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Immunomodulatory properties of a lemon-quince preparation (Gencydo®) as an indicator of anti-allergic potency

Carsten Gründemann^{a,*}, Menelaos Papagiannopoulos^c, Evelyn Lamy^b, Volker Mersch-Sundermann^b, Roman Huber^a

^a Center for Complementary Medicine, Department of Environmental Health Sciences, University Medical Center Freiburg, 79106 Freiburg, Germany

^b Department of Environmental Health Sciences, University Medical Center Freiburg, Germany

^c Corporate Analytical Services, WELEDA AG, Schwäbisch Gmünd, Germany

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ABSTRACT

Introduction: Gencydo®, a combination of lemon (*Citrus limon*) juice and aqueous quince (*Cydonia oblonga*) extract has been used traditionally in anthroposophical medicine for treating patients with allergic rhinitis or asthma. Because there are no reports about the mode of action, we investigated the anti-allergic effects of this preparation *in vitro* by using cell lines and primary cells in various biological and immunological endpoints.

Materials and methods: The release of soluble mediators from basophilic cells, mast cells and lung epithelial cells, which are essential for the initiation of early- and late-phase allergic reactions, was analyzed in relation to the synthetic anti-allergic drugs azelastine and dexamethasone. In addition, the impact of Gencydo® on the viability and activation of GM-CSF-activated eosinophil granulocytes was investigated. **Results and discussion:** Gencydo® reduced the degranulation and histamine release of IgE-activated basophilic cells and mast cells and inhibited the IgE- and PMA/A23187-induced increases in IL-8, TNF- α and GM-CSF production in mast cells. The effects were comparable to that of the used concentration of azelastine and dexamethasone. Furthermore, Gencydo® partially blocked eotaxin release from human bronchial epithelial cells, but has no impact on the viability and activation of GM-CSF-activated eosinophil granulocytes.

In conclusion, these results give a rational base for the topical use of Gencydo® in treatment of allergic disorders through the down regulation of soluble mediators, which are essential for the initiation and maintenance of allergic reactions.

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Introduction

There is a marked rise in allergic diseases, including asthma and rhinitis in Europe. The characteristics of an allergic response is mediated through allergens by cross linking of IgE-Fc ϵ RI complexes on mast cells or basophils surface and this leads to the “early-phase” allergic reaction within minutes. This immediate response is caused by the activity of the mast cells and basophils released histamine and other toxic mediators, which provoke a rapid increase in vascular permeability and contraction of smooth muscles in target organs of the allergic reaction, like the nose (rhinitis) and the lungs (asthma) (Pearlman 1999). Furthermore, mast cells represent a potential source of cytokines (e.g. TNF-alpha and GM-CSF) and chemokines (e.g. IL-8) discharged in this early-phase and orchestrate the “late-phase” of an allergic reaction. This delayed stage

which peaks some hours later and implies recruitment and activation of inflammatory cells, as lymphocytes, neutrophil or eosinophil granulocytes at sites sensitized to the allergen (Gould and Sutton 2008). Attracted and activated eosinophil granulocytes can cause significant damage to tissue and the delayed reaction is a major cause of serious long-term illnesses, like asthma (Macfarlane et al. 2000).

The usual treatments of allergies are antihistamines, intranasal steroids, nasal cromoglicate-based drugs and leukotriene modifiers which are used in therapeutic algorithms (Greiner and Meltzer 2006), although rather they may have side effects, especially in children (Juniper et al. 2005). Immunotherapy, including sublingual immunomodulation is efficacious in a limited subgroup of patients only (Saltoun and Avila 2008). Although effective conventional remedies are available, approximately 30% of patients with allergies in Europe use complementary therapies, to avoid side-effects (Mainardi et al. 2009). One of these complementary therapies is Gencydo®, which is composed of lemon (*Citrus limon*) juice and an aqueous quince (*Cydonia oblonga*) extract. This combination of

* Corresponding author. Tel.: +49 761 270 8322; fax: +49 761 270 8323.

E-mail address: carsten.gruendemann@uniklinik-freiburg.de (C. Gründemann).

lemon and quince is based on a traditional use in anthroposophical medicine. Gencydo® is a product with marketing authorization and it has been investigated in an open study in patients with grass pollen allergy, showing clearly a symptom improvement of patients (Baars and De Bruin 2005). An observational study with 140 seasonal allergic rhinitis patients also reported a positive outcome without adverse effects (Rother and Eexle 2008). Moreover, *in vitro* studies with peripheral blood lymphocytes of allergic and non allergic donors showed immunomodulatory properties of a lemon-quince preparation (Baars and Savelkoul 2008). Despite its widespread clinical use and marginal observational studies (De Bruin and Baars 2001), there are no reports about the mode of anti-allergic action.

The aim of the present study was to analyse the effects of Gencydo® on the release of soluble mediators from basophilic cells, mast cells and lung epithelial cells, which are essential for the initiation of early- and late-phase allergic reactions and for the recruitment and activation of different leukocyte populations to the site of allergen exposure. In addition, we wanted to know whether Gencydo® has an impact on the viability and activation of GM-CSF-activated eosinophil granulocytes.

Materials and methods

Plant material

Gencydo® (Weleda AG, Schwäbisch Gmünd, Germany) is composed of lemon juice (*Citrus limon* succus) and an aqueous quince extract (*Cydonia oblonga* fructus rec. 1:2.1). The origins of the lemon and quince fruits were Sicily (Italy) and Wetzgau (Germany), respectively. The fresh fruits were kept refrigerated and sent to Weleda Schwäbisch Gmünd, Germany where the identity was analyzed by macroscopic confirmation and processed immediately upon arrival. The lemon fruits were squeezed by mechanical pressing in a roller mill to obtain *Citrus limon* juice. The quince fruits were homogenized and extracted with water by aid of an ultrasonic bath for 10 min with occasional swirling of the solution to obtain aqueous *Cydonia oblonga* extract. Gencydo® 3% injection solution contains per millilitre 24–36 mg of a lemon juice (*Citrus limon*, succus), corresponding to 1.95 mg of fruit acids (calculated as citric acid) and 30 mg of an aqueous quince extract (*Cydonia oblonga*, fructus rec. 1:2.1). Additionally, Gencydo® contains purified water and sodium chloride. The ingredients were sterile filtered, mixed together, ampouled and sent to the laboratory in Freiburg, Germany, where the cell biological experiments were performed. For each experiment, a fresh ampoule of Gencydo® was used.

HPLC–MS analysis of the lemon-quince preparation

The analyses for the profiling of the natural phytochemicals in the lemon-quince preparation Gencydo® were carried out by HPLC coupled with electrospray ion trap/orbitrap multistep fragmentation mass spectrometry. The Accela High Speed liquid chromatography system consisted of an Accela pump with integrated solvent degasser, an Accela Autosampler with built-in column oven, and an Accela photodiode array detector equipped with a 10 mm flow cell. An LTQ Orbitrap XL hybrid FT mass spectrometer equipped with a heated electrospray ionization source was connected to the LC system. The data were acquired with the XCalibur mass spectrometry data system (all Thermo Scientific, Dreieich, Germany).

For the chromatographic separation, a Hypersil Gold column with a length of 100 mm and an internal diameter of 2.1 mm with a particle diameter of 1.9 µm (Thermo Scientific) was set to a tem-

perature of 25 °C. Gradient elution was carried out at a constant flow rate of 300 µl/min with LC–MS-grade water (Fisher Scientific, Fair Lawn, NJ, USA) with an addition of 0.1% by volume of formic acid (Sigma–Aldrich, Steinheim, Germany) as mobile phase A and LC–MS-grade acetonitrile (Fisher Scientific) as mobile phase B as follows: elution started at 100% mobile phase A with a linear gradient to 100% mobile phase B after 20 min, followed by a 5 min wash with 100% mobile phase B and a 5 min reequilibration with the initial condition. 5 µl of each of the sample solutions was injected into the LC–MS-system and the UV–Vis absorption spectra were monitored at a wavelength range from 235 to 600 nm.

The mass spectrometer was operated in the negative mode. The settings for the ionization of the compounds eluting from the liquid chromatograph (without flow split) were: electrospray voltage 3 kV, sheath gas 40 arbitrary units, auxiliary gas 5 arbitrary units, sweep gas 0 arbitrary units, ESI heater temperature off, capillary temperature at 300 °C, source CID 10 V, injection waveforms off, capillary voltage –35 V, and tube lens –110 V. Mass spectra were acquired in the *m/z*-range from 220 to 1000 mass units. Multistep ion trap fragmentation was performed in the dependent scan mode for the MS²- and MS³-spectra.

Identification of individual compounds was conducted using the UV–Vis-spectra (where applicable), the chemical elemental composition calculated from the exact mass measurement of the Orbitrap, and the fragmentation patterns and masses of the corresponding product ions in comparison with data from the quince preparation's respective ingredients, reference compounds and literature data.

Cell cultures and primary human material

RBL-2H3 cells were obtained from H. Pircher (Freiburg, Institut of Medical Microbiology and Hygiene, Division of Immunology, Germany) and grown in DMEM. For optimal degranulation capacity, individual clones were generated by limiting dilution procedure. HMC-1 cells were kindly provided by J.H. Butterfield (Rochester, MN, USA) and grown in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany). BEAS-2B cells were a gift from M. Idzko (Department of Pneumology, Freiburg, Germany) and grown in DMEM/F12 medium (Invitrogen, Karlsruhe, Germany). Each medium was supplemented with 10% heat-inactivated FCS (PAA, Coelbe, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Karlsruhe, Germany) and the cells were cultured at 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere. All experiments conducted with human material were approved by the Ethics committee of the University of Freiburg.

Assays for cell viability

Cell viability was determined using the WST-1 assay (Roche, Mannheim, Germany) according to the manufacturer's instructions or by the trypan blue exclusion test (Strober 2001). Cytotoxicity is expressed as percentage of cell viability of treated cells compared to percentage of cell viability of untreated cells (=100%). A sample is considered to be cytotoxic to the cells if cell viability is <80%.

Mouse bone marrow cultures

Bone marrow-derived mast cells (BMMCs) were generated from mouse bone marrow from 6- to 8-week-old male mice with C57BL/6 background as described in the literature (Gimborn et al. 2005). The cells were kindly provided by Michael Huber (University Hospital Aachen, Germany) and were immediately used for experiments.

BMMC and RBL-2H3 activation

For degranulation studies, BMNCs (10^6) were preloaded with 0.15 $\mu\text{g/ml}$ and RBL-2H3 cells (2×10^5) with 0.5 $\mu\text{g/ml}$ IgE mAb with specificity for DNP (SPE-7; Sigma–Aldrich, Taufkirchen, Germany) for 12–18 h at 37 °C in complete medium with 10% FCS. The cells were then washed twice with Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.1% BSA in 100 mM HEPES, pH 7.4; all from Sigma–Aldrich, Germany) and treated (10 min, 37 °C) with Gencydo® or azelastine (Sigma–Aldrich, Taufkirchen, Germany) before stimulation (30 min, 37 °C) with 20 ng/ml DNP–HSA (Sigma–Aldrich, Taufkirchen, Germany).

 β -Hexosaminidase and histamine release assay

The supernatant was used to determine β -hexosaminidase or histamine release. Total β -hexosaminidase and histamine release was obtained by lysing the cells with 0.1% Triton-X 100 (in PBS) prior to removing the supernatant for measurement of β -hexosaminidase and histamine. To determine the amount of β -hexosaminidase activity released by BMMC or RBL-2H3 cells, 10 or 25 μl of supernatant and 90 or 50 μl of 1.2 mM β -hexosaminidase substrate (4-nitrophenyl-N-acetyl- β -D-glucosaminid; Sigma–Aldrich, Germany) in 0.1 M sodium acetate buffer (pH 4.5) were mixed in 96-well plates and incubated for 2 h at 37 °C. The reaction was completed by adding a 0.4 M glycine buffer (pH 10.7, 150 μl). Histamine content was measured according to the manufacturer's specification using a histamine ELISA assay kit (IBL International, Germany). The reaction product was measured at 405 nm (β -hexosaminidase) or 450 nm (histamine) using a microplate reader (Tecan, Austria), and percentage of β -hexosaminidase or histamine release was calculated as follows: release supernatant/(release supernatant + release pellet) $\times$ 100.

Activation and measurement of cytokine and chemokine production from mast cells

BMNCs (10^6) were preloaded with dinitrophenyl (DNP)-specific IgE mAb (SPE-7; Sigma–Aldrich, Taufkirchen, Germany) overnight at 37 °C in medium. Cells were washed with medium and treated (60 min, 37 °C) with the indicated concentrations of Gencydo® or dexamethasone (Sigma–Aldrich, Taufkirchen, Germany) before stimulation (5 h, 37 °C) with 20 ng/ml DNP–HSA (Sigma–Aldrich, Taufkirchen, Germany). HMC-1 cells (10^5) were preincubated (30 min) with indicated Gencydo® concentrations or dexamethasone followed by an additional 2.5 h stimulation with 40 nM Phorbol 12-myristate 13-acetate (PMA) and 1 μM A23187 (Sigma–Aldrich, Taufkirchen, Germany). Cytokine and chemokine productions were quantified using FlowCytomix™ Technology according to the manufacturer's specification (Bender MedSystems, Vienna, Austria).

Cytokine stimulation and detection of chemokine proteins from bronchial epithelial cells

BEAS-2B cells (2×10^4) were seeded into 96-well, flat-bottom culture plates and the medium was replaced by serum-free DMEM/F12 (Invitrogen, Mannheim, Germany) for 24 h. Cells were stimulated with 100 ng/ml human TNF- α and 100 ng/ml IL-4 (PeproTech, Germany) in the presence of indicated concentrations of Gencydo® or dexamethasone for 24 h in medium containing FCS. Culture supernatants from cytokine-stimulated BEAS-2B cells were harvested and Eotaxin and RANTES production were quantified by sandwich immunoassays using the protocol supplied by Invitrogen

(Mannheim, Germany).

Eosinophil isolation

Human eosinophils were isolated from the blood of adult healthy donors (blood eosinophil levels, 1–5%) obtained from the Blood Transfusion Centre (Freiburg, Germany). Venous blood was left to sediment in 6% dextran (Carl Roth, Karlsruhe, Germany) and the leukocyte-rich supernatant centrifuged on a two-layer Percoll gradient (densities, 1.0776 and 1.1026; 20 min, 500 $\times$ g, 20 °C; GE Healthcare Life Sciences, Freiburg, Germany). Neutrophils and contaminating lymphocytes were tagged with a micromagnetic bead cocktail (Human Eosinophil Enrichment Kit; StemCell Technologies, Germany). Eosinophils were purified by placing the cell suspension in a magnet (Purple EasySep® Magnet; StemCell Technologies, Grenoble, France). They were collected at a purity of at least 97%, analyzed by flow cytometry and viability was of at least 95%.

Determination of eosinophil apoptosis and CD69 and CCR3 expression

Isolated human eosinophils (10^5) were preincubated (60 min) with various concentrations of Gencydo®, Campothecin (100 μM) (Tocris, Bristol, UK) or Triton-X100 (0.5%) (Carl Roth, Karlsruhe, Germany) as controls in medium in FCS-precoated wells followed by further incubation for 23 h in presence or absence of 20 ng/ml human GM-CSF (PeproTech, Hamburg, Germany). The levels of apoptosis were determined using the Annexin V-FITC Apoptosis Detection kit according to the manufacturer's instructions (ebioscience, Frankfurt, Germany). After Annexin V staining, propidium iodide (PI; ebioscience, Frankfurt, Germany) was added and the cells were incubated for additional 10 min in the dark. Determination of CD69 and CCR3 surface expression were assessed using flow cytometry and FITC-labeled anti-CD69 and APC-labeled anti-CCR3 APC mAbs (ebioscience, Frankfurt, Germany).

Analysis of data

Data were analyzed using SigmaPlot® (Systat Software Inc., Erkrath, Germany) and presented as mean $\pm$ standard deviation and statistical significance was determined by Student's paired *t*-test and *P* values <0.05 (*) were considered statistically significant and <0.01 (**) were considered highly statistically significant.

Results*HPLC–MS profiling of flavonoids in Gencydo®*

HPLC–MS analysis for the identification of Gencydo® were targeted at the identification of the typical flavanon-glycosides known in lemon and flavonol-glycosides and caffeoylquinic acids which have been described in quince previously. In Gencydo®, eriocitrin and hesperidin were easily detected as the prominent polyphenols according to established literature data (Fig. 1A) and also caffeoylquinic acids (Fig. 1B) and flavonol-glycosides (Fig. 1C) could be detected, which are known to be the main polyphenolic compounds in lemon and quince.

In summary, the typical compounds of lemon and quince could be identified in the lemon-quince preparation by HPLC–MS unambiguously by the corresponding peaks in the proper mass traces and their specific MSⁿ-fragmentation patterns.

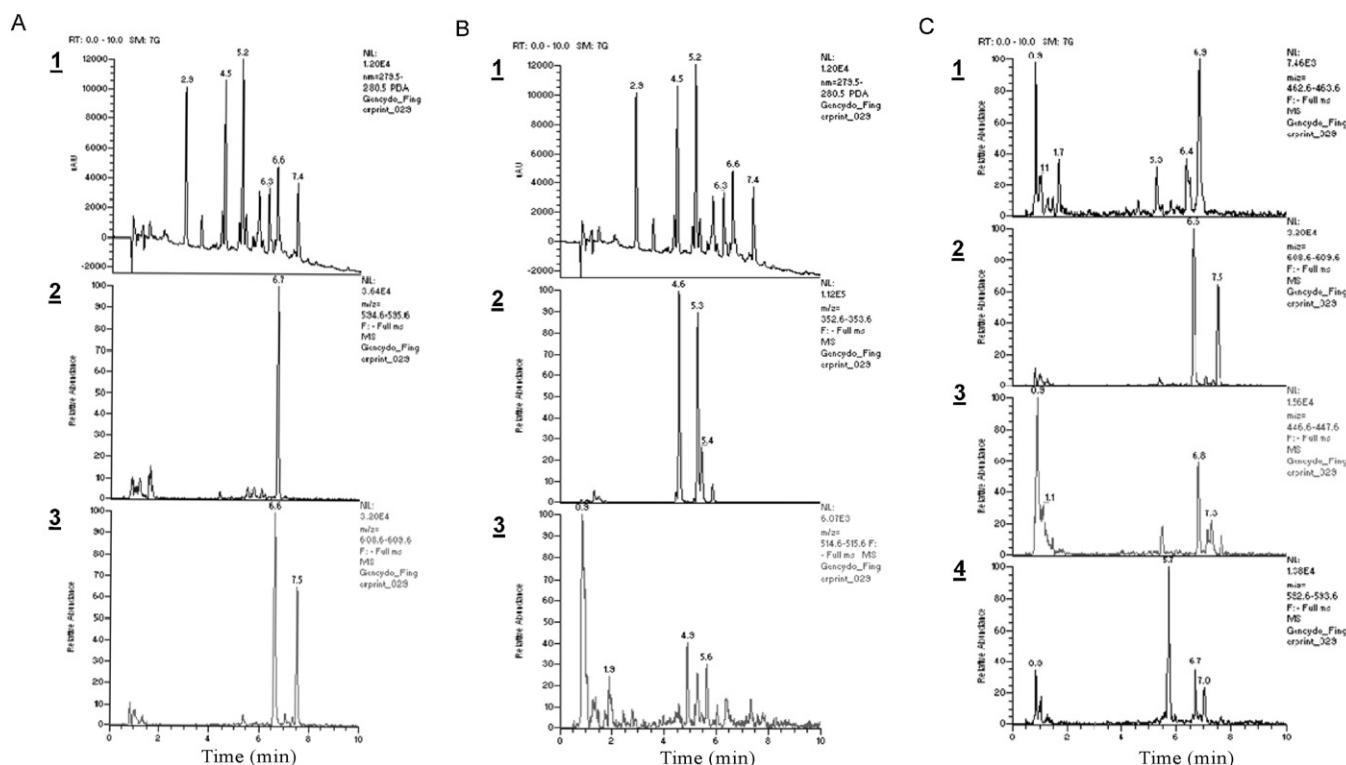


Fig. 1. (A) The lemon flavanon-glycosides in Gencydo®. Shown are (1) the UV-chromatogram at 280 nm, (2) the mass trace at m/z 595.1 with the peak of eriocitrin at 6.7 min, and (3) the mass trace at m/z 609.1 with the peak of hesperidin at 7.5 min (from upper line to lower line). (B) The cydonia caffeoylquinic acids in Gencydo®. Shown are (1) the UV-chromatogram at 280 nm, (2) the mass trace at m/z 353.1 with the peaks of the caffeoylquinic acid-isomers at 4.6, 5.3, and 5.4/5.5 min, and (3) the mass trace at m/z 515.1 with the peak of dicaffeoylquinic acid at 4.9 min (from upper line to lower line). (C) The cydonia flavonol-glycosides in Gencydo®. Shown are (1) the mass trace at m/z 463.1 with the peak of quercetin-galactoside at 6.8/6.9 min, (2) the mass trace at m/z 609.1 with the peak of quercetin-rutinoside at 6.6, (3) the mass trace at m/z 447.1 with the peak of kaempferol-glucoside at 6.8, and (4) the mass trace at m/z 593.1 with the peak of kaempferol-rutinoside at 5.6/5.7 min (from upper line to lower line). The second prominent peak in Gencydo® in the mass trace at m/z 609.1 at 7.5 min is the peak of hesperidin (see also (A) for comparison).

Gencydo® inhibits degranulation and histamine release

IgE-mediated activation caused a rapid release of β -hexosaminidase in basophilic cells and mast cells (Fig. 2A). Moreover we could show that Gencydo® inhibited the degranulation of IgE-activated basophilic cells (Fig. 2A, left panel) and mast cells (Fig. 2A, right panel). The influence of Gencydo® on histamine release of basophilic cells and mast cells after IgE/Ag stimulation was additionally investigated and the data in Fig. 2B illustrate that the indicated Gencydo® concentrations were also sufficient to inhibit the release of histamine in basophilic cells (Fig. 2B, left panel) and mast cells (Fig. 2B, right panel). Both effects were comparable to that of the used concentration of azelastine. Cell viability was measured by WST-1 assay and by trypan blue exclusion test and did not exhibit cytotoxicity at any tested concentrations of Gencydo® (Fig. 2C). Taken together, these results revealed that Gencydo® inhibits degranulation and histamine release of basophilic cells and mast cells.

Gencydo® inhibits cytokine and chemokine production from mast cells

Gencydo® was further analyzed whether it affects cytokine production of IgE/Ag-activated mouse mast cells in comparison to dexamethasone (Fig. 3). Following stimulation with antigen the results revealed an induction of TNF- α and GM-CSF production compared to non-stimulated cells (Fig. 3). The secretion of TNF- α (Fig. 3, left panel) was affected by the presence of 0.8 mg/ml Gencydo®, but lacked significant inhibition by lower concentrations of Gencydo®. In contrast, as shown in Fig. 3 (middle panel),

Gencydo® was found to inhibit IgE-mediated release of GM-CSF in a dose-dependent manner, without decreasing cell viability (Fig. 3, right panel) as much as dexamethasone.

To support the findings that Gencydo® has the capacity to modulate cytokine release, we stimulated HMC-1 cells with PMA and calcium ionophore A23187 and detected the production of IL-8 (Fig. 4, left panel) and TNF- α (Fig. 4, right panel). Treatment of cultures with dexamethasone significantly blocked the PMA/A23187-induced release of both IL-8 and TNF- α and with Gencydo® blocking was detected at 0.8 and 0.4 mg/ml, but not at 0.2 mg/ml (Fig. 4). Taken together, these data demonstrate that the lemon-quince preparation inhibits IL-8, TNF- α and GM-CSF production of mast cells which are essential for the orchestration of early- and late-phase allergic reactions.

Gencydo® modulates chemokine production from lung epithelial cells

To determine whether the production of RANTES and eotaxin from human bronchial epithelial cells can be influenced by Gencydo®, we stimulated BEAS-2B cells for 24 h with TNF- α and IL-4 (Fig. 5). As a control we used dexamethasone. RANTES and Eotaxin protein levels were detected in the supernatants of activated epithelial cells (Fig. 5). All indicated concentrations of the lemon-quince preparation do not affect RANTES release in comparison to dexamethasone (Fig. 5, left panel), however; the release of eotaxin showed a concentration-dependent inhibition at all concentrations of Gencydo® (Fig. 5, middle panel). These results indicate that Gencydo® has a potential to inhibit the production of eotaxin from human lung epithelial cells.

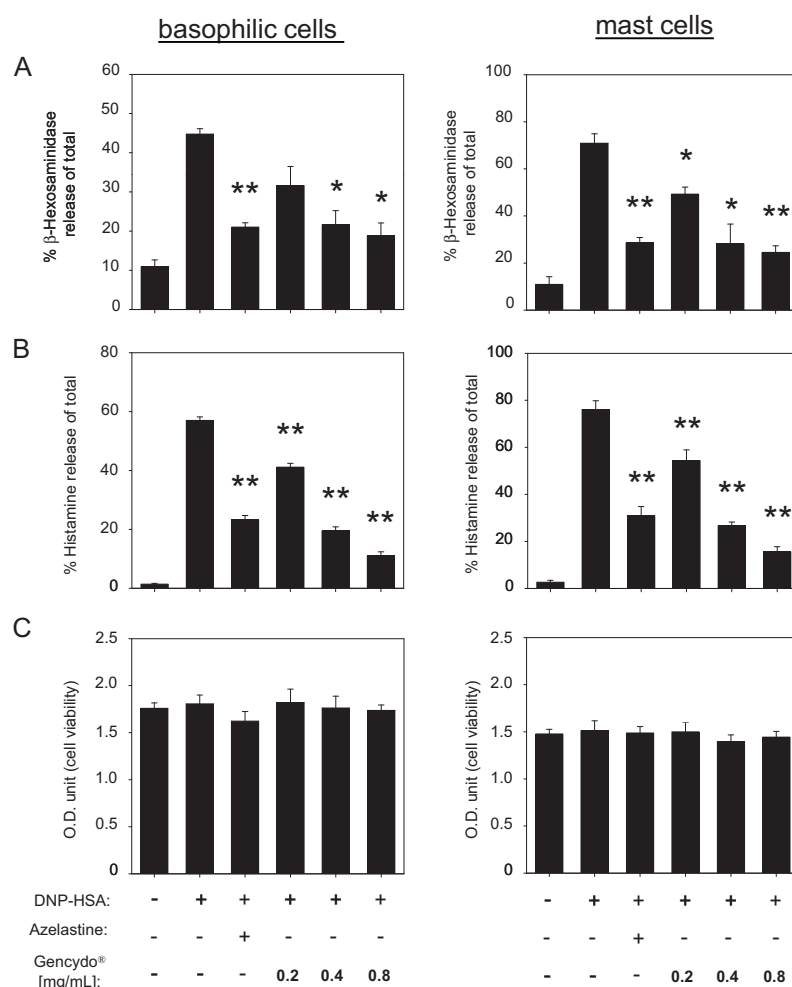


Fig. 2. Effects of Gencydo® on antigen-induced β -hexosaminidase and histamine release. (A) Basophilic cells or mouse mast cells were incubated with DNP-specific IgE. Cells were incubated for 10 min with concentrations of Gencydo® (0.2, 0.4, and 0.8 mg/ml) or azelastine (10^{-11} M) before stimulation with DNP-HSA. β -hexosaminidase release was expressed as percentage of total release of content (intra- and extracellular). Cells incubated with medium alone were used as controls. Data are expressed as mean values $\pm$ SE of three independent experiments. The asterisks represent significant differences from activated cells alone (* $P < 0.05$, ** $P < 0.01$). (B) Same as (A) except that histamine release was measured. Limit of quantification for the histamine ELISA was 1.3 ng/ml. Cell viability was assessed by WST-1 assay (C).

Gencydo® does not affect behaviour of human eosinophilic granulocytes

To investigate whether Gencydo® influenced the expression of the eotaxin receptor CCR3 on human eosinophils, we assessed

CCR3 expression by using flow cytometry (Fig. 6A). After a 24 h incubation period, GM-CSF induced no increase in CCR3 expression on eosinophils and was not modulated by co-incubation with different concentrations (0.2 mg/ml, 0.4 mg/ml, and 0.8 mg/ml) of Gencydo®. Furthermore, we tested the impact of Gencydo® on

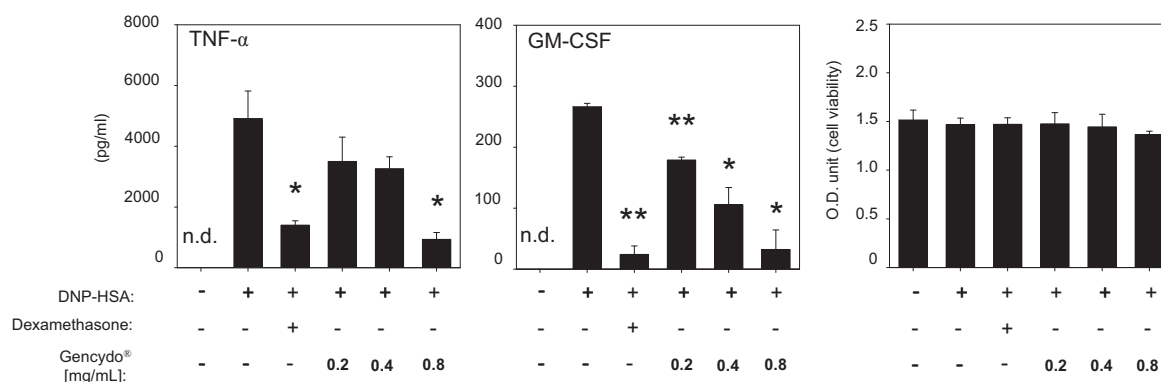


Fig. 3. Effects of Gencydo® on cytokine production from antigen-activated mouse mast cells. Mouse mast cells were sensitized with DNP-specific IgE antibody. Cells were pretreated for 60 min with concentrations of Gencydo® (0.2, 0.4, and 0.8 mg/ml) or dexamethasone (10^{-8} M) and stimulated with DNP-HSA. Cells incubated with medium alone were used as controls. The release of murine TNF- α (left panel) and murine GM-CSF (middle panel) were quantified using a commercial ELISA-based bead assay. Cell viability was detected by WST-1 assay (right panel). Data are expressed as mean values $\pm$ SE of three independent experiments. The asterisks represent significant differences from activated cells alone (* $P < 0.05$, ** $P < 0.01$). n.d., not detected in the assay. Limit of detection for the murine TNF- α and GM-CSF ELISA-based assays was 2.1 and 10.9 pg/ml, respectively.

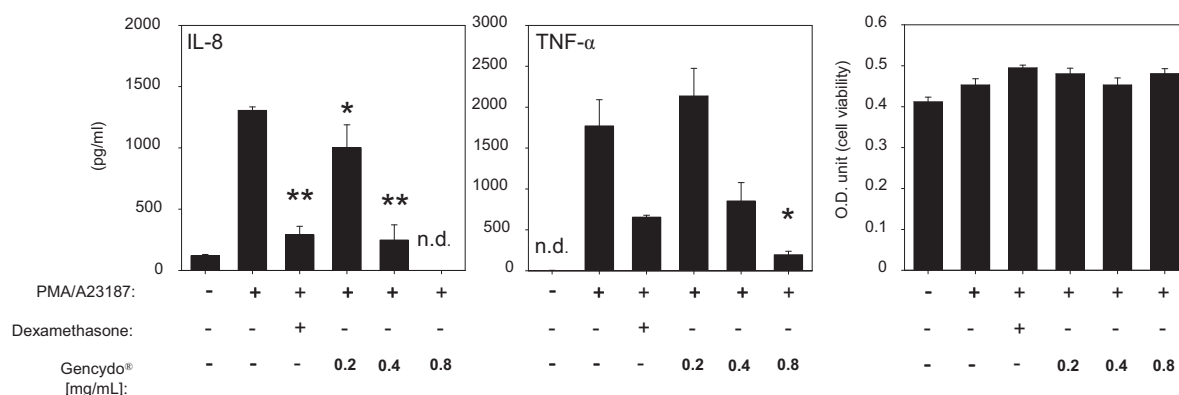


Fig. 4. Effects of Gencydo® on cytokine and chemokine production from antigen-independent activated human mast cells. Human mast cells were activated with PMA (Phorbol 12-myristate 13-acetate; 40 nM) and A23187 (calcium ionophore; 1 μ mol/l) for 2.5 h after pretreatment with different concentrations of Gencydo® (0.2, 0.4, and 0.8 mg/ml) or dexamethasone (10^{-8} M). Cells incubated with medium alone were used as controls. Supernatants were harvested and assayed for human IL-8 (left panel) and human TNF- α (right panel). Cell viability was detected by WST-1 assay (right panel). Data were expressed as mean values $\pm$ SE of three independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from PMA and A23187 alone. n.d., not detected in the assay. Limit of detection for the human IL-8 and TNF- α ELISA-based assay was 0.5 and 3.2 pg/ml, respectively.

apoptosis/necrosis induction. As depicted in Fig. 6B, it was shown that GM-CSF prolonged the survival of eosinophils, suggested by a reduction of apoptotic positive eosinophils, but Gencydo® had no effect on these processes. The flow cytometry analysis further demonstrated that GM-CSF activated the cells and therefore the number of CD69 positive eosinophils increased, but not in a significant manner (Fig. 6C). In summary, these results demonstrate that Gencydo® has no impact on the behaviour of GM-CSF-stimulated human eosinophils.

Discussion

Gencydo®, a combination of lemon juice with an aqueous quince extract has traditionally been used in anthroposophical medicine for treating patients with allergic rhinitis or asthma. Because there are no reports about the mode of action, we investigated the anti-allergic effects of Gencydo® by analyzing various biological and immunological endpoints in cell lines and primary cells in comparison to the synthetic drugs azelastine and dexamethasone. In this current report we showed that Gencydo® inhibited the release of soluble mediators from basophilic cells, mast cells and lung epithelial cells, which are essential for the initiation of early- and late-phase allergic reactions and for the recruitment of eosinophils

to the site of allergen exposure. Furthermore, we demonstrated that Gencydo® had no impact on the behaviour of GM-CSF-stimulated eosinophil granulocytes.

Our results reveal that Gencydo® inhibits degranulation and histamine release of basophilic cells and mast cells to that of the used concentrations of azelastine and suggested that Gencydo® is useful for the treatment of allergic disorders. The degranulatory capacity was measured by β -hexosaminidase release, since this enzyme is stored in the secretory granules. This assay has been widely used to monitor the capacity of potential new drugs to block basophilic and mast cell activation and degranulation (Ortega Soto and Pecht 1988; Aketani et al. 2001; Granberg et al. 2001). Furthermore, our results demonstrate that Gencydo® affected the secretion of inflammatory cytokines (IL-8 and TNF- α) from activated mast cells to that of the elected doses of dexamethasone. Reduction of inflammatory cytokines from mast cells is probably one of the key indicators of improvement of late-phase allergic symptoms (Hide et al. 1997). Additionally, in our study Gencydo® was found to inhibit IgE-mediated release of GM-CSF. GM-CSF is a cytokine that promotes eosinophil activation and survival. Eosinophils have been linked to airway inflammation in asthma and measurable levels were detected in bronchoalveolar lavage fluid from asthma patients (Broide et al. 1992). In this line, dur-

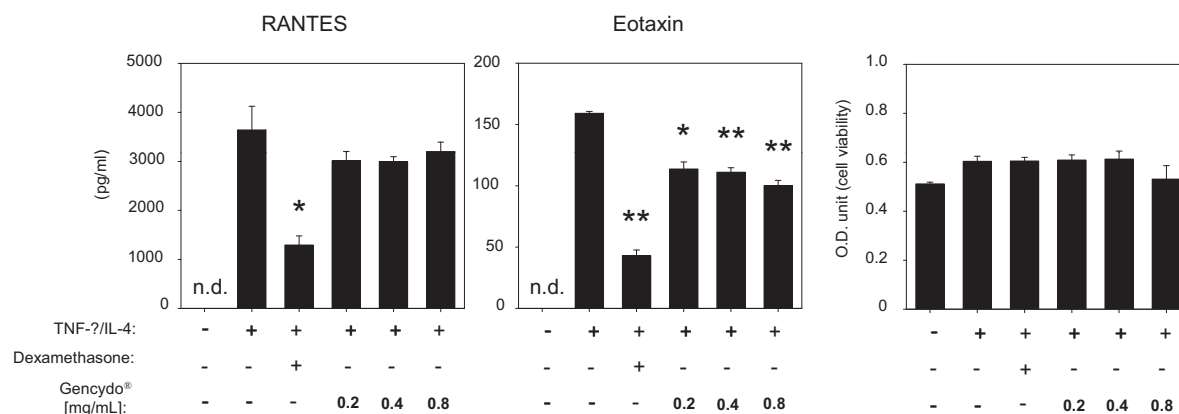


Fig. 5. Effects of Gencydo® on cytokine-induced chemokine release from human bronchial epithelial cells. Cells were starved for 24 h in medium without FCS. Cells were stimulated to induce chemokine production with 100 ng/ml human IL-4 and 100 ng/ml human TNF- α with different concentrations (0.2, 0.4, and 0.8 mg/ml) of Gencydo® or Dexamethasone (10^{-8} M). Cells incubated with medium alone were used as controls. After 24 h human RANTES (left panel) and human eotaxin (middle panel) were determined. Cell viability was detected by WST-1 assay (right panel). Data represent mean values $\pm$ SE of three independent experiments. The asterisks represent significant differences of treated cells in comparison to cytokine-activated cells alone (* $P < 0.05$, ** $P < 0.01$). n.d., not detected in the assay. Limit of detection for human RANTES and eotaxin ELISA-based assays was 3.0 and 2.2 pg/ml, respectively.

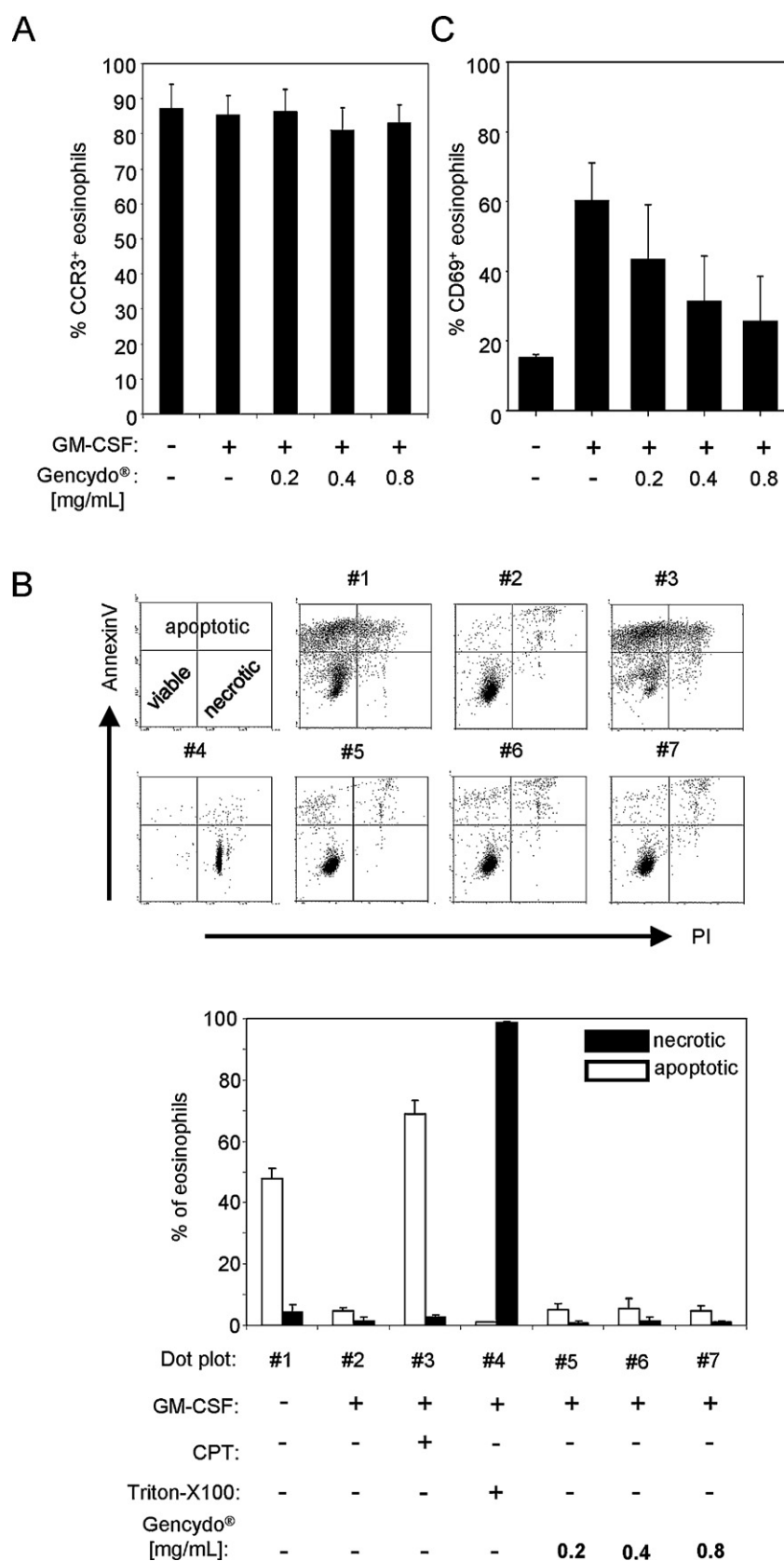


Fig. 6. Effects of Gencydo[®] on granulocyte macrophage-colony stimulating factor (GM-CSF)-stimulated human eosinophil granulocytes. Primary human eosinophils were activated with human GM-CSF (20 ng/ml) in FCS-precoated wells in the presence of Gencydo[®] (0.2, 0.4, and 0.8 mg/ml) (A–C), or for apoptosis/necrosis analysis additional with CPT (100 μ M) and Triton-X100 (0.5%) (B). Cells incubated with medium alone were used as negative controls. After 24 h eosinophils were stained with anti-CCR3 mAbs (A) and anti-CD69 mAbs (C). For apoptosis and necrosis analysis, cells were stained with Annexin V and propidium iodide (B). Data represent mean values $\pm$ SE of three experiments and additionally FACS dot plots from one experiment are shown (B).

ing an allergic response, eosinophils are recruited to the site of allergen exposure, attracted especially by eotaxin that is produced by the epithelium (Zimmermann et al. 2003; Horie et al. 1997). We showed that Gencydo® inhibits the production of eotaxin and therefore, Gencydo® has the capacity to reduce the attraction of eosinophils, but the effects are weaker than that of dexamethasone. Topical application of the lemon-quince preparation via inhalation may therefore be an interesting option and should be further investigated. To see whether Gencydo® affects eosinophil behaviour directly, we analyzed cell viability and eotaxin receptor (CCR3) expression. Additionally, the expression of the integrin CD69 on the surface of eosinophils was investigated, since this antigen is expressed on eosinophils at inflammatory sites and is discussed as a useful marker for activated eosinophils (Nishikawa et al. 1992). Hereby, we demonstrated that Gencydo® has no impact on eosinophil viability, CCR3 expression and activation.

In summary the data implicate that Gencydo® could affect chronic allergic disorders, like asthma by diminishing the recruitment of eosinophils mediated through regulating eotaxin release from the epithelium. This twofold effect in the early- and late-phase of an allergic reaction may be advantageous compared to antihistamines which are only effective against the early-phase allergic reaction and do not prevent the effects of cytokines which initiate the late-phase allergic reaction (Holgate and Polosao 2008).

Citrus limon and *Cydonia oblonga* are known to contain appreciable amounts of polyphenolic compounds. Several compounds of this diverse group of phytochemicals have attracted increasing interest in the past due to their physiological actions. Thus, the LC–MS-analyses of Gencydo® were targeted at the identification of the typical flavanon-glycosides known in lemon and flavonol-glycosides and caffeoylquinic acids which have been described in quince previously. Because of its high separation performance and the high selectivity of detection, the LC–MS-technique has been applied widely in the past to the detection of polyphenolics in complex matrices like plant extracts, and the multistep ion trap fragmentation allows an identification of individual compounds.

Citrus fruits are known to contain flavanon-glycosides with different individual compounds occurring in the different citrus fruit species. Lemon is known to contain mainly the flavanon-glycosides eriodictin (eriodictyol-7-O-rutinoside) and hesperidin (hesperitin-7-O-rutinoside). The phenolic compounds in quince have been reported to be mainly caffeoylquinic acids (3-, 4-, and 5-O-caffeoylquinic acid and 3,5-dicaffeoylquinic acid) and flavonol-glycosides (quercetin-3-galactoside, quercetin-3-rutinoside, kaempferol-3-glucoside, and kaempferol-3-rutinoside). Several of the numerous already detected components of the lemon-quince preparation's ingredients have been reported as anti-allergic substances and inhibited mediators which are essential for the initiation of early- and late-phase allergic reactions. (Kempuraj et al. 2005; Kawai et al. 2007; Park et al. 2008).

In Gencydo®, the typical polyphenolic compounds were identified by LC–MS unambiguously by the corresponding peaks with their specific masses and MSⁿ-fragmentation pattern.

From the literature we know, that glycosides given orally or systemically were found to be absorbed and eliminated very fast in humans (Scholz and Williamson 2007). In contrast to this, due to the local application of Gencydo®, the dose used *in vivo* is comparable to the doses used in our experiments. This relation to dose and the comparison to the anti-allergic drugs azelastine and dexamethasone speak for a clinical relevance of the demonstrated effects. In this current study we analyzed the whole lemon-quince extract (Gencydo®) and showed a significant inhibitory influence of Gencydo® on the release of different allergic mediators. To evaluate the possible chemical compounds which act as the therapeutic principle further investigations would be required and these data will then be published in a separate article.

In conclusion, our results give a rational base for the topical use of Gencydo® in treatment of allergic disorders through the down-regulation of soluble mediators which are essential for the initiation of early and late-phase allergic reactions. Its clinical efficacy has, however, to be proved in clinical placebo-controlled studies.

Conflict of interest

None.

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