

# Up-regulated Vitamin D Receptor by *Pelargonium Sidooides* Extract EPs® 7630 Contributes to Rhinovirus Defence in Bronchial Epithelial Cells

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## Research

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# Abstract

**Background:** The *Pelargonium sidoides* herbal drug preparation EPs®7630 reduces the severity of viral upper respiratory tract infections. Vitamin D a secosteroid also improves anti-viral host defence. The anti-viral effect of both compounds was linked to similar signalling pathways in immune and epithelial cells. This study assessed if EPs®7630 modifies vitamin D receptor (VDR) expression by human bronchial epithelial cells.

**Methods:** Bronchial epithelial cells were incubated with EPs®7630 over 48 hours before calcitriol stimulation and/or infection with Rhinovirus (RV)-16. The VDR expression and regulation was determined by Western-blotting. Intracellular signalling was studied by inhibition of mitogen activated protein kinases; Erk1/2, p38, and JNK. The anti-viral effect was assessed by immunofluorescence for RV-16 protein.

**Results:** Treatment with EPs®7630 up-regulated the expression of the VDR through activation of Erk1/2 and thereby increased the cells sensitivity to calcitriol, reflected as the increased shift of the VDR from the cytosol into the nucleus. Compared to cells not pre-treated with EPs®7630 this VDR shift occurred at a 5.3 times lower calcitriol concentration. EPs®7630 increased Erk1/2 MAPK dependent signalling, but reduced the phosphorylation of p38. It had not effect on the activation and expression of JNK. EPs®7630 pre-treatment improved the anti-viral effect of vitamin D on RV-16 infection by 2.1 folds compared to vitamin D alone or to untreated cells. Furthermore, EPs®7630 improved the differentiation of epithelial cells by upregulating E-cadherin expression through Erk1/2.

**Conclusions:** The results suggest that EPs®7630 increases host defence against rhinovirus infection by upregulating the VDR and thus increasing the response of epithelial cells to vitamin D.

## Highlights

EPs®7630 increases epithelial cell host defence against rhinovirus infection by upregulating the VDR.

## Background

Viral infections are the most frequent cause of respiratory ailments and present a severe health problem to children and elderly [1, 2]. EPs®7630 is a herbal drug preparation from the roots of *Pelargonium sidoides*, which effectively reduces the severity of symptoms in patients with acute bronchitis, acute sinusitis maxillaris, tonsillopharyngitis, or common cold, combined with a good tolerability [1–4].

The mechanisms by which EPs® 7630 reduces viral infection were investigated in several non-clinical trials. It has been reported that EPs® 7630 significantly reduced the ability of various viruses to attach to the host cells, or to prevent virus release from infected cells. In cell culture experiments, EPs® 7630 inhibited the attachment of HIV-1 to human immune cells, protecting them from viral entry [5], whereas protection of cells against influenza A was mediated via inhibition of hemagglutination and

neuraminidase activity [6]. In human bronchial epithelial cells, EPs® 7630 reduced Rhinovirus-16 (RV-16) replication by downregulating the expression of inducible co-stimulator (ICOS) and its ligand (ICOSL), as well as the surface calreticulin receptor, whereas host defence supporting protein  $\beta$ -defensin 1 and SOCS-1 were increased [7]. However, the intracellular signalling mechanism underlying the anti-viral effects of EPs® 7630 remained to be defined.

For type-A and -B Rhinoviruses, the intracellular adhesion molecule1 (ICAM1) is a major docking protein of host cells [8], required for viral infection of different host cells [7, 9]. Vitamin D receptor (VDR) activation has been shown to reduce the adherence of RV to ICAM1 [10]. In this context, low serum levels of vitamin D correlated with increased susceptibility to respiratory virus infection. In children, rhinovirus infection inversely correlated with vitamin D levels [11], and low serum vitamin D increased the sensitivity to RV infection [12]. Vitamin D deficiency correlated with the frequency of exacerbation caused by rhinovirus infection in chronic obstructive pulmonary disease (COPD) patients [13]. Furthermore, infection with either RV or Respiratory syncytial virus (RSV) reduced the expression of the VDR by human epithelial cells, but this was reversed by vitamin D supplementation [14].

The importance of the VDR in host defence is supported by reports that the VDR genotype affects the risk of upper respiratory tract infections. In children with asthma, a genome analysis indicated that the VDR is one of the most important factors that regulates the susceptibility to virus-induced upper respiratory tract infections [15]. In a cohort of 1462 adults from the UK, a link between the minor VDR allele, rs4334089 SNP and the susceptibility to upper respiratory tract infections was confirmed [16]. In children with severe bronchiolitis, SNP rs2228570 VDR together with SNPs of Toll like receptor 4 and Toll like receptor 2 was associated with increased risk of death [17].

The expression of the VDR is controlled by Erk1/2 mitogen activated protein kinase (MAPK) in muscle cells [18]. EPs® 7630 has been shown to differentially regulate Erk1/2 MAPK in immune cells, while suppressing p38 MAPK [19]. Hence, it seems feasible that EPs® 7630 could also affect VDR expression.

In this study, we assessed the effect of EPs® 7630 on the expression and activation of the VDR in bronchial epithelial cells, and determined its functional relevance for the anti-viral activity.

## Methods

**Primary bronchial epithelial cells and BEAS-2B cells:** Primary bronchial epithelial were isolated and characterised as described earlier [20]. Endo-bronchial tissue biopsies were obtained from six patients by endo-bronchoscopy for diagnostic reasons by the lung clinic (University Hospital Basel, Switzerland). All patients provided written informed consent for the use of one additional anonymised biopsy for scientific investigations. The study was approved by the local Institutional Ethical Committee (EKBB 05/06).

Epithelial cells were isolated by cell-type selective medium CnT-PR-A (CellnTec, Bern, Switzerland). BEAS-2B cells were grown in the same cell culture medium. The cell phenotype was monitored by phase

contrast microscopy and by staining for: positive E-cadherin and cytokeratin-13, as well as for negative fibronectin staining (Fig. 1a).

Drugs: EPs® 7630, a herbal drug preparation from the roots of *Pelagonium sidoides* (1:8–10), extraction solvent: ethanol 11% (w/w), supplied by Schwabe Pharma AG (6403 Küssnacht am Rigi, Switzerland) was dissolved in cell culture medium.

Vitamin D, calcitriol (Sigma-Aldrich Merck, Buchs, Switzerland), was dissolved in ethanol (100 µg/ml) before being added to the cell cultures at final concentrations ranging from concentrations of 0.1–10 µM, together with EPs® 7630.

High Pressure Liquid Chromatography: EPs® 7630 high pressure liquid chromatography -UV High Resolution Mass Spectrometry (HRMS): The HPLC-UV-HRMS chromatograms were recorded on an Thermo® Vanquish UHPLC coupled to an DAD and Thermo Orbitrap® Fusion mass detector using a Waters® Atlantis T3 (3 µM, 2 x 150 mm) column without pre-column. Eluent A consisted of 2.5% (v/v) acetonitrile and 0.5% (v/v) formic acid in water. Eluent B consisted of 5% (v/v) water and 0.5% (v/v) formic acid in acetonitrile.

At a flow rate of 0.2 mL/min, the gradient was as follows: from 0.0–10.0 min. linear for 0–5% eluent B, from 10.0–65.0 min. linear for 5–50% eluent B, from 65.0 to 66.0 min. linear for 50–100% eluent B, from 66.0 to 71.0 min. isocratic 100% eluent B column wash, from 71.0–72.0 min. linear from 100–0% eluent B followed by 8 min. equilibration period with 0% eluent B, resulting in a total run time of 80.00 min. UV detection wavelength of 280 nm and a column temperature of 40 °C were applied. The injection volume was 4 µL of a 5 mg/mL *Pelargonium sidoides* extract EPs® 7630 dissolved in eluent A. HRMS based peak assignment was performed using ACDLabs Spectrus Processor Software v2017.2.1.

Cell treatment: Confluent epithelial cells were stimulated with EPs® 7630 (0.1–10 µg/ml), or calcitriol, or the combination of both, for up to 48 hours before being infected with 1 MOI of RV-16 as depicted in Fig. 1b.

For EPs® 7630-induced VDR expression, cells were pre-treated for 24 or 48 hours with EPs® 7630 (10 µg/ml). To determine the effect of EPs® 7630 and calcitriol on VDR translocation pre-treated cells were exposed to increasing concentration of vitamin D (calcitriol 0.1–10 µM in DMSO). The expression of the VDR was determined by Western-blotting in total protein extracts. In some experiments, the EPs® 7630 pre-treated cells were infected with 1 MOI RV-16 for up to 48 hours. Total proteins were collected over 4 days and analysed as described below for protein expression, or cells grown on cover slips were fixed with 2% formaldehyde for 2 x 5 min. followed by staining and fluorescence microscopy for RV16 protein expression (Fig. 1b).

Rhinovirus infection and detection: The RV-16 strain used was described earlier (Oliver et al 2008). Cells were seeded in 8-well chamber slides (Thermo Fisher Scientific, Switzerland), and at 80–90% confluence they were infected with RV16 (1x multiplicity of infection: MOI) by 5 min centrifugation (200 x g). Cells

were continued under standard cell culture conditions for up to 4 days. RV16 infection was monitored by immunofluorescence for anti-RV16 antibody (cat# 18758, QED-Bioscience Inc. San Diego, USA). Cells were fixed by formalin (4% in PBS, 2 x 5 minutes), washed twice (PBS), and permeabilised (5 min., 0.01% TWEEN-100 in PBS). Unspecific antibody binding was blocked (30 min., 2% bovine serum albumin in PBS) and afterwards incubated with the anti-RV16 antibody (1:100 dilution, overnight, (4°C). Cells were washed 3 x (PBS), followed by incubation with anti-mouse FITC labelled antibody (Abcam, Switzerland, 1 hour, room temperature). Nuclei were stained by DAPI for cell counting (Thermo Fisher Scientific). The number of RV16 positive cells was determined after 3 washes (PBS) by immunofluorescence microscopy (EVOS FLoid cell imaging station, Thermo Fisher Scientific). All experiments were performed in a Bio-Safety-Level-II laboratory.

**Cytosolic - nuclear protein translocation:** Confluent epithelial cells were treated with EPs® 7630 (10 µg/ml) for 48 hours before being stimulated with increasing concentrations of vitamin D (0.1–10 mM) over various time periods (0, 3, 6, and 24 hours). The cell compartmental distribution of the VDR was determined by immunofluorescence staining using EVOS cell imaging system (Thermo Fisher Scientific, Waltham, USA).

**Western-blotting:** Cells were lysed in RIPA buffer, or as cytosolic and nuclear proteins. The protein content was quantified by BCA (Thermo Fisher Scientific). Denatured proteins (10µg) were size-fractionated by electrophoresis (8–16% SDS–PAGE, Thermo Fisher Scientific), and transferred onto PVDF membranes. Unspecific binding of antibodies was blocked by 30 min incubation of the membranes with 2%bovine serum albumin in phosphate buffer saline (PBS) containing 0.05% TWEEN-20. Proteins were detected by incubating the membranes with one of the primary antibodies to either the VDR, Erk1/2 MAPK, p38 (α, β, γ, δ), JNK, E-cadherin, or GAPDH (all: Abcam Plc, Cambridge, UK) for overnight at 4°C. Following 3 washes with blocking buffer, the membranes were incubated with secondary species-specific HRP conjugated antibodies (Abcam). Protein bands were visualised by chemiluminescence, applying SuperSignal West Dura substrate (Thermo Fisher Scientific) and documented by c300 (Azure Biosystems, Dublin, USA).

**Immunofluorescence:** Epithelial cell were seeded on 8-well PCA-slides (cat 94.6140.802, Sarstedt, Sevelen, Switzerland) and allowed to adhere overnight. Cells were then treated with medium alone, or by increasing concentrations of EPs® 7630 (0.01-10 µg/ml) over various durations up to 5 days. Cells were fixed in 4% paraformaldehyde (in PBS, 2×5 min), and immuno-fluorescence staining was performed as described earlier [7]. Nuclei were stained by DAPI.

**Statistics:** The Null-hypothesis was: No modification of protein expression, activation or location by any treatment compared to untreated cells. The Null-hypothesis was tested by ANOVA, Student's t-test and sub-sequent Mann-Whitney U-test as appropriate; p-value < 0.05 was accepted as significant.

## Results

## **Analysis of EPs® 7630 components by high pressure liquid chromatography (HPLC)**

Figure 2 shows a typical HPLC-UV-HRMS profile of EPs® 7630. The chemical structure of the different benzopyranones contained in EPs® 7630 are assigned by small letters (d – o) and the chemical structure analysis is provided in Table 1. More details of the EPs® 7630 constituents were published by others [21, 22].

## **VDR regulation by EPs® 7630 and the underlying signalling pathway**

Epithelial cells treated with EPs® 7630 increased the expression of the VDR in a concentration-dependent manner over 24 hours (Fig. 3a). There was no difference between primary epithelial cells or BEAS-2B cells. This effect became significant at concentration > 5 µg/ml EPs® 7630 after 24 hours (Fig. 3a). Kinetics of the EPs® 7630-induced VDR expression were studied with a fixed concentration of EPs® 7630 (10 µg/ml) over 48 hours which showed a continuous increase (Fig. 3b).

Based on earlier reports, the involvement of mitogen activated protein kinases in the EPs® 7630-mediated activation of the VDR expression was studied. In Fig. 3c the kinetic of the stimulating effect of EPs® 7630 on phosphorylation of Erk1/2 is shown. None of the other MAPKs was significantly modified by EPs® 7630 (data not shown). Blocking MAPK signalling pathways by specific chemical inhibitors, the stimulating effect of EPs® 7630 (10 µg/ml) on VDR expression was significantly reduced by PD98059 in a concentration dependent manner (Fig. 3d). In contrast, neither the inhibition of p38 MAPK by SB203580, nor that of JNK by SP600125 had any significant effect on EPs® 7630 induced VDR expression (Fig. 3d).

EPs® 7630 not only increased the overall expression of the VDR, but also induced its translocation into the nucleus (Fig. 4a). However, this effect was much lower compared to calcitriol-induced nuclear accumulation of the VDR (Fig. 4b). Both effects were concentration dependent. In untreated cells, the VDR was located mainly in the cytosol (Fig. 4c). Calcitriol significantly increased the number of cells with nuclear VDR, and this was further increased when the cells were also treated with EPs® 7630 (10 µg/ml) (Fig. 4c). When cells were treated with the combination of EPs® 7630 (10 µg/ml) and calcitriol, only Erk1/2 MAPK inhibition (PD98059) suppressed the accumulation of the VDR into the nucleus (Fig. 4d).

## **Calcitriol supports the anti-viral effect of EPs® 7630**

In a previous study, EPs® 7630 reduced the infection of epithelial cells by RV-16 [7]. This observation was confirmed by cell counting of the RV-positive stained BEAS-2B cells (Fig. 5a). Calcitriol alone also reduced RV-infection of BEAS-2B cells in a concentration-dependent manner as shown in Fig. 5b. When combined, the inhibitory effect of the two drugs on RV-16 infections was significantly improved, compared to calcitriol alone (Fig. 5c). This effect was rather additive than synergistic. Representative immunofluorescence photographs of the anti-viral effect of EPs® 7630, with and without calcitriol are shown in Fig. 5d.

## **EPs® 7630 increased epithelial cell differentiation**

E-cadherin is an indicator of epithelial cell maturation and is important for the cell-cell contact. Treating BEAS-2B cells with EPs® 7630 over 24 hours significantly increased the expression of E-cadherin in a concentration-dependent manner (Fig. 6a). When inhibiting MAPKs, only PD98059, the inhibitor of Erk1/2, significantly reduced the expression of E-cadherin in BEAS-2B cells treated with EPs® 7630 (Fig. 6b).

## Discussion

EPs® 7630 enhanced the expression of the VDR in human bronchial epithelial cells through Erk1/2 MAPK signalling. Increased VDR expression improved its activation by calcitriol and might thereby enhance the anti-viral effect of EPs® 7630.

The present study showed that EPs® 7630 activates Erk1/2 MAPK signalling pathway, but inhibited p38 MAPK signalling, and had no effect on JNK. The data is in line with earlier studies in human monocytes [19]. Erk1/2 MAPK was the major regulating signalling protein for EPs® 7630 induced expression of the VDR. This effect might occur through proanthocyanidins contained in EPs® 7630 [23]. In human granulosa cells, proanthocyanidins increased the expression of Erk1/2 MAPK and of hormones at higher concentrations [24]. Thereby another proanthocyanidin, resveratrol, increased the expression of the VDR in other conditions [25]. However, other studies reported that proanthocyanidins and their metabolites inhibited Erk1/2, p38 and JNK MAPKs, and thereby reduced the reproduction of the hepatitis B virus [26]. These studies indicate that the effect of proanthocyanidins on different MAPKs might be concentration dependent, or varied with cell type or virus.

The increased expression of the VDR by EPs® 7630 might also account for the improved differentiation of epithelial cells by increasing their barrier function and host defence mechanisms. Low vitamin D levels were associated with increased epithelial de-differentiation and remodelling in COPD patients [27]. In gingival keratinocytes, the VDR action maintained the expression of E-cadherin and thereby reduced tissue remodelling [28]. In cancer cells, VDR activation prevented epithelial mesenchymal transition and thereby maintained the epithelial cell character [29]. However, the contribution of the VDR to epithelial cell differentiation might vary during the course of maturation [30].

In this study, EPs®7630 increased the expression of E-cadherin in non-differentiated bronchial epithelial cells by activating Erk1/2 MAPK. This observation is in line with the above-described EPs® 7630 -induced activation of Erk1/2 MAPK in other cell types [19]. It has to be verified if this maturation in epithelial cells involves the VDR. In line with these results, E-cadherin expression was suppressed by RV-infection in a human bronchial epithelial cell line and resulted in EMT [31]. The authors suggested that this mechanism might contribute to the thickness of the basal membrane in asthma. Furthermore, RV infection delayed the repair of TGF- $\beta$ -induced epithelium injury, which correlated with reduced epithelial cell differentiation, and low E-cadherin expression [32]. Thus, an increase of E-cadherin expression indicates that EPs® 7630 might reduce the thickening of the airway wall in airway infection or inflammation. However, this effect has to be confirmed by clinical studies and in animal models.

## Conclusion

This study indicates that the protective effect of EPs® 7630 against rhinovirus infection might involve the upregulation of the VDR and the improved epithelial cell differentiation.

## List Of Abbreviations

COPD chronic obstructive pulmonary disease

DAPI 4',6-diamidin-2-phenylidol

ERK extracellular signal regulated kinase

ICAM1 intracellular adhesion molecule 1

JNK C-Jun-N-terminal kinase

MAPK mitogen activated protein kinases

p38 protein 38

RV rhinovirus

SNP single nucleotide polymorphism

TGF- $\beta$  transforming growth factor  $\beta$

VDR vitamin D receptor

## Declarations

### Ethics approval:

The study was approved by the local Institutional Ethical Committee (EKBB 05/06).

### Consent for publication:

not applicable

### Availability of data and materials:

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests:

The authors declare that they have no competing interests. The sponsor (Dr. Willmar Schwabe GmbH & Co.KG) had neither influence on the study design nor on the data analysis and interpretation.

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### **Authors' contributions:**

Study concept and design was done by MR and MT. Experiments and data analyses were performed by MR, and QS. Manuscript writing and editing was done by MR, QS, and MT. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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### **Conflict of Interest:**

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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## Table

Due to technical limitations, table 1 docx is only available as a download in the Supplemental Files section.

## Figures

Fig 1.A

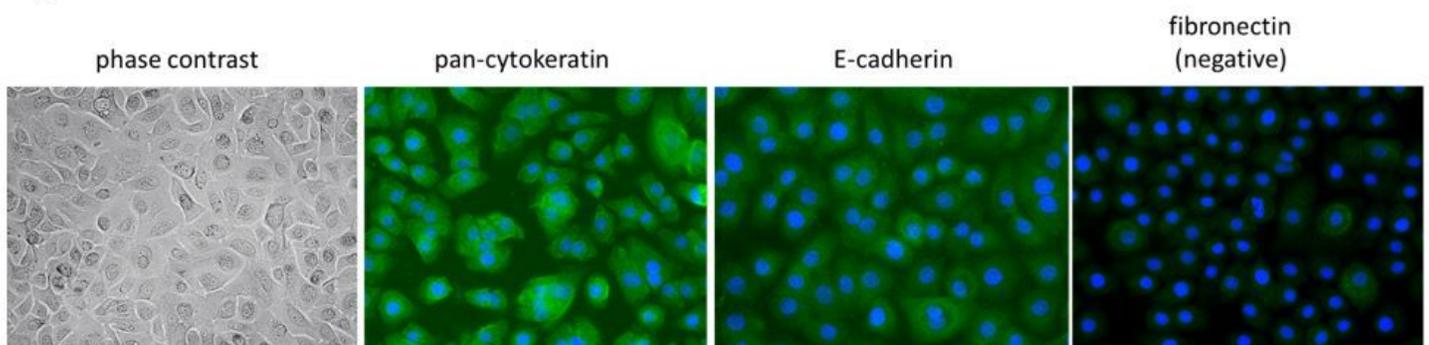
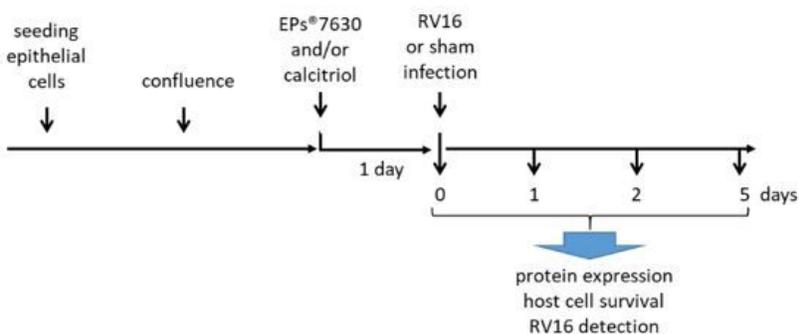


Fig 1.B



## Figure 1

Cell characterisation and treatment. (a) Cell type characterisation by phase contrast microscopy and positive immunofluorescence pan-cytokeratin, E-cadherin, and fibronectin (negative control). (b) Treatment scheme of cells. Confluent epithelial cells were pre-treated with either EPs® 7630 or calcitriol

alone, as well as with the combination before begin infected with 1 MOI RV-16. RNA and protein were isolated over 4 days. Non-infected cells were used to calculate changes of protein expression at the corresponding time points.

Fig 2

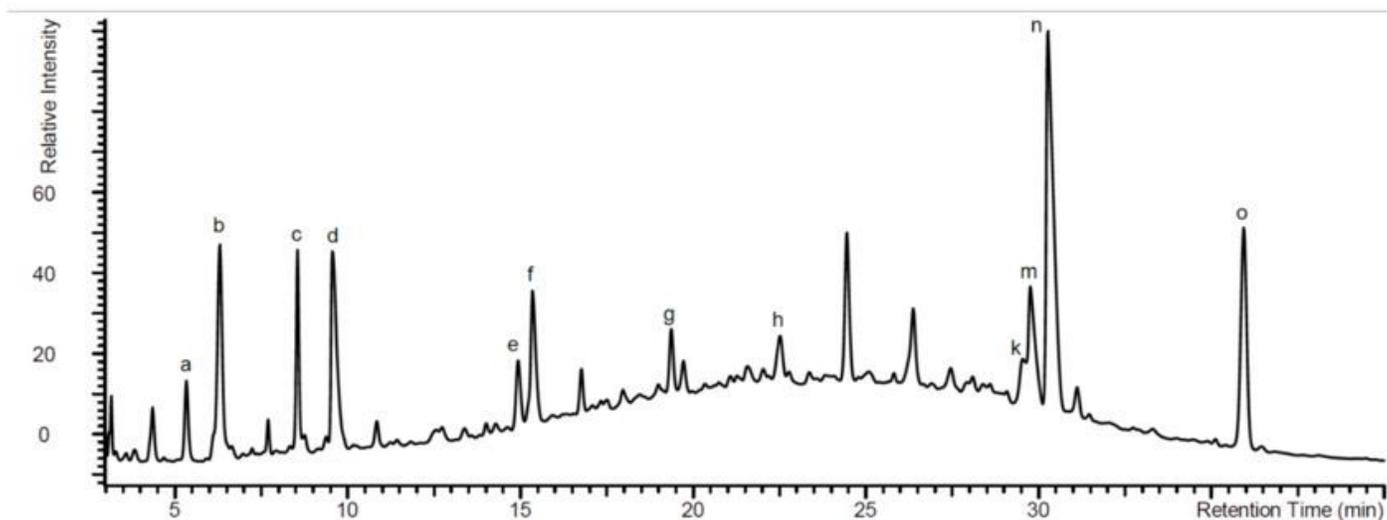
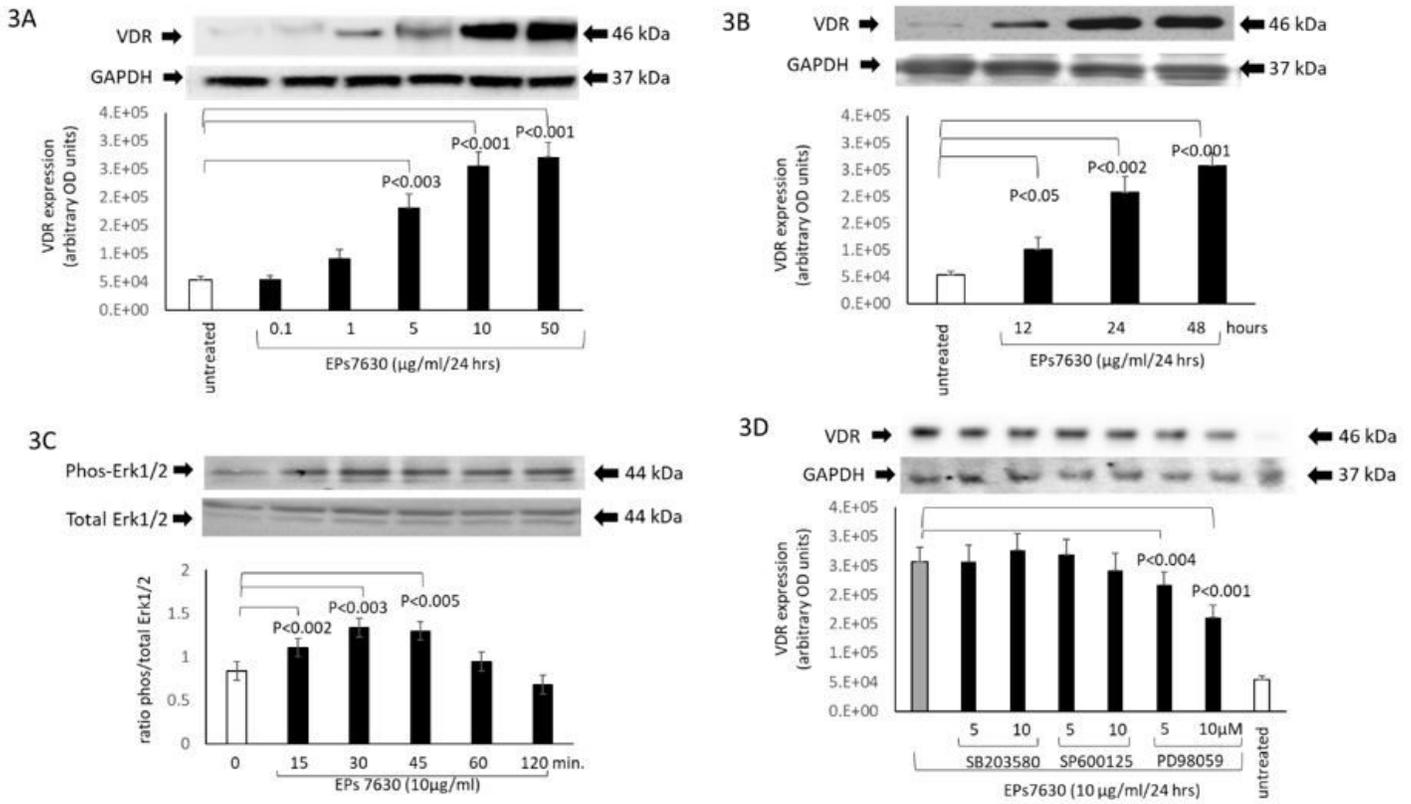


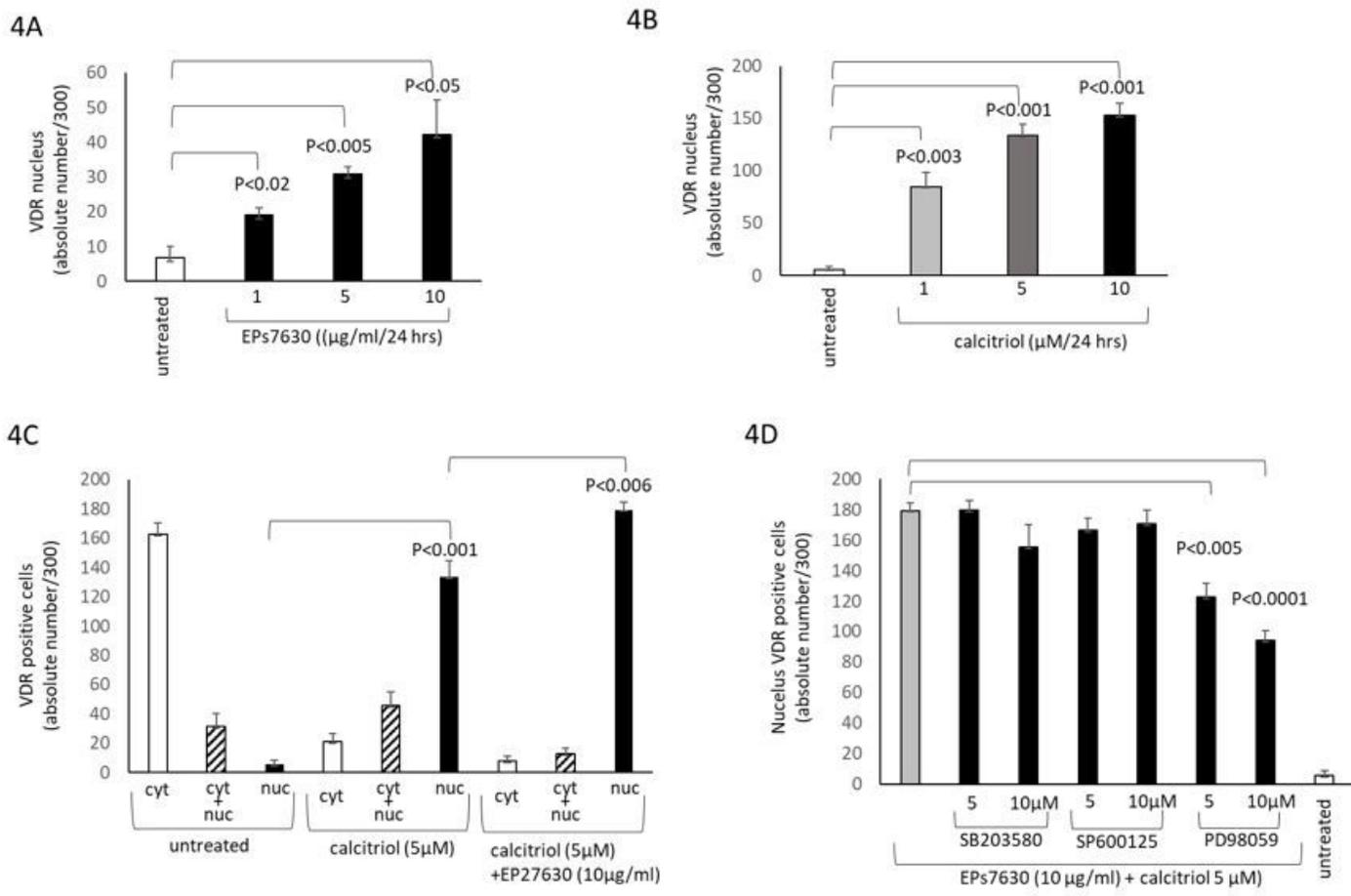
Figure 2

HPLC fingerprint detected by UV at 280nm. The major peaks were assigned by analysis of HRMS data, and are given as follows: (a) adenosine 3',5'-cyclic monophosphate, (b) guanosine 3',5'-cyclic monophosphate, (c) 1-methylguanosine 3',5'-cyclic monophosphate, and (d) benzopyranones, the assigned structures are given in table 1.



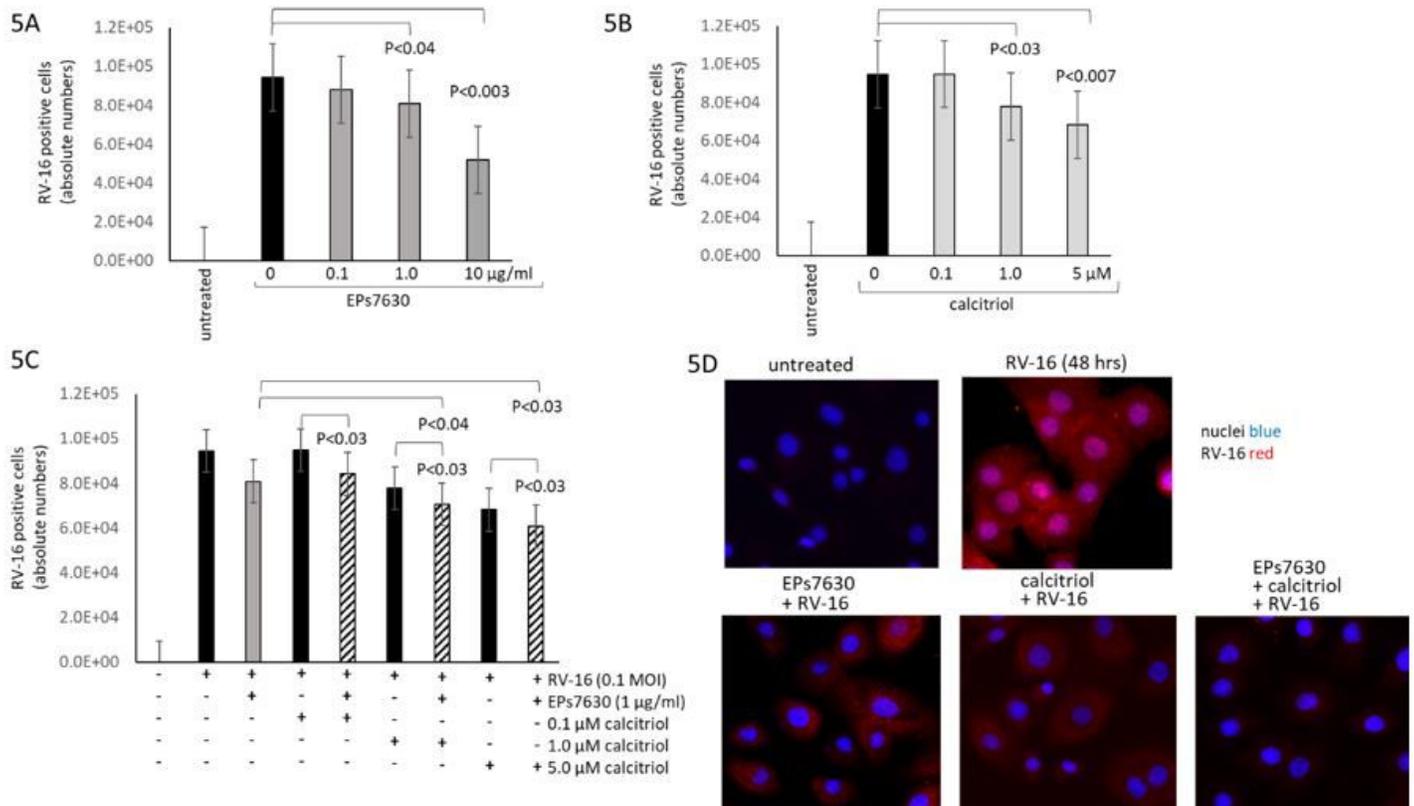
**Figure 3**

EPs® 7630 modifies VDR expression. (a) Representative Western-blot and image analyses of VDR expression in primary bronchial epithelial cells (n=4) stimulated with increasing concentrations of EPs® 7630 over 24 hours. Bars represent mean±SEM. (b) Kinetic of EPs® 7630 induced VDR expression over 48 hours (n=4). (c) The effect of EPs® 7630 on the phosphorylation of Erk1/2 MAPK over 120 minutes. (d) The effect of inhibitors for JNK, p38, and Erk1/2 on EPs® 7630 induced VDR expression (n=4). Representative Western-blot are depicted above the corresponding bars. Similar results were obtained in BEAS-2B cells (triplicates).



**Figure 4**

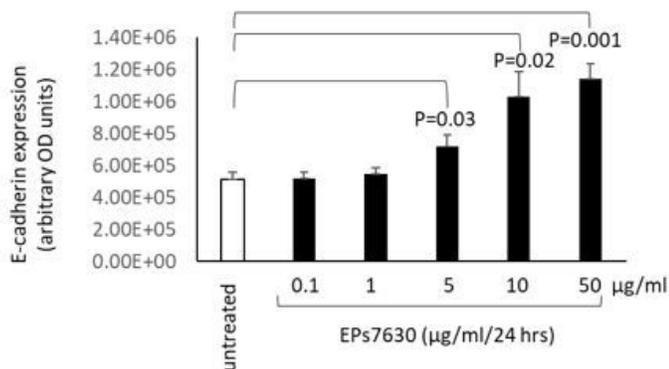
EPs® 7630 and calcitriol activate the VDR. (a) Concentration dependent increase of nuclear VDR by EPs® 7630 over 24 hours in primary human bronchial epithelial cells (n=4). (b) Concentration dependent increase of nuclear VDR by calcitriol over 24 hours (n=4). (c) The effect of combined EPs® 7630 with calcitriol on the ratio of cytosolic versus nuclear VDR accumulation (n=4) over 24 hours. (d) The effect of MAPK inhibitors for JNK (SP600125), p38 (SB203580), and Erk1/2 (PD98059) on EPs® 7630 and calcitriol induced VDR expression (n=3). Bars represent mean $\pm$ SEM. Similar results were obtained in BEAS-2B cells (triplicates).



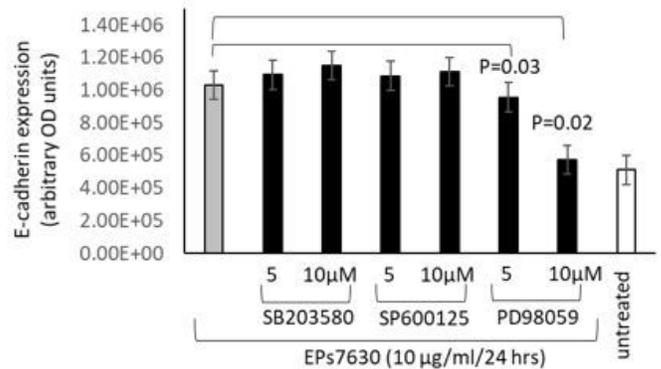
**Figure 5**

The effect of EPs® 7630 and calcitriol on RV-16 infection in BEAS-2B cells. (a) Concentration dependent effect of EPs® 7630 on RV-16 staining in BEAS-2B cells. (b) Concentration dependent effect of calcitriol on RV-16 staining in BEAS-2B cells. (c) The effect of combined EPs® 7630 (fixed concentration) with calcitriol (increasing concentrations) on RV-16 staining on BEAS-2B cells. Bars represent mean±SEM of triplicate experiments. (d) Representative immunofluorescence photographs of RV-16 positive epithelial cells in the presence and absence of EPs® 7630 (1 µg/ml), or calcitriol (1 µM), or the combination of both (EPs® 7630 1 µg/ml + calcitriol 1 µM).

**Fig 6A**



**B**



**Figure 6**

EPs® 7630 increases E-cadherin expression through Erk1/2 MAPK in BEAS-2B cells. (a) Concentration effect of EPs® 7630 on the expression of E-cadherin over 24 hours in BEAS-2B cells. (b) The effect of inhibitors for JNK, p38, and Erk1/2 on EPs® 7630 induced E-cadherin expression. Bars represent mean±SEM of triplicate experiments.

## Supplementary Files

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- [Graphicabstract.jpg](#)
- [Table1.docx](#)