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Erucin exhibits vasorelaxing effects and antihypertensive activity by H₂S-releasing properties

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Background and Purpose: Hydrogen sulfide (H₂S)-releasing agents are viewed as potential antihypertensive drugs. Recently, natural isothiocyanates emerged as original H₂S-donor agents. Among them, erucin, present in some edible cruciferous plants, shows suitable H₂S-releasing properties and features of "druggability." The aim of this work was to investigate the erucin-mediated release of H₂S inside vascular cells, its vasorelaxing effects, and activity on BP of normo and hypertensive animals.

Experimental Approach: Intracellular H₂S-release and the hyperpolarizing effect of erucin were tested using fluorescent dye, in human aortic smooth muscle cells (HASMCs). Its direct vasorelaxing effect and ability to inhibit noradrenaline-induced vasoconstriction were evaluated on endothelium-intact or -denuded rat aortic rings. Its vasodilator properties were tested in coronary arteries using Langendorff-perfused rat hearts. Finally, erucin's antihypertensive activity was evaluated in vivo in normotensive and spontaneously hypertensive rats (SHRs) by recording systolic BP using the tail-cuff method.

Key Results: Erucin induced the release of H₂S inside HASMCs. Moreover, erucin hyperpolarized the membrane of HASMCs membrane in a concentration-dependent manner. It induced vasodilatation of rat aortic rings, in endothelium-denuded vessels. This effect was further improved by the presence of endothelial NO. When pre-incubated with rat aortic rings, erucin induced concentration-dependent inhibition of noradrenaline-induced vasoconstriction. Erucin did not affect basal coronary flow but restored the flow to normal in pre-contracted coronary vessels. Finally, in vivo, erucin decreased systolic BP in SHRs by about 25%, and restored the BP to values observed in normotensive rats.

Conclusions and Implications: Erucin is an H₂S donor endowed with vasorelaxing and antihypertensive effects.

Abbreviations: AngII, angiotensin II; BKCa, large-conductance calcium-activated potassium channels; CF, coronary flow; DADS, diallyldisulfide; DiBac4(3), bisoxonol dye bis(1,3-dibutylbarbituric acid); Emax, maximal vasorelaxing efficacy; HASMCs, human aortic smooth muscle cells; L-NAME, N^ω-nitro-L-arginine methyl ester; NA, noradrenaline; Psys, systolic blood pressure; SHR, spontaneously hypertensive rat; WSP-1, Washington State Probe-1

1 | INTRODUCTION

Hypertension is still the most widespread chronic disease, it is the main cause of life-threatening cardiovascular complications, such as stroke, coronary artery disease, and heart failure, and it is the principal risk factor for premature mortality worldwide. A further rise in prevalence of hypertension is expected, because of economic growth, life-style, and increased life expectancy (Kumar, 2013). Nowadays, this sneaky silent killer is estimated to be responsible for about 10 million deaths every year (Achelrod, Wenzel, & Frey, 2015; Noubiap et al., 2018). However, adequate control of correct levels of blood pressure is associated with an effective reduction in complications and mortality (Sundström et al., 2015; Xie et al., 2016). Consistently, many pharmacological targets useful for treating hypertension have been identified and a huge number of drugs of proven efficacy and safety is currently available. However, in spite of this wide and heterogeneous choice of pharmacological interventions, a significant number of hypertensive patients cannot achieve blood pressure control (Myat, Redwood, Qureshi, Spertus, & Williams, 2012; Pierdomenico et al., 2005; Sarafidis, Georgianos, & Bakris, 2013). In particular, the prevalence of true resistant hypertension (i.e., the resistance that occurs in fully adherent patients treated with well-designed therapies and multiple antihypertensive drugs) is estimated to be about 10% (Noubiap et al., 2018). Therefore, the discovery of original pharmacological mechanisms and novel antihypertensive drug candidates is still a timely and compelling issue.

Since the discovery of **hydrogen sulfide (H₂S)** as an endogenous gasotransmitter, the potential utility of this small gaseous molecule in cardiovascular pharmacology has emerged (Bucci & Cirino, 2011; Martelli et al., 2012). Indeed, H₂S acts as a vasorelaxing mediator and is a key regulator of vascular tone homeostasis (Gheibi, Jeddi, Kashfi, & Ghasemi, 2018). In vascular tissues, physiological levels of this gasotransmitter are mainly biosynthesized by **cystathionine γ-lyase (CSE)**, although the contribution of the other two H₂S-generating enzymes **cystathionine β-synthase (CBS)** and **3-mercaptopyruvate sulfurtransferase (MPST)** cannot be excluded (Yang & Wang, 2015). An altered biosynthesis of H₂S is presently known to account for “H₂S-rich” or “H₂S-poor” diseases, associated with an excess or deficiency of H₂S respectively (Szabo & Papapetropoulos, 2017). In particular, H₂S deficiency is likely to have a relevant significant role in hypertension (Ahmad et al., 2015; Meng, Ma, Xie, Ferro, & Ji, 2015). Indeed, an association between a deficiency in H₂S and hypertension has been clearly demonstrated in rodents (Yan, Du, & Tang, 2004; Yang et al., 2008) and even in humans. In fact, reduced H₂S concentrations have been detected in plasma of grade 2 and 3 hypertensive patients (Sun, Xi, Yang, Ma, & Tang, 2007), in patients with portal hypertension (Wang et al., 2014) and in maternal hypertension and preeclampsia (Wang et al., 2013). Conversely, the administration of exogenous H₂S by suitable H₂S donors can be considered as a useful innovative strategy for the pharmacological treatment of hypertension. Actually, different chemotypes of H₂S donors exhibited vasorelaxing activity and blood pressure lowering effects in several scientific studies (Szabo & Papapetropoulos, 2017). The importance of most of these compounds

What is already known

- Hydrogen sulfide (H₂S) donors are potential antihypertensive drugs.
- Natural isothiocyanates are H₂S donors.

What this study adds

- The natural isothiocyanate erucin releases H₂S inside vascular cells.
- Erucin evokes vasorelaxing and antihypertensive effects mediated by H₂S.

What is the clinical significance

- Erucin may represent an attractive drug candidate for the treatment of hypertension.

has been to elucidate the molecular mechanism of action of H₂S and understand the pathophysiological roles of H₂S in the regulation of cardiovascular function. Nevertheless, for clinical applications an ideal H₂S donor should combine well-balanced H₂S-releasing properties (in particular, slow and long-lasting release) with other essential pharmacological “druggability” features, such as good oral bioavailability, safety, known biotransformation, and safe metabolites.

In a recent paper, we first reported the H₂S-releasing properties of different natural isothiocyanates, which are phytochemical compounds typical of cruciferous plants (family of *Brassicaceae*; Citi et al., 2014). These properties were tested by “cell-free” electrochemical approaches, but no pharmacological evaluation of their cardiovascular effects was carried out. Among the various isothiocyanates, erucin seems to be worthy of pharmacological investigation, because of its favourable characteristics. Indeed, erucin (Figure 1) showed slow and long-lasting release of H₂S. Moreover, erucin is present in edible plants, such as *Eruca sativa* Mill. (salad rocket), which are widely used in human nutrition and are not associated with any evidence of toxicity. Different pharmacokinetic studies on erucin have been carried out in animals and humans. These studies demonstrated that erucin is well absorbed after oral administration; they also described the metabolites of erucin derived from its biotransformation (Clarke et al., 2011; Platz et al., 2015).

The aim of this study was to evaluate the potential H₂S-releasing activity of erucin in vascular smooth muscle, investigate its typical H₂S-mediated effects in vascular smooth muscle cells and in isolated vessels, and finally, assess the in vivo effects of erucin in a suitable experimental model of hypertension.

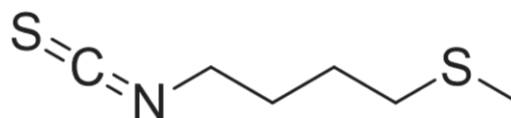


FIGURE 1 Molecular structure of erucin

2 | METHODS

2.1 | Cell cultures

Human aortic smooth muscle cells (HASMCs, Cat.no: C-007-5C) were purchased from Thermo Fisher (Waltham, MA, USA) and were cultured following the manufacturer's instructions. Briefly, HASMCs were cultured in Medium 231 (Thermo Fisher) supplemented with Smooth Muscle Growth Supplement (Thermo Fisher), 1% of 100 units·ml⁻¹ penicillin and 100 mg·ml⁻¹ streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂. Cells were split 1:2 twice a week and used until passage 16.

2.2 | Intracellular H₂S-release measurement

HASMCs were cultured up to about 90% confluence and 24 hr before the experiment cells were seeded onto a 96-well clear-bottom black plate, pre-coated with gelatin 1% (from porcine skin, Sigma-Aldrich), at a density of 30 × 10³ per well. After 24 hr, to allow cell attachment, 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'-xanthen]-6'-yl-2(pyridin-2-yl)disulfanyl benzoate); Cayman Chemical, Ann Arbor, MI, USA). After the 30 min incubation of HASMCs with WSP-1 (allowing cells to up-load the dye), the supernatant was removed and replaced with different solutions of erucin (CREA, Bologna, Italy) dissolved in standard buffer (HEPES, 20 mM; NaCl, 120 mM; KCl, 2 mM; CaCl₂·2H₂O, 2 mM; MgCl₂·6H₂O, 1 mM; glucose, 5 mM; and pH 7.4, at room temperature) at three increasing concentrations (30, 100, and 300 μM). WSP-1 is a specific and highly sensitive dye for H₂S detection; indeed, when it reacts with H₂S, it releases a fluorophore detectable with a spectrofluorometer at λ = 465–515 nm. The change in fluorescence (expressed as fluorescence index) was monitored every 5 min for 35 min, by means of an EnSpire (PerkinElmer, Waltham, MA, USA) spectrofluorometer and it was calculated as the difference between the fluorescence at time t, after the addition of erucin or vehicle (DMSO 1%, Sigma-Aldrich), and the basal fluorescence. The AUC was also calculated. On the bases of previous experiments (Barresi et al., 2017), diallyldisulfide (DADS, Sigma-Aldrich) 300 μM was used as slow H₂S-donor reference compound. Six different experiments (n = 6) were performed, each carried out in six replicates. The results are expressed as mean ± SEM.

2.3 | Evaluation of HASMC membrane hyperpolarization

HASMCs were seeded onto a 96-well black plate, clear-bottom pre-coated with gelatin 1% (Sigma-Aldrich), at density of 72 × 10³ cells per well. After 24 hr, the medium was replaced, and cells were incubated for 1 hr with the bisoxonol dye bis(1,3-dibutylbarbituric acid) (DiBac4(3), 2.5 μM, Sigma-Aldrich) dissolved in a standard buffer. This membrane potential-sensitive dye DiBac4(3) allowed us a non-

electrophysiological measurement of changes in cell membrane potential as already described (Martelli et al., 2014). An increase in fluorescence, due to an inward flow of the dye, is a consequence of membrane depolarization; conversely, a decrease in fluorescence, consequent to an outward flow of the dye, reflects membrane hyperpolarization. The spectrofluorometric measurement was set at excitation and emission wavelengths of 488 and 520 nm respectively (Multiwells reader, Enspire, PerkinElmer). NS1619 (10 μM, Sigma-Aldrich), a well-known activator of BK_{Ca} potassium channels, was used as a reference hyperpolarizing drug. After the basal fluorescence had been measured, cells were treated with different concentrations of erucin (30, 100, and 300 μM), NS1619, or vehicle (DMSO 1%), and changes in fluorescence were recorded for 35 min. The relative reduction in fluorescence, consequent to membrane hyperpolarization, was recorded every 2.5 min and was expressed as (F_t - F₀)/F₀, where F₀ represents the basal fluorescence and F_t is the fluorescence at time t after the administration of the test compounds. Moreover, the AUC was also calculated. The results were expressed as a % of the AUC induced by NS1619 10 μM. Six different experiments (n = 6) were performed, each carried out in six replicates.

2.4 | 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). The experiments were authorized by the Ethical Committee of the University of Pisa (Protocol number 0037321/2013) and by the Italian Ministry of Health (authorization number 751/2018-PR). All the animals were housed in humidity- and temperature-controlled rooms (22°C and 50%, respectively) with 12-hr light/dark cycles, water, and food availability ad libitum. Three animals were housed in each cage. All efforts to reduce and minimize the number of animals and their suffering were carried out. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. A randomization of animals between groups and treatments was carried out. The investigators responsible for data analysis were blind to which animals represent which treatments and controls.

2.5 | Functional assays on aortic rings

Adult male normotensive Wistar rats (300–350 g, Envigo, Huntingdon, UK, RGD Cat# 2312511, RRID:RGD_2312511) were given an overdose of sodium thiopental (100 mg·kg⁻¹ i.p., Pentothal MSD, Kenilworth, NJ, USA), killed by cervical dislocation and bled. Thoracic aorta was rapidly excised and freed of extraneous tissues. When required for the experimental procedures, the endothelial layer was removed by gently rubbing the intimal surface of the aortae with a hypodermic needle. The aorta was cut in 5-mm-wide rings which were

suspended, under a preload of 2 g, in organ baths, containing 20 ml of Tyrode solution (NaCl, 136.8 mM; KCl, 2.95 mM; CaCl₂·2H₂O, 1.80 mM; MgSO₄·7H₂O, 1.05 mM; NaH₂PO₄·H₂O, 0.41 mM; NaHCO₃, 11.9 mM; and glucose, 5.5 mM), thermostated at 37°C, and continuously gassed with Cloxicarb, a mixture of O₂ (95%) and CO₂ (5%). An isometric transducer (Grass FTO3, Harvard Apparatus, Holliston, MA, USA), connected to a preamplifier (Buxco Electronics, Troy, NY, USA) and with software for data acquisition (BIOPAC Systems Inc., MP 100, Goleta, CA, USA), was used to record changes in tension. After an equilibration period of 60 min, the endothelial maintenance or removal were confirmed by the administration of ACh (10 μM) to KCl (25 mM)-precontracted vascular rings. As regards the endothelium-intact aortic rings, a relaxation ≥70% of the KCl-induced contraction was considered representative of a suitable presence of functional endothelium; while, the rings, exhibiting a relaxation <70%, were discarded. Conversely, for the endothelium-removed aortic rings, a relaxation <10% of the KCl-induced contraction represented an acceptable lack of the endothelial layer; while, the rings, showing a relaxation ≥10%, were discarded.

In the first set of experiments, designed to evaluate the direct vasorelaxing activity of erucin, after about 45 min from the confirmation of the endothelium presence/removal, the aortic rings were contracted with KCl 25 mM and when the contraction had reached a stable plateau, erucin was added cumulatively (100 nM–100 μM). When required, the inhibitor of the biosynthesis of endothelial **nitric oxide (NO)**, N^ω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) 100 μM, was added 20 min before KCl. Preliminary experiments demonstrated that the KCl (25 mM)-induced contractions remained in a stable tonic state for at least 60 min. The maximal vasorelaxing efficacy (E_{max}) was presented as the maximal vasorelaxing response achieved with the highest concentration of erucin (100 μM) and expressed as a percentage (%) of the contractile tone induced by KCl. Potency is expressed as pEC₅₀, calculated as negative logarithm of the molar concentration of erucin evoking an effect = 50% of E_{max}. The parameters for efficacy and potency are expressed as mean ± SEM, from aortae of six animals (n = 6) for each different treatment.

In a separate set of experiments, the inhibition of noradrenaline (NA)-induced vasoconstriction by erucin was evaluated. In particular, 45 min after the confirmation of endothelium removal, the endothelium-denuded aortic rings were contracted with 60mM KCl, as a reference depolarizing stimulus evoking an almost full contraction of vascular smooth muscle. After a plateau state had been reached, the preparations were washed, and after 45 min of re-equilibration, rings were exposed to different concentrations of erucin (10, 30, and 100 μM) or the corresponding vehicle (DMSO 1%) for 20 min. Thereafter, a cumulative concentration–response curve for NA (1 nM–1 μM) was performed. In order to rule out a potential toxic effect and/or to test the viability of the vascular musculature and the “reversibility” of the inhibitory effect of erucin, rings were extensively washed. Then, the concentration–response curve to NA (1 nM–1 μM) was repeated. NA concentration–vasocontraction response curves were analysed by the Hill equation, and the efficacy of NA expressed as maximal contractile response, calculated as a percentage (%) of the

contractile tone previously induced by 60 mM KCl. The parameter of potency pE₅₀, was calculated as the negative logarithm of the molar concentration of NA evoking a half-maximal response. The results for the efficacy and potency of NA are expressed as mean ± SEM, of six experiments (n = 6) for each different treatment.

2.6 | 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 and placed in Krebs solution (NaHCO₃, 25 mM; NaCl, 118.1 mM; KCl, 4.8 mM; MgSO₄, 1.2 mM; CaCl₂·2H₂O, 1.6 mM; KH₂PO₄, 1.2 mM; and glucose, 11.5 mM) at 4°C bubbled with cloxicarb to stop the heart contracting and reduce oxygen consumption. Then, the hearts were rapidly mounted in a Langendorff apparatus (Radnoti, Monrovia, USA) and perfused with Krebs solution gassed with cloxicarb at 37°C at constant pressure (70–80 mmHg). 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 erucin on the basal flow: briefly, the hearts were perfused with Krebs solution containing erucin at cumulatively increasing concentrations (0.1, 1, 10, and 100 μM, for 20 min, each concentration). Another set of experiments was dedicated to demonstrate the effects of erucin on CF of hearts precontracted with angiotensin II (AngII). These hearts, after the initial 20 min of equilibration, 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 erucin (0.1, 1, 10, and 100 μM, for 20 min, each concentration) were administered (in the constant presence of AngII 0.1 μM). Preliminary experiments demonstrated that perfusion with AngII 0.1 μM caused a rapid fall in the CF, stable for at least 2 hr. 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.

2.7 | In vivo measurement of blood pressure on normotensive and spontaneously hypertensive rats

The effects of erucin and its vehicle (DMSO 1 ml·kg⁻¹) on BP were tested on 12-week-old male normotensive (Wistar) and spontaneously hypertensive rats (SHR; 300–350 g), which represents one of the best models for essential hypertension. Briefly, rats 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, erucin 10 mg·kg⁻¹ or the corresponding vehicle were administered i.p. to different groups (normotensive vs. SHR), each composed of six rats. Starting from the administration of the test compounds, the Psys

values were recorded, for 2 hr after 10, 30, 60, 90, and 120 min. Basal Psys was expressed as a mean of the four measurements carried out in each rat before the administration of test compounds. Change in Psys, recorded after erucin or vehicle administration, is expressed as a percentage of the basal Psys and also calculated as mean value of the five recordings (at 10, 30, 60, 90, and 120 min) carried out after drug administration. BP measurements were carried out in six animals per group ($n = 6$ for each treatment both in normotensive and SHR groups).

2.8 | Data and statistical analysis

All the experimental data were analysed by a computer fitting procedure (GraphPad Prism 6.0, La Jolla, CA, USA, RRID:SCR_002798) and expressed as mean \pm SEM of at least six independent experiments. All the group sizes were designed to be homogeneous. ANOVA and Student's *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. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology.

2.9 | Materials

Cell culture reagents Medium 231 (Cat.no.: M-231-500) and Smooth Muscle Growth Supplement (Cat.no.: S-007-25) were from Thermo Fisher while penicillin/streptomycin solution (Cat.no.: P4333), gelatin (Cat.no.: G1890, CAS Number: 9000-70-8), DADS (Cat.no.: SMB00378, CAS Number: 2179-57-9), DMSO (Cat.no.: D 2650, CAS Number: 67-68-5), DiBac4(3) (Cat.no.: D8189, CAS Number: 70363-83-6), NS1619 (Cat.no.: N170, CAS Number: 153587-01-0), ACh chloride (Cat.no.: A9101, CAS Number: 60-31-1), KCl (Cat.no.: P9333, CAS Number: 7447-40-7), L-NAME (Cat.no.: N5751, CAS Number 51298-62-5), NA hydrochloride (Cat.no.: A7256, CAS Number 55-27-6), AngII (Cat.no.: A9525, CAS Number 4474-91-3), and all the salts and glucose (Cat.no.: G8270, CAS Number 50-99-7) used for preparation of Buffer standard, Tyrode, and Krebs solutions were from Sigma-Aldrich. WSP-1 (Cat.no.: 11179, CAS Number: 1352750-34-5) was from Cayman Chem. Purified erucin (CAS Number: 4430-36-8) was kindly provided by the Italian Council for Agricultural Research and Economics, Research Centre for Cereal and Industrial Crops (Bologna, Italy).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro et al., 2017; Alexander, Striessnig et al., 2017).

3 | RESULTS

3.1 | Erucin exhibits intracellular H₂S-release in HASMCs

Erucin was able to release H₂S inside cells. Indeed, the addition of several concentrations of erucin to HASMCs caused a significant, gradual, and concentration-dependent increase in WSP-1 fluorescence (indicating the generation of intracellular H₂S), until the reaching of a plateau, (Figure 2a). The AUC of the H₂S-release was also calculated and highlighted a clear concentration-dependent release. The amount of H₂S released from the highest concentration of erucin (300 μ M) reached about 70% of the value obtained after the administration of the reference H₂S-donor DADS (300 μ M; Figure 2b).

3.2 | Hyperpolarizing effect of erucin on HASMC membrane

The hyperpolarizing effect of erucin on HASMCs pre-incubated with DiBac4(3) was recorded for 35 min until a plateau was obtained, and compared with the effect of a well-known hyperpolarizing drug, that is, the BK_{Ca} activator, NS1619 10 μ M (Figure 3a). As shown in Figure 3a and in the correlated AUC graph (Figure 3b), the hyperpolarizing effect of erucin on HASMC membrane was significant versus vehicle, concentration-dependent and, at the highest concentration (300 μ M), it was also comparable (106 \pm 2% of the NS1619 hyperpolarizing effect) with that of the reference drug, confirming that the novel H₂S-donor erucin is a hyperpolarizing drug.

3.3 | Direct vasorelaxing effect on rat aortic rings

Erucin evoked endothelium-independent vasorelaxing effects, since it caused vasodilatation of endothelium-denuded rat aortic rings precontracted with KCl ($E_{max} = 35 \pm 6\%$; $pEC_{50} = 4.95 \pm 0.07$). However, this vasorelaxing effect was significantly increased when erucin was tested on endothelium-intact rat aortic rings precontracted with KCl ($E_{max} = 56 \pm 4\%$; $pEC_{50} = 5.63 \pm 0.08$). In order to confirm a possible influence of the endothelial function on the vasorelaxing effect of erucin, its vascular effects were also evaluated in endothelium-intact rat aortic rings pre-incubated with L-NAME 100 μ M, an inhibitor of **endothelial NOS (eNOS)** biosynthesis. The experiments performed in the presence of L-NAME showed a reduction in the maximal vasorelaxing effect ($E_{max} = 26 \pm 7\%$; $pEC_{50} = 5.27 \pm 0.11$) leading to a curve being obtained that was almost superimposable on the curve produced by erucin in endothelium-denuded rat aortic rings (Figure 4).

3.4 | Inhibition of NA-induced vasoconstriction

Besides its direct vasodilating effect, erucin also inhibited the NA-evoked vasoconstriction. In the vehicle pretreated endothelium-denuded aortic rings, NA induced a sustained vasoconstriction, with

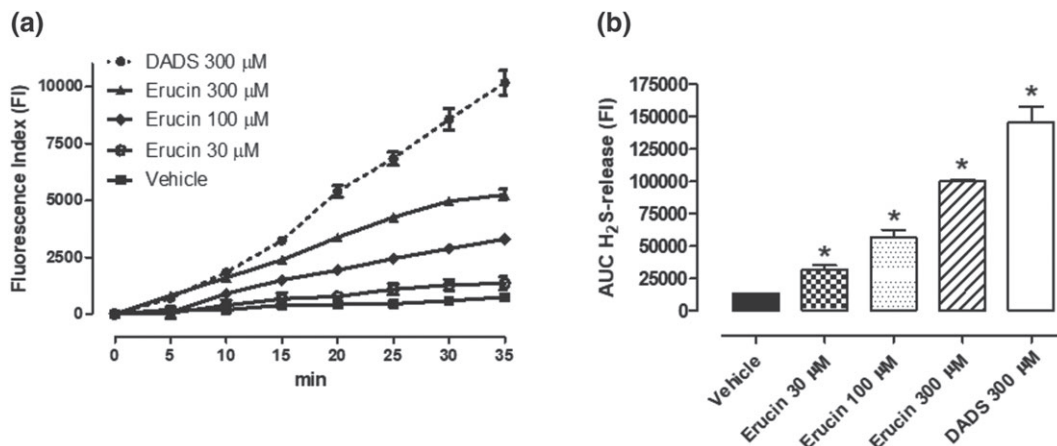


FIGURE 2 Fluorometric recording of erucin-mediated H₂S-release inside HASMCs. (a) Time course of the fluorometric recordings of H₂S released by vehicle, erucin 30, 100, and 300 μM and DADS 300 μM, during 35 min of observation: the increase in H₂S is expressed as FI. (b) The histograms show the total amount of H₂S released by vehicle, erucin 30, 100, and 300 μM and DADS 300 μM, in the 35 min of observation time, expressed as AUC. The vertical bars represent SEM, six different experiments were performed, each with six replicates ($n = 6$). The asterisks indicate a statistically significant difference from vehicle (* $P < 0.05$)

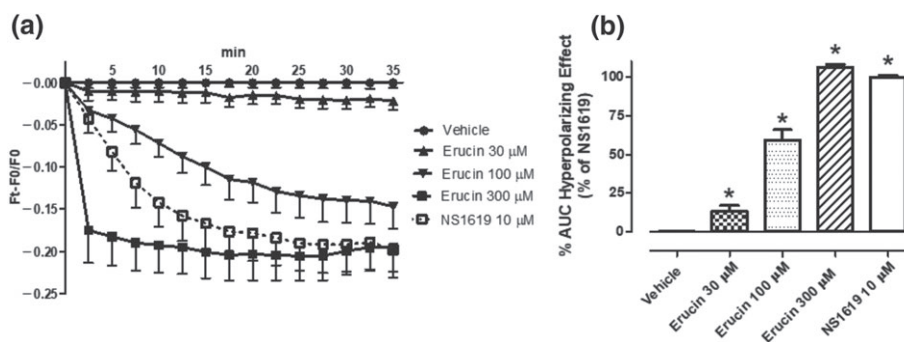


FIGURE 3 Hyperpolarizing effects in HASMCs. (a) Time-course spectrofluorometric recording of changes in HASMCs membrane potential, followed for 35 min, expressed as $(F_t - F_0)/F_0$ and induced by vehicle, erucin 30, 100, and 300 μM and the hyperpolarizing reference drug, NS1619 10 μM. (b) Corresponding AUC of the hyperpolarizing effect of vehicle, erucin 30, 100, and 300 μM and NS1619 10 μM. Data are expressed as mean \pm SEM. Six different experiments were performed, each with six replicates ($n = 6$). The asterisks indicate a significant difference from the effect evoked by vehicle (* $P < 0.05$)

an E_{max} of 100 ± 2 and a potency value (pEC_{50}) of 7.44 ± 0.04 . Pre-treatment with increasing concentrations of erucin (10, 30, and 100 μM) caused a progressive inhibition of both the potency and efficacy of NA. Indeed, pre-incubation of the aortic rings with erucin (10, 30, and 100 μM) led to concentration-dependent and significant inhibitory effects. In particular, the increasing concentrations of erucin produced a similar reduction in the efficacy of NA by about 25–30%, but different effects on NA potency (pEC_{50}) of 7.20 ± 0.10 , 6.94 ± 0.06 , and 6.69 ± 0.04 , for erucin 10, 30, and 100 μM respectively (Figure 5a). Noteworthy, the inhibitory effect of erucin at the different concentrations was fully reversible; indeed, after an organ washout, a full recovery of the contractile effects of NA was observed (Figure 5b).

3.5 | Evaluation of the functional effects on the CF

The basal CF in Langendorff-perfused rat hearts was $13.08 \pm 1.92 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$. The administration of 0.1, 1, 10, and 100

μM erucin did not affect the basal values of CF (Figure 6a). A different trend was observed when AngII (0.1 μM) was administered to the rat hearts. Indeed, in the presence of AngII, the CF was significantly reduced to $81.8 \pm 3.3\%$ of the basal value, and the administration of 0.1, 1, 10, and 100 μM erucin, in the continuous presence of AngII, led to a progressive increase in CF, which was finally restored to the basal CF value, when 100 μM erucin was added ($101.9 \pm 4.1\%$ of the CF basal value; Figure 6b).

3.6 | Effect of erucin on systolic BP in SHR and normotensive rats

On the basis of previous results obtained in vascular smooth muscle, in vivo BP measurements have been carried out in SHR and normotensive rats. Normotensive Wistar rats showed basal Psys of $133 \pm 2 \text{ mmHg}$, and i.p. administration of vehicle did not alter the basal Psys value. The administration of erucin $10 \text{ mg} \cdot \text{kg}^{-1}$ i.p. caused

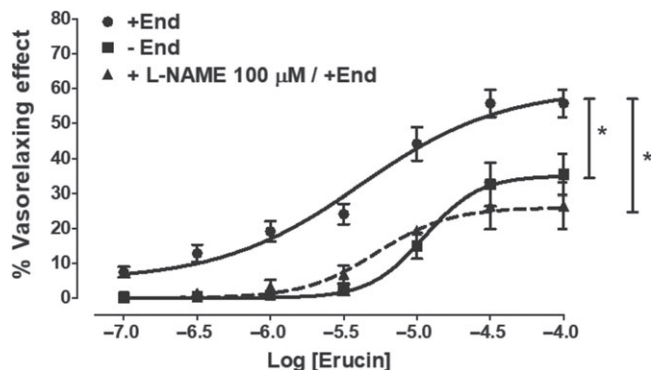


FIGURE 4 Direct vasorelaxing effects. Concentration–response curves, relative to the vasorelaxing effects evoked by erucin, in endothelium-intact and -denuded rat aortic rings, precontracted with KCl 25 mM. In endothelium-intact aortic rings, the vasorelaxing effects have been recorded either in the absence or presence of L-NAME 100 μM (inhibitor of the NO-biosynthesis). The vertical bars indicate the SEM. Six different experiments were performed, each with six replicates ($n = 6$). The asterisks indicate a significant difference from the curve obtained on endothelium-intact aortic rings ($*P < 0.05$)

only a slight decrease in P_{sys} , which was significantly different from the vehicle at three recording times (10, 30, and 120 min; Figure 7a). However, the overall mean change in P_{sys} was not significant ($-9.98 \pm 6.2\%$; Figure 7c). This behaviour was completely different when erucin and vehicle were administered into SHR. Indeed, SHR exhibited basal P_{sys} of 177 ± 3 mmHg and, as already observed for normotensive rats, the i.p. administration of vehicle did not alter the basal P_{sys} . In contrast, the administration of erucin $10 \text{ mg}\cdot\text{kg}^{-1}$ i.p. to SHR caused a clear and significant decrease in P_{sys} both in the curve of time recordings and in the bars reporting the overall mean change in P_{sys} ($-23.9 \pm 3.8\%$; Figure 7b,c).

4 | DISCUSSION

After it was discovered that H_2S has multiple pathophysiological roles and the potential therapeutic application of H_2S -donor drugs in “ H_2S -poor” pathologies was realized, much effort has been devoted to the characterization of new H_2S -releasing chemical moieties. In this broad panorama of heterogeneous chemical structures, we identified the isothiocyanate group as an interesting moiety. Indeed, in previous studies, we found that some synthetic arylisothiocyanates behaved as H_2S donors and caused clear H_2S -mediated vasorelaxing effects. Noteworthy, the release of H_2S from these aryl-isothiocyanates was not a spontaneous process but was triggered by the presence of organic thiols (e.g., **L-cysteine** or **GSH**), as often documented for other classes of H_2S donors, such as allyl polysulfides (Benavides et al., 2007), thioamides (Martelli, Testai, Citi, et al., 2013), iminotioethers (Barresi et al., 2017), or GYY4137 (one of the best characterized H_2S donors; Martelli, Testai, Citi, et al., 2013). This thiol-dependent profile was considered as a

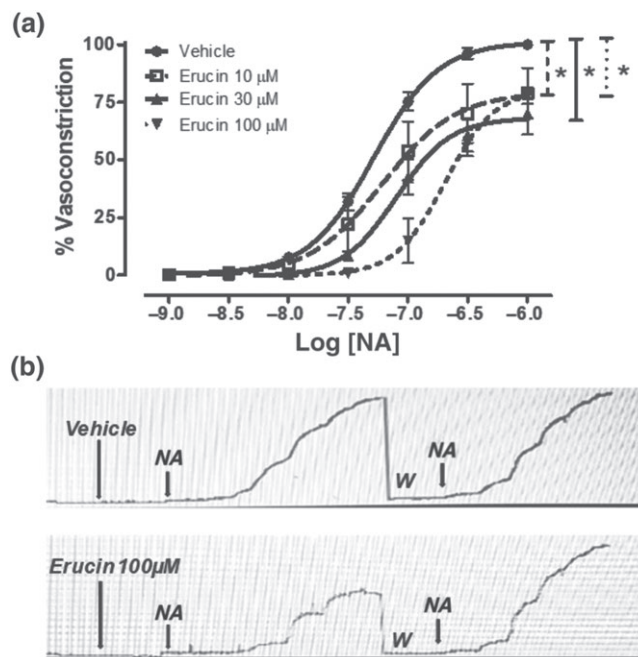


FIGURE 5 Inhibition of NA-induced vasoconstriction. (a) Concentration–response curves to NA, obtained in endothelium-denuded rat aortic rings pre-incubated with vehicle or erucin at the concentrations of 10, 30, and 100 μM . The effects are expressed as a % of the contractile responses previously induced by the administration of KCl 60 mM. The vertical bars indicate the SEM. Six different experiments were performed, each with six replicates ($n = 6$). The asterisks indicate a significant difference from the NA-induced vasoconstriction curve obtained on endothelium-denuded aortic rings pre-incubated with the vehicle ($*P < 0.05$). (b) Representative microdynamometric tracings obtained in two different aortic rings, demonstrating that the inhibitory effects of erucin were fully reversible. In the upper panel, one aortic ring was pre-incubated with vehicle (20 min); thereafter, cumulative increasing concentrations of NA, evoking concentration-dependent vasoconstriction, were added. The aortic ring was submitted to washout (W) and adequate equilibration time to recover again the resting tension. Then, the aortic ring was treated again with vehicle (20 min); thereafter, cumulative increasing concentrations of NA, evoking concentration-dependent vasoconstriction, were added again. In the lower panel, the other aortic ring was pre-incubated with erucin 100 μM (the highest concentration, for 20 min); thereafter, cumulative increasing concentrations of NA, evoking reduced concentration-dependent vasoconstriction, were added. The ring was submitted to washout (W) and an adequate equilibration time to recover again the resting tension. Finally, the aortic ring was treated with vehicle (20 min); thereafter, cumulative increasing concentrations of NA, evoking concentration-dependent vasoconstriction, were added again. In the lower panel, complete recovery of the vasoconstriction effects of NA is evident

useful feature of a “smart” H_2S donor (Calderone, Martelli, Testai, Citi, & Breschi, 2016), as it would ensure that the release of H_2S does not occur spontaneously and in an undifferentiated way but is particularly facilitated in just the cell cytosol, where the highest concentrations of organic thiols are present: GSH (in 1–10 mM range) and cysteine (in 30–200 μM range; Tian et al., 2014)

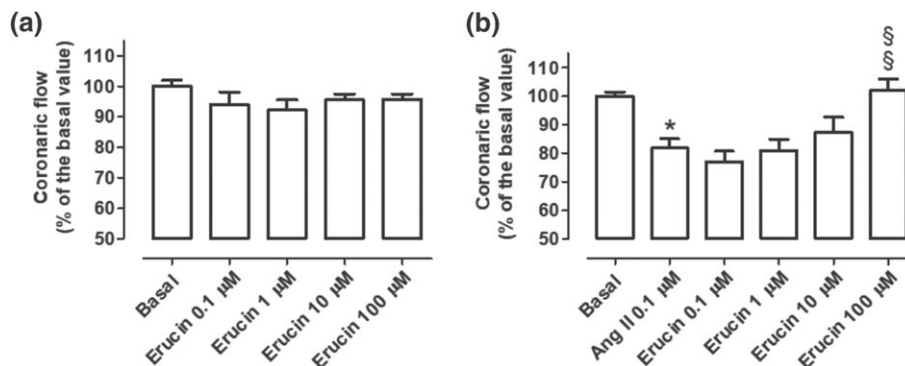


FIGURE 6 Effects on coronary flow. Changes in CF (expressed as a % of the basal CF) induced by the perfusion of increasing concentrations of erucin, in the absence (a) or in the constant presence (b) of AngII, as a co-administration. The vertical bars indicate the SEM. Six different experiments were performed, ($n = 6$). The asterisks indicate a significant difference from the basal CF ($*P < 0.05$) while the symbols [§] indicate a significant difference from the CF recorded in the presence of AngII 0.1 μM ($^{\S}P < 0.05$)

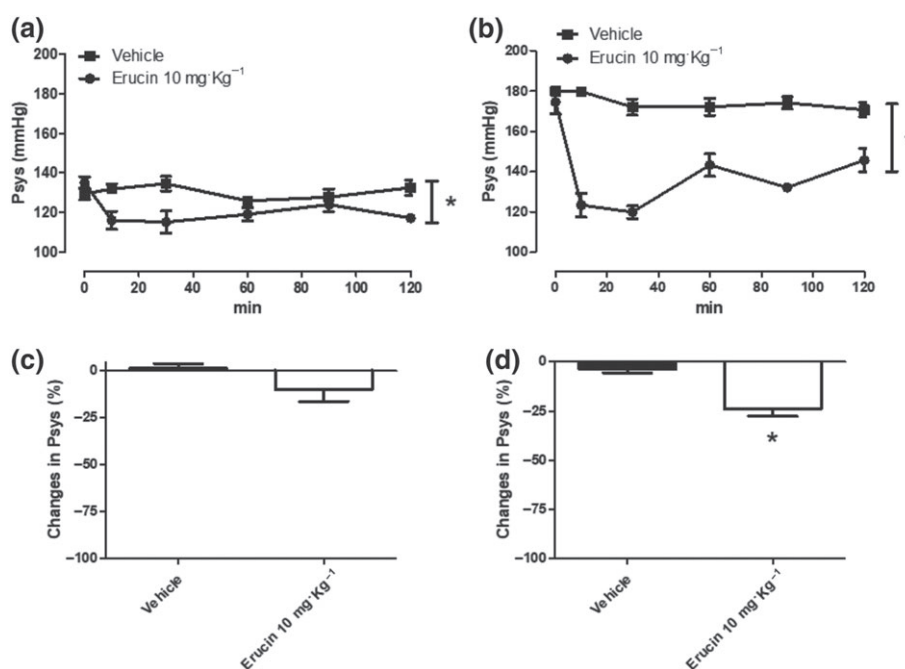


FIGURE 7 Effects on blood pressure. Time-course of changes in systolic blood pressure values, expressed in mmHg in normotensive (a) and SHR rats (b) after i.p. administration of vehicle (DMSO 1 ml·kg⁻¹) or erucin 10 mg·kg⁻¹, recorded for 2 hr, at 10, 30, 60, 90, and 120 min. The vertical bars indicate the SEM. Six different experiments were performed for each treatment ($n = 6$). The asterisks indicate a significant difference from Psys trend curve obtained after the administration of vehicle ($*P < 0.05$). Overall mean change in Psys (expressed as a % of the basal Psys values) in normotensive (c) and SHRs (d), recorded in the 2 hr after i.p. administration of vehicle (DMSO 1 ml·kg⁻¹) or erucin 10 mg·kg⁻¹. The vertical bars indicate the SEM. Six different experiments were performed for each treatment ($n = 6$). The asterisks indicate a significant difference from changes in Psys (%) obtained after the administration of vehicle ($*P < 0.05$)

In this context, we have previously demonstrated that erucin, a natural isothiocyanate, typically present in many edible cruciferous plants, behaved as a smart H₂S donor, since it exhibited slow, long-lasting, and thiol-dependent release of H₂S; however, this result was obtained in a “cell-free” experimental model in vitro (Citi et al., 2014). Therefore, the first objective of this work was to test the ability of erucin to enter vascular smooth muscle cells and promote the generation of H₂S using the intracellular pool of organic thiols. DADS was used as a reference H₂S donor for this experiment, since it shares

some physicochemical analogies with erucin (relatively low molecular weight, no charge, lipophilicity). In contrast, the use of other well-known H₂S donors, such as the inorganic sulfides, did not seem to be appropriate, because of the large differences in terms of physicochemical properties and of H₂S-releasing rates. As detected by the spectrofluorimetric assay with the WSP-1 probe, the incubation of HASMCs with erucin promoted a slow and progressive increase in H₂S levels, in a concentration-dependent way. In particular, incubation with the highest concentration of erucin (300 μM) led to an

intracellular production of H₂S which was about 75% of that produced by an equimolar concentration of DADS. However, it should be noted that the functional group isothiocyanate can release a single H₂S molecule; instead, DADS generates two H₂S molecules, as elegantly demonstrated by Benavides et al. (2007).

Next, we evaluated whether erucin evoked typical H₂S-mediated vascular responses. As reported above, H₂S is a pivotal regulator of vascular tone, through its vasorelaxing effect. Indeed, by means of "S-sulphydration," that is, the post-translational modification of cysteine residues of target proteins (Paul & Snyder, 2012), H₂S promotes multiple mechanisms of action responsible for its vasodilator activity, against the vasoconstrictor effects evoked by different stimuli, which were either receptor-mediated or non-receptor-mediated. Among them, the modulation of several types of vascular potassium channel, with consequent membrane hyperpolarization in vascular smooth muscle cells, plays a relevant role. For instance, H₂S-mediated vasorelaxation was very early attributed to the activation of vascular **ATP-sensitive potassium channels** (K_{ir}6.2) (Zhao, Zhang, & Lu, 2001). In addition, the activation of **K_v7 voltage gated potassium channels** has been more recently proposed as a further important and peculiar mechanism accounting for the vasorelaxing effects of H₂S (Martelli, Testai, Breschi, et al., 2013; Schleifenbaum et al., 2010). Therefore, we decided to evaluate the effects produced by erucin on the membrane potential of HASMCs cells. In this kind of experiment, NS1619 was selected as a reference drug, because it is a powerful activator of vascular **large-conductance calcium-activated potassium channels** (K_{Ca}1.1), which can promote a massive hyperpolarization of the cell membrane (Calderone, 2002). Through the potential-sensitive probe DiBac4(3), it was shown that erucin causes a concentration-dependent membrane hyperpolarization in HASMCs, thus exhibiting typical H₂S-mediated vascular effects. Noteworthy, the hyperpolarizing effects produced by the highest concentration of erucin (300 μM) were equivalent to those produced by the reference drug. The identification of the specific potassium channel type(s) involved in this hyperpolarizing effect will be the object of future electrophysiological studies.

Concerning the H₂S-mediated functional effects, erucin exhibited endothelium-independent vasorelaxing activity, since it evoked concentration-dependent vasorelaxation of endothelium-denuded aortic rings. The vasorelaxing effects were further increased when erucin was administered to endothelium-intact rat aortic rings. However, when erucin was added to endothelium-intact rat aortic rings pretreated with L-NAME (inhibitor of eNOS), the vasorelaxing effects were almost equivalent to those recorded in the endothelium-denuded vessels. Thus, the H₂S-mediated vasorelaxing effect of erucin is endothelium-independent but is significantly improved by the presence of endothelium and in particular by the presence of endothelial NO. Indeed, data on the possible interactions between H₂S and NO are numerous and sometimes conflicting (Cirino, Vellecco, & Bucci, 2017; Nagpure & Bian, 2016). However, it is currently accepted that the overall interplay between the two gasotransmitters (at physiological concentrations) is positive and acts through multiple mechanisms, such as the formation of active hybrid species, the H₂S-induced mobilization of NO from stable "pools," the increased activity of eNOS

through calcium mobilization and PI3K/Akt signalling, and the synergistic effects on cGMP (NO activates the guanylate cyclase, while H₂S inhibits **PDE5** and activates **cGMP-dependent protein kinases**; Szabo, 2016). Alongside its direct vasodilator effects, erucin significantly inhibited the vasoconstriction induced by the adrenoceptor agonist NA on endothelium-denuded rat aortic rings. Noteworthy, these effects were reversible, confirming the involvement of pharmacological mechanisms of action, rather than possible tissue damage induced by toxicity.

The role of endogenous H₂S in the physiological regulation of CF is still an underdeveloped field of research. Recent experimental findings indicate that endogenous H₂S may have a modest role in regulating canine coronary microvascular resistance (Casalini et al., 2014), while the presence of H₂S-producing enzymes (in particular, mercaptopyruvate sulfotransferase) was observed in rodent coronary arteries (Kuo et al., 2016). However, irrespective of the potential role of endogenous H₂S, in the above experimental studies, the administration of exogenous H₂S donors always caused a reduction in vascular resistance and a significant increase in CF. This feature had been observed previously for synthetic aryl-isothiocyanate H₂S donors (Martelli et al., 2014; Testai et al., 2015). These effects suggest a possible utility for H₂S donors in coronary artery diseases. In the present study, the possible vasorelaxing effects of erucin were also confirmed in the coronary arteries. The perfusion with erucin did not change the flow of coronary arteries with a basal vascular tone, since the vascular smooth muscle of these vessels is likely to be already relaxed. In contrast, when the coronary vessels were precontracted with AngII, erucin promoted a significant increase in the flow in concentration-dependent fashion, bringing it back to baseline levels.

Although there is a paucity of data on H₂S and human hypertension, many experimental studies indicate that an increase in H₂S levels induced by suitable H₂S donors may be a rational and effective therapeutic approach for hypertensive patients (Van Goor, van den Born, Hillebrands, & Joles, 2016). Indeed, different chemotypes of H₂S donors exhibit antihypertensive effects in different experimental models of hypertension. For instance, early on it was observed that administration of the well-known H₂S-donor GYY4137 caused a marked decrease in Psys in SHR, while it caused lower (albeit significant) hypotensive effects in normotensive animals (Li et al., 2008). Sodium hydrosulfide (rapid H₂S donor) has also been shown to exhibit antihypertensive effects in SHR (Ahmad et al., 2012). Novel H₂S-donor iminothioethers have been recently reported to cause a significant reduction in Psys in a rat experimental model of hypertension, induced by pharmacological inhibition of eNOS (Barresi et al., 2017). Therefore, in order to translate the above experimental observations towards a reliable experimental model of pathology and therefore to identify a possible clinical application of erucin, in the last step of this study we evaluated the effects of erucin on the arterial pressure of normotensive and hypertensive rats. In normotensive animals, inhibitors of H₂S biosynthesis are known to increase BP (Yan et al., 2004), suggesting that the vascular regulatory mechanisms are fully activated by endogenous H₂S. Consistently, in our study, erucin had only a negligible influence on the BP of normotensive rats. In contrast, the

expression/activity of H₂S-producing enzymes is significantly reduced in SHR and thus the H₂S-mediated regulatory mechanisms are defective (Bucci et al., 2014; Yan et al., 2004). Noteworthy, in SHR, the administration of erucin (i.e., the H₂S supplementation with this H₂S donor) caused a marked reduction in Psys, bringing it back to the normotensive levels.

5 | CONCLUSIONS

The isothiocyanate erucin is naturally present in some edible cruciferous plants, commonly used in human nutrition and in functional diets, and this would exclude serious toxicity concerns. Several studies have already examined the main pharmacokinetic characteristics of erucin, highlighting its appreciable oral bioavailability and providing a detailed description of its metabolites. In these studies, no significant toxicity of erucin or erucin-derived metabolites has been reported. In the present study, we demonstrated for the first time that erucin can be considered as an original H₂S donor, with interesting vascular effects and, in particular, with significant BP lowering effects in hypertensive animals and negligible effects in normotensive ones. Therefore, an adequate pharmaceutical development of erucin or of improved erucin analogues may actually pave the way for a new class of H₂S-donor antihypertensive drugs, with reliable “druggability” and thus with the real possibility of clinical use.

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AUTHOR CONTRIBUTIONS

A.M. and E.Pi. performed the research. A.M. and V.Ca. designed the research study. E.Pa., L.U., and L.L. provided purified Erucin. L.T., V.Ci., and O.L.M. contributed to preliminary studies. A.M. and V.Ca. wrote the manuscript. A.M., V.Ca., L.D.M., C.G., M.C.B., and M.B. analysed the data and revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This [Declaration](#) acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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