was studied in a rat heart cell culture medium, using Dulbecco's modified Eagle's medium collected from H9c2 cell culture on the third day. H9c2 cardimyocyte cells were purchased from ATCC, CRL-1446, LGC Standards GmbH Wesel, Germany. BM-164 (0.5 mg) was dissolved in 5 mL of medium and the H_2S release was detected from this solution by the sensor.

2.3. Measurement of antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH radical scavenging activity is employed to investigate the antioxidant capacity of BM-164 according to the method reported by Shimamura et al. (2014) with a few modifications. DPPH is a stable free radical with a deep violet color. Upon receiving an electron or a hydrogen radical, DPPH's color fades to yellow, indicating its reduced form.

Hydrolysis of dithioesters, including dithioacetates, catalyzed by various aspecific esterases found in the cardiovascular system can lead to the formation of a thiol and eventually H2S (Gyöngyösi et al., 2021; Liu and Orgel, 1997). In our study, we used a freshly prepared supernatant of the tissue homogenate from a frozen control rat heart in PBS buffer to induce H2S release from BM-164, and the antioxidant activity profile of BM-164 was evaluated with and without the addition of the heart tissue homogenate.

In principle, a mixed solution of methanol (1.2 mL) and 0.1 M Tris–HCl buffer (pH = 7.4, 800 μL) was selected as blank. DPPH solution (1 mL) was added to a mixture of methanol (200 μL) and 0.1 M Tris–HCl

buffer (800 μL). After mixing, the absorbance at 517 nm was recorded by a UV-1600 PC VWR spectrophotometer (VWR, China). To avoid any possible interference from the tissue homogenate with the assay, the absorbance by adding different volumes of the supernatant of the tissue homogenate to the aforementioned mixture was checked. As a result, two volumes, 5 and 20 μL, which displayed no activity were chosen.

2.3.1. DPPH assay procedure

To a mixture of BM-164 solution in PBS buffer (200 μL) and 0.1 M Tris–HCl buffer (800 μL), DPPH (1 mL) (BM-164 without tissue homogenate group) the supernatant of the heart homogenate (5 or 20 μL) (supernatant-containing groups) was added. The resultant solution was mixed immediately, and the absorbance was measured. The experiment was continued for a total of 120 min, and the absorbance values were taken at min of 1, 15, 30, 60, and 120. All three studied groups were tested at concentrations ranging from 500 to 0.49 μM. Ascorbic acid (vitamin C) served as the positive control and the reference compound. Each experiment was conducted in triplicate, and the antioxidant activity was evaluated based on the absorbance at 517 nm.

The inhibition ratio (IR, %) was calculated as the following:

$$Inhibition\ Ratio\ (\%) = \left(\frac{Ac - As}{Ac}\right) * 100$$

Where Ac = the absorbance if methanol was added instead of the sample, and As = the absorbance if the testing sample solution was added.

The concentration required to cause a 50 % inhibition of the free radical (IR_{50}, μM) was calculated by plotting the percent of inhibition against the sample concentration.

2.4. Cell culture and cellular toxicity assay

The cellular toxicity assay was carried out on H9c2 cardiomyocyte cells (ATCC, CRL-1446, LGC Standards GmbH Wesel, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10 % FBS (fetal bovine serum) and 1 % penicillin-streptomycin combination at 37 °C in a humidified incubator in 5 % CO_2 95 % air atmosphere. The cells were left for one day in a cell culture flask establish a 60 to 70 % confluent layer. Ascorbic acid and BM-164 were dissolved into the medium and cytotoxicity was measured by MTT [3-(4,5-dimethylthiazol 2-yl)-2,5-(diphenyltetrazolium bromide)] assay.

The cells were seeded into 96 well cell culture plates, with an average 3000 cells/well and treated with ascorbic acid or BM-164 3 μM, 10 μM, 30 μM, 100 μM 300 μM and 500 μM, respectively, for 24 h. After the treatments, MTT solution (0.5 mg/ml final concentration) was added to each well and incubated for 3 h at 37 °C, and then, the medium was discarded and replaced by isopropyl alcohol to dissolve the formed formazan crystals. Absorbance was measured by Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Ratastie, Finland) at 570 and 690 nm. The coloration of the solution was quantified by the measurement of the absorbance (570 nm) subtracted from the background absorbance (690 nm). The values were expressed relative to the absolute control (100 % cell viability). 10 % DMSO was used as positive control, which significantly reduced the cell viability in the cell culture. The absorbance values were measured four times and averaged.

2.5. Experimental design of the ex vivo studies

The goal of the experiments was to examine the potential beneficial effects of H_2S releasing, modified ascorbic acid (BM-164) with regards to ischemia/reperfusion (I/R) injuries in isolated heart model (Langendorff, 1895) (Scheme 1).

After the anesthesia of the rats, the heart was excised and cannulated on a Langendorff-device via the aorta. Each heart was perfused by modified Krebs-Henseleit buffer (Krebs and Henseleit, 1932) (KHB). Following a brief (5 min) equilibration period, measurements of CF and HR were carried out. During the pre-treatment phase (10 min) KHB containing 30 μM of BM-164 or ascorbic acid was added in the experimental groups, meanwhile coronary flow (CF) and heart rate (HR) were registered at 1st, 5th and 10th min, and H_2S release was measured from the CF effluent at each time point, and then, the hearts were subjected to 30 min of global ischemia (ISA) followed by 120 min of reperfusion. When the buffer perfusion restarted, the incidence of reperfusion-induced ventricular fibrillation (VF) was recorded, and the measurements of CF, HR and H_2S values were carried out at 1, 5, 10, 30, 60, 90 and 120 min of the reperfusion period, respectively.

2.6. Experimental animals

Male Sprague-Dawley rats (400–500 g; anesthesia: ketamine/xylazine: 50/10 mg/kg i.p.) were used for the experiments. All experiments and the handling of the animals were carried out in accordance with the EU regulation (EU Directive 63/2010) and the Hungarian laws (The Hungarian Animal Protection Act, Law XVIII/1998), together with ethical approvals registered by the University of Debrecen Committee of Animal Welfare (Reg. Nr.: 6/2019/UDCAW).

2.7. Isolated heart preparation and cardiac function assessment

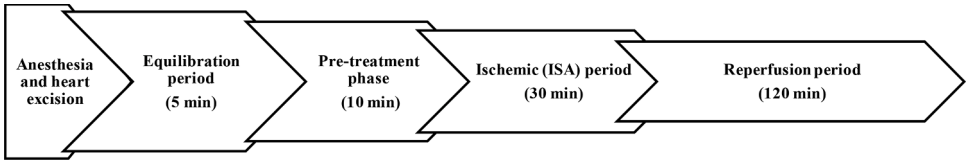
The rats were anesthetized, injected by 400 U of heparin i.p., the chest cavity was opened, the heart was excised and placed into modified Krebs-Henseleit bicarbonate buffer. The next step was to cannulate the heart via the aorta and perfuse in Langendorff-mode at a constant pressure equivalent to 71 mmHg (10 kPa). The perfusion medium contains the following ingredients in Krebs-Henseleit buffer solution: 118 mM sodium chloride, 4.7 mM potassium chloride, 1.7 mM calcium chloride, 25 mM sodium bicarbonate, 0.36 mM potassium biphosphate, 1.2 mM magnesium sulfate, 10 mM glucose. Coronary flow was directly measured, and heart rates were registered during the experiment with a pressure transducer and recorded by a software (LabChart Pro 8.1; AD Instruments, Sydney, Australia).

2.8. Detection of H_2S release from coronary flow

The release of H_2S was detected by an amperometric, H_2S specific sensor as described in Chapter 2.2. The signal induced by H_2S values was measured from the CF effluent by immersing the tip of the sensor in 5 mL effluent-buffer solution and the value of the stabilized signal was recorded.

2.9. Measurement of infarct size

The infarct size was measured by TTC (2,3,5-triphenyltetrazolium



Scheme 1. Experimental design of the ex vivo study.

chloride) (Sigma-Aldrich, Inc., St. Louis, MO, USA) staining (Czegledi et al., 2019). At the end of the experiment, the hearts were perfused by 50 mL freshly prepared TTC solution (1 % TTC in phosphate buffer solution) over 5 min and stored at −80 °C until the evaluation. The hearts were taken out of −80 °C, sliced into 2 mm thick slices, and left to slowly defrost. Following the defrosting, the slices' weight was measured individually, and digitized by an Epson J232D flat-bed scanner. The image analysis was carried out by ImageJ (National Institute of Health, Bethesda, MD, USA) image processing and analysis software. We measured the staining of the tissue slices by pixel and calculated the infarcted area (white staining) ratio to the risk area (the entire scanned section) for each heart. This ratio then was multiplied by the slices' weight, and the total infarcted volume of the heart was expressed in percentage (%).

2.10. Protein determination in isolated rat hearts

The expression level of proteins in rat heart tissues was measured using Western blot analysis. For protein extraction approximately 300 mg of heart tissues were homogenized by using a polytron homogenizer in ice cold tissue lysis buffer supplemented with protease and phosphatase inhibitor cocktail. Total protein extraction was carried out using M-PER tissue lysis kit (Thermo Fisher Scientific, Waltham, MA, USA) and the lysis was performed on ice for 30 min, and then, samples were centrifuged at 20 000 rpm at 4 °C for 20 min. Supernatants were collected for protein concentration measurement. Protein concentration was evaluated by BCA Protein Assay Kit (Thermo Fisher Scientific).

2.11. Western blot analysis

Equal amount of protein (40 μg) from each sample was diluted with 4X Laemmli buffer, boiled at 95 °C for 8 min and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (TGX Stain-FreeTM FastCastTM Acrylamide Kit, 12 %, Bio-Rad, Hercules, CA, USA). Molecular weight marker Precision Plus Protein Dual Color Standards (Bio-Rad, #1610374) was used, and gels were transferred on a PVDF membrane. Non-specific binding sites were blocked in 5 % nonfat dry milk/Tris-buffered saline with 0,1 % Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with specific primary antibodies against the following autophagic and apoptotic proteins: LC-3 (1:1000), p62 (1:1000), Beclin-1 (1:1000), caspase-3 (1:1000), AMPK/p-AMPK (1:500), Tom-20 (1:1000). HPRT (hypoxanthine-guanine phosphoribosyltransferase) (1:1000) was used as the loading control, and AMPKα for pAMPKα (Dzhalilova et al., 2022). Antibodies were obtained from Cell Signaling Technology (Boston, MA, USA), and Novus Biologicals (Litleton, Colorado, USA) detailed description of antibodies is listed in Table S1 (Supplementary Information).

After extensive washing, membranes were incubated with anti-rabbit horseradish-peroxidase-conjugated secondary antibody (HRP-linked anti-rabbit IgG, 1:5000, Cell Signaling Technology) for 1 h at room temperature. Signal intensities for each protein band were detected using Clarity Western ECL Substrate (Bio-Rad, California, USA). The optical density of bands was measured by using the ChemiDoc Touch Imaging System (Bio-Rad, California, USA). The level of the protein of interest was normalized against HPRT using the Bio-Rad Image Lab 5.2.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Finally, the gels were also stained with Coomassie Blue (Welinder and Ekblad, 2011) in order to verify that the same amount of applied protein was run and transferred to the PVDF (polyvinylidene difluoride) membranes (Fig. S2 A and B. Supplementary information).

2.12. Statistical analysis

The statistical analysis was carried out by GraphPad Prism (GraphPad Prism 8.4.3., GraphPad Software Inc, Boston, MA, USA). Analysis of variance (ANOVA) and *t*-tests were done making comparison between the groups. For the incidence of reperfusion induced arrhythmias Chi-squared test was performed to compare the results obtained in the experimental groups. Probability value (p) less than 0.05 was considered as statistically significant.

3. Results

3.1. Design and synthesis of BM-164

BM-164 is a H_2S donor ascorbic acid derivative prodrug containing a dithioacetate moiety. It was prepared from 6-deoxy-6-bromoascorbic acid (**2**) which was obtained from ascorbic acid (**1**) according to the literature, by treatment with hydrogen bromide in glacial acetic acid (Bock et al., 1981). Nucleophilic substitution reaction of the bromo derivative **2** with potassium dithioacetate afforded **BM-164** (Scheme 2).

The dithioester-containing **BM-164** can release H_2S under experimental conditions when it reacts with cysteine via a native chemical ligation mechanism (Scheme 3a), resulting in a thiol (**ii**), a cysteine-derived dihydrothiazole (**v**) and H_2S (Cerda et al., 2019; Vass et al., 2023). On the other hand, dithioacetates can also undergo hydrolysis catalyzed by native aspecific esterases (Scheme 3b), resulting in a thiol and a thiolacetic acid (**viii**), which is spontaneously oxidized to a disulfide (**ix**); the reaction of the latter disulfide with native amines results in one equivalent of acetamide (**x**) and half equivalent of H_2S (Gyöngyösi et al., 2021; Liu and Orgel, 1997).

3.2. Investigation of H_2S donating property of BM-164

BM-164 releases H_2S in biological milieu either by hydrolysis catalyzed by aspecific esterase enzymes or by a cysteine-triggered pathway as previously discussed. Rat heart cell culture medium (DMEM) collected from H9c2 cell culture on the third day was used to model the biological environment. Using the H_2S selective sensor, we found that the reducing property of ascorbic acid (vitamin C) can affect the sensor, since ascorbic acid in aqueous solution is easily oxidized by oxygen dissolved in water. Therefore, the culture medium (5 mL) was deoxygenated with nitrogen gas bubbled through the aqueous solution and thus used for the study. Upon addition of BM-164 (0.5 mg), immediate H_2S evolution was observed, reaching a maximum in approximately 5 min (Fig. 1, black curve). In a control experiment, when 0.5 mg of ascorbic acid was dissolved in the deoxygenated medium and examined with the H_2S-selective sensor, a continuous low-intensity signal was observed (orange curve, Fig. 1).

The H_2S donor property of BM-164 was also investigated in a non-biological environment in PBS buffer, when no H_2S release was detected (see in the Supplementary Information Fig. S3). These results show that either esterases or cysteine and amines present under physiological conditions are essential for the degradation of the dithioacetic acid moiety of BM-164, resulting in the release of H_2S.

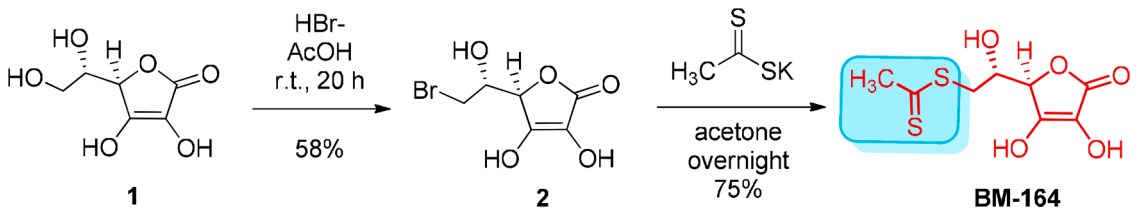
3.3. Antioxidant activity of BM-164

Since both ascorbic acid (vitamin C) and H_2S (Gęgotek and Skrzydlewska, 2023; Kanagy et al., 2017) have strong antioxidant properties, a H_2S-donor ascorbic acid derivative such as BM-164 (Fig. 2) is expected to have an additive antioxidant effect. Cardiovascular tissue homogenate (hereafter referred to as extract or triggering extract) was used to induce H_2S release from BM-164, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was chosen to investigate its antioxidant properties.

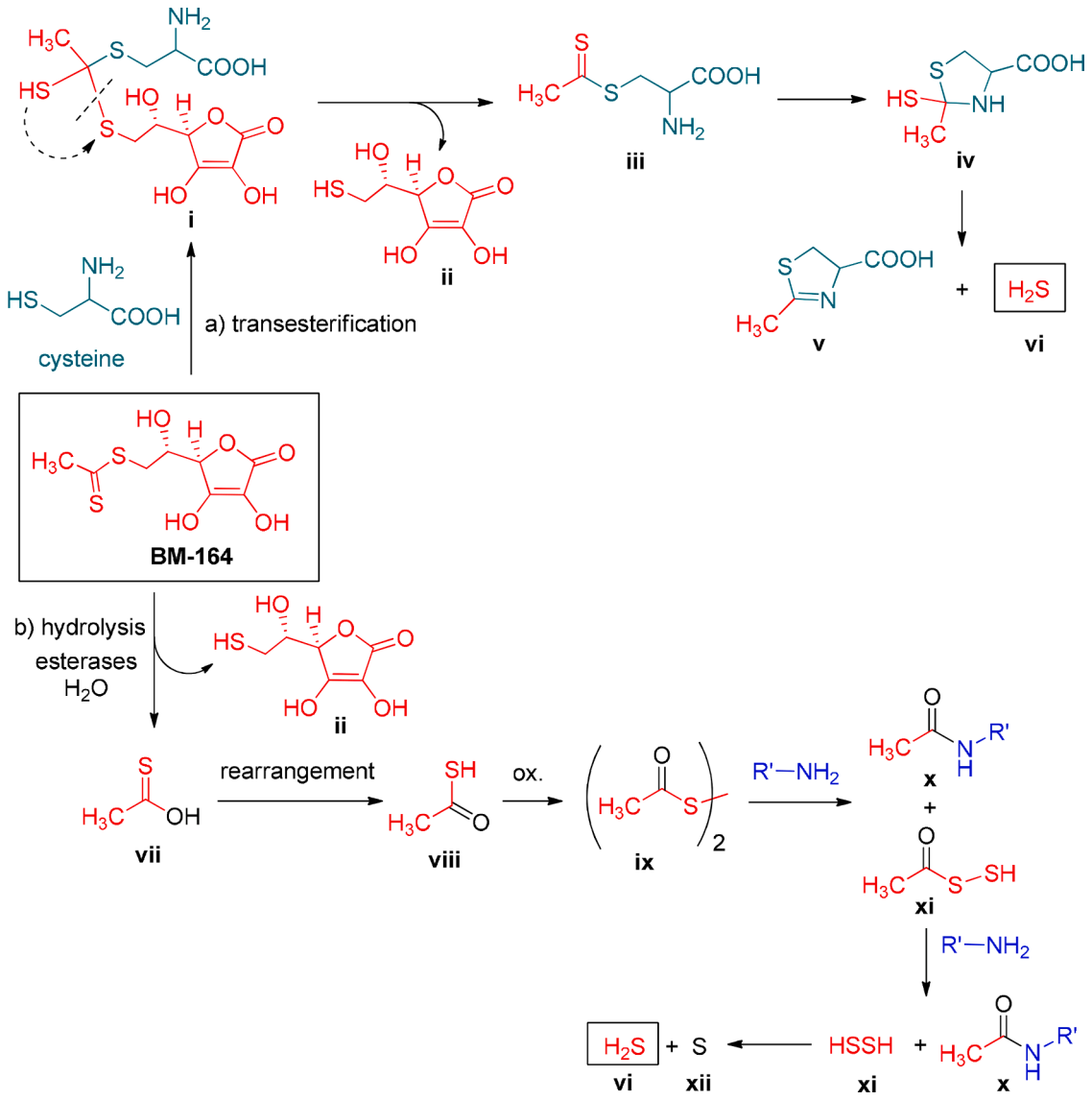
3.4. DPPH assay

3.4.1. Extract absorbance determination

Antioxidant activity is abundant in biological systems protecting them against the toxicity of reactive oxygen species (ROS)



Scheme 2. Synthesis of **BM-164**. The H_2S-releasing dithioacetate group is highlighted in blue.



Scheme 3. Two possible ways of H_2S release from 6-dithioacetyl-ascorbic acid **BM-164** under physiological conditions; (a) cysteine-mediated H_2S release via a transesterification mechanism (b) H_2S release by aspecific esterase-catalyzed hydrolysis followed by disulfide formation and reaction with native amines.

(Dubois-Deruy et al., 2020). Such activity of the triggering heart extract used in the present study can also be expected, which may result in an incorrect interpretation of the radical scavenging activity of the tested compound. To avoid this, the extract can only be used in a volume in which it has no or negligible radical scavenging capability. Therefore, we studied the antioxidant properties of the different volumes of the extract, ranging from 50 to 5 µL, in a DPPH assay (Fig. S4).

We found that the extract resulted in an inhibition ratio of 22 % and 7 % of the DPPH$^{\bullet}$ free radical if added in 50 and 25 µL, respectively, compared to the inhibitory effect of ascorbic acid, while the antioxidant effect was negligible at amounts of 20 µL and below (inhibition ratio of 2.5 %). Therefore, to assess the antioxidant activity of the compound investigated, maximum and minimum volumes of 20 µL and 5 µL of the homogenized heart extract were selected, respectively.

3.4.2. *DPPH antioxidant activity*

We have studied the concentration and time dependence of the antioxidant effect of ascorbic acid and BM-164, the latter was studied i) alone without the triggering heart extract, ii) with 5 µL of extract and iii) with 20 µL of extract. The obtained results are shown in Fig. 3.

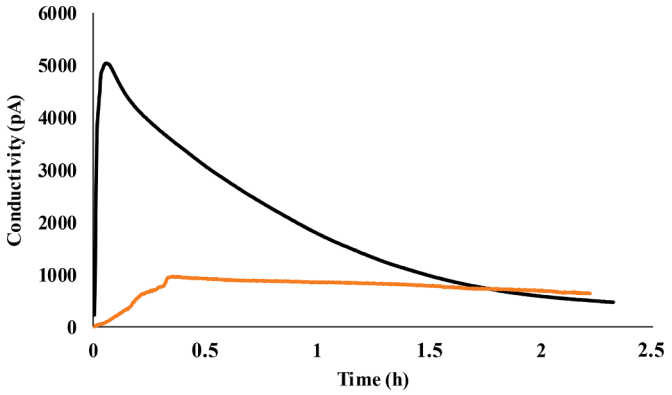


Fig. 1. Black curve: H_2S release of BM-164 in the medium derived from H9c2 cardiomyoblast cells. Orange curve: signal in the presence of ascorbic acid in the medium.

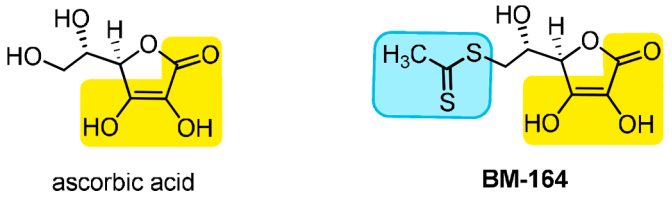


Fig. 2. The structure of ascorbic acid (reference compound) and BM-164. Colored boxes indicate the molecular parts responsible for the antioxidant properties. The reductone part is highlighted in yellow, the H_2S-releasing moiety is highlighted in blue.

Antioxidant activity of ascorbic acid shows a maximum value in the range of 125 to 500 μM (saturation plateau), however further dilution strongly reduces its antioxidant activity. This concentration dependence was observed without change throughout the 120 min interval (Fig. 3A–E).

Compound BM-164 without a triggering extract displays high antioxidant activity at a concentration of 500 μM, which strongly decreases in the first 30 min in diluted solutions (Fig. 3A–C). Interestingly, after 1 h, this sharp reduction in antioxidant capacity due to the dilution and a concentration-inhibition relationship similar to the samples containing the triggering extract can be seen (Fig. 3D and E). The obtained results can be explained by the slow degradation of the dithioester group of BM-164 in the test solution, which results in the evolution of H_2S, but in a delayed manner compared to the samples containing the biological extract.

BM-164 with 20 μL of the homogenized myocardial tissue (extract) showed a higher antioxidant activity than without the extract, except for at the highest concentration in the first 30 min. The antioxidant effect of BM-164 in the presence of 5 μL extract showed exactly the same concentration and time dependence as the sample containing 20 μL, but in the first 30 min the activity of the 5 μL extract-containing sample was significantly at a lower level (Fig. 3A–E).

Fig. 4 shows the time and concentration dependence of the antioxidant effect of BM-164 + 20 μL extract compared to the antioxidant effect of ascorbic acid. Fig. 4 clearly demonstrates that the DPPH inhibitory activity of BM-164 in the presence of the extract continuously increases during the 120 min time due to the continuous release of H_2S originated from BM-164. The result of this additive effect of H_2S, the antioxidant activity of BM-164 surpasses that of ascorbic acid at lower concentrations, from about 60 μM. However, in high concentrations (in the range of 100 to 500 μM), the unmodified ascorbic acid has a higher antioxidant capacity in comparison with BM-164. A probable explanation for this is that at high concentrations the effect of the reductone part dominates, and the electron-withdrawing dithioacetyl substituent in BM-164

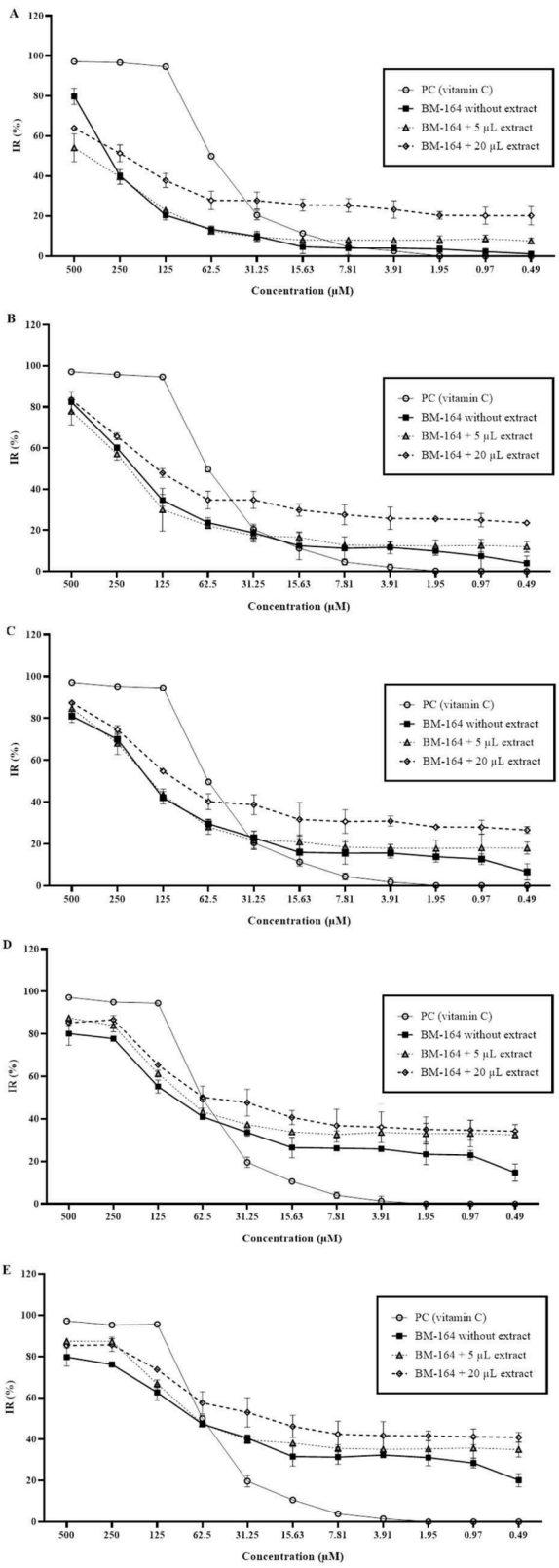


Fig. 3. DPPH inhibition ratio of BM-164 alone and with the medium compared to the positive control (PC, vitamin C) at (A): 1 min, (B): 15 min, (C): 30 min, (D): 60 min, and (E): 120 min. Error bars show the standard deviation of the mean value ($n = 3$). Results express mean ± SD. IR = inhibition ratio.

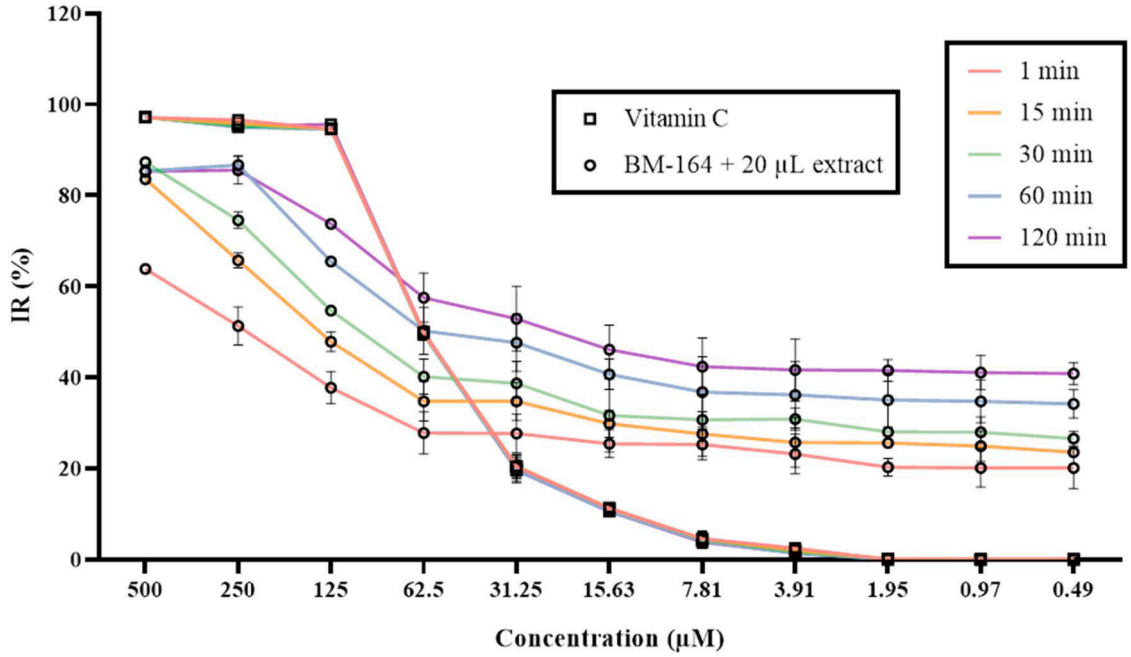


Fig. 4. DPPH inhibition ratio of BM-164 alone and with the extract (homogenized tissue) compared to ascorbic acid (vitamin C) at 1, 15, 30, 60, and 120 min. Error bars show the standard deviation of the mean value ($n = 3$). Results express mean ± SD; IR = inhibition ratio.

somewhat reduces the antioxidant activity of the reductone part.

Table 1 shows the antioxidant capacity of the groups expressed as IR_{50} (μM). During the 120 min time-frame, BM-164 with 20 μL of the homogenized myocardial extract showed the lowest IR_{50} values of 21.45 – 3.01 μM. Additionally, as discussed earlier, both BM-164 alone and with 5 μL of the extract exhibited an increase in their antioxidant activity after 1 h. Table 1 shows that these groups displayed lower IR_{50} values (22.80–11.04 μM and 13.14 – 8.77 μM, respectively) compared to the group treated with vitamin C, which had an IR_{50} value of around 55 μM throughout the entire time course.

It should be noted that both 5 μL and 20 μL of extract (homogenized tissue) were added to the unmodified ascorbic acid and DPPH decolorization and monitored for 120 min. The extract did not affect the antioxidant activity of ascorbic acid, indicating that the added extract has no effect on the reductive endiol system and is only effective on the dithioester moiety of BM-164.

3.5. Cell viability assay

The cytotoxicity of BM-164 was evaluated in H9c2 cells in comparison with ascorbic acid at six different concentrations (Fig. 5). The results show that 10 μM and 30 μM of BM-164 do not cause significant toxic effect, however, 100 μM, 300 μM and 500 μM of BM-164 resulted in increasing cytotoxicity. The same concentrations of ascorbic acid do not produce the same effect. Therefore, the use of BM-164 in 30 μM concentration was selected for the isolated heart experiments.

Table 1
IR_{50} values (μM) of BM-164 alone and with the extract (homogenized tissue) compared to ascorbic acid (vitamin C).

Time (min)	Vitamin C	BM-164 without extract	BM-164 + 5 μL extract	BM-164 + 20 μL extract
0	55.30	204.3	122.6	21.45
15	55.37	109.8	103.9	20.89
30	55.44	69.28	66.77	18.10
60	56.22	22.80	13.14	7.21
120	55.71	11.04	8.77	3.01

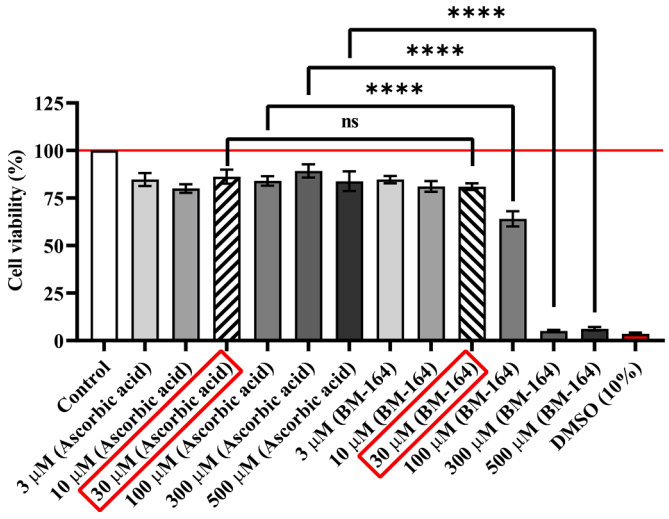


Fig. 5. Evaluation of BM-164 and ascorbic acid cytotoxicity. H9c2 cells were treated with BM-164 and ascorbic acid from 3 to 500 μM concentrations, respectively, for 24 h. The cell viability was detected using MTT assay and calculated as the percentage of cell surviving drug exposure. Data were expressed as the mean ± SEM, $n = 8$ wells in each group. **** $p < 0.001$ in comparison with the same concentrations of BM-164 to ascorbic acid; ns: not significant. Control: cell viability in untreated H9c2 cells (100 %).

3.6. Detection of H_2S in the perfusion effluent of the heart

H_2S release was detected directly from the CF effluent during the isolated rat heart experiment by a H_2S selective sensor (Fig. 6). After the 30 min ischemic period, the H_2S content in the effluent was higher (BM-164 > control > ascorbic acid) than before the induction of ischemia. This elevated level of H_2S is due to endogenous processes; in the presence of ascorbic acid, endogenous H_2S production was lower compared to the control group, while the H_2S level measured in the BM-164 treated group exceeded that of the control group due to the additional effect of H_2S released from BM-164. During reperfusion, from the 30th min, H_2S

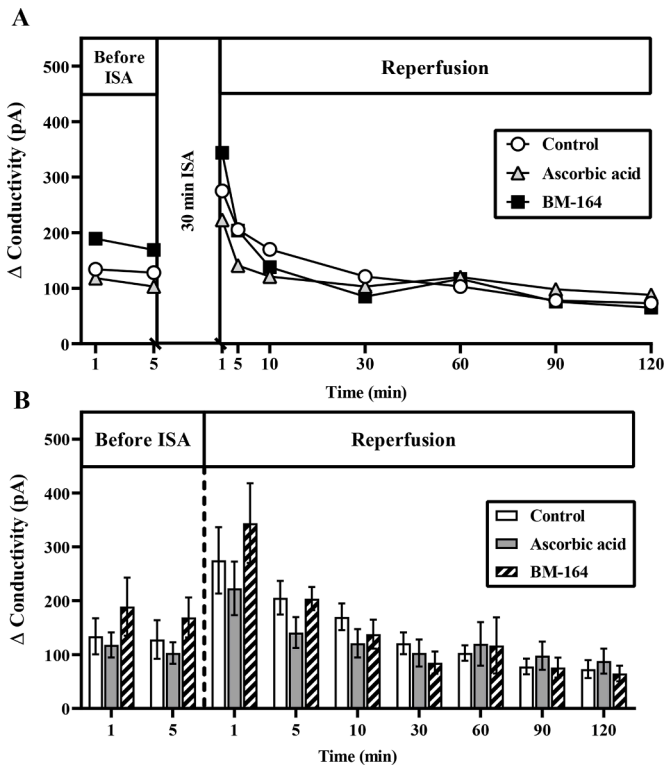


Fig. 6. The changes in H_2S levels during the experiments. (A): The time-course of H_2S release in each group before ischemia (Before ISA) and during the reperfusion period. (B): Mean ± SEM values of the H_2S release; $n = 6$ at each time point.

levels of each group returned to the normal range measured before the induction of ischemia (ISA).

3.7. Incidence of ventricular fibrillation and detection of infarct size

The isolated rat hearts were subjected to 30 min of global ischemia followed by 120 min of reperfusion. The incidence of VF was highest in the ischemic control group (79 %) in accordance with previous experience and literature data (Vass et al., 2023). This was followed by the ascorbic acid group (55 %) and the lowest values were detected in the BM-164 treated group (31 %). The BM-164 treatment significantly reduced the incidence of VF from 79 % to 31 % compared to the control group ($p < 0.05$) (Fig. 7A).

The infarcted areas were the largest in the control group (28.1 ± 4.2 %), the second highest in the ascorbic acid group (19.25 ± 6 %) and the lowest in the BM-164 treated group (13.8 ± 1.8 %). The difference between the control and the BM-164 group proved to be significant, $*p < 0.05$ (Fig. 7B).

3.8. Coronary flow and heart rate

Fig. 8 shows the coronary flow (CF; Fig. 8A) and heart rate (HR; Fig. 8B) during the equilibration, pre-treatment, and the reperfusion period. Regarding CF values, there were no significant differences between the groups. The graphs show a decreasing trend during the reperfusion phase, however lower CF values in the control group *versus* the treated groups were registered. These gradually decreasing tendencies can be considered valid in this kind of experimental model. However, the BM-164 treated hearts showed the highest CF recovery values during reperfusion, although those were not at a statistically significant level.

HR did not show a significant change in the drug treated groups in comparison with the drug-free control group. The lowest HR values were

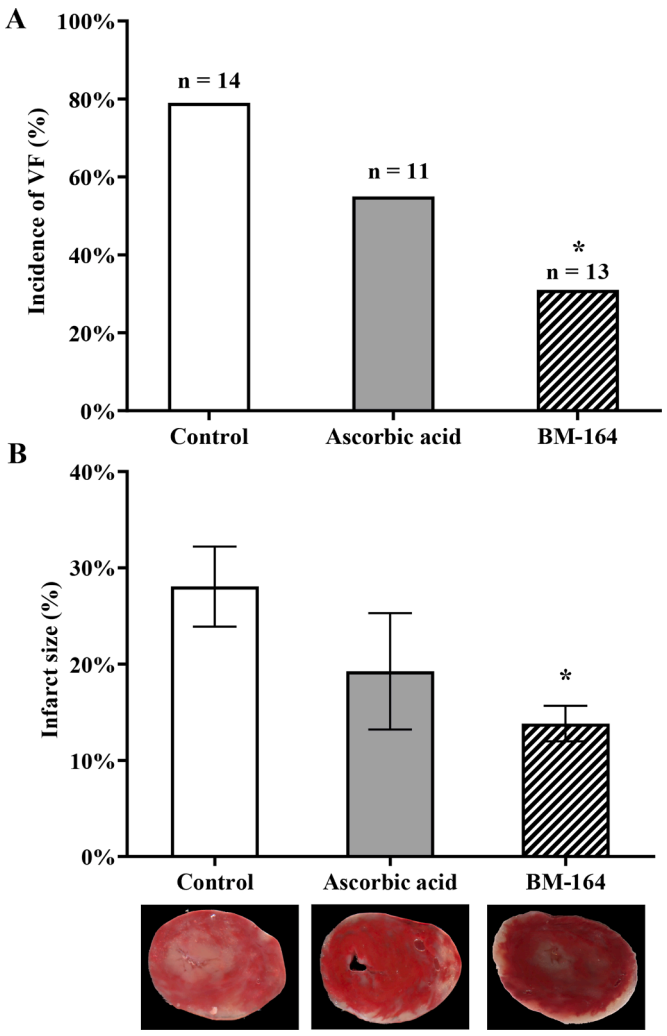


Fig. 7. (A): The incidence of the reperfusion-induced ventricular fibrillation (VF) after 30 min of the ischemic period; (B) Representative infarct size in the control and treated groups are shown. (infarcted area in white, and non-ischemic region stained in red) $*p < 0.05$ BM-164 vs. control; mean ± SEM.

registered in the control group and no significant differences were detected between the control and treated groups (Fig. 8B).

3.9. Effect of BM-164 treatment on autophagy/apoptosis related protein expression

Previous studies demonstrated (Zhang et al., 2021) that H_2S-donors significantly improved cardiac function, including through pathways regulating autophagic and apoptotic processes. In the present study we examined the impact of the new H_2S-donor, BM-164, on the expression of several autophagic and apoptotic proteins. To explore whether BM-164 treatment promoted or inhibited autophagy, the levels of the basic autophagic marker proteins were measured by Western blot. It was investigated how BM-164 treatment affected the ischemia reperfusion (I/R) induced injury; the hearts were divided into the following two groups: hearts showing VF after ischemia-reperfusion and hearts did not develop VF during reperfusion (no VF). For comparisons, the samples were run in parallel with an ischemic, untreated control group and a group treated with ascorbic acid or BM-164. Three samples obtained from three different hearts were run for each group/gel for protein expression analysis.

Representative Western blot results (relative protein expressions normalized to HPRT housekeeping protein) are shown in Fig. 9; results

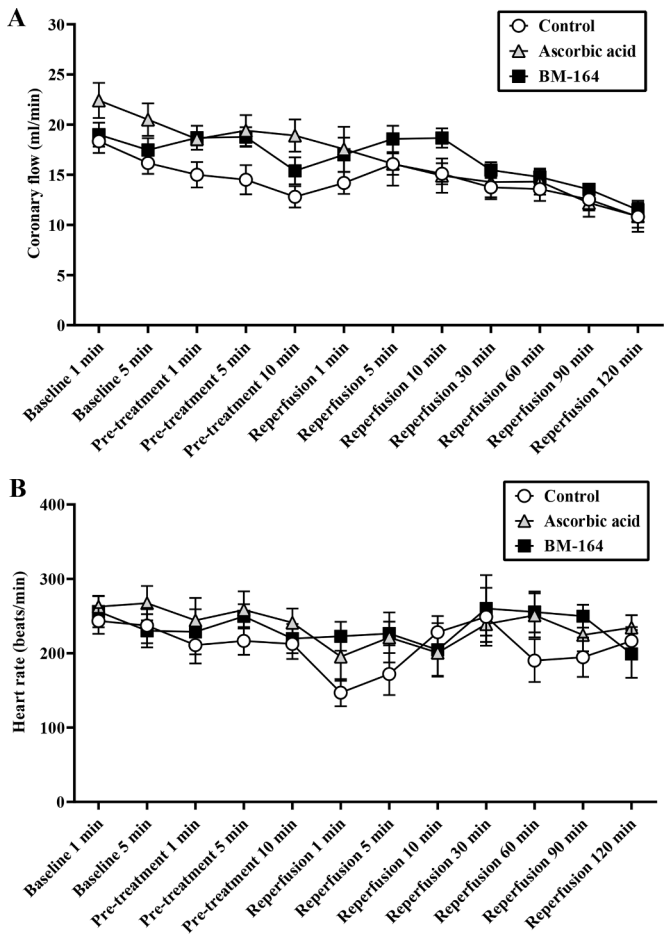


Fig. 8. (A): Coronary flow (ml/min) values during the experiments. (B): Heart rate (beats/min) values during the study; $n = 11$ at each time point; mean ± SEM.

obtained from no VF samples are shown on *panel A*, and results obtained from VF samples are shown on *panel B* (Fig. 9.1A and B).

Expression of total LC-3, an autophagosomal membrane protein used as a marker of initiation of autophagosome formation, was significantly increased in BM-164-treated hearts that did not developed VF upon reperfusion. The expression level of two forms of LC-3, LC-3-I, which is found in the cytoplasm, and LC-3-II, which is membrane-bound and is converted from LC-3-I to initiate formation of the autophagosome were quantified. Both expressions of LC-3-I and LC-3-II were significantly elevated in BM-164 treated I/R non-fibrillated group compared to the untreated ischemic control hearts (Fig. 9.2C, E, G). The results presented in the right panel show that in fibrillated hearts only LC-3-II but no LC-3-I was significantly elevated by the treatment with BM-164 compared to control hearts (Fig. 9.2D, F, H).

Levels of Beclin-1, the signal of the onset of autophagy process, were significantly elevated in all BM-164 treated hearts compared to non-treated ischemic groups (Fig. 9.3I and J).

Interestingly, the level of p62, a well-known substrate of autophagic processes, was significantly increased only in the BM-164 treated hearts that showed VF upon reperfusion in comparison with the other groups. BM-164 treatment also enhanced the level of p62 in I/R non-fibrillated hearts, although it did not reach a statistically significant level (Fig. 9.3K and L).

The level of pAMPK stimulates the autophagic flux by contributing to autophagosome maturation, which was significantly increased only in the BM-164 treated I/R group in hearts developed ventricular fibrillation (Fig. 9.3M and N) upon reperfusion.

The mitophagy often follows the autophagy, therefore, we also studied the changes of Tom20 protein expression (translocase of outer mitochondrial membrane 20 protein) that characterizes this process. This protein expression was detected both in I/R (VF) and I/R (no VF) groups compared to the ischemic/reperfused drug-free control hearts. Ascorbic acid-treated group also showed an elevated level of Tom20 protein in comparison with the drug-free control hearts (Fig. 9.3O and P). An apoptotic marker, caspase-3 expression slightly increased in the fibrillated hearts in response to BM-164 treatment, however, the increase of this protein expression was not at a significant level (Fig. 9.3Q and R).

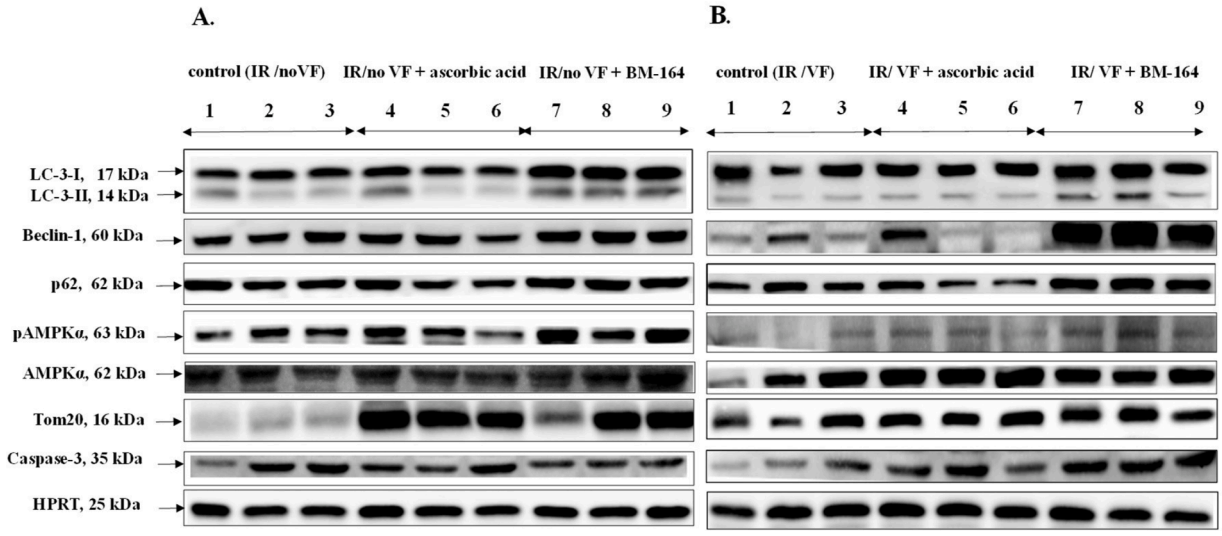


Fig. 9.1. Western blot analysis of proteins associated with autophagy and apoptosis (A, B.). The hearts before the induction of ischemia-reperfusion (I/R) were perfused by Krebs-Henseleit buffer (ischemic control), 30 μM of BM-164 or ascorbic acid for 10 min. After I/R the hearts divided into two groups; hearts without ventricular fibrillation (no VF) (panel A) and hearts developed ventricular fibrillation (VF) (panel B) were processed for protein expression analysis. Equal amount of proteins (40 μg) obtained from tree different hearts in all groups [control (1,2,3), ascorbic acid (4,5,6) and BM-164 (7,8,9)] were separated by 12 % SDS gel electrophoresis. After blotting, the membranes were incubated with specific antibodies. All signal intensities are shown in panel A and B.

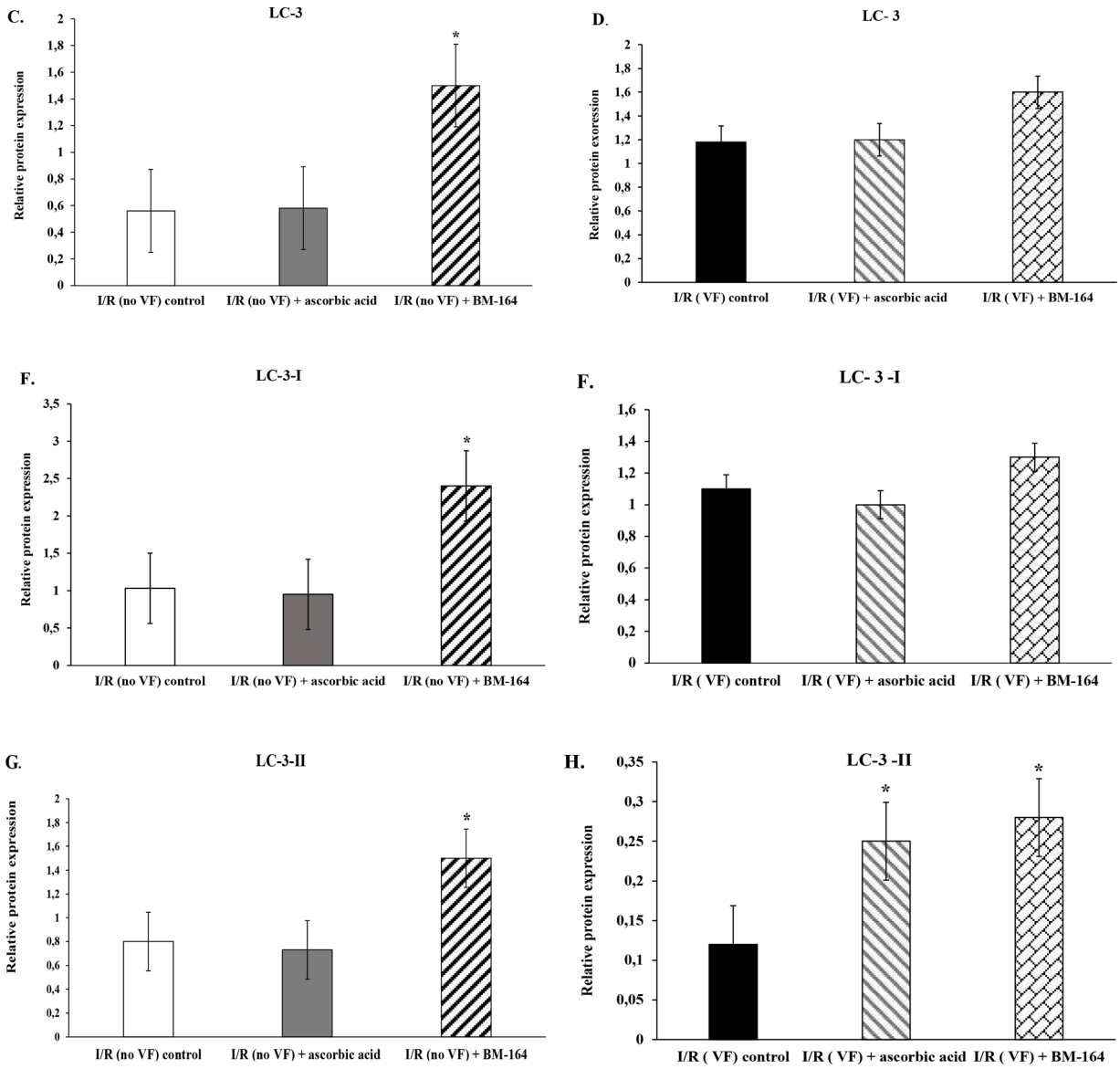


Fig. 9.2. Quantitative analysis of LC-3 protein expression detected by Western blot. Signal intensities for each protein band detected are shown in Fig. 9.1 (see panel A and B above) were quantified using the ChemiDoc Touch Imaging System and the levels of LC-3, LC-3-I, LC-3-II were normalized against HPRT (Bio-Rad Image Lab 5.2.1 software). The results detected in non-fibrillated hearts (no VF) are shown on a left side (C, E, G). The right side of the panel (D, F, H) presents protein expression of LC-3, LC-3-I, LC-3-II detected in fibrillated hearts (VF) in each group (ischemic control, 30 μM ascorbic acid and 30 μM BM-164), $^*p < 0.05$ compared to the appropriate control group; mean ± SD; $n = 3$ in each group.

4. Discussion

The ischemia/reperfusion-induced injury is a major contributing factor in several pathological processes in the development of cardiovascular diseases (Buono et al., 2019; Lu et al., 2015). The discontinuation of the coronary flow, and subsequent starvation of the myocardium causes local necrosis/apoptosis in the affected areas, leading to decreased cardiac functions. The increase of reactive oxygen species, metabolites, the destruction of red blood cells and endothelium are major concerns in these cases (Frangogiannis, 2015).

A previous study Vass et al. (2023) demonstrated that BM-88, the ibuprofen based organic H_2S donor, 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. 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 mean of controlling this potentially life threatening lethal ventricular arrhythmia. Based upon these experiments our group decided to synthesize and study the effects of a new H_2S-releasing ascorbic acid derivative, BM-164. Ascorbic acid (vitamin C) is an essential and natural abundant antioxidant with excellent water solubility; therefore, it was chosen as the parent molecule of our new H_2S donor. We found that BM-164 is stable in aqueous solution (UV/VIS spectra in the Supplementary Information as Fig. S5) and releases H_2S under experimental conditions within a 90 min period. The antioxidant activity of BM-164 was investigated by DPPH inhibition assay in the presence of homogenized heart tissue, which was used to mimic physiological conditions initiating H_2S-release. We found that during the 120 min of the experiment, the antioxidant activity of BM-164 increased continuously, due to the fact that the antioxidant effect of the releasing H_2S was added to the inherent antioxidant effect of the enediol unit (reductone part) of BM-164. Considering that the donation of H_2S from BM-164 ceases after 90 min (Fig. 1), it is surprising that the DPPH

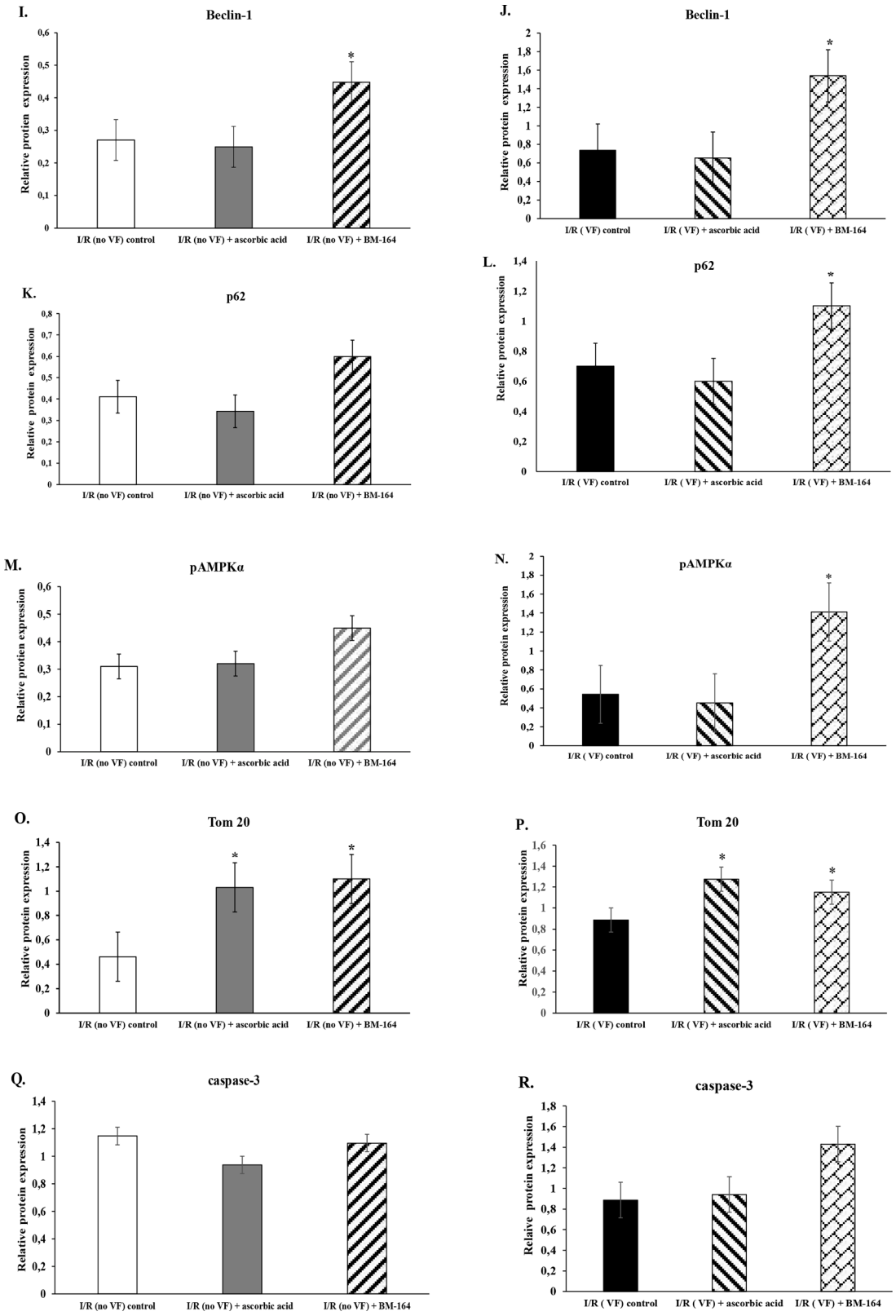


Fig. 9.3. Quantitative analysis of Beclin-1, p62, pAMPKα, Tom20 and caspase-3 expression level detected by Western blot. Signal intensities were quantified and normalized against HPRT using the Bio-Rad Image Lab 5.2.1 software. The results detected in non-fibrillated hearts (no VF) are shown on a left side (I, K, M, O, Q), while J, L, M, P, H graphs represent protein expression levels of Beclin-1, p62, pAMPKα, Tom20 and caspase-3 detected in the fibrillated hearts (VF), $*p < 0.05$ compared to the appropriate control group; mean ± SD; $n = 3$ in each group.

inhibitory activity of BM-164 increases following this time period. This prolonged increase in antioxidant capacity can be explained by the specific structure of 6-thioascorbic acid, the metabolite of BM-164; in addition to the reductone part, this metabolite also contains a thiol functional group, which makes it a self-regenerating antioxidant. Additionally, it is possible that H_2S donation is slower under the conditions of the DPPH assay than in the rat heart cell culture medium. Most importantly, at the concentration used in *ex vivo* studies (30 μM), BM-164 showed a significant antioxidant effect, which is more potent than using ascorbic acid.

The H_2S release was measured from the CF effluent obtained from isolated hearts, and the values showed that after 30 min of ISA the highest H_2S concentration was observed in the BM-164 treated group, which was followed by the control and the ascorbic acid treated groups. The elevated levels can be explained by endogenous processes, which were highest in the BM-164 due to the added effect of H_2S released from this compound.

The cytotoxicity of BM-164 was evaluated in H9c2 cells in comparison with ascorbic acid using six different concentrations. The results showed that 10 μM and 30 μM of BM-164 did not cause significant toxic effect, however, 100 μM, 300 μM and 500 μM of BM-164 resulted in increased cytotoxicity, respectively. The same concentrations of ascorbic acid did not produce similar cardiotoxic effect. Therefore, the use of 30 μM BM-164 was appropriate for the isolated heart experiments, which is in line with the blood plasma levels of ascorbic acid (23–114 μM/L) (Hagel et al., 2018).

The incidence of ventricular fibrillation after 30 min of ISA was highest in the drug-free control group in comparison with the ascorbic acid and/or BM-164 group. These results are in line with our previous studies (Vass et al., 2023) with an ibuprofen based H_2S releasing drug (BM-88), showing that BM-164 has a beneficial effect in preventing the development of arrhythmias. The size of the infarcted areas is showing the aforementioned tendencies that the lowest amount of damaged heart tissue was detected in the BM-164 treated group at the end of the designed experiments.

Macroautophagy (herein after referred to as autophagy) is a self-defense mechanism for the removal of dysfunctional organelles and damaged proteins in order to maintain the physiological homeostasis of cells (Despotović et al., 2022; Li et al., 2016). During the sequestration process, the lipidated form of the microtubule-associated protein1 light chain 3 protein (LC-3-II) incorporates in the autophagosomal membrane. Therefore, the amount of LC-3-II protein in the cell serves as a marker of autophagosome formation indicating the initial process of the early autophagy. Beclin-1 also plays a crucial role, initiating autophagy processes and coordinating their possible cytoprotective effects, which combats the process of apoptosis that is parallel or competes with autophagic cell death (Li et al., 2016). It is well known that Beclin-1 and LC-3 are involved in the nucleation phase, while p62 as a cargo receptor is consumed during the elongation phase (Au et al., 2017). It is also of interest to note that a role of p62 in removal of molecules „marked" by SQSTM1/p62, following interaction with LC-3 and deliver all „sequestrated" molecules to autophagosomes for degradation (Au et al., 2017; McKnight et al., 2014; Nezis and Stenmark, 2012).

The stress induced I/R in the heart results in multidirectional cell-damaging mechanisms with increased reactive oxygen and nitrogen radicals in cells' environment, therefore, to restore heart function „acute cell repair" mechanisms are needed. Several reports highlight that adaptive induction of autophagy contributes to the mitigation of cardiac damage during ischemia/reperfusion-induced injury (Ma et al., 2012; Popov et al., 2023; Yan et al., 2005). Our goal was to synthesize a drug candidate with a potential cardioprotective effect. For this reason, we investigated the effects of BM-164 on cell-protective mechanisms, focusing primarily on autophagy, namely whether BM-164 induces or inhibits the processes described above. The expression levels of LC-3, Beclin-1 and p62 proteins were analyzed by Western blot analyses upon reperfusion in fibrillated and non-fibrillated hearts.

It is revealed that the levels of LC-3-II and Beclin-1 markedly increased in the group of hearts treated with BM-164 showing the initiation of autophagosome formation. Interestingly, the expression levels of p62 were significantly increased in the hearts treated with BM-164 in both fibrillated and non-fibrillated groups. The literature reports show that p62 expression was decreased if the autophagy flux was successfully induced (Sánchez-Martín and Komatsu, 2018). We argue that increased p62 level - not decreased- in our experiments indicates that autophagy alone is not able to remove degradation products and only partially fulfill its "sweeping" task, presumably handing over this task to the processes of apoptosis and necrosis in the cell. Our results indicate that autophagy processes are activated only upstream (LC-3 and Beclin-1) and not downstream to p62 protein expression.

Increased level of pAMPK contributes to autophagosome maturation, which change was observed at significant amount in BM-164 treated fibrillated hearts only, and reports have demonstrated that AMPK can inhibit the development of heart failure by promoting autophagic processes (Li et al., 2018; Xie et al., 2015).

The studied autophagy-related proteins showed remarkable changes only in the hearts treated with the H_2S-releasing BM-164, while the same concentration of ascorbic acid did not produce any significant change in the detected amount of LC-3, Beclinl-1, p62 or pAMPK, respectively, compared to the drug-free ischemic reperfused controls. This finding suggests that the increased level of autophagy may be primarily caused by the release of H_2S (Lv et al., 2021). Correlation between the H_2S release and the increased level of autophagy markers are not surprising, since several studies have described the existing effects of both endogenously generated H_2S and exogenously introduced H_2S-releasing molecules on autophagy processes (Wu et al., 2018). In a study conducted in mice, the expression levels of Beclin-1 and other autophagy-related proteins were significantly decreased following the treatment with a H_2S-releasing molecule, NaHS, where autophagy was excessively activated by alcohol consumption, indicating that H_2S released from NaHS can intensively downregulate autophagy (Liang et al., 2017). Our results in the I/R model show that the released H_2S from BM-164 increases rather than decreases the expression of autophagic markers.

The literature data suggest that the effects of H_2S donors on autophagy processes are bidirectional (Lazado et al., 2023; Wu et al., 2018). Recent studies show that some of the pathways are involved in the pro-autophagic effect of H_2S, while on the other hand, there are several signaling pathways that play important roles in the anti-autophagic effect of H_2S (Lv et al., 2021; Magli et al., 2021), which may give an acceptable explanation for our results. Another possible explanation of the obtained results could be that an increased intensity of autophagy may cause the destruction of the autophagic cells formed during ischemia and the loss of injured cardiac cells could promote pathological heart muscle remodeling/regenerative processes. Elucidating the exact mechanism obtained under *in vitro* conditions, additional *in vivo* experiments are needed to be done. We believe that the regulatory role of H_2S donors in autophagy might be influenced by the structure of the H_2S donor and the kinetics of their release. This phenomenon can cause different effects at the cellular level, which was published in various biological systems. For example, other researchers revealed that a slow-releasing H_2S donors morpholine (4-methoxyphenyl)(morpholino) phosphinodithioate (GYY4137) produced more substantial transcriptomic changes than the fast-releasing salt, sodium hydrosulfide (NaHS) (Lazado et al., 2023). Therefore, since the BM-164 continuously releases H_2S, it can explain the increased expression of some autophagic proteins compared to the extensive downregulative effect of fast-releasing NaHS (Lazado et al., 2023; Liang et al., 2017).

Mitophagy (mitochondrial autophagy) cannot be completely separated from autophagy, and often follows it. Tom20 protein level expression is one of its indicators of mitophagy. We revealed a significantly increased expression of this protein in both the I/R (VF) and I/R (no VF) groups compared to the ischemic/reperfused drug-free control hearts. In the group treated with ascorbic acid, the level of Tom20

protein was also significantly increased compared to the drug-free control group. The Tom20 complex has been reported to be important for Bax mitochondrial localization to induce cytochrome c release, thus, Tom20 is a novel downstream factor of ROS signaling to mitochondria through Tom20-Bax-caspase-3 pathway (Zhou et al., 2018). Caspase-3 is a predominant effector in apoptosis (Kovacs et al., 2001) and studies have shown that caspase-3 is essential to autophagic activity too. Interactions among the crucial proteins of autophagy and apoptosis, underline the importance of the crosstalk existing between autophagy and apoptosis, therefore, we analyzed the expression of caspase-3 in all groups after the treatment with ascorbic acid and BM-164. The results show that caspase-3 expression did not differ significantly upon administration of ascorbic acid or its derivative, BM-164. However, there is evidence in the literature that ascorbic acid can induce dose-dependent and caspase-independent apoptosis, which is possibly might be the reason of non-caspase-3 induction after the aforementioned treatments (Szarka et al., 2021).

5. Conclusions

In the present study it was chosen to investigate the effects of a novel H_2S-releasing ascorbic acid derivative BM-164 on the myocardium in a controlled, well-established ischemia/reperfusion model in comparison with ascorbic acid. We conclude that exerted beneficial therapeutic effects are mediated via autophagic/apoptotic processes in cardiac ischemia. However, for elucidating its precise mechanism of action before considering for application, additional preclinical studies and more experimental data under *in vivo* conditions are required. Both ascorbic acid and BM-164 treatment showed a cardioprotective effect compared to the ischemic/reperfused drug-free control group. Furthermore, BM-164 treatment afforded a better cardioprotection in this regard, showing that the newly synthetized H_2S-releasing ascorbic acid derivative, BM-164, possesses cardioprotective effects as low as 30 μM concentration. In conclusion, our findings underscore a potential therapeutic promise of BM-164, as H_2S donor, over the use of ascorbic acid as a vitamin and antioxidant agent for the management of ischemia/reperfusion-induced injuries. It is reasonable to note that data obtained under *in vivo* conditions may lead to different conclusions in comparison with the accuracy of our *in vitro* results.

Funding

This study was supported by the grants of National Research, Development and Innovation Office (NKFIH-K-124719), Hungary, and HUN-REN-Office for Supported Research Groups (HUN-REN-DE Pharmamodul Research Team), Hungary.

Institutional review board statement

The experiments were carried out in accordance of the EU regulation (EU Directive 63/2010) and the Hungarian laws (The Hungarian Animal Protection Act, Law XVIII/1998) about animal experimentations, and with ethical approvals registered by the University of Debrecen Committee of Animal Welfare (Reg. Nr.: 6/2019/UDCAW).

Data availability statement

All data presented in this study are available in the main text and the Supplementary Information.

CRediT authorship contribution statement

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Declaration of competing interest

All authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106721.

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