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Safety-assessment of 3-methoxyquercetin as an antirhinoviral compound for nasal application: effect on ciliary beat frequency

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Abstract

It has been shown that 5,7,3′,4′-tetrahydroxy-3-*O*-methylflavone (3-MQ) exhibits antipicornaviral activity. In order to explore the potential of 3-MQ as an antirhinoviral compound for nasal application, the effect of 3-MQ on the ciliary beat frequency (CBF) of human nasal epithelial cells was studied in vitro in the absence or presence of solubility/absorption enhancers (hydroxypropyl-β-cyclodextrin (HP-β-CD) or polysorbate 80). Nasal epithelial cells were obtained by protease digestion of surgical specimens of human nasal polyps, and used at confluency. The effect of 3-MQ (2, 10, and 20 μg/ml), HP-β-CD (1, 3, and 10% (w/v)), polysorbate 80 (0.1, 0.3, and 1% (w/v)), and of the combination of 3-MQ with 3% HP-β-CD or 1% polysorbate 80, on the CBF was determined by computerized microscope photometry 15 min after incubation with the test compounds; recovery was determined 35 min after rinsing. HP-β-CD at 1 and 3% did not affect CBF; a reversible decrease (by 37%) was observed at 10%. Polysorbate 80 caused a reversible cilio-inhibitory effect of 40, 53, and 49% at 0.1, 0.3, and 1%, respectively. At 2 and 10 μg/ml, 3-MQ showed a reversible cilio-stimulatory effect of 18 and 14%, respectively. Combined with 3% HP-β-CD, the reversible cilio-stimulatory effect of 2 μg/ml 3-MQ was preserved, while 10 and 20 μg/ml 3-MQ did not affect the CBF. The combination of polysorbate 80 (1%) and 3-MQ decreased the CBF, which could be attributed to the presence of polysorbate 80. In conclusion, no ciliotoxic effect could be observed for 3-MQ (up to 20 μg/ml) in the absence or presence of HP-β-CD (3%). The potential of this combination as an antirhinoviral formulation for nasal application will be further explored.

Keywords: Human nasal epithelial cells; Ciliary beat frequency; 3-Methoxyquercetin

1. Introduction

3-Methoxyquercetin (5,7,3′,4′-tetrahydroxy-3-*O*-methylflavone, 3-MQ) is a naturally occurring 3-methoxyflavone (Fig. 1) with pronounced antiviral

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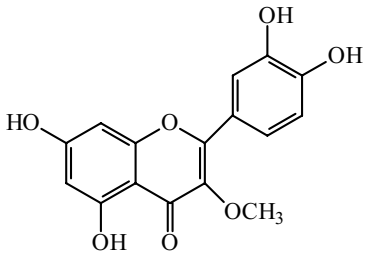

Fig. 1. Chemical structure of 3-MQ.

and moderate anti-inflammatory and antioxidant activity (Middleton et al., 2000). 3-MQ was first isolated from plant extracts of *Euphorbia grantii* Oliver (Euphorbiaceae) (Van Hoof et al., 1984; De Meyer et al., 1991) but it has also been chemically synthesized (De Meyer, 1989). Naturally occurring and chemically synthesized 4′-hydroxy 3-*O*-methylflavones possess antiviral activity against Picornaviruses: Poliovirus type I, Coxsackie type B2 and B4, and Human Rhinovirus type 15 and 81 (Van Hoof et al., 1984; Tsuchiya et al., 1985; De Meyer et al., 1991; Vanden Berghe et al., 1993). The antipicornaviral activity of the isolated and synthesized 3-MQ has been tested in vitro on cell lines and in vivo on mice (Van Hoof et al., 1984). In cell cultures of Green monkey kidney cells (VERO cells), 90% inhibition of Poliovirus type I and Coxsackie B4 has been observed at concentrations of about 0.01 μg/ml; the effect remained unchanged upon increasing the concentration of 3-MQ up to 25 μg/ml. At a concentration of 5 μg/ml, 3-MQ has also shown antiviral activity against vascular stomatitis virus. 3-MQ was well tolerated in vitro by VERO cells and human skin fibroblasts over a 5-day exposure, and the 50% cytotoxic concentration (TC_{50}) was determined to be 40 μg/ml (Van Hoof et al., 1984). Deng (1998) reported antipolioviral activity of chemically synthesized 3-MQ in the range of 0.5–10 μg/ml and a TC50 of 25 μg/ml. 3-MQ recently obtained on large-scale by a semi-synthesis from a natural occurring product ‘Rutin’, exhibited activity in the same concentration range (1.0–12.5 μg/ml) against Poliovirus type I, Coxsackie B2 and Human Rhinovirus type 81 (unpublished data).

Studies on the mechanism of antiviral action of 3-MQ have shown that 3-MQ inhibits an early step of virus replication (between 1 and 1.5 h post-infection) reducing viral RNA and protein synthesis (Vlietinck et al., 1986; Vrijsen et al., 1987; Vanden Berghe et al., 1993). 3-MQ did not have any extracellular virucidal effect against Poliovirus and Coxsackie virus (Van Hoof et al., 1984).

Structure–activity relationship studies have shown that 3-methoxyl and 5-hydroxyl groups of the flavone skeleton are necessary for specific antirhinoviral activity, while 3-methoxyl and 4-hydroxyl groups are responsible for the antipolioviral effect (Tsuchiya et al., 1985).

The “common cold” is an universally recognized short illness and a major cause of acute morbidity in all age ranges. The main symptoms involve the upper respiratory tract and the nasal symptoms usually predominate (Chilvers et al., 2001). The rhinoviruses are the major known cause, responsible for one third of the colds in adults (Winther, 1994). There is no efficient specific therapy against the rhinoviruses, which account of more than 100 different immunotypes. Because of its pronounced antipicornaviral and moderate anti-inflammatory and antioxidant activity (Pelzer et al., 1998; De Meyer et al., 1991; Cos et al., 1998), 3-MQ has a potential as an antirhinoviral product for nasal application.

A prerequisite for nasally applied formulations is that drugs and additives in the dosage forms do not interfere with normal nasal functioning, such as the nasal mucociliary clearance system, which is particularly susceptible to damage (Merkus et al., 1991). This can have serious consequences because mucociliary transport is one of the most important local defence mechanisms of the respiratory tract (Jorissen et al., 2000). The coordinated ciliary activity is essential for efficient mucociliary clearance, and the ciliary beat frequency (CBF) is one of the basic functional ciliary parameters (Jorissen et al., 2000). Hence, it is important to investigate the influence of drug formulations on CBF to establish the safety of nasally administered drugs and various formulation excipients such as preservatives, and absorption enhancing compounds (Merkus et al., 2001). Within the framework of the development of a nasal drug delivery system for 3-MQ, we have studied the effect of 3-MQ with or without absorption enhancers (hydroxypropyl-β-cyclodextrin (HP-β-CD) or polysorbate 80) on the CBF of human nasal epithelial cells in vitro.

2. Materials and methods

2.1. Chemicals and materials

3-MQ was synthesized at the Laboratory for Pharmaceutical Technology and Biopharmacy (University of Antwerp, Belgium) based on previously described methods (Boers et al., 1997; Deng et al., 1997; Deng, 1998). The purity of 3-MQ amounted to 94% (HPLC analysis). Pronase XIV, choleratoxin, glycocholate, and penicillin–streptomycin solution (10,000 IU/ml and 10,000 μg/ml, respectively) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). DMEM-Ham's F12 1:1 medium, Ultroser G and NU-serum were obtained from Life Technologies Ltd. (Paisley, UK). HP-β-CD was purchased from Roquette (Lestrem, France), polysorbate 80 from Federa (Belgium), and chlorocresol from UCB (Leuven, Belgium). Tissue culture Iwaki Glass 6-well plates (9.4 cm^2 growth area) were obtained from International Medical (Belgium), and 25 cm^2 tissue culture flasks from Falcon, Becton Dickinson (Oxnard, CA).

2.2. Cell isolation and culture

Surgical specimens of human nasal epithelial tissue were obtained during elective surgery of nasal polyps from seven patients. Human nasal epithelial cells were isolated according to the procedure described by Jorissen et al. (1989), which includes pronase treatment and differential attachment on plastic. The human nasal epithelial tissues were rinsed three times in saline solution (0.9% NaCl) and were enzymatically dissociated using 0.1% pronase solution in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin for a period of 16–24 h at 4 °C. At the end of the pronase incubation, the large pieces of tissue were removed, and the protease activity was inhibited with 10% NU-serum. The cells were washed three times in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2% Ultroser G, and 10 ng/ml choleratoxin by centrifugation (800 rpm, 5 min, 4 °C). After the last centrifugation, the cell pellets were resuspended in 10 ml of the above mentioned complete medium and incubated for 1 h in a 25 cm^2 plastic tissue culture flask in a CO_2 incubator (5% CO_2–95% air, 37 °C) to allow selective attachment of the contaminating fibroblasts and macrophages. The cell number was determined with a Coulter Multisizer counter (Northwell, UK). The cells were plated in 0.2% rat tail collagen pre-coated 6-well plates (106 μl/cm^2) at a density of 5.3×10^4 cells/cm^2 in a final volume of 3 ml medium, and incubated at 37 °C in an atmosphere of 5% CO_2–95% air. The medium was changed 24 h after plating and subsequently every second day. Human nasal epithelial cell cultures were used for CBF measurements at days 6–7 after plating, when microscopically confluent layers, consisting of ciliated and non-ciliated cells were obtained.

2.3. Cell treatment

3-MQ (2, 10, and 20 μg/ml), HP-β-CD (1, 3, and 10% (w/v)), polysorbate 80 (0.1, 0.3, and 1.0% (w/v)), 3-MQ (2–20 μg/ml) with 3% (w/v) HP-β-CD, 3-MQ (2–20 μg/ml) with polysorbate 80 (1% (w/v)), glycocholate (0.5% (w/v)) and chlorocresol (0.005% (w/v)) were dissolved in cell-culture medium. All the solutions were freshly prepared on the day of the experiment. Glycocholate and chlorocresol were used as reference cilio-stimulatory and cilio-inhibitory compounds, respectively.

The cells were preconditioned for 30 min at room temperature (23–24 °C). CBF was measured between 15 and 25 min after exposure to the test compounds (1 ml/well). To investigate whether the effect on CBF is reversible after withdrawal of xenobiotic exposure, the CBF was also determined between 35 and 45 min after rinsing of the cell layer (3 × 1 ml medium in 2 min) and incubation with medium. The control cells were incubated with medium only and treated in the same way.

2.4. Ciliary beat frequency measurement

The CBF was determined at 23–24 °C by a computerized microscope photometry system as described by Jorissen et al. (1989), using Leitz MDV C2 equipment. The signal from the fluctuations of light intensity, caused by beating cilia was transduced to an electrical signal, amplified and transmitted to a personal computer. A light beam with a frequency of 500 Hz was used, and the signal was measured for a period of 1 min. The recorded signal was analyzed by

performing time spectral analysis using Fast Fourier Transformation on the waveform obtained. Ten periods from the signal obtained for 1 min were analyzed for each cell. The highest peak of the first harmonic within these time segments represented the mean beat frequency of the cilia. The CBF of 10 different cells was measured per group in a given experiment.

2.5. Statistical analysis

The results are expressed as percentages of the corresponding control and presented as mean percentage ± S.D. The data were statistically analyzed by Student's *t*-test or one-way ANOVA followed by Dunnett's post-test. $P < 0.05$ was considered as significant. The statistical method used in a given analysis is specified in figure legends.

3. Results and discussion

Different in vitro models of nasal epithelial cells and tissues from humans and animal species have been developed to study the effect of xenobiotics on CBF (reviewed by Agu et al., 2002). Agu et al. (1999) reported the suitability of human nasal epithelial cells cultured in both monolayer and subsequent monolayer-suspension culture systems (Jorissen et al., 1989) for screening the effect of pharmaceutical compounds on ciliary beating. Because similar results have been obtained with both culture systems (Agu et al., 1999), the monolayer culture of human nasal epithelial cells was selected for the purpose of this study. This culture system is less time-consuming, not so complicated, and gives reproducible and reliable results. The main disadvantage of the monolayer cultures is the unstable ciliary activity with time in culture (Yoshitsugu et al., 1994; Rhee et al., 2001; Agu et al., 2001) which requires the cultures to be used for CBF measurement at well defined time point after plating. In order to verify the reliability of the cell-culture system used in this study (human nasal epithelial cells cultured in immersed monolayers for 6–7 days) for testing the effect of xenobiotics on CBF, the effect of reference cilio-stimulatory (glycocholate) and cilio-inhibitory (chlorocresol) compounds was tested. Because it is also important to investigate whether the effect on CBF is reversible after withdrawal of xenobiotic exposure (Hermens et al., 1990), the CBF was measured between 15 and 25 min after exposure to the test compounds and after rinsing and subsequent incubation (35 min) with medium only. These time points were selected, because intranasally applied substances are rapidly cleared from the nose, with a clearance half-life of approximately 15–30 min (Andersen and Proctor, 1983; Soane et al., 1999).

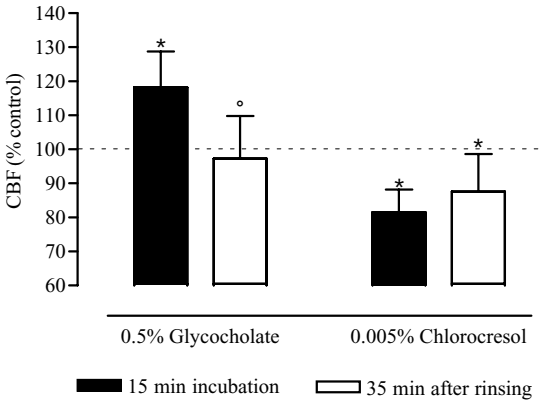

Fig. 2. Effect of 0.5% (w/v) glycocholate (reference cilio-stimulatory compound) and 0.005% (w/v) chlorocresol (reference cilio-inhibitory compound) on CBF of human nasal epithelial cells after 15-min exposure and 35 min after rinsing. The results are expressed as percentages of the corresponding time-point control and presented as mean percentage ± S.D. Control CBF values (Hz): 15 min, 8.9 ± 1.1; after rinsing, 11.0 ± 1.4; $n = 9$–10 cells. $^{*}P < 0.05$ vs. corresponding time-point control, $^{\circ}P < 0.05$ vs. corresponding 15 min; Student's *t*-test.

The absorption enhancer glycocholate (0.5% (w/v)) increased CBF by 18% after 15 min of exposure (Fig. 2). This cilio-stimulatory effect was reversible upon rinsing and incubation with medium for 35 min. These findings were in agreement with the results of Agu et al. (1999). The lipophilic preservative chlorocresol has been shown to impair CBF in chicken and rat tracheal explants and human nasal epithelial cells in vitro (Batts et al., 1990; Joki et al., 1996; Agu et al., 1999). The cilio-inhibitory effect of chlorocresol in these reports varied between 30 and 70%, depending on the concentration(s) tested and the model used. Our results showed a cilio-inhibitory effect (by 19%) of 0.005% (w/v) chlorocresol in human nasal epithelial cells in vitro. The effect was partially reversible. These results and the normal range of mean CBF values measured in control (untreated) cells (9.1 ± 1.7)

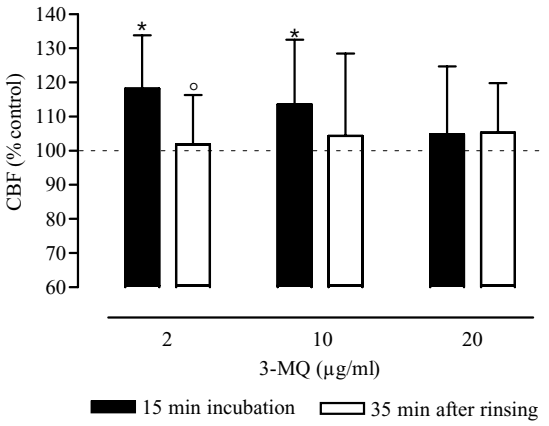

Fig. 3. Effect of 3-MQ (2, 10, and 20 μg/ml) on CBF of human nasal epithelial cells after 15-min exposure and 35 min after rinsing. The results are expressed as percentages of the corresponding time-point control and presented as mean percentage ± S.D. Control CBF values (Hz): 15 min, 7.9 ± 1.3; after rinsing, 9.5 ± 1.9; n = 27–30 cells from three different patients. $^{*}P < 0.05$ vs. corresponding time-point control, one-way ANOVA, Dunnett's post-test; $^{\circ}P < 0.05$ vs. corresponding 15 min; Student's t-test.

confirmed the reliability of the in vitro cell-culture system used for CBF determination.

The effect of 3-MQ on CBF is shown in Fig. 3. At the lowest concentration tested, 3-MQ showed a cilio-stimulatory effect on human nasal epithelial cells in vitro after a 15-min exposure. CBF increased by 18 and 14% at 2 and 10 μg/ml, respectively. This effect was reversible, and after rinsing and incubation with medium the CBF returned to the control level. No significant changes in CBF were observed at 20 μg/ml under the experimental conditions employed. Similar cilio-stimulatory effects of low concentrations of ethanol and capsaicin have been described (Delamanche et al., 2001). The mechanism of the observed cilio-stimulatory effect at low 3-MQ concentrations is not clear, although a speculative suggestion can be made. The ability of quercetin to increase intracellular cAMP concentration has been shown by Mucsi and Pragai (1985) in Hep-2 cells and chicken embryo fibroblasts, and a direct relationship between the antiviral activity of quercetin and its ability to raise the cAMP appeared to exist. This effect has been related to the inhibition by quercetin of cAMP phospodiesterases, enzymes responsible for the degradation of cAMP (Ferrell et al., 1979). Studies on ciliary motility focus on the cAMP pathway and the stimulation of CBF by cAMP analogues (Lansley et al., 1992). Increase in intracellular cAMP has been associated with increased CBF in human respiratory epithelium (Di Benedetto et al., 1991). Wyatt et al. (1998) showed that agents that increase cAMP and activate cAMP-dependent protein kinase (PKA) result in stimulation of CBF in bovine airway epithelium. Therefore, a cAMP-related mechanism of CBF stimulation by 3-MQ could be speculatively suggested.

The small volume (25–200 μl) of the nasally applicable dose necessitates high drug solubility and potency (Tirucherai et al., 2002). Because the water solubility of 3-MQ is low ($S_0 = 21.7$ μg/ml), a pharmaceutical dosage form requires the inclusion of a solubility enhancing agent. In this study, two solubility enhancers (HP-β-CD or polysorbate 80) were evaluated. In addition to enhancing the solubility, they may also promote the absorption of the 3-MQ. Because the enhancers should be devoid of any ciliotoxicity, and because the feasibility of nasal drug administration may depend largely on their effects on the ciliated epithelial tissue (Hermens et al., 1990) the effect of different concentrations of HP-β-CD and polysorbate 80 on CBF was also tested.

Cyclodextrins are capable of forming noncovalent inclusion complexes with lipophilic compounds, resulting in an increased water solubility, enhanced stability, increased bioavailability and therapeutic efficiency of the complexed drug (Hermens and Merkus, 1987; Loftsson and Brewster, 1996; Rajewski and Stella, 1996). In the experimental system of human nasal epithelial cells, HP-β-CD used at concentrations 1 and 3% (w/v) did not affect CBF (Fig. 4A). At a concentration of 10% (w/v), HP-β-CD reduced the CBF by 37% of the control value within 15 min. The cilio-inhibitory effect was completely reversible upon rinsing. Agu et al. (2000) reported a very mild cilio-inhibitory effect of 10% HP-β-CD (by 9%) within 30 min in suspension culture of human nasal epithelial cells. It has been found that the effect of xenobiotics on CBF was more pronounced in monolayer culture in comparison to the suspension culture, which could be explained by the absence of basolateral membrane exposure to test solution in the suspension culture system (Agu et al., 1999). In chicken embryo, trachea in vitro Merkus et al. (1991) reported a 35% decrease in CBF after 10-min incubation with 5%

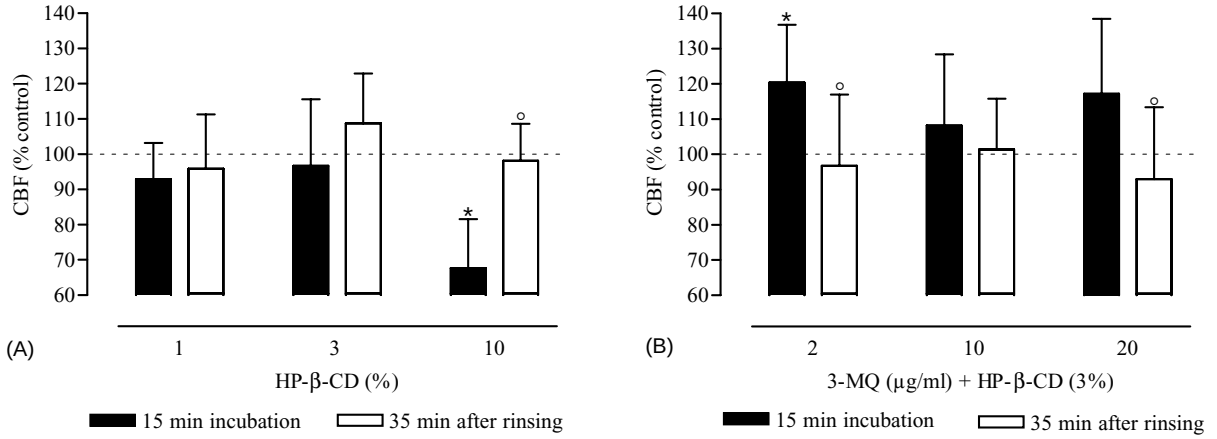

Fig. 4. Effect of HP-β-CD (1, 3, and 10% (w/v)) (A) and the combination of 3% (w/v) HP-β-CD with 3-MQ (2, 10 or 20 μg/ml) (B) on CBF of human nasal epithelial cells after 15-min exposure and 35 min after rinsing. The results are expressed as percentages of the corresponding time-point control and presented as mean percentage ± S.D. (A) Control CBF values (Hz): 15 min, 10.0 ± 1.6; after rinsing, 10.2 ± 1.2; $n = 6$–10 cells. (B) Control CBF values (Hz): 15 min, 7.9 ± 1.3; after rinsing, 10.3 ± 1.4; $n = 9$–10 cells. $^{*}P < 0.05$ vs. corresponding time-point control, one-way ANOVA, Dunnett's post-test; $^{\circ}P < 0.05$ vs. corresponding 15 min; Student's *t*-test.

HP-β-CD. No rat nasal mucosa damage was observed after 30- and 60-min exposure to 10% HP-β-CD (Asai et al., 2002). The reported differences could be attributed to species-related sensitivity as well as to experimental models used. Although the mechanism of cyclodextrins-induced adverse effects has not been well established, the complexation of membrane lipid molecules (e.g. cholesterol) has been suggested to be involved (Rajewski and Stella, 1996).

As 3% (w/v) HP-β-CD did not affect CBF, further studies were performed at this concentration. The effect of different 3-MQ concentrations in the presence of 3% (w/v) HP-β-CD on CBF is shown on Fig. 4B. A reversible increase in CBF by 20% was observed after 15-min incubation with 2 μg/ml 3-MQ and 3% (w/v) HP-β-CD. This effect did not differ from the CBF values measured in the presence of 3-MQ alone, suggesting that the cilio-stimulatory effect of the combination could be attributed to 3-MQ. The combinations of 3% (w/v) HP-β-CD with 10 and 20 μg/ml 3-MQ did not show significant differences in CBF values compared to the control. These results illustrate the safety of the combination of 3-MQ with HP-β-CD and support the use of HP-β-CD in concentrations up to 3% (w/v) as solubility/absorption enhancer in a pharmaceutical preparation for nasal application of 3-MQ.

The non-ionic surfactant polysorbate 80 is used in formulations for oral application. Polysorbate 80 forms micelles in aqueous solution as well as biological fluids (e.g. plasma), and can increase the solubility and stability of drugs entrapped in the micelles (Van Zuylen et al., 2001). Its absorption enhancement properties are also related to modulation of tight junctions and inhibition of the efflux transporter P-glycoprotein (Cornaire et al., 2000). In this study, the effect of polysorbate 80 (0.1, 0.3, and 1% (w/v)) and the combination of 1% (w/v) polysorbate 80 with 3-MQ (2, 10 or 20 μg/ml) on CBF of human nasal epithelial cells was studied (Fig. 5).

Polysorbate 80 applied alone showed a pronounced cilio-inhibitory effect, decreasing CBF values within 15 min by 40, 53, and 49% compared to the control at polysorbate 80 concentrations of 0.1, 0.3, and 1% (w/v), respectively (Fig. 5A). CBF recovered after rinsing and did not differ significantly from the control values after 35-min incubation with medium, showing the reversibility of the cilio-inhibitory effect. Although a reversible effect was observed, the inhibition of CBF at very low polysorbate 80 concentrations (0.1% (w/v)) could be of clinical significance, particularly if chronic nasal drug application is required. Severe nasal damage has been described in rats in vivo, after nasal application of the non-ionic

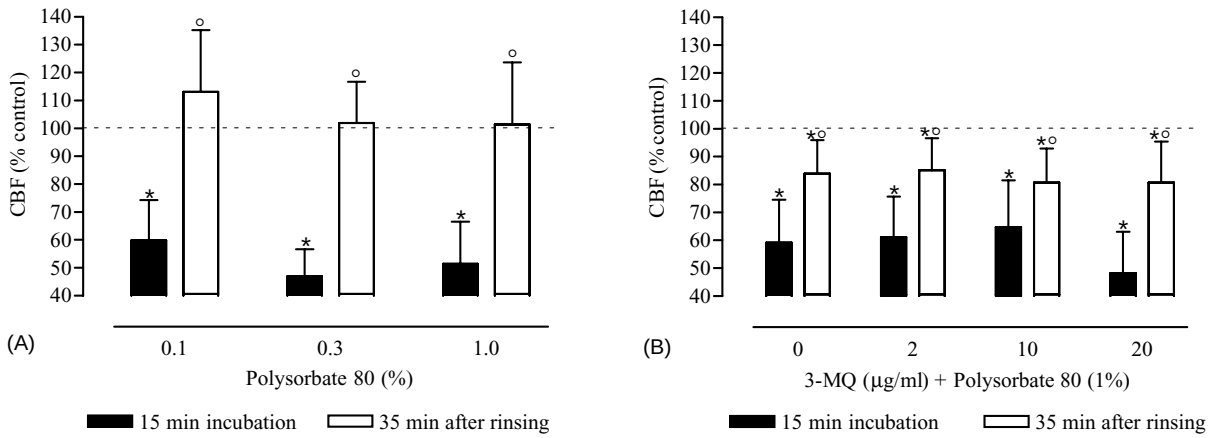

Fig. 5. Effect of polysorbate 80 (0.1, 0.3, and 1% (w/v)) (A) and the combination of 1% (w/v) polysorbate 80 with 3-MQ (2, 10 or 20 μg/ml) (B) on CBF of human nasal epithelial cells after 15-min exposure and 35 min after rinsing. The results are expressed as percentages of the corresponding time-point control and presented as mean percentage ± S.D. (A) Control CBF values (Hz): 15 min, 9.2 ± 1.7; after rinsing, 8.5 ± 1.6; $n = 10$ cells. (B) Control CBF values (Hz): 15 min, 8.3 ± 1.5; after rinsing, 10.0 ± 1.2; $n = 10$ cells. $^{*}P < 0.05$ vs. corresponding time-point control, one-way ANOVA, Dunnett's post-test; $^{\circ}P < 0.05$ vs. corresponding 15 min; Student's *t*-test.

surfactant polyoxyethylene-9-lauryl ether (Zhou and Donovan, 1996).

The incubation of human nasal epithelial cells with the combination of 1% (w/v) polysorbate 80 and 3-MQ (2, 10 or 20 μg/ml) decreased CBF significantly at all 3-MQ concentration tested (Fig. 5B). This effect was partially reversible and can be associated with the presence of polysorbate 80 in the incubation medium. Polysorbate 80 (1% (w/v)) alone inhibited CBF by 41%, and this effect did not change in the presence of 3-MQ. The different degree of reversibility of the cilio-inhibitory effect induced by polysorbate 80 observed in the two experiments (Fig. 5A and B) could not be explained, but might be due to inter-individual variability in the sensitivity to polysorbate 80 induced ciliary effects.

The in vitro and in vivo comparison of CBF measurements often shows that the deleterious effects of topical drugs seen in vitro, cannot be demonstrated in vivo. This could be due to the fact that in vitro, the cells are totally immersed in the tested compound, while in vivo the ciliated epithelium is protected by the mucus barrier and mucociliary clearance (Van der Baan, 2000; Merkus et al., 2001). This difference may lead to an overestimation of the in vitro toxicity of the test compounds. Nevertheless, the in vitro methods remain a valuable screening technique at an early stage in the process of discovery and development of safe nasal drug formulations.

In conclusion: (i) no ciliotoxic effect could be observed for 3-MQ (up to 20 μg/ml) in the absence or presence of HP-β-CD (3% (w/v)); (ii) the safety of HP-β-CD (3% (w/v)) as solubility/absorption enhancer in pharmaceutical preparations for nasal application has been confirmed; and (iii) polysorbate 80 cannot be recommended as a component of formulations for nasal application. These results have shown the potential of 3-MQ alone or in combination with the absorption enhancer HP-β-CD as an antirhinoviral compound for nasal application.

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