An extract from the medicinal plant *Phyllanthus acidus* and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis

Marisa Sousa, Jiraporn Ousingsawat, Roswitha Seitz, Supaporn Puntheeranurak, Ana Regalado, André Schmidt, Tiago Grego, Chaweewan Jansakul, Margarida D. Amaral, Rainer Schreiber, Karl Kunzelmann

JO,RS, SP,RS,KK Institut für Physiologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany

MS,AR,AS,TG,MA Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisboa and Centre of Human Genetics, National Institute of Health Dr. Ricardo Jorge, Lisboa, Portugal

CJ Department of Physiology, Prince of Songkla University, Hat-Yai, Thailand
**Running Title:** Cellular effects of *Phyllanthus acidus*

**Corresponding author:**
Prof. Dr. Karl Kunzelmann
Institut für Physiologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany
Tel: +49 (0) 941 943 4302, Fax: +49 (0) 941 943 4315 email: uqkkunze@mailbox.uq.edu.au

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**List of non-standard abbreviations:**
ATP = adenosine 5′-triphosphate; BAPTA-AM = 1,2-bis (2 aminophenoxy) ethan-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; BIM = bisindolylmaleimide; cAMP = cyclic adenosine monophosphate; CFTR = cystic fibrosis transmembrane conductance regulator; DIDS = 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC-PX = 1,3-Dipropyl-8-cyclopentylxanthine IBMX = 3-isobutyl-1-methylxanthine; MRS2179 = 2′-Deoxy-N6-methyl adenosine 3′,5′-diphosphate; 8-SPT = 8-sulfophenyltheophylline; PPADS = pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; UDP = uridine 5′-diphosphate; U73122 = 1-[6-((17b-3-Methoxyestra-1,3,5(10)-triien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.
Abstract

According to previous reports flavonoids and nutraceuticals correct defective electrolyte transport in cystic fibrosis (CF) airways. Traditional medicinal plants from China and Thailand contain phyto-flavonoids and other bioactive compounds. We examined herbal extracts of the common Thai medicinal Euphorbiaceous plant *Phyllanthus acidus* (*P. acidus*) for their potential effects on epithelial transport. Functional assays by Ussing chamber, patch-clamping, double electrode voltage-clamp and Ca\(^{2+}\) imaging demonstrate activation of Cl\(^{-}\) secretion and inhibition of Na\(^{+}\) absorption by *P. acidus*. No cytotoxic effects of *P. acidus* could be detected. Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl\(^{-}\) secretion by increasing both intracellular Ca\(^{2+}\) and cAMP. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid. Additional experiments in human airway cells and CFTR expressing BHK cells and *Xenopus* oocytes confirm the results obtained in native tissues. Cl\(^{-}\) secretion was also induced in tracheas of CF mice homozygous for F508del-CFTR and in F508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects defective electrolyte transport in CF airways by parallel mechanisms including i) increasing the intracellular levels of second messengers cAMP and Ca\(^{2+}\), thereby activating Ca\(^{2+}\) - dependent Cl\(^{-}\) channels and residual CFTR-Cl\(^{-}\) conductance; ii) stimulating basolateral K\(^{+}\) channels; iii) redistributing cellular localization of CFTR; iii) directly activating CFTR; and v) inhibiting ENaC through activation of CFTR. These combinatorial effects on epithelial transport may provide a novel complementary nutraceutical treatment for the CF lung disease.
Introduction

Cystic fibrosis (CF) is an autosomal recessive disease with high frequency among the Caucasian population. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. One mutation alone, F508del-CFTR is present in at least one allele in approximately 90% of CF patients (Bobadilla et al., 2002). CF is characterized by deficient Cl⁻ transport and enhanced airway Na⁺ absorption, mediated by epithelial Na⁺ channels (ENaC) along with other abnormalities in ion transport. Pharmacological interventions attempt to correct defective ion transport among other pulmonary phenotypes. Recent strategies make use of natural food components because of their ready accessibility and low toxicity (deCarvalho et al., 2002; Bjarnsholt et al., 2005; Egan et al., 2004). These compounds act in different ways, such as correcting the trafficking defect of mutant CFTR or potentiating residual CFTR activity (Moran & Zegarra-Moran, 2005; Kunzelmann & Mall, 2003; Van Goor et al., 2006).

Medicinal plants have been the basis for traditional pharmacology for many centuries. Around 500 different herb-based medicines have been counted in Thailand. They are used for the treatment of a variety of diseases such as cardiovascular failure, diabetes and cancer. In addition their diuretic, anti-inflammatory, anti-asthmatic and anti-hypertensive properties are exploited and some are used as dietary supplements and in sport medicine (Mueller-Oerlinghausen et al., 1971; Panthong et al., 1986). An increasing number of Thai medicinal plants are taken to laboratories for purification and analysis. Through this approach, a number of novel compounds have been identified (Kanchanapoom et al., 2001; Wolfender et al., 2001). The extract of the traditional medicinal plant *Phyllanthus acidus* (*P. acidus*; Mayom) has been shown to be enriched with adenosine (Fig. 1) (Cohen et al., 1997). Therefore, we have assessed the effects of this extract on the adenosine receptor system in mouse airways and in human airway epithelial cells. In particular, effects on A₁ and A₂B receptors were examined using pharmacological inhibitors 8-SPT, alloxazine, and DPC-PX. Stimulation of
these receptors has been demonstrated to activate both Ca^{2+} dependent and cAMP (CFTR) regulated Cl^{-} channels and to affect the epithelial Na^{+} channel ENaC, while other were unable to detect effects of adenosine on Cl^{-} secretion in CF tissues (Clancy et al., 1999). Apart from adenosine, *P. acidus* also contains other components, which are likely to affect electrolyte transport in the airways, such as the flavonoid kaempferol and 2,3-dihydroxybenzoic acid (DHBA) (Li & Wang, 2004; Illek & Fischer, 1998). We compared the effects of *P. acidus* with the effect of commercially purchased adenosine, kaempferol and DHBA and dissected out the underlying signaling pathways and the conductances affected. The present data indicate that extracts from *P. acidus* activate electrolyte secretion in epithelial tissues by means of intracellular second messengers and by directly increasing membrane expression and activity of ion channels. Thus, medicinal plant extracts from *Phyllanthus acidus* may represent a novel and effective tool to correct defective electrolyte transport in CF.
Methods

Preparation of the extract: Leaves of *Phyllanthus acidus* were collected in Songkhla Province, Thailand. Fresh leaves were simmered at 60° C for 3 h in water. The clear solution of the extract was simmered at 50 °C to reduce its volume to 50%, followed by partition extraction with water-saturated n-butanol. The n-butanol phase was collected and evaporated *in vacuo* and lyophilized. The extract was further purified by column chromatography as described previously (Jansakul et al., 1999). Identification was made by Prof. P. Sirirugsa, Dept. of Biology, Faculty of Science, Prince of Songkla University and Prof. K. Hostettmann, Laboratoire de Pharmacognosie et Phytochimie, Ecole de Pharmacie, Universite de Geneve, Geneve, Switzerland. The amounts of adenosine, kaempferol, and hypogallic acid (dihydrobenzoic acid, DHBA) present in the extract were (mg) 355.9, 697.4, and 1004.6, respectively. Multiple lots of the extract were prepared and used for experiments. The extract was used at concentrations of 1 – 100 µg/ml.

Ussing chamber recordings: Tracheas were removed from normal mice (C57BL/6, Charles Rivers, Germany, animal facility University of Queensland) and mice homozygous for F508del-CFTR mice (Prof. Dr. B. Scholte, Institute of Cell Biology and Genetics, The Erasmus University Rotterdam) after sacrificing the animals by cervical dislocation. After removing connective tissues, tracheas were opened by a longitudinal cut. Tissues were put immediately into an ice cold buffer solution of the following composition (mmol/l): NaCl 145, KCI 3.8, D-glucose 5, MgCl2 1, HEPES 5, Ca-gluconate 1.3. The tissues were mounted into a perfused micro Ussing chamber with a circular aperture of 0.95 mm². Apical and basolateral surfaces of the epithelium were perfused continuously at a rate of 5 - 10 ml/min (chamber volume 2 ml). The bath solution contained (mmol/l): NaCl, 145; KH₂PO₄, 0.4; K₂HPO₄, 1.6; D-glucose, 5; MgCl₂, 1; Heps 5; and Ca-gluconate, 1.3. pH was adjusted to 7.4 and all experiments were carried out at 37 °C under open circuit conditions. Transepithelial resistance (Rₑₑₑ) was determined by applying short (1 s) current pulses (I = 0.5 µA) and the corresponding changes in Vₑₑₑ (Vₑₑₑ) and basal Vₑₑₑ were
recorded continuously. Values for the transepithelial voltage ($V_{te}$) were referred to the serosal side of the epithelium. The equivalent short-circuit current ($I_{sc}$) was calculated according to Ohm’s law from $V_{te}$ and $R_{te}$ ($I_{sc} = V_{te} / R_{te}$).

**Cell culture:** Human bronchial epithelial cells (16HBE14o-) and human CF airway epithelial cells homozygous for F508del-CFTR (CFBE) were kindly provided by Prof. Dr. D.C. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA, USA) and were grown at 37 °C in modified Eagle culture media containing (in mmol/l): D-glucose 5, L-glutamine 2, 100 g/l fetal calf serum, 100 mg/l penicillin / streptomycin in an atmosphere of 5% CO₂ and 95% O₂. Transfected Baby Hamster Kidney (BHK) cells were grown in the presence of 500 µM methotrexate.

**Patch-Clamp:** Cells were mounted on the stage of an inverted microscope (IM35, Zeiss, Oberkochen, Germany) and kept at 37°C. The bath was continuously perfused with Ringer solution at a rate of 5 - 10 ml/min. Patch-clamp experiments were performed in fast whole-cell configuration. The patch pipettes had an input resistance of 2 - 4 MΩ when filled with a solution containing (mmol/l) KCl 30, K-gluconate 95, NaH₂PO₄ 1.2, Na₂HPO₄ 4.8, EGTA 1, CaCl₂ 0.726, MgCl₂ 1.034, D-glucose 5, ATP 1 (32 Cl). The pH was adjusted to 7.2, the Ca²⁺ activity was 0.1 µmol/l. The access conductance was monitored continuously and was larger than 50 nS. Currents (voltage-clamp) and voltages (current-clamp) were recorded using a patch-clamp amplifier (EPC 7, List Medical Electronic, Darmstadt, Germany). Data were continuously stored on a computer hard disc. At regular intervals, membrane voltages ($V_c$) were clamped in steps of 10 mV from -100 mV to +40 mV. Conductances (G) were calculated according to Ohm’s law.
Intracellular Ca²⁺ concentration: For measurements of the intracellular Ca²⁺ concentration, cells were perfused with Ringer solution (mmol/l: NaCl 145; KH₂PO₄ 0.4; K₂HPO₄ 1.6; Glucose 5; MgCl₂ 1; Ca²⁺-Gluconat 1.3) at 37°C. Cells were loaded with 5 µM Fura-2 AM (Molecular Probes) in OptiMEM (Invitrogen) with 0.02% pluronic (Molecular Probes) for 1h at RT. Fura-2 was excited at 340/380 nm, and emission was recorded between 470 and 550 nm using a CCD-camera (CoolSnap HQ, Visitron Systems, Germany). Fluorescence was measured continuously using an inverted microscope IMT-2 (Olympus, Germany) and a high speed polychromator system (VisiChrome, Visitron Systems, Germany). Experiments were controlled and analyzed using the software package Meta-Fluor (Universal Imaging, USA). All optical filters and dichroic mirrors were from AHF (Tübingen, Germany).

cRNAs for CFTR, ENaC subunits and P2Y₂: cDNAs encoding rat α,β,γ ENaC (kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland), wt-CFTR and F508del-CFTR and the purinergic P2Y₂ receptor were linearized in pBluescript with NotI or MluI, and in vitro transcribed using T7, T3 or SP6 promotor and polymerase (Promega, USA). After isolation from adult female Xenopus laevis frogs (Xenopus express, South Africa), oocytes were dispersed and defolliculated by a 45 min treatment with collagenase (type A, Boehringer, Germany). Subsequently, oocytes were rinsed and kept at 18 °C in ND96-buffer (in mmol/l): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55), supplemented with theophylline (0.5 mmol/l) and gentamicin (5 mg/l).

Double electrode voltage-clamp: Oocytes were injected with cRNA (1 - 10 ng) after dissolving in 47 nl double-distilled water (Nanoliter Injector WPI, Germany). Water injected oocytes served as controls. 2 - 4 days after injection, oocytes were impaled with two electrodes (Clark Instruments) which had a resistance of < 1 MΩ when filled with 2.7 mol/l KCl. Using two bath electrodes and a virtual-ground head stage, the voltage drop across R₁
was effectively zero. Membrane currents were measured by voltage-clamping of the oocytes (Warner oocyte clamp amplifier OC725C) in intervals from -90 to +30 mV, in steps of 10 mV, each 1 s. Amiloride-sensitive conductances ($G_{Amil}$) were used in the present report to express the amount of whole-cell conductance that is inhibited by 10 µmol/l amiloride. During the whole experiment, the bath was continuously perfused at a rate of 5 -10 ml/min. All experiments were conducted at room temperature (22 °C).

**Viability assay and Western blot:** Twenty-four hours after seeding BHK cells, culture medium was changed, methotrexate removed and *Phyllanthus* extract was added. Forty-eight hours later, cells were collected, washed once with PBS, re-suspended in bovine serum albumin (BSA) solution (0.5 mg/ml in PBS) and stained with calcein AM and ethidium homodimer-1 (Live/Dead® Viability/Cytotoxicity kit, Molecular Probes-Invitrogen, Carlsbad, CA, USA). Membrane-permeant calcein-AM (excitation/emission = 494/517 nm) is cleaved by esterases in living cells to yield cytoplasmic green fluorescence while membrane-impermeant ethidium homodimer-1 (ex/em = 528/617 nm) labels nucleic acids of membrane-compromised cells with red fluorescence. Flow cytometry analysis was carried out at excitation of 488 nm in a FACScalibur flow cytometer, (BD Biosciences, Bedford, MA, USA). For Western blot cells were lysed after treatment, 30-50 µg total protein was separated by SDS-PAGE and transferred onto nitrocellulose filters. Filters were probed with the anti-CFTR monoclonal antibody M3A7 (Chemicon, cat no. MAB3480, Temecula, CA, USA).

**Pulse-chase and immunoprecipitation experiments:** After treatment cells were starved for 30 min in methionine-free modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA, USA). Cells were then pulse-labeled for 30 min in the same medium with 150 µCi/ml $[^{35}S]$-methionine (ICN, Costa Mesa, CA, USA) as described previously (Farinha & Amaral, 2005).
After chasing for 0, 0.5, 1, 2 and 3 h in modified Eagle’s medium supplemented with fetal bovine serum (Gibco) and 1 mM non-radioactive methionine, cells were lysed in 1 ml RIPA buffer and immunoprecipitated. Briefly, samples were centrifuged at 14,000 g for 30 min and the supernatant was incubated overnight at 4°C with 1.5 µg anti-CFTR M3A7 antibody. Then, 25 µg Protein-G agarose beads (Roche, Basel, Switzerland) were added for a further 4 h at 4°C, beads were washed 4 times using 1 ml RIPA buffer and protein was eluted for 1 h at room temperature after addition of 80 µl cracking buffer: 0.5 mM dithiothreitol (Sigma); 0.001% (w/v) bromophenol blue (Merck, Darmstadt, Germany); 5% (v/v) glycerol (Merck); 1.5% (w/v) SDS; and 31.25 mM Tris, pH 6.8. Samples were electrophoretically separated on 7% (w/v) polyacrylamide gels. Quantification of the core-glycosylated form of wt- or F508del-CFTR (band B) at a given chase time \( t \) was estimated as the percentage given by the ratio of the amount of the band B at that chase time (\( P \)) over its amount at chase time zero (\( P_o \)), i.e., at the end of the pulse period. Similarly, maturation efficiency, was determined by the appearance of the fully-glycosylated form (band C) also as a percentage given by the ratio of \( P \), the amount of band C at time \( t \), over \( P_o \), the amount of band B at the start of the chase (\( t = 0 \)).

**Iodide efflux assay:** Iodide efflux experiments were performed by a standard protocol using an ion-selective electrode. Briefly, cells were incubated for 1 h in loading buffer containing (mM): 136 NaI; 3 KNO₃; 2 Ca(NO₃)₂; 11 glucose; and 20 HEPES, adjusted to pH 7.4 with NaOH. Cells were thoroughly washed with efflux buffer (136 mM 6 NaNO₃ replacing NaI in the loading buffer) to remove extracellular iodide and then equilibrated in 2.5 ml efflux buffer for 1 min. The efflux buffer was changed at 1 min intervals. Four minutes after anion substitution, cells were exposed to 10 µM forskolin and 50 µM genistein for 4 min. The amount of iodide in each 2.5 ml sample of efflux buffer was determined using an iodide-
selective electrode (Mettler Toledo, Columbus, OH, USA). Cell loading and measurements were performed at room temperature.

**Materials and statistical analysis:** All used compounds were of highest available grade of purity. 3-isobutyl-1-methylxanthine (IBMX), forskolin, ATP, adenosine, UDP, PPADS, Suramin, DIDS, BAPTA-AM, amiloride, carbachol, CPA, kaempferol, 8-SPT, dihydrobenzoic acid, alloxazine, DPC-PX, MRS2179 were all from Sigma (Deisenhofen, Germany). U-73122 was from Calbiochem (Nottingham, UK). Students t test p values <0.05 were accepted to indicate statistical significance (*).
Results

Activation of ion transport by *P. acidus* extract via stimulation of adenosine receptors in mouse trachea: A major component of *P. acidus* is adenosine, which is known to activate ion transport in airway epithelial cells. We therefore compared the effects of adenosine and the *P. acidus* extract on electrolyte transport in mouse airways. Both adenosine (100 µM) and *P. acidus* (100 µg/ml) induced a transient and steady-state Cl⁻ secretion, when applied to the luminal side of the epithelium (Fig. 1A). The effects of both adenosine and *P. acidus* were dose-dependent and did not saturate in the concentration range examined (Fig. 1B,C). The non-selective inhibitor of adenosine receptors 8-SPT (10 µm) completely inhibited the effects of adenosine on ion transport. The secretory response of *P. acidus* was largely reduced and only part of the transient response remained (Fig. 1A,D,E). The transient and steady-state responses are likely due to activation of both Ca²⁺- and cAMP-mediated Cl⁻ secretion. Mouse trachea is dominated by Ca²⁺-activated Cl⁻ secretion, but also contains cAMP-activated CFTR Cl⁻ channels. Stimulation of A₁ adenosine receptors increases cytosolic Ca²⁺, while activation of A₂B receptors enhances intracellular cAMP. The A₁ receptor antagonist DPC-PX (200 nM) inhibited both transient and steady-state Iₘ activated by *P. acidus* extract, while the A₂B receptor antagonist alloxazine (10 µM) blocked only steady-state responses, e.g. cAMP-mediated Cl⁻ secretion (Fig. 1F,G). Moreover, Cl⁻ secretion was also induced by *P. acidus* in mouse colonic (ΔIₑₛₜ = 61 ± 7.3 µA/cm²; n = 6) and nasal (ΔIₑₛₜ = 151 ± 17.3 µA/cm²; n = 6) native epithelia.

We also examined possible effects of the *P. acidus* extract on other purinergic receptors. Stimulation of luminal P2Y₂ receptors with ATP or UTP (both 100 µM), or inhibition of P2Y receptors with PPADS or suramin (both 100 µmol/l) did not interfere with the ability of *P. acidus* to induce Cl⁻ secretion. Moreover, a role of P2Y₁ receptors in the effects of *P. acidus* on epithelial transport was unlikely, since *P. acidus* had similar effects in the presence of the
specific P2Y₁ agonist MRS2179 (10 µmol/l) (data not shown). Taken together, our data indicate that activation of A₁ and A₂B receptors contributes substantially to the observed induction of Cl⁻ secretion by *P. acidus* extract.

**Activation of Ca²⁺ and cAMP dependent Cl⁻ secretion by *P. acidus* in mouse trachea:**
Luminal stimulation of mouse and human airways with adenosine, increases intracellular cAMP and leads to a steady CFTR – dependent Cl⁻ secretion (Huang et al., 2001). We found that the transient I_{sc} and a substantial part of the steady state I_{sc} activated by adenosine (100 µM) in mouse airways is inhibited by niflumic acid (NFA, 10 µM), an inhibitor of Ca²⁺ activated Cl⁻ channels (Fig. 2A,B). Thus, adenosine activates both cAMP and Ca²⁺ dependent Cl⁻ secretion. Similar to adenosine, both transient and steady secretion induced by *P. acidus* were inhibited by NFA and DIDS, another blocker of Ca²⁺ activated Cl⁻ channels (Fig. 2C,D).

We further examined whether *P. acidus* also activates cAMP-dependent CFTR Cl⁻ channels. To that end, we pre-stimulated mouse airways with IBMX (100 µM) and forskolin (2 µM) and found that Cl⁻ secretion induced by *P. acidus* was significantly reduced. Moreover, application of the CFTR inhibitor glibenclamide also reduced steady state I_{sc} induced by *P. acidus* (Fig. 2F). Thus, *P. acidus* activates two luminal Cl⁻ channels, CFTR and a Ca²⁺-activated Cl⁻ channel of unknown molecular identity. The effects of *P. acidus* were not limited to mouse trachea, since *P. acidus* also activated I_{sc} of 161 ± 17.5 µA/cm² (n = 6) and 48 ± 8.7 µA/cm² (n = 6) when applied to mouse nasal epithelium and proximal colon, respectively.

We further demonstrated that transient Cl⁻ secretion induced by *P. acidus* is due to an increase in intracellular Ca²⁺. Thus we emptied endoplasmic reticulum (ER) Ca²⁺ stores with 10 µmol/l cyclopiazonic acid (CPA), which induced a transient I_{sc}. In the presence of CPA, the transient Cl⁻ secretion induced by 100 µg/ml *P. acidus* was largely reduced (Fig. 3A,B). Activation of
Cl− secretion was also reduced when intracellular Ca2+ was chelated with 10 µmol/l BAPTA-AM, or when phospholipase C was inhibited using U73122 (10 µmol/l) (Fig. 3C,D). Ca2+ signaling in airways is compartmentalized, i.e. basolateral stimulation of Cl− secretion by carbachol (CCH, 100 µM) activates Cl− secretion through activation of basolateral K+ channels, rather than luminal Ca2+ activated Cl− channels (Huang et al., 2001). Thus, luminal application of P. acidus extract did not interfere with stimulation of basolateral M3 receptors by carbachol (Fig. 3E,F). Moreover, basolateral application of P. acidus induced smaller effects on ion transport, when compared to luminal application (Fig. 3G).

Activation of Cl− secretion by kaempferol and DHBA: P. acidus extract contains the flavonoid kaempferol and hypogallic acid (dihydrobenzoic acid; DHBA) (Li & Wang, 2004). Both compounds induced a dose dependent Cl− secretion, albeit smaller than that activated by adenosine (Fig. 5). Substantial amounts of the Cl− secretion induced by kaempferol and DHBA were inhibited by the Cl− channel blocker NFA. We then asked, whether the effects of P. acidus extract on epithelial ion transport could be reproduced by a mixture of the isolated components adenosine, kaempferol and DHBA. As shown in Fig. 5, the mixture demonstrated similar, albeit larger effects than those produced by the P. acidus extract. Thus the effects of P. acidus are reproduced by a defined mixture of isolated components.

P. acidus activates Cl− secretion in human airway epithelial cells and overexpressing oocytes: We obtained further evidence for the activation of Cl− currents by P. acidus through whole-cell patch-clamp experiments with human airway epithelial cells (16HBE). The cells were voltage-clamped and exposed to 10 µg/ml P. acidus, which activated a whole-cell current. The effects of P. acidus extract were compared with the well known secretagogue ATP (100 µM) (Fig. 6A,B). Whole-cell conductances activated by either P. acidus or ATP were inhibited by removal of Cl− from the extracellular bath solution (data not shown). Intracellular
Ca^{2+} concentrations were directly measured in 16HBE cells, using the Ca^{2+}-sensitive dye Fura-2. As shown in Fig. 6C,D both ATP and *P. acidus* enhanced intracellular Ca^{2+} concentrations.

*P. acidus* also induced Cl⁻ secretion in overexpressing cells. Oocytes from *Xenopus laevis* endogenously express Ca^{2+}-activated Cl⁻ channels. As shown in Fig. 7A, *P. acidus* (100 µg/ml) induced a transient Cl⁻ secretion, probably due to the activation of endogenous Ca^{2+}-activated Cl⁻ channels in *Xenopus* oocytes. DIDS (100 µM) completely suppressed current activation by *P. acidus* (Fig. 7A,B). In contrast to non-injected oocytes, where *P. acidus* only transiently activated Cl⁻ secretion, oocytes overexpressing wild-type (wt) CFTR exhibited both transient and steady-state Cl⁻ currents, when exposed to *P. acidus* (Fig. 7C). Current activation was significant when compared to the effects of the phosphodiesterase inhibitor IBMX (1 mM), which increases intracellular cAMP (Fig. 7C,D). Thus, in *Xenopus* oocytes *P. acidus* activates endogenous Ca^{2+}-activated Cl⁻ channels and overexpressed CFTR Cl⁻ channels. Numerous reports have demonstrated inhibition of ENaC during activation of CFTR. In fact, lack of ENaC inhibition by mutant CFTR has been proposed as a mechanism for enhanced Na⁺ absorption in CF (Stutts et al., 1995). We thus coexpressed CFTR and the epithelial Na⁺ channel ENaC in *Xenopus* oocytes and found amiloridesensitive Na⁺ currents under control conditions (Fig. 7E). Activation of Ca^{2+}-dependent and CFTR Cl⁻ currents by *P. acidus* (100 µg/ml) inhibited amiloride sensitive Na⁺ channels (Fig. 7E,F). Moreover, inhibition of ENaC was not observed by *P. acidus* when CFTR was inhibited by the specific inhibitor 172, thus showing that ENaC currents were not directly inhibited by *P. acidus*. In other words, *P. acidus* had no direct effect on Na⁺ currents in the absence of CFTR activity.

We further examined if *P. acidus* also activates mutant CFTR, which carries the most common mutation F508del. Stimulation of F508del-CFTR with IBMX (1 mM) and forskolin
(2 µM) activated a small but significant Cl− current in *Xenopus* oocytes. Activation of F508del-CFTR currents was significantly augmented after incubation of the oocytes with *P. acidus* (100 µg/ml) for 24 h. This was not observed when oocytes were incubated in control Ringer solution (Fig. 7G-I). Moreover, in preliminary experiments with F508del-CFTR overexpressing BHK cells, F508del-CFTR was membrane-rescued by low temperature (26°C). In these cells *P. acidus* induced a large I− efflux, suggesting activation of mutant CFTR by *P. acidus* (data not show). This result suggests that membrane-rescued F508del-CFTR can be activated by *P. acidus*. Moreover, *P. acidus* may increase membrane expression by redistribution of F508del-CFTR from intracellular compartments (Lim et al., 2004).

*P. acidus is not toxic for mammalian cells and acts as a potentiator of CFTR:* Using a viability/cytotoxicity test (see Methods) we examined whether *P. acidus* exerts any toxic effect on mammalian cells (Fig.8). Following 48 h incubation with *P. acidus* in the 50-200 µg/ml concentration range, BHK cells stably expressing wt-CFTR or F508del-CFTR were analyzed by flow cytometry. Graphs represent bivariate frequency distributions of red-fluorescent (585 nm) ethidium homodimer-1-stained dead cell population (y axis, arbitrary units) over green-fluorescent (530 nm) calcein-stained live cell population (x axis, arbitrary units). The fraction of live cells was larger than 95% under all conditions, indicating that *P. acidus* is not toxic for mammalian cells, up to a concentration of 200 µg/ml (Fig. 8A,B). The cells continued to divide normally in the presence of the extract (data not shown).

We examined steady-state levels of expression of wt-CFTR and F508del-CFTR in BHK cells by Western blot and did not find any significant changes following incubation with *P. acidus* extract up to a concentration of 200 µg/ml. Only wt-CFTR levels were slightly increased after 48h incubation with *P. acidus*. In metabolic pulse-chase experiments, we examined the turnover rate of the core-glycosylated form (band B) of wt-CFTR and F508del-CFTR, which
was not affected by 100 µg/ml *P. acidus* (Fig.8B and 8D). Moreover, *P. acidus* did not alter the efficiency of CFTR maturation, i.e. conversion of band B to band C when processing efficiency was assessed by densitometry (Fig.8C). In order to assess a potentiator effect of *P. acidus* on wt-CFTR when expressed in mammalian cells, we performed iodide efflux assays in BHK cells stably expressing F508del-CFTR, which was membrane rescued by low temperature (26°C) (Denning et al., 1992). In fact, the forskolin / genistein activated iodide efflux was enhanced after acute application of *P. acidus* (Fig. 8D). Interestingly, the delay of activation, which is typically observed for F508del-CFTR, was corrected by *P. acidus* (Fig. 8D), suggesting a correction of the gating defect of this most common CFTR-mutant.

We further examined, if *P. acidus* and its isolated components are able to activate Cl⁻ secretion in tracheas of CF mice homozygous for the most common CFTR- mutation F508del. Ussing chamber recordings demonstrate that adenosine, a mix of adenosine, kaempferol and DHBA, and *P. acidus* activate Cl⁻ secretion in CF tracheas (Fig. 9A,B). These results were further confirmed in patch clamp experiments with human airway epithelial cells (CFBE), homozygous for F508del-CFTR. Both, a mix of the individual components (each at 100 µM) as well as *P. acidus* (100 µg/ml) activated a whole cell Cl⁻ current and depolarized membrane voltages (Fig. 9C,D). Taken together, *P. acidus* exhibits multiple pro-secretory effects on epithelial electrolyte transport in normal and CF airways. The results strongly suggest that *P. acidus* acts as a potentiator of wtCFTR and F508del-CFTR. It may therefore represent a novel therapeutic strategy to circumvent the defect in electrolyte transport observed in CF epithelial tissues (Fig. 10).
Discussion

Complementary treatment of the CF lung disease by nutraceuticals: Defective electrolyte transport is a major cause of severe lung disease in cystic fibrosis. Various therapeutic interventions have been developed to counteract abnormal ion transport caused by a lack of Cl⁻ secretion and hyperabsorption of electrolyte. Pharmacological strategies have been reviewed in several recent articles (Kerem, 2005; Boucher, 2004; Kunzelmann & Mall, 2003). A major step forward in identifying new therapeutic small molecules is high-throughput quantitative screening for CFTR activators (potentiators) and correctors (Ma et al., 2002; Pedemonte et al., 2005; Van Goor et al., 2006). Although bioactive molecules are discovered by this procedure, it is nevertheless both time and cost-intensive and may typically require 7 years or longer for analysis of the mechanism of action, evaluation, pre-clinical and clinical testing before FDA approval is obtained. Alternatively, compounds which have already received FDA approval, such as phenyl butyrate or aminoglycosides, or common food components and plant constituents, could be tested for their potential therapeutic benefits, as they can be much faster in the clinical setting.

Phytoflavonoids such as genistein have been extensively tested and have been proven to activate CFTR (Illek & Fischer, 1998; Mall et al., 2000; Suaud et al., 2002; Suaud et al., 2001; Hwang et al., 1997). Flavonoids also restore functional interactions between mutant F508del-CFTR or G551-CFTR and ENaC (Suaud et al., 2001). Genistein is currently under investigation in a phase I pilot study in co-administration with phenylbutyrate. Also other dietary flavonols such as quercetin and kaempferol have been identified as activators of Cl⁻ secretion (Cermak et al., 1998). The effects of the spice curcumin have been inconsistent among different groups who tested this compound, but are nevertheless currently under examination in a phase I clinical trial (Egan et al., 2004; Berger et al., 2004; Song et al., 2004). Another study has demonstrated opening of CFTR Cl⁻ channels by vitamin C (L-ascorbate) (Fischer et al., 2004). Vitamin C was identified as a biological regulator of CFTR-mediated Cl⁻ secretion. Although citrus limonoids were found to increase Cl⁻ conductance in
epithelial cells to an extent comparable to genistein (deCarvalho et al., 2002), we were unable to detect significant effects of L-ascorbate in mouse trachea (data not shown). This is probably due to the relatively low levels of CFTR expression in this tissue.

**Constituents of the herbal plant P. acidus enhance electrolyte secretion:** Plant extracts from *P. acidus* contain various bioactive compounds, such as adenosine, kaempferol and hypogallic acid. The effects of these compounds include: i) increasing the intracellular second messengers cAMP and Ca\(^{2+}\) and thereby activating CFTR and Ca\(^{2+}\)-dependent Cl\(^{-}\) channels; ii) activating CFTR directly, as demonstrated for flavonoids; iii) increasing membrane expression of CFTR; iv) enhancing the driving force for luminal Cl\(^{-}\) exit by activating basolateral K\(^{+}\) channels; v) reducing ENaC activity through activation of CFTR, thereby reducing NaCl absorption and preventing dehydration of the airway surface liquid (ASL) (Fig. 10).

The components of *P. acidus* have been shown to affect membrane ion transport in previous studies. Apart from activating CFTR directly, flavonoids have also been shown to inhibit endoplasmic reticulum Ca\(^{2+}\)-ATPase and to stimulate mitochondrial Ca\(^{2+}\) uptake (Montero et al., 2004), which may affect ER chaperones and thus CFTR membrane traffic. Flavonoids also lead to a favorable redistribution of F508del-CFTR within cellular compartments, without directly affecting processing of the protein (Lim et al., 2004). This may explain why *P. acidus* had only modest effects on biogenesis of CFTR, but activated F508del-CFTR currents after incubation of oocytes or acute application to F508del-CFTR expressing BHK cells. Adenosine and other xanthines have been found to bind and activate mutant and wt-CFTR directly. Moreover, adenosine activates purinergic A\(_{1}\) and A\(_{2B}\) receptors, thereby increasing intracellular Ca\(^{2+}\) and cAMP. Hypogallic acid induced Ca\(^{2+}\)-dependent Cl\(^{-}\)...
secretion, an effect that had been demonstrated for another component of *P. acidus*, caffeic acid (Lin et al., 2004).

Previous studies demonstrated that the P2Y receptors agonist ATP had only short-term effects on ion transport in the airways, due to inactivation by rapid hydrolysis. It is unlikely that the effects of *P. acidus* are short-lasting, since its components are more stable and will probably not be removed from the airway surface as effectively. Subsequent studies in a mouse model will have to compare the effects of local vs. systemic application. These studies should also examine pharmacokinetics of absorption and pharmacodynamics of these compounds, which are currently not known.

**Ethnopharmacology – a new source for CF therapeutics?** The present study identified bioactive components in herbal extracts of *P. acidus*. In a recent elegant study, a growth deficient yeast strain was used as a drug discovery surrogate bioassay, in order to identify natural plant products restoring Cl− channel function (deCarvalho et al., 2002). During the course of this study limonoids were identified as F508del-CFTR correctors. In previous studies with the extract from another medicinal plant, *Randia siamensis*, we also found effects on ion transport properties in mouse trachea (Jansakul et al., 1999). Extracts from *R. siamensis* induced Cl− secretion by activation of Ca2+-dependent Cl− channels. Similar to *P. acidus*, *R. siamensis* also contains flavonoids and other bioactive compounds, such as pseudoginsenosides. Ginsenosides and pseudoginsenosides are active ingredients of the ginseng root (Blumenthal, 2001) which have been shown to stimulate Ca2+-activated Cl− channels by activation of phospholipase C and mobilization of intracellular Ca2+ (Choi et al., 2001). Moreover, ginsenoside Re has been shown to increase NO, which activates K+ and Ca2+ channels as well as Cl− secretion via wt-CFTR and mutant F508del-CFTR (Bai et al., 2004; Lee et al., 2004; Kamosinska et al., 1997; Dong et al., 1995). Taken together, the use of
natural plant products provides new avenues for the treatment of CF. *P. acidus* extract can thus be used to enhance the activity of CFTR mutants with residual function or, in combination with compounds that rescue mutants with traffic defects such as F508del-CFTR, to further stimulate the Cl\(^{-}\) channel activity of these mutants.

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Footnotes

R.S. and K.K. share senior authorship. M.S. and J.O. contributed equally to the present work.

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Legends for Figures

Fig. 1: Activation of ion transport by *P. acidus* extract via stimulation of adenosine receptors in mouse trachea. A) Original Ussing chamber recording of the transepithelial voltage $V_{te}$ in mouse trachea. Effects of *P. acidus* (*P. a.*, 100 µg/ml) and adenosine (ade, 100 µM) in the presence or absence of the non-selective inhibitor of adenosine receptors, 8-SPT (10 µM). B) Concentration-dependent activation of steady-state (stead) and transient (trans) short-circuit currents ($I_{sc}$) by luminal application of adenosine. C) Concentration-dependent activation of steady-state and transient short-circuit currents ($I_{sc}$) by luminal application of *P. acidus*. D) Summary of transient and steady state $I_{sc}$ induced by adenosine in the absence or presence of 8-SPT. E) Summary of transient and steady state $I_{sc}$ induced by *P. acidus* in the absence or presence of 8-SPT. F) Summary of transient and steady state $I_{sc}$ induced by *P. acidus* in the absence or presence of DPC-PX (200 nM). G) Summary of transient and steady state $I_{sc}$ induced by *P. acidus* in the absence or presence of alloxazine (10 µM). * indicate significant difference (paired t-tests). (n) = number of experiments.

Fig. 2: Adenosine and *P. acidus* extract activate $Cl^-$ secretion via increase of intracellular $Ca^{2+}$ and cAMP in mouse trachea. A) Original recordings of the effects of adenosine (ade, 100 µg/ml) in the absence or presence of the $Cl^-$ channel blocker niflumic acid (NFA, 100 µmol/l) in mouse trachea. B) Summary of transient and steady state $I_{sc}$ induced by adenosine in the absence or presence of NFA (100 µM). C) Summary of the transient and steady-state $I_{sc}$ activated by *P. acidus* in the absence or presence of NFA. D) Summary of the transient and steady-state $I_{sc}$ activated by *P. acidus* in the absence or presence of DIDS (100 µM). E) Summary of the transient and steady-state $I_{sc}$ activated by *P. acidus* in the absence or presence of pre-stimulation with IBMX (100 µM) and forskolin (2 µM) (cAMP). F) Summary of the transient and steady-state $I_{sc}$ activated by *P. acidus* in the absence or presence of...
glibenclamide (glibencl, 100 µM). * indicates significant difference (paired t-test). (n) = number of experiments.

**Fig. 3:** *P. acidus extract increases intracellular Ca$^{2+}$ by luminal store depletion in mouse trachea.* A) Original Ussing chamber recordings of the effects of *P. acidus* (*P. a*, 100 µg/ml) in mouse trachea, in the absence or presence of the Ca$^{2+}$ ATPase inhibitor cyclopiazonic acid (CPA, 10 µM). B) Summary of the transient and steady state $I_{sc}$ activated by *P. acidus* in the absence or presence of CPA. C) Summary of the transient and steady state $I_{sc}$ activated by *P. acidus* in the absence or presence of BAPTA-AM. D) Summary of the transient and steady-state $I_{sc}$ activated by *P. acidus* in the absence or presence of the phospholipase C inhibitor U73122. E) Original recording of the effects of basolateral stimulation with carbachol (CCH, 100 µg/ml) in mouse trachea, in the absence or presence of *P. acidus*. F) Summary of the effects of CCH on $I_{sc}$ in the absence or presence of the *P. acidus*. G) Concentration-dependent activation of $I_{sc}$ by basolateral stimulation with adenosine. * indicates significant difference (paired t-test). # indicates significant difference when compared to the effects of *P. acidus* (unpaired t-test). (n) = number of experiments.

**Fig. 4:** *The *P. acidus* components kaempferol and DHBA activate Cl$^{-}$ secretion in mouse trachea, which is partially inhibited by NFA.* A) Original Ussing chamber recording of the effects of kaempferol (100 µM) in the absence or presence of NFA (100 µM). B) Summary of the $I_{sc}$ activated by kaempferol in the absence or presence of NFA. C) Original recording of the effects of DHBA (100 µM) in the absence or presence of NFA (100 µM). D) Summary of the $I_{sc}$ activated by DHBA in the absence or presence of NFA. E) Concentration-dependent activation of steady state $I_{sc}$ by luminal application of kaempferol. F) Concentration-dependent activation of steady state $I_{sc}$ by luminal application of DHBA.* indicates significant difference (paired t-test). (n) = number of experiments.
**Fig. 5:** *P. acidus* extract and co-application of isolated components have similar effects on ion transport in mouse trachea. A) Original recordings show the effects of *P. acidus* extract and of a mixture of the pure *P. acidus* components: adenosine, kaempferol and DHBA. B) Summary of the transient (trans, filled symbols) and steady state (stead, open symbols) $I_{sc}$ activated by *P. acidus* (dashed line) or the mixture of components (solid line). * indicates significant difference (paired t-test). (n) = number of experiments.

**Fig. 6:** *P. acidus* extract activates Cl$^{-}$ secretion and increases intracellular Ca$^{2+}$ in human airway epithelial cells. A) Activation of a whole-cell current in a human airway epithelial (16HBE) by *P. acidus* (100 µM). B) Summary of the effects of *P. acidus* and comparison with the effects of ATP (100 µM) on whole-cell conductance (Gm). C) Original recording of the 340/380 fluorescence ratio (cytosolic Ca$^{2+}$ concentration) and effects of *P. acidus* and ATP. D) Summary of the 340/380 fluorescence ratio changes induced by *P. acidus* and ATP. * indicates significant difference (paired t-test). (n) = number of experiments.

**Fig. 7:** *P. acidus* extract activates CFTR and Ca$^{2+}$-dependent Cl$^{-}$ conductance and inhibits Na$^{+}$ conductance in Xenopus oocytes. A) Current recording from a non-injected Xenopus oocyte obtained by double-electrode voltage clamp. Transient activation of endogenous Ca$^{2+}$-activated Cl$^{-}$ currents by *P. acidus* (100 µg/ml) and effects of DIDS (100 µM). B) Summary of the effects of *P. acidus* in the absence or presence of DIDS. C) Current recording from a CFTR expressing Xenopus oocyte. Activation of non-transient CFTR whole-cell currents by *P. acidus* and IBMX (1 mM). D) Summary of the effects of *P. acidus* and IBMX in CFTR expressing oocytes. E) Current recording from a CFTR / ENaC co-expressing Xenopus oocyte. Inhibition of Na$^{+}$ conductance (ENaC) by amiloride (A, 10 µM) and reduced effects of amiloride after stimulation of CFTR by *P. acidus*. F) Summary of the effects of amiloride
before and after activation of CFTR by *P. acidus*. G) Activation of whole-cell currents by IBMX and forskolin (F) in F508del-CFTR expressing *Xenopus* oocytes after 24 h control incubation. H) Activation of whole-cell currents by IBMX (1 mM) and forskolin (2 µM) in F508del-CFTR expressing *Xenopus* oocytes after 24 h incubation in *P. acidus* extract (100 µg/ml). I) Summary of the whole-cell Cl\(^-\) conductance activated by IBMX/F in *P. acidus*-incubated oocytes or control oocytes. * indicates significant difference (paired t-test). # indicates significant difference of the effects of amiloride or IBMX/ forskolin (F), respectively (paired t-test). (n) = number of experiments.

**Fig. 8:** *P. acidus* extract is not cytotoxic and activates F508del-CFTR. Flow cytometry viability assay of BHK cells stably expressing A) wt-CFTR or B) F508del-CFTR, following 48 h incubation with different concentrations of *P. acidus* as indicated in each panel. Graphs represent bivariate frequency distributions of red-fluorescent (585 nm) ethidium homodimer-1-stained dead cell population (y axis, arbitrary units) over green-fluorescent (530 nm) calcein-stained live cell population (x axis, arbitrary units). The population of live cells was larger than 95% in each assay. C) Effect of *P. acidus* (50 µg/ml, 48 h) on the turnover and processing of wt- and F508del-CFTR. BHK cells stably expressing wt- or F508del-CFTR were pulse-labeled for 30 min, chased for the indicated times and lysates were immunoprecipitated with an anti-CFTR antibody. Panels show turnover of core-glycosylated CFTR (band B) of wt- and F508del-CFTR and appearance of fully-glycosylated wtCFTR (band C). Each experiment was performed at least three times. D) (10 µM/ 50 µM, black line). Cells were grown at 37°C or 26°C, in the absence or presence of different concentrations of (*P.a.*) as indicated. Data indicate means ± SEM (n = 4).

**Fig. 9:** *P. acidus* activates Cl\(^-\) secretion in CF tracheas and human CF airway epithelial cells.
A) Original Ussing chamber recordings of $V_{te}$ in tracheas of mice homozygous for F508del-CFTR. Black boxes indicate effects of DHBA, kaempferol, adenosine, a mix of all three components and of extract. B) Summary of the short circuit currents activated by the individual components, the mix of components and extract. C) Whole cell current traces activated by a mix of the three isolated components of and of extract. Cells were held at their membrane voltage and voltage clamped ± 50 mV. D) Summary of the whole cell conductances activated by the mix and extract. * indicates significant difference (paired t-test). (n) = number of experiments.

**Fig. 10** Transport model of an airway epithelial cell and effects of *Phyllanthus acidus* and its major constituents adenosine, kaempferol, and dihydrobenzoic acid (DHBH).
Fig. 3

A

B

C

D

E

F

G
Fig. 5

A. 

B. 

\( V_e \) (mV)

\( \Delta_{SC} \) (\( \mu A/cm^2 \))

\( P. acidus \) 

mix

5 min

1 min

(5)

trans (mix)

stead (mix)

trans \( (P. a) \)

stead \( (P. a) \)

mix (\( \mu M \))

\( P. acidus \) (\( \mu g/ml \))
Fig. 6

A

P. acidus

B

G (nS)

(9) ATP

(4) P. acidus

C

P. acidus  ATP

D

Fluorescence Ratio (340/380 nm)

1.18

1.16

1.14

1.12

1.10

1.08

1 min

∆Fluorescence Ratio (340/380 nm)

0.25

0.20

0.15

0.10

0.05

0.00

(4) P. acidus  ATP

peak

plateau

peak

plateau
Fig. 10

P. acidus

adenosine

kaempferol

DHBA

A_2B

ENaC

Na^+

Cl^-

CFTR

CACC

ER Mito

Nucleus

cAMP

Ca^{2+}

K^+

K^+

Cl^-

Basolateral

Luminal