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NO–Steroids: Potent Anti-inflammatory Drugs with Bronchodilating Activity *in Vitro*

Dominique Tallet,* Piero Del Soldato,† Nicole Oudart,* and Jean-Luc Burgaud†,[1]
**Laboratoire de Pharmacologie, Faculté de Pharmacie de Limoges, 2 rue du Docteur Marcland, 87025 Limoges cedex, France; and †NicOx, Gaia II, B.P. 313, 2455 route des Dolines, 06906 Sophia-Antipolis, France*

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The synthesis of nitric oxide (NO) releasing anti-inflammatory molecules is an innovative strategy to design novel anti-inflammatory drugs. These compounds slowly release NO, via an enzymatic pathway conferring new biological activities. Here we report the potent anti-inflammatory profile and the bronchodilator effect of nitro-derivatives of steroids, prednisolone, especially. The experiments were performed on guinea pig trachea or perfused bronchioles precontracted by methacholine. We demonstrated for the first time that unlike the parent compounds which produced weak bronchodilation at the maximum used dose (10^{-4} M), NO–steroids caused a significant bronchodilating activity up to 70% of the maximal relaxation induced by 10^{-4} M papaverine. This effect was epithelium- and endogenous-independent but cGMP-dependent. Taken together these data suggest that NO–steroids possessed a more potent anti-inflammatory activity than native compounds coupled with a concentration-dependent bronchodilating activity. Further studies are required to determine if NO–steroids will be effective as anti-inflammatory agents in the clinic.

Key Words: **NO–steroids; nitric oxide; airways; guinea pig; bronchodilation.**

Bronchial asthma is widely recognized as a chronic inflammatory disorder of the airways. In the course of an inflammation response, many types of inflammatory cells such as lymphocytes (especially T2 lymphocytes), mast cells, eosinophils interact with each other in a manner controlled by the so-called cytokine network.

Therapeutic drugs used for treatment of diseases of the airways may generally be divided into drugs aimed at reducing the primary airway inflammation (e.g., steroids) and drugs aimed at causing bronchodilatation (e.g., β-adrenoceptor agonists such as salbutamol). Interestingly, production of nitric oxide (NO) has also been found to be elevated in a number of airway diseases, whilst, inhaled NO has been suggested as a useful therapy to induce airway dilation (1–4). Inhaled NO may be beneficial, by reducing inflammatory cell adhesion and infiltration into the airways as well as by promoting bronchodilatation (5). Taken together these observations suggest that NO-linked drugs, for instance, NO–steroids, may be very effective therapy for airway diseases such as asthma and chronic obstructive pulmonary disease (COPD).

Steroid effects are produced by multiple mechanisms of actions underlying the initiation and maintenance of the host inflammatory response. This is perhaps not surprising, since it has been calculated that almost 1% of the genes present in the nucleus of a human cell can be affected by steroids. Therefore, steroid actions may be considered as the end point of a potent alteration of protein expression, both in terms of downregulation or induction of their expression. The synthesis of a range of pro-inflammatory cytokines, enzymes, chemokines, and adhesion molecules are inhibited by steroids, whereas others, e.g., those of lipocortin 1, β_2-adrenergic receptor are induced by these drugs (6). More recent work has shown that steroids affect the action of a pivotal transcription factor, nuclear factor-κB (NF-κB), which controls the synthesis of several pro-inflammatory mediators: steroids reduce the effect of this and other transcription factors again in several ways, including direct binding of the complex steroid/receptor to the transcription factor (as in the case of AP-1) or the induction of endogenous inhibitors which trap the specific transcription factor in an inactive status (such as for IκB and NF-κB) (7).

On the other hand, long-term use of glucocorticoids is associated with multiple side effects, affecting the bone and hypothalamopituitary axis especially. Moreover, the administration of larges doses of steroids often necessary to produce a therapeutic response result from excessive action on electrolyte balance on

[1] To whom correspondence and reprint requests should be addressed. Fax: 33 (0)4 92 38 70 30. E-mail: burgaud@nicox.com.

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other aspects of metabolism, including gluconeogenesis, the action on tissue repair and healing, and an inhibitory effect on the secretion of corticotrophin by the anterior lobe of the pituitary gland. Although the aerosol steroids are administered in low doses because of their high topical activity, there are local side effects, as well as concern for certain systemic side effects. Three systemic effects of concern with inhaled steroids have been HPA suppression, loss of bone density, and growth retardation in children. Suppression of HPA function is nonexistent or small at low doses of inhaled aerosol steroids but increases with higher doses. Although higher inhaled doses of steroid have a greater risk of adrenal suppression, the dose at which the risk for toxicity outweights the beneficial effect of an inhaled steroid is not known. There are questions about the effect of inhaled steroids on growth when used with prepubertal children, growth retardation was observed in studies with beclomethasone dipropionate. Moreover, even if there are not clear evidence, data showed the effects of inhaled glucocorticoids on bone density and osteoporosis in asthma. More potent novel steroids with less side effects, used at a lower dose, are now of an important need.

In the present study, we present the effects of novel steroids with a new pharmacological profile. Adding a chemical moiety able to release nitric oxide (NO) to conventional steroids (NO–steroids), we synthetized new chemical entities which demonstrate greater maximal anti-inflammatory effects with a bronchodilating activity.

MATERIALS AND METHODS

To demonstrate the new pharmacological profile and the interest in the use of NO–steroids on airways we showed that NO derivatives of hydrocortisone (NCX-1004), dexamethasone, (NCX-1005), prednisolone (NCX-1010 and NCX-1015) and budesonide (NCX-1020) could induce bronchodilation in the same mechanism. In order to simplify the presentation of the data, we have used NO–prednisolone (NCX 1015) and NO–budesonide (NCX-1020) as example products.

NO–steroids synthesis. The NicOx technology was applied for the synthesis of all NO–steroids. To avoid redundancy, we will describe the synthesis of one compound: NCX-1015, the NO-derivative of prednisolone.

NCX-1015 (prednisolone 21-[(4′-nitrooxymethyl)benzoate]) was synthetised at NicOx S.a. laboratories, in Milan (Italy). The batches were prepared following a two-step synthesis with a overall yield of about 75%. A solution of prednisolone (33.3 mmol) in tetrahydrofurane was added with 49.9 mmol of 4-(chloromethyl)benzoyl chloride and triethylamine. The reaction was stirred for 24 h, and the solvent evaporated under vacuum. Following treatment of the residue with ethyl acetate and water, and removal of the insoluble material, the intermediate I (prednisolone 21-(4′-chlorobenzoate) was obtained by anidrification with sodium sulfate and concentration under reduced pressure (31.19 mmol, yeald 97%). This intermediate was then treated with silver nitrite (43.66 mmol), acetonitrile (100 ml) and tetrahydrofurane (200 ml) in the dark for 35 h. The precipitate was filtered off, the solvent evaporated under vacuum and the residue purified by silica gel chromatography, and cristallised from tetrahydrofurane. The final product (prednisolone 21-[(4′-nitrooxymethyl)benzoate) was obtained as a white powder, with a molecular weight of 539.59 and a melting point of 231–235°C. The structure of NCX-1015 was confirmed by ^{1}H NMR, ^{13}C NMR and infrared analyses (Fig. 1).

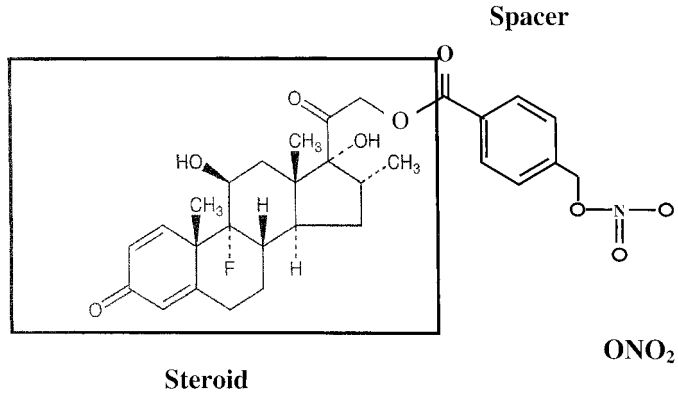

FIG. 1. Chemical structure of NO–prednisolone (NCX 1015).

Isometric technique. Durkin–Hartley guinea pigs of either sex weighing 400–500 g were anesthetized with urethane (1.5 g · kg^{-1} i.p.) and killed by cutting off the carotid artery. The trachea was immediately removed and placed in modified Krebs'-Henseleit solution. This solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 24.9, and glucose 11, and was gassed with 5% CO_2/95% O_2 with a pH of 7.3–7.45. One trachea ring (2 mm large) was cut and transferred to an organ bath, through which flowed oxygenated buffer solution (5 ml/min; 37°C), and equilibrated under a constant tension of 2*g* for 30 min before being precontracted with 3×10^{-6} M methacholine. When the contraction was stable, increasing concentration of steroids or NO–steroids was added to the organ bath in a cumulative manner. When the action of L-NAME (NO synthase inhibitor) or ODQ (soluble guanylate cyclase inhibitor) were tested, drugs were added to the perfusate reservoir at concentrations of 10^{-4} or 5×10^{-6} M, respectively, 45 min prior testing the effect of NO–steroids or steroids. At the end of each test, 10^{-4} M papaverine was added to the perfusate reservoir to induce a maximal relaxation as an internal reference.

Steroids and NO–steroids were dissolved in dimethyl sulfoxide (1% v/v final concentration). Preliminary tests showed 4% DMSO did not induce any relaxant effect on guinea-pig trachea.

Perfusion technique. We used the perfusion technique previously described (8) in guinea pig perfused bronchioles. Briefly, Durkin–Hartley guinea pig of either sex were anesthetized with urethane (1.5 g/kg, i.p.) and bled. The lungs were quickly removed and placed in the same buffer solution as in the isometric technique. This solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 24.9, and glucose 11, and was gassed with 5% CO_2/95% O_2 with a pH of 7.3–7.45.

An intact bronchiolar segment of the pulmonary cardiac lobe was cannulated with a short, polished hypodermic needle. The cannulated bronchiole was then placed in an organ bath at 37°C and perfused at a constant rate of 1.0 ml min^{-1} by means of a peristaltic pump. The bronchiolar segments were allowed to equilibrate for 90 min in the buffer solution before tests. Increasing concentrations of drugs were added to the perfusate reservoir in a cumulative manner. The interval between consecutive concentrations did not exceed 10–15 min.

At the end of each test, 10^{-4} M papaverine was added to the perfusate reservoir to induce a maximal bronchodilation as an internal reference. Steroids and NO–steroids were dissolved in polyethylene glycol 400 (1% v/v final concentration).

Preliminary tests showed PEG did not induce any relaxant effect on guinea-pig perfused bronchioles.

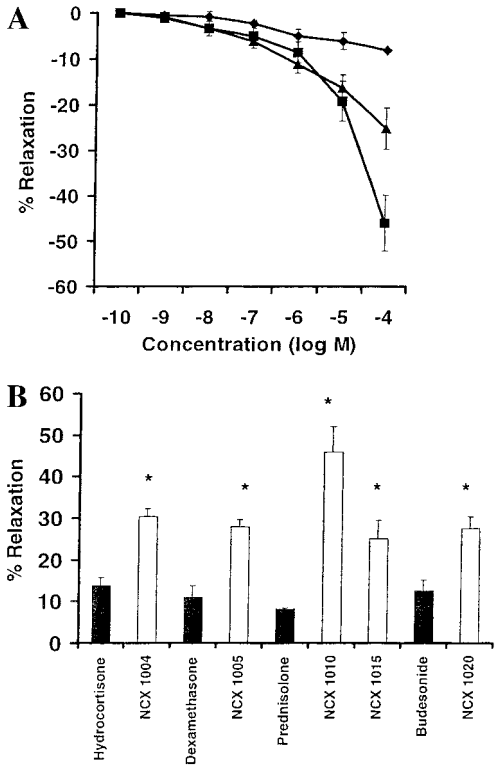

FIG. 2. (A) Concentration–response curves for prednisolone (◆), NCX 1010 (■), and NCX 1015 (▲) in guinea pig tracheal rings. (B) Comparison of mean maximal relaxant effects (E_{max}, mg) to 10^{-4} M hydrocortisone, dexamethasone, prednisolone, budesonide and their nitro-derivatives in guinea pig tracheal rings. Each point is the mean of at least 6 animals and vertical lines show SEM. *$P < 0.05$ vs native steroid.

Data analysis. Relaxation is expressed as a percentage of the maximum relaxation induced by 10^{-4} M papaverine. All parameters were compared by analysis of variance when we compared results obtained with different treatments (9). Difference was considered significant when $P < 0.05$. Dispersion of values about the mean were indicated by the standard error of mean (SEM).

RESULTS

Unlike Steroids, NO–Steroids Induced Bronchodilation

In guinea-pig trachea and perfused bronchiole preparations, methacholine 3×10^{-6} M caused a 50% maximal contraction to increase tension by 1423 ± 93 mg ($n = 50$) and perfusion pressure 26.4 ± 2.9 mm Hg ($n = 50$), respectively. There was no effect of epithelium removal upon the response to the muscarinic agonist on any tissue. The presence of L-NAME (10^{-4} M) or soluble guanylate cyclase inhibitor ODQ (5×10^{-6} M) did not alter baseline tone or the contractile response to methacholine. On guinea-pig trachea and bronchioles, the maximal relaxation for 10^{-4} M papaverine was 2409 ± 117 mg ($n = 50$) and 27.1 ± 2.2 mm Hg ($n = 50$), respectively. At the end of the experiments on trachea with or without epithelium, we noted that papaverine induced equivalent relaxation in both preparations.

In tracheal rings precontracted with methacholine, steroids induced a weak relaxation of the airways smooth muscle cells. In the opposite, NO–steroids caused a concentration-dependent relaxation (Fig. 2). The response to NO–steroids was not modified by the presence in the organ bath of 10^{-4} M ML-NAME or by the lack of epithelium (Fig. 4A). In contrast the presence of 5 μM ODQ blocked by about 80–90% the relaxation induced by the NO derivatives of steroids. Steroids had no significant effect on the methacholine precontracted guinea-pig trachea (Fig. 3B).

In perfused bronchioles, as in tracheal rings, NO–prednisolone and NO–budesonide elicited a concentration-dependent relaxation with a higher potency than on trachea preparation, reaching 73 ± 7% of 10^{-4} M papaverine-induced maximum relaxation (Fig. 4). In

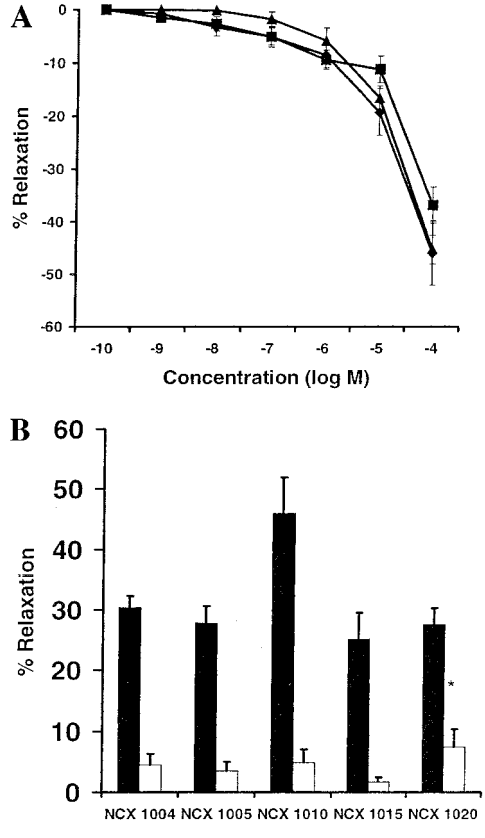

FIG. 3. (A) Response of control (◆), in presence of L-NAME (■) and without epithelium (▲) guinea pig tracheal rings to NO–prednisolone (NCX 1010). Each point represents mean ± SEM for at least 6 animals. (B) Comparison of mean maximal of relaxant effects (E_{max}, mg) to 10^{-4} M NO–hydrocortisone (NCX 1004), NO–dexamethasone (NCX 1005), NO–prednisolone (NCX 1010; NCX 1015), and NO–budesonide (NCX 1020) in guinea pig tracheal rings in the absence (closed bars) or in the presence (opened bars) of ODQ. Each point is the mean of at least 6 animals and vertical bars show SEM. *$P < 0.05$ vs control.

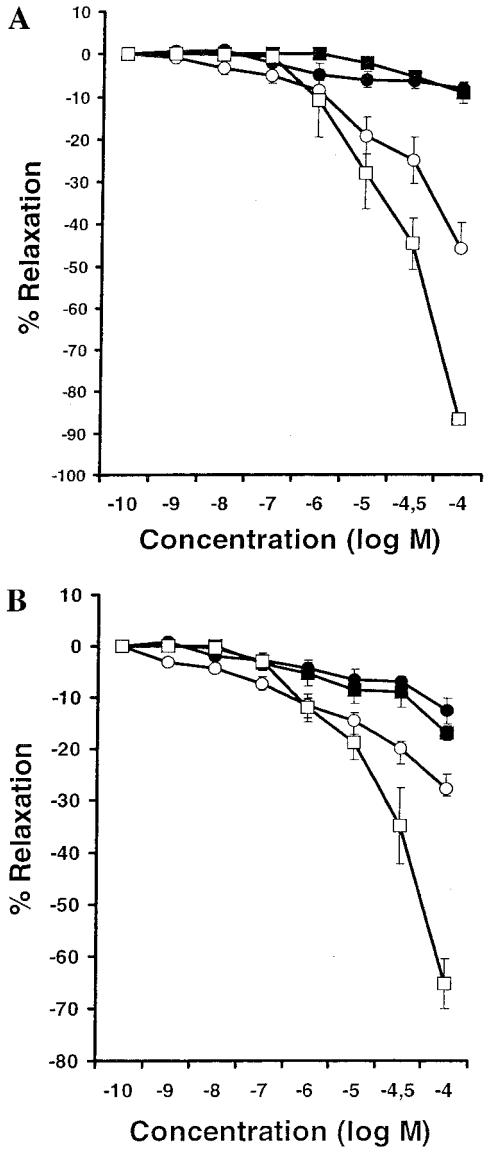

FIG. 4. (A) Response of guinea pig tracheal rings (circles) and guinea pig perfused bronchioles (squares) to prednisolone (closed symbols) and NCX 1010 (open symbols). (B) Response of guinea pig tracheal rings (circles) and guinea pig perfused bronchioles (squares) to budesonide (closed symbols) and NCX 1020 (open symbols). Each point is the mean of at least 6 animals and vertical bars show SEM. $^{*}P < 0.05$ vs control.

comparison, steroids did not show any activity on the methacholine precontracted guinea-pig bronchioles.

DISCUSSION

NO appears to play an important role in regulating several biological functions in the lung, including modulation of pulmonary and bronchial smooth muscle tone. In addition, NO plays an important role in nonspecific host defense and has antimicrobial activity against a wide variety of pathogens (10).

Small amounts of inhaled NO gas, diffused directly from the alveoli into vascular smooth muscle, induce selective pulmonary vasodilation and reverse hypoxic pulmonary vasoconstriction in lambs and human volunteers fully awake (11). The avid binding of NO molecules to hemoglobin, and its subsequent inactivation within the pulmonary circulation, probably explains the lack of systemic effects (12). NO activates soluble guanylate cyclase and thereby causing rapid and pronounciated relaxation of the vascular smooth muscle (13, 14). Common nitrovasodilators act by releasing NO (15) and inducing relaxation in a wide variety of vascular and nonvascular smooth muscle cells. In airway smooth muscle, NO is thought to induce relaxation through formation of cGMP as in vascular smooth muscle (16). Inhaled NO causes bronchodilation in guinea pigs after induction of bronchoconstriction by continuous infusion of metacholine. The effect is rapid, dose-dependent, and occurs at concentration as low as 5 parts per million (ppm). Bronchoconstriction occurs within minutes of ceasing NO inhalation. Similar results have been reported in rabbits after bronchoconstriction induced by inhaled aerosolized methacholine (17), in dogs during histamine and methacholine challenge, and in pigs during continuous infusion of methacholine (18). The main target of inhaled NO molecules is likely to be the airway smooth muscle, where it activates soluble guanylate cyclase to produce cGMP leading to relaxation (19). Furthermore, there is a nonhomogeneous response to chemical mediators along the bronchial tree; central and peripheral airways respond differently to various stimuli, and this distribution could influence the bronchodilator potency of inhaled NO.

The differential effect of inhaled NO on lung resistance and dynamic compliance in mechanically ventilated guinea pigs when various NO–steroids are used suggests that inhaled NO has the capacity to reverse obstruction in large airways. This interpretation is in agreement with the observation: nitroglycerin and sodium nitroprusside are less effective in relaxing small as compared to large airways *in vitro* (20). This is in marked contrast with the similarity of responsiveness to isoproterenol observed in variously sized bovine airways. Another possible explanation of the lesser potency of inhaled NO to relax small airways is that NO gas is scrubbed from the airstream in such a manner that at low concentration, little NO actually reaches distal airways. Epithelium may also provide a diffusion barrier or could act as a metabolic sink for the inhaled NO molecules.

Using 10^{-4} M NO–steroids we demonstrated for the first time *in vitro* that NO-released moiety is able to reduce by 75–90% the bronchoconstriction induced by methacholine in guinea-pig perfused bronchioles whereas the same compounds relaxed only by 25–40% the precontracted trachea. We can explain these data

by the fact that, in opposite to conventional NO-donors, NO–steroids release slowly the NO moiety which can act for a longer period on the smooth muscle cells stimulating the soluble guanylate cyclase.

Nitro-compound adducts form readily under physiological conditions. Moreover, they are potent activators of soluble guanylate cyclase (sGC) by NO and nitrovasodilators. 5×10^{-6} M ODQ (inhibitor of the sGC) blocked by 90% the activity of NO–steroids on guinea-pig trachea confirming that the relaxant effects of NicOx's compounds are due to the activation of the guanylate cyclase in the smooth muscle cells. Moreover, these compounds have half-lives significantly greater than that of NO. The formation of NO-compounds derivatives may be viewed as a mean of stabilizing NO in a unique bioactive form, potentially facilitating its transport in tissues and, importantly in the lung, mitigating the toxicity arising from its reaction with oxygen and superoxide anion (21).

In vivo experiments, further, require taking into account the mechanisms of airway obstruction, reflex activation, as well as physical components such as mucus hypersecretion and airway wall edema, further impeding pulmonary mechanics induced by each bronchoconstrictor agonist. These observations raise the possibility that delivering a NO-compound aerosol may be a more physiological method than inhaled NO to activate the cGMP pathway in airway smooth muscle.

There are a number of reasons supporting the hypothesis that addition of an NO releasing moiety to a GC structure will provide beneficial "added value" to the anti-inflammatory actions of the GC nucleus. For instance, arteriole dilation and an antiadhesive effect on circulating leukocytes are both phenomena requiring NO-dependent cGMP formation (22).

To test our hypothesis that NO could improve the anti-inflammatory activity of steroids, we linked NO to prednisolone, a corticosteroid of moderate potency and of therapeutic value in the clinic, at position 21 to obtain NCX 1010 and NCX 1015. Position 21 was chosen because substitution of this position does not inhibit the biological activity of the native molecule. In a recent paper, Perretti *et al.* demonstrated that NCX-1015 dose-dependently induced, in a higher manner than prednisolone, the steroid sensitive cell surface marker CD163 in human peripheral blood mononuclear cells. In the zymosan peritonitis model, NCX-1015 was more active than prednisolone in suppressing neutrophil extravasation (ED_{50} of 5.5 and 25.8 μmol/kg, respectively), nitrite accumulation (ED_{50} of 1.38 and 22.2 μmol, respectively) and release of chemokine KC (ED_{50} of 5.5 and 27.7 μmol/kg, respectively) as determined at the 4 h time point. NCX-1015 given orally was found to be equally active to inhibit the release of interleukin-1β or prostaglandin E2. The activity of NCX-1015 was mimed by the co-administration of prednisolone with the nitric oxide donor NOC-18 or sodium nitroprusside. In a chronic model of granulomatous tissue inflammation, administration of NCX-1015 (13.9 μmol/kg) from day 1 after induction of inflammation was more effective than prednisolone in reducing the granuloma dry weight, and this was associated to a lower anti-angiogenic effect (23).

However, despite the large clinical applications of steroids and in line with what reported for NSAID, therapeutic management of long term pathologies with steroids is linked to a series of unwanted side effects. The side-effects of steroids are manifested after medium to long term therapy and can be manifold, including suppression of the hypothalamus-pituitary axis function, Cushing's syndrome and osteoporosis. In addition, steroids may also aggravate gastro-intestinal damage, especially if associated in long term therapies with other gastrolesive agents, such as NSAIDs. At least one rationale of adding a ONO_2 group to the steroid structure is that NO releasing agents decrease osteoclast activation: therefore, with NO–steroids we may directly target one of the major problems associated with steroid therapy (see below for more details). Similarly, liberation of the NO moiety in an *in vivo* microenvironment is expected to produce clear and drastic beneficial effects on the GI tract, by analogy with what reported for NO-NSAIDs (24–26). In addition, the potential synergism between NO and steroid molecules locally released in a damage tissue, may allow to obtain a new pharmacological profile (bronchodilating activity), and a significant reduction in doses required to effectively control the outcome of an inflammatory pathology. The possibility to reduce the therapeutically effective doses of steroids seems another appealing mean to prevent side-effects on bone resorption and gastrointestinal tract outlined above.

In conclusion, the results obtained from these studies provide the first evidence that an optimal balance of anti-inflammatory activity and NO release can be achieved with NO–steroids; they also support the concept that the NO-releasing derivative of steroids may support therapeutic potential in the control of asthma and Chronic Obstructive Pulmonary Diseases progression. In other terms, the potential clinical implications of these findings are important, steroids being the most potent anti-inflammatory drugs we possess up to now. Using NO–steroids, we found a way to reduce the overall dosage of such compounds whilst maintaining their potency and developing new pharmacological activities.

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