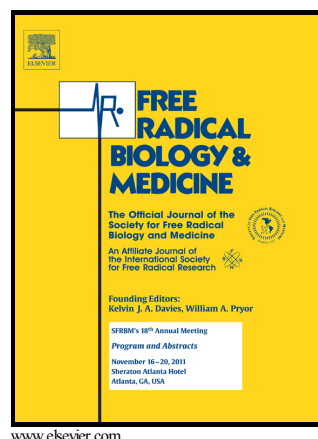


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Cyclic nitroxide radicals attenuate inflammation and hyper-responsiveness in a mouse model of allergic asthma

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Abstract

The effects of stable cyclic nitroxide radicals have been extensively investigated both *in vivo* and *in vitro* demonstrating anti-inflammatory, radioprotective, anti-mutagenic, age-retardant, hypotensive, anti-cancer and anti-teratogenic activities. Yet, these stable radicals have not been evaluated in asthma and other airway inflammatory disorders. The present study investigated the effect of 4-hydroxy-

2,2,6,6-tetramethyl-piperidine-N-oxyl (TPL) and 3-carbamoyl-proxyl (3-CP) in a mouse model of ovalbumin (OVA)-induced allergic asthma. Both 3-CP and TPL were non-toxic when administered either orally (1% w/w nitroxide-containing chow) or *via* intraperitoneal (IP) injection (~300 mg/kg). Feeding the mice orally demonstrated that 3-CP was more effective than TPL in reducing inflammatory cell recruitment into the airway and in suppressing airway hyper-responsiveness (AHR) in OVA-challenged mice. To characterize the optimal time-window of intervention and mode of drug administration, 3-CP was given orally during allergen sensitization, during allergen challenge or during both sensitization and challenge stages, and *via* IP injection or intranasal instillation for 3 days during the challenge period. 3-CP given *via* all modes of delivery markedly inhibited OVA-induced airway inflammation, expression of cytokines, AHR and protein nitration of the lung tissue. Oral administration during the entire experiment was the most efficient delivery of 3-CP and was more effective than dexamethasone a potent corticosteroid used for asthma treatment. Under a similar administration regimen (IP injection before the OVA challenge), the effect of 3-CP was similar to that of dexamethasone and even greater on AHR and protein nitration. The protective effect of the nitroxides, which preferentially react with free radicals, in suppressing the increase of main asthmatic inflammatory markers substantiate the key role played by reactive oxygen and nitrogen species in the molecular mechanism of asthma. The present results demonstrate the therapeutic potential of nitroxides for the treatment of asthma.

Abbreviations: airways hyper-responsiveness, AHR; bronchoalveolar lavage fluid, BALf; 3-carbamoyl pyrrolidine N-oxyl (3-carbamoyl-proxyl), 3-CP; dexamethasone, Dex; electron paramagnetic resonance, EPR; hematoxylin and eosin, H&E; interleukin, IL; 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl, TPL; intraperitoneal, IP; 3-nitrotyrosine, 3-NT; ovalbumin, OVA; periodic acid-schiff, PAS; phosphate buffer saline, PBS

Keywords

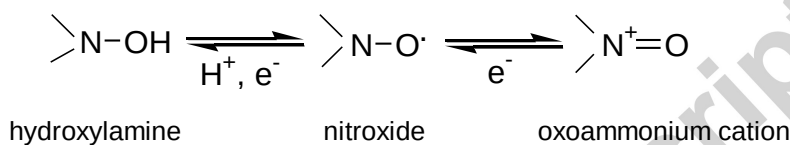
Nitroxide; Antioxidant; Asthma; Oxidative stress; Mouse; Reactive nitrogen species

Introduction

Allergic asthma is a chronic disease characterized by airway hyper-responsiveness (AHR), reversible airflow obstruction, airway inflammation and remodeling. Pathophysiological features include inflammation of the airways with increased number of infiltrating eosinophils, mast cells, lymphocytes and other cell types together with release of multiple inflammatory mediators and cytokines [1-3]. Oxidative stress is a key component in the pathogenesis of asthma and other airways inflammatory disorders [4-12]. The close association between oxidative stress and airways inflammation is reflected by increased production of reactive oxygen species (ROS), reactive nitrogen species (RNS) [7, 9, 13-15], and an inverse correlation between endogenous antioxidants levels and airway inflammation [4, 5, 8, 11, 16-18]. The role of oxidative stress in airway inflammation suggests that antioxidant therapy may be of value in the treatment of asthma.

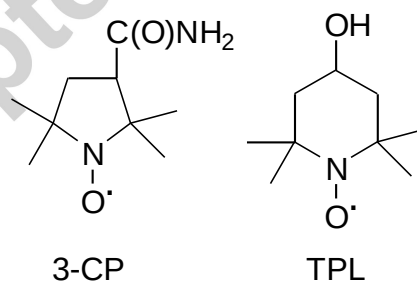
Dietary supplementation of common antioxidants such as vitamins C and E, selenium and flavonoids have been tested for several decades in an attempt to prevent or treat asthma and other airway inflammatory disorders [5, 8, 10, 17-26]. Yet, the reported efficacy has been equivocal at best [23, 27], and on the whole disappointing [18, 20, 23, 25, 28], though data from a mouse asthma model demonstrated that γ -tocopherol and α -lipoic acid suppressed both airway inflammation and airway hyper-responsiveness (AHR) [29-31].

Unlike common antioxidants, stable cyclic nitroxide radicals ($>\text{N}-\text{O}^\bullet$) do not react with most diamagnetic molecules, but readily detoxify radicals including $\text{HO}_2^\bullet$ [32, 33], $^\bullet\text{OH}$ [34], $^\bullet\text{NO}_2$ [35], $\text{CO}_3^{\bullet-}$ [36], carbon-centered radicals [37-39], peroxy radicals [40] and thiyl radicals [41] without giving rise to secondary radicals. Instead, they yield their respective oxoammonium cation, which is readily reduced back to the nitroxide, and can modify oxidative and nitrosative damage acting both as reducing and oxidizing species while being continuously recycled (Scheme 1).



Scheme 1. Three oxidation states of nitroxides.

Cyclic nitroxide radicals are stabilized by methyl groups at the α -position as demonstrated in Scheme 2 for the five-membered pyrrolidine 3-CP and the six-membered piperidine TPL ring structures.



Scheme 2. Structures of 3-carbamoyl-proxyl (3-CP), and 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (TPL).

The protective effect of nitroxides both *in vitro* and *in vivo* has been investigated in several experimental models beginning in the 1990s [42] demonstrating anti-inflammatory, radioprotective, anti-mutagenic, age-retardant, hypotensive, anti-cancer and anti-teratogenic activities [43, 44]. They

can be administered by any route (e.g., topical, oral, intravenously (IV), intramuscularly (IM), intraperitoneal (IP), nasal instillation, aerosol inhalation), readily permeating cellular and mitochondrial membranes and crossing the blood-brain barrier, while distributing instantaneously throughout all tissues and cellular compartments. The toxicological profile of nitroxides demonstrates that the therapeutic window in mice is extremely wide [45-47]. To date, however, nitroxides have not been tested in asthma or in other airway inflammatory disorders. In this study, we evaluated the efficacy of TPL and 3-CP in a murine model of asthma. TPL is the most studied nitroxide, but the half-life of 5-membered ring nitroxides such as 3-CP *in vivo* are longer because they are less susceptible to bio-reduction [48, 49], and their clearance rates are lower than those of 6-membered ring structures [50-52].

Materials and methods

Materials

Grade V chicken ovalbumin (OVA), TPL and dexamethasone (Dex) were purchased from Sigma-Aldrich, methacholine was purchased from Spectrum Chemicals, New Brunswick, NJ. 3-CP and TPL were purchased from Sigma-Aldrich. Nitroxide-containing chow (1% w/w) were prepared by dissolving 500 g chow in a minimum volume of water containing 5 g nitroxide and 5 g commercial chicken soup powder, preparing the chow and drying it at 37 °C. Animals were reluctant to eat nitroxide-containing chow until chicken soup powder was added and during three weeks they gained weight.

Mice

BALB/c mice were bred in a specific-pathogen free animal facility at Hadassah-Hebrew University Medical Center with unlimited access to sterilized chow and water. The Hebrew University–Hadassah

Medical School Animal Ethics Committee approved all experimental protocols. Eight to 10 mice were used for each group.

Allergen challenge protocol

Mice aged 8 to 10 weeks were sensitized and challenged with OVA to induce allergic inflammation.

Mice received IP injections of 10 μ g OVA and 1 mg Al(OH)₃ in 0.5 mL saline at days 1 and 10. One week after the last sensitization (on days 14 – 18), the mice were challenged with OVA aerosols (2% in saline) for 20 min each day for 3 days while animals of the control group were challenged with saline (Fig. 1).

Mice were fed *ad libitum* with either standard or with nitroxide-containing standard mouse chow (1% (w/w)). The effects of the nitroxides were investigated by feeding the mice of the experimental groups *ad libitum* diet of nitroxide-containing chow during both OVA-induced sensitization and challenge stages (days -2 – 19), during the sensitization period alone (days -2 – 11) or during the challenge period alone (days 13 – 19). For other experimental groups, 3-CP was administered IP *via* three daily injections (200 μ L, 0.15 M, ~300 mg/kg) 0.5 h prior to OVA challenge or by nasal instillation (50 μ L, 0.3 M, ~150 mg/kg) twice a day 0.5 h prior to the challenge with OVA aerosol and 6 h later (without exceeding its maximally tolerated dose; previously established to be 300 - 500 mg/kg [53, 54]). Both 3-CP and TPL were non-toxic when administered orally or via IP injection to untreated OVA-challenged mice during the entire experiment (22 days). Dex was administered using 3 daily IP injections (1 mg/kg) 0.5 h prior to the challenge with OVA aerosol.

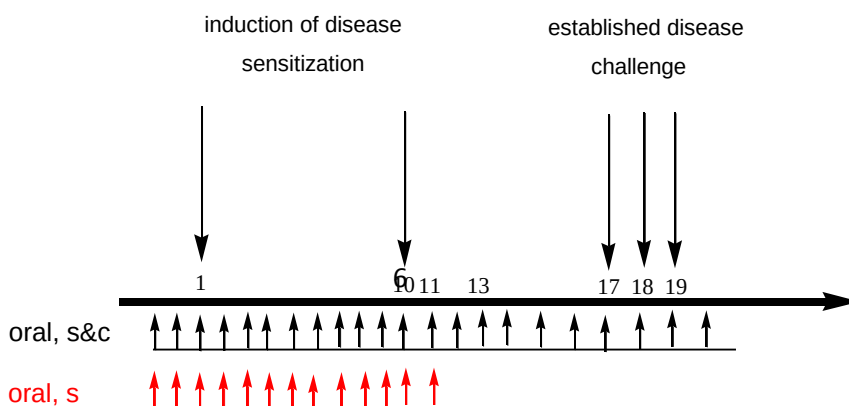


Figure 1. Schematic overview of nitroxide administration protocols

Electron paramagnetic resonance (EPR)

EPR spectra were recorded using a JEOL X band JES-RE3X spectrometer operating at 9.5 GHz with center field set at 3287 G, 100 kHz modulation frequency, 1 G modulation amplitude and 4 – 16 mW incident microwave power. Blood samples or tissue samples were diluted with 0.5 mL phosphate buffer (40 mM, pH 7.4) or water, inserted into a flexible capillary placed in a quartz tube installed within the EPR spectrometer cavity and assayed for residual nitroxide. A similar procedure was used to determine the residual nitroxide in tissue homogenates of various organs, which were corrected for blood contamination spectrophotometrically by following the absorption of oxyhemoglobin at 400 – 600 nm. After monitoring the EPR signal of any residual nitroxide, $K_3Fe(CN)_6$ at 2 mM final concentration was added to the sample to oxidize the hydroxylamine to the respective nitroxide and the EPR signal was monitored again yielding the total concentration of the nitroxide & hydroxylamine. The intensities of the EPR signal were compared to those monitored for 100 μ M nitroxide standard solution.

Assessment of airways hyper-responsiveness (AHR)

Twenty-four hours after the last challenge, mice were anesthetized using ketamine/xylazine, tracheostomized, and ventilated with a FlexiVent (SCIREQ, Montreal, Quebec, Canada). After baseline determination of airway resistance, mice were challenged with 0 to 64 mg/mL methacholine

nebulized directly into the ventilatory circuit using an AeroNebLab nebulizer (SCIREQ). Two models of respiratory mechanics were used to assess lung resistance (R): the linear first-order single compartment model and the constant-phase model. All data points were collected with FlexiVent software (SCIREQ). Results were expressed as relative increase in R over base line values.

Analysis of the bronchoalveolar lavage fluid (BALf)

BALf was centrifuged at 1000 rpm for 10 min and the supernatants were stored at -20°C for further examination. Cell pellets were resuspended in 1 mL saline and aliquots of 100 μL were used for cytopspin preparations using a Shandon cytopspin (Thermo Fisher Scientific, Waltham, MA, USA), and stained using Diffquik stain (Dade Diagnostica, Munich, Germany). A total of 200 cells were counted and classified for each slide. Total BAL cells were counted using a Newbauer Chamber (Marien-feld, Lauda-Konigshofen, Germany). Results are expressed as number of cells per mL of BALf.

ELISA

Cytokine levels (IL-4, IL-5, IL-13, IFN- γ , TGF- β) in BALf and lung tissue were measured by commercially available kits (PeproTech Asia, Rehovot, Israel). Lung tissue was homogenized in ELISA buffer, centrifuged and supernatant was used for examination. BALf measurements were performed without any additional modification. Protein nitration assay in lung tissue was carried out using a commercially available kit (Abcam, USA) according to manufacturer recommendations.

Histology

The left lung was fixed by intrabronchial infusion with 4% formaldehyde. Cross-sections were prepared from the lobe of the lung, and were stained with hematoxylin and eosin (H&E) and periodic

acid-Schiff (PAS) to evaluate inflammation and mucus producing cells, respectively. The degree of inflammation and amount of mucus producing epithelial cells were graded on a numeric scale of 0 to 5.

Data Analysis

Means and standard error of mean (SEM) values are given for each group. Either one-way or two-way ANOVA followed by Bonferroni posttest were used to compare groups. $P < 0.05$ was considered statistically significant.

Results

Nitroxide residual level

Nitroxides given by almost any route distribute virtually instantaneously throughout all tissues, and therefore, do not require early administration [48, 55-57]. However, nitroxides do not accumulate but are rather progressively depleted *via* reduction to their respective hydroxylamines and clearance from the circulation [48-52, 54]. EPR spectroscopy was used to assay the residual level of the nitroxides in the blood and tissue including the lung, liver, kidney, heart, spleen and heart of treated mice. All mice contained significant concentrations of the nitroxide after eating the nitroxide-containing chow (1% w/w). Typical results are presented in Fig. 2A.

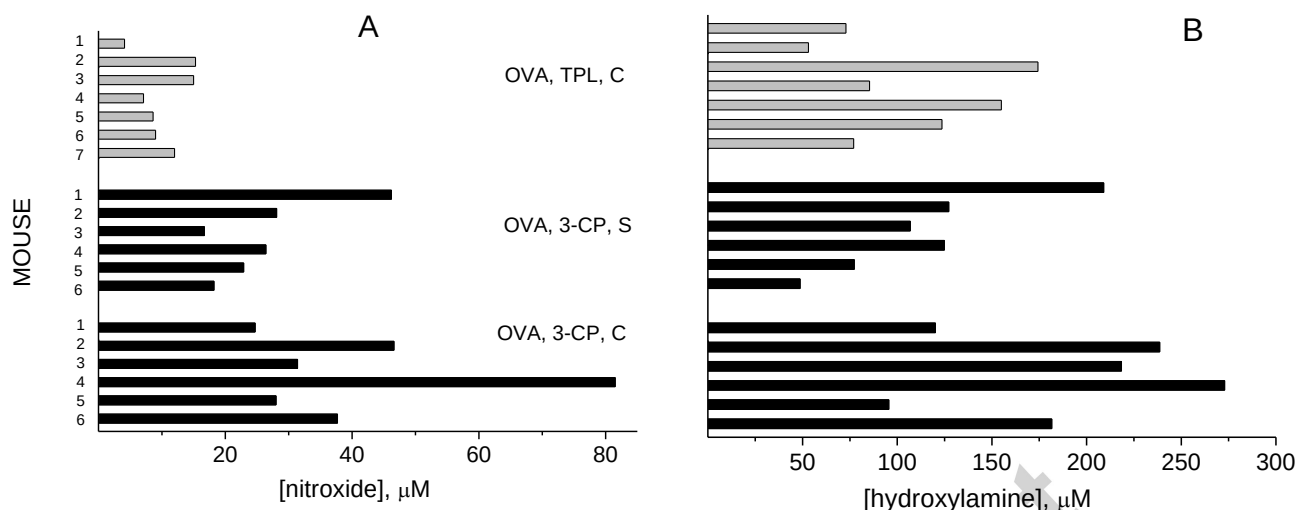


Figure 2. Residual concentrations of 3-CP and TPL (A) and of their respective hydroxylamines (B) in whole blood of mice orally fed with nitroxide-containing chow (1% w/w) determined using EPR spectroscopy. Blood samples were taken from different groups of OVA-challenged mice during the sensitization stage (S) and challenged stage (C). The intensities of the EPR signal was measured, and immediately thereafter, $\text{K}_3\text{Fe}(\text{CN})_6$ at 2 mM was added to the sample to re-oxidize the hydroxylamine to nitroxide and the EPR signal was monitored again yielding the total concentration of nitroxide and hydroxylamine.

The residual concentration of 3-CP in the lungs was about 65% of that in the blood, and significantly higher than in the heart, spleen, liver, kidney and brain in that order. The residual concentrations of their respective hydroxylamines were higher than those of the therapeutically active nitroxide radical (Fig. 2B).

Upon nasal instillation (50 μL , 0.3 M) the residual concentration of 3-CP in the blood, lung tissue and other organs decreased exponentially with time. Fig. 3 demonstrates that the rate of the reduction/clearance of 3-CP from the lung is similar to that from the blood, and that the absolute concentration of the nitroxide in the lung tissue is about twice higher than in the blood. The half-life of the nitroxide in the lung tissue is about 80 min, and therefore the treatment of the OVA-challenged mice included nasal instillation twice a day 0.5 h before and 6 h after the OVA challenge.

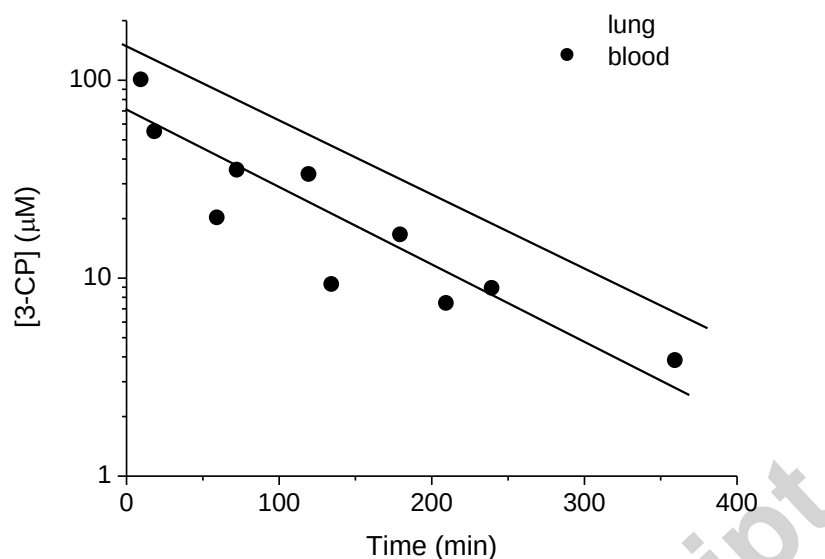


Figure 3. Time-dependent depletion of residual concentrations of 3-CP in the blood and lung tissue after nasal instillation (~150 mg/kg).

3-CP is more potent than TPL in attenuating airway inflammation and AHR

BAL and bronchial submucosal inflammation are characteristic features of asthma. OVA inhalation markedly increased total cell and particularly eosinophil counts compared with those in mice treated by saline aerosol control. Oral administration of 3-CP was significantly more effective than TPL in attenuating the increase in the total inflammatory cell and eosinophil counts in BALf compared with those in mice of the nitroxide vehicle control (Fig. 4).

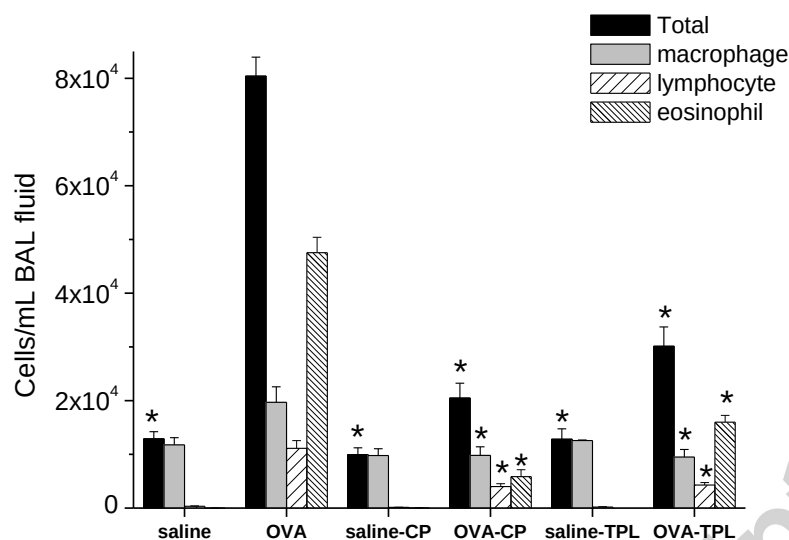


Figure 4. Inflammatory cell counts in OVA-challenged mice in the absence and presence of 3-CP or TPL administered orally (1% w/w) during the sensitization and challenge periods. Saline represents untreated mice and saline-CP and saline-TPL mice that received IP and inhaled saline together with oral drug for base line drug effect. Values are means $\pm$ SEM, 8 - 10 mice per group. * $P < 0.05$ compared to OVA group.

The increase in lung resistance in response to methacholine was attenuated more effectively by 3-CP than by TPL during oral administration along the entire experiment as demonstrated in Fig. 5.

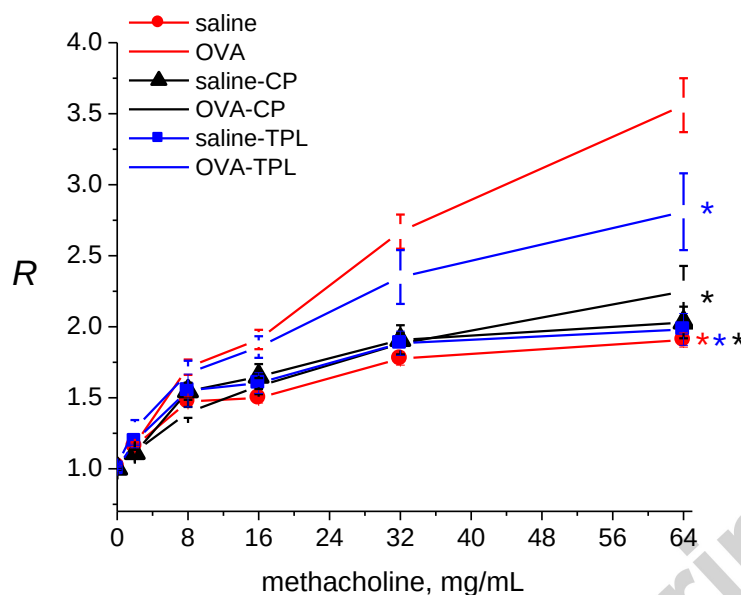


Figure 5. Increase in lung resistance (R) in response to methacholine measured in mice sensitized and challenged with OVA with and without oral nitroxides (1% w/w) during the sensitization and challenge periods. Saline represents untreated mice and saline-CP and saline-TPL mice that received IP and inhaled saline together with oral drug for base line drug effect. Values are means $\pm$ SEM, 8 - 10 mice per group. * $P < 0.05$ compared to OVA group.

Efficacy of 3-CP given at different stages of the disease by different routes of administration, and the comparison to corticosteroids

3-CP administered orally (1% w/w) during both the sensitization and challenge stages reduces the total cells and eosinophils counts more effectively compared to delivery during either stage alone. 3-CP significantly attenuated airway inflammation even when administered only during sensitization, and more importantly, even when administered only during challenge after the mice had already been sensitized to OVA. Oral administration of 3-CP was more effective than IP injection (~ 300 mg/kg, before the OVA challenge) or intranasal instillation (~ 150 mg/kg before the OVA challenge) (Fig. 6). Corticosteroids are considered the most potent and effective anti-inflammatory therapy for asthma. Oral administration of 3-CP for the entire sensitization and challenge period was more effective than Dex (1 mg/kg, IP, 0.5 h before the OVA challenge) in reducing BAL inflammation ($p = 0.034$) and was comparable to Dex even when given orally only during the sensitization or only during the challenge

phase of the experiment. No significant differences in BAL inflammatory cell counts were found between OVA-challenged mice treated with Dex and those treated with 3-CP even when given in an equivalent manner (Fig. 6).

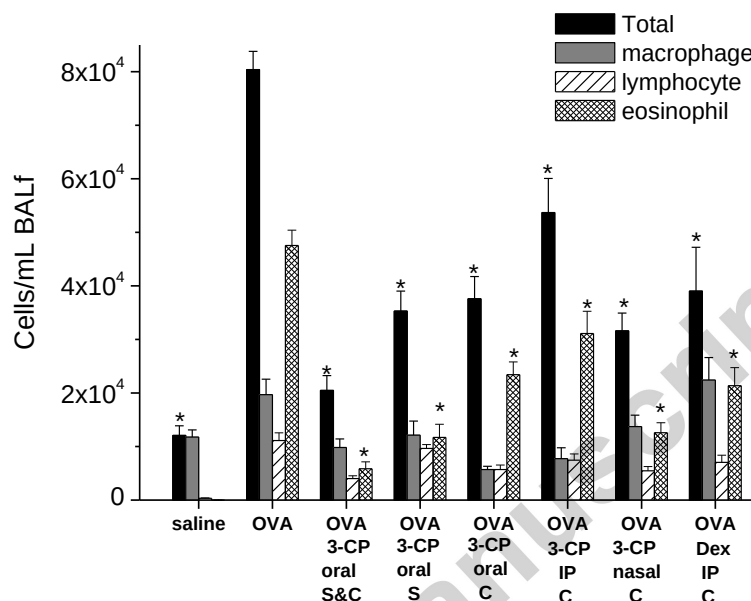


Figure 6. Inflammatory cell counts in OVA-induced allergic asthmatic mice in the absence and presence of 3-CP administered orally (1% w/w nitroxide-containing chow), via IP injection (~300 mg/kg) or via intranasal instillation (~150 mg/kg) during the sensitization period (S), challenge period (C) or both periods (S&C). Dex represents IP administration (1 mg/kg) during the challenge period. Saline represents untreated mice. Values are means $\pm$ SEM, 8 - 10 mice per group. *P < 0.05 compared to OVA group.

3-CP significantly reduced dramatically AHR using all modes of delivery except when administered orally only during the sensitization period (Fig.7). 3-CP was more effective than Dex even under the same administration regimen (Fig. 7).

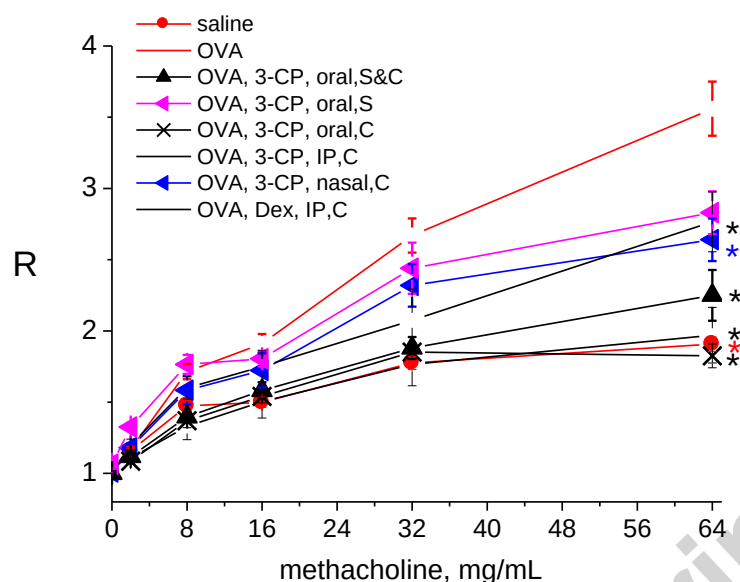


Figure 7. Increase of lung resistance (R) in response to methacholine measured in mice sensitized and challenged with OVA without and with 3-CP administered orally (1% w/w nitroxide-containing chow), via IP injection (~ 300 mg/kg) or via intranasal instillation (~ 150 mg/kg) during the sensitization period (S), challenge period (C) or both periods (S&C). Dex represents IP administration (1 mg/kg) during the challenge period. Saline represents untreated mice. Values are means $\pm$ SEM, 8 - 10 mice per group. * $P < 0.05$ compared to OVA group; OVA-OVA 3-CP oral (S), $P = 0.0573$.

Effect of 3-CP on the expression of TGF- $\beta 1$, IL-4, IL-5, IL-13 and IFN- γ

TGF- $\beta 1$ is a multifunctional cytokine that plays key roles in diverse biological processes, including tissue remodeling and repair [58, 59]. In human chronic lung diseases like asthma, TGF- $\beta 1$ expression increases while TGF- $\beta 1$ increased expression in asthma patients correlates with disease severity and the degree of peribronchial fibrosis [60]. The effects of 3-CP administered orally or via IP injection on the expression of IL-4, IL-5 and IFN- γ in the BALf are demonstrated in Figs 8A, 8B and 8C. The effects of 3-CP administered orally, via IP injection or intranasal instillation and the comparison to the effect of Dex on the expression of IL-13 and TGF- β are demonstrated in Figs 8D and 8E, respectively. The effects of the drugs in the lung tissue are summarized in Table 1.

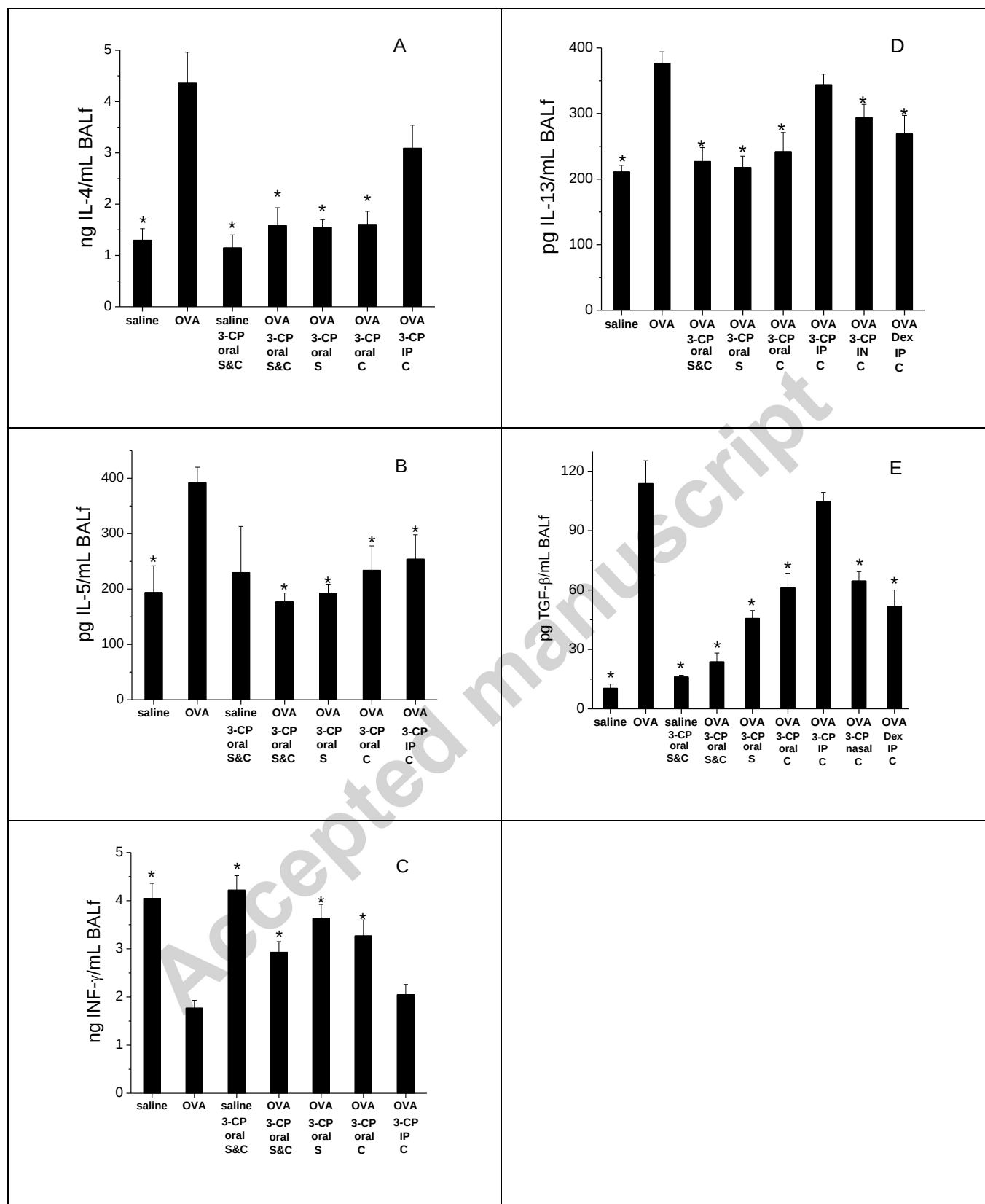


Figure 8. Effect of 3-CP on the expression of IL-4 (A), IL-5 (B), and INF-γ (C) in BALf of OVA-induced allergic asthmatic mice in the absence and presence of 3-CP administered orally (1% w/w

nitroxide-containing chow) or via IP injection (~300 mg/kg) during the sensitization stage (S), challenge stage (C) or both stages (S&C). Figs 8D and 8E show the effect of 3-CP on the expression of IL-13 and TGF- β 1 also when administered via intranasal instillation (~150 mg/kg) and the effect of IP injection of Dex (1 mg/kg) during the challenge period. Saline represents untreated mice and saline-oral-S&C mice that received IP and inhaled saline together with oral 3-CP. Values are means $\pm$ SEM, 8 - 10 mice per group. *P < 0.05 compared to OVA group.

Table 1. Effect of 3-CP on IL-4, IL-5, IL-13 and IFN- γ levels in lung tissue (ng/mg) administered during the sensitization period (S), challenge period (C) or both periods (S&C). *P < 0.05 compared to OVA group.

	IL-13	IL-4	IL-5	IFN- γ	TGF- β 1
saline	17.7 $\pm$ 1.0(*)	10.9 $\pm$ 1.4(*)	16.0 $\pm$ 0.9(*)	61.0 $\pm$ 1.7(*)	5.8 $\pm$ 0.7(*)
OVA	27.3 $\pm$ 1.5	28.7 $\pm$ 1.9	35.1 $\pm$ 1.5	38.9 $\pm$ 2.7	12.8 $\pm$ 1.6
saline, oral, S&C	22.0 $\pm$ 1.5(*)	19.9 $\pm$ 0.6(*)	19.8 $\pm$ 0.5(*)	64.2 $\pm$ 2.8(*)	8.4 $\pm$ 0.9(*)
OVA, oral, S&C	20.9 $\pm$ 1.1(*)	18.9 $\pm$ 1.9 (*)	25.1 $\pm$ 4.1(*)	56.0 $\pm$ 2.3(*)	8.2 $\pm$ 0.8(*)
OVA, oral, S	22.0 $\pm$ 0.7(*)	25.6 $\pm$ 1.4	31.5 $\pm$ 0.7	37.8 $\pm$ 2.7	7.9 $\pm$ 1.0(*)
OVA, oral, C	19.0 $\pm$ 2.7(*)	22.4 $\pm$ 2.6	30.4 $\pm$ 3.2	53.0 $\pm$ 3.1(*)	9.3 $\pm$ 1.8
OVA, IP, C	14.0 $\pm$ 1.1(*)	17.0 $\pm$ 1.5(*)	25.0 $\pm$ 3.3(*)	51.7 $\pm$ 1.9(*)	9.2 $\pm$ 0.7

Effect of 3-CP on the level of protein nitration

Lung homogenates were assayed for 3-nitrotyrosine (3-NT) levels. OVA challenge significantly increased pulmonary levels of 3-NT compared to those in mice of saline aerosol control group without and with the drug. 3-CP administered through all modes of delivery markedly suppressed 3-NT levels compared to those in the vehicle control and those treated with Dex (Fig. 9). Even under a similar administration regimen, the effect of 3-CP was greater than that of Dex.

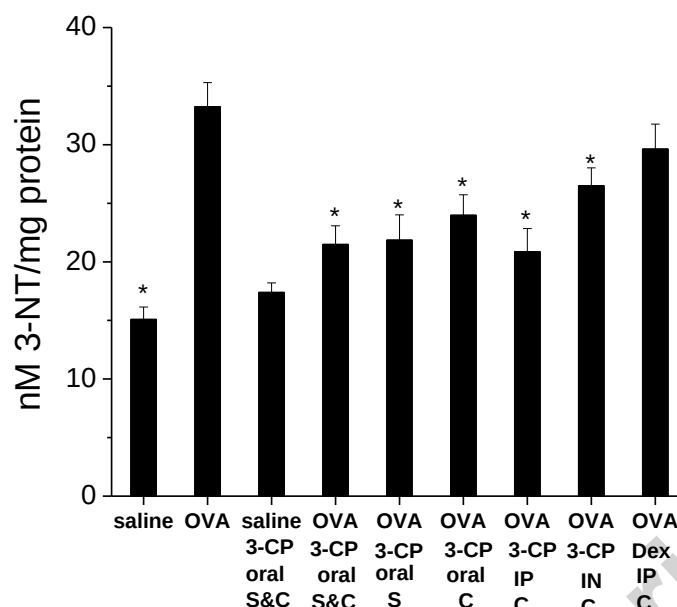


Figure 9. Effect of 3-CP on the level of protein nitration (3-NT) in lung tissue of OVA-induced allergic asthmatic mice in the absence and presence of 3-CP administered orally (1% w/w nitroxide-containing chow), via IP injection (~300 mg/kg) or intranasal instillation (~150 mg/kg) during the sensitization stage (S), challenge stage (C) or both stages (S&C). The last column shows the effect of IP injection of Dex (1 mg/kg) during the challenge period. Saline represents untreated mice and saline-oral-S&C mice that received IP and inhaled saline together with oral 3-CP. Values are means $\pm$ SEM, 8 - 10 mice per group. *P < 0.05 compared to OVA group.

Effect of 3-CP on lung histopathology

Upon histopathological examination, intense inflammation accompanied with infiltrating eosinophils and mononuclear cells surrounding the bronchi was evident in the lungs of asthmatic mice, and this was greatly diminished by the oral administration of 3-CP (Fig. 10).

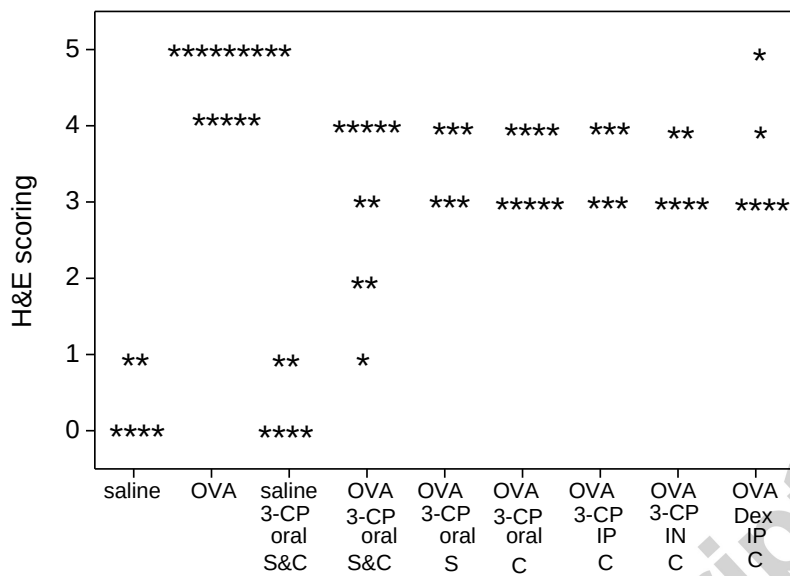


Figure 10. Effect of 3-CP and Dex on hematoxylin and eosin (H&E) staining of lung tissue of OVA-induced allergic asthmatic mice in the absence and presence of 3-CP administered orally (1% w/w nitroxide-containing chow), via IP injection (~300 mg/kg) or intranasal instillation (~150 mg/kg) during the sensitization stage (S), challenge stage (C) or both stages (S&C). Saline represents untreated mice and saline-oral-S&C mice that received IP and inhaled saline together with oral 3-CP. OVA-saline, $p < 0.001$; OVA-OVA 3-CP oral (S&C), $p < 0.001$; OVA-OVA 3-CP oral (S), $p = 0.046$; OVA-OVA 3-CP oral (C), $p = 0.007$; OVA-OVA 3-CP IP (C), $p = 0.046$; OVA-OVA 3-CP IP (C) IN, $p = 0.011$; OVA-OVA Dex IP (C), $p = 0.046$.

Periodic acid-schiff (PAS) staining and the degree of mucin production measured from PAS staining are demonstrated in Fig. 11.

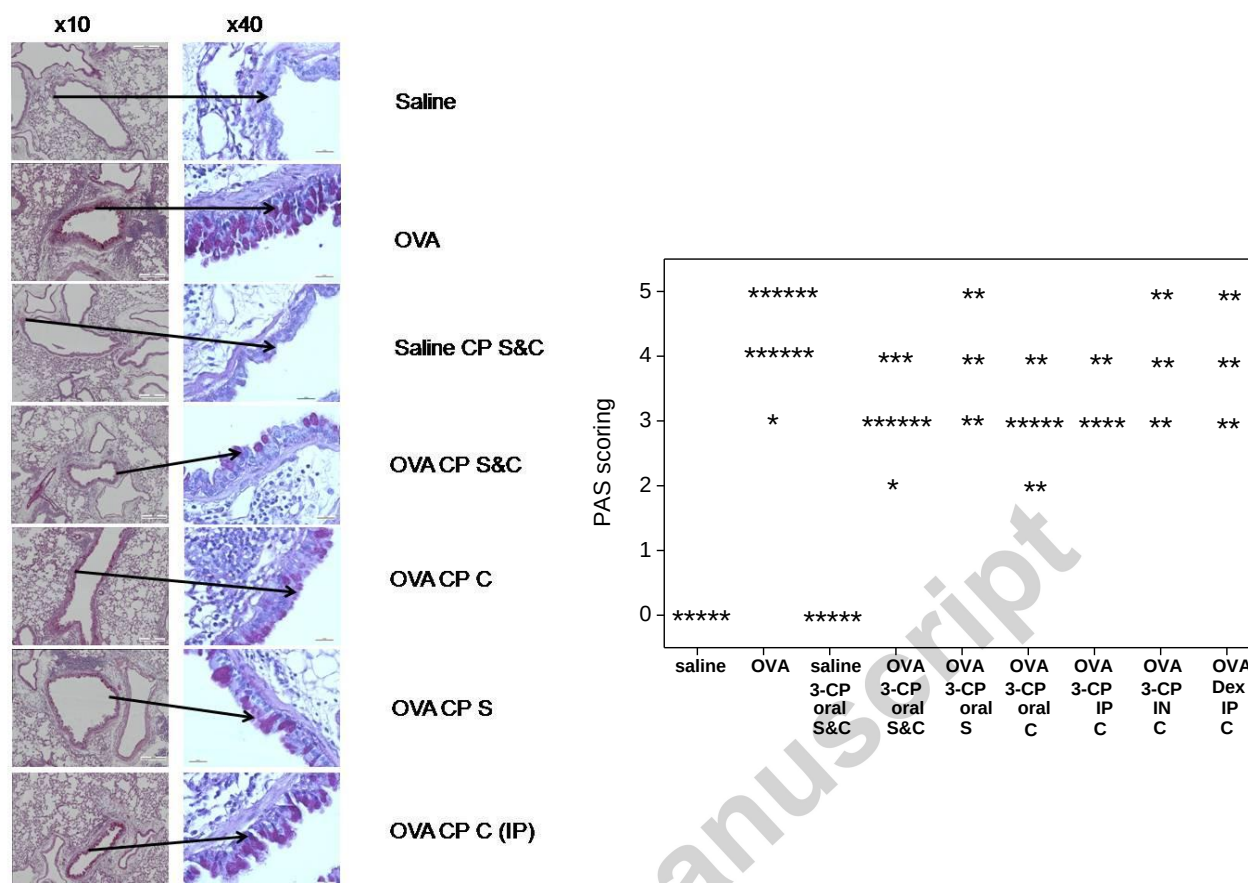


Figure 11. Effect of 3-CP and Dex on PAS staining of lung tissue of OVA-induced allergic asthmatic mice in the absence and presence of 3-CP administered orally (1% w/w nitroxide-containing chow), via IP injection (~300 mg/kg) or intranasal instillation (~150 mg/kg) during the sensitization stage (S), challenge stage (C) or both stages (S&C). Saline represents untreated mice and saline-oral-S&C mice that received IP and inhaled saline together with oral 3-CP. OVA-saline, $p < 0.001$; OVA-OVA 3-CP oral (S&C), $p = 0.003$; OVA-OVA 3-CP oral (C), $p = 0.002$; OVA-OVA 3-CP IP (C), $p = 0.046$.

Discussion

This study demonstrates that TPL and 3-CP attenuate inflammation and AHR in a mouse model of OVA-induced allergic asthma. 3-CP is more effective than TPL most probably due the longer half-life of 5-membered than 6-membered ring nitroxides *in vivo* [50-52]. The drug is effective when

delivered orally, IP and by nasal instillation. Oral administration during the entire experiment was the most efficient delivery of 3-CP. 3-CP was effective when administered only during the challenge phase of the model and even when administered only after allergen sensitization during the challenge phase of the model.

In asthma, exposure to allergen up regulates production of ROS and RNS. Higher levels of oxidative stress eventually overwhelm the antioxidant defenses in the lungs and lead to induction of proinflammatory cytokines, chemokines, and adhesion molecules. Further increases in oxidative stress inflict direct toxic effects causing cellular apoptosis and necrosis and oxidative lung injury. It has been observed in patients with severe asthma that oxidative stress is a major contributing factor to resistance to steroid therapy [61]. In the mouse model of allergen induced "asthma", OVA inhalation dramatically enhanced protein nitration implying the involvement of nitrosative damage to the lungs [7, 9, 13-15]. Given the importance of oxidative injury including protein nitration induced in asthmatic inflammation, drugs that effectively target this phenomenon are likely be of value in the treatment of the disease and particularly in chronic severe asthma.

TPL has been long known to protect animals from injuries associated with oxidative damage and inflammatory conditions, which are characterized by the increased production of ROS and RNS, respectively. It is assumed that nitroxides attenuate tissue injury under inflammatory conditions mainly because of their ability to react rapidly with free radicals. Moreover, nitroxides are the most efficient scavengers of $\cdot\text{NO}_2$ [33, 35], which is the exclusive direct mediator of protein nitration [62]. While acting as antioxidants, nitroxides are oxidized to their corresponding oxoammonium cation, which in turn, can be recycled back to the nitroxides by cellular reductants thus acting catalytically.

Although most of the nitroxide administered to the mouse is rapidly reduced to its respective hydroxylamine, the residual nitroxide radical was sufficient to inhibit the increase in inflammation characteristic of allergen induced "asthma" in mice. The increase in AHR and in the number of inflammatory cells, particularly eosinophils, was attenuated by both 3-CP and TPL administered

orally, although 3-CP was more effective, most probably due to its longer half-life *in vivo* [48, 49, 52]. During oral administration the drug persists during the entire experiment, although at low steady-state concentration, and therefore oral administration is more effective than IP injection or nasal instillation on several asthmatic parameters including AHR, eosinophilia, protein nitration and some cytokines in BALf.

Corticosteroids are considered the most effective anti-inflammatory drugs available and have served as the cornerstone of asthma therapy for several decades. Patients with severe asthma may become refractory to corticosteroid treatment and at present, there is no effective therapeutic option for these patients. In the present study, 3-CP was more effective than Dex in attenuating AHR and in reducing inflammation when given orally during both sensitization and challenge phases of the experiment. Under a similar administration regimen (IP injection before the OVA challenge), the effect of 3-CP and Dex on inflammatory cell counts were similar and even greater on AHR. OVA inhalation dramatically enhanced protein nitration implying the involvement of nitrosative damage to the lungs [7, 9, 13-15]. In contrast to 3-CP, which blocked this effect, Dex failed to suppress the dramatic 3-NT formation in the lung of asthmatic mice in agreement with a previous report [63]. These findings suggest that a therapeutic strategy targeting protein nitration may be an alternative to steroids in refractory patients and may, by blocking inflammatory pathways not responsive to Dex, also have synergistic effects when delivered together with corticosteroids.

3-CP was effective even when delivered only during the challenge phase of the model. This has important clinical implications as patients are only diagnosed and started on therapy once they have established disease. Nevertheless, administration of the drug throughout the experiment was the most effective route, suggesting that treatment is likely to be most effective when received earlier in the course of the disease.

Both "natural" (vitamins and foods) and synthetic antioxidant therapy has been evaluated extensively in many different disease states including asthma and has been universally disappointing.

This is in sharp contrast to expectations based on the biology of these diseases in which oxidation and nitration are clearly pathogenic. Failure of antioxidant therapy has led to skepticism and a dampening of the enthusiasm for this approach. Our findings in the present study support the notion that the key to effective antioxidant therapy lies in identifying the appropriate molecule, such as 3-CP, which allows for easy administration, rapid and extensive tissue distribution and molecular "renewal" or recycling, resulting in sustained tissue antioxidant activity.

In conclusion, 3-CP decreases oxidative injury in mice airway and effectively suppresses allergic inflammation and AHR increase in acute murine models of asthma most efficiently when it is administered orally during the entire experiment. This implies that oxidative stress plays an important role in the asthmatic airway during the sensitization as well as challenge stages. The clinical implications of this strategy have to be proven considering the roles played by deleterious radicals under normal and pathological conditions.

References

1. Bousquet, J.; Jeffery, P.K.; Busse, W.W.; Johnson, M. Asthma - From bronchoconstriction to airways inflammation and remodeling. *Am. J. Resp. Crit. Care Med.* 161:1720-1745; 1990.

2. Locksley, R.M. Asthma and Allergic Inflammation. *Cell* 140:777-783; 2010.
3. Kim, H.Y.; DeKruyff, R.H.; Umetsu, D.T. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nature Immun.* 11:577-584; 2010.
4. Mohan, I.K.; Das, U.N. Oxidant stress, anti-oxidants, nitric oxide and essential fatty acids in bronchial asthma. *Med. Sci. Res.* 25:307-309; 1997.
5. Dworski, R. Oxidant stress in asthma. *Thorax* 55:S51-S53; 2000.
6. Li, N.; Hao, M.Q.; Phalen, R.F.; Hinds, W.C.; Nel, A.E. Particulate air pollutants and asthma - A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin. Immunol.* 109:250-265; 2003.
7. Andreadis, A.A.; Hazen, S.L.; Comhair, S.A.A.; Erzurum, S.C. Oxidative and nitrosative events in asthma. *Free Radic. Biol. Med.* 35:213-225; 2003.
8. Rubin, R.N.; Navon, L.; Cassano, P.A. Relationship of serum antioxidants to asthma prevalence in youth. *Am. J. Respir. Crit. Care Med.* 169:393-398; 2004.
9. Kirkham, P.; Rahman, I. Oxidative stress in asthma and COPD: Antioxidants as a therapeutic strategy. *Pharmacol. Therapeut.* 111:476-494; 2006.
10. Riedl, M.A.; Nel, A.E. Importance of oxidative stress in the pathogenesis and treatment of asthma. *Curr. Opin. Allergy Clin. Immunol.* 8:49-56; 2008.
11. Sackesen, C.; Ercan, H.; Dizdar, E.; Soyer, O.; Gumus, P.; Tosun, B.N.; Buyuktuncer, Z.; Karabulut, E.; Besler, T.; Kalayci, O. A comprehensive evaluation of the enzymatic and nonenzymatic antioxidant systems in childhood asthma. *J. Allergy Clin. Immunol.* 122:78-85; 2008.
12. Zuoa, L.; Otenbakera, N.P.; Rosea, B.A.; Salisbury, K.S. Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma. *Mol. Immunol.* 56:57-63; 2013.
13. Kasperska-Zajac, A.; Rogala, B. Nitric oxide (NO) in the pathophysiology of asthma and interstitial lung diseases. *Postepy Hig. Med. Dosw.* 55; 2001.

14. Kharitonov, S.A.; Barnes, P.J. Nitric oxide, nitrotyrosine, and nitric oxide modulators in asthma and chronic obstructive pulmonary disease. *Curr. Allergy Asthma Rep.* 3:121-129; 2003.
15. Maniscalco, M.; Sofia, M.; Pelaia, G. Nitric oxide in upper airways inflammatory diseases. *Inflamm. Res.* 56:58-69; 2007.
16. Kelly, F.K.; Mudway, J.; Blomberg, A.; Frew, A.; Sandstrom, T. Altered lung antioxidant status in patients with mild asthma. *Lancet* 354:482-483; 1999.
17. Picado, C.; Deulofeu, R.; Lleonart, R.; Agusti, M.; Mullol, J.; Qunito, L.; Torra, M. Dietary micronutrients/antioxidants and their relationship with bronchial asthma severity. *Allergy* 56:43-49; 2001.
18. Allan, K.; Kelly, F.J.; Devereux, G. Antioxidants and allergic disease: a case of too little or too much? *Clin. Exp. Allergy* 40:370-380; 2010.
19. McNally, N. Preliminary report on the use of vitamin C in asthma. *J. Ir. Med. Assoc.* 33:175-178; 1953.
20. Greene, L.S. Asthma and oxidant stress - Nutritional, environmental, and genetic risk-factors. *J. Am. Coll. Nutr.* 14:317-324; 1995.
21. Greene, L.S. Asthma, oxidant stress, and diet. *Nutrition* 15:899-907; 1999.
22. Fogarty, A.; Lewis, S.A.; Scrivener, S.L.; Antoniak, M.; Pacey, S.; Pringle, M.; Britton, J. Oral magnesium and vitamin C supplements in asthma: a parallel group randomized placebo-controlled trial. *Clin. Exp. Allergy* 33:1355-1359; 2003.
23. Misso, N.L.A.; Thompson, P.J. Oxidative stress and antioxidant deficiencies in asthma: potential modification by diet. *Redox Rep.* 10:247-255; 2005.
24. Kelly, F.J. Vitamins and respiratory disease: antioxidant micronutrients in pulmonary health and disease. *Proc. Nutr. Soc.* 64:510-526; 2005.
25. Dunstan, J.A.; Breckler, L.; Hale, J.; Lehmann, H.; Franklin, P.; Lyons, G.; Ching, S.Y.L.; Mori, T.A.; Barden, A.; Prescott, S.L. Supplementation with vitamins C, E, beta-carotene and

- selenium has no effect on anti-oxidant status and immune responses in allergic adults: a randomized controlled trial. *Clin. Exp. Allergy* 37:180-187; 2007.
26. Braskett, M.; Riedl, M.A. Novel antioxidant approaches to the treatment of upper airway inflammation. *Curr. Opin. Allergy Clin. Immunol.* 10:34-41; 2010.
 27. Murr, C.; Schroecksnadel, K.; Winkler, C.; Ledochowski, M.; Fuchs, D. Antioxidants may increase the probability of developing allergic diseases and asthma. *Med. Hypotheses* 64:973-977; 2005.
 28. Hernandez, M.; Zhou, H.B.; Zhou, B.Q.; Robinette, C.; Crissman, K.; Hatch, G.; Alexis, N.E.; Paden, D. Combination treatment with high-dose vitamin C and alpha-tocopherol does not enhance respiratory-tract lining fluid vitamin C levels in asthmatics. *Inhal. Toxicol.* 21:173-181; 2009.
 29. Cho, Y.S.; Lee, J.; Lee, T.H.; Lee, E.Y.; Lee, K.U.; Park, J.Y.; Moon, H.B. a-Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *J. Allergy Clin. Immunol.* 114:429-435; 2004.
 30. Wagner, J.G.J., Q.; Harkema, J.R.; Ames, B.N.; Illek, B.; Roubey, R.A.; Peden, D.B. g-Tocopherol prevents airway eosinophilia and mucous cell hyperplasia in experimentally induced allergic rhinitis and asthma. *Clin. Exp. Allergy* 38:501-511; 2008.
 31. Mabalirajan, U.; Aich, J.; Leishangthem, G.D.; Sharma, S.K.; Inda, A.K.; Ghosh, B. Effects of vitamin E on mitochondrial dysfunction and asthma features in an experimental allergic murine model. *J. Appl. Physiol.* 107:1285-1292; 2009.
 32. Goldstein, S.; Merenyi, G.; Russo, A.; Samuni, A. The role of oxoammonium cation in the SOD-Mimic activity of cyclic nitroxides. *J. Am. Chem. Soc.* 125:789-795; 2003.
 33. Goldstein, S.; Samuni, A.; Hideg, K.; Merenyi, G. Structure-activity relationship of cyclic nitroxides as SOD mimics and scavengers of nitrogen dioxide and carbonate radicals. *J. Phys. Chem. A* 110:3679-3685; 2006.

34. Samuni, A.; Goldstein, S.; Russo, A.; Mitchell, J.B.; Krishna, M.C.; Neta, P. Kinetics and mechanism of hydroxyl radical and OH-adduct radical reactions with nitroxides and with their hydroxylamines. *J. Am. Chem. Soc.* 124:8719-8724; 2002.
35. Goldstein, S.; Samuni, A.; Russo, A. Reaction of cyclic nitroxides with nitrogen dioxide: The intermediacy of the oxoammonium cations. *J. Am. Chem. Soc.* 125:8364-8370; 2003.
36. Goldstein, S.; Samuni, A.; Merenyi, G. Reactions of nitric oxide, peroxynitrite, and carbonate radicals with nitroxides and their corresponding oxoammonium cations. *Chem. Res. Toxicol.* 17:250-257; 2004.
37. Asmus, K.D.; Nigam, S.; Willson, R.L. Kinetics of nitroxyl radical reactions. A pulse-radiolysis conductivity study. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* 29:211-219; 1976.
38. Beckwith, A.L.J.; Bowry, V.W.; Ingold, K.U. Kinetics of nitroxide radical trapping .1. Solvent effects. *J. Am. Chem. Soc.* 114:4983-4992; 1992.
39. Bowry, V.W.; Ingold, K.U. Kinetics of nitroxide radical trapping .2. Structural effects. *J. Am. Chem. Soc.* 114:4992-4996; 1992.
40. Goldstein, S.; Samuni, A. Kinetics and mechanism of peroxy radical reactions with nitroxides. *J. Phys. Chem. A* 111:1066-1072; 2007.
41. Goldstein, S.; Samuni, A.; Merenyi, G. Kinetics of the reaction between nitroxide and thiyl radicals: Nitroxides as antioxidants in the presence of thiols. *J. Phys. Chem. A* 112:8600-8605; 2008.
42. Samuni, A.; Krishna, C.M.; Riesz, P.; Finkelstein, E.; Russo, A. A novel metal-free low-molecular weight superoxide-dismutase mimic. *J. Biol. Chem.* 263:17921-17924; 1988.
43. Soule, B.P.; Hyodo, F.; Matsumoto, K.-i.; Simone, N.L.; Cook, J.A.; Krishna, M.C.; Mitchell, J.B. The chemistry and biology of nitroxide compounds. *Free Radic. Biol. Med.* 42:1632-1650; 2007.

44. Soule, B.P.; Hyodo, F.; Matsumoto, K.I.; Simone, N.L.; Cook, J.A.; Krishna, M.C.; Mitchell, J.B. Therapeutic and clinical applications of nitroxide compounds. *Antioxid. Redox Signal.* 9:1731-1743; 2007.
45. Samuni, A.M.; Barenholz, Y. Site-activity relationship of nitroxide radical's antioxidative effect. *Free Radic. Biol. Med.* 34:177-185; 2003.
46. Jubeh, T.T.; Nadler-Milbauer, M.; Barenholz, Y.; Rubinstein, A. Local treatment of experimental colitis in the rat by negatively charged liposomes of catalase, TMN and SOD. *J. Drug Target.* 14:155-163; 2006.
47. Wasserman, V.; Kizelsztejn, P.; Garbuzenko, O.; Kohen, R.; Ovadia, H.; Tabakman, R.; Barenholz, Y. The antioxidant tempamine: In vitro antitumor and neuroprotective effects and optimization of liposomal encapsulation and release. *Langmuir* 23:1937-1947; 2007.
48. Swartz, H.M.; Sentjurs, M.; Morse, P.D. Cellular metabolism of water-soluble nitroxides: Effect on rate of reduction of cell/nitroxide ratio, oxygen concentrations and permeability of nitroxides. *Biochem. Biophys. Acta - Mol. Cell Res.* 888:82-90; 1986.
49. Iannonea, A.; Hua, H.; Tomasib, A.; Vanninib, V.; Swartz, H.M. Metabolism of aqueous soluble nitroxides in hepatocytes: Effects of cell integrity, oxygen, and structure of nitroxides. *Biochem. Biophys. Acta - General Subjects* 991:90-96; 1989.
50. Utsumi, H.; Muto, E.; Masuda, S.; Hamada, A. In vivo ESR Measurements of free radicals in whole mice. *Biochem. Biophys. Res. Commun.* 172:1342-1348; 1990.
51. Takeshita, K.; Utsumi, H.; Hamada, A. ESR Measurement of radical clearance in lung of whole mice. *Biochem. Biophys. Res. Commun.* 177:874-880; 1991.
52. Komarov, A.M.; Joseph, J.; Lai, C.-S. In vivo pharmacokinetics of nitroxides in mice. *Biochem. Biophys. Res. Commun.* 201:1035-1042; 1994.

53. Hahn, S.M.; Tochner, Z.; Krishna, C.M.; Glass, J.; Wilson, L.; Samuni, A.; Sprague, M.; Venzon, D.; Glatstein, E.; Mitchell, J.B. Tempol, a stable free radical, is a novel murine radiation protector. *Cancer Res.* 52:1750-1753; 1992.
54. Hahn, S.M.; DeLuca, A.M.; Coffin, D.; Krishna, C.M.; Mitchell, J.B. In vivo radioprotection and effects on blood pressure of the stable free radical nitroxides. *Int. J. Radiat. Oncol. Biol. Phys.* 42:839-842; 1998.
55. Griffeth, L.K.; Rosen, G.M.; Rauckman, E.J.; Drayer, B.P. Pharmacokinetics of nitroxide NMR contrast agents. *Invest. Radiol.* 19:553-562; 1984.
56. Halpern, H.J.; Peric, M.; Cheng, Y.; Barth, E.D.; Chandramouli, G.V.R.; Makinen, M.; Rosen, G.M. In vivo spin label murine pharmacodynamics using low frequency electron paramagnetic resonance imaging. *Biophys. J.* 71:403-409; 1996.
57. Ferrara, M.; Colacicchia, S.; Gualtieria, G.; Santini, M.T.; Sotgiu, A. Whole mouse nitroxide free radical pharmacokinetics by low frequency electron paramagnetic resonance. *Biochem. Biophys. Res. Commun.* 166:168-173; 1990.
58. Homer, R.J.; Elias, J.A. Airway remodeling in asthma: therapeutic implications of mechanisms. *Physiology (Bethesda)* 20:28-35; 2005.
59. Lee, C.G., Kang, H.R., Homer, R.J., Chupp, G., Elias, J.A. Transgenic modeling of transforming growth factor-beta(1): role of apoptosis in fibrosis and alveolar remodeling. *Proc. Am. Thorac. Soc.* 3:418-423; 2006.
60. Minshall, E.M., Leung, D.Y., Martin, R.J., Song, Y.L., Cameron, L., Ernst, P., Hamid, Q. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 17, 326–333. 17:326-333; 1997.
61. Barnes, P.J. Corticosteroid resistance in airway disease. *Proc. Am. Thorac. Soc.* 1:264-268; 2004.

62. Goldstein, S.; Czapski, G.; Lind, J.; Merenyi, G. Tyrosine nitration by simultaneous generation of (NO)-N-center dot and O-2(center dot) under physiological conditions - How the radicals do the job. *J. Biol. Chem.* 275:3031-3036; 2000.
63. Ho, W.E.; Cheng, C.; Peh, H.Y.; Xu, F.; Tannenbaum, S.T.; Ong, C.N.; Wong, W.S.F. Anti-malarial drug artesunate ameliorates oxidative lung damage in experimental allergic asthma. *Free Radic. Biol. Med.* 53:498-507; 2012.

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Highlights

- Nitroxides decrease oxidative injury in murine models of allergic asthma.
- 3-carbamoyl-proxyl is better than 2,2,6,6-tetramethyl-piperidine-N-oxyl.
- Oral administration during sensitization & challenge was the most effective route.
- Oxidative stress plays an important role during sensitization and challenge stages.

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