Pharmacological Research 159 (2020) 105039

Contents lists available at ScienceDirect

Pharmacological Research

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

Searching for novel hydrogen sulfide donors: The vascular effects of two thiourea derivatives

Valentina Citi[a], Alma Martelli[a], Mariarosaria Bucci[b], Eugenia Piragine[a], Lara Testai[a], Valentina Vellecco[b], Giuseppe Cirino[b], Vincenzo Calderone[a,*]

[a] Department of Pharmacy, University of Pisa, via Bonanno, 6 - 56125, Pisa, Italy
[b] Department of Pharmacy, University Federico II, via Montesano, 49 - 80131, Naples, Italy

ARTICLE INFO

Keywords:
Hydrogen sulfide
H_2S-donor
Vasorelaxation
HASMCs
Thioureas

ABSTRACT

The gasotransmitter hydrogen sulfide (H_2S) is involved in the regulation of the vascular tone and an impairment of its endogenous production may play a role in hypertension. Thus, the administration of exogenous H_2S may be a possible novel and effective strategy to control blood pressure. Some natural and synthetic sulfur compounds are suitable H_2S-donors, exhibiting long-lasting H_2S release; however, novel H_2S-releasing agents are needed to improve the pharmacological armamentarium for the treatment of cardiovascular diseases.

For this purpose, N-phenylthiourea (PTU) and N,N'-diphenylthiourea (DPTU) compounds have been investigated as potential H_2S-donors. The thioureas showed long-lasting H_2S donation in cell free environment and in human aortic smooth muscle cells (HASMCs). In HASMCs, DPTU caused membrane hyperpolarization, mediated by activation of K_{ATP} and Kv7 potassium channels. The thiourea derivatives promoted vasodilation in rat aortic rings, which was abolished by K_{ATP} and Kv7 blockers. The vasorelaxing effects were also observed in angiotensin II-constricted coronary vessels.

In conclusion, thiourea represents an original H_2S-donor functional group, which releases H_2S with slow and long lasting kinetic, and promotes typical H_2S-mediated vascular effects. Such a moiety will be extremely useful for developing original cardiovascular drugs and new chemical tools for investigating the pharmacological roles of H_2S.

1. Introduction

The gasotransmitter hydrogen sulfide (H_2S) is involved in the regulation of the homeostasis of the cardiovascular system, where it is mainly biosynthesized by the pyridoxal-5-phosphate dependent enzyme cystathionine-γ-lyase (CSE), from the amino acid L-cysteine. However, the enzymes cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfotransferase/cysteine amino transferase (3-MST/CAT) also contribute to cardiovascular production of H_2S [1,2]. At physiological concentrations, H_2S evokes direct vasorelaxing responses through different mechanisms that include the activation of different ion channels, such as the ATP-sensitive potassium channels (K_{ATP}) [3,4], the large conductance calcium-activated potassium channel (BK_{Ca}) [5] and the voltage-gated potassium channels Kv7 [6,7], and the inhibition of phosphodiesterase-5 with the consequent increase of intracellular cGMP [8,9].

Consistently, reduced levels of endogenous H_2S have been found in hypertensive animals and humans, suggesting that H_2S could be involved in blood pressure regulation [10,11].

In particular, genetic modulations of CSE, CBS or 3-MST levels are effective means to experimentally investigate the cardiovascular actions of H_2S. CSE−/− mice showed higher blood pressure than that of CSE−/+ mice, suggesting that a deficiency of CSE/H_2S may contribute to hypertension [12]. However, it has been controversially reported

Abbreviations: CSE, cystathionine gamma-lyase; K_{ATP}, ATP-sensitive potassium channels; BK_{Ca}, large conductance calcium-activated potassium channel; Kv7, voltage-gated potassium channels; GYY4137, morpholin-4-ium-4-methoxyphenyl-morpholino-phosphinodithioate; HASMC, human aortic smooth muscle cells; PTU, N-phenylthiourea; DPTU, N, N'-diphenylthiourea; DPU, N,N'-diphenylurea; 4-COOH-PITC, 4-carboxy-phenyl isothiocyanate; DADS, diallyl disulfide; DATS, diallyl trisulfide; DMSO, dimethyl sulfoxide; WSP-1, Washington State Probe-1, 3'-methoxy-3-oxo-3H-spiro(isobenzofuran-1, 9'-xanthen)-6'yl2(pyridin-2-yldisulfanyl); Ach, acetylcholine; DiBac4(3), bis-(1, 3-dibutylbarbituric acid)trimethine oxonol; NS1619, 1, 3- dihydro-1-(2-hydroxy-5-(trifluoromethyl)phenyl)-5-(trifluoromethyl)-2H-benzimidazol-2-one 31

* Corresponding author.
E-mail address: vincenzo.calderone@unipi.it (V. Calderone).

https://doi.org/10.1016/j.phrs.2020.105039
Received 22 May 2019; Received in revised form 15 June 2020; Accepted 16 June 2020
Available online 18 June 2020

that C57BL/6 J mice with CSE deficiency actually exhibit normal blood pressure [13]. This discrepancy may be due to differences in their genetic background.

Noteworthy, insufficient H_2S enzymatic production has been also associated with cardiovascular complications in experimental models of diabetes mellitus [14]. In particular, in diabetic patients also show an enhanced H_2S consumption due to oxidative inactivation of H_2S has been reported, leading to implications in the pathophysiology of cardiovascular disease [15,16].

Since the insufficient production/activity of endogenous H_2S may play a causal role in hypertension, the role of H_2S has been mostly studied by inhibiting its physiological production [17] or using exogenous source of H_2S which could be considered as possible strategy in controlling blood pressure [18,19].

Ideal H_2S-donors for clinical purpose should be endowed with slow H_2S-releasing profile, ensuring a constant and long-lasting H_2S concentration at physiological level [20]. The synthetic H_2S-donor compounds GYY4137 [21] and many natural polysulfide compounds from garlic (*Allium sativum* L.) [22] were early prototypical slow H_2S-donors. Thereafter, some heterogeneous H_2S-releasing chemical moieties, such as natural isothiocyanates [23–25], synthetic isothiocyanates [26–30], thioamides [31], iminothioethers [32], thiadiazolidindiones [33] or polysulfide molecules [34–36] were reported to be slow releasing H_2S compound able to exert many beneficial effects [17,37]. However, the discovery of novel H_2S-releasing chemical moieties is still a compelling issue, in order to improve the pharmacological armamentarium for the treatment of cardiovascular diseases and facilitate the project of new H_2S-based drugs.

Thiourea functional group is a sulfur moiety, whose potential H_2S-releasing properties have never been investigated until now. Noteworthy, the blood pressure lowering effects of some thiourea derivatives have been already described, even if the mechanisms of action were quite elusive stressing the necessity of further mechanistic pharmacological studies [38,39].

In this paper, thiourea functional group has been investigated as a novel potential source of H_2S by using two thiourea derivatives such as N-phenylthiourea (PTU) and N,N'-diphenylthiourea (DPTU), shown in Fig. 1. Evaluation of their H_2S-donor profile in both "cell free" environment and in human aortic smooth muscle cells (HASMCs) have been performed. Then, in order to assess the pharmacological effects of PTU and DPTU on vascular tissues, different pharmacological approaches have been used. In detail, the hyperpolarizing activity of the two thioureas has been evaluated in HASMCs, while the vasorelaxant effect has been explored in isolated rat aortic rings and in angiotensin-II precontracted coronary vessels of Langendorff-perfused rat hearts.

2. Materials and methods

2.1. Determination of H_2S by amperometry

The H_2S-release properties of PTU (Sigma-Aldrich, N-Phenylthiourea, PubChem CID: 676454) and DPTU (Sigma-Aldrich, 1-Benzyl-3-Phenylthiourea, PubChem CID: 668300) have been evaluated by amperometric approach, through an Apollo-4000 Free Radical Analyzer (World Precision Instrument -WPI) detector and H_2S-selective electrodes. 4-COOH-PITC [26] (FluorChem, 4-isothiocyanatobenzoic Acid, PubChem CID: 242946) and GYY4137 [40] (abcam - morpholin-4-ium-4-methoxyphenyl-morpholino-phosphinodithioate PubChem CID: 53393943) were used as H_2S-donor reference compounds. The experiments were carried out at room temperature in a PBS buffer adjusted to pH 7.4. The H_2S-selective electrode was equilibrated in 10 mL of the PBS buffer, after establishing a stable baseline. Then, 100 µl of a dimethyl sulfoxide (DMSO – Sigma-Aldrich) solution of the tested compounds was added at the final concentration of the 1 mM and 1% of the vehicle DMSO. The generation of H_2S was observed for 25 min. When required by the experimental protocol, an excess of L-cysteine (Sigma-Aldrich) 4 mM was added, before the H_2S-donors. The relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was determined by calibration curves with increasing concentrations of NaHS (Cayman Chemical) (1 µM, 3 µM, 7 µM, 10 µM) at pH 4.0. The lower limit of reliable quantitative determination was 0.5 µM. The curves relative to the progressive increase of H_2S vs time, following the incubation of the tested compounds, were analyzed by a fitting curve using the software GraphPad Prism 6.0, obtaining Cmax (the highest concentration achieved in the recording time) and HCT (Half Concentration Time, i.e. the time required to achieve 50 % of Cmax). The values of Cmax and HCT were calculated by a computer fitting procedure.

2.2. Cell line

HASMCs were cultured in Medium 231 (Life technologies) supplemented with Smooth Muscle Growth Supplement (SMGS, Life Technologies) and 1% of 100 units/mL penicillin and 100 mg/mL

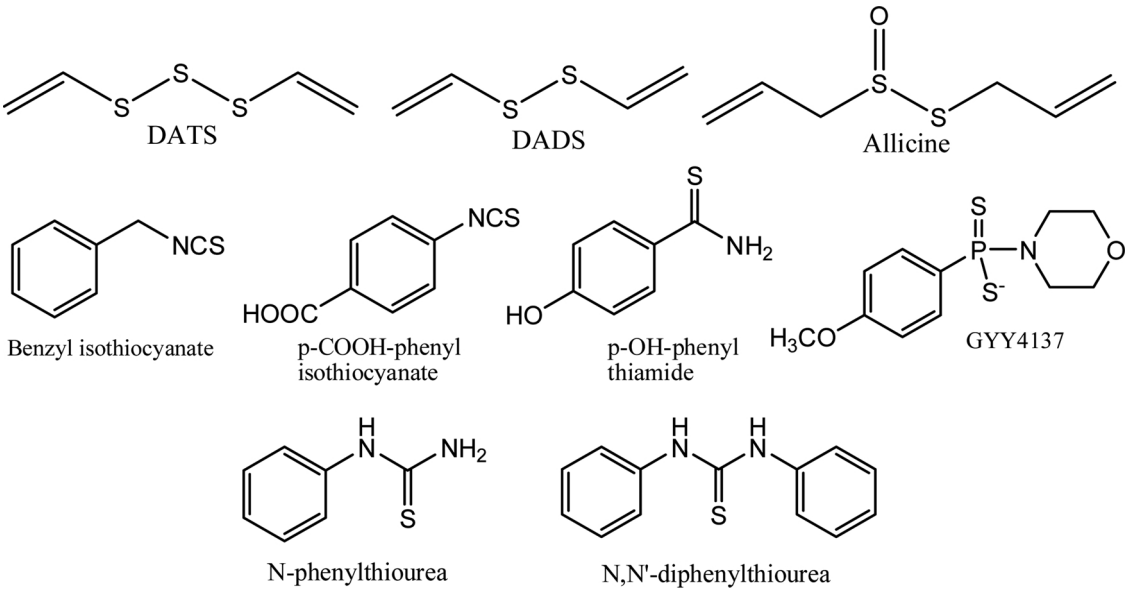

Fig. 1. Structures of some known and novel H_2S-donors.

streptomycin (Sigma Aldrich) in tissue culture flasks at 37 °C in a humidified atmosphere and 5% CO_2. HASMCs were cultured up to about 90 % confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatin 1% (from porcine skin, Sigma Aldrich), at density of 72 × 10^3 per well. Cells were split 1:2 twice a week and used until passage 18.

2.3. Evaluation of H_2S release on HASMCs

After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 30 min in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2{\cdot}2H_2O$ 2 mM, $MgCl_2{\cdot}6H_2O$ 1 mM, Glucose 5 mM, pH 7.4, at room temperature) containing the fluorescent dye WSP-1 (Washington State Probe-1, 3'-methoxy-3-oxo-3H-spiro (isobenzofuran-1,9'-xanthen)-6'yl2(pyridin-2-yldisulfanyl) benzoate, Cayman Chemical) at the concentration of 100 μM [32,41,42]. Then the supernatant was removed and replaced with a solution of the tested compounds or the 4-COOH-PITC as a known H_2S-donor [26] in buffer standard. When WSP-1 reacts with H_2S, it releases a fluorophore detectable with a spectrofluorometer at excitation and emission wavelengths of 465 − 515 nm. The increasing of fluorescence (expressed as fluorescence index = FI) was monitored for 30 min, using a spectrofluorometer (EnSpire, Perkin Elmer).

2.4. Animal procedures

All the experimental procedures 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). The experiments were authorized by the Ethical Committee of the University of Pisa (Protocol number 0037321/2013). The blood pressure evaluation were authorized by the Ministry of Health of Italy (Protocol number 108/2020-PR).

2.5. Evaluation of the functional effects on rat aortic rings. Study in vitro on isolated rat aortic rings

To determine a possible vasodilator mechanism of action, the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). Rats were sacrificed by cervical dislocation under overdose of sodium pentobarbital and bled. Thoracic aorta was immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intimal surface of the aortae with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2.2H_2O$ 1.80; $MgSO_4.7H_2O$ 1.05; $NaH_2PO_4.H_2O$ 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with Clioxicarb, a mixture of O_2 (95 %) and CO_2 (5%). Changes in tension were recorded by an isometric transducer (Grass FTO3), connected with a preamplifier (Buxco Electronics) and with a software for data acquisition (BIOPAC Systems Inc., MP 100). After an equilibration period of 60 min, the endothelial removal was confirmed by the administration of acetylcholine (ACh, 10 μM) to KCl (25 mM)-precontracted vascular rings. A relaxation < 10 % of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation ≥10 % (i.e. significant presence of the endothelium), were discarded. Then, 45 min after the confirmation of the endothelium removal, the aortic preparations were recontracted by KCl 25 mM and when the contraction reached a stable plateau, the tested compounds or the reference H_2S-donor 4-COOH-PITC were cumulatively added (0.1 μM – 1 mM). Preliminary experiments showed that the KCl (25 mM)-induced contractions remained in a stable tonic state for at least 40 min [43]. The vasorelaxing efficacy (Emax) was defined as maximal vasorelaxing response achieved with the highest concentration (1 mM) of the tested compounds and was expressed as a percentage (%) of the contractile tone induced by KCl. The parameter of potency was expressed as pEC50, calculated as negative Logarithm of the molar concentration evoking a half-maximal vasorelaxing effect.

The parameters of efficacy and potency were calculated by a computer fitting procedure (software: GraphPad Prism 6.0) and expressed as mean ± standard error, from aortae of 6–10 animals.

2.6. Effects on coronary flow in Langendorff-perfused rat hearts

The animals were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and bled. After the opening of the chest, the hearts were quickly excised and placed in Krebs solution at 4 °C (composition mM: $NaHCO_3$ 25.0, NaCl 118.1, KCl 4.8, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}2H_2O$ 1.6, KH_2PO_4 1.2, glucose 11.5) equilibrated with 95 % O_2 5% CO_2. Rapidly, the ascending aorta was cannulated, the heart was mounted on a Langendorff apparatus and perfused with Krebs solution (thermostated at 37 °C and continuously bubbled with a gas mixture of 95 % O_2 and 5% CO_2). Perfusion was carried out at constant pressure (70–80 mmHg). Then, a bolus of heparin (100 UI i.p.) was administered to prevent blood clotting. The above procedure was completed within about 2 min. In order to monitor the functional parameters, a water-filled latex balloon connected to a pressure transducer (Bentley Trantec, mod 800) 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. The Rate Pressure Product (RPP) and the dP/dt were continuously monitored by a computerized Biopac system (California, USA), in order to discard hearts showing severe arrhythmia or unstable RPP and dP/dt values, during the equilibration period. Coronary flow (CF) was volumetrically measured at 5 min intervals and expressed as ml/min, normalized by the heart weight. After a 20 min equilibration period, angiotensin II (AngII) 0.1 μM was administered through the perfusion. Once a stable contractile coronary artery tone was obtained (evaluated as a reduction of the CF), cumulatively increasing concentrations of PTU or DPTU (1 μM–100 μM, 20 min of perfusion for each concentration) were administered (in the constant presence of AngII 0.1 μM). The reference H_2S-donor 4-COOH-PITC was added at the concentration of 100 μM [26]. Preliminary experiments demonstrated that the treatment with AngII 0.1 μM alone caused a rapid decrease of the CF, which reached and maintained a stable level for at least 2 h [44].

Changes in CF were recorded after the pharmacological treatments and were expressed as a % of the basal values measured in the last 5 min of the equilibration period. The parameters were evaluated as mean ± standard error, from hearts of 6–10 animals.

2.7. Evaluation of the membrane hyperpolarizing effects on HASMCs

The membrane hyperpolarizing effects were evaluated on HASMCs by spectrofluorometric methods, as already described [6,45]. HASMCs were cultured up to about 90 % confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatin 1% (from porcine skin, Sigma Aldrich), at density of 72 × 10^3 per well. After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2{\cdot}2H_2O$ 2 mM, $MgCl_2{\cdot}6H_2O$ 1 mM, Glucose 5 mM, pH 7.4, at room temperature) containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich) 2.5 μM [46]. This membrane potential-sensitive dye DiBac4(3) allowed us to measure the cell membrane potential; in fact, this lipophilic and negatively-charged oxonol dye shuffles between cellular and extracellular fluids in a membrane potential-dependent manner (following the Nernst laws), thus allowing to assess changes in membrane potential by means of spectrofluorometric recording. In particular, an increase of fluorescence, corresponding to an inward flow of the dye,

reflects a membrane depolarization; in contrast, a decrease in fluorescence, due to an outward flow of the dye, is linked to membrane hyperpolarization. The spectrofluorometric recording is carried out at excitation and emission wavelengths of 488 and 520 nm, respectively (Multiwells reader, Enspire, PerkinElmer). NS1619 (Sigma-Aldrich) at the concentration of 10 μM [47], was used as reference drug, since it evokes membrane hyperpolarizing effects with highest potency and efficacy [32], while 4-COOH-PITC was used as a H_2S-donor. When required by the experimental procedures, after the assessment of base-line fluorescence, channel blockers were incubated for 20 min; the tested compounds were added, and the trends of fluorescence was followed for 40 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as:

$(Ft - F0)/F0$

where F0 is the basal fluorescence before the addition of the tested compounds, and Ft is the fluorescence at time t after their administration. The value corresponding to the maximum hyperpolarizing effect were expressed as % of that induced by NS1619 10 μM.

2.8. In vivo measurement of blood pressure

The effects of DPTU and its vehicle (DMSO 1 mL·kg − 1) were tested on blood pressure on an experimental model of hypertension, induced by the administration of L-NG-nitroarginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase. Male 12-week-old normotensive Wistar rats (300–350 g) were anaesthetized with sodium thiopental 60 mg kg^{-1} i.p. and after the administration of the anaesthetic, they were kept on a heated platform (about 30 °C) for 20 min to induce a slight vasodilatation of the tail artery, in order to allow an easier recording of the basal systolic BP (Psys) with the "tail-cuff" method by a BP recorder (BP-2000 Blood Pressure Analysis System, Series II, Visitech System, Apex NC, USA). Basal level of Psys was recorded for 20 min, at 5 min intervals. Then, the rats received an i.p. injection of 100 mg/kg L-NAME, and the Psys increase was further monitored for 20 at 5 min intervals. Then, DPTU 1 mg Kg-1, 3 mg kg − 1 and 10 mg Kg^{-1} or the corresponding vehicle were administered i.p. to different groups, each composed of six rats. Starting from the administration of the tested compounds, the Psys values were recorded, for 30 at 5 min intervals. Basal Psys was expressed as a mean of the four measurements carried out in each rat before the administration of L-NAME. L-NAME-induced hypertensive Psys was expressed as a mean of the four measurements carried out in each rat after the administration of L-NAME. Change in systolic blood pressure, recorded after the drug administration, was expressed as percentage of the L-NAME-increased Psys and calculated as mean value of the six recordings carried out after the drug administration. Blood pressure measurements were carried out in 6 animals/group.

2.9. Statistical analysis

Experimental data were analyzed by a computer fitting procedure (software: GraphPad Prism 6.0) and expressed as mean ± standard error; three different experiments were performed, each carried out in three replicates. ANOVA and Student *t*-test were selected as statistical analyses; when required, the Bonferroni post hoc test was used. $P < 0.05$ was considered representative of significant statistical differences.

3. Results

3.1. Amperometric measurement of H_2S-release

As already reported [31], the incubation of the well-known slow-releasers of H_2S (4-COOH-PITC [26] and GYY4137 [40]) require the presence of cysteine (4 mM) to get a detectable generation of H_2S (Table 1). Interestingly, PTU and DPTU led to H_2S release both in the absence and in the presence of L-cysteine with a relatively slow kinetic, although the presence of L-cysteine slightly but not significantly improved the H_2S formation. Indeed, PTU exhibited Cmax of H_2S release of about 8 μM, while DPTU showed lower H_2S formation (Cmax of about 3 μM) independently of L-cysteine (Table 1, Fig. 2).

Table 1
H_2S-releasing profile of the tested compounds. The table shows the values of Cmax and HCT of H_2S-release obtained with the indicated compounds (1 mM) and recorded by the amperometric analysis in the absence (-L-Cys) or in the presence (+L-Cys) of an excess of L-cysteine (4 mM). Data are expressed as means ± standard error. n.d. = not detectable.

	+ L-cysteine 4 mM		− L-cysteine	
	Cmax (μM)	HCT (min)	Cmax (μM)	HCT (min)
4-COOH-PITC	34.9 ± 3.3	5.2 ± 0.3	< 1	n.d.
GYY4137	10.3 ± 2.6	2.5 ± 0.8	< 1	n.d.
PTU	8.99 ± 0.42	6.23 ± 0.49	7.69 ± 0.51	10.25 ± 0.93
DPTU	3.46 ± 0.16	5.26 ± 0.69	3.30 ± 0.08	5.97 ± 0.74

3.2. Evaluation of H_2S-release in HASMCs

A further investigation about the H_2S releasing properties of PTU and DPTU has been carried out using cultured HASMCs, for assessing the H_2S formation into the cells without adding any exogenous thiol.

In particular, the H_2S generation was detected in HASMCs by spectrofluorometric measurements using the dye 3′-methoxy-3-oxo-3H-spiro-(isobenzofuran-1,9′-xanthen)-6′–yl–2-(pyridin-2-yldisulfanyl) benzoate (Washington State Probe-1,WSP-1), which selectively binds H_2S, determining an increase in the fluorescence values. The incubation of both PTU and DPTU determined a significant concentration-dependent H_2S formation into the cells compared to the vehicle (DMSO 1%), but with different rate: DPTU 1 mM showed an increase in fluorescence values, and thus in H_2S formation, about two-fold higher than PTU indicating that the DPTU can easier cross the cell membrane and explicate the H_2S-donor feature. 4-COOH-PITC (100 μM) has been use as a known H_2S releasing molecule and showed a marked increase in the fluorescence index, thus indicating the intracellular H_2S formation (Fig. 3).

3.3. Direct vasorelaxing effects

PTU produced weak vasorelaxing effects, with a low efficacy (Emax = 37.71 ± 1.91). In contrast, DPTU showed a full vasorelaxing efficacy

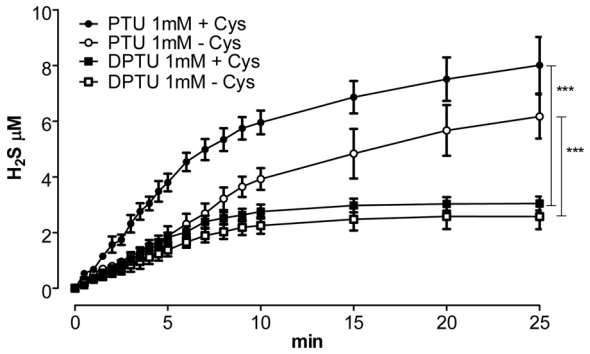

Fig. 2. Evaluation of H_2S-release kinetic of PTU and DPTU in the absence (-L-Cys) or in the presence of L-Cys 4 mM (+ L-Cys). In a cell free environment both PTU and DPTU display a slow release of H_2S that resulted independent of L-cysteine addition. Three different experiments were carried out, each in triplicate (*** $P < 0.001$).

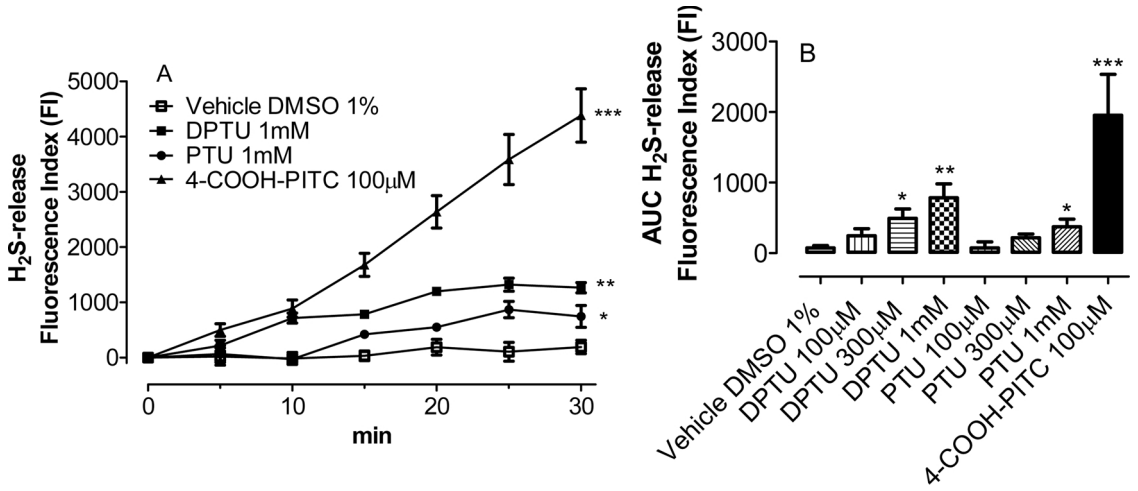

Fig. 3. H_2S release measured into HASMCs. A) Time-course of the intracellular H_2S formation (expressed as WSP-1 fluorescence) following the administration of vehicle, PTU 1 mM, DPTU 1 mM and 4-COOH-PITC 100 μM as known H_2S-donor to HASMCs. **B**) Cumulative H_2S formation (expressed as area under the curve of the WSP-1 fluorescence in the recording time) produced by the incubation of vehicle or different concentrations of PTU and DPTU and 4-COOH-PITC (100 μM). Data were expressed as mean ± standard error. Three different experiments were carried out, each in triplicate. Student *t*-test has been applied to calculate the significance level. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 2
The table reports the parameters of Emax and pEC50 of the vasorelaxing effects recorded on KCl 25 mM pre-contracted endothelium denuded rat aortic rings, without blockers, in the presence of Kv7 blocker (+ XE991 10 μM) and in the presence of K_{ATP} blocker (+ Gli 10 μM). Data are expressed as mean ± SEM (n.c. = not calculated).

	No-blocker		+ XE991 10 μM		+ Gli 10 μM	
	Emax	pEC50	Emax	pEC50	Emax	pEC50
PTU	37.7 ± 1.9	n.c.	21.4 ± 2.6	n.c.	22.7 ± 6.1	n.c.
DPTU	99.7 ± 0.8	4.5 ± 0.1	82.3 ± 1.6	3.4 ± 0.1	93.1 ± 0.1	3.4 ± 0.1
4-COOH-PITC	89.9 ± 2.3	4.1 ± 0.1	7.4 ± 3.8	n.c.	6.2 ± 2.2	n.c.
DPU	77.7 ± 5.3	3.7 ± 0.1	–	–	–	–

(Emax = 99.67 ± 0.84) and a pEC50 of 4.52 ± 0.03 (Table 2). The Kv7 blocker XE991 and the K_{ATP} blocker glibenclamide, both at the concentration of 10 μM, significantly abolished the vasodilator effect of the two thioureas.

4-COOH-PITC, used as reference H_2S-donor, showed an almost full vasorelaxing effect (Emax = 89.9 ± 2.34) and both XE991 and glibenclamide totally abolished the vasodilator effect (Fig. 4. Table 2). To further investigate the role of H_2S released by DPTU, the vasorelaxing effect of N,N'-diphenylurea (DPU) has been evaluated. As reported in Fig. 4D, the incubation of increasing concentration of DPU exerted a mild vasodilation, reaching as maximum effect about the 77 % (Table 2) (Fig. 4 and Fig. 5).

3.4. Evaluation of the functional effects on the coronary flow

The basal CF in Langendorff-perfused rat hearts was 11.1 ± 0.34 mL/min/g. The perfusion of Ang II 10 μM led to a decrease of CF of about 25 %. The "add-on" perfusion of PTU, in the constant presence of AngII, did not exert any changes in CF (Fig. 6 A). In contrast, "add-on" perfusion of DPTU, in the constant presence of AngII, caused a concentration-related improvement of the CF, leading (at the concentration 10 μM) to a complete recovery of the basal coronary flow and, thus, abolishing the AngII mediated vasoconstriction (Fig. 6 B). 4-COOH-PITC at the concentration of 100 μM showed a marked recovery of the basal coronary flow as expected.

3.5. Evaluation of membrane hyperpolarization of HASMCs

The effects of DPTU have been evaluated on the membrane potential of cultured human vascular smooth muscle cells (HASMCs), taking 1,3- dihydro-1-(2-hydroxy-5-(trifluoromethyl)phenyl)-5-(trifluoromethyl)-2H-benzimidazol-2-one 31 (NS1619), as reference hyperpolarizing agent, and 4-COOH-PITC (100 μM) as reference H_2S-donor [47].

The DPTU caused a significant and concentration-dependent membrane hyperpolarization of HASMCs. The highest concentration of DPTU induced a hyperpolarizing response of about the 50 % compared to the reference compound NS1619 (Fig. 7). Therefore, this concentration has been selected to further investigate the involvement of K_{ATP} and Kv7 channels in the DPTU hyperpolarizing response. For this purpose, glibenclamide and XE991 (K_{ATP} and Kv7 blockers, respectively) have been used. As reported in Fig. 7, the two blockers completely abolished the hyperpolarizing effect induced by DPTU, confirming the involvement of Kv7 and K_{ATP} potassium channels in DPTU activity.

The 4-COOH-PITC (100 μM) showed a significant hyperpolarization effect comparable to DPTU 1 mM. This effect was completely abolished only by XE991, while glibenclamide did not exert any effect (Fig. 7).

3.6. Effect of DPTU on systolic blood pressure

Normotensive Wistar rats showed basal systolic pressure (Psys) of 110 ± 4 mmHg. Intraperitoneal administration of L-NAME (100 mg/kg) caused a significant increase of Psys to 165 ± 2 mmHg. The administration of vehicle (DMSO 1 mL/kg i.p.) did not cause any significant change of Psys in rats with L-NAME-induced hypertension. In contrast, DPTU showed a clear concentration dependent antihypertensive effect, reaching the maximum effect at the dose of 10 mg/Kg reducing the blood ressure by about 25 % (Fig. 8).

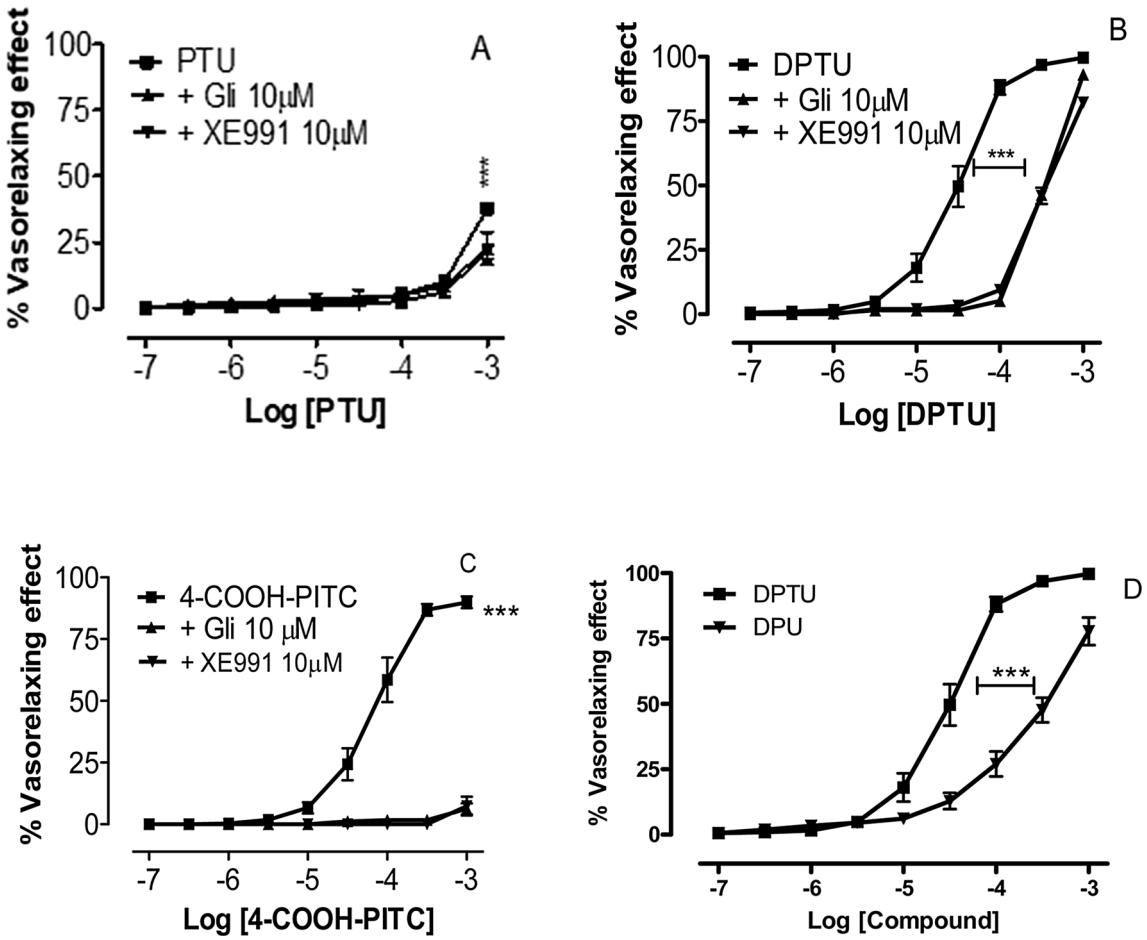

Fig. 4. Vasorelaxing effect of PTU, DPTU, DPU and 4-COOH-PITC in rat aortic rings. The graphs show the concentration-response curves, relative to the vasorelaxing effects evoked by PTU (**A**), DPTU (**B**), 4-COOH-PITC (**C**) and DPU (**D**) in endothelium-denuded rat aortic rings pre-contracted with KCl 25 mM. The vasorelaxing effects of the compounds have been also recorded in the presence of the Kv7-blocker XE991 10 μM or the K_{ATP} blocker glibenclamide 10 μM. The vertical bars indicate the SEM. Two-way ANOVA analysis showed that the incubation with each potassium channel blockers led to significant inhibition of the effects of the thiourea derivatives ($P < 0.001$).

4. Discussion

Hypertension is an epidemic and progressively growing disease, affecting about one billion patients across the globe and, despite of the availability of numerous classes of antihypertensive drugs, it is still the most common risk factor for death in the world. Therefore, the discovery of novel and effective antihypertensive strategies is still a compelling issue [48].

H_2S is emerging as an important endogenous gasotrasmitter that plays a pivotal role in regulating the vascular smooth muscle tone and blood pressure. Consistently, a lack of enzymatic production of endogenous H_2S is associated with hypertension. Thus, original H_2S-release moieties can be viewed as novel and innovative antihypertensive drugs.

However, Di Villa Bianca and colleagues reported that NaHS produced a dual action with vasoconstriction at lower doses, and vasodilatation at very high doses. Since the effect of NaHS were reduced by apamin/charybdotoxin and phospholipase A2 and cytochrome P450 inhibitors, the authors proposed that H_2S may promote a cytochrome P450 derivative of arachidonic acid. [49]. Furthermore, H_2S seems to

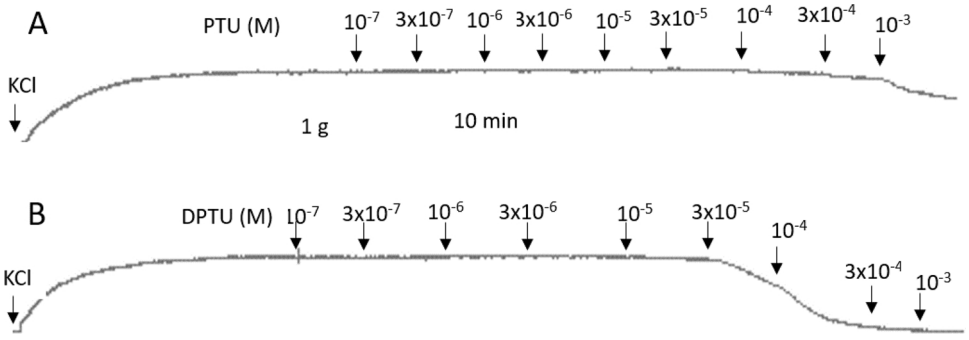

Fig. 5. Microdynamometric recordings. The figure shows two representative microdynamometric traces of the vasorelaxing effects promoted by PTU (panel A) and DPTU (panel B). The aortic rings were pre-contracted by KCl 25 mM, then increasing concentrations of the thiourea compounds were added cumulatively.

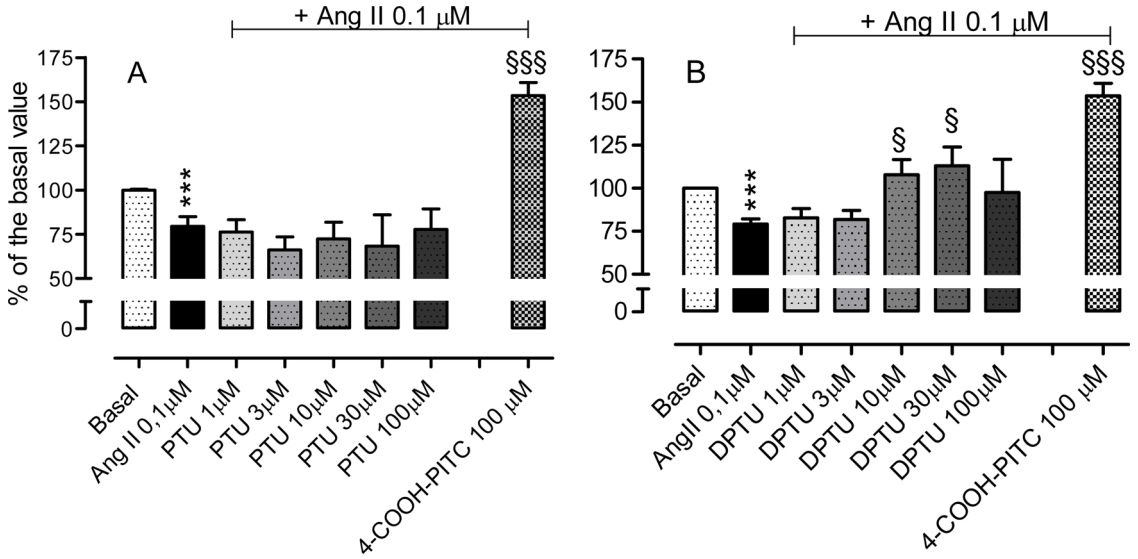

Fig. 6. Effects on coronary flows of PTU, DPTU and 4-COOH-PITC. The graphs show the effects on coronary flows induced by the perfusion of increasing concentrations of PTU (**A**) or DPTU (**B**) in the presence of AngII; 4-COOH-PITC (100 μM) was used as reference H_2S-donor compound. Changes of CF are expressed as a % of basal values. The vertical bars indicate the SEM (* indicates the basal value vs Ang II; *** $P < 0.001$; § indicates Ang II vs treatment; § $P < 0.05$; §§§ < 0.001).

have different effect considering the blood vessel types. Indeed, H_2S induces vasoconstriction of cerebral arteries. This effect seems to be due to the reduction of cAMP level in cerebral vascular smooth muscle cells. The study shows a novel and important profile of H_2S effect on cerebral artery, which provides a new view on understanding the physiological regulation of cerebrovascular tone [50].

In the present study, the H_2S-donor properties of two aromatic thiourea derivatives PTU and DPTU have been evaluated by amperometric measurements in aqueous buffer, in order to quantify and investigate the kinetics of H_2S-release. As reported also in previous studies, 4-COOH-PITC [26] and GYY4137 [40] (which were used as reference slow H_2S-donors) exhibited slow H_2S-releasing properties in the presence of L-cysteine; while, the H_2S release was dramatically reduced in the absence of L-cysteine [31]. In this "cell free" amperometric assay, both the thioureas showed significant H_2S-releasing effects, but PTU led to the formation of a higher amount of H_2S. However, both the thioureas showed a slow kinetic of H_2S-release with a stable steady state; such an important feature is currently considered as an indispensable requirement for a "smart" H_2S-donor, since it may avoid the side effects due to a fast H_2S generation typical of the sulfide and hydrosulfide salts [15]. Noteworthy, both the thiourea compounds showed a relevant difference from the reference compounds 4-COOH-PITC and GYY4137 since H_2S release was L-cysteine independent.

The H_2S-releasing properties of the two aromatic thioureas have been also evaluated in HASMCs cultured cells, through a specific dye (WSP-1) able to accumulate into the cells and selectively react with the gasotrasmitter. This spectrofluorometric assay revealed that the DPTU released higher amount of H_2S into HASMCs, probably due to its

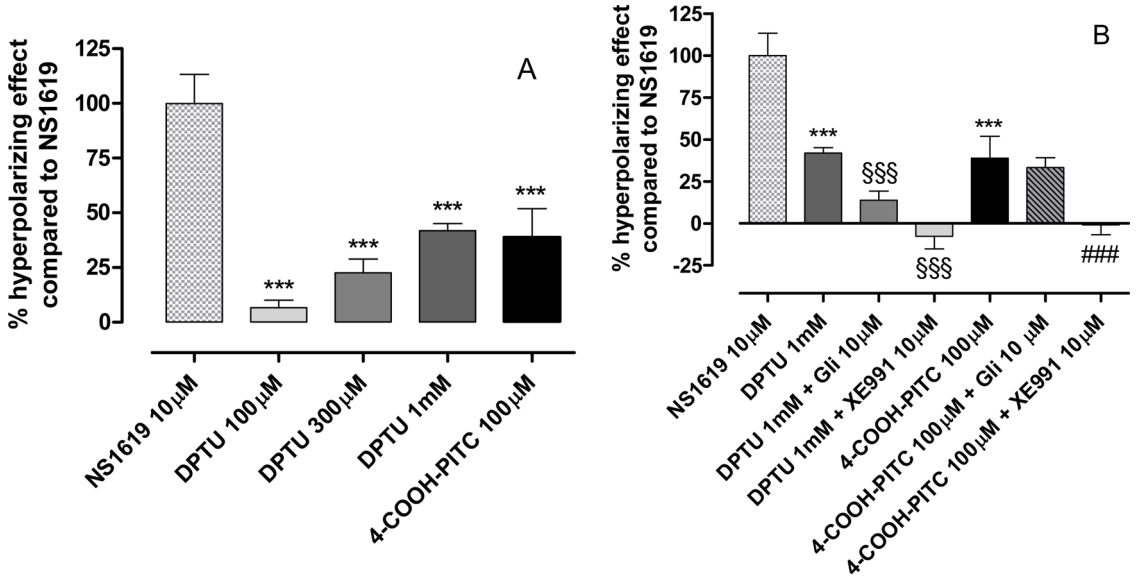

Fig. 7. Hyperpolarizing effect of DPTU and 4-COOH-PITC on HASMCs membrane. A) The graph shows the concentration-dependent hyperpolarizing effect of DPTU and the hyperpolarizing effect of 4-COOH-PITC (100 μM) on cell membrane of HASMCs; effects are expressed as a % of the hyperpolarization evoked by the reference compound NS1619. **B**) The incubation of XE991 completely abolishes the hyperpolarizing effect induced by both DPTU and 4-COOH-PITC, while the incubation with glibenclamide only significantly inhibits the hyperpolarizing effect induced by DPTU. Data are expressed as mean ± SEM. Six different experiments were performed, each in six replicates. * indicates significant differences vs NS1619 (*** = $P < 0.001$); § indicates significant differences vs DPTU 1 mM (§§§ = $P < 0.001$); # indicates significant differences vs 4-COOH-PITC (100 μM) (### = $P < 0.001$).

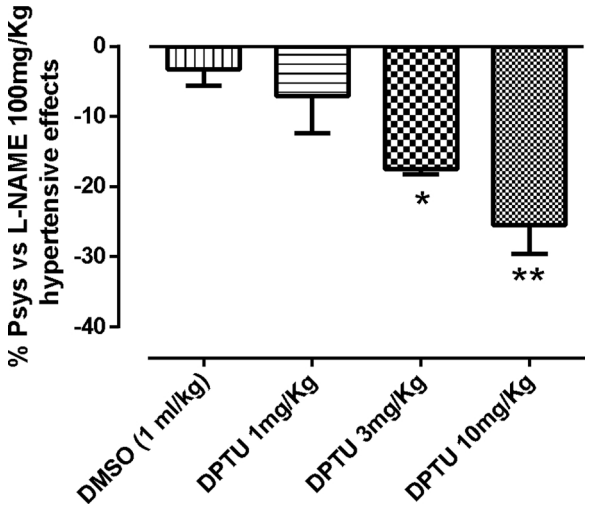

Fig. 8. Effects on systolic blood pressure. Changes in Psys (expressed as a % of the L-NAME-induced hypertensive Psys), following the i.p. administration of DPTU 1, 3 and 10 mg/Kg. The asterisk indicates significant difference vs vehicle (* $P < 0.05$; ** $P < 0.01$).

lipophilic nature that may allow an easier crossing of the cell membrane. Indeed, the theoretical LogP values for DPTU and PTU are 2.79 and 0.57, respectively (calculated with the software ALOGP 2.1) [48,51]. This observation further confirms that, aside the actual H_2S-releasing properties, the pharmacokinetic profile can strongly influence the efficacy and the potency of H_2S-donor compounds.

Endogenous H_2S directly acts on the tone of blood vessels and regulates the homeostasis of the cardiovascular function through the relaxing effects on vascular smooth muscle. Since the two thiourea derivatives exhibited clear H_2S releasing properties in vascular smooth muscle cells, their vascular effects were investigated also in functional tests. In particular, their vasorelaxing properties have been tested in endothelium-denuded rat aortic rings.

In agreement with the H_2S-release shown in HASMCs, DPTU showed a full vasorelaxing effect, superimposable to the reference H_2S-donor 4-COOH-PITC, while PTU exhibited only a partial vasorelaxing activity. Again, a different pharmacokinetic profile, and in particular a different ability to cross the cell membrane and to accumulate and release H_2S into the cells, may be the main factor accounting for the difference in the vasorelaxing efficacy. Furthermore, to provide definitive evidence that relaxation is due to the H_2S released by DPTU, also the vasorelaxing effect of DPU has been evaluated. As reported in Fig. 4D, DPU resulted to promote a milder vasorelaxing response than the sulfur DPTU, confirming that H_2S may be the crucial vasorelaxing mediator. However, a slight vasorelaxation can be noted also for DPU. This is not surprising because DPU derivatives were designed by Palle C. et al. as potassium channel activators. Nevertheless, the substitution of the oxygen with sulfur atom makes DPTU a more potent vasorelaxing molecule [52].

The vasorelaxing effects of the DPU and DPTU were also assessed in the coronary vascular bed. Again, only the DPTU caused an increase of the basal coronary flow (even if more restrained compared to 4-COOH-PITC), counteracting the coronary vasoconstriction induced by Ang II, while the incubation of PTU did not produce any significant effect.

Among the different molecular targets of H_2S within the vasculature, different sub-classes of potassium channels have been widely recognized and accounted for the H_2S-induced vasodilation [31]. In particular, activation of K_{ATP} potassium channels was early identified as a key mechanism, responsible for the H_2S-vasorelaxing effect [53]. Thereafter, a possible role of BK_{Ca} potassium channels has been reported [54]. More recently, Martelli et al. demonstrated that even vascular Kv7 potassium channels play a strong role in mediating the H_2S-induced hyperpolarization of vascular smooth muscle cells and the consequent vasorelaxant effect [6]. Therefore, the activation of K_{ATP} and Kv7 potassium channels by novel putative H_2S-donors can be viewed as a convincing evidence that this gasotransmitter is the ultimate player in their vasorelaxing response [19,24,25]. As concerns PTU and DPTU, our results clearly demonstrate that glibenclamide and XE991 (K_{ATP} and Kv7 blockers, respectively) significantly but not totally antagonized the vasorelaxing effect of these thioureas in the functional experiments on endothelium-denuded aortic rings. Interestingly, a clear difference between the 4-COOH-PITC and DPTU profiles emerged. Although 4-COOH-PITC and DPTU are two H_2S-donors, glibenclamide and XE991 completely inhibited the vasorelaxing effect induced by 4-CPI (Fig. 4C), while only a partial inhibition of the vasorelaxing response can be noted for DPTU (Fig. 4B). This difference is probably indicating some sort of mechanistic difference. In fact, as reported by Palle C. et al., diphenylurea derivatives were designed as potassium channel activators, including Kv, K_{ATP} and KB_{Ca} [52]. This "aspecific" mechanism of the aromatic urea structural-related compounds could explain the partial inhibition of glibenclamide and XE991 in the vasorelaxing response.

Consistently, DPTU induced a concentration-dependent hyperpolarization of the membrane potential of cultured HASMCs and, again, this effect was completely abolished by pre-incubating glibenclamide and XE991, confirming that the activation of K_{ATP} and Kv7 potassium channels play a pivotal electrophysiological role in the hyperpolarizing response of vascular cells. Finally, the *in vivo* antihypertensive effect of DPTU Finally, the *in vivo* antihypertensive effects of DPTU were evaluated in an experimental model of L-NAME-induced hypertension. In this experimental protocol, the slow H_2S-donor DPTU evoked marked hypotensive activity in a concentration dependent manner.

5. Conclusion

In conclusion, thiourea derivatives are known for their vasorelaxant effect [55], but this work demonstrated that the thiourea functional group is an H_2S-donor moiety, able to release the H_2S with a slow and long lasting kinetic and to exert the vasorelaxing effect typical of the gasotrasmitter. Given the versatility of this chemical group, such characteristics can be considered very useful in order to develop novel and promising cardiovascular drugs and new chemical tool for investigating the pharmacological roles of H_2S. Even if the *in vivo* antihypertensive effects of DPTU were clearly demonstrated in the experimental model of L-NAME-induced hypertension, the use of spontaneously hypertensive rats would further clarify the beneficial effects of this compound.

Declaration of Competing Interest

None.

Acknowledgements

This work has been funded by the Italian MIUR (Ministry of Instruction, University and Research) in the program "Project of Relevant National Interest" (PRIN, 2015, protocol 201532AHAE_005 – Principal Investigator: Prof. Giuseppe Cirino).

References

[1] L.L. Pan, X.H. Liu, Q.H. Gong, H.B. Yang, Y.Z. Zhu, Role of cystathionine gamma-lyase/hydrogen sulfide pathway in cardiovascular disease: a novel therapeutic strategy? Antioxid. Redox Signal. 17 (1) (2012) 106–118.
[2] R. Wang, The gasotransmitter role of hydrogen sulfide, Antioxid. Redox Signal. 5 (4) (2003) 493–501.
[3] W. Zhao, J. Zhang, Y. Lu, R. Wang, The vasorelaxant effect of H_2S as a novel endogenous gaseous KATP channel opener, EMBO J. 20 (21) (2001) 6008–6016.
[4] W. Zhang, C. Xu, G. Yang, L. Wu, R. Wang, Interaction of H_2S with calcium

permeable channels and transporters, Oxid. Med. Cell. Longev. 2015 (323269) (2015).
[5] O. Jackson-Weaver, J.M. Osmond, M.A. Riddle, J.S. Naik, L.V. Gonzalez Bosc, B.R. Walker, et al., Hydrogen sulfide dilates rat mesenteric arteries by activating endothelial large-conductance Ca^{2+}-activated K^+ channels and smooth muscle Ca2+sparks, Am. J. Physiol. Heart Circ. Physiol. 304 (11) (2013) H1446–54.
[6] A. Martelli, L. Testai, M.C. Breschi, K. Lawson, N.G. McKay, F. Miceli, et al., Vasorelaxation by hydrogen sulphide involves activation of Kv7 potassium channels, Pharmacol. Res. 70 (1) (2013) 27–34.
[7] A. Martelli, L. Testai, A. Marino, M.C. Breschi, F. Da Settimo, V. Calderone, Hydrogen sulphide: biopharmacological roles in the cardiovascular system and pharmaceutical perspectives, Curr. Med. Chem. 19 (20) (2012) 3325–3336.
[8] M. Bucci, A. Papapetropoulos, V. Vellecco, Z. Zhou, A. Zaid, P. Giannogonas, et al., cGMP-dependent protein kinase contributes to hydrogen sulfide-stimulated vasorelaxation, PLoS One 7 (12) (2012) e53319.
[9] M. Bucci, A. Papapetropoulos, V. Vellecco, Z. Zhou, A. Pyriochou, C. Roussos, et al., Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity, Arterioscler. Thromb. Vasc. Biol. 30 (10) (2010) 1998–2004.
[10] G. Meng, Y. Ma, L. Xie, A. Ferro, Y. Ji, Emerging role of hydrogen sulfide in hypertension and related cardiovascular diseases, Br. J. Pharmacol. 172 (23) (2015) 5501–5511.
[11] H. Yan, J. Du, C. Tang, The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats, Biochem. Biophys. Res. Commun. 313 (1) (2004) 22–27.
[12] G. Yang, L. Wu, B. Jiang, W. Yang, J. Qi, K. Cao, et al., H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase, Science 322 (5901) (2008) 587–590.
[13] I. Ishii, N. Akahoshi, H. Yamada, S. Nakano, T. Izumi, M. Suematsu, Cystathionine gamma-Lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury, J. Biol. Chem. 285 (34) (2010) 26358–26368.
[14] C. Szabo, Roles of hydrogen sulfide in the pathogenesis of diabetes mellitus and its complications, Antioxid. Redox Signal. 17 (1) (2012) 68–80.
[15] K. Suzuki, M. Sagara, C. Aoki, S. Tanaka, Y. Aso, Clinical implication of plasma hydrogen sulfide levels in Japanese patients with type 2 diabetes, Intern. Med. 56 (1) (2017) 17–21.
[16] K. Suzuki, G. Olah, K. Modis, C. Coletta, G. Kulp, D. Gero, et al., Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function, Proc. Natl. Acad. Sci. U. S. A. 108 (33) (2011) 13829–13834.
[17] A. Martelli, V. Citi, L. Testai, S. Brogi, V. Calderone, Organic isothiocyanates as hydrogen sulfide donors, Antioxid. Redox Signal. 32 (2) (2020) 110–144.
[18] P.M. Snijder, A.R. Frenay, R.A. de Boer, A. Pasch, J.L. Hillebrands, H.G. Leuvenink, et al., Exogenous administration of thiosulfate, a donor of hydrogen sulfide, attenuates angiotensin II-induced hypertensive heart disease in rats, Br. J. Pharmacol. 172 (6) (2015) 1494–1504.
[19] H. van Goor, J.C. van den Born, J.L. Hillebrands, J.A. Joles, Hydrogen sulfide in hypertension, Curr. Opin. Nephrol. Hypertens. 25 (2) (2016) 107–113.
[20] V. Calderone, A. Martelli, L. Testai, V. Citi, M.C. Breschi, Using hydrogen sulfide to design and develop drugs, Expert Opin. Drug Discov. 11 (2) (2016) 163–175.
[21] P. Rose, B.W. Dymock, P.K. Moore, GYY4137, a novel water-soluble, H_2S-releasing molecule, Methods Enzymol. 554 (2015) 143–167.
[22] D. Liang, H. Wu, M.W. Wong, D. Huang, Diallyl trisulfide is a fast H_2S donor, but diallyl disulfide is a slow one: the reaction pathways and intermediates of glutathione with polysulfides, Org. Lett. 17 (17) (2015) 4196–4199.
[23] V. Citi, A. Martelli, L. Testai, A. Marino, M.C. Breschi, V. Calderone, Hydrogen sulfide releasing capacity of natural isothiocyanates: is it a reliable explanation for the multiple biological effects of Brassicaceae? Planta Med. 80 (8-9) (2014) 610–613.
[24] E. Lucarini, L. Micheli, E. Trallori, V. Citi, A. Martelli, L. Testai, et al., Effect of glucoraphanin and sulforaphane against chemotherapy-induced neuropathic pain: Kv7 potassium channels modulation by H2 S release in vivo, Phytother. Res. 32 (11) (2018) 2226–2234.
[25] V. Citi, E. Piragine, E. Pagnotta, L. Ugolini, L. Di Cesare Mannelli, L. Testai, et al., Anticancer properties of erucin, an H2 S-releasing isothiocyanate, on human pancreatic adenocarcinoma cells (AsPC-1), Phytother. Res. 33 (3) (2019) 845–855.
[26] A. Martelli, L. Testai, V. Citi, A. Marino, F.G. Bellagambi, S. Ghimenti, et al., Pharmacological characterization of the vascular effects of aryl isothiocyanates: is hydrogen sulfide the real player? Vascul. Pharmacol. 60 (1) (2014) 32–41.
[27] A. Marino, A. Martelli, V. Citi, M. Fu, R. Wang, V. Calderone, et al., The novel H_2S donor 4-carboxy-phenyl isothiocyanate inhibits mast cell degranulation and renin release by decreasing intracellular calcium, Br. J. Pharmacol. 173 (22) (2016) 3222–3234.
[28] L. Testai, A. Marino, I. Piano, V. Brancaleone, K. Tomita, L. Di Cesare Mannelli, et al., The novel H_2S-donor 4-carboxyphenyl isothiocyanate promotes cardioprotective effects against ischemia/reperfusion injury through activation of mitoKATP channels and reduction of oxidative stress, Pharmacol. Res. 113 (Pt A) (2016) 290–299.
[29] S. Sestito, S. Daniele, D. Pietrobono, V. Citi, L. Bellusci, G. Chiellini, et al., Memantine prodrug as a new agent for Alzheimer's Disease, Sci. Rep. 9 (1) (2019) 4612.
[30] S. Rapposelli, L. Gambari, M. Digiacomo, V. Citi, G. Lisignoli, C. Manferdini, et al., A Novel H_2S-releasing Amino-Bisphosphonate which combines bone anti-catabolic and anabolic functions, Sci. Rep. 7 (1) (2017) 11940.
[31] A. Martelli, L. Testai, V. Citi, A. Marino, I. Pugliesi, E. Barresi, et al., Arylthioamides as H_2S donors: l-Cysteine-activated releasing properties and vascular effects in vitro and in vivo, ACS Med. Chem. Lett. 4 (10) (2013) 904–908.
[32] E. Barresi, G. Nesi, V. Citi, E. Piragine, I. Piano, S. Taliani, et al., Iminothioethers as hydrogen sulfide donors: from the gasotransmitter release to the vascular effects, J. Med. Chem. (2017), https://doi.org/10.1021/acs.jmedchem.7b00888.
[33] B. Severino, A. Corvino, F. Fiorino, P. Luciano, F. Frecentese, E. Magli, et al., 1,2,4-Thiadiazolidin-3,5-diones as novel hydrogen sulfide donors, Eur. J. Med. Chem. 143 (2018) 1677–1686.
[34] G. Ercolano, P. De Cicco, F. Frecentese, I. Saccone, A. Corvino, F. Giordano, et al., Anti-metastatic properties of Naproxen-HBTA in a murine model of cutaneous melanoma, Front. Pharmacol. 10 (66) (2019).
[35] S. Fortunato, C. Lenzi, C. Granchi, V. Citi, A. Martelli, V. Calderone, et al., First examples of H_2S-releasing glycoconjugates: stereoselective synthesis and anticancer activities, Bioconjug. Chem. 30 (3) (2019) 614–620.
[36] E. Mitidieri, T. Tramontano, D. Gurgone, V. Citi, V. Calderone, V. Brancaleone, et al., Mercaptopyruvate acts as endogenous vasodilator independently of 3-mercaptopyruvate sulfurtransferase activity, Nitric Oxide 75 (2018) 53–59.
[37] V. Citi, E. Piragine, L. Testai, M.C. Breschi, V. Calderone, A. Martelli, The role of hydrogen sulfide and H_2S-donors in myocardial protection against ischemia/reperfusion injury, Curr. Med. Chem. 25 (34) (2018) 4380–4401.
[38] B. Loev, P.E. Bender, H. Bowman, A. Helt, R. McLean, Jen T. Amidines, 3. Thioureas possessing antihypertensive activity, J. Med. Chem. 15 (10) (1972) 1024–1027.
[39] J.W. Tilley, P. Levitan, R.W. Kierstead, M. Cohen, Antihypertensive (2-aminoethyl) thiourea derivatives. 1, J. Med. Chem. 23 (12) (1980) 1387–1392.
[40] M. Whiteman, A. Perry, Z. Zhou, M. Bucci, A. Papapetropoulos, G. Cirino, et al., Phosphinodithioate and phosphoramidodithioate hydrogen sulfide donors, Handb. Exp. Pharmacol. 230 (2015) 337–363.
[41] B. Peng, W. Chen, C. Liu, E.W. Rosser, A. Pacheco, Y. Zhao, et al., Fluorescent probes based on nucleophilic substitution-cyclization for hydrogen sulfide detection and bioimaging, Chemistry 20 (4) (2014) 1010–1016.
[42] A. Martelli, E. Piragine, V. Citi, L. Testai, E. Pagnotta, L. Ugolini, et al., Erucin exhibits vasorelaxing effects and antihypertensive activity by H_2S-releasing properties, Br. J. Pharmacol. (2019), https://doi.org/10.1111/bph.14645.
[43] M. Magnon, V. Calderone, A. Floch, I. Cavero, Influence of depolarization on vasorelaxant potency and efficacy of Ca2+ entry blockers, K+ channel openers, nitrate derivatives, salbutamol and papaverine in rat aortic rings, Naunyn Schmiedebergs Arch. Pharmacol. 358 (4) (1998) 452–463.
[44] L. Testai, V. D'Antongiovanni, I. Piano, A. Martelli, V. Citi, E. Duranti, et al., Different patterns of H_2S/NO activity and cross-talk in the control of the coronary vascular bed under normotensive or hypertensive conditions, Nitric Oxide 47 (2015) 25–33.
[45] A. Martelli, G. Manfroni, P. Sabbatini, M.L. Barreca, L. Testai, M. Novelli, et al., 1,4-Benzothiazine ATP-sensitive potassium channel openers: modifications at the C-2 and C-6 positions, J. Med. Chem. 56 (11) (2013) 4718–4728.
[46] A. Yamada, N. Gaja, S. Ohya, K. Muraki, H. Narita, T. Ohwada, et al., Usefulness and limitation of DiBAC4(3), a voltage-sensitive fluorescent dye, for the measurement of membrane potentials regulated by recombinant large conductance Ca2+-activated K+ channels in HEK293 cells, Jpn. J. Pharmacol. 86 (3) (2001) 342–350.
[47] A. Martelli, V. Citi, V. Calderone, Vascular effects of H_2S-Donors: fluorimetric detection of H_2S generation and ion channel activation in human aortic smooth muscle cells, Methods Mol. Biol. 2007 (2019) 79–87.
[48] I.V. Tetko, G.I. Poda, Application of ALOGPS 2.1 to predict log D distribution coefficient for Pfizer proprietary compounds, J. Med. Chem. 47 (23) (2004) 5601–5604.
[49] R. d'Emmanuele di Villa Bianca, R. Sorrentino, C. Coletta, E. Mitidieri, A. Rossi, V. Vellecco, et al., Hydrogen sulfide-induced dual vascular effect involves arachidonic acid cascade in rat mesenteric arterial bed, J. Pharmacol. Exp. Ther. 337 (1) (2011) 59–64.
[50] W.R. Dunn, S.P. Alexander, V. Ralevic, R.E. Roberts, Effects of hydrogen sulphide in smooth muscle, Pharmacol. Ther. 158 (2016) 101–113.
[51] I.V. Tetko, V.Y. Tanchuk, Application of associative neural networks for prediction of lipophilicity in ALOGPS 2.1 program, J. Chem. Inf. Comput. Sci. 42 (5) (2002) 1136–1145.
[52] C.J. Palle, D. Diphenylurea Derivatives Useful As Potassium Channel Activators, WO2006064015A3 (2006), p. 45.
[53] Y. Cheng, J.F. Ndisang, G. Tang, K. Cao, R. Wang, Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats, Am. J. Physiol. Heart Circ. Physiol. 287 (5) (2004) H2316–23.
[54] X.Y. Tian, W.T. Wong, N. Sayed, J. Luo, S.Y. Tsang, Z.X. Bian, et al., NaHS relaxes rat cerebral artery in vitro via inhibition of l-type voltage-sensitive Ca2+ channel, Pharmacol. Res. 65 (2) (2012) 239–246.
[55] I.S. Zorkun, S. Sarac, S. Celebi, K. Erol, Synthesis of 4-aryl-3,4-dihydropyrimidin-2(1H)-thione derivatives as potential calcium channel blockers, Bioorg. Med. Chem. 14 (24) (2006) 8582–8589.