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Hybrids between H_2S-donors and betamethasone 17-valerate or triamcinolone acetonide inhibit mast cell degranulation and promote hyperpolarization of bronchial smooth muscle cells

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ABSTRACT

Glucocorticoids represent the standard gold treatment of inflammation in asthmatic patients. More recently, H_2S has been described to exert positive effect on this disease. Bearing in mind that an improved pharmacological activity and a reduced toxicity can be obtained through hybridization of different molecules, simultaneously modulating multiple targets, we designed and synthesized novel betamethasone 17-valerate and triamcinolone acetonide hybrids with well-known H_2S-donor moieties.

Synthesized compounds have been evaluated for the potential H_2S-releasing profile both in cell-free environment and into the cytosol of bronchial smooth muscle cells (BSMCs). The two hybrids 4b and 5b were investigated by molecular modelling studies and results indicated that the steric accessibility of the isothiocyanate carbon atom can account for their different H_2S releasing properties.

Furthermore, the most promising derivatives 4b and 5b have been tested for inhibitory effect on mast cell degranulation and for the ability to induce cell membrane hyperpolarization in BSMCs. Significant inhibitory effect on mast cell degranulation was assessed, resulting to reduce β-hexosaminidase release more efficiently than the corresponding native drugs. Both compounds determined a massive membrane hyperpolarization of BSMCs and proved to be 4-fold more effective compared to reference compound NS1619. These effects represent an enrichment of the pharmacological activity of the native drugs.

1. Introduction

Asthma is a clinical syndrome characterized by heterogeneous features that affects more than 300 million people worldwide [1]; according to the World Health Organization, the number of patients is certainly higher due to under-diagnosis and its prevalence, severity, and mortality vary globally [2,3].

Asthma comprehends various pathological conditions affecting both the bronchial smooth muscle and mast cell degranulation causing cough, shortness of breath and variable degrees of airflow limitation, and inflammation which lead to appreciable worsening in quality of life [4]. Generally, asthma may have both allergic and non-allergic etiopathogenesis: in the first case, inflammation is mainly triggered by type 2 immune response, mediated by the release of pro-inflammatory mediators, while non-allergic asthma is promoted by inflammation caused by viral infections and is characterized by a prevalent neutrophilic component [5,6].

The standard gold treatment of inflammation in asthmatic patients is represented by glucocorticoids which are the most effective drugs for the pharmacological treatment and management of persistent asthma, leading to significative reduction of morbidity and mortality [7–9]. However, long-term treatment with

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glucocorticoids may expose patients to local side effects, such as sore throat, hoarse voice and opportunistic oral candidiasis infection, and systemic side effects, such as immunodepression (higher risk of developing pneumonia), reduction in bone mineralization and hydric retention [10–12].

Hydrogen sulfide (H_2S) has historically been known as a toxic and environmental polluting gas, but is now well documented as an important, endogenously produced physiological mediator [13]. Indeed, H_2S is involved in the physiological regulation of cardiovascular, respiratory and immune systems; as well, imbalanced H_2S production/activity is likely to play a pathogenetic role in important pathological conditions, such as hypertension, heart failure, diabetes, inflammation, neurodegeneration, atherosclerosis, sepsis, and asthma [14].

Molecules able to donate H_2S have been described to exert positive effects on asthma, by reducing airway eosinophil infiltration and lung oxidative stress, promoting a protective effect against airway epithelium and endothelium damage caused by an allergic reaction [15]. Moreover, H_2S induces bronchial relaxation by activating different classes of potassium channels expressed on the surface of bronchial smooth muscle cells, helping to restore the patency of respiratory tract in acute asthma events [16–18].

Hybridization of different molecules is a widely exploited strategy in medicinal chemistry that is focused onto the combination of two different compounds (or parts of them) in a new, single derivative. This approach has been reported as an effective strategy in the development of compounds able to modulate multiple targets [19,20].

The hybridization is generally obtained by combining two or more pharmacophoric units from different bioactive compounds, or in other words, two or more known bioactive compounds into a single compound. By this approach, medicinal chemists develop a new chemical entity (by direct linkage, spacer linkage, fusing the moieties, etc) that is believed to retain the pre-selected characteristics of the original templates, and exert improved pharmacological activity and/or reduced toxicity [21].

In this experimental work, we designed and synthesized novel H_2S-donor hybrids, using as native drugs triamcinolone acetonide and betamethasone 17-valerate, for their wide use in the clinical practice in the treatment of asthma, adding well-known H_2S-donor moieties (thioamide [22], isothiocyanate [23], dithiolthione [24] and dithioate [25] portions), linked by means of a succinic spacer. Indeed, merging glucocorticoids to moieties able to donate H_2S could be useful for exploiting the beneficial effect of H_2S in asthma disease, counteracting the side effect of glucocorticoids. To do this, and starting from purely synthetic considerations, we decided to adopt a procedure already used for the preparation of prodrugs of dexamethasone, where succinic anhydride provided the corresponding succinate derivative in high yield [26].

H_2S is also endowed with antiviral effect since it regulates the host response to viral infections, reduces the massive production of pro-inflammatory mediators leading to hypothesizing its employment also in Sars-Cov-2 infections [27].

Interestingly, the H_2S-donor moiety isothiocyanate has been previously used for hybridization [28–30]. For instance, it has been used for the development of a H_2S-donor hybrid of alendronate H_2S-donor, which is a bisphosphonate used for the treatment of osteoporosis, for counteracting the progressive loss of strength of bone in chronic treatment with [31].

The synthetic approach for the development of these new compounds has involved the use of microwave flash heating in order to shorten reaction times and to increase the obtained yields. In fact, in recent years, our interests have been directed to the application of microwave irradiation in the field of small molecules [32–34].

Synthesized compounds have been evaluated for the potential H_2S-releasing profile both in cell-free environment and into the cytosol of bronchial smooth muscle cells (BSMCs). Furthermore, the most promising derivatives have been tested for the evaluation of the inhibitory effect on mast cell degranulation and for the ability to induce cell membrane hyperpolarization in BSMCs, which is an event that lead to broncho-dilation.

2. Chemistry

2.1. Synthesis of hybrid derivatives

Chemical structures of compounds **4a-4d** and **5a-5d** are reported in Table 1 and the synthetic strategy for their synthesis is reported in Scheme 1.

The synthetic procedure for the preparation of compounds **4a-4d** and **5a-5d** has been based on the conversion of Betamethasone 17-valerate (**1a**) or Triamcinolone acetonide (**1b**) into the corresponding hemi-succinated esters, by treatment with an excess of succinic anhydride and catalytic amount of DMAP in anhydrous pyridine.

The intermediates **2a** and **2b** were subjected to a condensation reaction between the carboxylic function and the phenolic function of the different H_2S-donors (**3a**-**3d**), via EDC in the presence of DMAP, thus obtaining the final compounds **4a-4d** and **5a-5d.**

H_2S-donors **3a** and **3b** (4-hydroxythiobenzamide or TBZ and 4-isothiocyanatophenol or HPI, respectively) are commercially available; compound **3c** (ADT-OH) was obtained following with modifications a synthetic procedure previously described by us [35], based on the reaction between *trans*-anethole and sulfur in DMF.

H_2S-donor **3d** (ethyl 4-hydroxybenzodithioate or HBTA) was synthesized using recently published procedures [25].

3. Results and discussion

3.1. Amperometric evaluation of H_2S releasing properties

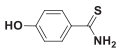
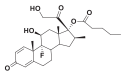
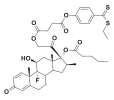
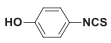
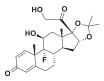
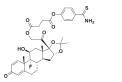
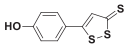
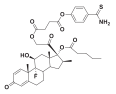
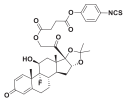
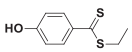
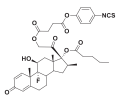
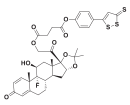
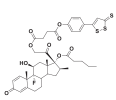
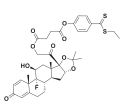
The H_2S-release of the H_2S-donor moieties and the novel synthesized hybrid molecules were investigated *in vitro* by an amperometric assay in the absence of biological substrates. This approach allows to obtain a peculiar description of the H_2S-releasing process thanks to a real-time assessment of the H_2S release. In table 2 are reported the data of the highest amount of H_2S reached in the recording time of 30 min (Cmax) from the tested compounds 100 μM in the different experimental conditions (in the absence (−L-Cys) or in the presence (+L-Cys) of an excess of L-cysteine (4 mM) that was added to mimic the endogenous presence of free thiols). Generally, in the absence of L-Cys very low H_2S release has been recorded for all the tested compounds, confirming the H_2S-releasing feature showed by other H_2S-donors described in the literature [36,37]. In particular, in the absence of L-Cys, the H_2S generation from the hybrid drugs was almost negligible, while the H_2S-donor moieties showed low but clear release of H_2S.

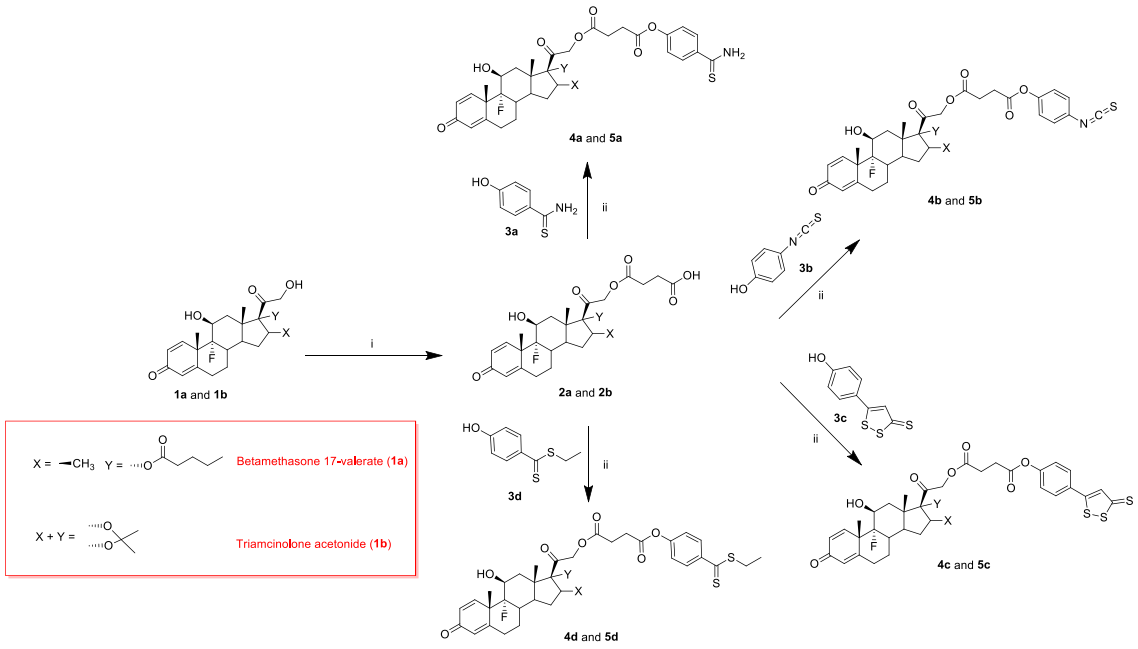
The data reported in Table 1 indicate that, in the presence of organic thiols (L-Cys), most of the tested compounds behaved as H_2S donors, albeit with different features in the quantitative aspects that can be related to their chemical structures.

The presence of an excess of L-Cys (4 mM) increased the H_2S generation from almost all the tested molecules. Comparing the results obtained for the two different series of derivatives, we evidenced that when TBZ was used as H_2S-donor the amount of produced gasotransmitter was equivalent in the two hybrids (2.0 ± 0.3 vs 1.9 ± 0.4 for **4a** and **5a**, respectively).

On the other hand, HBTA demonstrated an impressive capability to produce H_2S in the presence of L-Cys ($3.5\ \mu M \pm 0.6$); however,

Table 1
H_2S-releasing rate: values of Cmax of H_2S-release obtained with H_2S-donors, with parent drugs and with hybrids **4a-4d** and **5a-5d** (100 μM), recorded in the absence (-L-Cys) or in the presence (+L-Cys) of an excess of L-Cysteine (4 mM). Data are expressed as means ± SEM.

Compd.	Structure	H_2S release (μM)		Compd.	Structure	H_2S release (μM)		Compd.	Structure	H_2S release (μM)	
		+-L-Cys	–L-Cys			+-L-Cys	–L-Cys			+-L-Cys	–L-Cys
TBZ 3a		1,6 ± 0,3	0.3 ± 0.1	**Betametasone 17-valerate 1a**		–	–	**4d**		0.9 ± 0.2	<0.1
HPI 3b		1,6 ± 0,2	0.9 ± 0.3	**Triamcinolone acetonide 1b**		–	–	**5a**		1.9 ± 0.4	<0.1
ADT-OH 3c		1,8 ± 0,5	1,0 ± 0,3	**4a**		2,0 ± 0,3	<0.1	**5b**		2,8 ± 0,6	<0.1
HBTA 3d		3.5 ± 0.6	0.8 ± 0.3	**4b**		0,4 ± 0,1	<0.1	**5c**		0.5 ± 0.2	<0.1
				4c		0,3 ± 0,1	<0.1	**5d**		1.6 ± 0.3	<0.1



Scheme 1. Reagents and conditions i) succinic anhydride (3 eq.), DMAP (0.1 eq.), anhydr. pyridine, rt, 12h. ii) EDC (1.5 eq.), DMAP (0.1 eq.), anhydr. THF, rt, 12h.

when this moiety was bonded with bethametasone 17-valerate (compound **4d**) or with triamcinolone acetonide (compound **5d**) the data obtained were of 0.9 μM ± 0.2 and 1.6 μM ± 0.3, respectively.

The best result was obtained with compound **5b** which produced a significant increase in H_2S release compared to the "free" H_2S-donor moiety with a value of 2.8 ± 0.6 μM. Indeed, the introduction of the triamcinolone moiety is able to improve the reactivity with L-Cys of HPI, contrarily to what observed for the other hybrid compounds in which the H_2S release activity is smaller or comparable to the "free" H_2S-donor. The opposite effect is observed when the HPI moiety is combined with betamethasone17-valerate (**4b**), leading to a decrease in H_2S release upon reaction with L-Cys both compared to **5b** and the "free" H_2S-donor moiety. In our previous works isothiocyanates have been demonstrated to represent a promising H_2S-donor moiety [38,39] and the multi-step mechanism leading to H_2S release in the presence of L-Cys has been demonstrated by Lin and colleagues [40]. According to such a mechanism, the carbon atom of the isothiocyanate group undergoes two subsequent nucleophilic attacks upon reaction with L-Cys, the first by the sulfur atom and the second by the nitrogen atom, leading to an ITC-cysteine adduct, facilitating the release of

H_2S. Accordingly, it is likely that steric accessibility to the isothiocyanate moiety can account for the different H_2S releasing properties observed for the two hybrids **4b** and **5b** and we challenged this hypothesis by means of molecular modelling studies (see below).

The L-Cys mediated effects caused progressive and time-related "slow" H_2S releasing profiles from almost all the compounds. The graphics showing the H_2S-kinetic release for each compound incubated in the presence or in the absence of L-Cys are reported in supporting information (Fig. 2SI). Although we did not determine the chemical stability of our compounds in the presence of L-Cys, however, considering the differences in H_2S release observed comparing the "free H_2S donors" and the molecular hybrids (Table 1), we could at least exclude the possibility that the aryl-ester bond was hydrolysed in the totality of the hybrids before H_2S release.

Noteworthy, the pharmacological effect of H_2S-releasing molecules is not directly related only with the quantity of the H_2S donation because even small a low but long-lasting H_2S generation can exert beneficial effects. This has been reported for slow H_2S-donors, such as isothiocyanates and some aryl-thioamides and other H_2S-donors [22,38]. In particular, the effects of H_2S-releasing molecules on inflammatory response are clearly modulated by the rate of the H_2S release [41].

3.2. Intracellular H_2S release in BSMC

The amperometric assay aims at defining the profile of potential H_2S release compounds, showing that many of sulfur compounds may be considered as "smart" donors: they behave as H_2S-releasing molecules in biological environments like the cytosol where they can react with endogenous organic thiols but they are relatively stable in water and don't release H_2S. However, the amperometric assay was performed only in buffered aqueous solution in the presence or in the absence of L-Cys. Therefore, for demonstrating that these compounds are able to release H_2S also in biological substrates, a fluorometric assay has been performed using bronchial smooth muscle cells (BSMCs), without the addition of exogenous thiols. The intracellular H_2S release was detected using the dye WSP-1, which selectively and irreversibly reacts with H_2S. BSMCs have been chosen because they represent an *in vitro* reliable model for the evaluation of the activity of the bronchial muscle. Spectrofluorometric recordings showed that the addition of the vehicle caused a slight increase of fluorescence index (FI), probably due to endogenous production of H_2S. In contrast, the addition of DADS (diallyl disulfide, reference H_2S-donor) to BSMCs preloaded with the fluorescent dye WSP-1 led to a significant increase of fluorescence (FI, fluorescence index), indicating a clear significant formation of H_2S ($P < 0.01$ vs vehicle).

Results obtained with "free" H_2S-donors are reported for comparison both in Figs. 1 and 2: the addition of ADT-OH 100 μM and 300 μM and TBZ 100 μM to WSP1-preloaded BSMCs led to a slight and not significant increase of FI. Increasing concentration of TBZ (300 μM), led to a significant increase in FI even higher if compared to the reference compound DADS. HPI showed a massive increase in H_2S intracellular donation when incubated 100 μM and 300 μM.

The intracellular H_2S release profiles following the incubation of Betamethasone 17-valerate hybrids are reported in Fig. 1. In particular, compound **4a** 100 μM and 300 μM promoted a mild but significant H_2S release; compound **4b** 100 μM and 300 μM when incubated into BSMCs led to appreciable H_2S intracellular release, while **4c** 100 and 300 μM did not cause any significant increase of fluorescence.

Fig. 2 shows the results about the intracellular H_2S release after the incubation of Triamcinolone acetonide hybrids. The incubation of compounds **5a** 100 μM and 300 μM and compound **5c** 100 μM did not cause any significant increase of fluorescence, while **5c** 300 μM promoted only a mild increase in fluorescence index. The incubation of **5b** 100 and 300 μM both evoked a significant increase in the intracellular level of H_2S, even higher if compared to DADS.

Generally, the addition of the moieties to the native corticosteroid drugs led to different H_2S-profiles. In particular, the moieties TBZ and HPI, with the exception of ADT-OH which wasn't able to release H_2S into the cytosol of BSMCs, led to a massive H_2S release. The corresponding corticosteroid derivatives showed a weaker H_2S-donor profile. This is probably due to the different lipophilicity/solubility which influence the capacity of the compounds to enter the cells and exert the H_2S donation.

Looking at the results obtained on the intracellular H_2S-releasing rate, the "best" compounds resulted to be the HPI-derivatives. Indeed, both the single moiety HPI and the triamcinolone hybrid **5b** showed the most promising H_2S donating profiles since they are able to enter the cells, react with the endogenous thiols (mainly L-Cys and glutathione) and lead to an appreciable increase in H_2S levels.

Unfortunately, the moiety HBTA and the corresponding hybrid drugs **4d** and **5d** have not been fluorometric tested due to an unexpected reaction with the probe. Indeed, the compounds are intrinsically coloured and this feature probably interferes with the dye WSP-1 (data not showed). For these molecules therefore the available rate of H_2S release is only that recorded through amperometric evaluation.

Regarding the stability of the aryl ester bond (−C(O)−O-aryl) in the BMSC cells, due to the differences in H_2S releasing properties of the "free" H_2S-donors compared to the corresponding hybrids (Figs. 1 and 2), we can exclude, as in amperometric assays, the hypothesis of a complete compound hydrolysis before H_2S formation,.

3.3. Molecular modelling studies

According to the multistep mechanism proposed by Lin et al. for the reaction of the isothiocyanate group with L-Cys [40], we hypothesized that the steric accessibility of the isothiocyanate moiety could be responsible for the different H_2S releasing properties of the two hybrids **4b** and **5b** (Table 1), characterized by the presence of the Betamethasone 17-valerate and Triamcinolone acetonide moiety, respectively.

To investigate this issue, **4b** and **5b** were firstly subjected to an in depth conformational analysis based on a stochastic search algorithm (see Experimental section for details). This led to the identification of the most probable (i.e., higher occurrence rate) energetically allowed (within 5 kcal/mol from the global minimum conformer) conformational families of each compound. Compound **4b** showed six possible orientations of the isothiocyanate group with respect to the glucocorticoid moiety (family i-vi; Table 1SI), while compound **5b** resulted in fifteen families corresponding to as many orientations of the isothiocyanate group with respect to the glucocorticoid moiety (family i-xv; Table 2SI). Thus, the steric hindrance caused by the presence of the bulky and flexible valerate chain at position 17 limited the conformational freedom of the isothiocyanate group of **4b** with respect to that of **5b**, which presented the smaller and more rigid 16, 17-cyclic acetal substituents.

Then, in order to compare the steric accessibility of isothiocyanate carbon atom in the two hybrids, we calculated the solvent accessible surface area (SASA) of the isothiocyanate carbon atom in the energetically allowed conformational families of compounds **4b** and **5b**. The resulting SASA values were compared with the maximum SASA value calculated for the isothiocyanate carbon atom (full accessibility; Fig. 1SI) and accordingly reported as SASA

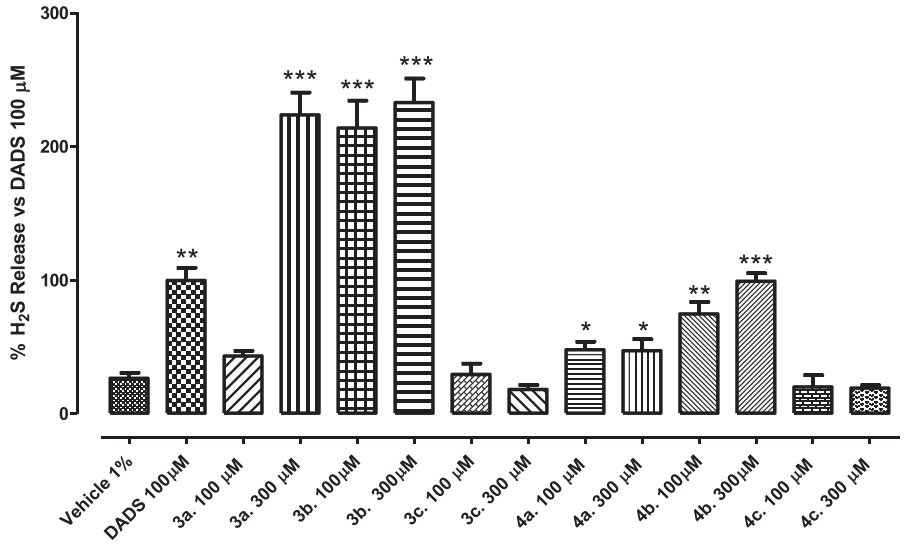


Fig. 1. Cumulative H_2S formation (expressed as area under the curve of the WSP-1 fluorescence in the recording time) produced by the incubation of vehicle or different concentrations of the tested compounds. Data were expressed as mean ± SEM setting DADS H_2S release as 100%. Three different experiments were carried out, each in triplicate. One-way ANOVA post-test Bonferroni has been applied to calculate the significance level. (*P < 0.05; **P < 0.01; ***P < 0.001).

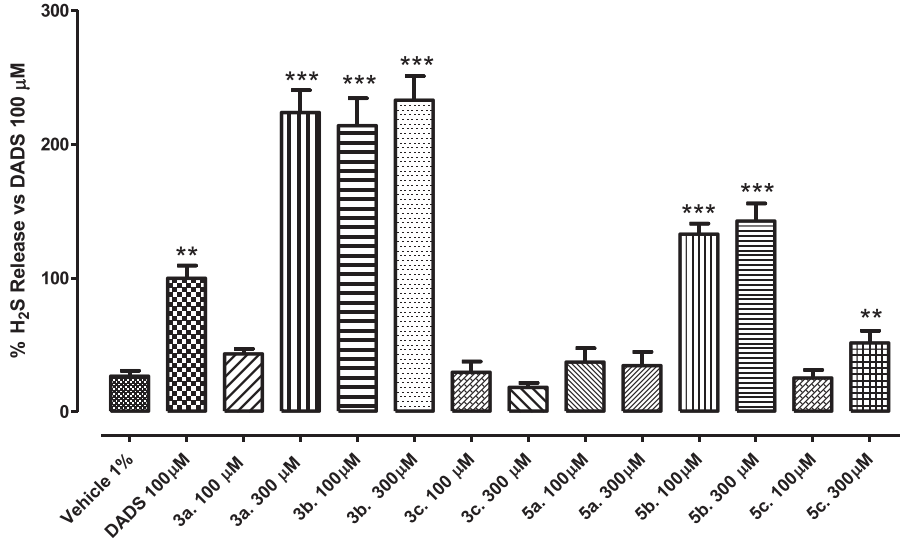


Fig. 2. Cumulative H_2S formation (expressed as area under the curve of the WSP-1 fluorescence in the recording time) produced by the incubation of vehicle or different concentrations of the tested compounds. Data were expressed as mean ± SEM setting DADS H_2S release as 100%. Three different experiments were carried out, each in triplicate. One-way ANOVA post-test Bonferroni has been applied to calculate the significance level. (**P < 0.01; ***P < 0.001).

decrease percentage (Table 1SI and Table 2SI). A SASA decrease higher than 25% corresponded to conformational families characterized by the steric inaccessibility of the isothiocyanate carbon from at least one side of the double bond (Fig. 3A).

It resulted that the most populated conformational family of compound **4b** (i.e., family ii; occurrence rate: 39%) presented a reduced steric accessibility of the isothiocyanate carbon (i.e., SASA decrease = 27%; Fig. 3A) while that of **5b** presented only 3% of SASA decrease (i.e., family ix; occurrence rate: 11%; Fig. 3B).

This trend is confirmed considering all the conformers within 5 kcal/mol from GM. Indeed, compound **4b** presented 69% of conformers within 5 kcal/mol from GM with a SASA decrease >25% (Table 1SI). On the contrary, compound **5b** showed 92% of the conformers within 5 kcal/mol from GM with a SASA decrease for the isothiocyanate carbon atom <25% (Table 2SI).

The above results indicate that the substituents at positions 16 and 17 affect both the conformational freedom and the steric accessibility of the H_2S donor moiety, likely affecting its ability to react with L-Cys. Accordingly, the less hindered HPI hybrid obtained from triamcinolone acetonide (**5b**) resulted to be able to release a greater amount of H_2S with respect the corresponding HPI hybrid obtained from Betamethasone 17-valerate (**4b**) (Table 1).

Finally, the higher ability to release H_2S of compound **5b** with respect to the "free" H_2S donor HPI could be explained considering that H_2S formation rate is dependent on the nature of the substituent bound to the isothiocyanate group and a strong electron-

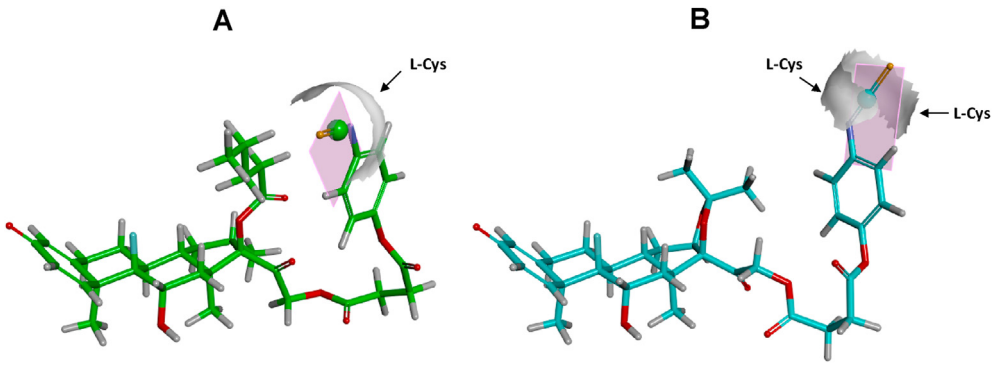


Fig. 3. Lowest energy conformers of the most populated conformational families of compound **4b** (green, A; SASA decrease = 27%) and **5b** (cyan, B; SASA decrease = 3%). The calculated solvent accessible surface area (SASA) of the isothiocyanate carbon atom is shown as grey transparent surface. The plane of the isothiocyanate group is displayed and coloured in pink. The isothiocyanate carbon atom is evidenced as ball. The compounds are coloured by atom type (O: red, N: blue, S: yellow and H: white). The putative L-Cys approach from one side (A) or both sides (B) of the double bond plane is indicated by a black arrow.

donating group, such as the hydroxyl group, increases the electronic density on the isothiocyanate group thus reducing its reactivity [40].

3.4. Inhibition of mast cell degranulation

FcεRI–receptor activation triggers mast cell degranulation by promoting a tyrosine kinase cascade. Interestingly, Mxarino et al. [42] showed that H_2S releasing compounds reduce the antigen-induced RBL-2H3 degranulation by targeting the downstream proteins of the FcεRI pathway.

Accordingly, we next investigated the inhibitory effects of HPI and of its hybrid compounds **4b** and **5b** and the relative native drugs (Bethametasone 17-valerate and Triamcinolone acetonide) in an antigen-mediated mast cell degranulation model. These compounds have been selected considering their clear and satisfactory ability to donate H_2S inside the cells.

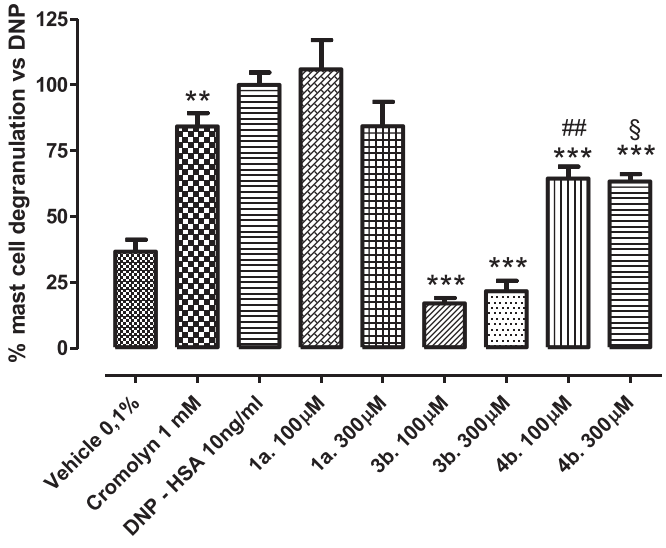


Fig. 4. Effect on RBL-2H3 degranulation. Cells were sensitized with DNP-IgE and after 24h incubated for 5 min with the tested compounds, cromolyn (1 mM) or the vehicle (DMSO 0.1%). Cell degranulation was induced by DNP. Triton-X-100 was added, to elicit cell lysis and exhaustive release of β-HEX (data not sown). The release of β-HEX was measured at 405 nm. One-way ANOVA plus Bonferroni has been used as statistical analysis. * indicates significant differences vs DNP-HSA setted as 100% (** = P < 0.01; *** = P < 0.001); § indicates significant differences vs **1a** 300 µM (§ = P < 0.01); # indicates significant differences vs **1a** 100 µM (## = P < 0.01).

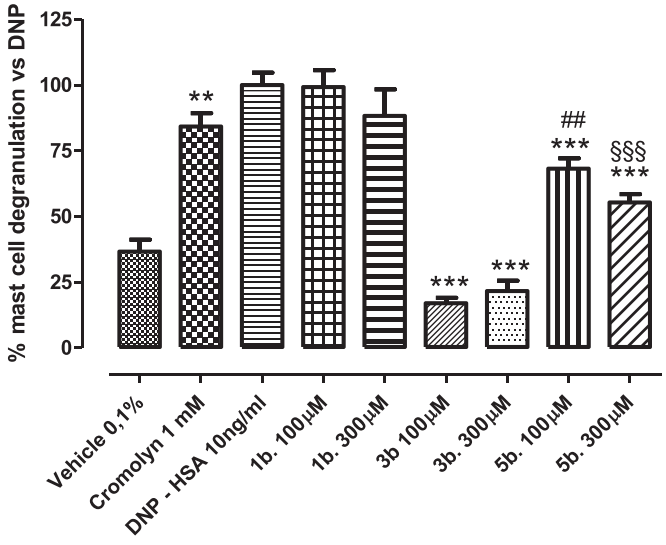


Fig. 5. Effect on RBL-2H3 degranulation. Cells were sensitized with DNP-IgE and after 24h incubated for 5 min with the tested compounds, cromolyn (1 mM) or the vehicle (DMSO 0.1%). Cell degranulation was induced by DNP. Triton-X-100 was added, to elicit cell lysis and exhaustive release of β-HEX (data not sown). The release of β-HEX was measured at 405 nm. One-way ANOVA plus Bonferroni has been used as statistical analysis. * indicates significant differences vs DNP-HSA setted as 100% (** = P < 0.01; *** = P < 0.001); § indicates significant differences vs **1b** 300 µM (§§§ = P < 0.001); # indicates significant differences vs **1b** 100 µM (## = P < 0.01).

The addition of the antigen DNP-HSA to pre-sensitized RBL-2H3 cells caused a significant degranulation highlighted as β-hexosaminidase release confirming the activation of FcεRI pathway (Figs. 4 and 5). Cromolyn has been chosen as reference compound whose inhibitory effect on mast cell degranulation is well documented in the literature, being a clinically used drug for the treatment of allergic asthma [43]. Indeed, cromolyn stabilizes the mast cell membrane, preventing their degranulation and reducing the consequent release of histamine. In DNP-HAS activated RBL-2H3 cells, cromolyn significantly inhibited β-hexosaminidase release by 20%. The treatment with HPI 100 µM and 300 µM promoted a remarkable and impressive inhibition of the RBL-2H3 degranulation induced by DNP-HSA, reducing β-hexosaminidase release by about 75%.

In contrast, betamethasone 17-valerate (Fig. 4) and triamcinolone acetonide (Fig. 5) exhibit only a mild and not significant inhibitory activity on the degranulation induced DNP-HSA if

incubated 300 μM. The effect of long-lasting treatment with corticosteroid on mast cell degranulation is related to post-transcriptional mechanism leading to reduction of the expression of FcεRI on the surface of mast cell membrane as reported by Yamaguchi and colleagues [44]. In the model used in this paper, corticosteroid are incubated 5 min before the antigenic stimulus, probably not enough to promote those intracellular modifications responsible for the pharmacological activity of corticosteroid drugs.

Contrarily, the hybrid compounds **4b** (Figs. 4) and **5b** (Fig. 5) of 100 and 300 μM showed a significant inhibitory effect on mast cell degranulation, reducing β-hexosaminidase release more efficiently than the corresponding native drugs.

These results suggest that the molecules able to release H_2S (HPI and compounds **4b** and **5b**), reduce the activation of FcεRI receptor directly and rapidly acting on the molecular target, rather than involving intracellular modifications. Merging the H_2S-donor moiety to the corticosteroid drugs could lead to a double time-dependent pharmacodynamic activity: H_2S could promote a fast inhibition of mast cell degranulation since its target it's the already expressed FcεRI receptor, while the native corticosteroid drug could reduce the expression of the receptor contributing to the lately beneficial effect for the treatment of allergic pathologies.

3.5. Bronchial smooth muscle cell membrane hyperpolarizing effect

The relaxant activity of H_2S is mediated by different mechanisms but the activation of bronchial ATP-sensitive potassium (K_{ATP}) channels seems to play a relevant role.

In this view, it was interesting to evaluate the effects of the moiety HPI and of hybrids **4b** and **5b** on the membrane potential of cultured BSMCs taking 1,3−dihydro−1- [2-hydroxy−5-(trifluoromethyl)phenyl]−5-(trifluoromethyl)-2H−benzimidazole−2-one (NS1619), a well-known potassium channel activator, as reference hyperpolarizing agent [45]. HPI showed a significant hyperpolarizing effect compared to vehicle, but assisting to lower values if compared to the NS1619-induced hyperpolarizing effect.

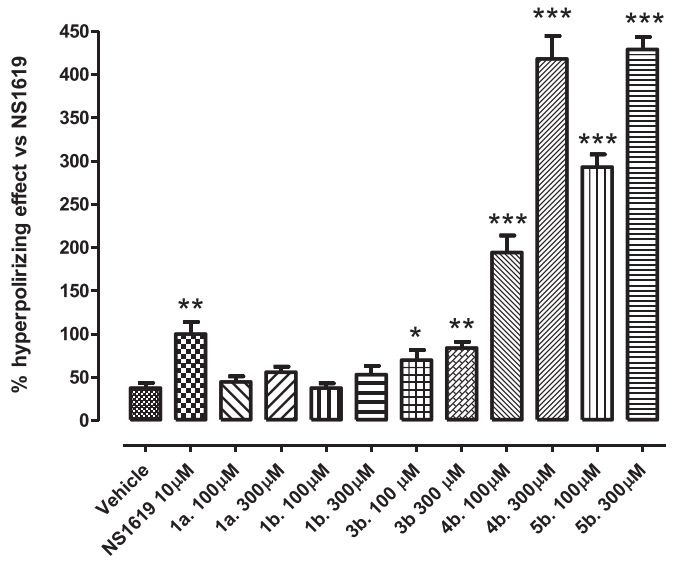


Fig. 6. − Hyperpolarizing effect of the tested compounds on BSMCs membrane. The graph shows the hyperpolarizing effect of the tested compounds on cell membrane of BSMCs; effects are expressed as % of the hyperpolarization evoked by the reference compound NS1619. Data are expressed as mean ± SEM. Six different experiments were performed each in six replicates. One-way ANOVA post-test Bonferroni has been applied to calculate the significance level (*P < 0.05, **P < 0.01,***P < 0.001).

Interestingly, both the hybrid compounds **4b** and **5b** tested at 100 and 300 μM caused a significant and massive membrane hyperpolarization of BSMCs (Fig. 6) by 4-fold higher compared to NS1619 when incubated at the maximal concentration. Contrarily, the two native drugs caused a slight but not significant cell membrane hyperpolarization, indicating that this effect is mainly due to the addition of the H_2S donor moiety to the corticosteroid drugs.

Looking at the results of the intracellular donation of H_2S, the HPI moiety resulted to be a stronger H_2S-donor if compared to the corresponding H_2S-hybrid glucocorticoids. Surprisingly, the results about the hyperpolarizing efficacy followed an opposite trend, since the hybrid molecules exerted a more efficient effect. The hypothesis which may explain this marked difference between the hyperpolarizing effect of HPI and both the hybrid derivatives could be addressed to the direct airway relaxant activity of some glucocorticoids. Indeed, Nabishah and colleagues [46] reported that rats treated with either dexamethasone or cortisone for 7 days showed a reduced contractile response of isolated bronchial smooth muscle preparations to acetylcholine. Another research investigated the effect of glucocorticoids changes in intracellular Ca^{2+} concentration in airway smooth muscle cells and demonstrated that 24 h-pretreatment with dexamethasone reduced the subsequent calcium-mobilizing response to bradykinin [47]. Furthermore, Schramm and Grunstein [48] demonstrated that methylprednisolone increases rabbit airway smooth muscle relaxation by potentiating the electrogenic Na^+/K^+ ATPase pump.

The tested H_2S-donor hybrid molecules, being made up by the H_2S-donor portion which is able to open different classes of potassium channels and by the “native” corticosteroid drug, may promote a marked hyperpolarizing effect because of these different mechanism of action.

4. Conclusion

In this work, novel H_2S-donor hybrid corticosteroids have been developed for merging the beneficial effect of H_2S on the respiratory tract to the anti-inflammatory pharmacological effect of corticosteroids. In particular, we demonstrated that the hybridization process led to the synthesis of compounds able to release H_2S both in aqueous solution containing free thiols and when incubated with BSMCs. Although all the compounds showed appreciable H_2S-releasing properties in presence of biological substrates, some differences regarding the amount of the generation of H_2S emerged and the HPI hybrid **5b** resulted the most active one. Molecular modelling studies allowed to demonstrate that the steric accessibility of the isothiocyanate moiety is responsible for the different H_2S releasing properties observed for the two hybrids **4b** and **5b**, characterized by the presence of the Betamethasone 17-valerate and Triamcinolone acetonide moiety, respectively.

Ashma symptoms are often related to a massive mast cell degranulation, leading to the increase of pro-inflammatory mediators which contribute to the hypercontractility of bronchial smooth muscles. Interestingly, the two HPI hybrid molecules **4b** and **5b** promoted significant reduction of mast cell degranulation and hyperpolarization of BSMCs in *in vitro* models, which can be viewed as an enrichment of the pharmacological activity of the native drugs.

The idea of preparing H_2S-corticosteroid hybrids to be employed in the treatment of respiratory tract diseases such as asthma can be considered an interesting therapeutic strategy. Indeed, adding the H_2S releasing moiety provides further pharmacological effects to native drugs and more importantly could prevent those side effect linked to long-lasting treatment with corticosteroid drugs.

The effects described using *in vitro* models should be further strengthen by *in vivo* models, but these preliminary data may

already pave the way for exploiting the hybridization process with H_2S-donor moieties for increasing the pharmacological activity of already employed drugs or counteracting specific side effects.

Future studies are planned in order to investigate the potential use of the new hybrids as *in vivo* prodrugs including the investigation of the stability at differing pH values and in human serum.

5. Experimental section

5.1. Materials and methods

Betamethasone 17-valerate and Triamcinolone acetonide were purchased from Carbosynth Ltd (UK); all the reagents, solvents or other chemicals were obtained from Merck. Reactions were stirred at 400 rpm by Heidolph MR Hei-Standard magnetic stirrer. Solutions were concentrated with a Buchi R-114 rotary evaporator at low pressure. All reactions were followed by TLC carried out on Merck silica gel 60 F254 plates with fluorescent indicator on the plates and were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Melting points, determined using a Buchi Melting Point B-540 instrument, are uncorrected and represent values obtained on re-crystallized or chromatographically purified material. Mass spectra of final products were performed on LTQ Orbitrap XL™ Fourier transform mass spectrometer (FTMS) equipped with an ESI ION MAX™ (Thermo Fisher, San José, USA) source operating in positive mode. Elemental analyses were carried out on Carlo Erba model 1106; analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. All spectra were recorded in DMSO-d_6. Chemical shifts are reported in ppm. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), dd (double doublet), t (triplet), m (multiplet), bs (broad singlet).

5.2. General procedure for the synthesis of 21-hemisuccinate glucocorticoids

5.2.1. Betamethasone17-valerate-21-succinate (2a)

1.0g (2.10 mmol) of betamethasone 17-valerate **1a** was dissolved in anhydrous pyridine (30 mL) and 0.63g (6.30 mmol) of succinic anhydride and 25 mg (0.21 mmol, 1 eq) of DMAP were then added. After being stirred overnight at room temperature, the mixture was evaporated under reduced pressure. The resulting residue was then treated with 20 mL of water and the mixture was stirred for 20 min and then centrifuged. The obtained precipitate was washed again with H_2O and filtered. Product **2a** (1.12 g) was obtained as a white powder. Yield 93%. m.p. 191.1–192.5 °C.

^{1}H NMR (DMSO-d_6) δ 12.26 (bs, 1H, COOH), 7.27 (d, J = 10.1, 1H), 6.22 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.03 (bs, 1H), 5.53 (d, J = 4.3, 1H), 4.70 (d, J = 16.8, 1H), 4.48 (d, J = 16.8, 1H), 4.22 (m, 1H), 2.63 (t, J = 5.8, 2H), 2.61–2.58 (m, overlapped, 1H), 2.49 (t, J = 5.8, 2H), 2.39 (m, 2H), 2.35 (m, 1H), 2.23 (m, 1H), 2.07 (m, 1H), 1.88–1.85 (m, 2H), 1.71 (d, J = 13.3, 2H), 1.52 (m, overlapped, 2H), 1.50 (s, 3H), 1.38–1.36 (m, 1H), 1.35 (t, overlapped, J = 7.4, 3H), 1.27–1.22 (m, overlapped, 7H), 1.12–1.06 (m, 1H), 0.86–0.81 (m, overlapped, 6H). ^{13}C NMR (DMSO-d_6) δ 14.03, 16.66, 20.01, 22.12, 23.41 (d, $^3J_{C\text{-}F}$ = 5.4, C-18), 26.53, 28.03, 28.83, 29.34, 30.63, 33.42 (d, $^2J_{C\text{-}F}$ = 19.2, C-8), 34.15, 34.88, 36.66, 46.65, 47.87, 48.20 (d, $^2J_{C\text{-}F}$ = 22.8, C-10), 68.11, 70.50 (d, $^2J_{C\text{-}F}$ = 35.6, C-11), 93.89, 101.39 (d, $^1J_{C\text{-}F}$ = 175.5, C-9), 124.60, 129.50, 152.96, 167.17, 171.74, 173.60, 174.41, 185.70, 198.70. ESI-MS $[M+H]^+$ *m/z* calc. 576.65 for $C_{31}H_{41}FO_9$ found 577.2.

5.2.2. Triamcinolone acetonide-21-succinate (2b)

The desired product **2b** has been obtained following the same procedure reported for compound **2a** starting from 1.0 g (2.30 mmol) of triamcinolone acetonide **1b**, 0.69g (6.90 mmol) of succinic anhydride and 28 mg (0.23 mmol, 0.1 eq.) of DMAP. White powder (yield 1.15g, 93%). m.p. 183.2–184.6 °C.

^{1}H NMR (DMSO-d_6) δ 12.36 (bs, 1H, COOH), 7.29 (d, *J* = 10.1 Hz, 1H), 6.24 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.02 (s, 1H), 5.48 (d, *J* = 3.9 Hz, 1H), 5.16 (d, J = 17.8, 1H), 4.87 (d, J = 5.1, 1H), 4.76 (d, J = 17.8, 1H), 4.20 (m, 1H), 2.68 (t, J = 5.8, 2H), 2.64 (m, 1H), 2.52 (t, J = 5.8, 2H), 2.50–2.45 (m, overlapped, 2H), 2.34 (dd, J = 13.5, J = 3.6, 1H), 2.03 (d, J = 13.5, 1H), 1.94 (m, 1H), 1.82 (m, 1H), 1.72 (d, J = 12.9, 1H), 1.59 (m, 1H), 1.54 (m, 1H), 1.49 (s, 3H, CH_3), 1.37 (m, overlapped, 1H), 1.35 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 0.83 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ 16.52, 23.29 (d, $^3J_{C\text{-}F}$ = 5.6, C-18), 25.89, 26.72, 27.84, 28.80, 29.09, 30.50, 30.90, 32.89 (d, $^2J_{C\text{-}F}$ = 19.5, C-8), 33.51, 36.26, 43.00, 45.55, 48.16 (d, $^2J_{C\text{-}F}$ = 22.4, C-10), 67.62, 70.54 (d, $^2J_{C\text{-}F}$ = 38.3, C-11), 81.67, 97.48, 101.43 (d, $^1J_{C\text{-}F}$ = 176.5, C-9), 111.35, 124.72, 129.57, 152.70, 167.05, 172.05, 173.61, 186.35, 203.49. ESI-MS $[M+H]^+$ *m/z* calcd 534,57for $C_{28}H_{35}FO_9$; found 535.2.

5.3. Synthesis of compounds 4a-4d and 5a-5d

5.3.1. 4-Carbamothioylphenyl-(2-((9R,10S,11S,13S,16S,17R)-9-fluoro-11-hydroxy-10,13,16-trimethyl-3-oxo-17-(pentanoyloxy)-6,7,8,9,10,11,12,1314,15,16,17-dodecahydro-3H-cyclopenta[a] phenanthren-17-yl)-2-oxoethyl) succinate

5.3.1.1. (Betamethasone-17-valerate-21-succinate-TBZ, 4a). To a solution of **2a** (1.0 g, 1.7 mmol) in anhydrous tetrahydrofuran (30 mL), 4-hydroxythiobenzamide (TBZ, **3a**, 0.26 g, 1.7 mmol) and DMAP (0.02 g, 0.17 mmol) were added. The reaction mixture was kept on ice bath stirring under nitrogen for 10 min and EDC (0.49 g, 2.85 mmol) was then added. The mixture was stirred under nitrogen atmosphere at room temperature for 12 h. The solvent was evaporated and the residue was purified by silica gel column chromatography (ethyl acetate/n-hexane, 6/4, v/v). Compound **4a** was crystallized from diethyl ether/n-hexane (1/1, v/v). Yellow powder. Yield 0.87 g, 72%. m.p. 120.3–122.1 °C.

^{1}H NMR (DMSO-d_6) δ 9.87 (bs, 1H, NH_2), 9.50 (bs, 1H, NH_2), 7.91 (d, J = 8.6, 2H), 7.28 (d, J = 10.2, 1H), 7.15 (d, J = 8.6, 2H), 6.22 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.00 (bs, 1H), 5.53 (d, J = 4.3, 1H), 4.74 (d, J = 16.7, 1H), 4.53 (d, J = 16.7, 1H), 4.22 (m, 1H), 2.88 (t, J = 5.8, 2H), 2.80 (t, J = 5.8, 2H), 2.61–2.58 (m, 1H), 2.39 (m, 2H), 2.35 (m, 1H), 2.25 (m, 1H), 2.08 (m, 1H), 1.87–1.83 (m, 2H), 1.74 (d, J = 13.2, 2H), 1.52 (m, overlapped, 2H), 1.50 (s, 3H), 1.38–1.36 (m, 1H), 1.35 (t, d = 7.4, 3H), 1.27–1.22 (m, overlapped, 7H), 1.12–1.06 (m, 1H), 0.86–0.81 (m, overlapped, 6H). ^{13}C NMR (DMSO-d_6) δ 14.03, 16.66, 20.01, 22.12, 23.41 (d, $^3J_{C\text{-}F}$ = 5.4, C-18), 26.53, 28.03, 28.83, 29.34, 30.63, 33.42 (d, $^2J_{C\text{-}F}$ = 19.2, C-8), 34.15, 34.88, 36.66, 46.65, 47.87, 48.20 (d, $^2J_{C\text{-}F}$ = 22.8, C-10), 68.11, 70.50 (d, $^2J_{C\text{-}F}$ = 35.6, C-11), 93.89, 101.43 (d, $^1J_{C\text{-}F}$ = 175.8, C-9), 121.63, 124.60, 129.19, 129.50, 137.50, 152.96, 153.07, 167.21, 170.93, 171.74, 174.41, 185.70, 198.80, 199.50. ESI-MS $[M+H]^+$ *m/z* calc. 711.28 for $C_{38}H_{46}FNO_9S$ found 712.0.

5.3.2. 2-((9R,10S,11S,13S,16S,17R)-9-fluoro-11-hydroxy-10,13,16-trimethyl-3-oxo-17-(pentanoyloxy)-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta-[a]phenanthren-17-yl)-2-oxoethyl (4-isothiocyanatophenyl) succinate

5.3.2.1. (Betamethasone-17-valerate-21-succinate-HPI, 4b). Compound **4b** has been obtained following the same procedure reported for **4a** starting from **2a** (1.0g, 1.7 mmol) and 4-hydroxyphenyl isothiocyanate (HPI, **3b**, 0.26 g, 1.7 mmol). The residue was purified by silica gel column chromatography ($CHCl_3$/ethyl acetate, 8/2, v/v). The obtained compound **4b** was crystallized from n-hexane. White solid. Yield 0.80 g, 66%. m.p. 89.5–92.0 °C. ^{1}H NMR (DMSO-d_6) δ 7.48 (d, J = 8.6, 2H), 7.28 (d, J = 10.2, 1H), 7.12 (d, J = 8.6, 2H), 6.22 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.00 (s, 1H), 5.53 (d, J = 4.3,

1H), 4.74 (d, J = 16.7, 1H), 4.53 (d, J = 16.7, 1H), 4.22 (m, 1H), 2.88 (t, J = 5.8, 2H), 2.80 (t, J = 5.8, 2H), 2.61–2.58 (m, 1H), 2.39 (m, 2H), 2.35 (m, 1H), 2.25 (m, 1H), 2.08 (m, 1H), 1.87–1.83 (m, 2H), 1.74 (d, J = 13.2, 2H), 1.52 (m, overlapped, 2H), 1.50 (s, 3H), 1.38–1.35 (m, 1H), 1.27–1.22 (m, overlapped, 7H), 1.12–1.06 (m, 1H), 0.86–0.81 (m, overlapped, 6H). ^{13}C NMR (DMSO-d_6) δ 14.02 16.65, 19.99, 22.10, 23.40 (d, $^{3}J_{C-F}$ = 5.4, C-18), 26.53, 28.04, 28.82, 29.30, 30.63, 33.40 (d, $^{2}J_{C-F}$ = 19.1, C-8), 34.14, 34.87, 36.65, 43.70, 46.65, 47.85, 48.16 (d, $^{2}J_{C-F}$ = 22.6, C-10), 68.05, 70.42 (d, $^{2}J_{C-F}$ = 35.2, C-11), 93.86, 101.37 (d, $^{1}J_{C-F}$ = 175.6, C-9), 123.77, 124.59, 127.62, 128.00, 129.43, 134.13, 159.78, 153.97, 167.17, 170.91, 171.73, 174.48, 185.60, 198.88. ESI-MS $[M+H]^+$ *m/z* calc. 709.82 for $C_{38}H_{46}FNO_9S$ found 710.6.

5.3.3. 2-((9R,10S,11S,13S,16S,17R)-9-fluoro-11-hydroxy-10,13,16-trimethyl-3-oxo-17-(pentanoyloxy)-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

5.3.3.1. (Betamethasone-17-valerate-21-succinate-ADT, 4c). Compound **4c** has been obtained following the same procedure reported for **4a** starting from **2a** (1.0 g, 1.7 mmol) and 5-(*p*-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH, **3c**, 0.39g, 1.7 mmol). The obtained residue was purified by silica gel column chromatography (ethyl acetate/n-hexane, 6:4, v/v). Compound **4c** was crystallized from n-hexane. Orange powder. Yield 0.81g, 61%. m.p. 105.5–107.0 °C. ^{1}H NMR (DMSO-d_6) δ 7.97 (d, J = 8.6, 2H), 7.83 (s, 1H), 7.32 (d, J = 8.6, 2H), 7.29 (d, J = 10.1, 1H), 6.24 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.02 (s, 1H), 5.56 (d, J = 4.3, 1H), 4.74 (d, J = 16.7, 1H), 4.53 (d, J = 16.7, 1H), 4.22 (m, 1H), 2.88 (t, J = 5.8, 2H), 2.80 (t, J = 5.8, 2H), 2.61–2.58 (m, 1H), 2.39 (m, 2H), 2.35 (m, 1H), 2.25 (m, 1H), 2.08 (m, 1H), 1.87–1.83 (m, 2H), 1.74 (d, J = 13.2, 2H), 1.52 (m, overlapped, 2H), 1.50 (s, 3H), 1.38–1.35 (m, 1H), 1.27–1.22 (m, overlapped, 7H), 1.12–1.06 (m, 1H), 0.86–0.81 (m, overlapped, 6H). ^{13}C NMR (DMSO-d_6) δ 14.02, 16.66, 20.00, 22.11, 23.42 (d, $^{3}J_{C-F}$ = 5.4, C-18), 26.54, 28.03, 28.83, 29.38, 30.63, 33.43 (d, $^{2}J_{C-F}$ = 19.2, C-8),34.15, 34.87, 36.66, 43.69, 46.65, 47.87, 48.18 (d, $^{2}J_{C-F}$ = 22.6, C-10), 68.11, 70.50 (d, $^{2}J_{C-F}$ = 35.3, C-11), 93.89, 101.38 (d, $^{1}J_{C-F}$ = 175.8, C-9), 123.49, 124.63, 129.08, 129.31, 129.50, 136.26, 152.95, 153.85, 167.21, 170.86, 171.77, 173.15, 174.38, 185.64, 198.81, 215.96. ESI-MS $[M+H]^+$ *m/z* calc. 784.22 for $C_{38}H_{46}FNO_9S$ found 785.1.

5.3.4. 4-((ethylthio)carbonothioyl)phenyl-(2-((9R,10S,11S,13S,16S,17R)-9-fluoro-11-hydroxy-10,13,16-trimethyl-3-oxo-17-(pentanoyloxy)-6,7,8,9,10,11,12,13,14,15, 16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl) succinate

5.3.4.1. (Betamethasone-17-valerate-21-succinate-HBTA, 4d). Compound **4d** has been obtained following the same procedure reported for **4a** starting from **2a** (1g, 1.7 mmol) and ethyl 4-hydroxybenzodithioate (HBTA, **3d**, 0.34 g, 1.7 mmol). The residue was purified by silica gel column chromatography ($CHCl_3$/ethyl acetate, 9/1, v/v). The obtained compound **4d** was crystallized from n-hexane. Pink powder. Yield 0.81g, 63%. m.p. 103.0–104.5 °C. ^{1}H NMR (DMSO-d_6) δ δ 8.01 (d, J = 8.6, 2H), 7.28 (d, J = 10.2, 1H), 7.24 (d, J = 8.6, 2H), 6.24 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.02 (s, 1H), 5.57 (d, J = 4.3, 1H), 4.74 (d, J = 16.7, 1H), 4.53 (d, J = 16.7, 1H), 4.22 (m, 1H), 3.39 (q, J = 7.4, 2H), 2.88 (t, J = 5.8, 2H), 2.80 (t, J = 5.8, 2H), 2.39 (m, 2H), 2.35 (m, 1H), 2.25 (m, 1H), 2.08 (m, 1H), 1.87–1.83 (m, 2H), 1.74 (d, J = 13.2, 2H), 1.52 (m, overlapped, 2H), 1.50 (s, 3H), 1.38–1.36 (m, 1H), 1.35 (t, d = 7.4, 3H), 1.27–1.22 (m, overlapped, 7H), 1.12–1.06 (m, 1H), 0.86–0.81 (m, overlapped, 6H).^{13}C NMR (DMSO-d_6) δ 12.60, 14.03, 16.64, 19.98, 22.10, 23.40 (d, $^{3}J_{C-F}$ = 5.4, C-18), 26.54, 28.02, 28.83, 29.36, 30.63, 31.63, 33.43 (d, $^{2}J_{C-F}$ = 19.2, C-8), 34.15, 34.88, 36.67, 43.69, 46.64, 47.87, 48.18 (d, $^{2}J_{C-F}$ = 22.6, C-10), 68.08, 70.50 (d, $^{2}J_{C-F}$ = 35.3, C-11), 93.89, 101.38 (d, $^{1}J_{C-F}$ = 175.8, C-9), 122.52, 124.54, 128.28, 129.51, 142.44, 152.81, 154.48, 167.15, 170.80, 171.75, 174.32, 185.67, 198.79. ESI-MS $[M+H]^+$ *m/z* calc. 757.94 for $C_{38}H_{46}FNO_9S$ found 760.2.

5.3.5. 4-Carbamothioylphenyl-(2-((6aS,6bR,7S,8aS,8bS,11aR)-6b-fluoro-7-hydroxy-6a,8a,10,10-tetramethyl-4-oxo-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho [2′,1′:4,5]indeno[1,2-d] [1,3]dioxol-8b-yl)-2-oxoethyl) succinate

5.3.5.1. (Triamcinolone acetonide-21-succinate-TBZ, 5a). To a solution of **2b** (1g, 1.9 mmol) in anhydrous tetrahydrofuran (30 mL), 4-hydroxythiobenzamide (TBZ, **3a**, 0.29 g, 1.9 mmol) and DMAP (0.02 g, 0.19 mmol) were added. The reaction mixture was kept on ice bath stirring under nitrogen for 10 min and EDC (0.49 g, 2.85 mmol) was then added. The mixture was stirred under nitrogen atmosphere at room temperature for 12 h. The solvent was evaporated and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 9/1, v/v). Compound **5a** was crystallized from diethyl ether/n-hexane (1/1, v/v). Yellow powder. Yield 0.69g, 54%. m.p. 179.0–181.0 °C. ^{1}H NMR (DMSO-d_6) δ 9.88 (bs, 1H, NH_2), 9.51 (bs, 1H, NH_2), 7.92 (d, J = 8.8, 2H), 7.28 (d, *J* = 10.1 Hz, 1H), 7.14 (d, J = 8.8, 2H), 6.23 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.01 (s, 1H), 5.49 (d, *J* = 3.9 Hz, 1H), 5.21 (d, J = 17.8, 1H), 4.88 (d, J = 5.1, 1H), 4.82 (d, J = 17.8, 1H), 4.21 (m, 1H), 2.91 (t, J = 5.8, 2H), 2.85 (t, J = 5.8, 2H), 2.64 (m, 1H), 2.50–2.45 (m, overlapped, 2H), 2.34 (dd, J = 13.5, J = 3.6, 1H), 2.06 (d, J = 13.5, 1H), 1.96 (m, 1H), 1.83 (m, 1H), 1.73 (d, J = 12.9, 1H), 1.59 (m, 1H), 1.54 (m, 1H), 1.50 (s, 3H, CH_3), 1.37 (m, overlapped, 1H), 1.36 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 0.83 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ 16.44, 22.99 (d, $^{3}J_{C-F}$ = 5.6, C-18), 25.75, 26.47, 27.54, 28.68, 29.30, 30.88, 33.16 (d, $^{2}J_{C-F}$ = 19.5, C-8), 33.60, 37.26, 43.00, 45.55, 48.11 (d, $^{2}J_{C-F}$ = 22.4, C-10), 65.83, 67.93, 71.79 (d, $^{2}J_{C-F}$ = 38.3, C-11), 81.87, 97.48, 100.03 (d, $^{1}J_{C-F}$ = 176.5, C-9), 111.59, 121.43, 122.64, 125.17, 128.46, 129.87, 133.65, 136.70, 151.81, 153.29, 165.76, 170.45, 171.70, 186.35, 201.30, 203.49. ESI-MS $[M+H]^+$ *m/z* calcd 670.76 for $C_{35}H_{40}FNO_9S$; found 670.9.

5.3.6. 2-((6aS,6bR,7S,8aS,8bS,11aR)-6b-fluoro-7-hydroxy-6a,8a,10,10-tetramethyl-4-oxo-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho-[2′,1′:4,5]-indeno[1,2-d] [1,3]dioxol-8b-yl)-2-oxoethyl (4-isothiocyanatophenyl) succinate

5.3.6.1. (Triamcinolone acetonide-21-succinate-HPI, 5b). Compound **5b** has been obtained following the same procedure reported for **5a** starting from **2b** (1.0 g, 1.9 mmol) and 4-hydroxyphenyl isothiocyanate (HPI, **3b**, 0.29 g, 1.9 mmol). The residue was purified by silica gel column chromatography ($CHCl_3$/ethyl acetate, 7/3, v/v). Compound **5b** was crystallized from n-hexane. White solid. Yield 0.66g, 52%. m.p. 206–207 °C. ^{1}H NMR (DMSO-d_6) δ 7.48 (d, J = 8.8, 2H), 7.26 (d, *J* = 10.1 Hz, 1H), 7.12 (d, J = 8.8, 2H), 6.21 (dd, *J* = 10.1, 1.8 Hz, 1H), 5.99 (s, 1H), 5.47 (d, *J* = 3.9 Hz, 1H), 5.16 (d, J = 17.8, 1H), 4.83 (d, J = 5.1, 1H), 4.77 (d, J = 17.8, 1H), 4.17 (m, 1H), 2.85 (t, J = 5.8, 2H), 2.81 (t, J = 5.8, 2H), 2.60 (m, 1H), 2.47–2.43 (m, overlapped, 2H), 2.30 (dd, J = 13.5, J = 3.6, 1H), 2.02 (d, J = 13.5, 1H), 1.90 (m, 1H), 1.78 (m, 1H), 1.68 (d, J = 12.9, 1H), 1.55 (m, 1H), 1.51 (m, 1H), 1.46 (s, 3H, CH_3), 1.33 (m, overlapped, 1H), 1.32 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 0.80 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ 16.50, 23.25 (d, $^{3}J_{C-F}$ = 5.6, C-18), 25.79, 26.74, 27.93, 28.74, 29.25, 30.53, 32.85 (d, $^{2}J_{C-F}$ = 19.5, C-8), 33.46, 36.41, 43.25, 45.61, 48.17 (d, $^{2}J_{C-F}$ = 22.3, C-10), 67.80, 70.64 (d, $^{2}J_{C-F}$ = 36.6, C-11), 81.56, 97.54, 101.44 (d, $^{1}J_{C-F}$ = 176.5, C-9), 111.33, 123.70, 124.69, 127.61, 127.97, 129.47, 134.18, 149.81, 152.89, 167.00, 170.84, 171.72, 185.61, 203.78. ESI-MS $[M+H]^+$ *m/z* calcd 668.74 for $C_{35}H_{38}FNO_9S$; found 668.9.

5.3.7. 2-((6aS,6bR,7S,8aS,8bS,11aR)-6b-fluoro-7-hydroxy-6a,8a,10,10-tetramethyl-4-oxo-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho-[2′,1':4,5]-indeno[1,2-d] [1,3]dioxol-8b-yl)-2-oxoethyl (4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl) succinate (triamcinolone acetonide-21-succinate-ADT, 5c)

Compound **5c** has been obtained following the same procedure reported for **5a** starting from **2b** (1.0 g, 1.9 mmol) and 5-(*p*-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH, **3c**, 0.43 g, 1.9 mmol). The obtained residue was purified by silica gel column chromatography (CH_2Cl_2/ethyl acetate, 8/2, v/v). Compound **5c** was crystallized from diethyl ether. Orange solid. Yield 0.83g, 59%. m.p. 222.0–224.0 °C. ^{1}H NMR (DMSO-d_6) δ 7.96 (d, J = 8.8, 2H), 7.81 (s, 1H), 7.28 (overlapped, 2H), 7.27 (overlapped, 1H), 6.22 (dd, *J* = 10.1, 1.8 Hz, 1H), 6.00 (s, 1H), 5.46 (d, *J* = 3.9 Hz, 1H), 5.18 (d, J = 17.8, 1H), 4.85 (d, J = 5.1, 1H), 4.79 (d, J = 17.8, 1H), 4.18 (m, 1H), 2.89 (t, J = 5.8, 2H), 2.83 (t, J = 5.8, 2H), 2.60 (m, 1H), 2.47–2.43 (m, overlapped, 2H), 2.31 (dd, J = 13.5, J = 3.6, 1H), 2.03 (d, J = 13.5, 1H), 1.92 (m, 1H), 1.80 (m, 1H), 1.70 (d, J = 12.9, 1H), 1.55 (m, 1H), 1.52 (m, 1H), 1.47 (s, 3H, CH_3), 1.33 (m, overlapped, 1H), 1.32 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ 16.51, 23.25 (d, $^3J_{C\text{-}F}$ = 5.6, C-18), 25.82, 26.76, 27.92, 28.76, 29.35, 30.54, 32.85 (d, $^2J_{C\text{-}F}$ = 19.5, C-8), 33.48, 36.43, 43.27, 45.62, 48.20 (d, $^2J_{C\text{-}F}$ = 22.3, C-10), 67.82, 70.67 (d, $^2J_{C\text{-}F}$ = 36.6, C-11), 81.57, 97.55, 101.40 (d, $^1J_{C\text{-}F}$ = 176.5, C-9), 111.35, 123.43, 124.69, 129.11, 129.27, 129.48, 136.24, 152.88, 153.81, 167.01, 170.77, 171.75, 173.12, 185.61, 203.77, 215.92. ESI-MS $[M+H]^+$ *m/z* calcd 743.89 for $C_{37}H_{39}FO_9S_3$; found 744.3.

5.3.8. 4-((ethylthio)carbonothioyl)phenyl-(2-((6aS,6bR,7S,8aS,8bS,11aR)-6b-fluoro-7-hydroxy-6a,8a,10,10-tetramethyl-4-oxo-2,4,6a,6b,7,8,8a,8b,11a,12,12a,12b-dodecahydro-1H-naphtho[2′,1':4,5]indeno[1,2-d] [1,3]dioxol-8b-yl)-2-oxoethyl) succinate (triamcinolone acetonide-21-succinate-HBTA, 5d)

Compound **5d** has been obtained following the same procedure above reported for **5a** starting from **2b** (1.0 g, 1.9 mmol) and ethyl 4-hydroxybenzodithioate (HBTA, **3d**, 0.38 g, 1.9 mmol). The residue was purified by silica gel column chromatography (ethyl acetate/n-hexane, 6/4, v/v). Compound **5d** was crystallized from n-hexane. Pink solid. Yield 0.76 g, 56%. m.p. 179.0–180.0 °C. ^{1}H NMR (DMSO-d_6) 8.00 (d, J = 8.8, 2H), 7.26 (d, *J* = 10.1 Hz, 1H), 7.22 (d, J = 8.8, 2H), 6.21 (dd, *J* = 10.1, 1.8 Hz, 1H), 5.99 (s, 1H), 5.46 (d, *J* = 3.9 Hz, 1H), 5.18 (d, J = 17.8, 1H), 4.85 (d, J = 5.1, 1H), 4.79 (d, J = 17.8, 1H), 4.18 (m, 1H), 3.35 (q, J = 7.4, 2H), 2.89 (t, J = 5.8, 2H), 2.83 (t, J = 5.8, 2H), 2.60 (m, 1H), 2.47–2.43 (m, overlapped, 2H), 2.31 (dd, J = 13.5, J = 3.6, 1H), 2.03 (d, J = 13.5, 1H), 1.92 (m, 1H), 1.80 (m, 1H), 1.70 (d, J = 12.9, 1H), 1.55 (m, 1H), 1.52 (m, 1H), 1.47 (s, 3H, CH_3), 1.33 (m, overlapped, 4H), 1.32 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ 12.57, 16.50, 23.26 (d, $^3J_{C\text{-}F}$ = 5.6, C-18), 25.81, 26.76, 27.93, 28.74, 29.32, 30.53, 31.61, 32.86 (d, $^2J_{C\text{-}F}$ = 19.5, C-8), 33.48, 36.42, 43.26, 45.62, 48.18 (d, $^2J_{C\text{-}F}$ = 22.3, C-10), 67.81, 70.63 (d, $^2J_{C\text{-}F}$ = 36.6, C-11), 81.57, 97.56, 101.33 (d, $^1J_{C\text{-}F}$ = 175.7, C-9) 111.35, 122.44, 124.69, 128.30, 129.48, 142.39, 152.89, 154.41, 167.02, 170.73, 171.76, 185.61, 203.76, 227,26. ESI-MS $[M+H]^+$ *m/z* calcd 715.86 for $C_{37}H_{43}FO_9S_2$; found 716.2.

5.4. Molecular modeling

Molecular modelling calculations were performed on High Performance Computing Cluster (HPCC) and molecular modelling graphics were carried out on a personal computer equipped with Intel(R) Core(TM) i7-4790 processor.

Compounds **4b** and **5b** were built using the Small Molecule tool of Discovery Studio 2017 (Dassault Systèmes BIOVIA, San Diego). Atomic potentials and charges were assigned using the CHARMm force field [49]. The conformational space of the compounds was sampled using the random search algorithm Boltzmann Jump for the random generation of a maximum of 400 conformations. By applying this method, each random perturbation is either accepted or rejected according to the Metropolis selection criterion with a ratio according to the Boltzmann distribution (T = 300K). An energy threshold value of 20 kcal/mol was used as selection criteria. The generated structures were then subjected to MM energy minimization until the maximum RMS derivative was less than 0.01 kcal/Å, using Conjugate Gradient [50] as minimization algorithm and the Generalized Born implicit solvent model with a solvent dielectric constant value of 80 [51]. Resulting conformers were ranked by their conformational energy. Conformers within 5 kcal/mol from the global minimum (ΔEGM≤ 5 kcal/mol) were classified into families according to their values of root mean squared deviation (RMSD; heavy atoms). In particular, pairwise RMSDs were calculated between all the conformers and the set of conformers characterized by a RMSD value < 3 Å were grouped in the same conformational family. The occurrence rate of each conformational family was calculated. The solvent accessible surface area (SASA) of the isothiocyanate carbon atom of the lowest energy conformer of each family was calculated and visually analysed. The calculated SASA values were compared to the maximum SASA value of the isothiocyanate carbon atom (i.e, 15.2 Å) and the rate of SASA decrease was accordingly calculated.

5.5. Pharmacological procedures

5.5.1. Amperometric determination of H_2S release

The H_2S-generating properties of the tested compounds have been evaluated by an amperometric approach, through an Apollo-4000 Free Radical Analyzer (WPI) detector and H_2S-selective mini-electrodes at room temperature as reported previously [52]. Briefly, a "PBS buffer 10 × " was prepared ($NaH_2PO_4 \cdot H_2O$ 1.28 g, $Na_2HPO_4 \cdot 12H_2O$ 5.97 g, and NaCl 43.88 g in 500 mL of H_2O) and stocked at 4 °C. Immediately before the experiments, the "PBS buffer 10 × " was diluted in distilled water (1:10) to obtain the assay buffer (AB); pH was 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 μL of a dimethyl sulfoxide (DMSO) solution of the tested compounds was added (final concentration of the H_2S-donors 100 μM; final concentration of DMSO in the AB 1%). The generation of H_2S was observed for 30 min. When required by the experimental protocol, 4 mM L-cysteine was added, before the H_2S-donors. The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was determined by opportune calibration curves with increasing concentrations of NaHS (1, 3, 5, and 10 μM) at pH 4.0. The lower limit of reliable quantitative determination was 0.3 μM.

5.5.2. Cell culture

Human bronchial smooth muscle cells (BSMCs) were cultured in Medium 231 (Life technologies) supplemented with Smooth Muscle Growth Supplement (SMGS, Life Technologies) and 1% of 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich) in tissue culture flasks at 37 °C in a humidified atmosphere and 5% CO_2. BSMCs were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatin 1% (from porcine skin, Sigma Aldrich), at density of 72 × 10^3 per well. Cells were split 1:2 twice a week and used until passage 18. RBL-2H3 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in Minimum Essential Medium (MEM - Sigma-Aldrich) supplemented with 10% of Fetal Bovine Serum (FBS - Sigma-Aldrich) and 1% streptomycin/penicillin (Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 atmosphere.

5.5.3. Intracellular H_2S release

BSMCs were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96 well clear bottom black plate at a density of 72 × 10^3 per well. After 24 h, the medium was replaced and cells were incubated for 30 min with a 100 μM solution of the fluorescent dye WSP-1 (Washington State Probe, 1,3′-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthen]-6′-yl 2-(pyridine-2-yl-disulfanyl benzoate) that is highly sensitive for H_2S detection [53,54]. Then, the supernatant was removed and replaced with different solutions of the teste compounds dissolved in standard buffer (HEPES 20 mM; NaCl, 120 mM; KCl, 2 mM; $CaCl_2 \cdot 2H_2O$, 2 mM; $MgCl_2 \cdot 6H_2O$, 1 mM; glucose, 5 mM; and pH 7.4, at room temperature) at the concentration of 100 and 300 μM. The change in fluorescence (expressed as fluorescence index measured at λ = 465−515 nm) was monitored every 5 min for 60 min, by means of a spectrofluorometer. On the bases of previous experiments [55,56], diallyl disulfide (DADS, Sigma-Aldrich) 100 μM was used as slow H_2S-donor reference compound. Six different experiments (n = 6) were performed, each carried out in three replicates. The results are expressed as mean ± SEM.

5.5.4. β-hexosaminidase (β-HEX) release assay

The β-hexosaminidase (β-HEX) release was detected as a reliable indicator of mast cell degranulation. This assay was carried out on rat basophilic RBL-2H3 cell line. After reaching 80% confluence, RBL-2H3 cells were seeded into a 96-well plate at 37 °C at the density of 72000 cells for well and incubated for 24h at 37 °C in a humidified 5% CO_2 atmosphere to allow cell attachment. FcεRI-mediated degranulation: RBL-2H3 were sensitized with an overnight anti-dinitrophenylated-human serum albumin (DNP-HSA), IgE treatment (0.50 μg/mL), and subsequently, MEM was replaced with phenol-free DMEM supplemented with 1 mg/mL bovine serum albumin (BSA). Vehicle (DMSO 0.1%), cromolyn (1 mM) - a well-known mast cell stabilizer - and the tested compounds (100 and 300 μM) were incubated for 5 min at 37 °C. Cells were then treated with DNP (10 ng/mL) to induce the degranulation. TRITON-X-100 0.1%, was used to cause exhaustive release of β-exosaminidase (β-HEX) (data not shown). One hour later the degranulation stimuli (DNP), 50 μL of supernatants from each well were collected and added to 50 μL of p-nitrophenyl-N-acetyl-β-D-glucosaminide 1.4 mM in citrate buffer 0.2 M, pH 4.2. The enzymatic reaction was terminated after 1 h by adding 100 μL/well of Trizma solution 0.3 M pH 9.4. The release of β-HEX was measured at 405 nM in a multiplate reader (EnSpire, PerkinElmer, Milan, Italy).

5.5.5. Evaluation of the membrane hyperpolarizing effects on BSMCs

The membrane hyperpolarizing effects were evaluated on BSMCs by spectrofluorometric methods, as already described [54]. BSMCs were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatin 1% (from porcine skin, Sigma Aldrich), at density of 72 × 10^3 per well. After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard (see above) containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich) 2.5 μM [54]. This membrane potential-sensitive dye DiBac4(3) allowed us to measure the cell membrane potential; in fact, this lipophilic and negatively-charged oxonol dye shuffles between cellular and extracellular fluids in a membrane potential-dependent manner (following the Nernst laws), thus allowing to assess changes in membrane potential by means of spectrofluorometric recording. In particular, an increase of fluorescence, corresponding to an inward flow of the dye, reflects a membrane depolarization; in contrast, a decrease in fluorescence, due to an outward flow of the dye, is linked to membrane hyperpolarization. The spectrofluorometric recording is carried out at excitation and emission wavelengths of 488 and 520 nm, respectively (Multiwells reader, Enspire, PerkinElmer). NS1619 (Sigma-Aldrich) at the concentration of 10 μM, was used as reference drug, since it evokes membrane hyperpolarizing effects with highest potency and efficacy [45]. After the assessment of base-line fluorescence, the tested compounds were added, and the trends of fluorescence was followed for 40 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as:

$$(Ft - F0)/F0$$

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

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113517.

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