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A novel H_2S releasing-monastrol hybrid (MADTOH) inhibits L-type calcium channels†

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A new alleged monastrol-H_2S releasing hybrid, named **MADTOH**, was designed based on the structure of monastrol (**M**) and 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**) and synthesized in 7.8% overall yield. **MADTOH** was shown to be an H_2S donor under physiological conditions. In addition, the hybrid causes a decrease in global calcium transient in cardiomyocytes similar to nifedipine (**NIFE**), taken as a positive control. Whole-cell voltage-clamp showed that **MADTOH** decreases L-type Ca^{2+} current in isolated ventricular cardiomyocytes.

1. Introduction

Voltage-dependent calcium channels are important regulators of neurotransmission and membrane ion conductance in which modulations of these channels influence a plethora of intracellular events. L-Type Ca^{2+} channels (CaV1.2 isoform) are critical in the excitation–contraction coupling in cardiomyocytes,[1] comprising the main pathway of Ca^{2+} influx in myocardial cells. Thus, Ca^{2+} entry through these channels is the main trigger to SR Ca^{2+} release. The control and maintenance of Ca^{2+} homeostasis plays a fundamental role in myocardial physiology and pathology.[2] Indeed, alterations in the expression and function of L-type calcium channels have been implicated in a variety of cardiovascular diseases such as atrial fibrillation,[3] cardiac hypertrophy,[4] heart failure[5,6] and ischaemic heart disease.[7] Furthermore, administration of L-type calcium channel blockers (CCBs) is recognized as an effective therapy for hypertension,[8] cardiac hypertrophy,[9] arrhythmia,[10] congestive heart failure,[7] and myocardial ischemia.[11]

Three main classes of CCBs are reported according to the structure of the pharmacophore group namely arylalkylamines, benzothiazepines and 1,4-dihydropyridines (1,4-DHPs; Fig. 1).[12] The 1,4-DHP scaffold was the most investigated among the CCBs, in which chemical modifications were carried out at almost every position (including the DHP moiety).[13] The 4-aryl-3,4-dihydropyrimidin-2(1*H*)-ones(thiones) (DHPM) derivatives (Fig. 1) were determined to be the most potent and metabolically stable CCBs.[12,14] Monastrol (**M**; Fig. 1), the most studied DHPM, was reported in the 1990s as a potent anticancer agent.[15] Compound **M** was later revealed as an inhibitor of centrin,[16] a protein pivotal for the duplication of the centrosome. A role as a urease[17,18] and L-type calcium channel inhibitor[19,20] has also been documented.

Hydrogen sulfide (H_2S), an endogenously generated gaseous transmitter, is a key player in the modulation of calcium channels, besides regulating cardiac functions and preventing myocardial infarction, hypertrophy, arrhythmia, fibrosis and

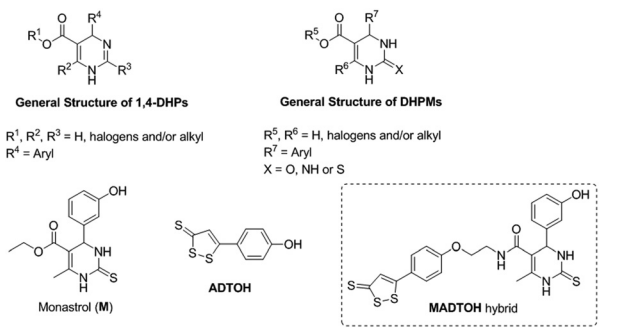

Fig. 1 Chemical structures of 1,4-dihydropyridines (1,4-DHPs) and 4-aryl-3,4-dihyropyrimidin-2(1*H*)-ones(thiones) (DHPM), monastrol (**M**), 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**) and the hybrid (**MADTOH**).

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ischemia–reperfusion injury (I/R).[21,22] In fact, exogenous H_2S was shown to be cardioprotective.[23–26] H_2S was demonstrated to inhibit L-type calcium channels in cardiomyocytes,[26] an effect that depends on the number of sulfhydryl groups.[27]

The use of hybrid molecules is a strategy that enhances and improves biological proprieties based on two or more pharmacophoric groups.[28,29] The building block derivatives from Lawesson's reagent, 1,2-dithiole-3-thiones (DTT), and arylthioamides were successfully used as a H_2S releasing agent.[29,30] 5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**, Fig. 1), a DTT derivative, has already been used for the hybridization of several modified drugs that are currently undergoing preclinical testing.[31–36] The mechanism by which H_2S is released from **ADTOH** is not fully elucidated. Giustarini and coworkers (2010) speculated that the mechanism of H_2S release from ADTOH involves two possible pathways: (i) H_2S could be formed from the hydrolysis of a thionyl group followed by the breakdown of the S–S bond to yield the dithiol derivative or (ii) the dithiol derivative is formed first following the hydrolysis of the thionyl group.[37]

The blocking action of monastrol (**M**) and H_2S on calcium channels prompted us to use the design and synthesis of a monastrol-H_2S releasing hybrid (**MADTOH**; Fig. 1) as a theoretically dual CCB agent. The nucleus **ADTOH** was chosen as an H_2S donor core to be attached to the **M** moiety. Hybrid **MADTOH** and its precursors were tested as an H_2S donor and on ventricular cardiomyocytes to investigate their effect as CCB inhibitors.

2. Materials and methods

All reagents were purchased from Sigma Aldrich. Tetrahydrofuran (THF) and acetonitrile (ACN) were dried according to Perrin and Amarengo.[38] Chemical reactions were monitored using thin layer chromatography (POLYGRAM-UV2540, 20 mm MACHEREY – NAGEL; 20 × 20 cm). Silica gel (technical grade, pore size 60 Å, 70–230 mesh, 63–200 μm) was used for flash column chromatography. The melting point of the synthesized compounds was determined on a GEHAKA-PF1500 device. Infrared (IR) spectra were acquired in a Spectrometer One PerkinElmer as KBr pellets at 400–4000 cm^{-1}. Mass spectra were recorded on a SHIMADZU LC-ITTOF instrument. NMR was recorded on Bruker AVANCE DPX 200 and Bruker AVANCE-III 400 instruments. Chemical shifts (in ppm) are reported relative to residual protic solvent resonances.

2.1. Synthesis of monastrol-H_2S releasing hybrid MADTOH

2.1.1. Monastrol (M)[39]. Ethyl acetoacetate (**1**; 62.0 mmol), 3-hydroxybenzaldehyde (**2**; 41.0 mmol), thiourea (**3**; 62.0 mmol) and *p*-toluenesulfonic acid (PTSA; 2.2 mmol) were dissolved in 34 mL of ethanol. The mixture was stirred at room temperature for 8 h following the addition of aliquots of water (*ca.* 1 mL each) until the reaction became turbid. The reaction mixture was maintained under magnetic stirring for 20 min and the precipitate was filtered and dried under vacuum for 2 h. Monastrol (**M**) was obtained as a white solid in 57% yield (6.8 g–23.3 mmol).

*Monastrol (**M**).* M.p. 180–183 °C [lit. = 183–185 °C];[40] IR (KBr, cm^{-1}): 3308, 3184, 3010, 1668, 1474, 1286, 1192. ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.11 (t, 3H, J = 7.0 Hz), 2.28 (s, 3H), 4.01 (q, 2H, J = 7.0 Hz), 5.09 (d, 1H, J = 2.8 Hz), 6.63–6.67(m, 3H), 7.11 (t, 1H, J = 7.0 Hz), 9.45 (s, 1H), 9.59 (brs, 1H), 10.29 (brs, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 14.1, 17.3, 54.1, 59.7, 100.9, 113.4, 114.7, 117.1, 129.6, 144.9, 157.6, 165.3, 174.3. HRMS calcd for $C_{14}H_{16}N_2O_3S$ [M + 1] 293.096; found 293.1116 (error: −5.32 ppm), calcd for [M + Na] 315.078; found 315.0683 (error: 3.08 ppm).

2.1.2. 4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (4)[41]. Monastrol (**M**; 1.7 mmol) and KOH (5.0 mmol) were dissolved in 6 mL of water. The mixture was stirred at room temperature for 3 days. After the completion of the reaction, a small amount of 2.0 mol L^{-1} HCl (*ca.* 0.5 mL each) was added to the mixture until it became turbid. The mixture was then maintained under magnetic stirring for 20 min. The precipitate formed was filtered, washed with distilled water (3 × 10 mL) and dried under vacuum for 2 h. Compound **4** was obtained as a yellowish solid in 80% yield (359.4 mg–1.3 mmol).

*4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (**4**).* M.p. 195–198 °C; IR (KBr, cm^{-1}): 3308, 3192, 3130, 1672, 1586, 1482, 1268, 1186. ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.26 (s, 3H), 5.07 (brs, 1H), 6.63 (brs, 3H), 7.10 (t, 1H, J = 8Hz), 9.52 (brs, 2H), 10.19 (brs, 1H), 12.14 (brs, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 17.2, 54.1, 101.5, 113.4, 114.6, 117.2, 129.6, 144.4, 144.9, 157.5, 167.1, 174.3. HRMS calcd for $C_{12}H_{12}N_2O_3S$ [M − 1] 263.0491; found 263.0446 (error: 1.71 ppm).

2.1.3. 5-(4-Methoxyphenyl)-3*H*-1,2-dithiole-3-thione (9)[42]. (*E*)-1-Methoxy-4-(prop-1-en-1-yl)benzene (**8**; 80 mmol) and sulfur (560 mmol) were dissolved in 70 mL of dimethylacetamide (DMA). The mixture was heated until reflux and magnetically stirred for 6 h. The excess of sulfur was removed and the resulting solution was cooled to 0 °C for 5 h. The precipitate formed was recrystallized from ethyl acetate furnishing the desirable product as an orange solid in 30% yield (5.8 g–24.0 mmol).

*5-(4-Methoxyphenyl)-3H-1,2-dithiole-3-thione (**9**).* M.p. 106–108 °C [lit. 118 °C];[42] IR (KBr, cm^{-1}): 3080, 1602, 1176; ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.84 (s, 3H), 7.05 (d, 2H, J = 3 Hz), 7.74 (s, 1H), 7.84 (d, 2H, J = 3 Hz). ^{13}C NMR (50 MHz, DMSO-d_6): δ 55.6, 115.0, 123.6, 128.9, 134.10, 162.6, 173.7, 214.7. HRMS calcd for $C_{10}H_8OS_3$ [M + 1] 239.973; found 240.9889 (error: 3.07 ppm).

2.1.4. 5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADTOH)[43]. A mixture of 5-(4-methoxyphenyl)-3*H*-1,2-dithiole-3-thione (**9**) (4.2 mmol) and pyridine hydrochloride (Py·HCl) (24.0 mmol) was heated and magnetically stirred for 20 min in a microwave reactor (CEM®) with 4 min of ramp until 160 °C (300 W power rating). After complete consumption of **9**, attested by TLC analysis, hot water was slowly added to the reaction mixture. The solid formed was solubilized in 10% m/v K_2CO_3 solution (80 mL) and extracted with dichloromethane (5 × 250 mL). The organic layer was concentrated, dried under vacuum for 4 h and

ADTOH obtained as an orange solid in 60% yield (570.3 mg–2.5 mmol).

*5-(4-Hydroxyphenyl)-3H-1,2-dithiole-3-thione (**ADTOH**).* M.p. 178–180 °C [lit. 172–174 °C];[43] IR (KBr, cm^{-1}): 3158, 3050, 1608, 1476, 1197; ^{1}H NMR (200 MHz, DMSO-d_6) δ 6.90 (d, 2H, J = 7 Hz), 7.70 (s, 1H), 7.78 (d, 2H, J = 7 Hz), 10.52 (s, 1H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 116.4, 122.2, 129.2, 133.5, 161.7, 174.3, 214.4. HRMS calcd for $C_9H_6OS_3$ [M − 1]: 224.9503; found [M − 1]: 224.9477 (error: 1.16 ppm).

2.1.5. *tert*-Butyl(2-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)-ethyl)carbamate (10)[44,45]. 2-Bromoethan-1-amine (**6**) (47.0 mmol) was dissolved in 40 mL of dioxane and maintained at 0 °C under magnetic stirring. Di-*tert*-butyl dicarbonate (**5**) (27.0 mmol) was added to the reaction mixture followed by slow addition (during 5 min) of triethylamine (25.0 mmol). The reaction was kept under magnetic stirring at 0 °C for 40 min. Afterwards, the system was warmed up to room temperature and kept under such conditions for 48 h. The liquid layer obtained after filtration was concentrated under vacuum and the residue formed was solubilized in dichloromethane (100 mL) and washed with water (2 × 50 mL). The organic solvent was removed under vacuum to furnish *tert*-butyl-(2-bromoethyl)carbamate (**7**) as a yellowish viscous liquid in 73% yield (4.8 g–21.3 mmol). Carbamate **7** was used in the subsequent steps with no further chemical characterization. Briefly, 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (2.0 mmol) (**ADTOH**), KI (2.9 mmol) and Cs_2CO_3 (6.0 mmol) were dissolved in anhydrous ACN. The mixture was heated to reflux for 20 min followed by addition of 6.0 mmol of carbamate **7** prepared in anhydrous ACN. Forty-eight hours later, the residue formed from the reaction concentration under vacuum was solubilized in chloroform (40 mL) and extracted with 0.4 mol L^{-1} NaOH (2 × 30 mL). The organic layer was dried using $MgSO_4$ and the solvent removed under vacuum. The residue obtained was purified by column chromatography using methanol/chloroform (1 : 100) as the eluent. The carbamate **10** was obtained as an orange solid in 79% yield (583.8 mg–1.6 mmol).

*tert-Butyl(2-(4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)ethyl)carbamate (**10**).* M.p. 116–117 °C; IR (KBr, cm^{-1}): 3044, 1694, 1602, 1078; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.44 (s, 9H), 3.54 (q, 2H, J = 5.2 Hz), 4.07 (t, 2H, J = 5.2), 5.02 (brs, 1H), 6.94 (d, 2H, J = 8.8 Hz), 7.35 (s, 1H), 7.58 (d, 2H, J = 8.8). ^{13}C NMR (50MHz, $CDCl_3$): δ 28.5, 40.0, 67.6, 79.8, 115.5, 124.5, 128.7, 134.7, 155.9, 162.0, 172.9, 215.1. HRMS calcd for $C_{16}H_{19}NO_3S_3$ [M + 1]: 370.0605; found [M + 1]: 370.0600 (Error: 0.14 ppm); calcd for [M + Na] 392.0425; found [M + Na]: 392.0445 (error: 0.51 ppm).

2.1.6. 5-(4-(2-Aminoethoxy)phenyl)-3*H*-1,2-dithiole-3-thione hydrochloride (11). *tert*-Butyl(2-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)ethyl)carbamate (**10**) (1.0 mmol) was solubilized in ethyl acetoacetate followed by addition of 4 mol L^{-1} of HCl (10 mL). The reaction mixture was maintained for 3 h under magnetic stirring at room temperature and then at 4 °C for other 5 h. The precipitate obtained was filtrated, washed in distilled water (3 × 15 mL) and dried under vacuum. Compound **11** was obtained as an orange solid in 86% yield (263.0 mg–0.86 mmol).

*5-(4-(2-Aminoethoxy)phenyl)-3H-1,2-dithiole-3-thione hydrochloride (**11**).* M.p. 226–227 °C; IR (KBr, cm^{-1}): 3434, 3200, 3046, 1602, 1200; ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.22 (brs, 2H), 4.31 (brs, 2H), 7.11 (d, 2H, J = 8.0 Hz), 7.74 (s, 1H), 7.88 (d, 2H, J = 8.0 Hz), 8.46 (brs, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 38.1, 64.7, 115.7, 124.2, 129.0, 134.4, 161.2, 173.6, 214.8. HRMS calcd for $C_{11}H_{12}NOS_3^+Cl^-$ [M + 1]: 270.0076; found [M + 1]: 270.0027 (error: 2.00 ppm).

2.1.7. 4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-*N*-(2-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)ethyl)-1,2,3,4-tetrahydropyrimidine-5-carboxamide (MADTOH). 4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (**4**) (0.7 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.7 mmol) were dissolved in anhydrous THF (10 mL). Five minutes later, a solution of 5-(4-(2-aminoethoxy)phenyl)-3*H*-1,2-dithiole-3-thione hydrochloride (**11**) (0.3 mmol) prepared in *N*,*N*-disopropylethylamine (DIPEA) (2.3 mmol) was added to this mixture. The reaction was magnetically stirred for 24 h at room temperature and the residue formed was purified by column chromatography using ethanol/chloroform (1 : 10) as the eluent. The monastrol-H_2S releasing (**MADTOH**) hybrid was obtained as an orange solid in 64% yield (231.0 mg–0.44 mmol).

*4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-N-(2-(4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)ethyl)-1,2,3,4-tetrahydropyrimidine-5-carboxamide (**MADTOH**).* M.p. 206–208 °C; IR (KBr, cm^{-1}): 3200, 2968, 2868, 2880, 1602, 1198, 1180; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.01 (s, 3H), 3.37–3.54 (m, 2H), 4.05 (t, 2H, J = 6 Hz), 5.18 (d, 1H, J = 3.2 Hz), 6.61–6.64 (m, 3H), 7.03 (d, 2H, J = 8.8 Hz), 7.75 (brs, 1H), 7.86 (d, 2H, J = 8.8 Hz), 7.97 (t, 1H, J = 6 Hz), 9.28 (s, 1H), 9.41 (s, 1H), 9.81 (brs, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 16.3, 38.2, 54.8, 66.4, 106.8, 113.2, 114.5, 155.5, 166.9, 123.7, 129.0, 129.3, 134.2, 134.7, 144.5, 157.5, 161.8, 166.3, 173.8, 174.1, 214.8. HRMS calcd for $C_{23}H_{21}N_3O_3S_4$ [M − 1]: 515.0466; found [M − 1]: 516.0425 (error: 2.31 ppm).

2.2. *In vitro* assay of H_2S release

The ability of monastrol (**M**), 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**), 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-*N*-(2-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)ethyl)-1,2,3,4-tetrahydropyrimidine-5-carboxamide (**MADTOH**) and nifedipine (**NIFE**) to release H_2S was investigated using a TBR4100 Free Radical Analyzer (WPI, USA) equipped with an ISO-H2S-2 amperometric sensor (detection limit $\leq$5 nmol L^{-1}). Each compound (10 μmol L^{-1}) was prepared in Tyrode solution and analyzed at 0, 3, 6 and 12 min after preparation by individually adding 50 μL of each solution to 100 mmol L^{-1} PBS. The amount of H_2S released from the solutions at the specified times was determined using a standard curve of Na_2S and the equation H_2S = [Na_2S]/3.04173977, according to the manufacturer's instructions.

2.3. *Ex vivo* effects of MADTOH on L-type calcium channels

2.3.1. Animal model. This study was carried out with 10 to 12 week-old male C57BL/6 mice (n = 10 animals). The animals were housed in the animal facility, kept at controlled temperature (22–24 °C) under 12 h photoperiod. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Federal University of Minas Gerais (UFMG, Brazil) under protocol – CEUA183/2020. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication 86–23, revised 1985; nih.gov/regs/index.htm).

2.3.2. Ventricular cardiomyocyte isolation. After appropriate anesthesia (ketamine 100 mg kg^{-1}, xylazine 10 mg kg^{-1}, intraperitoneal), the animals were euthanized by rapid cervical dislocation. Briefly, the hearts were perfused *via* the Langendorff method with Digestion Buffer containing 1.2 mg mL^{-1} of collagenase type 2 (Worthington Biochemical Corporation) (DB: 130 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 25 mmol L^{-1} HEPES, 0.5 mmol L^{-1} $MgCl_2$, 0.33 mmol L^{-1} NaH_2PO_4, 22 mmol L^{-1} glucose; 50 μmol L^{-1} $CaCl_2$ and insulin 100 U mL^{-1}, pH = 7.4 adjust with NaOH). Ventricular myocytes were stored in regular Tyrode solution at pH 7.4 (140 mmol L^{-1} NaCl, 4 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 1.8 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} glucose and 5 mmol L^{-1} HEPES) until they were used.[46]

2.3.3. Intracellular measurement of H_2S. Cardiomyocytes were incubated at 37 °C with 25 μmol L^{-1} WSP-5 (Cayman Chemical) prepared in Tyrode solution. After 30 min, the cardiomyocytes were washed in Tyrode solution to remove the excess of the H_2S probe. Samples were analyzed by confocal microscopy (Zeiss LSM 880 Meta) at the Center for Acquisition and Processing of Images (Institute of Biological Sciences, UFMG) to verify changes in fluorescence. The software ImageJ (NIH) was used for the analyses of micrographies.

2.3.4. Intracellular Ca^{2+} transients. Ventricular cardiomyocytes were incubated at room temperature with 6 μmol L^{-1} Fluo-4/AM (Thermo Fisher Scientific) prepared in Tyrode solution. After 30 min, the cells were washed in Tyrode solution to remove the excess Ca^{2+} probe. Intracellular Ca^{2+} transients were evoked by electrical stimulation of the cardiomyocytes using field stimulating platinum electrodes with voltage pulses of 5 ms, 40 V intensity and biphasic pulses at 1 Hz. The scanning image was acquired by a confocal microscope Zeiss LSM 880 Meta and 63× oil objective. Cardiomyocytes were scanned with a 512 pixel line at 1.6 ms and sequential scans were obtained to create two-dimensional images with time on the x-axis. ImageJ software (NIH) was used for image analyses.

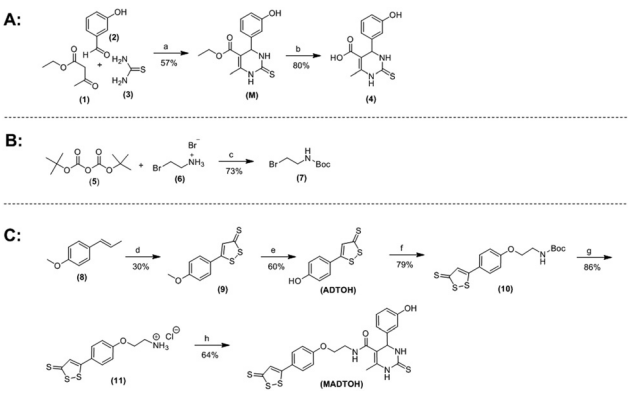
2.3.5. Measurement of L-type Ca^{2+} current in isolated left ventricular cardiomyocytes. Electrophysiological recordings were obtained by whole-cell voltage-clamp mode using an EPC-10 patch-clamp amplifier (HEKA Electronics, Germany). After achieving whole-cell configuration, equilibrium between the pipette solution and the intracellular environment was achieved in 3–5 min. Pipette resistances of 1–2 MΩ and electronic correction for a series resistance (R_s) of 50–60% were applied to each measurement. Cardiomyocytes presenting R_s higher than 8 MΩ were discarded. The composition of the internal solution was 120 mmol L^{-1} CsCl, 20 mmol L^{-1} TEACl, 5 mmol L^{-1} NaCl, 10 mmol L^{-1} HEPES and 5 mmol L^{-1} EGTA (pH 7.2) and the external solution was 140 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 1.8 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} HEPES and 10 mmol L^{-1} glucose (pH 7.4). The time course of the ICa,L peak current was recorded in the absence and presence of **M**, **ADTOH** and **MADTOH** (each at 10 μmol L^{-1}) to evaluate the acute effect of these xenobiotics. Pre-pulses from a holding potential (−80 mV to −40 mV), for 50 ms, were applied to inactivate Na^+ and/or T-type Ca^{2+} channels. A test pulse (0 mV) was then applied for 300 ms to measure ICa, L, and time between sweeps was set at 10 s.

2.3.6. Statistical analysis. In intracellular measurement of H_2S and Ca^{2+} transients experiments, data are presented as mean ± SEM of at least 3 independent experiments, and for statistical comparison, we used one-way ANOVA followed by Newman–Keuls *post hoc* test. For the L-type Ca^{2+} current experiments, data are presented as mean + SD of at least 3 independent experiments, and for statistical comparison, we used a paired Student's t-test. The level of significance was set to values of $p < 0.05$.

3. Results and discussion

3.1. Synthesis of the monastrol-H_2S releasing hybrid MADTOH

The monastrol-H_2S releasing hybrid **MADTOH** was constructed by connecting the building blocks **4** and **ADTOH** using the linker **7** (Panels A and C, Scheme 1). Initially, **4**, the corresponding carboxylic acid of monastrol (**M**) was prepared by a Biginelli reaction of the ethyl acetoacetate (**1**), 3-hydroxybenzaldehyde (**2**) and thiourea (**3**) catalyzed by *p*-toluenesulfonic acid (PTSA) (Panel A, Scheme 1).[39] Further treatment of **M** under basic conditions[41] furnished the desirable carboxylic acid **4** with 45% overall yield (2 steps). The preparation of **ADTOH** (the other coupling partner)



Scheme 1 Convergent synthesis of the monastrol-H_2S releasing hybrid **MADTOH**. Reagents and reactions conditions: (a) PTSA, ethanol, r.t., 8 h; (b) KOH, H_2O, r.t., 3 d; (c) dioxane, 0 °C, TEA, then r.t. 48 h; (d) S_8, DMA, reflux, 6 h; (e) Py·HCl, MWI, 160 °C, 20 min; (f) **7**, KI, Cs_2CO_3, ACN, reflux, 48 h; (g) HCl (4 mol L^{-1}), EtOAc, r.t., 3 h; (h) **4**, DCC, THF, DIPEA, r.t., 24 h.

was done by initially treating (*E*)-1-methoxy-4-(prop-1-en-1-yl)benzene (**8**) and sulfur in dimethylacetamide to obtain **9**[42] in 30% yield (Panel C, Scheme 1). The demethylation of **9** using pyridine hydrochloride (Py·HCl) under microwave irradiation (MWI) at 160 °C for 20 min[43] afforded **ADTOH** in 60% yield. The next step comprised the synthesis of the linker **7** (Panel B, Scheme 1), prepared by treating the amine **6** with di-*tert*-butyl dicarbonate (**5**) in the presence of trimethylamine (TEA) at room temperature for 48 h (73% of yield).[47] Both **ADTOH** and the linker **7** were treated with an excess of Cs_2CO_3 and KI under reflux in ACN for 48 h at room temperature to provide the *N*-BOC-protected amine **10** with 79% yield (Panel C, Scheme 1). An acid-mediated BOC-deprotection of **10** finally led to the desired amine hydrochloride **11** (86% yield). Then, **11** was coupled to **4** to obtain the new monastrol-H_2S releasing hybrid **MADTOH** in 64% yield (Panel C, Scheme 1). The convergent strategy herein used provided the target hybrid **MADTOH** in an overall yield of 7.8% (Scheme 1). In conclusion, from the point of view of our synthetic approach to **MADTOH**, a new monastrol-H_2S releasing molecule was successfully synthesized and such compounds may present a dual inhibitory effect on L-type calcium channels.

3.2. *In vitro* H_2S releasing studies

Solutions of **ADTOH**, **MADTOH**, **M** and **NIFE** were prepared in Tyrode solution to test the hypothesis that the allegedly monastrol-H_2S releasing hybrid herein synthesized (**MADTOH**) is able to release H_2S under physiological conditions. The use of a highly sensitive H_2S electrode and an amperometric approach allowed it to be unequivocally demonstrated that **MADTOH**, during the analyzed time, is an H_2S donor in an isotonic solution with interstitial fluid similar to that observed for **ADTOH**, the well-known H_2S donor used for the synthesis of the monastrol hybrid (Fig. 2). Monastrol (**M**) does not release H_2S under the same experimental conditions tested for **MADTOH**. As expected, no detectable H_2S was released from Tyrode solution or nifedipine (Fig. 2). Therefore, the strategy used to link a known H_2S donor (**ADTOH**) to **M** effectively provided a new molecule eligible to release H_2S donor under physiological conditions.

3.3. *Ex vivo* effects of MADTOH on calcium channels

The H_2S production was also evaluated *ex vivo* by incubating left ventricular cardiomyocytes exposed to test molecules (**MADTOH**, **M** or **ADTOH**) with WSP-5 for 10–20 min. WSP-5 is a substance that in the presence of H_2S emits fluorescence.[48] As expected, **ADTOH** induced a significant increase in H_2S (25%; $p < 0.05$; Fig. 3). Likewise, **MADTOH** enhanced H_2S production in left ventricular myocytes by 17% ($p < 0.05$; Fig. 3) while **M** had no significant effect on the H_2S production ($p < 0.05$; Fig. 3).

Hydrogen sulfide (H_2S) is described as an important modulator of cardiovascular function.[22,49,50] Ca^{2+} is the major ion that orchestrates excitation–contraction coupling (E–C coupling) in cardiomyocytes. To investigate the effect of **MADTOH**, **ADTOH** and **M** on global Ca^{2+} transients, ventricular myocytes were incubated with the Ca^{2+} sensitive fluorescent dye (Fluo-4/AM), paced at 1 Hz and visualized by confocal microscopy. The peak Ca^{2+} transient magnitude on ventricular myocytes was also monitored after treatment with **NIFE**, as the structure of this representative member of 1,4-dihydropyridines (1,4-DHPs) is closely related to that of **MADTOH**. The peak Ca^{2+} amplitude decreased

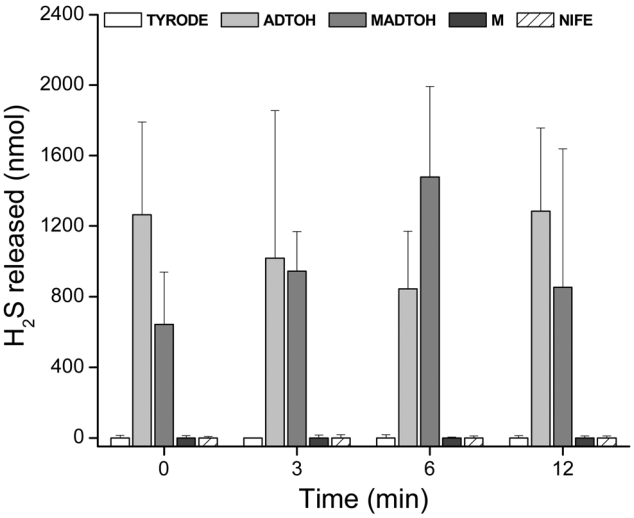


Fig. 2 Extent of H_2S release from a hybrid constituted of monastrol (**M**) and **ADTOH**, (**MADTOH**) under physiological conditions as a function of time. **ADTOH**, **MADTOH**, **M** and nifedipine (**NIFE**) were prepared at 10 μmol L^{-1} in Tyrode solution and the release of H_2S (if any) was monitored in the interval 0–12 min after solution preparation. The values are the means + SD of experiments carried out in triplicate.

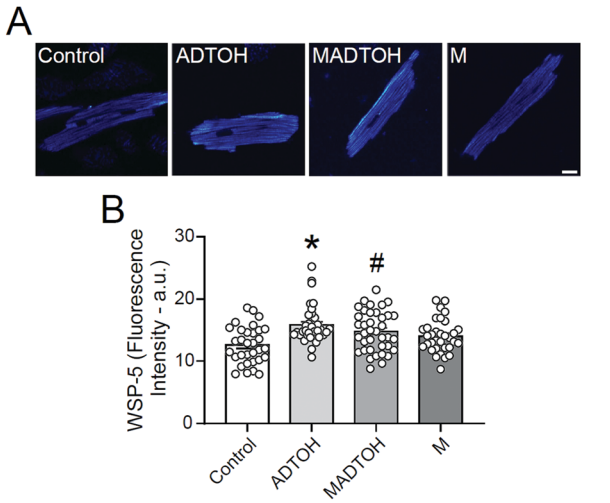


Fig. 3 5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**) and the monastrol-H_2S releasing (**MADTOH**) hybrid increase H_2S production in ventricular cardiomyocytes. (A) Representative confocal images showing WSP-5-loaded cardiomyocytes treated with **ADTOH** (10 μmol L^{-1}), **MADTOH** (10 μmol L^{-1}) or **M** (10 μmol L^{-1}). (B) Averaged-WSP-5 fluorescence increase in ventricular myocytes after **ADTOH** or **MADTOH** treatment (10 μmol L^{-1}) for 10–20 min. ○, number of cardiomyocytes isolated from 3 hearts. Bar = 20 μm. *$p < 0.05$ *vs.* Control and **M**. #$p < 0.05$ *vs.* Control. Data are mean ± SEM and were analyzed by one-way ANOVA followed by Newman–Keuls *post hoc* test.

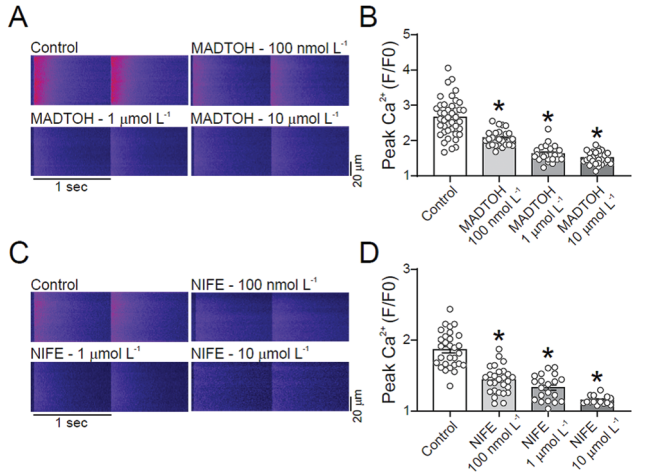


Fig. 4 **MADTOH** negatively affects the peak Ca^{2+} magnitude in ventricular cardiomyocytes. (A) Representative images of Ca^{2+} transients in control samples and cardiomyocytes treated with **MADTOH** (100 nmol L^{-1}–10 µmol L^{-1}). (B) Quantitative data for Ca^{2+} transient amplitude in cardiomyocytes treated with **MADTOH**. (C) Representative images of Ca^{2+} transients in control samples and cardiomyocytes treated with nifedipine (**NIFE**; 100 nmol L^{-1}–10 µmol L^{-1}). (D) Quantitative data for Ca^{2+} transient amplitude in cardiomyocytes treated with **NIFE**. ○, number of cardiomyocytes isolated from 2–3 hearts. Bar = 20 µm. *$p < 0.05$ *vs.* the other groups. Data are mean ± SEM and were analyzed by one-way ANOVA followed by Newman–Keuls *post hoc* test.

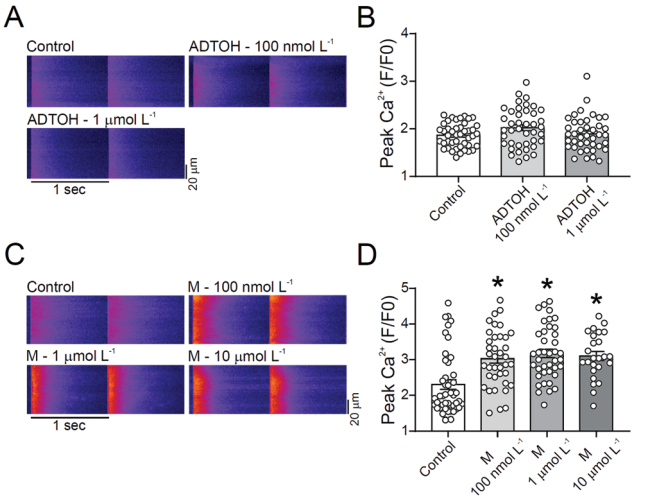


Fig. 5 Increment of peak Ca^{2+} in ventricular cardiomyocytes caused by monastrol (**M**). (A) Representative images of Ca^{2+} transients in control samples and cardiomyocytes treated with **ADTOH**. (B) Quantitative data for peak Ca^{2+} transient amplitude in cardiomyocytes treated with **ADTOH**. (C) Representative images of Ca^{2+} transients in control samples and cardiomyocytes treated with **M**. (D) Quantitative data for Ca^{2+} transient amplitude in cardiomyocytes treated with **M**. ○, number of cardiomyocytes isolated from 3 hearts. Bar = 20 µm. *$p < 0.05$ *vs.* control. Data are mean ± SEM and were analyzed by one-way ANOVA followed by the Newman–Keuls *post hoc* test.

in a concentration-dependent manner in ventricular cardiomyocytes exposed to **MADTOH** (−22% at 100 nmol L^{-1}; −28% at 1 µmol L^{-1}; −43% at 10 µmol L^{-1}; $p < 0.05$; Fig. 4A and B). Likewise, **NIFE** provoked a decrease in Ca^{2+} transient magnitude (−23% at 100 nmol L^{-1}; −29% at 1 µmol L^{-1} and −38% at 10 µmol L^{-1} $p < 0.05$; Fig. 4C and D).

Key intermediate molecules used in the synthesis of **MADTOH** were also tested for the effect on peak Ca^{2+} transient. Peak Ca^{2+} was not altered in cardiomyocytes upon treatment with **ADTOH**, regardless of the concentration tested, in comparison to the control (Fig. 5; $p > 0.05$). Notably, the treatment with **ADTOH** at 100 µmol L^{-1} impaired the viability of cardiomyocytes which, in turn, resulted in failure to record a Ca^{2+} transient. Surprisingly, **M** induced an increment in the peak Ca^{2+} (31% at 100 nmol L^{-1}; 37% at 1 µmol L^{-1}; 34% at 10 µmol L^{-1}; $p < 0.05$). Strikingly, the hybrid **MADTOH** shows an effect very distinctive of that of **ADTOH** or **M**, whose carbon skeletons are embedded in an **MADTOH** chemical structure.

Next, it was investigated the effects of **MADTOH** on L-type Ca^{2+} channels. Accordingly, L-type Ca^{2+} current (ICa,L) was measured in isolated left ventricular cardiomyocytes using the whole-cell voltage-clamp technique. **MADTOH** (10 µmol L^{-1}) led to a higher decrease in ICa,L peak when compared to **ADTOH** or **M** (Fig. 6). In fact, the ICa,L peak was decreased by 36% (from −1.1 ± 0.7 nA to −0.4 ± 0.2 nA; $p < 0.05$) 2 min after-incubation of cardiomyocytes with **MADTOH**. Overall, these results indicate that the decrease of Ca^{2+} influx in ventricular cardiomyocytes triggered by **MADTOH** was due to the inhibition of L-type Ca^{2+} channels.

Monastrol (**M**), at 0.1–100 µM, triggered an increase in Ca^{2+} transient amplitude in ventricular cardiomyocytes, although it was previously described as an inhibitor of a calcium channel in HEK293 cells (100 µM)[19] and aortic rings (10–100 µM).[20] This distinct effect observed for **M** on L-type calcium channels was also detected for nifedipine (**NIFE**)[51] and H_2S[52] when they were evaluated in different concentrations and/or cell lineages. Thus, we suggested that the inhibitory effect of **M** (0.1–100 µM) on L-type calcium channels from cardiomyocytes could be attributed for such compounds. In addition, **MADTOH** effects could be due to a synergism between **M** and **ADTOH** – the hybrid subunits.

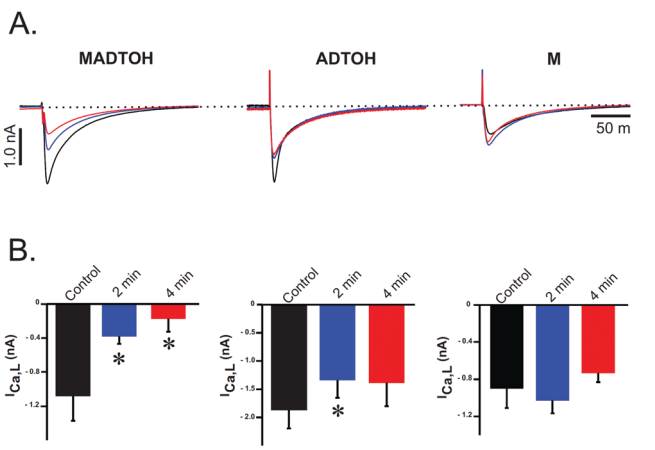


Fig. 6 Effects of **MADTOH** on L-type Ca^{2+} current. (A) Representative recording showing the L-type calcium current (ICa,L) in the control and after 2 or 4 min incubation with 10 µmol L^{-1} **MADTOH**, **ADTOH** or **M**. (B) Summarized data of **MADTOH** (left chart), **ADTOH** (middle chart) and **M** (right chart) on the ICa,L (n = 6, *$p < 0.05$). Data are mean + SD and were analyzed by a paired Student's t-test.

4. Conclusions

A new monastrol-H_2S-releasing hybrid (**MADTOH**) was successfully synthesized *via* a convergent strategy which provided the desired product in 7.8% overall yield. Opposed to monastrol (**M**), **MADTOH** efficiently decreased global calcium transient amplitude in cardiomyocytes by inhibiting L-type calcium channels. Notably, the intermediates **ADTOH** and **M** are less effective than the hybrid **MADTOH** in the control of Ca^{2+} homeostasis. Overall, the strategy of hybridization using the ADTOH moiety has proven to be promising for obtaining substances eligible to modulate calcium channels.

Conflicts of interest

There are no conflicts to declare.

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