H₂S donating corticosteroids: Design, synthesis and biological evaluation in a murine model of asthma

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HIGHLIGHTS

- Hydrogen sulfide is a fundamental endogenous gas-mediator in the respiratory system.
- Design and synthesis of SAIDs-hydrogen sulfide donors hybrids are described.
- Hydrogen sulfide release and chemical stability at differing pH were investigated.
- Prednisone-TBZ hybrid (compound 7) was selected for further evaluations.
- Prednisone-TBZ is a promising therapeutic option in allergic asthma treatment.

GRAPHICAL ABSTRACT

ABSTRACT

Introduction: Hydrogen sulfide (H₂S) is a fundamental biological endogenous gas-mediator in the respiratory system. It regulates pivotal patho-physiological processes such as oxidative stress, pulmonary circulation, airway tone and inflammation.
Objectives: We herein describe the design and synthesis of molecular hybrids obtained by the condensation of several corticosteroids with different hydrogen sulfide releasing moieties.
Methods: All the molecules are characterized for their ability to release H₂S both via amperometric approach and using a fluorescent probe. The chemical stability of the newly synthesized hybrid molecules has been investigated at differing pH values and in human serum.
Results: Prednisone-TBZ hybrid (compound **7**) was selected for further evaluations. The obtained results from the *in vitro* and *in vivo* studies clearly show evidence in favor of the anti-inflammatory properties of the released H₂S.

Lung diseases

Conclusions: The protective effect on airway remodeling makes the hybrid Prednisone-TBZ (compound **7**) as a promising therapeutic option in reducing allergic asthma symptoms and exacerbations.

Introduction

Asthma is one of the main chronic respiratory pathology, affecting both children and adults and represents one of the major causes leading to high health care costs due to the need of chronic pharmacological treatments [1,2].

Currently, anti-asthmatic therapy aims at controlling symptoms and reducing inflammation, symptomatic manifestations, and exacerbations, as well as maintaining lung function preventing acute attacks by chronic administration of corticosteroids [3,4]. However, chronic use of glucocorticoids is often related to serious adverse effects, among them cardiovascular disease, dyslipidemia, adrenal suppression, hyperglycemia, osteoporosis, and immunosuppression [5,6].

Hydrogen sulfide (H_2S) is reported to represent a fundamental biological endogenous gas-mediator involved in several patho-physiological conditions and largely synthesized in the airways by 5′ pyridoxal phosphate – dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in lung epithelial cells, in the nasal mucosa and submucosal glands [7]. In the respiratory airways, H_2S has been demonstrated to actively regulate fundamental patho-physiological processes such as oxidative stress, pulmonary circulation, airway tone and inflammation [8,9]. H_2S inhibits the transcription of the nuclear factor-κB (NF-kB) and prevents the release of cytokines, chemokines, and pro-inflammatory enzymes. Moreover, it acts as a scavenger of reactive oxygen species (ROS) and promotes the endogenous synthesis of antioxidant molecules and enzymes [10,11]. Generally, several studies supported the hypothesis that changes in the H_2S levels are related to the onset of chronic and acute inflammatory lung pathologies, such as asthma [12–14]. Consistently, emerging evidence highlights that the administration of molecules able to donate H_2S improves lung function and pulmonary flow rate [15].

Furthermore, H_2S facilitates electrolyte absorption in human bronchiolar epithelia due to Na^+/K^+-ATPase inhibition, opening of ATP-sensitive potassium channels (K_{ATP}) and Ca^{2+}-sensitive potassium channels. These properties promote the mucociliary activity, by enhancing the exclusion of external microorganisms and inducing bronchodilation [16]. In addition, H_2S breaks disulfide bonds of mucins in the respiratory tract, facilitating the expulsion of mucus, which is made less viscous [17].

Roviezzo and colleagues observed that Ovalbumin (OVA)-induced airway hyperresponsiveness is blunted by aerosolized NaHS. This suggests that a preventive treatment with a corticosteroid combined with an H_2S donor may counteract asthma clinically relevant symptoms through the inhibition of inflammatory cascade and by slowing down bronchial remodeling [13]. The anti-inflammatory activity of H_2S has been also confirmed using the slow H_2S-releasing compound GYY4137, which reduced pulmonary inflammation induced by LPS in rats, through the inhibition of the release of proinflammatory mediators (IL-1β, TNF, IL-6). Moreover, GYY4137 displayed a strong antioxidant effect since it was able to restore in lung tissues the activity of the antioxidant enzymes, such as superoxide dismutase and catalase, and hence by normalizing the GSH/GSSG ratio [18].

To nowadays H_2S-donor moieties have been exploited for designing many pharmacological entities and for several diseases [19–23]; prompted by our ongoing interest in this strategy [24], we designed new molecular hybrids in which H_2S-releasing substructures are joined to glucocorticoids in order to get a valid alternative in asthma care, by enhancing the classic anti-inflammatory effects of the "native" corticosteroids, with the promising H_2S properties.

In this research work, we describe the design, synthesis, and pharmacological investigation of glucocorticoids-H_2S donors obtained combining two of the most used corticosteroids, such as prednisone and dexamethasone, with two H_2S-donating moieties, such as 4-hydroxy-thiobenzamide (TBZ) and 5-(*p*-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH) (**5–8**; Table 1).

Additionally, the chemical stability of molecular hybrids, has been assessed both in phosphate buffered saline (PBS) pH 7.4, and in simulated gastric fluid (SGF) pH 1.2 without pepsin, to respectively mimic the physiological and gastric conditions. Furthermore, the enzymatic stability in human serum (HS) has also been investigated (Table 1). With the aim of predicting the hydrolytic pathways, for each hybrid we have evaluated quantitatively the various metabolites (i.e., the corresponding glucocorticoid, **1** or **2**, the H_2S-releasing compound, ADT-OH or TBZ, and the succinate derivative of glucocorticoid, **3** or **4**) that are formed during the incubation in simulated physiological solutions. The H_2S-releasing rate of the hybrid molecules was measured both amperometrically and in a biological environment by using a fluorescent probe. Finally, the biological activity of prednisone derivative **7** has been evaluated both *in vitro* and *in vivo*. These results undoubtedly show that the hybrid compound **7** may be as a promising therapeutic alternative in the management of allergic asthma patients.

Experimental

Chemistry

The reagents were purchased from Merck Life Science S.r.l. and Biosynth Carbosynth®. Melting points were evaluated on a Buchi Melting Point B-540 instrument. They are uncorrected and were evaluated on chromatographically purified or recrystallized compounds. The reactions were controlled by thin layer chromatography (TLC), using Merck silica gel 60 F254 plates preloaded with fluorescent indicator and visualized with UV light (254 nm). Silica gel column (Kieselgel 60) was employed for preparative chromatographic purifications. Na_2SO_4 was used to dry all the solutions which were concentrated with a Buchi R-114 rotary evaporator at low pressure. Homogeneity of the products was evaluated by analytical reversed-phase HPLC (RP-HPLC) on a Shimadzu-10 ADsp HPLC system by means of a Phenomenex Kinetex XB-C18 column, 5 μm, 4.6 × 250 mm and applying the gradient of acetonitrile (CH_3-CN) in 0.1% aqueous trifluoroacetic acid (TFA), from 0% to 100% in 25 min, at a flow rate of 1 mL/min. Analytical RP-HPLC indicated a purity > 98%. ^1H and ^{13}C NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument in dimethyl sulfoxide (DMSO d_6). Chemical shifts are described in ppm, while peak patterns are reported using the abbreviations: m (multiplet), t (triplet), d (doublet), s (singlet). API 2000 Applied Biosystem mass spectrometer was used to determine mass spectra of the final products.

Synthesis of glucocorticoids-H_2S donors (compounds **5–8**)

General procedure. The synthetic procedure employed to prepare glucocorticoids-H_2S donors (**5–8**) is described in Scheme 1.

Table 1

The table reports the structure of the designed compounds and their enzymatic and chemical stability at 37 °C. Chemical stability in SGF and PBS as percentage of remaining compound after 24 h of incubation. Stability in human serum as half-life (min). Results are reported as mean values ± SD (n = 3).

#	Structure	% remaining compd at 24 h		Half-life (min)
		SGF	PBS	Human Serum
5		99 ± 1	99 ± 2	12 ± 1
6		99 ± 1	99 ± 2	10 ± 1
7		99 ± 1	97 ± 1	15 ± 2
8		99 ± 1	99 ± 2	9 ± 2

1, Dexamethasone: R$_1$ = α-CH$_3$; R$_2$ = OH; X = F
2, Prednisone: R$_1$ = H; R$_2$ = ‖ ; X = H
                              O

3: R$_1$ = α-CH$_3$; R$_2$ = OH; X = F
4: R$_1$ = H; R$_2$ = ‖ ; X = H
                       O

5: R$_1$ = α-CH$_3$; R$_2$ = OH; X = F
7: R$_1$ = H; R$_2$ = ‖ ; X = H
                       O

6: R$_1$ = α-CH$_3$; R$_2$ = OH; X = F
8: R$_1$ = H; R$_2$ = ‖ ; X = H
                       O

Scheme 1. (i) succinic anhydride/DMAP/anhydrous pyridine; (ii) EDC/DMAP.

Dexamethasone (**1**) or prednisone (**2**) were converted to the corresponding 21-succinates (**3** and **4**) via succinic anhydride using a catalytic amount of 4-dimethylaminopyridine (DMAP) in anhydrous pyridine. The following coupling reaction of succinate derivative (**3** or **4**) with ADT-OH or TBZ, was performed in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and DMAP, producing the glucocorticoid derivatives (**5–8**).

4-(2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanoic acid (Dexamethasone succinate, 3). One gram (2.55 mmol) of dexamethasone **1** was dissolved in anhydrous pyridine (30 mL). Then 0.77 g (7.65 mmol) of succinic anhydride and DMAP (0.1 eq) were added at room temperature. The mixture was constantly stirred overnight and then evaporated under reduced pressure. The obtained residue was added with 20 mL of water, the resulting mixture was stirred for 20 min and then centrifuged. The obtained precipitate was washed again with H$_2$O and filtered affording 1.12 g of compound **3** as a white powder (yield of 89%); m. p. 188–189 °C.

4-(2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanoic acid (Prednisone succinate, 4). Following the procedure described above for **3**, com-

pound **4** was prepared starting from prednisone (**2**, 0.84 g, 8.37 mmol), succinic anhydride (1.0 g, 2.79 mmol) and DMAP (0.1 eq): yield 90%. m. p. 180–182.5 °C.

4-carbamothioylphenyl(2-((8S,9R,10S,11S,13S,14S,16R,17R) −9-fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15, 16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) succinate (Dexamethasone-succinate-TBZ, 5). Compound **3** (1 g, 2.0 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL). TBZ (0.613 g, 4.0 mmol) and DMAP (0.024 g, 0.2 mmol) were added. The resulting mixture was kept on ice bath, stirred under nitrogen for 10 min and EDC (0.575 g, 3.0 mmol) was then added. When the addition was completed, the mixture was brought at room temperature and stirred under nitrogen atmosphere for 3 h. The solvent was removed, and the obtained residue was purified by column chromatography on silica gel (ethyl acetate: *n*-hexane, 6:4). The following crystallization with *n*-hexane afforded the desired compound **5** as a yellow solid (0.98 g; yield 78%). m. p. 144.5–146.1 °C. ESI-MS (M+H)$^+$ *m/z* calcd 627.72 for C$_{35}$H$_{38}$FNO$_8$S; found 628.2.

^{1}H NMR (DMSO *d*$_6$) *δ* 9.87 (s, 1H), 9.50 (s, 1H), 7.91 (d, 2H, J = 8.61), 7.28 (d, 1H, J = 10.17), 7.14 (d, 2H, J = 8.61), 6.21 (d, 1H, J = 10.17), 5.99 (s, 1H), 5.39 (d, 1H, J = 4.30), 5.16 (s, 1H), 4.82–5.08 (dd, 2H, J = 17.61), 4.13 (s, 1H), 2.87 (t, 2H, J = 5.87, J = 6.26), 2.79 (t, 2H, J = 6.65, J = 5.48), 2.58–2.61 (m, 1H), 2.26–2.40 (m, 2H), 2.09–2.18 (m, 2H), 1.74–1.77 (m, 2H), 1.53–1.67

(m, 2H), 1.48 (s, 3H), 1.31–1.38 (m, 1H), 1.05–1.10 (m, 1H), 0.88 (s, 3H), 0.76 (d, 3H, *J* = 7.04).
^{13}C NMR (DMSO *d₆*) *δ* 205.11, 199.48, 185.73, 171.81, 170.92, 167.81, 153.10, 153.04, 137.52, 129.43, 129.18, 124.54, 121.61, 100.10, 90.94, 71.30, 68.70, 48.43, 48.21, 43.75, 36.13, 35.86, 32.35, 30.09, 29.36, 28.87, 27.96, 23.45, 16.70, 15.56.

2-((8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-10,13, 16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl (4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl) succinate (Dexamethasone-succinate-ADT, 6).
Following the same procedure reported above for **5**, compound **6** has been obtained starting from **3** (1 g, 2.0 mmol) and ADT-OH (0.904 g, 4.0 mmol). Yield: 1.13 g (81%) of product **6** as an orange solid. m. p. 134.8–135.1 °C. ESI-MS (M+H)⁺ *m/z* calcd 700.86 for C₃₅H₃₇FNO₈S₃; found 701.2.
^{1}H NMR (DMSO *d₆*) *δ* 7.96 (d, 2H, *J* = 8.61) 7.80 (s, 1H), 7.27–7.30 (m, 3H), 6.21 (d, 1H, *J* = 10.17), 5.96 (s, 1H), 5.73 (s, 1H), 5.47 (d, 1H, *J* = 10.17), 5.40 (s, 1H), 4.81–5.16 (dd, 2H, *J* = 18.00), 4.14 (s, 1H), 2.89 (t, 2H, *J* = 5.87, *J* = 6.26), 2.80 (t, 2H, *J* = 6.65, *J* = 5.48), 2.58–2.61 (m, 2H), 2.29–2.32 (m, 2H), 2.09–2.18 (m, 2H), 1.53–1.79 (m, 2H), 1.48 (s, 3H), 1.32–1.34 (m, 1H), 1.05–1.10 (m, 1H), 0.88 (s, 3H), 0.78 (d, 3H, *J* = 7.04).
^{13}C NMR (DMSO *d₆*) *δ* 205.13, 185.72, 173.15, 171.83, 170.85, 167.50, 153.85, 153.18, 136.26, 129.45, 129.28, 129.12, 124.56, 123.49, 90.94, 71.12, 70.76, 68.71, 48.44, 48.27, 43.75, 36.12, 35.87, 34.13, 32.35, 30.71, 29.41, 28.88, 27.72, 23.40, 16.70, 15.57.

4-carbamothioylphenyl (2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahy-dro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) succinate (Prednisone-succinate-TBZ, 7). Following the same procedure reported above for **5**, compound **7** has been obtained starting from **2** (2.0 mmol) and TBZ (0.613 g, 4.0 mmol). Yield 0.87 g (73%) of 7 as a yellow solid. m. p. 114.7–115.2 °C, ESI-MS (M+H)⁺ *m/z* calcd 593.20 for C₃₂H₃₅NO₈S; found 594.3.
^{1}H NMR (DMSO *d₆*) *δ* 9.87 (s, 1H), 9.51 (s, 1H), 7.91 (d, 2H, *J* = 6.26), 7.60 (d, 1H, *J* = 10.17), 7.14 (d, 2H, *J* = 6.26), 6.14 (d, 1H, *J* = 10.17), 6.00 (s, 1H), 5.85 (s, 1H), 4.83–5.00 (dd, 2H, *J* = 17.61), 3.13 (d, 1H, *J* = 13.69), 3.02 (t, 2H, *J* = 5.87, *J* = 6.26), 2.97 (t, 2H, *J* = 6.65, *J* = 5.48), 2.36 (t, 1H, *J* = 11.74, *J* = 9.78), 2.19 (t, 1H, *J* = 11.74, *J* = 9.78), 1.96–2.05 (m, 2H), 1.68–1.75 (m, 5H), 1.35 (s, 3H), 1.18–1.22 (m, 2H), 0.95–0.99 (m, 1H), 0.50 (s, 3H).
^{13}C NMR (DMSO *d₆*) *δ* 210.09, 205.11, 185.73, 171.81, 171.05, 167.81, 153.10, 153.04, 137.52, 129.43, 126.78, 125.04, 121.71, 100.12, 91.02, 71.30, 68.70, 48.43, 48.21, 43.75, 36.13, 35.91, 32.50, 30.24, 29.36, 28.77, 27.99, 23.45, 17.40.

2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a] phenanthren-17-yl)-2-oxoethyl (4-(3-thioxo-3H-1,2-dithiol-5-yl)phe-nyl) succinate (Prednisone-succinate-ADT, 8). Following the procedure above reported for **5**, compound **8** has been obtained starting from **2** (2.0 mmol) and ADT (0.904 g, 4.0 mmol). Yield 1.1 g (85%) of **8** as an orange solid. m. p. 127.7–128.5 °C. ESI-MS (M+H)⁺ *m/z* calcd 666.14 for C₃₄H₃₄O₈S₃; found 667.2.
^{1}H NMR (DMSO *d₆*) *δ* 7.95 (d, 2H, *J* = 8.61), 7.80 (s, 1H), 7.59 (d, 1H, *J* = 10.17), 7.28 (d, 2H, *J* = 7.82), 6.09 (d, 1H, *J* = 10.17), 5.99 (s, 1H), 5.81 (s, 1H), 4.82–4.99 (dd, 2H, *J* = 17.61), 2.89 (t, 2H, *J* = 5.87, *J* = 6.26), 2.79 (t, 2H, *J* = 6.65, *J* = 5.48), 2.35 (t, 1H, *J* = 11.74, *J* = 9.78), 2.19 (t, 1H, *J* = 11.74, *J* = 9.78), 1.97–2.03 (m, 4H), 1.62–1.77 (m, 5H), 1.35 (s, 3H), 1.17–1.22 (m, 2H), 0.49 (s, 3H).

^{13}C NMR (DMSO *d₆*) *δ* 210.10, 205.21, 199.48, 185.73, 171.81, 170.92, 167.81, 153.10, 153.04, 137.52, 129.43, 127.18, 124.54, 121.61, 100.10, 91.04, 71.30, 68.70, 48.43, 48.21, 43.75, 36.13, 34.86, 32.35, 31.21, 29.28, 28.92, 28.13, 23.58, 16.70, 15.96.

Chemical and enzymatic stability

Stability in simulated gastric fluid (SGF) and saline phosphate buffer (PBS)

Experiments were carried out as described by Magliocca *et al* [25]. Briefly, the compounds (**5–8**) were dissolved in DMSO (10 mM) and diluted in PBS or SGF without pepsin in glass tubes in triplicate to obtain the 50 μM final concentration (0.5% DMSO). The obtained solutions were incubated at 37 ± 0.5 °C for 24 h and at appropriate time intervals, the concentration of each compound and its relative metabolites was determined by RP-HPLC. For the calibration curves: standard solutions (r² > 0.99) in PBS or SGF in the concentration range 1–50 μM were used. HPLC analyses were performed as described below.

Stability in human serum

Experiments were carried out as previously described [26]. Briefly, the compounds (**5–8**) were dissolved in DMSO (10 mM) and the obtained solution was added to sterile-filtered human serum (Sigma-Aldrich) in glass tubes in triplicate at the concentration of 100 μM (1% of DMSO). After incubation times (0, 5′, 10′, 15′, 30′, 60′, 120′, 360′ and 24 h), 200 μL of each reaction mixture were collected and added to 200 μL of CH₃CN containing 0.1% TFA in order to deproteinize the serum. The samples were vortexed, sonicated for 3 min and then centrifuged for 5 min at 2500 g. Each clear supernatant was filtered by 0.45 μm PTFE (Alltech) and analyzed by RP-HPLC. For the calibration curves: standard solutions (r² > 0.99) in a mixture of CH₃CN/water 0.1% TFA in the concentration range 1–100 μM were used. HPLC analyses were performed as described below.

HPLC analysis

The RP-HPLC procedure allowed the separation and quantification of new hybrids (**5–8**) and their relative metabolites (i.e., the corresponding respective glucocorticoid, **1** or **2**, the H₂S-releasing compound, ADT-OH or TBZ, and the succinate derivative of glucocorticoid, **3** or **4**). HPLC analysis was performed with a HP 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped as reported by Sodano *et al* [27]. A Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 μm; Agilent) analytical column was used employing as mobile phase CH₃CN 0.1% TFA (solvent A) and water 0.1% TFA (solvent B), at a flow rate of 1 mL/min with gradient conditions (35% A until 3 min, from 35 to 80% A between 3 and 15 min, 80% A between 15 and 20 min, from 80% to 35% A between 20 and 25 min). The column effluent was monitored at 234, 250, and 300 nm referenced against 800 nm wavelength, depending on spectroscopic behavior of each compound.

In vitro evaluation

Amperometric measurement of H₂S release

The H₂S-generating properties of the hybrid molecules (compounds **5–8**) and the H₂S releasing moieties (TBZ and ADT-OH) have been evaluated at the concentration of 100 μM using an electrochemical approach, using Apollo-4000 Free Radical Analyzer (WPI) detector as reported previously [28–30]. The compounds have been evaluated in the presence or in the absence of L-Cysteine 4 mM in aqueous buffer pH 7.4.

Evaluation of physico-chemical properties

The evaluation of physico-chemical properties was performed using QikProp software (Schrödinger release 2018, Schrödinger, LLC, NY, 2018). Compounds were built in Maestro suite (Schrödinger release 2018, Schrödinger, LLC, NY, 2018) using the available drawing tools. The resulting structures were minimized employing Macromodel software (Schrödinger release 2018, Schrödinger, LLC, NY, 2018) and subsequently treated by LigPrep (Schrödinger release 2018, Schrödinger, LLC, NY, 2018) in order to obtain the most plausible protonation state at physiological pH (7.4 ± 0.2) as previously described [31,32]. Potential aggregator effects of the selected molecules were evaluated using the web-server Aggregator Advisor (http://advisor.bkslab.org/search/) [33].

Cell culture

BSMCs (Life technologies) and RBL-2H3 cells (ATCC)) were cultured as reported in technical data sheet. Media were supplemented with 1% pen/strep (Sigma-Aldrich) and cultured at 37 °C in a humidified 5% CO_2 atmosphere.

Intracellular H_2S release

BSMCs were seeded at a density of 30×10^3 cells per well onto a 96 well clear bottom black plate. The evaluation of intracellular H_2S release has been performed using the fluorescent dye WSP-1 (Washington State Probe) [32,34]. The hybrid molecules (compounds **5–8**) and the H_2S releasing moieties (TBZ and ADT-OH) have been incubated in BSMCs uploaded with the dye at the concentration of 100 and 300 μM. The details of the experimental procedures are reported in previous works [35,36]. Furthermore, diallyl disulfide (DADS, Sigma-Aldrich) 100 μM has been used as reference H_2S donor compound [28].

β-hexosaminidase (β-HEX) release assay

RBL-2H3 cells were seeded at a density of 72×10^3 cells per well into a 96-well plate. Cells were overnight sensitized with anti-dinitrophenylated-human serum albumin (DNP-HSA)-IgE (0.50 μg/mL), then the medium was substituted with DMEM containing 1 mg/mL bovine serum albumin (BSA). Vehicle (DMSO 0.1%), cromolyn (1 mM) and the "native" drug prednisone (**2**) the hybrid molecules (compounds **7** and **8**), the H_2S releasing moieties (TBZ and ADT-OH) (100 and 300 μM) were incubated for 5 min at 37 °C. Then, DNP (10 ng/mL) was used to trigger the degranulation. After one hour, 50 μL of supernatants were added to 50 μL of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide and the release of β-HEX was evaluated at 405 nm [37].

Evaluation of the membrane hyperpolarizing effects on BSMCs

The membrane hyperpolarizing effects on BSMCs were measured by spectrofluorometric method, using the anionic bisoxonol dye DiBac4(3) (Sigma Aldrich) 2.5 μM, which follows the cell membrane potential. Compounds **2** and **7** and the H_2S releasing moieties (TBZ and ADT-OH) were added at the concentration of 100 and 300 μM, and the fluorescence was measured for 40 min. NS1619 (Sigma-Aldrich) at the concentration of 10 μM, was used as reference drug. Data analysis is reported in previous works [38–40].

In vivo evaluation

Animals

All the *in vivo* protocols were approved by the São Leopoldo Mandic Animal Ethics Committee (license no. 2017/033). Male Balb/c mice (4–5 weeks old at the beginning of the experiments) were supplied by the Multi-Institutional Center of Bioterism (State University of Campinas – UNICAMP, Brazil) and kept at the local animal room under controlled temperature (22 ± 2 °C), 12/12 h

light/dark cycle and had free access to standard chow and tap water. Each experimental group comprised 5 animals.

Induction of allergic lung inflammation

Mice were sensitized by chronic exposure to an allergen, as previously described [41]. Briefly, the animals received subcutaneous injections (0.4 mL) of a suspension containing 100 μg ovalbumin (OVA - grade V) adsorbed on 4 mg aluminum hydroxide on days 1 and 11, and intranasal (i.n.) OVA (100 μg in 25 μL saline) on day 11. The sensitized animals were then intranasally exposed to the allergen over the next six weeks on two consecutive days per week (starting on days 19 and 20; non-challenged (control) mice received the same volume (25 μL) of saline solution. Four weeks after the last OVA challenge the animals were euthanized by the i.p. administration of 150 mg/kg sodium thiopental plus 10 mg/ml lidocaine, followed by exsanguination.

Treatments

The allergic animals were orally treated with equimolar doses of either prednisone (5.5 mg/kg) or the H_2S-releasing derivative (compound **7**; 9.1 mg/kg) 30 min before each i.n. OVA challenge; untreated allergic and control (non-allergic) mice received 1 mL/kg of aqueous 0.5% carboxymethyl cellulose (vehicle). Treatments went daily during the next 4 weeks after the last OVA challenge (i.e., until animal euthanasia).

Lung histology and morphometry

The lungs were removed and kept in 10% formaldehyde. The left lower lobe was sagittally sectioned, embedded in paraffin and cut into 5 μm sections. The sections were then deparaffinized and stained with either Masson's trichrome (for extracellular matrix evaluation), Sirius Red (for eosinophil visualization) or toluidine blue (for mast cell identification). Peribronchiolar smooth muscle actin was assessed by immunohistochemistry using an anti-α-actin monoclonal antibody (Abcam plc, UK). All the images were obtained using an optical microscope and were further analyzed using the ImageJ software.

Masson's trichrome stained sections were photographed at a 400x magnification. Extracellular matrix thickness was measured in 3 different areas of the same bronchiole and the average of the readings was recorded. For eosinophil and mast cell counting, the slides were analyzed in 5 distinct areas at a 100x magnification, and the readings were averaged. For the immunohistochemical analysis of peribronchiolar smooth muscle, >4 bronchioles per slide were photographed at a 200x magnification.

Results and discussion

Compounds **5–8** were prepared by standard solution methods. The glucocorticoid and the H_2S releasing moiety were connected through a succinic spacer, and the final desired compounds were obtained in excellent yields. The chemical stability of new glucocorticoids-H_2S donors (**5–8**) was determined at body temperature (37 °C) both at pH 7.4 in PBS and at pH 1.2 in SGF (without pepsin), to mimic the physiological and gastric environments, respectively.

All compounds exhibited a great chemical stability at the two pH conditions (Table 1). Particularly, the percentage of all hybrids (**5–8**) after 24 h in SGF remained over 99%; only about 1% was hydrolyzed to the succinate derivative of respective glucocorticoid. Their high hydrolytic stability in an acidic environment guaranteed the effectiveness of a possible oral administration and, at the same time, could improve their absorption already at the gastric level. In physiological pH condition (pH 7.4), the degradation of all compounds (**5–8**) after 24 h of incubation was lower than 3% and so

considered negligible; also, in this case the succinate derivative of respective glucocorticoid was the main degradation product.

To have successful hybrids with a dual anti-inflammatory activity, one for the presence of the glucocorticoid and the other for H_2S release, each hybrid molecule must regenerate the starting corticosteroid and, simultaneously, the H_2S-releasing moiety by enzymatic pathways. In our case, this condition was fulfilled, indeed the half-time ($t_{1/2}$) in human serum resulted to be 12 min for the compound **5**, 10 min for **6**, 15 min for **7**, 9 min for **8** (see Table 1). In every case, the esterase present in human serum hydrolyzed the ester bond between the H_2S-releasing compound (TBZ or ADT-OH) and the succinate derivative of prednisone or dexamethasone (**4** and **3**, respectively); from these last, the starting glucocorticoid (**1** or **2**) is slowly released (data not shown).

In vitro pharmacological evaluation

H_2S release: amperometric measurement

The H_2S-release of the H_2S-donor moieties and the novel synthesized hybrid molecules were investigated *in vitro* by an amperometric assay in the absence of biological substrates. This approach allows to obtain a peculiar description of the H_2S-releasing process thanks to a specific measurement of the H_2S release. The data of the highest amount of H_2S reached in the recording time of 30 min (C_{max}) from the tested molecules 100 μM are reported in Table 2, in different experimental conditions, such as without (−L-Cys) or with an excess (+L-Cys) of L-cysteine (4 mM) that was added to mimic the endogenous occurrence of free thiols. Generally, all the tested compounds had very low and almost appreciable H_2S generation without L-Cys, confirming the H_2S-donating profile displayed by other H_2S-donors described in the literature [42,43]. In the lack of L-Cys, the H_2S production from all the tested drugs was almost negligible (<0.4 μM), except for ADT-OH which showed H_2S-release of about 1 μM. The presence of L-Cys (4 mM) increased the H_2S generation from all the tested compounds. Contrarily, almost all the compounds showed L-cysteine mediated, gradual and time-dependent "slow" H_2S releasing profiles (data not shown).

Thinking about clinical purposes, ideal H_2S-donors should be provided with slow H_2S-donating profile, providing a long-lasting and constant H_2S generation at physiological level. The already described synthetic H_2S-donor GYY4137 and several natural polysulfides from garlic (*Allium sativum L.*) represent early slow H_2S-donors [44,45]. However, the design of innovative H_2S-releasing moieties is still a clear need, to expand the pharmacological options for the treatment of respiratory disorders. In this perspective, all the hybrid drugs here described showed appreciable H_2S-releasing features and compound **7** resulted to be the one able to release the highest amount of H_2S (about 5 μM).

Calculated physico-chemical properties

The output of the calculation is reported in Table 3. The result of this assessment showed a satisfactory solvent accessible surface area, calculated LogP, and calculated solubility (LogS), being the values for both compounds in the range indicated in the QikProp user manual. Moreover, the predicted apparent cell permeability using two computational models for Caco-2 and MDCK cells suggested that compounds. In general, although the calculated physico-chemical parameters are in the recommended range, as indicated in the QikProp user manual, the LogS and the LogP of compound **5** could preclude an efficient H_2S release. In particular, the value of these parameters could determine problems regarding a possible aggregation of this compound. In order to investigate this aspect, we performed a calculation using Aggregator Advisor. This *in silico* tool is able to identify compounds that are known to aggregate or may aggregate in biochemical assays, combining chemical similarity to known aggregators, and physical properties. Accordingly, the output of compound **7** did not show any alert regarding the possible aggregation. On the contrary, the result regarding compound **5** highlighted possible issues about the aggregation of this molecule. In fact, as described in the alert message due to the high calculated LogP, which is in the range reported for many other aggregators, compound **5** showed possible aggregation issues. In summary, the computational investigation indicated some concerns about the compound **5** that could be related to the limited capacity of releasing H_2S.

Intracellular hydrogen sulfide fluorometric measurement

The amperometric assay aimed at defining the profile of potential H_2S release compounds, showing that many sulfur compounds may be considered as "smart" donors: they act as H_2S-releasing molecules in organic situations as the intracellular compartment where they can react with endogenous organic thiols, but they are relatively stable in water and do not release H_2S. However, the amperometric assay was performed in buffered aqueous solution with or without L-Cys. Hence, for demonstrating that these compounds can release H_2S if added to biological substrates, a fluorometric assay was performed using bronchial smooth muscle cells (BSMCs), without the supplement of exogenous thiols. The intracellular H_2S release was detected using the dye WSP-1 [34]. BSMCs have been chosen because they represent an *in vitro* reliable model for the evaluation of the pharmacological responses of the bronchial muscle. The fluorescence emitted by the interaction between the dye and H_2S was quantitatively measured by a spectrofluorometric approach, recorded for 40 min and the obtained results are resumed in Fig. 1. The addition of the vehicle caused a slight increase in fluorescence index (FI), probably due to the endogenous production of H_2S. In contrast, the incubation of DADS 100 μM (reference H_2S donor) to BSMCs, led to a significant FI increase, indicating a clear significant production of H_2S (P < 0.01 vs vehicle). The addition of ADT-OH 100 and 300 μM and TBZ 100 μM to WSP-1-preloaded BSMCs led to a slight increase of FI that reached the statistical difference only for TBZ 100 μM. Increasing the concentration of TBZ (300 μM), led to a massive increase in FI even higher if compared to the reference compound DADS. The addition of the moieties to the native drugs led to different H_2S-profiles. Indeed, the incubation of compounds **6** and **8** (which are the hybrid drugs designed by adding as H_2S releasing moiety the ADT-OH to dexamethasone and prednisone, respectively), 100 and 300 μM, did not cause any significant increase in fluorescence.

The incubation of compounds **5** and **7** (which are the hybrid drugs designed by adding the TBZ H_2S releasing moiety to dexamethasone and prednisone, respectively), at concentrations of 100 and 300 μM, evoked a significant increase in the intracellular level of H_2S, even higher if compared to DADS.

Table 2

H_2S-generating characteristics of the tested molecules. The table shows the values of C_{max} of H_2S-release obtained with TBZ, ADT-OH, and the hybrid compounds at the concentration of 100 μM by the electrochemical analysis under different experimental conditions. Data are reported as means ± SEM.

Compound (100 μM)	H_2S-release (μM)	
	+ L-cysteine 4 mM	− L-cysteine
TBZ	1.6 ± 0.3	n.d.
ADT-OH	1.7 ± 0.5	1.1 ± 0.3
5	0.7 ± 0.1	n.d.
6	1.8 ± 0.5	0.4 ± 0.4
7	5.1 ± 1.5	0.4 ± 0.2
8	0.8 ± 0.1	0.5 ± 0.1

Table 3

Calculated physico-chemical properties for compounds **5** and **7** using QikProp software.

Cpd	SASA[a]	QPlogP[b]	QPlogS[c]	QPPCaco[d]	QPPMDCK[e]
5	969	3.42	−5.34	233	221
7	911	2.99	−4.62	246	237

[a] SASA predicted the total solvent accessible surface (range or recommended value for 95% of known drugs 300–1000).
[b] QPlogP predicted octanol/water partition coefficient (range or recommended value for 95% of known drugs −2–6.5).
[c] QPlogS predicted aqueous solubility in mol/dm^3 (range or recommended value for 95% of known drugs −6.5–0.5).
[d] QPPCaco predicted apparent Caco-2 cell permeability in nm/sec (range or recommended value for 95% of known drugs >500 great, <25 poor).
[e] QPPMDCK predicted apparent MDCK cell permeability in nm/sec (range or recommended value for 95% of known drugs >500 great, <25 poor).

Fig. 1. Intracellular hydrogen sulfide release. Graphs in panel A show the H_2S-kinetic release of each compound. Panel B shows the cumulative H_2S release calculated as AUC of the Fluorescence index measured for 40 min. Data are reported as mean ± SEM, comparing the data to diallyl disulfide H_2S release. One-way ANOVA post-test Bonferroni has been used as statistical analysis (n = 6). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Beside the H_2S-released amount, also the H_2S releasing kinetic plays a predominant role in mediating those anti-inflammatory effects reported in the literature using slow H_2S-donors. Contrarily, the administration of fast releasing molecules has been reported to induce important inflammatory reaction in mice [46]. The amperometric and fluorometric experimental procedures revealed important features of the tested compounds: all hybrid drugs can be considered as smart H_2S-donors because they release H_2S only in the presence of L-cysteine. The WSP-1 assay showed that only compound **7** was able release a satisfactory amount of H_2S inside the cells and for this reason, compound **7** has been selected for further pharmacological investigation.

Inhibition of mast cell degranulation

H_2S releasing molecules have been reported to reduce degranulation in basophilic leukemia cells of rat (RBL-2H3) treated with a

A

B

Fig. 2. Panel A. Evaluation of hexosaminidase release as marker of RBL-2H3 degranulation. Data are compared to the degranulation induced by DNP – HAS. One-way ANOVA plus Bonferroni has been used as statistical analysis. * indicates significant differences vs DNP-HSA as 100% (** = $P < 0.01$; *** = $P < 0.001$); # indicates significant differences of Prednisone (cmp **2**) 100 μM vs compound **7** 100 μM (# = $P < 0.05$); § indicates significant differences of Prednisone (cmp **2**) 300 μM vs compound **7** 300 μM (§§§ = $P < 0.001$). Panel B. Hyperpolarizing effect of the tested molecules on BSMCs membrane. The graph shows the concentration-dependent hyperpolarizing effect of the tested compounds on cell membrane of BSMCs; effects are compared to the reference compound NS1619. Data are reported as mean ± SEM. One-way ANOVA post-test Bonferroni has been used as statistical analysis (n = 6). * indicates the statistical difference vs NS1619 (*** $P < 0.001$); § indicates the statistical difference vs Prednisone (cmp **2**) 100 μM (§§§ $P < 0.001$); # indicates the statistical difference vs Prednisone (cmp **2**) 300 μM (### $P < 0.001$).

specific antigen by targeting downstream proteins of the FcεRI pathway, which is strictly involved in mast cell degranulation [37]. Respiratory diseases, including allergic asthmatic symptoms, comprehend the activation of mast cells promoting the release of those pro-inflammatory mediators, which promotes the hyper-reactiveness of bronchial smooth muscle cells [13]. Hence, we then explored the inhibitory effects of TBZ, compounds **2** (the native drug) and **7** (the hybrid drug) in an antigen-mediated mast cell degranulation model. The incubation of the antigen DNP-HSA to pre-sensitized RBL-2H3 cells triggered a clear degranulation measured by evaluating β-hexosaminidase release (Fig. 2 – Panel A). Cromolyn, a mast cell membrane stabilizer used in clinical practice and tested as reference compound, can inhibit mast cell degranulation of about 25%. TBZ 300 μM caused a significant inhibition of RBL-2H3 degranulation induced by DNP-HSA (Fig. 2 – Panel A). The hybrid **7**, at the concentration of 100 and 300 μM, showed a significant inhibitory effect on mast cell degranulation, resulting in reduced β-hexosaminidase release more efficiently than the native drug **2**, at 100 and 300 μM, which resulted to be totally inactive towards DNP-HSA induced degranulation.

Cell membrane hyperpolarization evaluation

The relaxant activity of H_2S accounts for heterogeneous mechanisms of action and among them many studies demonstrated that H_2S activates different classes of potassium channels promoting membrane hyperpolarization of BSMCs inducing bronchodilation [47]. Asthma is characterized by uncontrolled contraction of bronchial smooth muscle and it was thought stimulating to assess the effects of TBZ, compounds **2** (prednisone) and **7** on the membrane potential of cultured BSMC considering NS1619, a well-known BKCa potassium channel activator, as reference hyperpolarizing agent [48]. The chemical group TBZ (100 and 300 μM) caused only a slight hyperpolarizing effect (38% and 43%, respectively) compared to NS1619 10 μM. The hyperpolarizing effect of compound **2** (prednisone) results similar to that exerted by TBZ at the same concentration, but interestingly, the combination of TBZ and compound **2** massively increased the hyperpolarizing effect. Indeed, compound **7** promoted a clear and significant hyperpolarization in a concentration dependent manner of about 240% if incubated at the concentra-

tion of 100 μM, and of about 309% at the concentration of 300 μM, compared to the reference drug (Fig. 2 – Panel B). This synergic effect may be due to different targets of the two components: indeed, H_2S is able to activate different classes of potassium channels (voltage-gated potassium channels, ATP-sensitive potassium channel and large conductance calcium-activated potassium channels) through the sulfhydration of cysteine aminoacidic residues, leading to membrane hyperpolarization, while corticosteroids are responsible for the activation of large conductance calcium-activated potassium channels [47,49].

In vivo pharmacological evaluation

Chronic structural changes in the airways may be at least partly responsible for permanent hyperresponsiveness in asthma. These changes include increased airway wall thickness, subepithelial fibrosis, smooth muscle cell hyperplasia and hypertrophy, in addition to increased infiltration of inflammatory cells into the lung tissue, such as eosinophils and mast cells, which mediate this chronic allergic response [50]. In fact, as shown in the lung morphometry (Fig. 4, panels A and B) and histological images (Fig. 5), the chronic intranasal exposure of the animals to OVA caused a significant increase in the thickness of peribronchiolar smooth muscle (shown as α-actin immunoreactivity), as well as in the expression of subepithelial collagen, when compared to the saline-treated group. These tissue alterations agree with this murine model of asthma, as originally described by Hirota and colleagues [41] (see Fig. 3).

Despite treatments with equimolar doses of prednisone or its H_2S-releasing derivative - compound **7** (equivalent to 5.5 mg/kg prednisone) were efficient in reducing peribronchiolar smooth muscle and collagen density, compound **7** was significantly more efficient than the parent prednisone to reduce these parameters (as shown in Figs. 4A, B and 5).

As stated above, the presence of eosinophils and increased content of mast cells in the lung tissue are a hallmark of allergic diseases. As shown in Fig. 5, a rise in the number of peribronchiolar eosinophils and mast cells was observed in the parenchyma of the allergic mice, and the number of these cells was significantly reduced when the animals were treated with compound **7** or prednisone (Fig. 4A and B).

Fig. 3. Effect of treatment with either prednisone (Pred) or its H_2S-releasing derivative (compound **7**), at equimolar doses equivalent to 5.5 mg/kg prednisone, on OVA-induced allergic lung inflammation and remodeling in mice. Panel A: peribronchiolar collagen expression; panel B: smooth muscle α-actin expression.; panel C: peribronchiolar eosinophils; panel D: peribronchiolar mast cells. Differences among the group means were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons (n = 5 animals/group). ***$P < 0.001$ vs. Control; ###$P < 0.001$ vs. vehicle-treated allergic mice (OVA); $^{\delta\delta}P < 0.01$ vs Pred.

Fig. 4. Panels A, B, C, D represent the pulmonary morphometry showing the peribronchiolar collagen (in blue), as revealed by Masson's Trichrome staining. Panels E, F, G, H are representative images of the immunohistochemical reaction for smooth muscle α-actin (in brown). All the histological lung sections were analysed by optical microscopy (magnification: 200X; scale bar shown in the Saline group images: 100 μm).

Fig. 5. Panels A, B, C, D are representative images of the histological analysis of eosinophil infiltration around the bronchioles. The histological lung sections were stained with Sirius Red, under which conditions eosinophils present with red-coloured cytoplasm. Panels E, F, G, H are representative images of the histological analysis of mast cell infiltration into the lungs. The histological lung sections were stained with toluidine blue, under which conditions degranulated mast cells present with blue-coloured nucleus and green-coloured granules spread over the adjacent tissue. All the histological lung sections were analysed by optical microscopy (magnification was set at 400X for panels A-D and 200X for panels E-H).

Conclusion

The developed glucocorticoids-H_2S donors (**5–8**) demonstrated a prolonged chemical stability both at acidic and physiological pH. Moreover, in human serum, all hybrids showed to have a half-life of almost 10 min by releasing, at the same time, the corresponding succinate derivative of the glucocorticoid and TBZ or ADT-OH as H_2S donors. The H_2S releasing ability of the newly synthesized compounds was assessed by both an amperometric approach leading to the selection of compound **7** for further *in vitro* and *in vivo* analyses.

In particular, the evaluation of intracellular H_2S-release, confirmed that compound **7** was able to reach satisfactory levels of H_2S. Furthermore, compound **7** resulted to be more effective in inhibiting mast cell degranulation and in promoting BSMCs membrane hyperpolarization than prednisone, indicating adding the H_2S donor moiety could the pharmacological profile of corticosteroids.

For the *in vivo* experiments, the evaluation of the selected compound in a model of chronic allergic lung inflammation showed that the treatment with compound **7** is significantly more effective in reducing the peribronchiolar density of collagen as well as the thickness of the smooth muscle actin than prednisone. Both compound **7** and prednisone decreased the infiltration of eosinophils and mast cells in the airways to a similar extent. Therefore, due to the protective effect on airways remodelling, we consider that compound **7** can be a potentially useful therapeutic option for allergic asthma treatment.

Compliance with ethics requirements

All experiments involving animals were conducted according to the ethical policies and procedures approved by the São Leopoldo Mandic Animal Ethics Committee (license no. 2017/033).

Credit author statement

BS, GC, VS planned the study and coordinated the project. AC synthesized all the compounds. FFi, FFr, EM and EP performed the structural characterization of the compounds. BS, AC, FP and AP analyzed and discussed all the chemical data. MNM, HHAF,

FFC and CNC worked to the conception, design and execution of the in vivo pharmacological experiments, data acquisition, analysis, and interpretation. VCa, AM, VCi and EG designed the in vitro pharmacological experiments: AM and EG contributed to preliminary studies and VCi performed the research and wrote the in vitro pharmacological part of the manuscript. MGR, FS and BR designed and performed the evaluation of chemical and enzymatic stability of the compounds. SB calculated the physico-chemical properties. BS, AC, MNM, HHAF, AM, VCa and VCi drafted and revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.05.008.

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