Structure-activity relationships study of isothiocyanates for H₂S releasing properties: 3-Pyridyl-isothiocyanate as a new promising cardioprotective agent

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Abstract

A library of forty-five isothiocyanates, selected for their different chemical properties, has been evaluated for its hydrogen sulfide (H₂S) releasing capacity. The obtained results allowed to correlate several factors such as steric hindrance, electronic effects and position of the substituents to the observed H₂S production. Moreover, the chemical-physical profiles of the selected compounds have been studied by an in silico approach and from a combination of the obtained results, 3-pyridyl-isothiocyanate (25) has been selected as the most promising one. A detailed pharmacological characterization of its cardioprotective action has been performed. The results herein obtained strongly indicate 3-pyridyl-isothiocyanate (25) as a suitable pharmacological option in anti-ischemic therapy.
Introduction

The gasotransmitter hydrogen sulphide (H2S), an endogenous ubiquitous signalling molecule, is known for its beneficial effects on different mammalian systems [1]. H2S is mainly produced from l-cysteine via the catalytic activity of two pyridoxal-5'-phosphate dependent enzymes, known as cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) [2]. A third pathway, where 3-mercaptopruvate sulphur transferase (3-MST) and cysteine aminotransferase (CAT) [3–5] cooperate, is also responsible for H2S biosynthesis. Endogenous H2S is involved in many physiological processes and the role of H2S has been mostly studied by inhibiting its physiological production [6,7] or using exogenous sources of H2S.

H2S exhibits cardioprotective activity against ischemia/reperfusion (I/R) or hypoxic injury. The mechanisms of action accounting for this cardioprotective activity involve mitochondrial ATP-sensitive potassium channels (mitoKATP) [8], anti-apoptotic responses [9] and inhibition of type-5 phosphodiesterase [10]. Furthermore, H2S behaves as an antioxidant molecule, able to activate the Nrf-2-mediated machinery and reduce the reactive oxygen species, suggesting that also this effect may be involved in the protective properties against I/R injury [11].

Due to the intriguing biological activities of H2S in the cardiovascular function, compounds able to generate exogenous H2S, are viewed as promising cardioprotective agents. In this scenario, different classes of H2S-donors have been described in the literature, such as GYY4137 [12], thiadiazolidin-3,5-diones [13], arylthioamides [14], iminothioethers [15], mercaptopruvate [16], dithioates [17]. Furthermore, also natural [18,19] and synthetic [20] isothiocyanates are known to generate H2S with a slow kinetic and in an l-Cysteine dependent manner. In addition, hybrid molecules bringing an isothiocyanate portion were developed and investigated in neurodegenerative diseases [21–23].

Despite their numerous properties, natural isothiocyanates have some limitations: most of them, including sulforaphane, are volatile oils and particularly unstable at room temperature. In fact, they are spontaneously converted into several inactive intermediates with relatively high degradation rates [24].

Starting from these findings [25,26], we selected a series of natural and synthetic isothiocyanates, aiming at defining a structure-activity relationship correlating their structure and H2S-releasing properties. We selected forty-five aliphatic and aromatic isothiocyanates, commercially available, variously substituted both in terms of steric hindrance and electronic properties. Aromatic derivatives included compounds with electron-withdrawing and electron-donating substituents in ortho, meta and para positions with respect to the -SCN moiety. Pyridine (25) and naphthalene (37) derivatives were selected as ring equivalents of phenyl isothiocyanate (1). Among the selected compounds, 3-pyridyl-isothiocyanate (25) showed the highest H2S releasing ability. This compound has been employed in a different research field as starting material for the synthesis of cationic polymers for targeted delivery [27,28]. Here we describe it as an efficient H2S donor that was thoroughly studied for its pharmacological activity in different experimental models of I/R injury in rats.

Materials and methods

Substances

All isothiocyanates were commercial products purchased from Fluorochem (Hadfield, UK).

Amperometric approach

The H2S-generating properties of the tested compounds have been evaluated by an amperometric approach, through an Apollo-4000 Free Radical Analyzer (WPI) detector and H2S-selective mini-electrodes at room temperature, in phosphate buffer solution at pH 7.4 in the absence or in the presence of l-cysteine 4 mM, as reported previously [18]. The generation of H2S was observed for 30 min.

Molecules preparation and prediction of physicochemical properties

The three-dimensional structures of the selected molecules were built in Maestro molecular modelling environment (Maestro (Accelrys, San Diego, CA, USA)) and the model was subjected to energy minimization using MacroModel software (MacroModel, Schrödinger, LLC, New York, NY, 2018) as previously described [28,29]. Furthermore, LigPrep (LigPrep, Schrödinger, LLC, New York, NY, 2018) application was used to refine the chemical structures. The resulting compounds, saved as sdf file, were investigated for their physicochemical properties. This investigation was performed by the webserver FADrugs4.0 (http://fadrugs4.mti.univ-paris-diderot.fr/ access May 2019) [30].

Compound 25 intracellular H2S release measurement

H9c2 were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96 well clear bottom black plate at a density of 72 × 104 per well. After 24 h, the medium was replaced and cells were incubated for 30 min with a 100 μM solution of the fluorescent dye WSP-1 (Washington State Probe, 1,3‘-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9‘-xanthene]-6‘-yl-2‘-(pyridine-2-yl-disulfanyl benzolate) that is highly sensitive for H2S detection [31,32]. Then, the supernatant was removed and replaced with different solutions of compound 25 dissolved in standard buffer (HEPES 20 mM; NaCl, 120 mM; KCl, 2 mM; glucose, 5 mM; and pH 7.4, at room temperature) at three increasing concentrations (30, 100, and 300 μM). The change in fluorescence (expressed as fluorescence index measured at λ = 465–515 nm) was monitored every 5 min for 60 min, by means of a spectrofluorometer. On the bases of previous experiments [15,33], 4-carboxyphenylisothiocyanate (4-CPI, Sigma-Aldrich) 300 μM was used as slow H2S donor reference compound. Six different experiments (n = 6) were performed, each carried out in three replicates. The results are expressed as mean ± SEM.

Animal procedures and ethical statements

All the procedures involving animals were carried out following the guidelines of the European Community Council Directive 86-609 and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki, EU Directive 2010/63/EU for animal experiments). All the experiments were authorized by the Ethical Committee of the University of Pisa and by the Italian Ministry of Health (authorization number 45972/2016). All the animals were housed in humidity- and temperature-controlled rooms (22 °C and 50%, respectively) with 12 h light/dark cycles, water, and food availability ad libitum.

Measurement of coronary flow

Adult male normotensive Wistar rats (300–350 g) were anaesthetized with an overdose of sodium thiopental (100 mg kg⁻¹ i. p.); hearts were quickly excised, rapidly mounted in a Langendorff apparatus (Radnoti, Monrovia, USA) and perfused with Krebs solution (NaHCO3, 25 mM; NaCl, 118.1 mM; KCl, 4.8 mM; MgSO4, 1.2 mM; CaCl2, 2.5 mM; NaH2PO4, 1 mM; glucose, 5.6 mM; and pH 7.4, at room temperature) at a flow rate of 10 ml/min. Coronary blood flow (CBF) was monitored via a side-arm port of the Langendorff apparatus (Visitech, West Warwick, RI, USA) connected to a pressure transducer (Validyne, Northridge, CA, USA) and measured in triplicate by a computerized flowmeter (Visitech). Coronary flow was expressed as ml/min/100 g of tissue.
1.2 mM; CaCl2, 2H2O, 1.6 mM; KH2PO4, 1.2 mM; and glucose, 11.5 mM) gassed with cloxcarb at 37 °C at constant pressure (70–80 mmHg). As reported previously [34], coronary flow (CF) was volumetrically recorded every 5 min, expressed as mL·min⁻¹, and normalized to the heart weight (g). After a 20 min equilibration period, some hearts were selected to determine the effects of compound 25 on CF of hearts precontracted with angiotensin II (AngII). These hearts were perfused with AngII 0.1 µM, and at the onset of a stable coronary spasm (observed as a reduction in the CF), cumulatively increasing concentrations of compound 25 (1, 3, 10 and 30 µM, for 20 min) were perfused (in the constant presence of AngII 0.1 µM). Changes in CF are expressed as percentage (%) of the basal CF. Experiments were carried out on hearts from six animals (n = 6) for each different treatment.

Ex vivo ischemia/reperfusion injury

Male Wistar rats (260–350 g) were randomized in 8 groups and were treated with an intra peritoneal (i.p.) injection of different increasing doses of compound 25 180 µg·kg⁻¹, 60 µg·kg⁻¹, 20 µg·kg⁻¹ and 6.7 µg·kg⁻¹ or diazoxide 40 mg·kg⁻¹ (a mitochondrial opener) or 4-CPI 240 µg·kg⁻¹ or vehicle (DMSO) or 5-hydroxy decanoic acid (5-HD) 10 mg·kg⁻¹ for 20 min followed by compound 25 20 µg·kg⁻¹. After 2 h, all the animals were anaesthetized with sodium thiopental (100 mg·kg⁻¹ i.p.) and heparinized (100UI i.p.) to prevent blood clotting. After opening the chest, the hearts were quickly excised, mounted on a Langendorff apparatus as reported previously. A water-filled latex balloon connected to a pressure transducer (Bentley Transec, mod 800, UgoBasile, Comerio, Italy) was introduced into the left ventricle via the mitral valve and the volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5–10 mmHg during initial equilibration. After 30 min of equilibration, hearts were subjected to 30 min of global diastolic pressure of 5–10 mmHg and measuring the conversion of NADH to NAD+ at the wavelength 340 nm. The amount of released LDH has been evaluated by the possible reduction of the size of injured area due to the ischemic process with the experiments lasting 120 min of reperfusion (without the small amount recorded in the pre-ischemic phase), resulting from the AUC analysis (area under the curve of the LDH amount recorded) and related to 1 g of the heart weight.

Acute in vivo myocardial infarction

The cardioprotective effects of compound 25 were evaluated in vivo, in an experimental model more closely resembling the clinical condition of acute myocardial infarction. The experimental protocol for coronary occlusion-reperfusion was performed as described previously [35], with minor modifications. Two hours before the experimental procedure, rats received an i.p. injection (about 0.3 mL) of compound 25 (20 µg·kg⁻¹) or diazoxide 40 mg·kg⁻¹ (a mitochondrial opener) or 4-CPI 240 µg·kg⁻¹ or vehicle (DMSO). Then, rats were anaesthetized with sodium thiopental (70 mg·kg⁻¹ i.p.). The trachea was intubated and connected to a rodent ventilator (mod. 7025 UgoBasile, Comerio, Italy) for artificial ventilation with room air (stroke volume, 1 mL/100 g body weight; 70 S/min). Electrocardiogram (ECG) was continuously measured by lead II (Mindray, PM5000, 2 Biological Instruments, Varese, Italy). The chest was opened by a left thoracotomy. A 6–0 surgical needle was passed around the left anterior descending coronary artery (LAD), located between the base of the pulmonary artery and left atrium. The ends of the suture were passed through a polypropylene tube (PE50) to form a snare, allowing reversible artery occlusion. The acute infarction protocol consisted of 30 min occlusion/120 min reperfusion; successful occlusion was confirmed by observing regional cyanosis downstream of the ligation, and by ST elevation in the ECG recording. At the end of reperfusion, rats were euthanized by an overdose of sodium thiopental, hearts were quickly excised, mounted on a Langendorff apparatus and perfused for 10 min with Krebs solution at 37 °C to clean coronary blood vessels. Then, the atria and right ventricle were removed from the hearts. The left ventricular tissue was dried, frozen at –20 °C, and cut into 4–5 transverse slices from apex to base of equal thickness (about 2 mm). The slices were submitted to morphometric analysis. Experiments were carried out on hearts from six animals (n = 6) for each different treatment.

Morphometric analysis of the ischemic area

The potential cardioprotective effects of compound 25 have been evaluated by the possible reduction of the size of injured areas in compound 25-treated hearts submitted to I/R. The left ventricle was cut in 2 mm-wide slices which were immersed in a 1% aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 20 min at 37 °C and then in a 10% aqueous solution of formaldehyde. After 24 h, ventricular slices were photographed and analysed to highlight necrotic areas due to the ischemic process (visible as a white or light pink color) and the healthy areas (visible as a strong red due to the TTC reaction).

LDH activity measurement

The LDH was measured for dynamic monitoring of cellular damage with the experiments lasting 120 min of reperfusion. Coronary effluent samples were collected at the last 5 min of the pre-ischemic phase and every 5 min during the first 30 min of reperfusion and then every 10 min—a total of 13 samples were collected per heart. The flow rate was measured each time an LDH activity measurement was performed and submitted to morphometric assays. Experiments were carried out on hearts from six animals (n = 6) for each different treatment.

Results

Amperometric determination of H2S release

The compounds selected comprehend both aliphatic and aromatic isothiocyanates characterized by different substituents (electron donating/withdrawing and different steric hindrance groups). All the compounds were incubated at the concentration of 1 mM in the presence or in the absence of L-Cys 4 mM to evaluate their H2S releasing properties. The results about the amount of H2S produced are expressed as Cmax in enzymatic mU·g⁻¹ released in 120 min of reperfusion (without the small amount recorded in the pre-ischemic phase), resulting from the AUC analysis (area under the curve of the LDH amount recorded) and related to 1 g of the heart weight.

The cardioprotective effects of compound 25 were evaluated in vivo, in an experimental model more closely resembling the clinical condition of acute myocardial infarction. The experimental protocol for coronary occlusion-reperfusion was performed as described previously [35], with minor modifications. Two hours before the experimental procedure, rats received an i.p. injection (about 0.3 mL) of compound 25 (20 µg·kg⁻¹) or diazoxide 40 mg·kg⁻¹ (a mitochondrial opener) or 4-CPI 240 µg·kg⁻¹ or vehicle (DMSO). Then, rats were anaesthetized with sodium thiopental (70 mg·kg⁻¹ i.p.). The trachea was intubated and connected to a rodent ventilator (mod. 7025 UgoBasile, Comerio, Italy) for artificial ventilation with room air (stroke volume, 1 mL/100 g body weight; 70 S/min). Electrocardiogram (ECG) was continuously measured by lead II (Mindray, PM5000, 2 Biological Instruments, Varese, Italy). The chest was opened by a left thoracotomy. A 6–0 surgical needle was passed around the left anterior descending coronary artery (LAD), located between the base of the pulmonary artery and left atrium. The ends of the suture were passed through a polypropylene tube (PE50) to form a snare, allowing reversible artery occlusion. The acute infarction protocol consisted of 30 min occlusion/120 min reperfusion; successful occlusion was confirmed by observing regional cyanosis downstream of the ligation, and by ST elevation in the ECG recording. At the end of reperfusion, rats were euthanized by an overdose of sodium thiopental, hearts were quickly excised, mounted on a Langendorff apparatus and perfused for 10 min with Krebs solution at 37 °C to clean coronary blood vessels. Then, the atria and right ventricle were removed from the hearts. The left ventricular tissue was dried, frozen at –20 °C, and cut into 4–5 transverse slices from apex to base of equal thickness (about 2 mm). The slices were submitted to morphometric analysis. Experiments were carried out on hearts from six animals (n = 6) for each different treatment.
Table 1
Structures and parameters of Cmax of the forty-five isothiocyanates (1 mM) were determined after incubation in the assay buffer at physiological pH and temperature, in the absence or in presence of 4 mM l-Cysteine.

| Compd | Structure | H2S-release without l-cysteine 4 mM(Cmax μM) | H2S-release + l-cysteine (Cmax μM) |
|-------|-----------|---------------------------------------------|-----------------------------------|
| 1     | ![Structure](image1) | 1.214 ± 0.145 | 10.029 ± 1.206 |
| 2     | ![Structure](image2) | 1.041 ± 0 | 2.396 ± 0.515 |
| 3     | ![Structure](image3) | 2.386 ± 0.974 | 5.270 ± 0.557 |
| 4     | ![Structure](image4) | 0.364 ± 0 | 4.154 ± 0.537 |
| 5     | ![Structure](image5) | 0 | 0.29 ± 0.02 |
| 6     | ![Structure](image6) | 3.617 ± 1.030 | 13.335 ± 1.863 |
| 7     | ![Structure](image7) | 0 | 18.309 ± 2.220 |
| 8     | ![Structure](image8) | 0 | 6.33 ± 1.51 |
| 9     | ![Structure](image9) | 0 | 0.16 ± 0.01 |
| 10    | ![Structure](image10) | 5.351 ± 0 | 23.406 ± 3.506 |
| 11    | ![Structure](image11) | 0 | 1.93 ± 0.14 |
| 12    | ![Structure](image12) | 0.614 ± 0.267 | 20.066 ± 0.337 |
| 13    | ![Structure](image13) | 0.411 ± 0 | 5.732 ± 0.929 |
| 14    | ![Structure](image14) | 0.865 ± 0.303 | 6.422 ± 0.551 |
| 15    | ![Structure](image15) | 0.168 ± 0 | 19.028 ± 1.328 |
| 16    | ![Structure](image16) | 0 | 0.15 ± 0.02 |
| 17    | ![Structure](image17) | 5.355 ± 1.521 | 17.750 ± 2.961 |
| 18    | ![Structure](image18) | 0 | 21.575 ± 1.220 |
| 19    | ![Structure](image19) | 0 | 29.29 ± 1.85 |
| 20    | ![Structure](image20) | 0 | 1.2 ± 0.9 |
| 21    | ![Structure](image21) | 3.594 ± 0 | 36.919 ± 4.399 |
The ability to release H$_2$S follows the order ortho < meta < para for all the compounds with the only exception of chloro-, fluoro- and iodo-substituted derivatives that follow the order para < ortho < meta. The poor H$_2$S releasing profile of the o-, m- and p-iodo-phenylisothiocyanates results from the combination of steric factors (particularly important for the ortho- and meta-derivatives) and reactivity features. Aryl iodide, in fact, are very prone to C-S cross-coupling with aromatic and aliphatic thiols.

The most efficacious H$_2$S donor was compound 25, 3-pyridyl-isothiocyanate, where the electron-deficient nature of the pyridine ring, attributed to the electron-withdrawing, inductive, and mesomeric effects of the nitrogen atom, accounts for the high H$_2$S release. The molecular mechanism responsible for thiol activated H$_2$S release from isothiocyanate has been recently elucidated in detail [36]. It involves an intramolecular cyclization of the cysteine-ITC intermediate crucial for the H$_2$S releasing. The strongly electrophilic nature of the –SCN moiety is amplified by

### Table 1 (continued)

| Compd | Structure | H$_2$S-release without L-cysteine (4 mM) | H$_2$S-release + L-cysteine (C$_{max}$ μM) |
|-------|-----------|----------------------------------------|----------------------------------------|
| 22    | ![Structure](image) | 0 | 2.47 ± 0.14 |
| 23    | ![Structure](image) | 0.912 ± 0.432 | 22.158 ± 1.857 |
| 24    | ![Structure](image) | 0.59 ± 0.38 | 30.32 ± 1.37 |
| 25    | ![Structure](image) | 8.719 ± 1.555 | 65.436 ± 4.356 |
| 26    | ![Structure](image) | 0.612 ± 0 | 9.064 ± 0.444 |
| 27    | ![Structure](image) | 1.550 ± 0.441 | 7.411 ± 0.126 |
| 28    | ![Structure](image) | 0.786 ± 0 | 32.007 ± 3.404 |
| 29    | ![Structure](image) | 5.350 ± 1.000 | 37.713 ± 4.866 |
| 30    | ![Structure](image) | 1.876 ± 0 | 17.576 ± 2.997 |
| 31    | ![Structure](image) | 0 | 16.58 ± 0.32 |
| 32    | ![Structure](image) | 0 | 3.87 ± 0.42 |
| 33    | ![Structure](image) | 3.777 ± 0 | 19.517 ± 1.194 |
| 34    | ![Structure](image) | 0 | 1.19 ± 0.27 |
| 35    | ![Structure](image) | 0.635 ± 0.369 | 24.880 ± 4.449 |
| 36    | ![Structure](image) | 0 | 1.246 ± 0.891 |
| 37    | ![Structure](image) | 0.205 ± 0.005 | 0.36 ± 0.03 |
| 38    | ![Structure](image) | 0 | 2.08 ± 0.05 |
| 39    | ![Structure](image) | 0.674 ± 0.286 | 1.124 ± 1.015 |
| 40    | ![Structure](image) | 0 | 0 |
| 41    | ![Structure](image) | 2.541 ± 0.585 | 12.973 ± 0.981 |
| 42    | ![Structure](image) | 1.389 ± 0.586 | 0 |
| 43    | ![Structure](image) | 4.092 ± 0.413 | 0 |
| 44    | ![Structure](image) | 0.161 ± 0 | 0.461 ± 0 |
| 45    | ![Structure](image) | 0 | 0 |
In silico evaluation of physicochemical properties

The drug-like profile represents a critical property for assuring the advancement of a drug candidate into preclinical studies and clinical trials. Notably, at the beginning of drug discovery trajectory it is particularly important to prioritize potential hit compounds that could be suitable starting points for searching novel drug candidates. Accordingly, it is a common procedure, during the drug discovery pipeline, to consider some molecular descriptors for selecting potential candidate drugs with satisfactory physicochemical properties and ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) profile [29,37–39].

Accordingly, we performed an in silico analysis to determine which derivatives could be submitted to further cellular and in vivo studies. The calculation of the physicochemical properties, regarding the molecules reported in Table 1, was performed by means of the online server FAFDrugs4.0 and the output is reported in Table 2.

The calculation of the physicochemical properties of the molecules, along with some filters commonly used in pharma companies highlighted a conserved trend indicating that the selected compounds possess a satisfactory drug-like profile, although some properties can be improved including chemical complexity. According to this calculation all the compounds presented no violation of the Lipinski rules of five [40]. The analysis of the main PhysChem descriptors is reported in Fig. 1A.

The computational analysis revealed that for solubility issue compounds 5, 16, 22, 37 and 38 cannot be considered for further investigation. Moreover, also compounds 11, 15, 17, 28 and 34, presenting a calculated solubility under 4000 mg·L⁻¹, were not considered for additional pharmacological tests. The remaining compounds, although with different values, showed satisfactory solubility according to the different descriptors used in the calculation (Table 2). Supplementary analysis considering two filters used in Pharma companies (GSK 4_400 filter is related to the physicochemical properties openness and structure complexity. Other filters used are related to the in vivo assay. The in silico drug-like profile of the molecules presented in this work is particularly important as it can influence the drug’s activity and toxicity profile (Table 2).
**Fig. 1.** (A) Main PhysChem descriptors analysis; (B) Chemical structure of compound 25; (C) Radar plot positioning compound’s values within the selected filter ranges. Compound values (blue line) should fall within the Lipinski rule of five filter area (light blue) (pale blue and red); (D) Radar plot visualizing compound complexity. It involves the number of system ring, stereocenters, rotatable and rigid bonds, the flexibility (ration between rotatable and rigid bonds), the carbon saturation (sp3 ratio), and the maximum size of system rings; (E) Graph regarding oral bioavailability taking into account the model obtained with 466 orally bioavailable compounds extracted from the DrugBank database and 916 orally bioavailable compounds extracted from eDrugs library. The graph is obtained by applying the PCA (Principal Component Analysis) of the 15 principal physicochemical descriptors of these molecules (blue). Then, the compound analyzed (pink) is projected, in the same conditions, on the same chemical space; (F) Radar plot representing an oral absorption estimation: The compounds values are materialized by the blue line, which should fall within the optimal green area. The white area is the extreme maximum zone while the red one is the extreme minimum zone. These zones are obtained with the following descriptors ranges: logP (−2 to 5), Molecular Weight (150–500), iPSA (20–150), Rotatable Bonds (0–10), H-Bonds Acceptors (0–10) and Donors (0–5); (G) ADMET profiling for compound 25; (H) Pfizer 3_75 rule positioning. The output was generated by FAFDrugs4 webserver.

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Intracellular H₂S release in H9c2 cells

The H₂S generation was detected in H9c2 cells by spectrofluorometric measurements using the WSP-1 probe, which specifically and irreversibly interacts with H₂S. The fluorescence produced by this interaction was quantitatively recorded by a spectrofluorometric approach which showed that the addition of the vehicle did not cause any significant increase of fluorescence. In contrast, the addition of compound 25 at the concentration of 30, 100 and 300 μM to H9c2, preloaded with the fluorescent dye, led to a time and concentration-dependent increase of fluorescence (FI, fluorescence index), indicating a significant generation of H₂S (P < 0.001 vs vehicle). 4-CPI 300 μM was used as reference H₂S-donor and showed a comparable H₂S-release than compound 25 300 μM (Fig. 2).

Effects on the coronary flow in Angiotensin-II precontracted rat hearts

The basal CF in Langendorff-perfused rat hearts was 10.54 ± 0.42 mL/min/g. As expected, the perfusion with Angiotensin II (AngII, 0.1 μM) caused a significant reduction (about 25%) of the coronary flow in isolated rat hearts when compared to the basal CF. In the constant presence of AngII, the “add-on” perfusion with 1, 3, 10, 30 μM compound 25 led to a concentration related increase of CF up to the complete recovery of the basal coronary flow and, thus, abolishing the AngII-mediated vasoconstriction at the maximum concentration tested. 4-CPI 30 μM, used as reference H₂S-donor [11], led to a slightly higher increase in the coronary flow of about 110% (Fig. 3).

Effects on isolated rat heart subjected to I/R injury

I/R caused marked functional damage to isolated heart of vehicle-treated rats, with a significant reduction of myocardial vital tissue, evaluated by morphometric analysis showing about 40% of ischemic area, expressed as ratio of ischemic area to total left ventricular area (Ai/Avi, %). The treatment with 4-CPI 240 μg·kg⁻¹ reduced the damaged tissue of about 10% compared to vehicle. A comparable cardioprotective effect has been measured as reduction of the ischemic area in hearts of compound 25 180 μg·kg⁻¹-treated rats (equimolar dose of 4-CPI 240 μg ·kg⁻¹). Surprisingly, the reduction of compound 25 dose, further limited...
the I/R injury damage, exerting the maximum cardioprotective effect at 20 µg·kg⁻¹. Lower dose (compound 25 6.7 µg·kg⁻¹) lead to a reduction of the cardioprotective effect. In order to study the involvement of mitoK<sub>ATP</sub> channels in the cardioprotective effects evoked by compound 25, 5-HD 10 mg·kg⁻¹ has been administered 20 min before the treatment with compound 25 20 µg·kg⁻¹. 5-HD 10 mg·kg⁻¹ clearly limited the cardioprotective effect of the isothiocyanate, since the ischemic area was significant more extended compared to the treatment with compound 25 20 µg·kg⁻¹ alone (Ai/A<sub>LV</sub>% 34.6 ± 0.1 vs 5.3 ± 0.5; data expressed as mean ± SEM). This result strongly suggests that mitoK<sub>ATP</sub> is likely to be involved in the cardioprotective effects of H<sub>2</sub>S. To further confirm the involvement of mitoK<sub>ATP</sub> channels, administration of diazoxide 40 mg/Kg (a mitoK<sub>ATP</sub> opener) imitated the ability of cardioprotective and anti-ischemic effect of compound 25 (Fig. 4).

LDH activity measurement

The LDH activity was measured in the coronary effluent collected during the preischemic and reperfusion periods. There were no differences between the various groups in LDH released during the preischemic period (data not shown). However, during reperfusion, LDH rose progressively in the groups treated with vehicle and with 5-HD 10 mg·kg⁻¹ + compound 25 20 µg·kg⁻¹. In the perfusate of hearts from rat given compound 25 at different doses, the amount of LDH released during the reperfusion period was markedly reduced, reflecting the pattern of the Ai/A<sub>LV</sub>% of the rat hearts treated with vehicle. Compound 25 20 µg·kg⁻¹ was the most effective dose tested in reducing the LDH release. Diazoxide slightly reduced the amount of LDH in perfusate of hearts (Fig. 5).

![LDH activity measurement](image)

**Fig. 4.** The histograms show the morphometric analysis of the ischemic area observed in ventricular slices of rat hearts expressed as Ai/A<sub>LV</sub>% after I/R-induced injury. The bars refer to the different pharmacological pre-treatments: vehicle, 40 mg Kg⁻¹ diazoxide, 240 µg kg⁻¹ 4-CPI, 6.7, 20, 60, 180 µg kg⁻¹ compound 25 and 5-HD 10 mg kg⁻¹ + 20-µg kg⁻¹ compound 25. Data are expressed as mean ± standard error. Six different experiments were carried out. * = significantly different from vehicle (**P < 0.001; ***P < 0.01). Representative pictures of rat left ventricle slices and the infarct size for each treatment are reported upon the histograms. Representative changes of functional parameters of vehicle (A) and compound 25 20 µg·kg⁻¹ have been reported.
Infarction and myocardial ischemia are main causes of mortality in Western countries, and the identification of innovative pharmacological treatments to limit I/R-induced cardiac injury remains a challenging issue. The discovery of H2S as an endogenous gasotransmitter and the comprehension of its pivotal role in regulating cardiovascular function and in mediating cardioprotective effect, offered novel promising perspectives in this field of research [43]. In particular, H2S-releasing molecules, such as GYY4137 and 4-CPI, have shown significant protective effects in experimental models of myocardial I/R, suggesting that H2S-donors can actually be viewed as a promising class of anti-ischemic drugs [44]. In this paper, we evaluated the H2S-releasing properties of a small library of isothiocyanates, since this chemical moiety has been reported to offer novel promising perspectives in this field of research [45]. To investigate how the structure of the isothiocyanates affects their H2S releasing properties, several derivatives were selected and the H2S generation was amperometrically measured.

Isothiocyanates show l-Cys-dependent H2S-releasing properties. Accordingly, in this study we confirmed that the incubation of all the isothiocyanates led to the formation of negligible amounts of H2S in the absence of L-Cysteine. Contrarily, in the presence of l-Cysteine, the incubation of the tested compounds led to a slow release of H2S. Presently, the H2S-releasing properties derive from a combination of several factors such as steric hindrance, electronic effects and position of the substituents in association with the water solubility of the compounds. The aliphatic isothiocyanates [39–45] show a very little release of H2S, when compared to the aromatic ones. Only compound 41 (hexyl isothiocyanate), identified as one of the components of the extracts from several Brassicaceae, showed a more relevant ability of H2S releasing (Cmax = 12.973 μM). The relevance of the conjugation between -SCN moiety and the aromatic ring is demonstrated by the dramatic lack of H2S releasing properties of compound 36 (benzyl isothiocyanate). As a clear consequence of the steric hindrance, the presence of any substituent in ortho to -SCN moiety caused a dramatic fall in the H2S production (compounds 2, 3, 4, 5, 8, 9, 11). On the other hand, the exceptions of compounds 6, 7, 10 and 12, demonstrate that the presence of electron-withdrawing groups partially compensate this steric effect. When analyzing meta- and para-substituted compounds we have a clear overview of the electronic

Cardioprotective effect of compound 25 in vivo

Rats were pretreated with vehicle or compound 25 20 mg kg⁻¹ 2 h before 30 min of coronary occlusion and 2 h of reperfusion. In rats treated with compound 25 20 mg kg⁻¹, there was a significant reduction in myocardial ischemic area, measured as Ai/ALV% (40.3 ± 1.8 vs 19.0 ± 1.4, P < 0.001, data expressed as mean ± SEM). These findings indicate that compound 25 exerts cardioprotective effect on I/R-induced cardiac injury. Furthermore, also the cardioprotective effects of diazoxide and 4-CPI, mitoKATP opener and the H2S-donor reference drug respectively, have been evaluated. Both the compounds promoted a significant cardioprotection reducing the ischemic area of about 15% (Fig. 6).

Discussion

Fig. 6. Morphometric quantification of ischemia/reperfusion (I/R)-induced injury observed in ventricular slices of rat hearts, after acute myocardial infarction in vivo, after treatment with vehicle, 40 mg kg⁻¹ diazoxide, 240 μg kg⁻¹ 4-CPI, 20 μg kg⁻¹ compound 25. Data are expressed as mean ± standard error. Six different experiments were carried out. *** = significantly different from vehicle (P < 0.001). Representative pictures of rat left ventricle slices and the infarct size for each treatment are reported upon the histograms.
effects deprived from the steric ones. The meta- and para- derivatives, electron-donating substituted (13, 14, 16, 20, 26, 27, 32) and electron-withdrawing substituted (15, 17, 18, 19, 21, 23, 24, 28, 29, 30, 31, 33, 35) are, respectively, worse and better H2S donors with respect to 1. The poor H2S donor profile of the o-, m- and p-ido substituted derivatives (11, 22, 34) is due not only to steric and reactivity factors but also to the low water solubility of the three derivatives (Table 2). The scarce water solubility may also account for the negligible H2S production of compounds 5, 16, 37 and 38. On the other hand, the excellent H2S release of compound 25 arises certainly from the electron deficient nature of the pyridine ring but even the high water solubility of the compound plays an important role.

The observed behavior is strongly supported by the recently described molecular mechanism responsible for H2S release from isothiocyanates [36]. It implies a nucleophilic attack by the cysteine thiol group on the isothiocyanate moiety central carbon, leading to an ITC-cysteine adduct. An electron poor ring (such as pyridine or an electron withdrawing substituted aromatic ring) facilitates the intramolecular nucleophilic addition of the amino group leading to a 4,5-dihydrothiazole intermediate that produces H2S.

Irrespective of the mechanism of reaction, such an organic thiol-dependency of the H2S-releasing process is viewed as a particularly advantageous property, because it allows these compounds to behave as “smart” H2S-donors, able to release the gasotransmitter only in a biological environment.

Starting from the H2S-generation properties, in silico methods have been used in order to select the best candidate to be investigated about the cardioprotective properties.

The drug-like profile represents a critical property for assuring the advancement of a drug candidate into preclinical studies and clinical trials. Furthermore, the use of in silico methods to establish the potential drug-like profile for a given set of molecules, prioritizing only the most promising compounds, can limit the costs and time required for selecting potential drug candidates, also reducing the use of animals for the in vivo studies.

Based on this evaluation, compound 25 has been identified as suitable to proceed towards further pharmacological investigations considering both physicochemical properties, drug-like features and H2S-releasing rate.

The H2S-releasing properties of compound 25 have been firstly described by an amperometric method performed in phosphate intracellular release of H2S and it is not surprising that there is not a proportional correlation between the concentration of compound 25 and the levels of the intracellular H2S release. Indeed, the H2S release could be influenced by different factors. First, the test compound must cross the cell membrane and enter the cellular environment; these processes strongly depend on the physicochemical properties of the compound. Then, the test compound (that is a thiol-dependent H2S-donor) must react with the intracellular free thiols; this reaction is strongly influenced by the compound structure and by the stoichiometric rate compound/free thiols. Therefore, a linear relationship between the extracellular concentration of the H2S-donor and the intracellular release of H2S is not expected and can be only evaluated empirically.

Since H2S plays a pivotal role in the regulation of the vascular tone and in the cardio-protection process, the effect at the cardiac level of compound 25 has been further investigated in both ex vivo and in vivo experimental models.

Compound 25 promoted a clear vasorelaxing effect in the coronary vascular bed and effectively counteracted the coronary vasocostriction induced by AngII.

In an ex vivo experimental model of myocardial I/R (Langendorff-perfused rat hearts), i.p. pre-administration of compound 25, led to significant and evident limitation of tissue injury (the maximum effect has been measured at the dose of 20 µg kg−1), although no clear dose-dependency was observed. Starting from the lower dose of 6.7 µg kg−1 a dose-dependent cardioprotection can be observed up to 20 µg kg−1. But further increasing doses of compound 25, failed to show increasing cardioprotective effects, rather increase of the ischemic area can be observed, thus indicating a weaker cardioprotective effect. This behavior can be explained by the hormesis that can be represented by a “U-shape curve”. Indeed, the hormetic effect of both H2S and isothiocyanates is widely described in the literature [46].

Regarding the vasorelaxing effect and the cardioprotection of 4-CPI and compound 25, the different activity of the two compounds is explained by the different targets. H2S promotes vasodilation mainly by activating pharmacological targets expressed on the surface of the vascular smooth muscle cell membranes. For instance, such a vasodilatation effect involves the activation of sarcolemmal KATP, Kv7 and BKCa channels. As concerns the cardioprotective effects, they are mainly due to the activation of mitochondrial targets (i.e. to the triggering of ischemic preconditioning mechanisms). Therefore, pharmacokinetic factors (linked to the physicochemical features of the molecule) are likely to account for the differences observed in the concentration evoking vasodilator and cardioprotective effects.

Furthermore, 4-CPI 240 µg/kg exhibited a clear cardioprotective effect, but 72 µg/kg of 4-CPI failed to produce cardioprotective effects. The dose of 180 µg/kg compound 25 (equimolar dose of 4-CPI 240 µg/kg) had a similar cardioprotective effects with the 240 µg/kg of 4-CPI. However, the concentration of 20 µg/kg compound 25 exerted the maximum cardioprotective effect, while 72 µg/kg of CPI failed to produce cardioprotective effects. Although the two isothiocyanates can release H2S, they seem to behave in a different manner. The different efficacy and effect in the cardioprotection may be due to the physicochemical properties of the two compounds. The cardioprotection is mediated by intracellular channels (mitoKATP is a main actor in mediating the cardioprotection effect of H2S). Thus, the compounds have to cross the membrane and probably 4-CPI is able to exert a more efficient vasorelaxing effect, but it is worse in crossing the membrane failing to reach the mitochondrial specific target.

Further investigations about the molecular mechanism on the cardioprotective properties of compound 25 have been carried out by evaluating the involvement of mitoKATP channels whose activation has been previously reported to mediate beneficial effect in I/R injury models. Testai and colleagues [34] demonstrated that H2S and H2S-donors are able to activate mitoKATP channels suggesting that they may be a main target of the anti-ischemic effects evoked by this gasotransmitter. Indeed, in our experimental model, the mitoKATP blocker 5-HD (10 mg Kg−1) almost completely abolished the effects of compound 25, strongly suggesting that mitoKATP is likely to be involved in the cardioprotective effects of H2S.

The “bi-modal” curve is reflected also by the measurement of LDH activity in the perfusate effluent, confirming the tissue damage highlighted by the morphometric analysis.
Finally, the cardioprotective effects of compound 25 were tested in vivo, in an experimental model of acute myocardial infarction in rats, more closely resembling the clinical pattern of myocardial infarction. Also, in this model, compound 25 (20 μg kg⁻¹) exhibited cardioprotective effects.

Conclusion

A library of forty-five isothiocyanates was evaluated for its ability to release H₂S and then, by an in silico approach, compound 25 was identified as the most promising one. This derivative was thoroughly characterized for its cardioprotective function by means of ex vivo and in vivo assays. In conclusion, our results strongly suggest that isothiocyanate-based H₂S-releasing drugs, such as compound 25, can trigger a “pharmacological pre-conditioning” and could represent a suitable pharmacological option in anti-ischemic therapy.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

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Declaration of Competing Interest

The authors have declared no conflict of interest.

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