European Journal of Medicinal Chemistry 275 (2024) 116636

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Research paper

Isothiocyanate-Corticosteroid Conjugates against asthma: Unity makes strength

Antonia Scognamiglio [a,1], Ida Cerqua [a,1], Valentina Citi [b], Alma Martelli [b], Jacopo Spezzini [b], Vincenzo Calderone [b], Maria Grazia Rimoli [a], Federica Sodano [a], Giuseppe Caliendo [a], Vincenzo Santagada [a], Ferdinando Fiorino [a], Francesco Frecentese [a], Elisa Perissutti [a], Elisa Magli [c], Martina Simonelli [a], Angela Corvino [a,**], Fiorentina Roviezzo [a], Beatrice Severino [a,*]

[a] Department of Pharmacy, School of Medicine, University of Naples Federico II, Via D. Montesano, 49, 80131, Napoli, Italy
[b] Department of Pharmacy, University of Pisa, Via Bonanno, 6, I-56126 Pisa, Italy
[c] Department of Public Health, School of Medicine, University of Naples Federico II, Via Panzini, 5, 80131, Napoli, Italy

ARTICLE INFO

Handling Editor: Dr. Z Liu

Keywords:
hydrogen sulfide
Multi-target molecules
isothiocyanates
Steroidal anti-inflammatory drugs
Hybrids
Asthma

ABSTRACT

Asthma is a major noncommunicable disease, affecting both children and adults, and represents one of the major causes leading to high health care costs due to the need for chronic pharmacological treatments. The standard gold therapy of inflammation in asthmatic patients involves the use of glucocorticoids even if their chronic use is often related to serious adverse effects. Growing evidence suggests the biological relevance of hydrogen sulfide (H_2S) in the pathogenesis of airway diseases. Hence, aiming to associate the beneficial effects of steroidal anti-inflammatory drugs (SAIDs) to H_2S biological activity, we designed and synthesized novel multi-target molecules by chemically combining a group of glucocorticoids, usually employed in asthma treatment, with an isothiocyanate moiety, well-known for its H_2S releasing properties. Firstly, the synthesized compounds have been screened for their H_2S-releasing profile using an amperometric approach and for their *in vitro* effects on the degranulation process, using RBL-2H3 cell line. The physicochemical profile, in terms of solubility, chemical and enzymatic stability of the newly hybrid molecules, has been assessed at different physiological pH values and in esterase-rich medium (bovine serum albumin, BSA). The selected compound **5c**, through both its corticosteroid and H_2S releasing component, has been evaluated *in vivo* in experimental model of asthma. The compound **5c** inhibited *in vivo* all asthma features with a significative effect on the restoration of pulmonary structure and reduction of lung inflammation.

1. Introduction

Asthma is a major noncommunicable disease, affecting more than 300 million children and adults worldwide. Nowadays, the standard gold treatment of inflammation in asthmatics is represented by glucocorticoids which can effectively treat most patients, thus reducing overall morbidity and mortality over the past 30 years [1]. Nonetheless, approximately 5–10 % of patients have uncontrolled asthma with frequent exacerbations, which also cause a significant deterioration in quality of life. Such patients are classified as having severe uncontrolled asthma (SUA) and are not effectively treated with inhaled medications, thus managing the disease by using oral glucocorticoids. However, chronic use is often related to serious adverse effects, including cardiovascular disease, dyslipidaemia, adrenal suppression, hyperglycaemia, osteoporosis, and immune suppression [2]. Based on these considerations, there is a clear need to develop better alternative treatments that improve the quality of life of SUA patients.

Hydrogen sulfide (H_2S), identified as the third gaseous mediator, after nitric oxide (NO) and carbon monoxide (CO), is produced by many cell types in the mammals, including humans. In the lungs, H_2S

* Corresponding author.
** Corresponding author.
E-mail addresses: angela.corvino@unina.it (A. Corvino), bseverin@unina.it (B. Severino).
[1] These authors contribute equally.

https://doi.org/10.1016/j.ejmech.2024.116636
Received 27 May 2024; Received in revised form 20 June 2024; Accepted 27 June 2024
Available online 28 June 2024

production occurs mainly in pulmonary arteries, airway smooth muscle cells, lung primary fibroblasts, and endothelial cells [3]. H_2S has been demonstrated to actively regulate fundamental pathophysiological processes such as oxidative stress, pulmonary circulation, airway tone and inflammation [4]. As in other tissues and organs, H_2S is characterized by a hormetic behaviour: when H_2S is rapidly released, for example when NaHS is used, a pro-inflammatory response is evoked, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are massively produced. Contrarily, when H_2S is slowly released in an endogenous-like manner, inflammation and oxidative stress are counteracted. For this reason, the development of new molecules characterized by a slow H_2S release is of extreme importance for potential use in the clinic.

In this context, asthma has been related to an impairment of endogenous H_2S production, making the use of exogenous H_2S (as H_2S donors) a potential pharmacological strategy to restore the levels of this gaseous molecule for the treatment of pulmonary and other H_2S-related diseases [5]. In the past decades, a significant surge in interest for multi-target drugs due to their benefits in managing intricate illnesses and medical conditions associated with challenges in drug resistance. Indeed, the multi-target drug design strategies have been getting over the traditional design approach in the treatment of complex diseases [6]. Our research group has long been exploiting the hybridization as an effective strategy for the design and synthesis of new molecular entities, which have resulted in improved outcomes in several disease models such as asthma [7–9], atopic dermatitis [10], glaucoma [11], and melanoma [12].

The design strategy pursued involves the conjugation, either directly or by means of a spacer, of a commonly used drug with an H_2S donor. Among those employed are well-known H_2S-donors such as 4-hydroxythiobenzamide (TBZ), 5-(*p*-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH), 4-hydroxyphenyl-isothiocyanate (HPI) and ethyl 4-hydroxy-benzodithioate (HBTA). The main drawback associated with the use of these H_2S-releasing units is the reduced water solubility of the obtained hybrid that, very often, is less soluble than the parent compound. This problem is of concern for glucocorticoid hybrids, which themselves have poor water solubility. Starting from this consideration, we decided to couple a selection of SAIDs with glycine, affording the corresponding glycinates and then replacing the free amine group with an isothiocyanate functionality as a putative H_2S-donor moiety. This approach already explored for the preparation of memantine [13], bisphosphonate derivatives [14] and, metformin-isothiocyanate [15], should provide final compounds with a better solubility profile. Natural isothiocyanates (ITCs) are well-known H_2S donors, derived from cruciferous vegetables, which have been extensively studied for their cancer chemopreventive activity [16]. Moreover, natural (e.g. erucin) and synthetic ITCs have shown an interesting protective effect on the cardiovascular system, mainly due to their anti-inflammatory effect on the vascular endothelium [17–19]. Their ability to act as H_2S donors in a thiol-dependent manner has been investigated and the reaction pathways leading to H_2S generation have been demonstrated [20].

In this research work we describe the design, synthesis, characterization, and *in vitro/in vivo* pharmacological evaluation of H_2S-releasing glucocorticoids obtained by converting some of the most widely used corticosteroids, such as prednisone, dexamethasone, budesonide, betamethasone-17-valerate and desisobutyrylciclesonide (the active metabolite of prodrug ciclesonide) (Fig. 1), into the corresponding 2-isothiocyanatoacetates (compounds **1c-5c**; Table 1).

The physicochemical profile of the molecular hybrids **1c**-**5c** has been analyzed in terms of solubility, chemical stability at different pH values (7.4 and 1.2) and enzymatic stability in bovine serum albumin (BSA). For each molecular hybrid, the hydrolytic metabolites formed during incubation in the simulated physiological solutions, i.e., the corresponding 2-aminoacetate derivatives, **1b**–**5b**, and the starting glucocorticoids **1**–**5**, have been identified and quantified using newly validated chromatographic methods.

The H_2S-releasing rate of the hybrid molecules was measured through an amperometric approach, using H_2S-selective electrodes, in a cell free environment, in the presence or in the absence of the amino acid L-cysteine, added to mimic the endogenous presence of thiols. Furthermore, the antigen-induced degranulation inhibition of the synthesized compounds was evaluated in an *in vitro* model, using RBL-2H3 cell line, by measuring β−hexosaminidase activity. Finally, the biological activity of desisobutyrylciclesonide derivative **5c** has been evaluated *in vivo*. The results undoubtedly show that the hybrid **5c** inhibits asthma like features by improving the therapeutic efficacy of the parent compound.

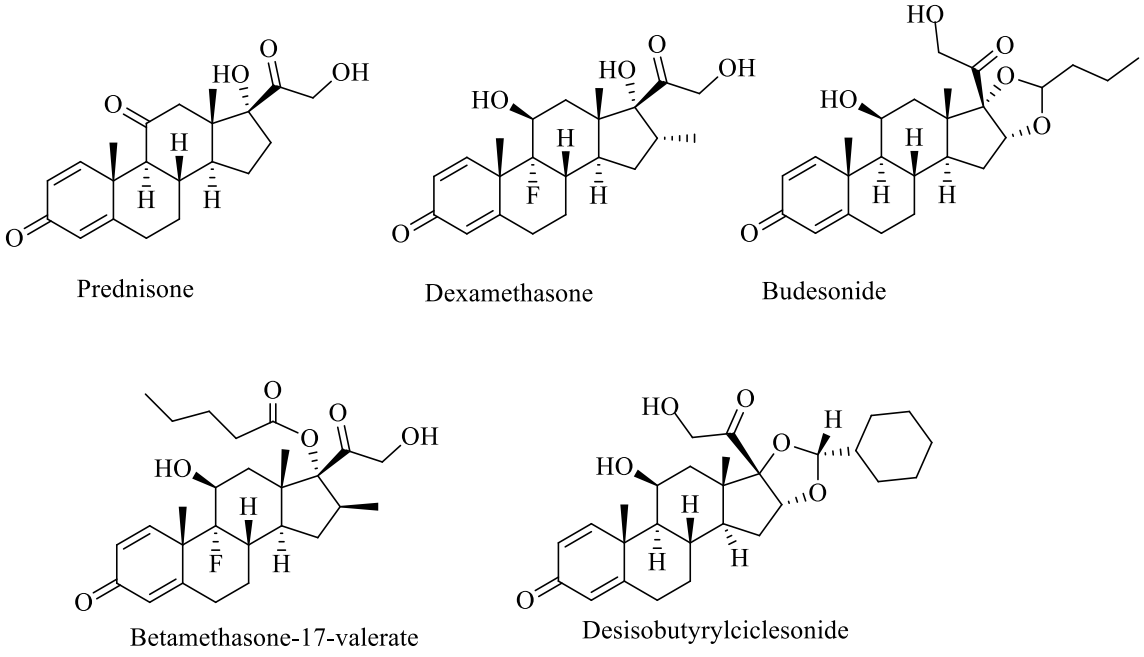
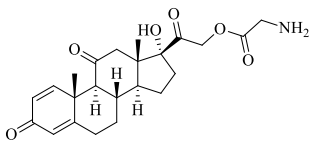
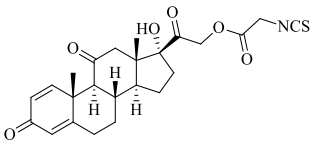
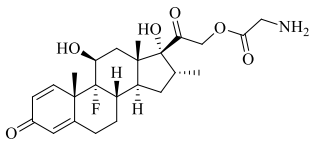
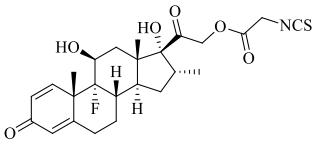
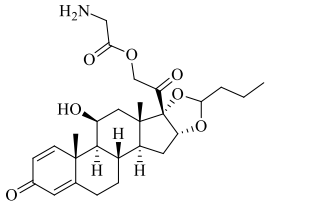
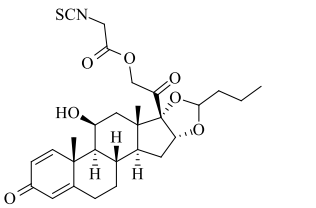
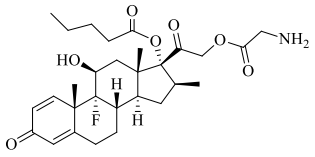
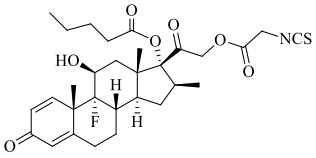
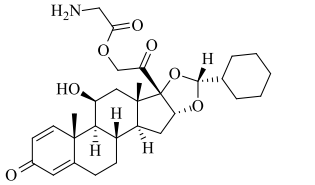
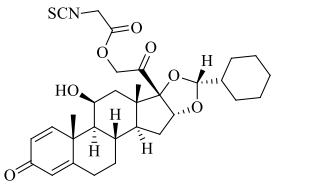


Fig. 1. Structures of the selected glucocorticoids.

Table 1
The table reports the structure of the designed compounds **1b-5b** and **1c-5c**.

#	Structure	#	Structure
1b		**1c**	
2b		**2c**	
3b		**3c**	
4b		**4c**	
5b		**5c**	

2. Results and discussion

2.1. Design and synthesis of 2-aminoacetates (1b–5b) and 2-isothiocyanatoacetates (1c-5c)

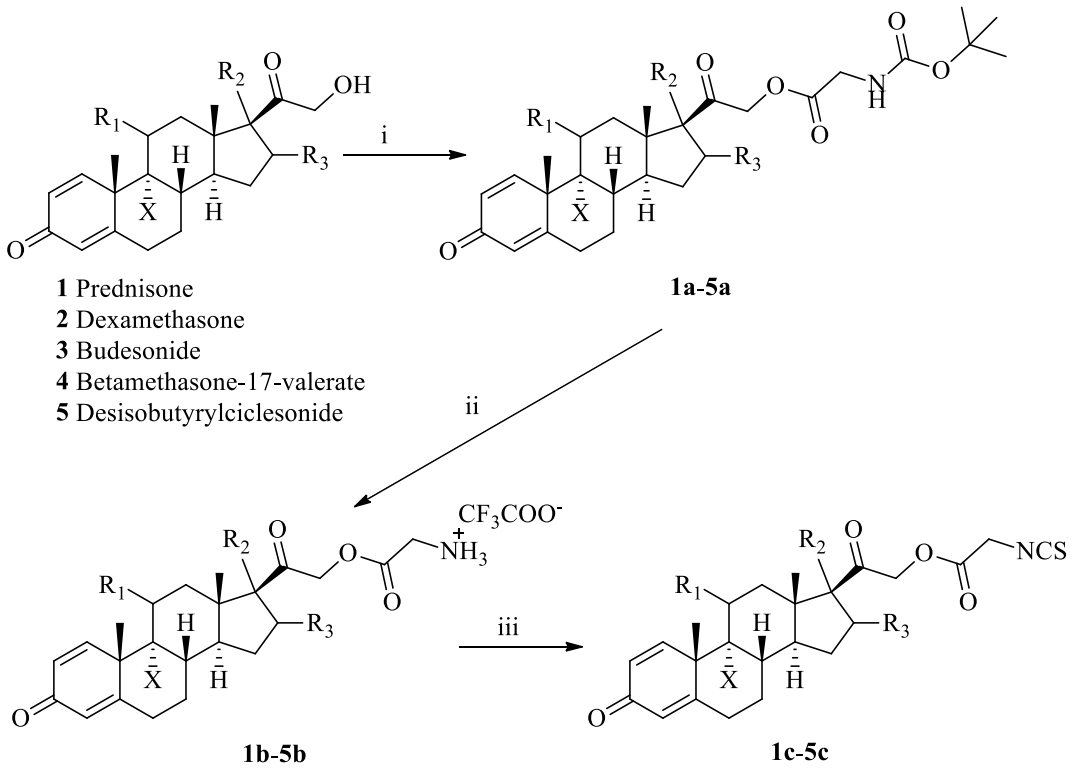
The chemical structures of compounds **1b-5b** and **1c-5c** are reported in Table 1. The synthetic strategy for the preparation of the compounds described here is depicted in Scheme 1.

The starting corticosteroid (Prednisone **1**, Dexamethasone **2**, Budesonide **3**, Betamethasone-17-valerate **4** and Desisobutyrylciclesonide **5**), was reacted with Boc-Gly-OH, via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/4-(dimethylamino)pyridine (EDAC•HCl/DMAP) in anhydrous tetrahydrofuran (THF). These reactions were carried out using a microwave oven (DISCOVER 2.0, CEM) especially designed for organic synthesis by placing the reagents and solvents in a sealed reactor specific for high-pressure reactions. The synthetic procedure was performed by following a microwave program consisting of appropriate ramping and holding steps and monitoring the temperature of the stirred reaction mixture using an iWave Sensor. The main advantage of this approach was that a short irradiation time of the reaction (10 min) provided compounds **1a-5a** in yields ranging from 82 % to quantitative and recoverable by crystallization. The same reactions, performed at room temperature, required 12 h, with yields ranging from 76 % to 87 % and a silica gel column purification to obtain a satisfying grade of purity. The 2-aminoacetates **(1b-5b)** have been obtained by removing the Boc protecting group through cleavage by trifluoroacetic acid (TFA). The synthetic strategy for the conversion of the amine group to isothiocyanate was optimized by varying the reaction solvent (DCM and acetone) and the molar ratios of the reagents to implement the yield, reduce the amount of thiophosgene used and facilitate the purification step. The optimized procedure, requiring 1.5 eq of thiophosgene, in the presence of 3 eq of Na_2CO_3, used acetone as the solvent and afforded yields in the range 30–37 %.

2.2. Physicochemical characterization

The hydrolytic susceptibility of molecular hybrids **1c-5c** was evaluated under various simulated physiological conditions. The chemical stability of **1c-5c** was first determined at body temperature and pH 7.4 in phosphate buffered saline (PBS) to mimic the blood environment, and at pH 1.2 in simulated gastric fluid (SGF-without pepsin) to simulate gastric environment. Briefly, the compounds **1c-5c** were dissolved in DMSO and diluted in the respective buffers to a final concentration of 100 μM. These solutions were maintained at 37° ± 0.5 °C and, at specific time intervals, their concentrations and corresponding metabolites were analyzed using UHPLC.

As reported in Table 2, all hybrid compounds were stable at acidic pH and compounds **3c-5c** even beyond 24 h of incubation in SGF. When the same hybrids **3c-5c** were incubated at body temperature (37 °C) and physiological pH (7.4) in PBS, they still displayed high stability; specifically, after 24 h of incubation, the formation of the two metabolites, the corresponding 2-aminoacetate derivatives, **3b**–**5b**, and the starting glucocorticoids **3**–**5** was negligible. On the contrary, the compounds **1c-2c** showed a higher percentage of hydrolysis at pH 7.4; therefore, their



Scheme 1. i) Boc-Gly-OH, EDAC•HCl, DMAP, 70 °C, MW; ii) 40 % TFA in DCM; iii) thiophosgene, Na_2CO_3, acetone, 0 °C.

Table 2
The table reports the chemical stability at 37 °C of the designed compounds **1c-5c** in SGF-without pepsin (pH = 1.2) and PBS (pH = 7.4) and the enzymatic stability in BSA.

Compd	Chemical		Enzymatic
	% Compd ±SD[a] or Half-Life[b]		
	SGF-without pepsin (pH = 1.2)	PBS (pH = 7.4)	BSA (3 % p/V)
1c	$t_{1/2}$ = 18 h	$t_{1/2}$ = 12 min	19 ± 2
2c	$t_{1/2}$ = 10 h	$t_{1/2}$ = 42 min	6 ± 1
3c	$t_{1/2}$ > 24 h	$t_{1/2}$ > 24 h	23 ± 1
4c	$t_{1/2}$ > 24 h	$t_{1/2}$ > 24 h	$t_{1/2}$ = 1 h
5c	$t_{1/2}$ > 24 h	$t_{1/2}$ > 24 h	$t_{1/2}$ = 1.4 h

[a] Results are expressed as mean ± SD (n = 3) after 10 min of incubation.
[b] Results are expressed as mean values (n = 3).

half-life resulted to be 12 and 42 min, respectively.

To be defined hybrid compounds with dual steroidal anti-inflammatory and H_2S activity, compounds **1c-5c** must be able to regenerate the starting glucocorticoid and H_2S-donor substructure through the action of enzymatic pathways. Therefore, the enzymatic stability of all hybrids was evaluated in an esterase-rich medium, i.e. bovine serum albumin (BSA). Specifically, each hybrid was incubated at 37 °C in BSA at a concentration of 200 μM and quantified under the same chromatographic conditions developed for chemical stability at different times over a 4 h period. As expected, the enzymes present in the medium rapidly metabolized the hybrids, releasing an appreciable amount of the starting glucocorticoids and a negligible amount of the corresponding 2-aminoacetate metabolites. The degradation of **4c-5c** into the respective glucocorticoids **4**–**5** was slower than that of **3c-2c-1c** into **3-2-1**, respectively, probably due to the difficult accessibility of hydrolysable bonds by enzymes.

To obtain a more comprehensive physiochemical profile of the hybrids of interest, their solubility was evaluated at 25 °C in water and in the media used for chemical stability tests, namely SGF (pH = 1.2) and PBS (pH = 7.4). Briefly, 5 mg of each test compound was suspended in 1 mL of the respective solutions. After 6 h of stirring, the mixtures were filtered, and the concentrations of the hybrids were analyzed using UHPLC. The solubility values, expressed in mg/L, are presented in Table 3.

2.3. In vitro pharmacological evaluation

2.3.1. Evaluation of the H_2S releasing properties

The H_2S-release of the novel synthesized isothiocyanate hybrid corticosteroids was evaluated *in vitro* by an amperometric assay in a cell free environment. Furthermore, also NaHS, as reference H_2S donor, was evaluated using this method. This approach has been widely used for the characterization of the kinetic of the H_2S-releasing process in the absence or in the presence of an excess of L-Cysteine [8,21]. This amino acid is added to the phosphate buffer to mimic the endogenous presence of free thiols. Indeed, several H_2S releasing moieties showed a thiol dependent H_2S release as demonstrated for diallyl disulfide and for some isothiocyanates [22,23]. NaHS, incubated at the concentration of 100 μM, generated about 20 μM of H_2S in the presence and in the absence of L-Cys. The H_2S release was characterized by a dramatic pick after 1 min of incubation, followed by a relatively rapid decrease in H_2S amount.

Table 3
The table reports the solubility of the designed compounds **1c-5c** in SGF-without pepsin (pH = 1.2), PBS (pH = 7.4) and water.

Compd	Solubility at 25 °C (mg/L)[a]		
	SGF-without pepsin (pH = 1.2)	PBS (pH = 7.4)	Water
1c	16.1	15.9	5.26
2c	<0.1	<0.1	0.14
3c	<0.1	<0.1	<0.1
4c	0.16	0.12	<0.1
5c	<0.1	0.25	0.55

[a] Results are expressed as mean values (n = 3, SD < 0.01).

This behavior is due to the nature of NaHS, which is a salt, completely soluble in aqueous solution, which quickly exhausts its ability to generate H_2S (Fig. S7, Supplementary Material). As reported in Fig. 2, all the tested compounds incubated at the final concentration of 100 μM slowly generated H_2S both in presence and in the absence of L-Cysteine, reaching approximately 2 μM (except for **4c**).

Thus, these derivatives spontaneously release H_2S probably because the more flexible glycine chain allows an easier interaction of the - NCS group with the nucleophile, supporting previous hypothesis concerning the fact that steric accessibility to the isothiocyanate moiety can account for the different H_2S releasing properties of the synthesized compounds [9].

Isothiocyanates, in fact, easily react, in physiological conditions, with thiols and with amines (1000 times faster with thiols than with amines). Moreover, they are subjected to hydrolysis in aqueous solutions mainly because of their reaction with OH^- ions [24]. Nevertheless, as reported in Table 2, the synthesized molecules, **1c-5c**, have excellent chemical stability, with $t_{1/2}$ values from 10 h to >24 h and from 12 min to >24 h at acidic and neutral pH, respectively. We showed previously [8] 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 nucleophiles (thiols and OH^- ions, in particular). Accordingly, the most hindered hybrids obtained from budesonide (**3c**), betamethasone 17-valerate (**4c**), and desisobutyrylciclesonide (**5c**) resulted to be the most stable both chemically and enzymatically, proposing them as the best candidates for further *in vitro* and *in vivo* studies, stated that the slow and long-lasting H_2S generating properties of these compounds represent fundamental features for considering a potential clinical tool for the management of allergic respiratory diseases.

2.3.2. Cytotoxicity testing

The cytotoxicity of the hybrid compounds was evaluated *in vitro* in RBL-2H3 cells after the incubation of the isothiocyanate hybrid corticosteroids. The compounds were incubated at a concentration of 100 μM for 24 h and the cell viability was assessed using the cell proliferation

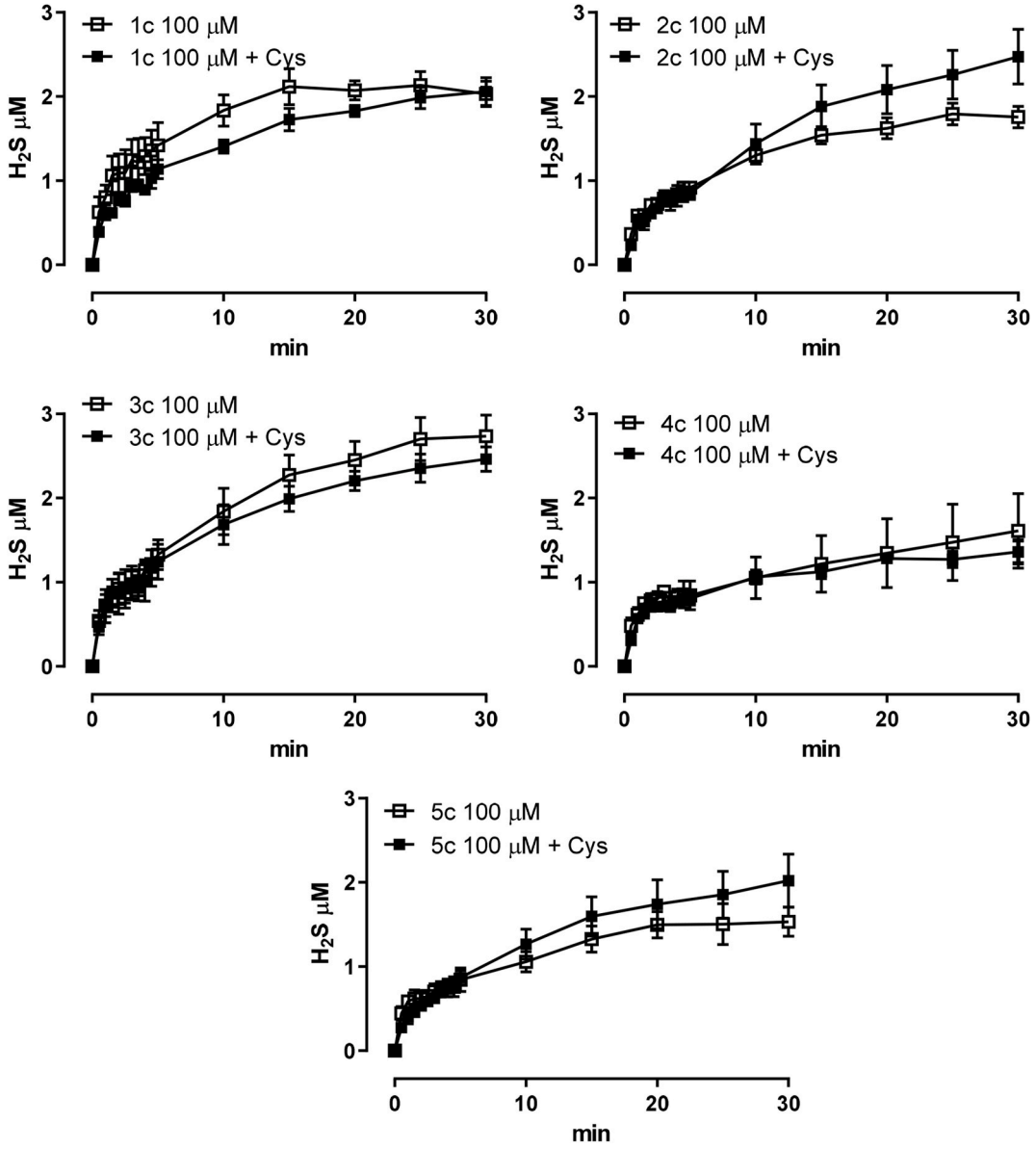


Fig. 2. Amperometric recordings reporting the increase of H_2S concentration over time after the incubation of the isothiocyanate hybrid corticosteroids at the concentration of 100 μM in the phosphate buffer, in the absence or in presence of L-cysteine 4 mM. Data are expressed as mean ± SEM. The vertical bars indicate the SEM.

reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulphonate) (Roche, Basel, Switzerland), that in living cells is cleaved to formazan. As shown in Fig. 3, compounds **1c**, **2c**, **3c** and **5c** did not show any reduction in cell viability compared to the vehicle (DMSO 0.1 %), while compound **4c** exhibited a significant cytotoxicity and was excluded from further experiments.

2.3.3. Inhibition of mast cell degranulation

Mast cell activation and degranulation determine the release of pro-inflammatory mediators, thus contributing to the development of respiratory diseases, including allergic asthmatic symptoms [25]. As reported in literature, H_2S-releasing compounds demonstrated to inhibit mast cell degranulation by the interaction with downstream proteins of the FcεRI pathway, therefore exerting protective properties against antigen-induced degranulation of RBL-2H3 cells [26]. The inhibitory effect against mast cell degranulation of compounds **1c**, **2c**, **3c** and **5c** was investigated in an antigen-mediated mast cell degranulation model. RBL-2H3 cells were pre-sensitized by the addition of the antigen DNP-HSA in order to trigger the degranulation, measured by the evaluation of β-hexosaminidase release (Fig. 4). Results were compared to the respective non-hybrid compounds and to cromolyn, a well-known molecule able to stabilize mast cell membrane and that is already used in the clinical practice. The obtained results show that DNP-HSA was able to effectively pre-sensitize RBL-2H3 cells, while cromolyn inhibited mast cell degranulation of about 25 %. The glycinates **1b**, **2b** and **5b** exerted protective properties comparable to the ones showed by cromolyn, but all the four newly synthesized isothiocyanate hybrid corticosteroids exhibited a stronger inhibition of mast cell degranulation compared to glycinates. Indeed, the hybrid corticosteroids showed a significantly higher protection compared also to cromolyn demonstrating that the addition of the H_2S-donor moiety was able to strongly enhance the inhibitory effect on mast cell degranulation.

2.4. In vivo pharmacological evaluation

The synthesized compounds have, therefore, shown the ability to inhibit mast cell degranulation with an enhanced effect compared to glycinate analogs. In order to evaluate the *in vivo* effects, we focused on compound **5c**, which exhibits greater chemical and enzymatic stability. Compound **4c**, while showing comparable stability, is much less water-soluble as well as cytotoxic. The dose range (1–2 mg/kg) to test *in vivo* was defined on the basis of the *in vivo* pharmacological activity of the parent compound ciclesonide.

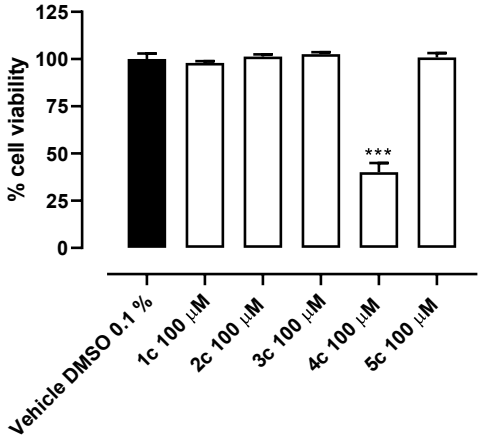


Fig. 3. Cytotoxicity evaluation of the isothiocyanate hybrid corticosteroids. Cells were treated with either vehicle (DMSO 0.1 %) or with compound **1c**, **2c**, **3c**, **4c**, and **5c** for 24 h. Cell viability was evaluated using WST-1 dye and expressed as percentage vs vehicle. Results are expressed with mean ± SEM. One way ANOVA followed by Bonferroni post-test was used for statistical analysis. *** indicates a significant difference compared to the Vehicle $p < 0.001$.

2.4.1. Compound 5c inhibits asthma like features

Sensitized mice were pre-treated with compound **5c** at a dose of 1 or 2 mg/kg and bronchi were used to assess bronchial reactivity to carbachol (Fig. 5A). Compound **5c** inhibited bronchial hyperreactivity in a dose-dependent manner (Fig. 5B). When used at 2 mg/kg, it restored bronchial reactivity to basal condition. The efficacy of **5c** at the higher dose in controlling bronchial hyperreactivity and airway fibrosis was confirmed by immunostaining that evidenced a significant reduction in the α-SMA positive muscle layer induced by OVA injection (Fig. 5C, arrow). Besides this, **5c** had an impact also on pulmonary inflammation (Fig. 6). Hematoxylin & Eosin (H&E) confirmed the allergen sensitization (Fig. 6A, b-b1) with an increase of cell infiltration (arrow), the thickening of airway smooth muscle layer (arrowhead) and detachment of the lamina propria (asterisk). All these damages were still present after treatment with the lowest dose (Fig. 6A, c-c1), but increasing the dose to 2 mg/kg was able to improve lung structure by acting on pulmonary inflammation (Fig. 6A, d-d1). In order to better define the pharmacological action of **5c**, IgE plasma levels and IL-13 lung expression were measured. **5c** at the 2 mg/kg dose significantly reduced IgE plasma level, confirming its immunosuppressive effect on the sensitization process (Fig. 6 BCE). Compound **5c** significantly and in a dose dependent manner reduced pulmonary IL-13 levels that well correlate with bronchial reactivity, confirming the improved ability to counteract OVA–induced AHR (Fig. 6C).

2.4.2. Compound 5c improves therapeutic efficacy of the parent compound

To define an amelioration of the pharmacological profile acquired by the corticosteroid following combination with an isothiocyanate moiety able to release H_2S, we treated OVA-sensitized mice with the equimolar dose of the parent compound **5b** (Fig. 7A). The functional study evidenced the inability of **5b** compared to **5c** to counteract OVA-induced hyperreactivity (Fig. 7B). Accordingly, only **5c** reduced OVA-induced α-SMA over-expression (Fig. 7C panel d–d1, arrow). The reduction of α-SMA expression suggests the acquired ability by **5c** to control OVA-induced airway remodeling as confirmed by inhibition of bronchial hyperreactivity. The improved action of **5c** was also confirmed in pulmonary inflammation (Fig. 8A).

Compound **5b** in no way prevents allergen-induced lung damage (Fig. 8A, c-c1). In contrast, **5c** restored the structure of the alveolar septa, bronchial epithelium, and airway smooth muscle (Fig. 8A, d-d1). IgE plasma level dosage showed a lower ability by **5b** to reduce IgE upregulation (Fig. 8B) and inefficacy in controlling pulmonary IL-13 upregulation (Fig. 8C).

3. Conclusion

Here we report a new series of glucocorticoid-H_2S donor hybrids (compounds **1c-5c**), designed for the purpose of combining in the same molecule the beneficial effects of hydrogen sulfide, at the lung level, with the known anti-inflammatory properties of corticosteroids, drugs of first choice in the therapy of chronic respiratory diseases, such as asthma and COPD. As H_2S-releasing unit, we chose isothiocyanate moiety with the idea of obtaining hybrids with better water solubility than previously synthesized derivatives, where the combination with TBZ, ADT-OH, HPI or HBTA had provided highly insoluble molecules [7–12]. We have achieved this result in fact, comparing the data for Pred-TBZ and Dex-TBZ derivatives, previously reported [7,8,10], with those of compounds **1c** and **2c**, reported here, an improvement in water solubility from <0.1 mg/L to 5.26 mg/L and from <0.1 mg/L to 0.14 mg/L is observed for prednisone and dexamethasone derivatives, respectively.

The hybridization approach led to the synthesis of compounds **1c-5c**, all of which can produce a slow and long-lasting release of H_2S, thus

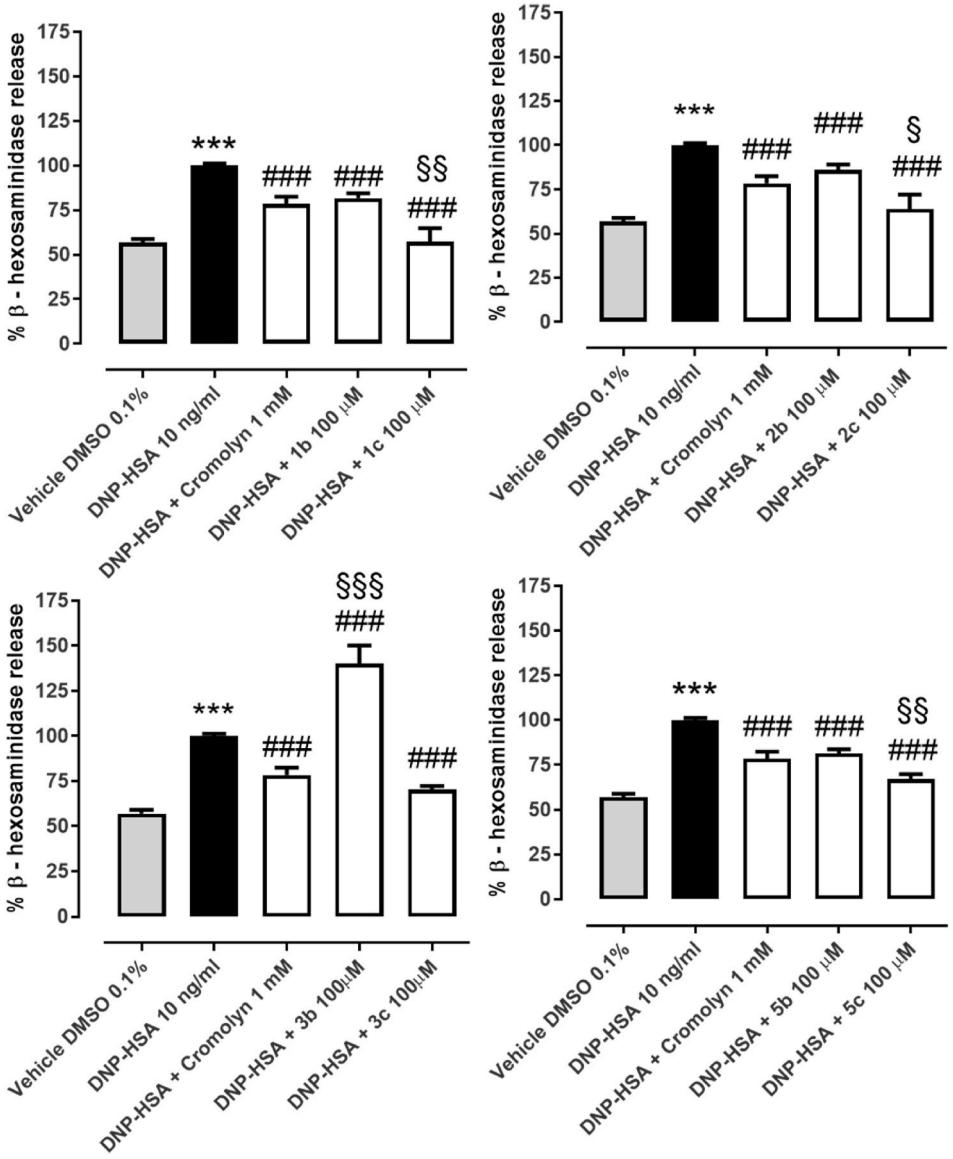


Fig. 4. Inhibition of mast cell degranulation showed as % of β-hexosaminidase release compared to DNP-HSA 10 ng/ml. Cells were treated with either vehicle (DMSO 0.1 %), DNP-HSA 10 ng/ml, DNP-HSA 10 ng/ml + Cromolyn 1 mM or DNP-HSA 10 ng/ml + hybrid or non-hybrid compound 100 μM. Results are expressed with mean ± SEM. One way ANOVA followed by Bonferroni post-test was used for statistical analysis. *** indicates a significant difference compared to the Vehicle $p < 0.001$, ### indicates a significant difference compared to DNP-HSA 10 ng/ml $p < 0.001$ and the symbol § indicates a significant difference compared to DNP-HSA 10 ng/ml + Cromolyn 1 mM, §$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$.

mimicking its physiological production, a necessary condition for exploiting the beneficial anti-inflammatory effects of hydrogen sulfide at the bronchial level.

In vitro evaluation, in an antigen-mediated mast cell degranulation model, also allowed us to verify that all of the synthesized compounds (with the exception of compound **4c**, which was excluded because of its cytotoxicity) were able to inhibit mast cell degranulation to a significantly greater extent than both glycinates and cromolyn.

Although the isothiocyanate group is very electrophilic and reactive, the combination with selected glucocorticoids brilliantly led to hybrids with improved water-solubility properties combined with surprising chemical stability and good enzymatic susceptibility. Compounds **3c-5c** were found to be the most chemically stable, and this is attributable to the presence of very bulky substitutions at positions 16 and 17, that protect the reactive functions from interaction with nucleophiles. This feature is highly desirable for a molecule designed as a prodrug and makes compounds **3c-5c** suitable for further *in vivo* evaluation.

Compound **5c**, selected for *in vivo* studies because of its higher water solubility and enzymatic stability, has been evaluated in OVA-sensitized mice. The compound was proven to inhibit the typical asthma features such as bronchial hyperreactivity and airway fibrosis. Moreover, the observed activity of compound **5c** is due to both its corticosteroid component and its ability to release H_2S, as demonstrated by *in vivo* experiments in which the effect of compound **5c** on bronchial hyperreactivity, restoration of pulmonary structure and reduction of lung inflammation was compared to the equimolar dose of the corresponding glycinate **5b**.

The design of multi-target drugs and, among these, hybridization of known drugs with H_2S-releasing moieties, has a great potential in the development of therapeutics for the management of complex diseases. In our case, the isothiocyanate group once again showed its usefulness as a H_2S releasing unit by providing derivatives characterized by surprising chemical stability and good enzymatic susceptibility. Among them, compound **5c** represents an interesting new therapeutic option for the treatment of inflammatory lung diseases.

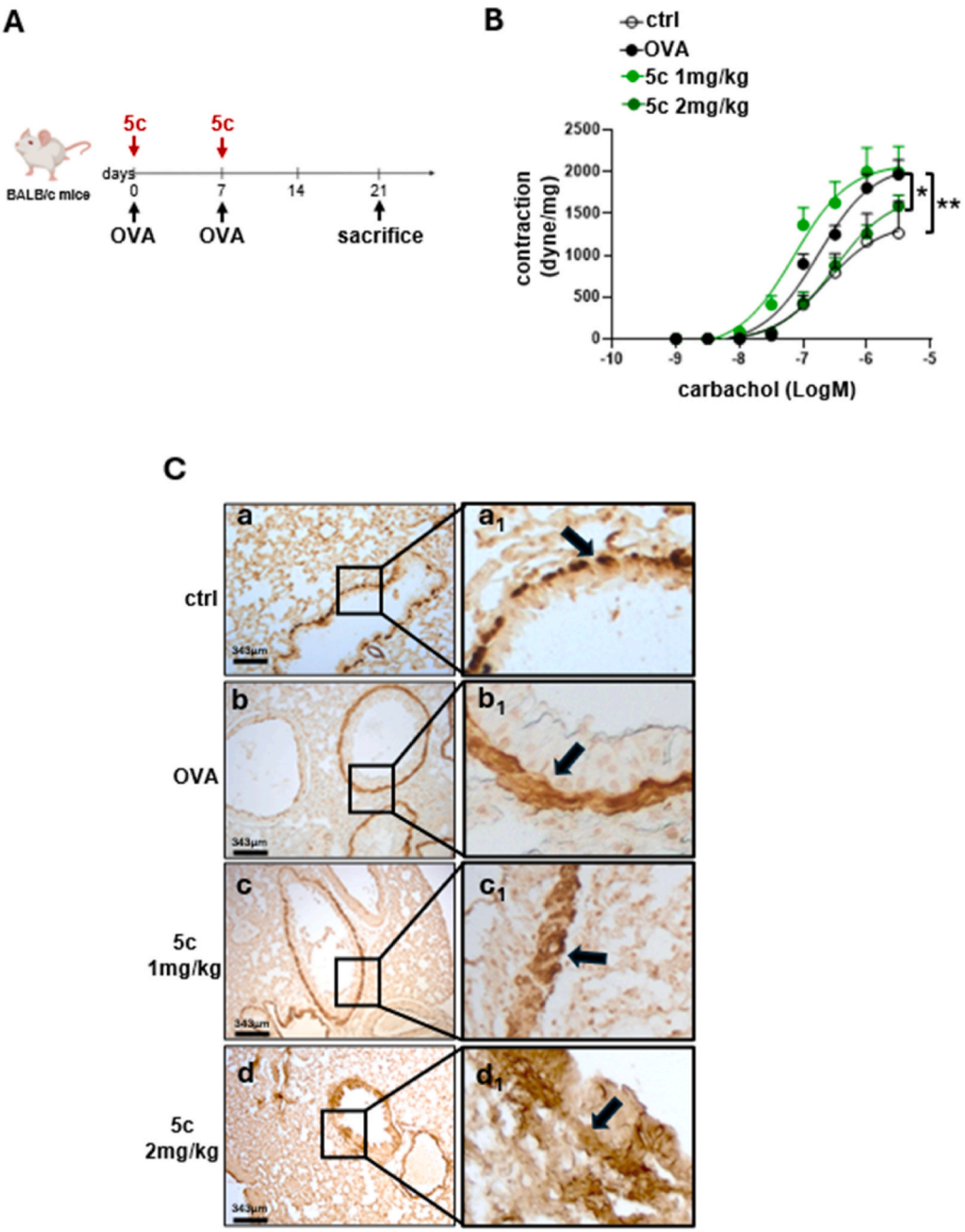


Fig. 5. Compound **5c** 2 mg/kg reduced bronchial hyperreactivity in mice sensitized with OVA. (A) **5c** (1 or 2 mg/kg) was intraperitoneally administrated 30 min before ovalbumin sensitization on days 0 and 7, mice were sacrificed on day 21. (B) Bronchial reactivity to carbachol. (C) Immunohistochemical analysis of α-SMA on lung slices. The statistical analysis used is two-way ANOVA plus Bonferroni *post-hoc* test (B). Significance is represented by *$p < 0.05$, **$p < 0.01$ N. animals for each group = 6 (B), 3 (C). Magnification: C, a–d: 20x, C, a_1-d_1: inset, detail of smooth muscle layer (arrow).

4. Experimental section

4.1. Synthesis

All reagents were commercial products purchased from Merck Life Science S.r.l. and Biosynth Limited. All reactions were followed by TLC, carried out on Merck silica gel 60 F_{254} plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Solutions were dried over Na_2SO_4 and concentrated with a Buchi R-114 rotary evaporator at low pressure. Melting points were determined using a Buchi melting point M560 apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Brucher Advance 700 MHz and 400 MHz instruments. Unless otherwise stated, 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), broad singlet (bs), d (doublet), doublet of doublets (dd), doublet of doublet of doublets (ddd), quadruplet of doublets (qd), t (triplet), quartet (q), m (multiplet). Mass spectra of intermediates and final products were performed on LTQ Orbitrap XL™ Fourier transform mass spectrometer (FTMS) equipped with an ESI ION MAX™ (Thermo Fisher Scientific, Waltham, MA, USA) source operating in positive mode. MeOH was used as solvent for compound infusion into LTQ Orbitrap source.

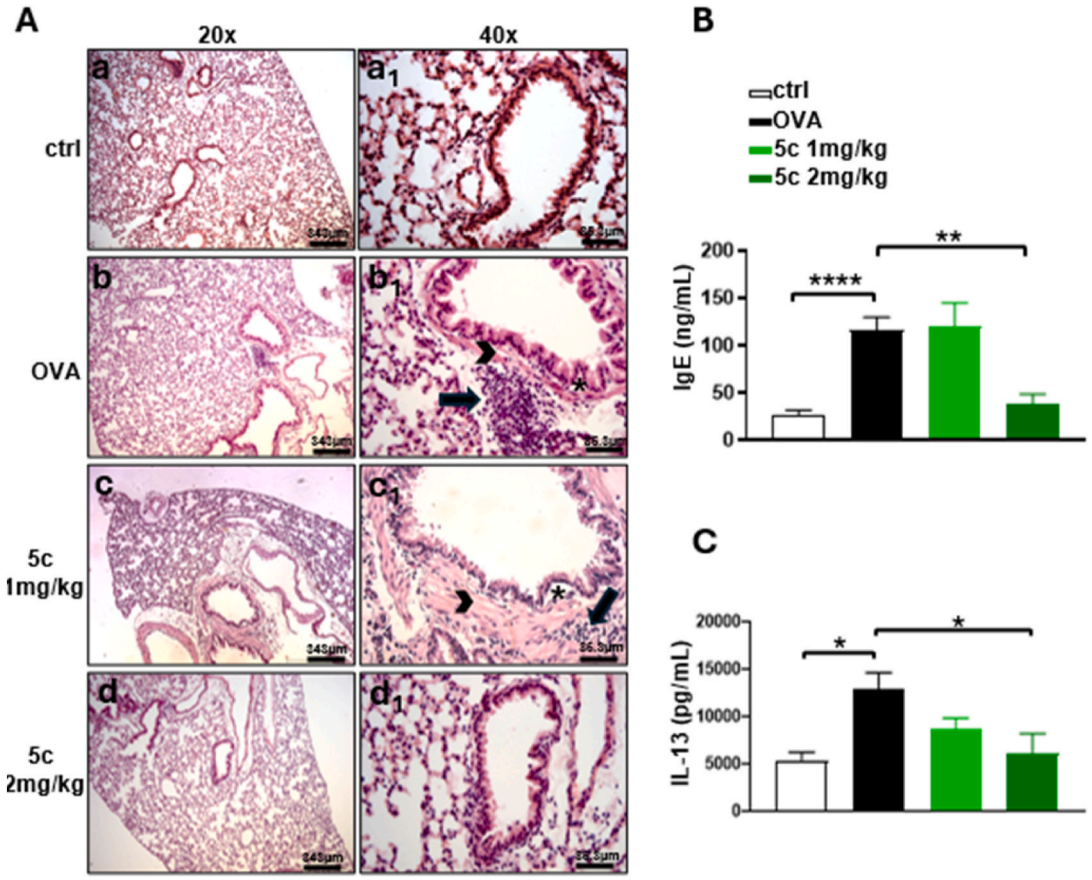


Fig. 6. Compound **5c** 2 mg/kg ameliorated pulmonary inflammation in mice sensitized with OVA. (A) Lung slices were stained for Hematoxylin & Eosin (H&E; arrowhead: smooth muscle layer; asterisk: detachment of bronchial epithelium; arrow: cell infiltration). (B) Plasma IgE level. (C) Pulmonary level of IL-13. Data were analyzed by one-way ANOVA plus Bonferroni (B, C). Significance is represented by *p < 0.05, **p < 0.01, ****p < 0.0001. N. animals for each group = 6 (B, C), 3 (A). Magnification: A, a–d: 20x, A, a1-d1: 40x.

4.1.1. 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 2-((tert-butoxycarbonyl)amino)acetate (1a)

A solution of prednisone (**1**, 100 mg, 0.279 mmol) in anhydrous tetrahydrofuran (5 mL) was placed in a 10 mL closed reaction vessel and EDAC•HCl (86 mg, 0.446 mmol), DMAP (55 mg, 0.446 mmol) and Boc-Gly-OH (73 mg, 0.418 mmol) were added. The closed reaction vessel was placed in the cavity of a CEM microwave reactor, run under pressure and irradiated according to the following parameters: T, 70 °C; ramp time, 2 min; hold time, 10 min; pressure, 150 psi; power, 100 W. After cooling to room temperature, the solvent was evaporated and the residue was washed with a 10 % citric acid solution, a 5 % $NaHCO_3$ solution and H_2O saturated with NaCl using dichloromethane as the organic solvent. The organic phase was dried with anhydrous Na_2SO_4, filtered, and evaporated by distillation at reduced pressure, to provide the desired product. The obtained compound **1a** was recrystallized from *n*-hexane providing a white solid product. Yield 143 mg, 100 %. m.p. 112.9–114.1 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 515.60 for $C_{28}H_{37}NO_8$; found 516.2.

^{1}H NMR (400 MHz, DMSO) δ 7.60 (d, J = 10.2 Hz, 1H), 7.27 (t, J = 6.1 Hz, 1H), 6.12 (d, J = 10.2 Hz, 1H), 6.02 (s, 1H), 5.84 (s, 1H), 5.00 (d, J = 17.8 Hz, 1H), 4.84 (d, J = 17.8 Hz, 1H), 3.79 (ddd, J = 31.7, 17.9, 6.2 Hz, 2H), 2.91 (d, J = 12.1 Hz, 1H), 2.60–2.51 (m, 1H), 2.42–2.33 (m, 2H), 2.20 (d, J = 11.8 Hz, 2H), 2.11–1.93 (m, 2H), 1.83–1.61 (m, 2H), 1.39 (s, 9H), 1.37 (s, 3H), 1.30–1.14 (m, 2H), 0.86 (t, J = 6.7 Hz, 1H), 0.51 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 210.60, 205.57, 185.54, 170.59, 167.66, 156.21, 155.57, 127.49, 124.24, 88.15, 78.75, 68.71, 59.26, 51.28, 49.82, 49.20, 42.42, 41.99, 35.99, 34.17, 33.60, 32.00, 28.62, 23.27, 19.22, 15.48.

4.1.2. 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 2-((tert-butoxycarbonyl)amino)acetate (2a)

The desired compound was prepared as described for **1a** starting from dexamethasone (**2**, 100 mg, 0.255 mmol), Boc-Gly-OH (67 mg, 0.382 mmol), EDAC HCl (78 mg, 0.408 mmol) and DMAP (50 mg, 0.408 mmol). Yield 137 mg, 97 %. m.p. 166.2–167.3 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 549.64 for $C_{29}H_{40}FNO_8$; found 550.2.

^{1}H NMR (400 MHz, DMSO) δ 7.33–7.22 (m,2H), 6.23 (dd, J = 10.1, 1.6 Hz, 1H), 6.01 (s, 1H), 5.40 (d, J = 4.3 Hz, 1H), 5.19 (s, 1H), 5.10 (d, J = 17.6 Hz, 1H), 4.82 (d, J = 17.6 Hz, 1H), 4.20–4.11 (m, 1H), 3.79 (qd, J = 17.9, 6.2 Hz, 1H), 2.93–2.83 (m, 1H), 2.62 (td, J = 13.3, 5.6 Hz, 1H), 2.44–2.27 (m, 2H), 2.23–2.07 (m, 2H), 1.83–1.72 (m, 1H), 1.69–1.52 (m, 1H), 1.49 (s, 3H), 1.39 (s, 9H), 1.34–1.30 (overlapped, 1H), 1.28–1.23 (m, 1H), 0.89 (s, 3H), 0.79 (d, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 205.03, 185.74, 170.40, 167.51, 156.22, 153.21, 129.46, 124.65, 101.72 (d, $^1J_{C\text{-}F}$ = 175.3 Hz, C-9), 90.92, 70.98 (d, $^2J_{C\text{-}F}$ = 36.9 Hz, C-11), 78.81, 68.69, 65.38, 48.43, 48.40 (d, $^2J_{C\text{-}F}$ = 22.9 Hz, C-10), 43.76, 42.01, 36.13, 35.81, 34.05 (d, $^2J_{C\text{-}F}$ = 19.3 Hz, C-8), 32.37, 30.73, 28.30, 27.75, 23.43 (d, $^3J_{C\text{-}F}$ = 5.6 Hz, C-19), 22.53, 16.72, 15.60, 14.43.

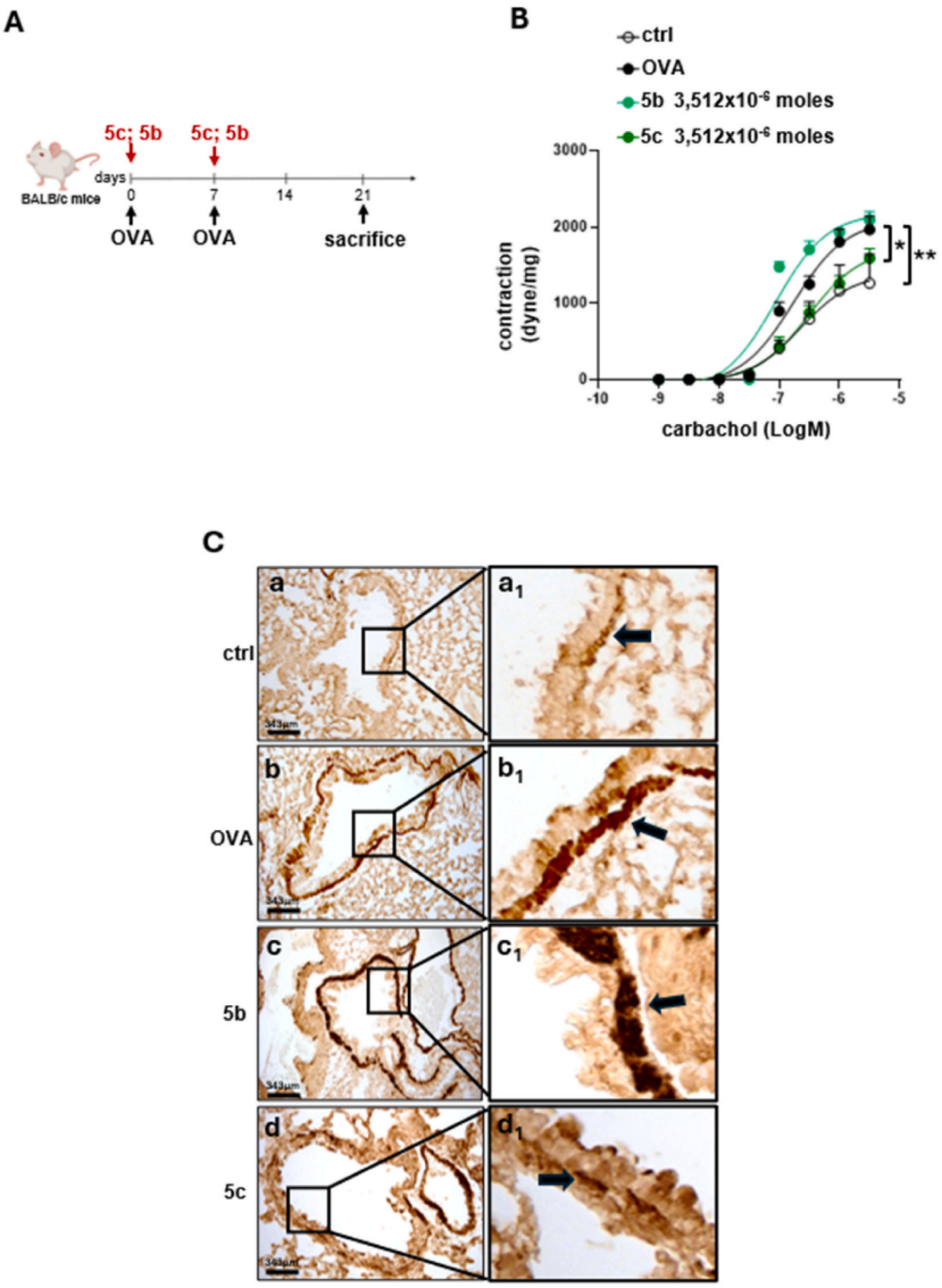


Fig. 7. Compound **5c** affected bronchial hyperreactivity in mice sensitized with OVA compared to the equimolar dose of **5b**. (A) **5c** or **5b** ($3{,}51 \times 10^{-6}$ mol) were intraperitoneally administrated 30 min before ovalbumin sensitization on days 0 and 7, mice were sacrificed on day 21. (B) Bronchial reactivity to carbachol. (C) Immunohistochemical analysis of α-SMA on lung slices. The statistical analysis used is two-way ANOVA plus Bonferroni post-hoc test (B). Significance is represented by *$p < 0.05$, **$p < 0.01$ N. animals for each group = 6 (B), 3 (C). Magnification: C, a–d: 20x, C, a1-d1: inset, detail of smooth muscle layer (arrow).

4.1.3. 2-((6aR,6bS,7S,8aS,8bS,11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-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 2-((tert-butoxycarbonyl)amino)acetate (3a)

The desired compound was prepared as described for intermediate **1a**, starting from budesonide (**3**, 100 mg, 0.232 mmol), Boc-Gly-OH (61 mg, 0.348 mmol), EDAC HCl (71 mg, 0.371 mmol) and DMAP (46 mg, 0.371 mmol). Yield 135 mg, 99 %. m.p. 134.2–135.4 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 587.71 for $C_{32}H_{45}NO_9$; found 588.2.

^{1}H NMR (400 MHz, DMSO) δ 7.36–7.25 (m, 2H), 6.17 (d, $J = 10.1$ Hz, 1H), 5.92 (s, 1H), 5.20 (t, $J = 4.8$ Hz, 1H), 5.13–5.00 (m, 2H), 4.89–4.62 (m, 3H), 4.35–4.27 (m, 1H), 3.89–3.71 (m, 2H), 2.64–2.51 (overlapped, 1H), 2.35–2.24 (m, 1H), 2.15–1.91 (m, 2H), 1.88–1.69 (m, 3H), 1.63–1.42 (m, 5H), 1.39 (s, 9H), 1.38–1.20 (m, overlapped, 7H), 1.05–0.92 (m, 3H), 0.91–0.82 (m, 7H). ^{13}C NMR (101 MHz, DMSO) δ 203.55, 201.91, 185.57, 170.68, 170.60, 170.41, 170.35, 156.90,

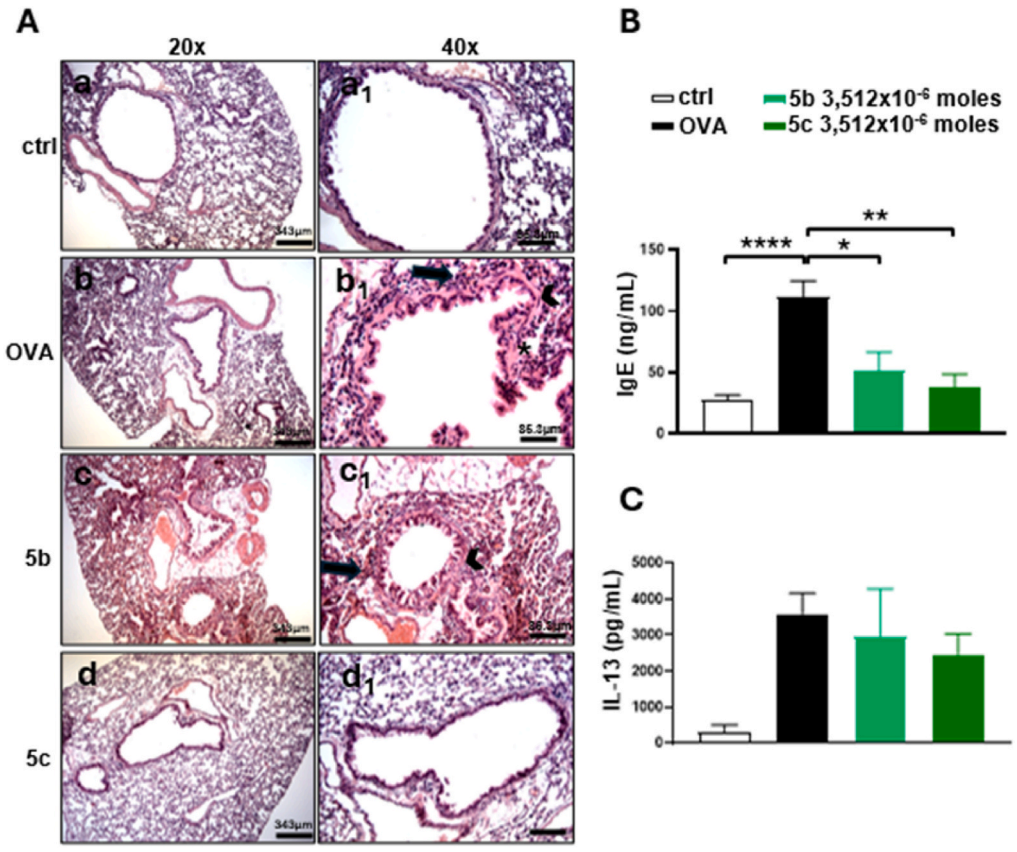


Fig. 8. Compound **5c** restored pulmonary structure and reduced lung inflammation compared to the equimolar dose of **5b**. (A) Lung slices were stained for Hematoxylin & Eosin (H&E; arrowhead: smooth muscle layer; asterisk: detachment of bronchial epithelium; arrow: cell infiltration). (B) Plasma IgE level. (C) Pulmonary level of IL-13. Data were analyzed by one-way ANOVA plus Bonferroni (B, C). Significance is represented by *p < 0.05, **p < 0.01, ****p < 0.0001. N. animals for each group = 6 (B, C), 3 (A). Magnification: A, a–d: 20x, A, a1-d1: 40x.

156.21, 127.59, 122.17, 122.12, 107.84, 104.24, 98.41, 97.69, 82.84, 81.67, 78.77, 68.52, 68.48, 67.80, 67.50, 55.46, 52.55, 49.88, 47.22, 46.10, 44.12, 41.95, 36.84, 34.90, 34.33, 33.96, 33.47, 32.75, 31.64, 31.42, 31.02, 30.37, 28.62, 28.29, 21.26, 21.23, 17.36, 16.97, 16.87, 14.32, 14.29.

4.1.4. (8S,9R,10S,11S,13S,14S,16S,17R)-17-(2-(2-((tert-butoxycarbonyl)amino)acetoxy)acetyl)-9-fluoro-11-hydroxy-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 pentanoate (4a)

The desired compound was prepared as described for intermediate **1a**, starting from betamethasone-17-valerate (**4**, 100 mg, 0.209 mmol), Boc-Gly-OH (55 mg, 0.313), EDAC HCl (64 mg, 0.334 mmol), DMAP (41 mg, 0.334 mmol). Yield 109 mg, 82 %. m.p. 107.1–108.3 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 633.33 for $C_{34}H_{48}FNO_9$; found 634.2.

^{1}H NMR (400 MHz, DMSO) δ 7.29 (d, J = 9.6 Hz, 1H), 6.24 (d, J = 10.1, 1H), 6.03 (s, 1H), 5.54 (d, J = 4.8 Hz, 1H), 4.72 (d, J = 16.8 Hz, 1H), 4.54 (d, J = 16.7 Hz, 1H), 4.22 (bs, 1H), 3.92–3.70 (m, 2H), 2.95 (s, 1H), 2.69–2.60 (m, 1H), 2.55–2.49 (m, overlapped, 1H), 2.47–2.30 (m, 3H), 2.29–2.19 (m, 1H), 2.16–1.99 (m, 1H), 1.93–1.79 (m, 3H), 1.77–1.63 (m, 1H), 1.56–1.51 (m, overlapped, 2H), 1.50 (s, 3H), 1.39 (s, 9H), 1.38–1.33 (overlapped, 1H), 1.33–1.27 (m, overlapped, 3H), 1.24 (d, J = 7.3 Hz, 2H), 1.18–1.06 (m, 1H), 0.90–0.81 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ 198.69, 185.68, 174.41, 170.32, 167.20, 156.23, 152.95, 129.51, 124.62, 101.39 (d, $^1J_{C\text{-}F}$ = 175.7 Hz, C-9), 93.84, 78.76, 70.52 (d, $^2J_{C\text{-}F}$ = 35.9 Hz, C-11), 68.09, 48.18 (d, $^2J_{C\text{-}F}$ = 22.7 Hz, C-10), 47.85, 46.64, 43.68, 41.97, 36.67, 34.87, 34.13, 33.41 (d, $^2J_{C\text{-}F}$ = 19.3 Hz, C-8), 30.62, 28.63, 28.27, 28.03, 26.52, 23.40 (d, $^3J_{C\text{-}F}$ = 5.4 Hz, C-19), 22.10, 19.99, 16.68, 14.02.

4.1.5. 2-((6aR,6bS,7S,8aS,8bS,10R,11aR,12aS,12bS)-10-cyclohexyl-7-hydroxy-6a,8a-dimethyl-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 2-((tert-butoxycarbonyl)amino)acetate (5a)

The desired compound was prepared as described for **1a** starting from desisobutyryl ciclesonide (**5**, 100 mg, 0.213 mmol), Boc-Gly-OH (56 mg, 0.319 mmol), EDAC HCl (65 mg, 0.340 mmol) and DMAP (42 mg, 0.340 mmol). Yield 139 mg, 99 %. m.p. 149.0–151.0 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 627.78 for $C_{35}H_{49}NO_9$; found 628.2.

^{1}H NMR (400 MHz, DMSO) δ 7.38–7.26 (m, 2H), 6.17 (dd, J = 10.1, 1.7 Hz, 1H), 5.92 (s, 1H), 5.02 (d, J = 17.7 Hz, 1H), 4.90–4.80 (m, 2H), 4.70 (d, J = 3.8 Hz, 1H), 4.39 (d, J = 4.1 Hz, 1H), 4.32 (s, 1H), 3.88–3.72 (m, 2H), 2.59–2.50 (m, overlapped, 1H), 2.35–2.25 (m, 1H), 2.14–1.96 (m, 2H), 1.83 (s, 2H), 1.73–1.47 (m, 10 H), 1.39 (overlapped, 12H), 1.29–0.86 (m, 10H), 0.84 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 203.61, 185.61, 170.61, 170.41, 156.91, 156.21, 127.56, 122.15, 106.81, 97.45, 81.42, 78.68, 68.51, 67.81, 55.44, 49.91, 46.21, 44.13, 41.95, 40.48, 34.41, 33.41, 31.60, 31.43, 30.40, 28.29, 27.04, 26.77, 26.34, 25.58, 25.53, 22.54, 21.29, 16.99, 14.44.

4.1.6. 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 2-aminoacetate (1b)

Compound **1a** (2 g, 3.8 mmol) was dissolved in a solution containing 40 % TFA in DCM (20 mL) and the reaction was stirred at room temperature for 2h. The reaction mixture was evaporated, diethyl ether was added to the residue and the precipitate was isolated by centrifugation obtaining the desired product. Yield 1.57 g, 99 %. m.p. 88.3–89.5 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 415.18 for $C_{23}H_{29}NO_6$; found 416.2.

^{1}H NMR (400 MHz, DMSO) δ 8.35 (s, 2H), 7.60 (d, J = 10.2 Hz, 1H),

6.12 (d, $J = 10.2$ Hz, 1H), 6.03 (s, 1H), 5.96 (bs, 1H), 5.13 (d, $J = 17.7$ Hz, 1H), 4.96 (d, $J = 17.7$ Hz, 1H), 3.98 (s, 2H), 2.92 (d, $J = 12.0$ Hz, 1H), 2.54–2.50 (overlapped, 2H), 2.43–2.34 (m, 2H), 2.27–2.13 (m, 2H), 2.11–1.93 (m, 2H), 1.83–1.76 (m, 2H), 1.44–1.36 (overlapped, 4H), 1.28–1.14 (m, 1H), 0.52 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 210.57, 205.24, 185.54, 168.08, 167.63, 155.50, 127.51, 124.27, 88.19, 69.82, 65.38, 59.23, 51.44, 49.83, 49.11, 42.39, 36.04, 34.22, 33.59, 31.98, 23.28, 19.22, 15.50.

4.1.7. 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 2-aminoacetate (2b)

The desired compound was prepared as described for intermediate **1b** starting from compound **2a** (2.47 g, 4.4 mmol). Yield 2 g, 99 %. m.p. 77.0–78.0 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 449.52 for $C_{24}H_{32}FNO_6$; found 450.2.

^{1}H NMR (400 MHz, DMSO) δ 8.35 (s, 2H), 7.32 (d, $J = 10.1$ Hz, 1H), 6.24 (dd, $J = 10.1$, 1.8 Hz, 1H), 6.02 (s, 1H), 5.48 (bs, 1H), 5.28 (overlapped, 1H), 5.26 (d, $J = 17.6$ Hz, 1H), 4.91 (d, $J = 17.6$ Hz, 1H), 4.17 (d, $J = 9.3$ Hz, 1H), 3.98 (s, 2H), 2.96–2.80 (m, 1H), 2.69–2.56 (m, 1H), 2.46–2.28 (m, 2H), 2.26–2.08 (m, 2H), 1.83–1.73 (m, 1H), 1.72–1.58 (m, 1H), 1.58–1.49 (m, 4H), 1.42–1.30 (m, 1H), 1.17–1.04 (m, 1H), 0.91 (s, 3H), 0.81 (d, $J = 7.2$ Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 204.68, 185.74, 167.86, 167.48, 158.63, 158.31, 153.16, 129.47, 124.50, 101.76 (d, $^1J_{C\text{-}F} = 175.7$ Hz, C-9), 91.02, 70.84 (d, $^2J_{C\text{-}F} = 36.7$ Hz, C-11), 69.77, 48.63, 48.38 (d, $^2J_{C\text{-}F} = 22.7$ Hz, C-10), 43.80, 36.2, 36.07, 34.05 (d, $^2J_{C\text{-}F} = 19.1$ Hz, C-8) 32.33, 30.73, 27.76, 23.43 (d, $^3J_{C\text{-}F} = 5.7$ Hz, C-19),16.76, 15.51.

4.1.8. 2-((6aR,6bS,7S,8aS,8bS,11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-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 2-aminoacetate (3b)

The desired compound was prepared as described for intermediate **1b** starting from compound **3a** (1.35 g, 2.2 mmol). Yield 1 g, 93 %. m.p. 92–93 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 487.59 for $C_{27}H_{37}NO_7$; found 488.2.

^{1}H NMR (400 MHz, DMSO) δ 8.37 (s, 2H), 7.34 (dd, $J = 9.5$, 6.3 Hz, 1H), 6.18 (d, $J = 9.6$ Hz, 1H), 5.93 (s, 1H), 5.48 5.29–5.16 (m, 2H), 5.05 (d, $J = 7.2$ Hz, 1H), 4.97–4.82 (m, 4H), 4.75–4.64 (m, 2H), 4.36–4.26 (m, 1H), 4.01 (s, 2H), 2.58–2.51(overlapped, 1H), 2.43–2.25 (m, 1H), 2.13–1.94 (m, 2H), 1.88–1.72 (m, 3H), 1.65–1.44 (m, 5H), 1.38 (d, $J = 3.2$ Hz, 3H), 1.37–1.28 (m, 4H), 1.17–1.06 (m, 1H), 1.05–0.93 (m, 2H), 0.91–0.82 (m, 9H). ^{13}C NMR (101 MHz, DMSO) δ 203.24, 201.49, 185.58, 170.66, 170.57, 167.91, 167.84, 156.87, 127.60, 122.19, 107.91, 104.34, 98.37, 97.70, 82.95, 81.80, 68.70, 68.41, 65.38, 55.47, 52.56, 49.88, 47.36, 46.29, 44.10, 36.80, 34.88, 34.32, 33.93, 33.49, 32.72, 31.62, 31.03, 30.38, 21.23, 17.39, 17.35, 17.02, 16.88, 14.32.

4.1.9. (8S,9R,10S,11S,13S,14S,16S,17R)-17-(2-(2-aminoacetoxy) acetyl)-9-fluoro-11-hydroxy-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 pentanoate (4b)

The desired compound was prepared as described for intermediate **1b** starting from compound **4a** (1.57 g, 2.5 mmol). Yield 1.1 g, 83 %. m.p. 77–79 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 533.64 for $C_{24}H_{32}FNO_6$; found 534.2.

^{1}H NMR (400 MHz, DMSO) δ 8.47 (s, 2H), 7.32 (d, $J = 9.0$ Hz, 1H), 6.25 (d, $J = 8.6$ Hz, 1H), 6.04 (s, 1H), 5.61 (s, 1H), 4.79 (d, $J = 16.5$ Hz, 1H), 4.69 (d, $J = 16.5$ Hz, 1H), 4.24 (bs, 1H), 3.97 (s, 2H), 2.66–2.61 (m, 1H), 2.46–2.32 (m, 4H), 2.27 (d, $J = 13.2$ Hz, 1H), 2.12–2.05 (m, 1H), 1.91–1.85 (m, 3H), 1.67 (d, $J = 13.3$ Hz, 1H), 1.55–1.50 (m, overlapped, 2H), 1.50 (s, 3H), 1.43–1.34 (m, 1H), 1.33–1.27 (m, overlapped, 2H), 1.25 (d, $J = 7.2$ Hz, 3H), 1.16–1.08 (m, 1H), 0.89 (s, 3H), 0.85 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 198.20, 185.67, 174.50, 167.89, 167.15, 152.91, 129.52, 124.64, 101.39 (d, $^1J_{C\text{-}F} = 176.1$ Hz, C-9), 93.79, 70.40 (d, $^2J_{C\text{-}F} = 35.6$ Hz, C-11), 69.01, 48.15 (d, $^2J_{C\text{-}F} = 22.9$ Hz, C-10), 47.92, 46.73, 43.71, 36.79, 34.86, 34.13, 33.40 (d, $^2J_{C\text{-}F} = 19.1$ Hz, C-8), 30.62, 28.03, 26.51, 23.39 (d, $^3J_{C\text{-}F} = 5.4$ Hz, C-19), 22.10, 19.98, 16.73, 14.03.

4.1.10. 2-((6aR,6bS,7S,8aS,8bS,10R,11aR,12aS,12bS)-10-cyclohexyl-7-hydroxy-6a,8a-dimethyl-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 2-aminoacetate (5b)

The desired compound was prepared as described for intermediate **1b** starting from compound **5a** (1.2 g, 1.9 mmol). Yield 0.945 g, 94 %. m.p. 78–80 °C. ESI-MS $(M + H)^+$ *m/z* calcd. 527.66 for $C_{30}H_{41}NO_7$; found 528.2.

^{1}H NMR (400 MHz, DMSO) δ 8.37 (s, 2H), 7.34 (d, $J = 10.1$ Hz, 1H), 6.18 (dd, $J = 10.1$, 1.8 Hz, 1H), 5.93 (s, 1H), 5.16 (d, $J = 17.7$ Hz, 1H), 4.92 (d, $J = 17.7$ Hz, 1H), 4.90 (overlapped, 1H), 4.72 (d, $J = 4.1$ Hz, 1H), 4.42 (d, $J = 4.2$ Hz, 1H), 4.34 (bs, 1H), 4.01 (s, 2H), 2.57–2.52 (m, 1H), 2.35–2.26 (m, 1H), 2.14–1.96 (m, 2H), 1.83 (s, 2H), 1.78–1.45 (m, 10 H), 1.39 (s, 3H), 1.23–0.90 (m, 10H), 0.88–1.81 (m, 5H). ^{13}C NMR (101 MHz, DMSO) δ 203.26, 185.60, 170.55, 167.93, 156.85, 127.63, 122.23, 107.00, 97.41, 81.58, 68.62, 68.44, 55.45, 49.95, 46.33, 44.11, 34.40, 33.42, 31.60, 30.40, 27.06, 26.80, 26.34, 25.56, 25.52, 21.26, 17.03.

4.1.11. 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 2-isothiocyanatoacetate (1c)

The mixture of compound **1b** (1 g, 2.4 mmol) and Na_2CO_3 (0.763 g, 7.2 mmol) in acetone was placed in an ice-bath and thiophosgene (277 μL, 3.6 mmol) was added dropwise. The reaction mixture was kept at 0 °C for an hour and then at room temperature for an hour. The precipitated solid was filtered, and the solvent evaporated by distillation at reduced pressure. The obtained residue was treated with water and extracted with dichloromethane. The organic phases were dried with anhydrous Na_2SO_4, filtered and evaporated. The crude product was purified on a silica gel column using DCM/MeOH 9:1 (v/v) as the eluent mixture. The collected fractions were dried by distillation and the residue was crystallized with *n*-hexane obtaining a white solid product. Yield 362 mg, 33 %. m.p. 140.2–141.6 °C. ESI-HRMS $(M + H)^+$ *m/z* calcd. 457.1559 for $C_{24}H_{27}NO_6S$; found 458.1632.

^{1}H NMR (700 MHz, DMSO-d_6) δ 7.62 (d, $J = 10.2$ Hz, 1H), 6.12 (dd, $J = 10.2$, 1.7 Hz, 1H), 6.03 (s, 1H), 5.89 (s, 1H), 5.12 (d, $J = 17.7$ Hz, 1H), 4.93 (d, $J = 17.7$ Hz, 1H), 4.83 (s, 2H), 2.92 (d, $J = 12.1$ Hz, 1H), 2.57–2.51 (m, 2H), 2.41–2.34 (m, 2H), 2.20 (dd, $J = 14.2$, 12.0 Hz, 2H), 2.06 (qd, $J = 11.3$, 3.3 Hz, 1H), 2.01–1.97 (m, 1H), 1.81–1.75 (m, 1H), 1.71–1.66 (m, 1H), 1.43–1.36 (m overlapped, 4H), 1.25–1.18 (m, 1H), 0.53 (s, 3H).^{13}C NMR (176 MHz, DMSO) δ 210.55, 205.15, 185.56, 167.69, 166.77, 155.58, 133.80, 127.50, 124.25, 88.16, 69.98, 59.23, 51.36, 49.84, 49.21, 46.61, 42.42, 35.98, 34.23, 33.59, 32.00, 23.28, 19.22, 15.54.

4.1.12. 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 2-isothiocyanatoacetate (2c)

Following the same procedure reported above for compound **1c**, compound **2c** has been obtained starting from **2b** (1 g, 2.2 mmol), Na_2CO_3 (0.707 g, 6.7 mmol) and thiophosgene (255 μL, 3.3 mmol). Yield (392 mg, 36 %). m.p. 228.7–229.8 °C**.** ESI-HRMS $(M + H)^+$ *m/z* calcd. 491.1778 for $C_{25}H_{30}FNO_6S$; found 492.1851.

^{1}H NMR (700 MHz, DMSO-d_6) δ 7.30 (d, $J = 10.1$ Hz, 1H), 6.23 (dd, $J = 10.1$, 1.8 Hz, 1H), 6.01 (s, 1H), 5.45 (d, $J = 4.2$ Hz, 1H), 5.24 (s, 1H), 5.19 (d, $J = 17.5$ Hz, 1H), 4.92 (d, $J = 17.5$ Hz, 1H), 4.83 (s, 2H), 4.16 (dd, $J = 7.3$, 2.9 Hz, 1H), 2.93–2.85 (m, 1H), 2.65–2.60 (m, 1H), 2.42–2.30 (m, 2H), 2.21–2.11 (m, 2H), 1.81–1.74 (m, 1H), 1.65 (q, $J = 11.4$ Hz, 1H), 1.55 (d, $J = 12.4$ Hz, 1H),1.50 (s, 3H), 1.41–1.31 (m, 1H),

1.12–1.05 (m, 1H), 0.90 (s, 3H), 0.80 (d, $J = 7.3$ Hz, 3H). ^{13}C NMR (176 MHz, DMSO-d_6) δ 204.67, 185.75, 167.52, 166.62, 153.20, 133.66, 129.48, 124.58, 101,74 (d, $^{1}J_{C\text{-}F} = 175.4$ Hz, C-9), 91.03, 70.93 (d, $^{2}J_{C\text{-}F} = 36.5$ Hz, C-11), 70.02, 48.58, 48.40 (d, $^{2}J_{C\text{-}F} = 22.9$ Hz, C-10), 46.61, 43.78, 36.15, 36.02, 34.04 (d, $^{2}J_{C\text{-}F} = 19.1$ Hz, C-8), 32.37, 30.72, 27.76, 23.44 (d, $^{3}J_{C\text{-}F} = 5.4$ Hz, C-19), 16.77, 15.57.

4.1.13. 2-((6aR,6bS,7S,8aS,8bS,11aR,12aS,12bS)-7-hydroxy-6a,8a-dimethyl-4-oxo-10-propyl-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 2-isothiocyanatoacetate (3c)

Following the same procedure reported above for compound **1c**, compound **3c** has been obtained starting from **3b** (0.800 g, 1.6 mmol), Na_2CO_3 (0.522 g, 4.9 mmol) and thiophosgene (185 μL, 2.4 mmol). Yield (309 mg, 37 %). m.p. 105.7–106.2 °C. ESI-HRMS $(M + H)^+$ *m/z* calcd. 529.2134 for $C_{28}H_{35}NO_7S$; found 530.2213.

^{1}H NMR (700 MHz, DMSO-d_6) δ 7.32 (dd, $J = 10.0$, 7.4 Hz, 1H), 6.18 (ddd, $J = 10.1$, 3.2, 1.9 Hz, 1H), 5.93 (s, 1H), 5.21 (t, $J = 4.9$ Hz, 1H), 5.17 (dd, $J = 17.6$, 9.7 Hz, 1H), 5.05 (d, $J = 7.5$ Hz, 1H), 4.93 (d, $J = 17.6$ Hz, 1H), 4.89–4.85 (m, 3H), 4.71 (d, $J = 4.9$ Hz, 1H), 4.68 (t, $J = 4.4$ Hz, 1H), 4.34–4.29 (m, 1H), 2.56–2.52 (m, overlapped, 1H), 2.33–2.27 (m, 1H), 2.14–2.07 (m, 1H), 2.10 (qd, $J = 11.1$, 4.1 Hz, 1H), 1.83 (dd, $J = 14.0$, 2.8 Hz, 2H), 1.80–1.73 (m, 1H), 1.64–1.43 (m, 5H), 1.39 (d, $J = 4.6$ Hz, 3H), 1.37–1.22 (m, 4H), 1.17–1.08 (m, 1H), 1.04–0.93 (m, 2H), 0.89 (d, $J = 4.0$ Hz, 2H), 0.89–0.86 (m, 7H). ^{13}C NMR (176 MHz, DMSO-d_6) δ 203.27, 201.57, 185.60, 170.70, 170.62, 166.66, 166.60, 156.93, 156.90, 133.82, 133.80, 127.60, 127.59, 122.18, 122.12, 107.89, 104.31, 98.40, 97.69, 83.00, 81.82, 69.07, 68.74, 68.49, 68.45, 55.44, 52.58, 49.87, 47.40, 46.56, 46.30, 44.14, 44.12, 36.80, 34.90, 34.33, 33.95, 33.51, 32.73, 31.62, 31.43, 31.03, 30.37, 22.54, 21.27, 21.24, 17.40, 17.01, 16.86, 14.44, 14.34, 14.31.

4.1.14. (8S,9R,10S,11S,13S,14S,16S,17R)-9-fluoro-11-hydroxy-17-(2-(2-isothiocyanatoacetoxy)acetyl)-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 pentanoate (4c)

Following the same procedure reported above for compound **1c**, compound **4c** has been obtained starting from **4b** (1 g, 1.87 mmol), Na_2CO_3 (0.596 g, 5.6 mmol) and thiophosgene (215 μL, 2.8 mmol). Yield (332 mg, 31 %). m.p. 122.5–123.1 °C. ESI-HRMS $(M + H)^+$ *m/z* calcd. 575.2353 for $C_{30}H_{38}FNO_7S$; found 576.2431.

^{1}H NMR (700 MHz, DMSO-d_6) δ 7.30 (d, $J = 10.1$ Hz, 1H), 6.25 (dd, $J = 10.1$, 1.5 Hz, 1H), 6.03 (s, 1H), 5.58 (d, $J = 4.8$ Hz, 1H), 4.86 (s, 2H), 4.80 (d, $J = 16.8$ Hz, 1H), 4.64 (d, $J = 16.8$ Hz, 1H), 4.23 (bs, 1H), 2.68–2.60 (m, 1H), 2.48–2.33 (m, overlapped, 4H), 2.25 (d, $J = 13.3$ Hz, 1H), 2.11–2.05 (m, 1H), 1.90–1.85 (m, 3H), 1.69 (d, $J = 13.2$ Hz, 1H), 1.54–1.51 (m, overlapped, 2H), 1.50 (s, 3H), 1.43–1.35 (m, 1H), 1.31–1.26 (m, overlapped, 3H), 1.25 (d, $J = 7.2$ Hz, 2H), 1.16–1.09 (m, 1H), 0.89 (s, 3H), 0.85 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (176 MHz, DMSO-d_6) δ 198.21, 185.69, 174.48, 167.20, 166.63, 152.94, 133.86, 129.52, 124.63, 101.38 (d, $^{1}J_{C\text{-}F} = 175.8$ Hz, C-9), 93.89, 70.47 (d, $^{2}J_{C\text{-}F} = 35.8$ Hz, C-11), 69.34, 48.17 (d, $^{2}J_{C\text{-}F} = 22.8$ Hz, C-10), 47.92, 46.70, 46.56, 43.70, 36.70, 34.85, 34.14, 33.40 (d, $^{2}J_{C\text{-}F} = 19.1$ Hz, C-8), 30.62, 28.03, 26.51, 23.41 (d, $^{3}J_{C\text{-}F} = 5.4$ Hz, C-19), 22.11, 19.99, 16.70, 14.03.

4.1.15. 2-((6aR,6bS,7S,8aS,8bS,10R,11aR,12aS,12bS)-10-cyclohexyl-7-hydroxy-6a,8a-dimethyl-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 2-isothiocyanatoacetate (5c)

Following the same procedure reported above for compound **1c**, compound **3c** has been obtained starting from **3b** (0.900 g, 1.7 mmol), Na_2CO_3 (0.542 g, 5.0 mmol) and thiophosgene (195 μL, 2.6 mmol). Yield (290 mg, 30 %). m.p. 146.2–147.1 °C. ESI-HRMS $(M + H)^+$ *m/z* calcd. 569.2447 for $C_{31}H_{39}NO_7S$; found 570.2522.

^{1}H NMR (700 MHz, DMSO-d_6) δ 7.33 (d, $J = 10.1$ Hz, 1H), 6.18 (dd, $J = 10.1$, 1.8 Hz, 1H), 5.93 (s, 1H), 5.11 (d, $J = 17.6$ Hz, 1H), 4.93 (d, $J = 17.6$ Hz, 1H), 4.89 (d, $J = 4.1$ Hz, 1H), 4.86 (s, 2H), 4.71 (d, $J = 4.9$ Hz, 1H), 4.42 (d, $J = 4.2$ Hz, 1H), 4.33 (bs, 1H), 2.56–2.52 (m, 1H), 2.30 (dd, $J = 13.3$, 2.6 Hz, 1H), 2.09 (qd, $J = 11.1$, 4.1 Hz, 1H), 2.00 (dd, $J = 7.6$, 5.1 Hz, 1H), 1.83 (d, $J = 2.7$ Hz, 2H), 1.71–1.49 (m, 10H), 1.39 (s, 3H), 1.30–1.22 (m, 2H), 1.21–0.91 (m, 8H), 0.89–0.86 (m, 5H). ^{13}C NMR (176 MHz, DMSO-d_6) δ 203.30, 185.61, 170.59, 166.65, 156.89, 133.78, 127.62, 122.21, 106.96, 97.41, 81.61, 69.00, 68.48, 55.44, 49.94, 46.56, 46.37, 44.13, 34.42, 33.45, 31.60, 31.43, 30.40, 27.03, 26.77, 26.35, 25.59, 22.54, 21.29, 17.03, 14.44.

4.2. Chemical and enzymatic stability

4.2.1. Stability in SGF and PBS

Experiments were conducted as described by Sodano et al. [27–30]. Briefly, a solution of each hybrid (**1c-5c**, 10 mM) in DMSO was added to SGF-without pepsin (pH = 1.2) or PBS (pH = 7.4, 50 mM) preheated to 37 °C. SGF and PBS buffers were prepared as previously described [27–29]. Resulting solutions (100 μM) were kept at 37 ± 0.5 °C for 24 h and at appropriate time intervals, each 20 μL aliquot was withdrawn and analyzed by UHPLC, as reported below. Chemical stability investigations were carried out on a large scale (5 mL), meaning that the aliquots were not replenished. All experiments were performed at least in triplicate. The results are expressed either as pseudo-first-order half-times ($t_{1/2}$) or as % of unmodified hybrid compound **1c-5c** after 10 min of incubation in SGF or PBS.

4.2.2. Stability in BSA

A solution of each hybrid (10 mM) in DMSO was added to a solution of 3 % BSA in PBS, preheated to 37 °C. The obtained solutions (200 μM) were incubated at 37 ± 0.5 °C and at specific time intervals, 300 μL of mixture was withdrawn and added to 300 μL of acetonitrile containing 0.1 % TFA in order to deproteinize the medium. Samples were sonicated using a 2210 Branson Ultrasonic, vortexed using a Vortex ZX4 shaker equipped with an infrared system from Hosmotic (Naples, Italy), and then centrifuged for 10 min at 2150*g* using an Allegra X–30R high-volume refrigerated centrifuge equipped with fixed-angle rotors from Beckman Coulter (Brea, CA, USA). Each clear supernatant was filtered by 0.45 μm PTFE filters (Alltech). 20 μL aliquots were withdraw and analyzed by UHPLC, as reported below. Enzymatic stability investigations were carried out on a large scale (5 mL), meaning that the aliquots were not replenished. All experiments were performed at least in triplicate. The results are expressed either as pseudo-first-order half-times ($t_{1/2}$) or as % of unmodified hybrid compound **1c-5c** after 10 min of incubation in BSA.

4.2.3. Solubility assay

Solubility was assessed in deionized water, SGF-without pepsin (pH = 1.2) and PBS (pH = 7.4, 50 mM). The experimental procedure was recently reported [27–29]. Briefly, the test solid compounds (5 mg) were added to 1 mL of water, PBS and SGF in glass tubes and shaken for 6 h at 25 °C. The suspensions were filtered through 0.45 μm PTFE filters (Alltech), and the resulting solutions were analyzed by means of UHPLC (see below). Experiments were performed in triplicate for each hybrid. Solubility is expressed as mg/L.

4.2.4. UHPLC analysis

The reverse-phase UHPLC procedure allowed separation and quantification of molecular hybrids **1c-5c** and the corresponding metabolites, the 2-aminoacetate derivatives **1b**–**5b** and the starting corticosteroids **1**–**5**. UHPLC analysis was performed with an UHPLC 1260 PRIME Infinity II chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary flexible pump (model G7104C), a membrane degasser (model G1328C), a diode-array detector (DAD, model G7115A), a fluorescence detector (FLD, model G7121B) integrated in the UHPLC 1260 Infinity II System. Data analysis was processed using a OpenLab LC ChemStation system (Agilent Technologies).

The analytical column was a Kinetex ® XB-C18 (250 × 4.6 mm, 5 μm, 100 Å, Phenomenex). The column was kept at room temperature; the mobile phase consisted of water 0.1 % TFA (solvent A) and acetonitrile 0.1 % TFA (solvent B). The injection volume was 20 μL and the flow rate was 1 mL/min. Depending on the hybrid, the gradient conditions changed and the column effluent was monitored at 220, 238, 240 and 242 nm, referenced against 400 nm wavelength, according to the spectroscopic behavior of each analyte. Quantification of compounds was calculated using calibration curves, the linearity of which was determined over a concentration range of 1.56–100 ppm ($r^2 > 0.99$). For compounds **1-1b-1c**: 20 % B until 1 min, from 20 to 50 % B between 1 and 15 min, 50 % B until 16 min, from 50 to 80 % B between 16 and 18 min, 80 % B until 23 min, from 80 to 20 % B between 23 and 25 min. For compounds **2-2b-2c** & **3-3b-3c** & **4-4b-4c**: 35 % B until 3 min, from 35 to 80 % B between 3 and 15 min, 80 % B until 20 min, from 80 to 35 % B between 20 and 25 min. For compounds **5-5b-5c**: 35 % B until 3 min, from 35 to 80 % B between 3 and 15 min, 80 % B until 20 min, from 80 to 35 % B between 20 and 27 min.

4.3. In vitro evaluation

4.3.1. Evaluation of H_2S releasing properties by amperometry

The evaluation of the potential H_2S-donating properties of H_2S donor corticosteroid derivatives (**1c, 2c, 3c, 4c, 5c**) has been performed by using the Apollo-4000 free radical analyzer (WPI) detector and H_2S-selective mini-electrodes. The H_2S-selective electrode was equilibrated in 2 ml of a standard PBS buffer pH 7,4, until the recovery of a stable baseline. Then, 20 μL of a DMSO solution of the tested H_2S-releasing compounds was added, at the final concentration of 100 μM; the final concentration of DMSO in the assay buffer was 1 %. The generation of H_2S was observed for 30 min. When required by the experimental protocol, L-Cysteine (final concentration 4 mM) was added 10 min before the addition of tested compounds. The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H_2S was previously determined by suitable calibration curves, which were obtained using the H_2S donor NaHS (1-3-5-10 μM) at pH 4.0. The curves relative to the progressive increase of H_2S vs time, following the incubation of the tested compounds, were analyzed following the same procedure previously described.

4.3.2. Cell culture

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.

4.3.3. Cytotoxicity assay

Cells were cultured up to about 80 % confluence in culture medium and 24 h before the experiment, 10^4 cells per well were seeded in 96-well plate. After 24 h to allow cell attachment, the medium was replaced, and the cells were treated for 24 h with the tested compounds at the final concentration of 100 μM or vehicle DMSO 0,1 %.

Cell viability was assessed using the Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolium]-1,3-benzene disulphonate) (Roche) based on the cellular cleavage of the WST-1 to formazan. WST-1 was added at 1:10 of the total volume and after 60 min of incubation at 37 °C, the absorbance was measured at 450 nm with a multiplate reader (Enspire; PerkinElmer).

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

After reaching 80 % confluence, RBL-2H3 cells were seeded into a 96-well plate at 37 °C at the density of 72 × 10^3 cells for well and incubated for 24h at 37 °C in a humidified 5 % CO_2 atmosphere to allow cell attachment. RBL-2H3 were sensitized with an overnight anti-dinitrophenylated-human serum albumin (DNP)-IgE treatment (0.50 μg/ml), and subsequently, MEM was replaced with phenol-free DMEM supplemented with 1 mg/ml BSA. Vehicle (DMSO 0.1 %), cromolyn (1 mM) - a well-known mast cell stabilizer - and the tested compounds were incubated for 5 min at 37 °C. Cells were then treated with DNP (10 ng/ml) to induce the degranulation. One hour later the degranulation stimuli, 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).

4.4. In vivo evaluation

4.4.1. Animals

Female BALB/c mice (20 g, 8 weeks, Charles River Laboratories) were fed with standard rodent chow and water and acclimated for 4 days at a 12 h light and 12 h dark schedule in a constant air-conditioned environment (21 ± 2 °C). Mice were randomly assigned to groups, and experiments were carried out during the light phase. Experimental procedures were conducted in conformity with Italian (D.L. 26/2014) and European (directive 2010/63/EU) regulations on the protection of animals used for scientific purposes and approved by the Italian Ministry.

4.4.2. OVA sensitization

BALB/c mice were treated with 0.4 ml s.c. of a suspension containing 100 μg of ovalbumin from chicken egg white (OVA, Sigma-Aldrich, Milan, Italy) absorbed to 13.3 mg of aluminum hydroxide gel (Merck KGaA, Darmstadt, Germany) or saline (control group) on days 0 and 7 [31,32]. Compound **5c** (1–2 mg/kg) or vehicle were administered ip 30 min before each OVA administration. The pharmacological effect of **5c** at the dose of 2 mg/kg was compared to the equimolar dose of **5b**. All animals were sacrificed at day 21 by an overdose of enflurane, and lungs, bronchi, and blood were collected for functional and molecular studies.

4.4.3. Bronchial reactivity

All mice were sacrificed on day 21 by enflurane overdose, exsanguinated, and once the lungs were removed, the main bronchi were rapidly dissected and cleaned from fat and connective tissue. Rings of 1–2 mm length were cut and mounted in 3 ml isolated organ baths containing Krebs solution, at 37 °C, oxygenated (95 % O_2 and 5 % CO_2), and connected to an isometric force transducer (type 7006, Ugo Basile, Comerio, Italy) associated to a Powerlab 800 (AD Instruments). Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to 0.5 g and the bathing solution was periodically changed. In each experiment, bronchial rings were previously challenged with carbachol ($1x10^{-6}$ M) (Sigma-Aldrich, Milan, Italy) and then a cumulative concentration-response curve of carbachol ($1x10^{-9}$M − $3x10^{-6}$ M) was performed to evaluate bronchial reactivity. Results are expressed as dyne per mg tissue [31,32].

4.4.4. Lung histology and immunohistochemistry

Left lung lobes harvested from mice were fixed in formalin 4 %, embedded in paraffin and 7 μm sections were cut. Lung slices were processed to remove paraffin and were rehydrated. Sections were stained with Hematoxylin and Eosin (H&E, Kaltek, Padua, Italy) to evaluate lung structure [31]. Other slices were processed and incubated with 3 % hydrogen peroxide (Sigma-Aldrich) for 15 min to quench the endogenous peroxidase activity. Non-specific interactions were reduced with 3 % BSA in PBS +0.1%Tween and tissue sections were incubated overnight with anti-actin α-smooth muscle antibody (A5228, Sigma, 1:100) or Isotype control. After rinsing with PBS 0.01 M, sections were incubated with Peroxidase AffiniPure Goat anti-Rabbit IgG (Jackson

ImmunoResearch, 1:500) for 1h at room temperature. Color development was visualized using 3,3′-diaminobenzidine Chromogen Solution (SIGMAFASTTM-DAB). Images were acquired by blinded operators using a Leica DFC320 video camera (Leica, Milan, Italy; magnification of 10x or 20x for H&E, 20x for α-SMA localization) connected to a Leica DM RB microscope using the Leica Application Suite software V.4.1.0.

4.4.5. Measurement of plasma IgE and pulmonary IL-13 levels

Blood was collected by intracardiac puncture using citrate as anticoagulant. Then plasma was obtained by centrifugation at 800×*g* at 4 °C for 10 min and immediately frozen at −80 °C. Total IgE levels were measured through an ELISA test using matched antibody pairs (BD Biosciences Pharmingen San Jose, CA). Lungs were isolated and homogenized in ice-cold PBS pH 7.4 (100 mg/ml, Sigma Aldrich, Milan, Italy) using a FastPrep tissue homogenizer (2 cycles of 20 s, 6 m/s; MP Biomedicals, DBA, Segrate, Italy). The homogenate was centrifuged (4 °C, 6000×*g*, 10 min). Commercially available ELISA test was used to measure the levels of IL-13 (DuoSet ELISA R&D systems).

4.4.6. Statistical analysis

The results are expressed as mean ± S.E.M of the mean of n observations, where n represents the number of animals or number of experiments performed at different days. In the experiments involving histology, the figures shown are representative of six animals. The results were analyzed by one-way or two-way ANOVA followed by Bonferroni post hoc tests. Post hoc tests were performed only if F achieved the $p < 0.05$ level of significance. A p-value less than 0.05 was considered significant.

CRediT authorship contribution statement

Antonia Scognamiglio: Writing – original draft, Methodology, Data curation. **Ida Cerqua:** Writing – original draft, Methodology, Data curation. **Valentina Citi:** Writing – original draft, Investigation, Data curation. **Alma Martelli:** Supervision, Formal analysis. **Jacopo Spezzini:** Methodology, Investigation. **Vincenzo Calderone:** Supervision, Conceptualization. **Maria Grazia Rimoli:** Validation, Formal analysis. **Federica Sodano:** Writing – original draft, Methodology, Investigation. **Giuseppe Caliendo:** Supervision, Conceptualization. **Vincenzo Santagada:** Validation, Supervision, Data curation. **Ferdinando Fiorino:** Validation, Formal analysis, Data curation. **Francesco Frecentese:** Visualization, Formal analysis, Data curation. **Elisa Perissutti:** Validation, Resources. **Elisa Magli:** Visualization, Validation, Data curation. **Martina Simonelli:** Methodology, Investigation. **Angela Corvino:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Fiorentina Roviezzo:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Beatrice Severino:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2024.116636.

References

[1] N.S. Khan, E. Rubin, B. McKenna, B.L. Palowitch, F. Sonnenberg, J. Argon, R. A. Panettieri Jr., The COVID-19 impact on severe uncontrolled asthma costs and biologic use, Allergy Asthma Proc. 44 (5) (2023) 340–344, https://doi.org/10.2500/aap.2023.44.230045.

[2] D. Liu, A. Ahmet, L. Ward, P. Krishnamoorthy, E.D. Mandelcorn, R. Leigh, J. P. Brown, A. Cohen, H. Kim, A practical guide to the monitoring and management of the complications of systemic corticosteroid therapy, Allergy Asthma Clin. Immunol. 9 (1) (2013) 30, https://doi.org/10.1186/1710-1492-9-30.

[3] Y. Suzuki, J. Saito, M. Munakata, Y. Shibata, Hydrogen sulfide as a novel biomarker of asthma and chronic obstructive pulmonary disease, Allergol. Int. 70 (2) (2021) 181–189, https://doi.org/10.1016/j.alit.2020.10.003.

[4] S. Khattak, Q.Q. Zhang, M. Sarfraz, P. Muhammad, E.E. Ngowi, N.H. Khan, S. Rauf, Y.Z. Wang, H.W. Qi, D. Wang, A. Afzal, X.Y. Ji, D.D. Wu, The role of hydrogen sulfide in respiratory diseases, Biomolecules 11 (5) (2021) 682, https://doi.org/10.3390/biom11050682.

[5] E. Magli, E. Perissutti, V. Santagada, G. Caliendo, A. Corvino, G. Esposito, G. Esposito, F. Fiorino, M. Migliaccio, A. Scognamiglio, B. Severino, R. Sparaco, F. Frecentese, H_2S donors and their use in medicinal chemistry, Biomolecules 11 (12) (2021) 1899, https://doi.org/10.3390/biom11121899.

[6] R.R. Ramsay, M.R. Popovic-Nikolic, K. Nikolic, E. Uliassi, M.L. Bolognesi, A perspective on multi-target drug discovery and design for complex diseases, Clin. Transl. Med. 7 (1) (2018) 3, https://doi.org/10.1186/s40169-017-0181-2.

[7] I. Cerqua, E. Granato, A. Corvino, B. Severino, D. D'Avino, M. Simonelli, E. Perissutti, A. Scognamiglio, D. Mirra, B. D'Agostino, G. Caliendo, A. Rossi, G. Cirino, C.M. Motta, F. Roviezzo, Prednisone-hydrogen sulfide releasing hybrid shows improved therapeutic profile in asthma, Front. Pharmacol. 14 (2023) 1266934, https://doi.org/10.3389/fphar.2023.1266934.

[8] A. Corvino, V. Citi, F. Fiorino, F. Frecentese, E. Magli, E. Perissutti, V. Santagada, V. Calderone, A. Martelli, E. Gorica, S. Brogi, F.F. Colombo, C. Nunes Capello, H. H. Araujo Ferreira, M.G. Rimoli, F. Sodano, B. Rolando, F. Pavese, A. Petti, M. N. Muscará, G. Caliendo, B. Severino, H_2S Donating corticosteroids: design, synthesis and biological evaluation in a murine model of asthma, J. Adv. Res. 35 (2022) 267–277, https://doi.org/10.1016/j.jare.2021.05.008.

[9] F. Giordano, A. Corvino, A. Scognamiglio, V. Citi, E. Gorica, C. Fattorusso, M. Persico, G. Caliendo, F. Fiorino, E. Magli, E. Perissutti, V. Santagada, B. Severino, R.C. Pavese, F. Petti, A. Martelli, V. Calderone, F. Frecentese, Hybrids between H_2S-donors and betamethasone 17-valerate or triamcinolone acetonide inhibit mast cell degranulation and promote hyperpolarization of bronchial smooth muscle cells, Eur. J. Med. Chem. 221 (2021), https://doi.org/10.1016/j.ejmech.2021.113517.

[10] S.A. Coavoy-Sánchez, A.R.A. Cerqueira, S.A. Teixeira, V. Santagada, G. Andreozzi, A. Corvino, A. Scognamiglio, R. Sparaco, G. Caliendo, B. Severino, S.K.P. Costa, L. C. Spolidorio, M.N. Muscará, Beneficial effects of two hydrogen sulfide (H_2S)-releasing derivatives of dexamethasone with antioxidant activity on atopic dermatitis in mice, Pharmaceutics 15 (7) (2023) 1907, https://doi.org/10.3390/pharmaceutics15071907.

[11] R. Sparaco, V. Citi, E. Magli, A. Martelli, E. Piragine, V. Calderone, G. Andreozzi, E. Perissutti, F. Frecentese, V. Santagada, G. Caliendo, B. Severino, A. Corvino, F. Fiorino, Design, synthesis and evaluation of novel molecular hybrids between antiglaucoma drugs and H_2S donors, Int. J. Mol. Sci. 23 (22) (2022) 13804, https://doi.org/10.3390/ijms232213804.

[12] G. Ercolano, P. De Cicco, F. Frecentese, I. Saccone, A. Corvino, F. Giordano, E. Magli, F. Fiorino, B. Severino, G. Cirino, A. Ianaro, Anti-metastatic properties of naproxen-HBTA in a murine model of cutaneous melanoma, Front. Pharmacol. 10 (2019) 66, https://doi.org/10.3389/fphar.2019.00066.

[13] S. Sestito, S. Daniele, D. Pietrobono, V. Citi, L. Bellusci, G. Chiellini, V. Calderone, C. Martini, S. Rapposelli, Memantine prodrug as a new agent for Alzheimer's Disease, Sci. Rep. 9 (1) (2019) 4612, https://doi.org/10.1038/s41598-019-40925-8.

[14] S. Rapposelli, L. Gambari, M. Digiacomo, V. Citi, G. Lisignoli, C. Manferdini, V. Calderone, F. Grassi, A Novel H_2S-releasing amino-bisphosphonate which combines bone anti-catabolic and anabolic functions, Sci. Rep. 7 (2017) 11940, https://doi.org/10.1038/s41598-017-11608-z.

[15] V. Citi, E. Barresi, E. Piragine, J. Spezzini, L. Testai, F. Da Settimo, A. Martelli, S. Taliani, V. Calderone, Anti-proliferative properties of the novel hybrid drug Met-ITC, composed of the native drug metformin with the addition of an isothiocyanate H_2S donor moiety, in different cancer cell lines, Int. J. Mol. Sci. 24 (22) (2023) 16131, https://doi.org/10.3390/ijms242216131.

[16] H.F. Gu, X.Y. Mao, M. Du, Metabolism, absorption, and anti-cancer effects of sulforaphane: an update, Crit. Rev. Food Sci. Nutr. 62 (13) (2022) 3437–3452, https://doi.org/10.1080/10408398.2020.1865871.

[17] A. Corvino, F. Frecentese, E. Magli, E. Perissutti, V. Santagada, A. Scognamiglio, G. Caliendo, F. Fiorino, B. Severino, Trends in H_2S-donors chemistry and their effects in cardiovascular diseases, Antioxidants 10 (3) (2021) 429, https://doi.org/10.3390/antiox10030429.

[18] V. Citi, A. Corvino, F. Fiorino, F. Frecentese, E. Magli, E. Perissutti, V. Santagada, S. Brogi, L. Flori, E. Gorica, L. Testai, A. Martelli, V. Calderone, G. Caliendo, B. Severino, Structure-activity relationships study of isothiocyanates for H_2S releasing properties: 3-Pyridyl-isothiocyanate as a new promising cardioprotective agent, J. Adv. Res. 27 (2020) 41–53, https://doi.org/10.1016/j.jare.2020.02.017.

[19] V. Ciccone, E. Piragine, E. Gorica, V. Citi, L. Testai, E. Pagnotta, R. Matteo, N. Pecchioni, R. Montanaro, L. Di Cesare Mannelli, C. Ghelardini, V. Brancaleone, L. Morbidelli, V. Calderone, A. Martelli, Anti-inflammatory effect of the natural

H_2S-donor erucin in vascular endothelium, Int. J. Mol. Sci. 23 (24) (2022) 15593, https://doi.org/10.3390/ijms232415593.
[20] Y. Lin, X. Yang, Y. Lu, D. Liang, D. Huang, Isothiocyanates as H_2S donors triggered by cysteine: reaction mechanism and structure and activity relationship, Org. Lett. 21 (15) (2019) 5977–5980, https://doi.org/10.1021/acs.orglett.9b02117.
[21] B. Severino, A. Corvino, F. Fiorino, P. Luciano, F. Frecentese, E. Magli, I. Saccone, P. Di Vaio, V. Citi, V. Calderone, L. Servillo, R. Casale, G. Cirino, V. Vellecco, M. Bucci, E. Perissutti, V. Santagada, G. Caliendo, 1,2,4-Thiadiazolidin-3,5-diones as novel hydrogen sulfide donors, Eur. J. Med. Chem. 143 (2018) 1677–1686, https://doi.org/10.1016/j.ejmech.2017.10.068.
[22] A. Martelli, L. Testai, V. Citi, A. Marino, F.G. Bellagambi, S. Ghimenti, M. C. Breschi, V. Calderone, Pharmacological characterization of the vascular effects of aryl isothiocyanates: is hydrogen sulfide the real player? Vascul, Pharmacol. 60 (1) (2014) 32–41, https://doi.org/10.1016/j.vph.2013.11.003.
[23] Y.R. Cai, C.H. Hu, Computational study of H_2S release in reactions of diallyl polysulfides with thiols, J. Phys. Chem. B 121 (26) (2017) 6359–6366, https://doi.org/10.1021/acs.jpcb.7b03683.
[24] L. Drobnica, P. Kristian, J. Augustin, The chemistry of the -NCS group, in: Chemistry of Cyanates and Their Thio Derivatives, vol. 2, John Wiley & Sons, Chichester, UK, 1977, p. 1003.
[25] G.H. Banafea, S. Bakhashab, H.F. Alshaibi, P. Natesan Pushparaj, M. Rasool, The role of human mast cells in allergy and asthma, Bioengineered 13 (3) (2022) 7049–7064, https://doi.org/10.1080/21655979.2022.2044278.
[26] A. Marino, A. Martelli, V. Citi, M. Fu, R. Wang, V. Calderone, R. Levi, The novel H_2S donor 4-carboxy-phenyl isothiocyanate inhibits mast cell degranulation and renin release by decreasing intracellular calcium, Br. J. Pharmacol. 173 (22) (2016) 3222–3234, https://doi.org/10.1111/bph.13583.
[27] F. Sodano, C. Cristiano, B. Rolando, E. Marini, L. Lazzarato, M. Cuozzo, S. Albrizio, R. Russo, M.G. Rimoli, Galactosylated prodrugs: a strategy to improve the profile of Nonsteroidal anti-inflammatory drugs, Pharmaceuticals 15 (5) (2022) 552, https://doi.org/10.3390/ph15050552.
[28] F. Sodano, L. Lazzarato, B. Rolando, F. Spyrakis, C. De Caro, S. Magliocca, D. Marabello, K. Chegaev, E. Gazzano, C. Riganti, A. Calignano, R. Russo, M. G. Rimoli, Paracetamol-galactose conjugate: a novel prodrug for an old analgesic drug, Mol. Pharm. 16 (10) (2019) 4181–4189, https://doi.org/10.1021/acs.molpharmaceut.9b00508.
[29] S. Magliocca, C. De Caro, L. Lazzarato, R. Russo, B. Rolando, K. Chegaev, E. Marini, M. Nieddu, L. Burrai, G. Boatto, C. Cristiano, D. Marabello, E. Gazzano, C. Riganti, F. Sodano, M.G. Rimoli, Aceclofenac-galactose conjugate: design, synthesis, characterization, and pharmacological and toxicological evaluations, Mol. Pharm. 15 (8) (2018) 3101–3110, https://doi.org/10.1021/acs.molpharmaceut.8b00195.
[30] F. Sodano, B. Avallone, M. Tizzano, C. Fogliano, B. Rolando, E. Gazzano, C. Riganti, S. Magliocca, M. Cuozzo, S. Albrizio, S, A. Calignano, C. Cristiano, R. Russo, M. G. Rimoli, Ketogal Safety profile in human primary Colonic epithelial cells and in mice, Pharmaceuticals 14 (2021) 1149, https://doi.org/10.3390/ph14111149.
[31] D. D'Avino, I. Cerqua, H. Ullah, M. Spinelli, R. Di Matteo, E. Granato, R. Capasso, L. Maruccio, A. Ialenti, M. Daglia, F. Roviezzo, A. Rossi, Beneficial effects of *Astragalus Membranaceus* (fisch.) bunge extract in controlling inflammatory response and preventing asthma features, Int. J. Mol. Sci. 24 (13) (2023) 10954, https://doi.org/10.3390/ijms241310954.
[32] K. Neukirch, K. Alsabil, C.P. Dinh, R. Bilancia, M. Raasch, A. Ville, I. Cerqua, G. Viault, D. Bréard, S. Pace, V. Temml, E. Brunner, P.M. Jordan, M.C. Marques, K. Loeser, A. Gollowitzer, S. Permann, J. Gerstmeier, S. Lorkowski, H. Stuppner, U. Garscha, T. Rodrigues, G.J.L. Bernardes, D. Schuster, D. Séraphin, P. Richomme, A. Rossi, A.S. Mosig, F. Roviezzo, O. Werz, J.J. Helesbeux, A. Koeberle, Exploration of long-chain vitamin e metabolites for the discovery of a highly potent, orally effective, and metabolically stable 5-LOX inhibitor that limits inflammation, J. Med. Chem. 64 (15) (2021) 11496–11526, https://doi.org/10.1021/acs.jmedchem.1c00806.