

Protective effects of valproic acid against airway hyperresponsiveness and airway remodeling in a mouse model of allergic airways disease

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Airway remodeling and airway hyperresponsiveness are major aspects of asthma pathology that are not targeted optimally by existing anti-inflammatory drugs. Histone deacetylase inhibitors have a wide range of effects that may potentially abrogate aspects of remodeling. One such histone deacetylase inhibitor is valproic acid (2-propylvaleric acid). Valproic acid is used clinically as an anti-epileptic drug and is a potent inhibitor of class I histone deacetylases but also inhibits class II histone deacetylases. We used valproic acid as a molecular model of histone deacetylase inhibition *in vivo* in chronic allergic airways disease mice with airway remodeling and airway hyperresponsiveness. Wild-type Balb/c mice with allergic airways disease were treated with valproic acid or vehicle control. Airway inflammation was assessed by bronchoalveolar lavage fluid cell counts and examination of lung tissue sections. Remodeling was assessed by morphometric analysis of histochemically stained slides and lung function was assessed by invasive plethysmography measurement of airway resistance. Valproic acid treatment did not affect inflammation parameters; however, valproic acid treatment resulted in reduced epithelial thickness as compared with vehicle treated mice ($p < 0.01$), reduced subepithelial collagen deposition ($p < 0.05$) and attenuated airway hyperresponsiveness ($p < 0.05$ and $p < 0.01$ for the two highest doses of methacholine, respectively). These findings show that treatment with valproic acid can reduce structural airway remodeling changes and hyperresponsiveness, providing further evidence for the potential use of histone deacetylase inhibitors for the treatment of asthma.

Introduction

Asthma consists of three important features: airway inflammation, airway remodeling and airway hyperresponsiveness (AHR). Airway inflammation is characterized by eosinophils, Th2 lymphocytes and mast cells in the airways and can in most cases be effectively treated by the mainstay therapies for asthma—inhaled corticosteroids. Alternatively, airway remodeling (defined as structural changes in the thickened and hypersecretory airway wall) and AHR (defined as increased bronchoconstrictor reactivity) are not well addressed by current therapies. Airway remodeling contributes to AHR in a number of ways. Remodeling may decrease the ability of the airway to respond to bronchodilators and the thickening of the airway wall due to the increased cell numbers is predicted to contribute to narrowing of the airway.

Histone deacetylase (HDAC) inhibitors have a number of properties that may be useful in treating airway remodeling in asthma. They are well known for slowing cell proliferation in

cancer, they may have anti-inflammatory properties including those mediated through regulatory T cells, anti-fibrotic effects and anti-angiogenic effects. One HDAC inhibitor, Trichostatin A, has been recently shown to reduce airway contraction.¹

Valproic acid (VPA) (2-propylvaleric acid) is a well-established substance used clinically as an anti-epileptic drug and mood-stabilizing drug, used in the treatment of bipolar disorder, migraine headaches, schizophrenia, neuropathic pain and, less commonly, major depression. VPA has exceptional potency for class I HDACs but can also inhibit class II enzymes.² VPA possesses multivalent properties such as the inhibition of voltage-dependent sodium channels and enhanced γ -aminobutyric acid (GABA) signaling (i.e., the chief inhibitory neurotransmitter in the mammalian CNS).^{3,4} However, the clinical use of VPA is restricted by its potentially deleterious, possibly multifactorial, teratogenicity and hepatotoxicity (due to the carboxylic moiety structure; HDAC inhibitory effects).^{5,6} Furthermore, other common side effects associated with VPA include tremor,

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sedation, alopecia and weight gain (up to 70% of adult patients gain 5–14 kg).⁷ In addition, *in vitro* studies have demonstrated transcriptional activation of adipogenic genes with reduction in HDAC activity.^{8,9} Saouaf et al. demonstrated that the use of VPA enhances the short half-life of FoxP3, its binding and effect on gene expression in regulatory T cells (Tregs) and increased Treg numbers in murine collagen-induced arthritis models.^{10,11} Tregs assist in AHR and Th2 mediated airway inflammation suppression, as shown in a mouse ovalbumin (OVA) allergic airways disease (AAD) model;¹² thus, through the use of VPA, Treg numbers and effects increase, indicating a potential role in the treatment of AAD. VPA has been shown to block angiogenesis, which has a pivotal role in airway remodeling in asthma, in animal models of the disease.^{13,14}

Valproic acid has potent anti-HDAC activity and, therefore, may be useful in the treatment of disease processes in asthma. We aimed to investigate the effects of systemic valproic acid treatment in a murine model of chronic allergic airways disease, which displays pathological features of human asthma in airway remodeling structural changes and AHR.

Results

Validation of the chronic allergic airways disease model. It was necessary to validate that OVA sensitization/challenge had been successfully established in the OVA sensitized-challenged mice treated with vehicle (OVA-VEH). Allergen-specific immunoglobulin E (OVA-specific IgE), BAL inflammatory cells and remodeling changes were assessed. Adequate OVA sensitization was verified by measurement of OVA-specific IgE in serum samples at the end of the 8-week OVA sensitization/challenge protocol. As expected, OVA-VEH mice demonstrated increased levels of OVA-specific serum IgE compared with saline control mice ($p < 0.001$; Fig. 1A). Total BAL cell counts were significantly increased in OVA-VEH mice as compared with the saline sensitized/challenged control mice (all $p < 0.001$; Fig. 1B). Mice sensitized and challenged with OVA displayed a thickened airway wall compared with saline (Fig. 1C). Airway inflammation was significantly increased in OVA sensitized/challenged mice treated with vehicle (OVA-VEH) as compared with the saline control mice ($p < 0.001$; Fig. 1D). These results confirmed that the allergic airways disease model was successfully established as previously reported in references 15–18.

Effect of valproic acid treatment on airway inflammation in chronic allergic airways disease. OVA-specific IgE levels in OVA sensitized/challenged mice treated with VPA (OVA-VPA) were similar to levels in OVA sensitized/challenged mice treated with vehicle (OVA-VEH), and levels in both the OVA-VPA and OVA-VEH groups were significantly higher than levels in saline sensitized/challenged control mice confirming successful OVA sensitization ($p < 0.001$; Fig. 1A).

Total and BAL cell counts were not significantly different when compared with the vehicle treated mice (OVA-VEH) (Fig. 1B). Tissue inflammation was also not significantly different (Fig. 1C and D). These results indicate that systemic (intraperitoneal) administration of VPA does not significantly inhibit

numbers of BAL inflammatory cells following sensitization/challenge with OVA at the completion of the chronic OVA challenge model.

Treatment with valproic acid suppresses airway remodeling changes in subepithelial collagen deposition and epithelial thickening but not goblet cell metaplasia in chronic allergic airways disease. Goblet cell percentages were determined in AB-PAS stained lung tissue sections, while airway epithelial thickness and subepithelial ECM thickness were determined in Masson trichrome stained lung tissue sections. Percentages of goblet cells in VPA treated mice were not significantly different than in vehicle treated mice (Fig. 2A), but remained significantly higher than in the saline sensitized/challenged mice ($p < 0.001$). The mean epithelial thickness was significantly lower in VPA-treated mice as compared with vehicle treated mice ($p < 0.01$), but remained higher than that observed in the saline sensitized/challenged mice ($p < 0.001$; Fig. 2B). Subepithelial extracellular matrix thickness was significantly lower in VPA-treated mice as compared with vehicle treated mice ($p < 0.05$), but remained higher than that observed in the saline sensitized/challenged mice ($p < 0.05$; Fig. 2C). These results suggest that VPA can regulate some aspects of airway remodeling (epithelial thickness and fibrosis) but not goblet cell hyperplasia.

Treatment with valproic acid attenuates AHR in chronic allergic airways disease. Anaesthetized tracheostomized mice were challenged with nebulized methacholine and the change in resistance from baseline was measured by invasive plethysmography. Methacholine-induced AHR was reduced in the OVA-VPA group as compared with the OVA-VEH group, with statistical significance reached at doses of 4 mg/ml and 8 mg/ml of methacholine ($p < 0.05$, $p < 0.01$; Fig. 3). These results show that VPA treatment can suppress AHR in the chronic allergic airways disease model.

Immunohistochemical analysis of TGFβ1 expression. TGFβ1 was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections (Fig. 4). Little constitutive staining for TGFβ1 was observed in the saline-sensitized and challenged control mice. In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGFβ1 was markedly increased. Strong cytoplasmic staining was present in bronchial epithelial cells, connective tissue cells in the lamina propria and adventitia and smooth muscle cells. Strong staining was also seen in inflammatory cells. Treatment with VPA resulted in markedly reduced staining for TGFβ1 in the lung tissue.

Immunohistochemical analysis of αSMA expression. αSMA was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections (Fig. 5). Strong cytoplasmic staining was present in airway smooth muscle cells. Treatment with VPA did not affect staining for αSMA in the lung tissue.

Immunofluorescence for Annexin V. Strong staining of Annexin V was found largely in bronchial epithelium and peribronchial inflammatory cells in mice treated with OVA-VEH. Weak Annexin V staining was present in mice treated with OVA-VPA. In contrast, Annexin V staining was not observed in the epithelium in saline control mice (Fig. 6).

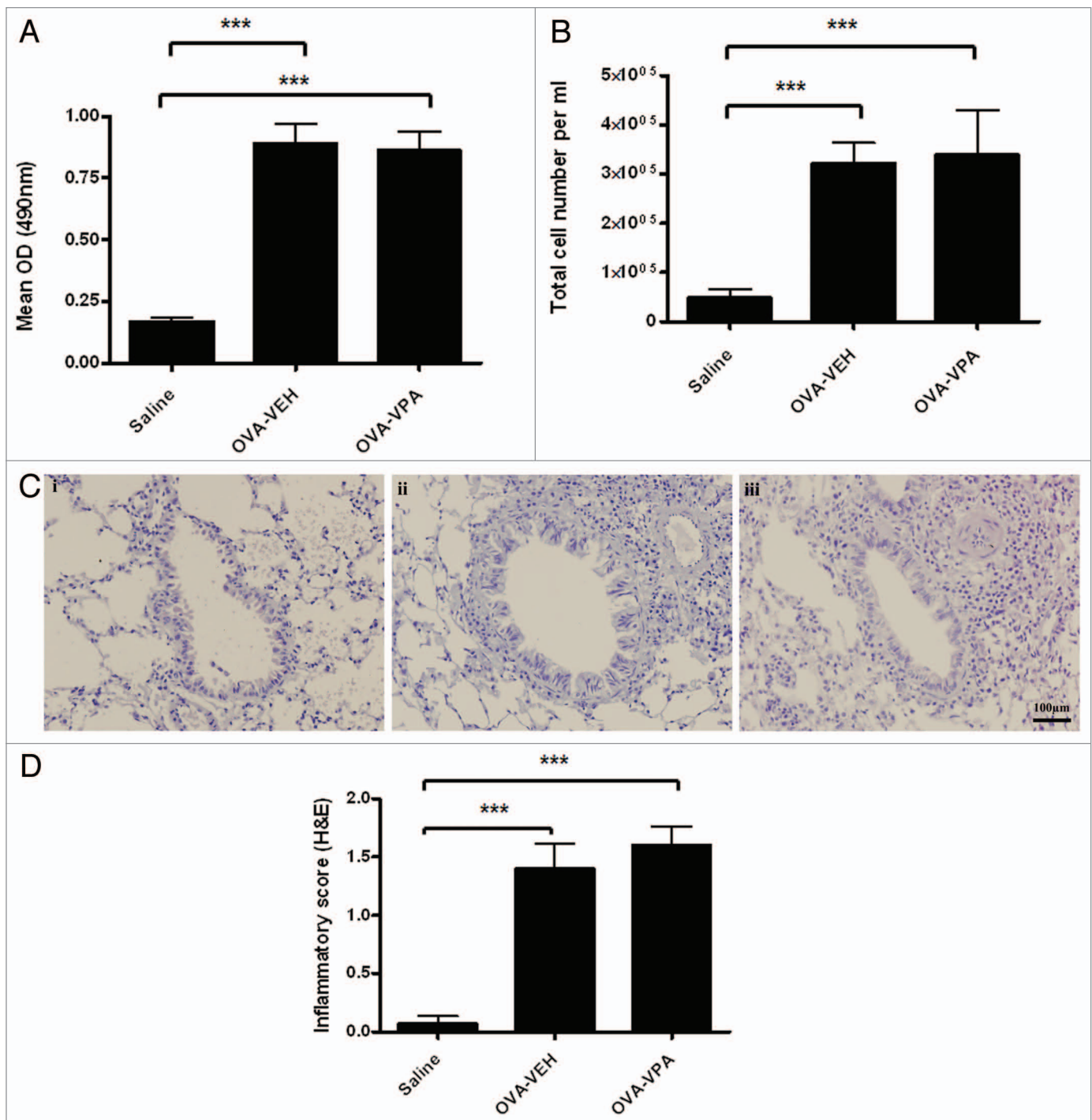


Figure 1. Biological effects of valproic acid on airway inflammation in chronic allergic airways disease. Serum levels of OVA-specific IgE in the chronic allergic airways disease model is shown (A). Enzyme-linked immunosorbent assay was performed on mice blood serum samples. OD values were read at 490 nm, with a reference wavelength of 650 nm. There was a significant increase in the OVA-VEH and the OVA-VPA sensitized groups with respect to saline group ($***p < 0.001$). In each group $n = 15$. Following sacrifice, BAL was performed and total inflammatory cell number per ml of BALF determined (B). The OVA-VEH and OVA-VPA groups showed a significant increase in total inflammatory cell numbers compared with the saline mice. ($***p < 0.001$). In each group $n = 15$. Representative photomicrographs of hematoxylin and eosin stained sections of mouse airways (C) show valproic acid attenuates epithelial thickening in an OVA-induced model of chronic AAD. Lung tissue sections were stained with H&E to observe remodeling and inflammatory cell infiltrate. Shown are representative lung tissue sections from mice treated with saline (1), OVA-VEH (2) or OVA-VPA (3). Images were acquired using an Olympus FSX100 microscope. Bar = 100 μm; x20 magnification. Inflammatory cell infiltration of hematoxylin and eosin stained sections of mouse airways was scored by two independent blinded investigators as 0 for no inflammation infiltration; 1 for 3 layers of inflammatory cells; 2 for 6 layers; 3 for 10 layers and congestion; or 4 for more than 10 layers of inflammatory cells and severe congestion (D). Both OVA-VEH and OVA-VPA had elevated inflammation scores as compared with saline mice. There was no significant difference between OVA-VEH and OVA-VPA groups (N.S.). ($***p < 0.001$). In each group $n = 15$.

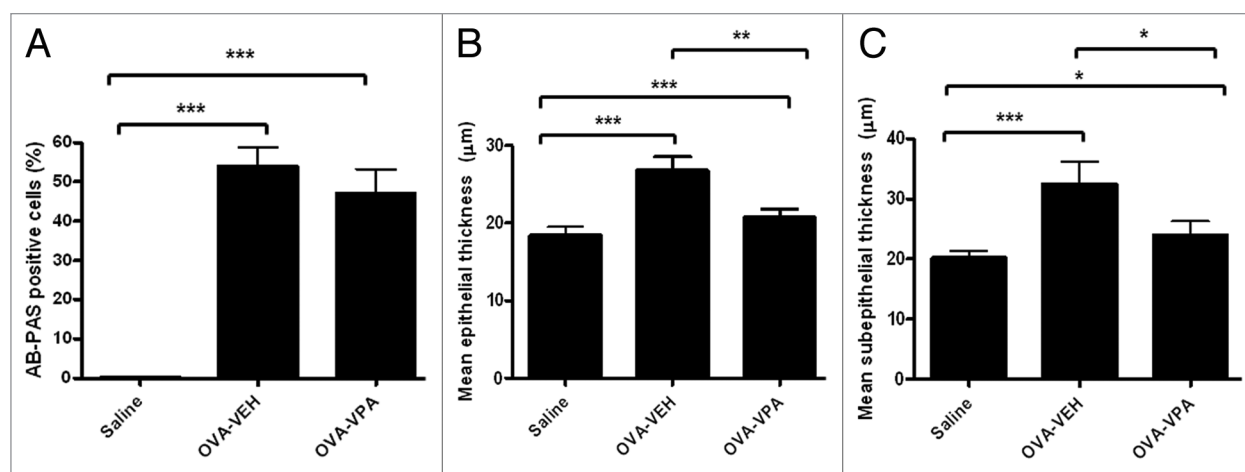


Figure 2. Percentage of AB-PAS positive goblet cells in mouse airways (A). Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with AB-PAS and the percentage of AB-PAS goblet cells determined. Both OVA-VEH and OVA-VPA groups had elevated goblet cell percentages as compared with saline mice. There was no significant difference between OVA-VEH and OVA-VPA groups (N.S.). (** $p < 0.001$). In each group $n = 15$. Morphometric analysis of epithelial thickness in the chronic AAD model (B). Airway epithelial thickness of Masson trichrome stained lung tissue sections were traced and assessed using morphometry software. The data represent mean epithelial thickness values obtained from mice in their respective treatment group. OVA-VEH mice had elevated epithelial thickness as compared with saline mice. OVA-VPA mice had significantly less epithelial thickness compared with OVA-VEH. (** $p < 0.01$, *** $p < 0.001$). In each group $n = 15$. Morphometric analysis of subepithelial collagen thickness in the chronic AAD model (C). Airway subepithelial collagen thickness of Masson trichrome stained lung tissue sections were traced and assessed using morphometry software. The data represent mean subepithelial collagen thickness values obtained from mice in their respective treatment group. OVA-VEH mice had elevated sub-epithelial collagen thickness as compared with saline mice. OVA-VPA mice had significantly less collagen deposition compared with OVA-VEH. (* $p < 0.05$, *** $p < 0.001$). In each group $n = 15$.

Discussion

Valproic acid is a branched short-chain fatty acid which alters activity of gamma amino butyrate in the brain and has several other effects that have lead to its widespread use in the treatment of epilepsy and related diseases.¹⁹ More recently, VPA has been shown to have HDAC inhibitory activity.¹⁹ There is considerable interest in HDAC inhibitors in the cancer field and in the treatment of inflammatory diseases. The HDAC inhibitor Trichostatin A has been shown to have anti-inflammatory effects in an acute OVA AAD model and to suppress airway reactivity in an *Aspergillus fumigatus* model of AAR.¹

In the current study we studied the effects of VPA using a chronic murine model of allergic airways disease to assess the effect of the drug on airway remodeling and AHR. Our findings indicate that VPA treatment suppressed AHR and inhibited the development of epithelial thickening and airway thickening due to fibrosis. TGF β protein expression in the airways of mice treated with VPA was decreased while α SMA marker for airway smooth muscle was not altered.

While much attention has been concentrated on the development and optimization of anti-inflammatory drugs for asthma pharmacology, other strategies may hold therapeutic potential. Valproic acid is predicted to have a number of beneficial effects on asthma pathology and is the subject of ongoing clinical trials.¹⁹ Lomia and colleagues conducted a clinical trial of VPA and carbamazepine (anti-convulsant and mood stabilizer used in epilepsy treatment) monotherapies.^{20,21} In this trial, the VPA monotherapy achieved stable remission of asthma symptoms in 11/14 subjects with moderate or severe asthma. Outcomes were

pulmonary function assessed by peak flow rates and incidence of asthma attacks. At the three-year follow-up all responders had continued the treatment and had normal peak flow rate. It was hypothesized that the treatment was successful due to the drugs targeting neurogenic paroxysmal and inflammatory mechanisms in asthma.²¹

The most interesting finding of the current study was that mice treated with VPA exhibited reduced methacholine induced AHR than mice in the OVA-VEH vehicle control group. AHR is the result of two processes in asthma: airway inflammation and airway remodeling.²² We found no significant reduction of lavage or lung tissue inflammation in mice treated with VPA. Others have found that VPA can reduce lung inflammation in acute lung injury and hemorrhagic shock models.^{23,24} This difference may reflect the level of inflammation in chronic disease and the treatment regimen used. We did however find a strong therapeutic effect on airway remodeling. Previous studies have shown an association between remodeling and AHR.²⁵ Furthermore, therapies that reduce remodeling may abrogate AHR.²⁵ Both subepithelial collagen thickness and epithelial thickness were reduced by VPA therapy. Fibrotic remodeling of the reticular basement membrane correlates with severity in endobronchial biopsies from asthma sufferers.²⁶ Subepithelial fibrosis contributes to the overall thickness of the airway wall that impacts AHR.^{27,28} Others have found that chronic treatment with VPA has decreased proliferation and transdifferentiation of hepatic stellate cells and has resulted in reduced carbon tetrachloride induced fibrosis.²⁹ We found less TGF β protein expression in mice treated with VPA than in OVA control mice. Airway fibrosis in asthma is due to extracellular matrix deposition by fibroblasts with differentiation

of myofibroblasts. These processes are regulated by TGF β , a potent profibrotic cytokine. TGF β is hypersecreted by epithelial cells and fibroblasts in the airway as well as the influx of inflammatory cells, including eosinophils in asthma.³⁰ The airway epithelium is recognized as playing a critical role in asthma.³¹ There is crosstalk between the airway epithelial cells and the underlying fibroblasts. This works bi-directionally; for example, the epithelial cells secrete profibrotic growth factors and cytokines while the proliferation and differentiation of epithelial cells can be influenced by an extracellular matrix composition that is characteristic of the diseased airway.³² In the current study we observed reduced Annexin V staining, a marker of cell apoptosis, particularly in the airway epithelial cells of mice treated with VPA. Airway epithelial cell apoptosis is seen in asthma and is highly significant as it is a marker of epithelial damage and, therefore, of epithelial activation and remodeling;³³ furthermore, corticosteroids can damage the airway epithelium by inducing apoptosis.³⁴ Therapies that circumvent epithelial apoptosis may be protective against epithelial activation and subsequent remodeling.

In the chronic AAD model VPA was administered intraperitoneally over a six-week period. The dose used was without any side effect in the mouse model as assessed by histopathology of representative organ samples, animal welfare scorings and body-weight (data not shown). In humans, VPA also has a proven safety profile but may have a number of side effects including aplastic anemia, hepatotoxicity and coagulopathies; it may also be teratogenic.¹⁹

In the current study we have demonstrated anti-remodeling effects of VPA in a chronic model of AAD that replicates many of the features of human asthma. Mice injected with VPA had reduced epithelial thickness and fibrosis. Treatment of remodeling may help to suppress AHR and improve lung function. This study provides evidence for the use of a HDAC inhibitor in treating airway disease.

Materials and Methods

Animals. Six-week-old female Balb/c mice were housed under specific pathogen free conditions and maintained on a fixed 12 h light, 12 h dark lighting schedule. Balb/c mice are known to demonstrate strong Th2 responses in ovalbumin (OVA)-induced chronic allergic airways disease (AAD) models.^{35–37} All experimental procedures were approved by the Murdoch Children's Research Institute Animal Ethics Committee and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

Mouse model of chronic allergic airways disease. An established model of OVA-induced AAD was used as previously described in references 15 and 16. This model includes many of the pathological features of human asthma including increased allergic responses indicated by increased immunoglobulin E against OVA (OVA-specific IgE), epithelial remodeling, goblet cell metaplasia, subepithelial collagen deposition (fibrosis) and AHR. The model does not display smooth muscle hyperplasia.^{15,16} Briefly, mice were sensitized with 10 μ g of grade V OVA

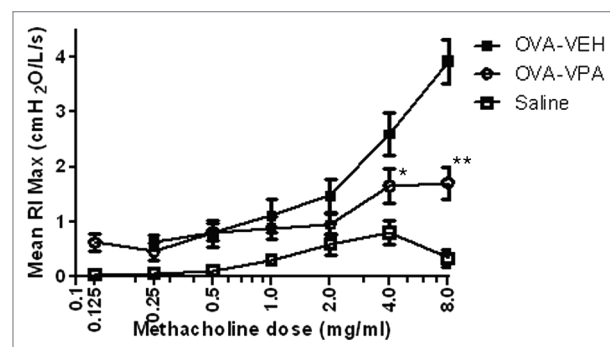


Figure 3. Airway hyperresponsiveness to methacholine in the chronic AAD model. Plethysmography was performed 48 h following the final treatment and nebulization. Increasing doses of methacholine was administered and maximal resistance values (cmH₂O/L/sec) after 5 min was recorded. OVA-VEH mice had elevated airway resistance at the highest methacholine dose as compared with saline mice. OVA-VPA had significantly lower airway resistance at the highest two methacholine dose compared with OVA-VEH. In each group n = 10. (*p < 0.05, **p < 0.01 v OVA-VEH).

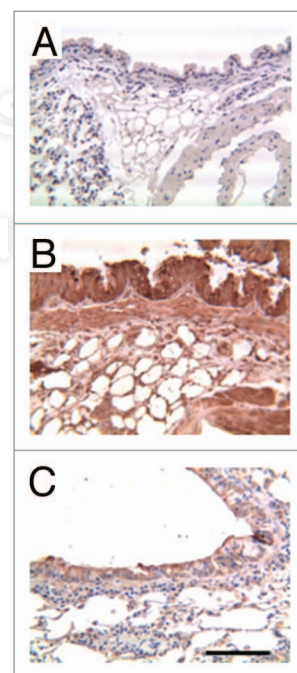


Figure 4. Immunohistochemical analysis of TGF β 1 expression. TGF β 1 was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections. Little staining for TGF β 1 was observed in the saline-sensitized and -challenged control mice (A). In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGF β 1 was markedly increased (B). Treatment with VPA resulted in reduced staining for TGF β 1 in the lung tissue (C). In each group n = 10. Bar equals 100 μ m.

(Sigma Chemical, St. Louis, MO) and 1 mg of aluminum potassium sulfate adjuvant (alum) in 500 μ l saline intraperitoneally on day 0 and 14 and then challenged with nebulized 2.5% (w/v) OVA in saline three days per week for six weeks to establish AAD.

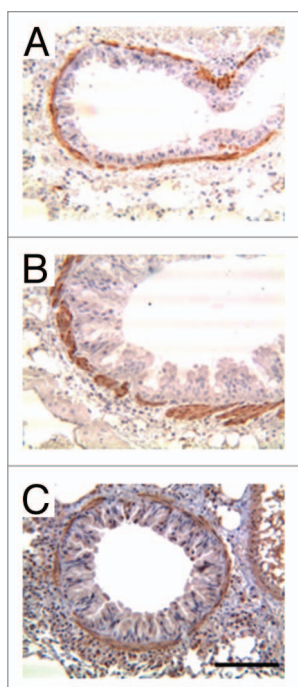


Figure 5. Immunohistochemical analysis of α SMA expression α SMA was investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections. Staining for α SMA was observed in the smooth muscle layer in the saline-sensitized and -challenged control mice (A), and in the lungs of mice receiving chronic OVA sensitization and challenge (B). Treatment with VPA did not alter staining for α SMA in the lung tissue (C). In each group $n = 10$. Bar equals 100 μ m.

Ovalbumin-exposed mice were treated with valproic acid (OVA-VPA, $n = 15$) or vehicle control (OVA-VEH, $n = 15$) intraperitoneally following each OVA nebulization (3 d per week for 6 weeks). A third group of mice, sensitized with saline/alum on days 0 and 14 and nebulized with saline 3 d per week for 6 weeks ($n = 15$), served as additional controls. Valproic acid (Sigma Chemical) was used at a dose of 100 μ l of 20 mg/ml in 20% ethanol/0.9% saline, which is known to be biologically active and has been used widely in experimental murine models of disease with minimal toxicity.³⁸

Methacholine-induced AHR. Forty-eight hours after the last OVA nebulization/treatment with valproic acid or vehicle, AHR was measured using a mouse invasive plethysmograph (Elan, Buxco Electronics, Troy, NY) as described previously in reference 15. Mice were anesthetized by intraperitoneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg), and tracheostomized. After baseline airway resistance had been recorded, increasing methacholine doses (0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml and 8 mg/ml) were delivered by nebulizer (Elan, Buxco Electronics, Troy, NY) and airway resistance change from baseline level were measured (Finepointe, Buxco Electronics) for three min after each dose.

Quantitation of serum OVA-specific IgE levels. Serum was obtained by lethal cardiac puncture of anesthetized mice after airway reactivity measurement and stored frozen at -70°C for

measurement of OVA-specific IgE by ELISA.^{15,17,39} OVA-specific IgE levels were expressed as mean optical density (OD) at 490 nm.

Bronchoalveolar lavage (BAL). Following measurement of airway reactivity, BAL was performed by three washes of 0.5 ml saline containing 5% (v/v) fetal calf serum and total and differential cell counts of inflammatory cells determined.^{15,40} Total viable cell counts were determined using a hemocytometer with trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes and monocytes were determined on cyto-spin smears of BAL samples (4×10^5 cells) from individual mice stained with DiffQuick (Life Technologies, Auckland, New Zealand) and identified by standard morphological criteria after counting 300 cells.

Tissue collection. Lung tissues were immediately dissected and weighed (total lung weight) and then separated into individual lobes for histological analysis.^{15,17,41}

Lung histopathology. The right lung was fixed in formalin, routinely processed and embedded in paraffin.^{15,17,41} Three micrometer lung tissue sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thicknesses, and Alcian blue-periodic acid Schiff (AB-PAS) for assessment of goblet cell percentages.^{15,17,41}

Airway tissue inflammation cell score. The degree of airway inflammatory cell infiltration was scored by two independent blinded investigators. The degree of airway inflammatory cell infiltration around the bronchi was scored as 0 for no inflammatory cell infiltration; 1 for 3 layers of inflammatory cells; 2 for 6 layers; 3 for 10 layers and congestion; or 4 for more than 10 layers of inflammatory cells and severe congestion. For each mouse, five airways were observed and the average scores were taken.

Morphometric analysis of structural changes. Morphometric analysis of lung tissue sections was performed as described previously in references 15, 17 and 42. Images of lung tissue sections were captured using a Digital camera (Q Imaging, Burnaby, BC, Canada). A minimum of five bronchi measuring 150–350 μ m luminal diameter were analyzed per mouse using Image Pro-Discovery software (Media Cybernetics, Silver Spring, MD), which was calibrated with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells using a digitizer (Aiptek, Irvine, CA) and calculating the mean distance between the lines by Image Pro-Discovery software (Media Cybernetics). Subepithelial collagen thickness was also measured by tracing around the outer extent of the total collagen layer in the submucosal region and around the basement membrane, and the mean distance between these lines calculated. For goblet cell determination the basement membrane length was measured by tracing in Image Pro-Discovery software (Media Cybernetics) and goblet cells were counted in AB-PAS stained sections and expressed as percentage of cells per 100 μ m of basement membrane.

Immunohistochemistry for TGF β 1. Sections of lung tissue were stained by immunohistochemistry to detect TGF β 1 protein expression. TGF β 1 was identified using rabbit polyclonal

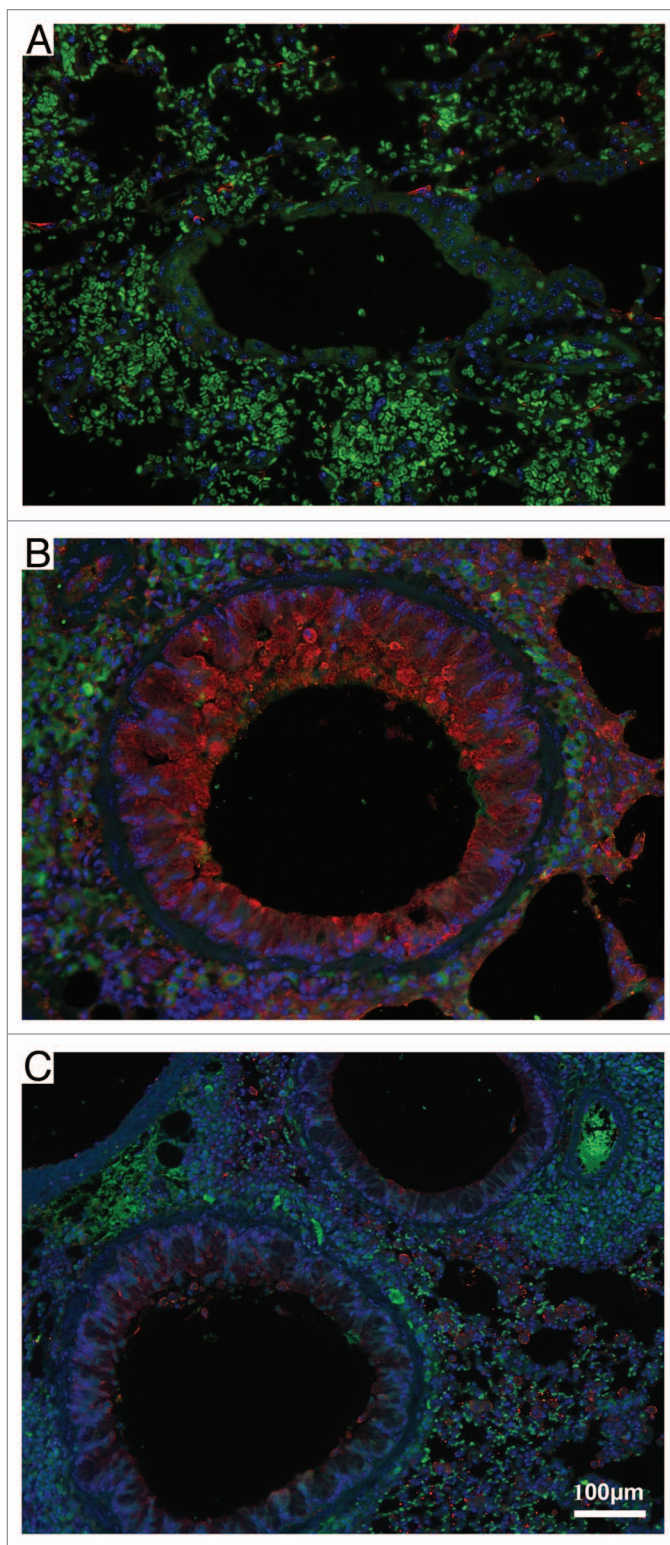


Figure 6. Immunofluorescence staining for a membrane apoptotic marker, Annexin V (red) is shown in representative mouse tissue sections from saline control (A), OVA-VEH (B) and OVA-VPA (C). Mouse monoclonal anti-histone deacetylase 8 (HDAC8; green) was used to highlight tissue structure. Strong staining of Annexin V was found largely in bronchial epithelium and peribronchial inflammatory cells in mice treated with OVA-VEH to induce a chronic model of AAD. Weak Annexin V staining was present in mice treated with OVA-VPA. In contrast, Annexin V staining was not observed in the epithelium in saline control mice. Merged image: nucleus stained with DAPI (blue), Annexin V (red), HDAC8 (green). Images were acquired using an Olympus BX61 fluorescence microscope with automated FVII camera. Bar = 100 μ m; x20 magnification.

Immunohistochemistry for α SMA. Formalin-fixed paraffin-embedded sections were stained for α SMA using immunohistochemistry to identify airway smooth muscle. Bound primary antibody, mouse monoclonal anti α SMA (Dako), was biotinylated and detected using streptavidin horseradish peroxidase (mouse on mouse Animal Research Kit; Dako). The chromagen 3,3-diaminobenzidine was used and sections counterstained with hematoxylin.

Immunofluorescence for Annexin V. Tissue sections were blocked for 1 h using Superblock (Thermo Scientific) at room temperature followed by a 5 min wash using 0.5% Tween 20, 0.1% Triton X-100 in phosphate buffered saline (PBS-TT). Tissues were exposed to primary monoclonal antibodies anti-Annexin V (rabbit; Epitomics) and anti-histone deacetylase 8 (mouse; Sigma), diluted in 1% BSA (1:500). Primary antibodies were incubated in a dark humidified chamber overnight. Following three 10 min washes in PBS-TT, tissues were incubated with secondary antibodies, goat anti-mouse Alexa 488 (Molecular Probes) and goat anti-rabbit 546 (Molecular Probes) diluted in 1% BSA (1:500) in a dark humidified chamber for one hour on a rotating platform. Following three 10 min washes in PBS-TT, tissues were mounted using Prolong Gold Antifade with DAPI (Invitrogen Molecular Probes). Slides were incubated overnight at 4°C before imaging. Images were acquired using an Olympus BX61 fluorescence microscope automated with FVII Camera.

Statistical analysis. Results were expressed as mean $\pm$ SEM with 95% confidence interval and analyzed using the Mann-Whitney test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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antibodies, sc-146, specific for TGF β 1 (Santa Cruz Biotechnology, Santa Cruz, CA). Bound primary antibody was detected using anti-rabbit EnVision (Dako, Glostrup, Denmark). The chromagen 3,3-diaminobenzidine was used and sections counterstained with hematoxylin.

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