

Hydrogen Sulfide Releasing Capacity of Natural Isothiocyanates: Is It a Reliable Explanation for the Multiple Biological Effects of Brassicaceae?

Authors

Valentina Citi¹, Alma Martelli¹, Lara Testai^{1,2}, Alice Marino¹, Maria C. Breschi¹, Vincenzo Calderone^{1,2}

Affiliations

¹ Dipartimento di Farmacia, Università di Pisa, Pisa, Italy

² Centro Interdipartimentale di Ricerca Nutraceutica e Alimentazione per la Salute "Nutrafood", Università di Pisa, Pisa, Italy

Key words

- hydrogen sulfide
- H₂S-donors
- Brassicaceae
- natural isothiocyanates
- glucosinolates

Abstract

Hydrogen sulfide is an endogenous pleiotropic gasotransmitter, which mediates important physiological effects in the human body. Accordingly, an impaired production of endogenous hydrogen sulfide contributes to the pathogenesis of important disorders. To date, exogenous compounds, acting as hydrogen sulfide-releasing agents, are viewed as promising pharmacotherapeutic agents. In a recent report, the hydrogen sulfide-releasing properties of some synthetic aryl isothiocyanate derivatives have been reported, indicating that the isothiocyanate function can be viewed as a suitable slow hydrogen sulfide-releasing moiety, endowed with the pharmacological potential typical of this gasotransmitter. Many isothiocyanate derivatives (deriving from a myrosinase-mediated transformation of glucosinolates) are well-known secondary metabolites of

plants belonging to the family Brassicaceae, a large botanical family comprising many edible species. The phytotherapeutic and nutraceutic usefulness of Brassicaceae in the prevention of important human diseases, such as cancer, neurodegenerative processes and cardiovascular diseases has been widely discussed in the scientific literature. Although these effects have been largely attributed to isothiocyanates, the exact mechanism of action is still unknown. In this experimental work, we aimed to investigate the possible hydrogen sulfide-releasing capacity of some important natural isothiocyanates, studying it *in vitro* by amperometric detection. Some of the tested natural isothiocyanates exhibited significant hydrogen sulfide release, leading us to hypothesize that hydrogen sulfide may be, at least in part, a relevant player accounting for several biological effects of Brassicaceae.

Introduction

Hydrogen sulfide (H₂S) is presently recognized as a pleiotropic endogenous gasotransmitter. At physiological concentrations, it is pivotally involved in the homeostatic regulation of the cardiovascular, respiratory, gastroenteric and nervous systems. Moreover, H₂S plays a significant modulatory role in inflammatory processes. Consistently, H₂S-releasing compounds are currently viewed as promising drugs for several diseases [1, 2]. Ideal H₂S-donors should generate H₂S at a relatively slow rate [1–3]. This pharmacological feature is exhibited by early examples of original synthetic H₂S-releasing agents, such as the phosphinodithioate derivative GYY4137 (morpholin-4-ium-4-methoxyphenyl-morpholino-phosphinodithioate), as well as aminothiols [4] and aryl thioamide [5] derivatives. Even some examples of natural H₂S-releasing derivatives are recog-

nized. For instance, organic polysulfides of Alliaceae, such as diallyl disulfide (DADS), are H₂S-donors, and such a property is thought to account for the most important biological and pharmacological effects of garlic [6].

In a recent paper of ours, a novel H₂S-releasing chemical moiety has been described: in that work, some synthetic aryl isothiocyanates have been demonstrated to act as H₂S-donor agents and to exhibit significant effects on vascular smooth muscle such as vasodilation related with the activation of vascular voltage-gated potassium channels [7], clearly attributable to the release of H₂S [8].

Notably, many isothiocyanates are naturally occurring compounds. They derive from a myrosinase-mediated transformation of glucosinolates, which are well-known secondary metabolites of plants mainly belonging to Brassicaceae, a large botanical family comprising many edible species,

received April 24, 2014
revised May 19, 2014
accepted May 20, 2014

Bibliography

DOI <http://dx.doi.org/10.1055/s-0034-1368591>
Planta Med 2014; 80: 610–613
© Georg Thieme Verlag KG
Stuttgart · New York ·
ISSN 0032-0943

Correspondence

Vincenzo Calderone
Università di Pisa
Dipartimento di Farmacia
via Bonanno, 6
I-56126 Pisa
Italy
Phone: + 39 502 21 95 89
Fax: + 39 502 21 96 09
calderone@farm.unipi.it

such as *Brassica oleracea* L. (var. *italica*, *gemmifera*, *botrytis*, *capitata*, and *acephala*), *Brassica nigra* (L.) Koch, *Sinapis alba* L. etc. Presently, glucosinolates, along with the corresponding isothiocyanates, are receiving greatest scientific interest for their numerous biological and pharmacological effects and useful applications in many aspects of human health [9]. It is worthy to note that there is an intriguing overlapping between many biological effects attributed to some Brassicaceae and those exhibited by the gasotransmitter H₂S.

Nevertheless, the possibly existing H₂S-releasing potential of this class of natural compounds has never been described.

In this paper, some important isothiocyanates, naturally occurring in many species of Brassicaceae, have been selected: allyl isothiocyanate (AITC, highly present in black mustard, *B. nigra*, 4-hydroxybenzyl isothiocyanate (HBITC, highly present in white mustard, *S. alba*), benzyl isothiocyanate (BITC, highly present in garden cress, *Lepidium sativum* L.) and erucin (ERU, present in different species such as broccoli, *B. oleracea* L., and rocket, *Eruca vesicaria* L.) (● Fig. 1). Moreover, the glucosinolate sinigrin (SIN, precursor of AITC) has been also selected for comparison purposes. The possible H₂S-releasing activity of the selected compounds has been investigated by a reliable amperometric approach which was already used for analogous studies [5,8].

Results and Discussion

The H₂S-releasing capacity is a pharmacologically relevant feature, already attributed to naturally occurring compounds, such as the diallyl polysulfides of garlic, but never reported for natural isothiocyanates. Indeed, in previous works of ours, the incubation of the reference drug DADS led to a negligible formation of H₂S in the absence of L-cysteine; but, in the presence of this amino acid, the incubation of DADS was followed by a relatively slow formation of H₂S, with C_{max} of about 20 μM (● Table 1). As concerns the isothiocyanates tested in the present work, ERU and BITC exhibited the weakest (albeit significant) H₂S-releasing properties. In particular, ERU and BITC behaved as very poor H₂S-donors both in the absence and in the presence of L-cysteine (● Table 1; ● Fig. 2 and 3).

AITC led to the formation of appreciable amounts of H₂S, but only in the presence of L-cysteine. Indeed, in the absence of L-cysteine, the incubation of AITC led to the formation of low concentrations of H₂S; in contrast, the presence of L-cysteine caused a significant improvement of the H₂S-releasing properties of AITC (● Table 1; ● Fig. 2 and 3). Such a L-cysteine-dependent release of H₂S, probably due to the nucleophilic behaviour of the amino acid, has been already observed for well-known H₂S-donors, such as GYY4137, DADS, and many synthetic thioamides and isothiocyanates [5,8]. In contrast, the allyl glucosinolate SIN was devoid of significant H₂S-releasing effects in both the experimental conditions (absence or presence of L-cysteine) (● Table 1; ● Fig. 2 and 3). This finding indicates that the isothiocyanate moiety is an obligatory requirement.

Among the few selected secondary metabolites of Brassicaceae, BITC was the most effective H₂S-donor, exhibiting significant and remarkable H₂S-release. In particular, in the absence of the L-cysteine, the incubation of HBITC was followed by a slow generation of appreciable levels of H₂S (about 10 μM). The co-presence of the amino acid led to a marked increase in the H₂S-release from HBITC (● Table 1; ● Fig. 4). The H₂S-releasing properties of BITC, recorded in the presence of L-cysteine, were equiva-

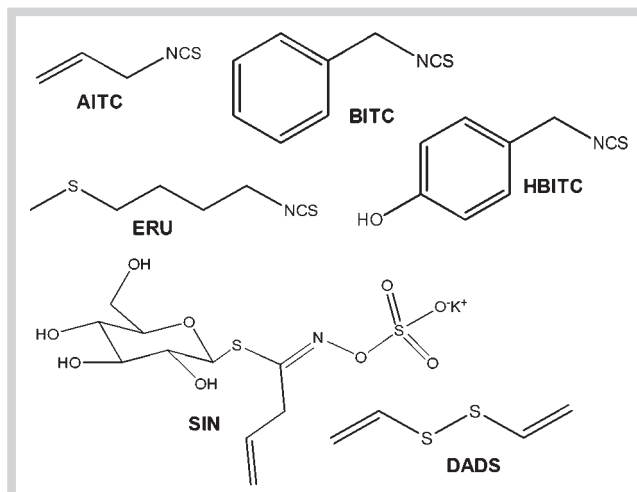


Fig. 1 Chemical structures of the tested compounds.

Table 1 C_{max} parameters of the hydrogen sulfide-donor compounds. The table summarizes C_{max} parameters relative to the H₂S-releasing effects exhibited by the tested compounds after their incubation in AB at physiological pH and temperature, in the absence (AB) or in the presence (AB+L-cys) of L-cysteine (4 mM); the results for DADS come from reference [5]. Data are expressed as means ± SEM.

Compound	AB C _{max} (μM)	AB + L-Cys C _{max} (μM)
DADS	< 0.5	18.9 ± 4.8
AITC	0.5 ± 0.2	6.2 ± 1.3
BITC	< 0.5	1.9 ± 0.4
ERU	0.5 ± 0.2	1.9 ± 0.1
HBITC	11.0 ± 0.8	17.9 ± 1.5
SIN	< 0.5	< 0.5

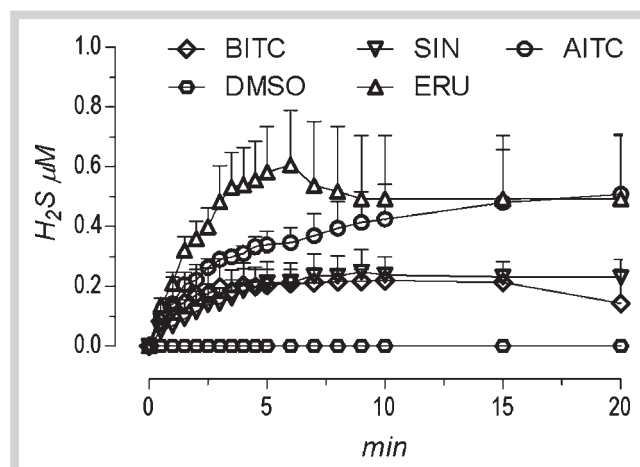


Fig. 2 Hydrogen sulfide-release from allyl isothiocyanate, erucin, benzyl isothiocyanate, and sinigrin in the absence of L-cysteine. The curves describe the increase of the H₂S concentration with respect to time, following the incubation of AITC, ERU, BITC, and SIN, in the absence of L-cysteine. H₂S was recorded by amperometry; the vertical bars indicate SEM.

lent to those exhibited by DADS in previous studies [5]; but, unlike DADS (which showed weaker H₂S-release in the absence of

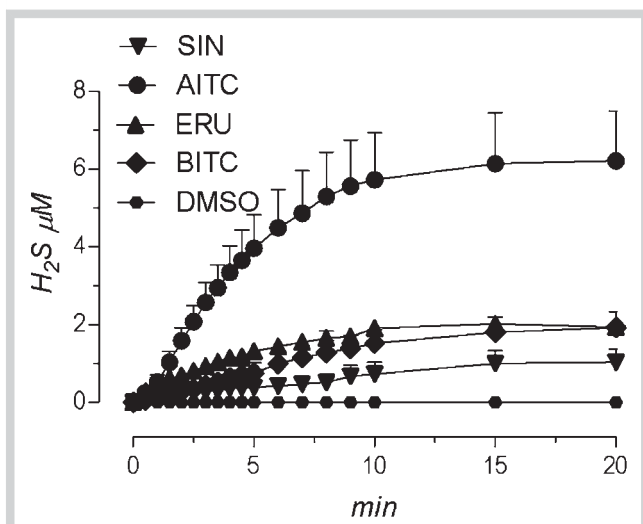


Fig. 3 Hydrogen sulfide-release from allyl isothiocyanate, erucin, benzyl isothiocyanate, and sinigrin in the presence of L-cysteine. The curves describe the increase of the H_2S concentration with respect to time, following the incubation of AITC, ERU, BITC, and SIN, in the presence of L-cysteine. H_2S was recorded by amperometry; the vertical bars indicate SEM.

L-cysteine), BITC behaved as a remarkable H_2S -donor even in the absence of this amino acid.

The nutraceutical and phytotherapeutic usefulness of Brassicaceae in the prevention of important human diseases, such as cancer, neurodegenerative processes, and cardiovascular diseases, has been widely discussed in the scientific literature [9]. Although these effects are reasonably attributed to isothiocyanates (mainly formed from the corresponding glucosinolates by the enzymatic activity of the plant enzyme myrosinase, but also by intestinal bacterial microflora in mammals [10]), the exact mechanism of action is still unknown.

This is the first experimental report demonstrating that some natural isothiocyanates behave as slow H_2S -releasing agent. Only in the last decade, H_2S has been recognized as an important pleiotropic gasotransmitter and has been defined as “the new nitric oxide” [11], since it is endowed with pivotal regulatory roles in almost all physiological systems in the human body and particularly in the cardiovascular one. Therefore, the H_2S -releasing properties of some natural isothiocyanates, as described in this paper, give insights for understanding the biological and pharmacological properties and the mechanism of action of many species of Brassicaceae. Moreover, on the basis of the growing scientific knowledge on the pathophysiological roles of H_2S , this finding may help to assess and identify the pharmaceutical and nutraceutical potential of naturally occurring glucosinolates/isothiocyanates.

Materials and Methods

Amperometric determination of H_2S and statistical analysis

The characterization of the potential H_2S -generating properties of the tested compounds has been carried out by an amperometric approach, through the Apollo-4000 free radical analyzer (WPI) detector and H_2S -selective mini-electrodes. The experiments have been carried out at room temperature. Following

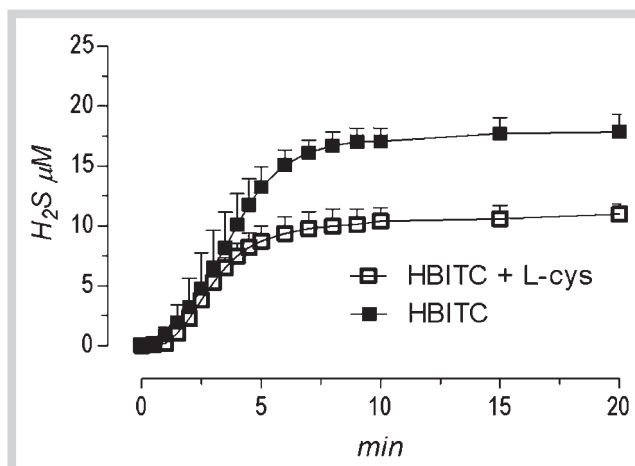


Fig. 4 Hydrogen sulfide-release from 4-hydroxybenzyl isothiocyanate. The curves describe the increase of the H_2S concentration with respect to time, following the incubation of HBITC, either in the absence (white squares) or in the presence of L-cysteine (4 mM) (black squares). H_2S was recorded by amperometry; the vertical bars indicate SEM.

the manufacturer's instructions, a “PBS buffer 10x” was prepared ($NaH_2PO_4 \cdot H_2O$ 1.28 g, $Na_2HPO_4 \cdot 12H_2O$ 5.97 g, NaCl 43.88 g in 500 mL H_2O) and stocked at 4 °C. Immediately before the experiments, the “PBS buffer 10x” was diluted using distilled water (1 : 10) to obtain the assay buffer (AB) and the pH adjusted to 7.4. The H_2S -selective mini-electrode was equilibrated in 10 mL of the AB, until the recovery of a stable baseline. Then, 100 μ L of a DMSO solution of all the tested H_2S -releasing compounds was added (the final concentration of the tested H_2S -donors was 1 mM; the final concentration of DMSO in the AB was 1%). The eventual generation of H_2S was observed for 20 min. Preliminary experiments demonstrated that DMSO 1% did not produce any interference on the amperometric recording. When required by the experimental protocol, L-cysteine (4 mM) was added before the addition of the H_2S -donors. The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was determined by suitable calibration curves, which were previously obtained by the use of increasing concentrations of NaHS (1 μ M, 3 μ M, 5 μ M, 10 μ M) at pH 4.0. The curves relative to the progressive increase of H_2S vs. time, following the incubation of the tested compounds, were analysed by the equation

$$C_t = C_{max} - (C_{max} \cdot e^{-k \cdot t})$$

where C_t is the instant concentration at time t , C_{max} is the highest concentration achieved in the recording time. The constant k is $0.693/t_{hc}$, where t_{hc} (time for half concentration) is the time required to reach a concentration of $1/2 C_{max}$. The values of C_{max} and t_{hc} were calculated by a computer fitting procedure (software: GraphPad Prism 5.0) and expressed as mean \pm standard error with at least 5 different curves performed for each compound. ANOVA and Student's t test was selected as statistical analysis; $p < 0.05$ was considered representative of significant statistical differences [8].

Substances

AITC, SIN, BITC and DADS have been purchased from Sigma-Aldrich; ERU and HBITC were purchased from Santa Cruz Biotechnology. The purity of the tested compounds was: AITC >97.0%, SIN >99.0%, BITC >98.0%, ERU >98.0%, HBITC >98.0%, and DADS >80.0%. All the tested compounds were dissolved at 100 mM in DMSO (Carlo Erba) and further diluted in aqueous solution. The solutions were prepared immediately before the experiments.

Acknowledgements

▼
This article was funded by the “Regional Health Research Program 2009” of Regione Toscana, Italy.

Conflict of Interest

▼
The authors declare no conflict of interest.

References

- 1 Martelli A, Testai L, Breschi MC, Blandizzi C, Viridis A, Taddei S, Calderone V. Hydrogen sulfide: novel opportunity for drug discovery. *Med Res Rev* 2012; 32: 1093–1130
- 2 Martelli A, Testai L, Marino A, Breschi MC, Da Settimo F, Calderone V. Hydrogen sulfide: biopharmacological roles in the cardiovascular system and pharmaceutical perspectives. *Curr Med Chem* 2012; 19: 3325–3336
- 3 Caliendo G, Cirino G, Santagada V, Wallace JL. Synthesis and biological effects of hydrogen sulfide (H₂S): development of H₂S-releasing drugs as pharmaceuticals. *J Med Chem* 2010; 53: 6275–6286
- 4 Zhao Y, Wang H, Xian M. L-Cysteine activated hydrogen sulfide (H₂S) donors. *J Am Chem Soc* 2011; 12: 15–17
- 5 Martelli A, Testai L, Citi V, Marino A, Pugliesi I, Barresi E, Nesi G, Rapposelli S, Taliani S, Da Settimo F, Breschi MC, Calderone V. Arylthioamides as H₂S donors: L-cysteine-activated releasing properties and vascular effects *in vitro* and *in vivo*. *ACS Med Chem Lett* 2013; 4: 904–908
- 6 Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, Kraus DW. Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci USA* 2007; 104: 17977–17982
- 7 Martelli A, Testai L, Breschi MC, Lawson K, McKay NG, Miceli F, Tagliatale M, Calderone V. Vasorelaxation by hydrogen sulphide involves activation of K(v)7 potassium channels. *Pharmacol Res* 2013; 70: 27–34
- 8 Martelli A, Testai L, Citi V, Marino A, Bellagambi FG, Ghimenti S, Breschi MC, Calderone V. Pharmacological characterization of the vascular effects of aryl isothiocyanates: Is hydrogen sulfide the real player? *Vascul Pharmacol* 2014; 60: 32–41
- 9 Dinkova-Kostova AT, Kostov RV. Glucosinolates and isothiocyanates in health and disease. *Trends Mol Med* 2012; 18: 337–347
- 10 Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol Biomarkers Prev* 1998; 7: 1091–1100
- 11 Sanderson K. Physiology: Emissions control. *Nature* 2009; 459: 500–502