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Safety assessment of selected cyclodextrins — effect on ciliary activity using a human cell suspension culture model exhibiting in vitro ciliogenesis

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Abstract

The objective of this study was to assess the cilio-inhibitory effect of a series of cyclodextrins using a human cell suspension culture system exhibiting in vitro ciliogenesis. Enzymatically released human nasal epithelial cells were cultured as sequential monolayer-suspension culture showing in vitro ciliogenesis. Ciliary beat frequency (CBF) was determined by computerized microscope photometry. Among the cyclodextrins investigated (γ-cyclodextrin, hydroxypropyl-β-cyclodextrin, anionic-β-cyclodextrin polymer, dimethyl-β-cyclodextrin and α-cyclodextrin), it was shown that after 30 min of exposure, γ-cyclodextrin (10% w/v), hydroxypropyl-β-cyclodextrin (10.0% w/v) and anionic-β-CD polymer (8.0% w/v) were not significantly cilio-inhibitory ($P > 0.05$). Similarly, CBF remained stable upon cell exposure to α-cyclodextrin (2.0% w/v) and dimethyl-β-cyclodextrin (1.0% w/v). However, higher concentrations of α-cyclodextrin and dimethyl-β-cyclodextrin resulted in mild to severe cilio-inhibition after 45 min of exposure. The effect of α-cyclodextrin (5.0% w/v; $54 \pm 4\%$ cilio-inhibition) was partially reversible while dimethyl-β-cyclodextrin (10% w/v; $36 \pm 4\%$ cilio-inhibition) was irreversible. The cilio-inhibition observed in this model was lower than reported for chicken trachea model. Given the fact that (1) irreversible cilio-inhibition observed in this study occurred only at concentrations exceeding those used in pharmaceutical formulations and/or at an unusual exposure time (45 min) and that (2) in an in vivo situation, dilution and mucociliary clearance contribute to further decrease in local concentrations of the applied compound, the results of this study confirm the safety of the cyclodextrins investigated as nasal absorption enhancers.

Keywords: Ciliary beat frequency; Cilio-toxicity; Cell culture; Absorption enhancers; Cyclodextrins

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1. Introduction

Since several years, intranasal administration of drugs is considered as an interesting route of extravascular delivery of drugs. It is especially considered as a feasible alternative to invasive parenteral administration. The nasal route is convenient to patients, thus increasing patients' compliance especially for patients on long-term medication (Quraishi et al. 1997). In addition, rich vasculature, high permeability of the nasal mucosa, and avoidance of hepatic and gastrointestinal metabolism (first pass effect) are obvious advantages for nasal drug administration. However, enzymatic degradation, the presence of a physical barrier imposed by mucus and nasal epithelium, as well as mucociliary clearance interferes with nasal drug absorption. For protein based drugs, there is also a possibility of peptide complexation by immunoglobulins leading to the formation of a complex with an increased molecular weight and thus lower transport (Duchêne and Ponchel, 1993).

In order to achieve adequate systemic delivery of proteins, peptides and other high molecular weight compounds through the nasal route, many compounds of widely varying chemical structures including bile acids, fusidate derivatives, fatty acids, phospholipids, cyclodextrins and chitosans have been investigated as potential nasal absorption enhancers. Ideally, absorption promoters should have an immediate and predictable duration of action; their effect on the barrier properties of the nasal mucosa should be reversed to normal following absorption of the drug, they should be systemically inert and devoid of any toxic, irritating or allergic activity; they should also not permit entry of potentially dangerous environmental materials and should be compatible with drugs and adjuvants in the preparation (Schipper et al., 1992). Consequently, absorption enhancement and toxicity are the key issues in the search and design of effective and safe drug formulations for the nasal route.

Different models of nasal epithelial cells from both human and animal species are currently used to study cilio-inhibition or toxicity of drugs and excipients in vitro (Van de Donk et al., 1980; Sisson et al., 1995; Joki et al., 1996). Loss of tissue viability with time, vestige of surgical trauma and anesthesia, and availability of human tissue are important considerations when using such models. Human adenoid tissue and chicken embryo trachea have been used to assess the effect of absorption enhancers on ciliary activity. (Hermens et al., 1990; Schipper et al., 1992); a correlation has been found between the use of human adenoid tissue and chicken embryo trachea to study the effect of pharmaceutical compounds on ciliary activity (Van de Donk et al., 1980). Extrapolating results obtained in laboratory animals to human airways could at times be problematic because of differences in animal and human airways (Riechelmann et al., 1994).

Culturing human nasal epithelial cells with techniques that allow long-term preservation of ciliary beating at a physiological range may alleviate some of the limitations associated with the use of intact tissue. For instance measuring ciliary activity in vitro after ciliogenesis using the sequential monolayer-suspension culture system offers the possibility for long-term study of the cilio-inhibitory effect of drugs and excipients because of preservation of ciliary beating in vitro for as long as 6 months. Also, the high reproducibility of the method, the normal qualitative and quantitative ciliary activity, the absence of cells other than epithelial cells and great quantity of cells that can be obtained from small biopsies are obvious advantages. (Jorissen et al., 1989, 1992; Jorissen and Bessems, 1995a,b). In a previous study, the monolayer and sequential monolayer-suspension culture systems were validated for the assessment of cilio-toxicity of pharmaceutical compounds (Agu et al., 1999).

Neither short nor long-term cell culture systems of human nasal epithelium has been exploited to investigate the cilio-inhibition or toxicity of absorption enhancers.

This study was aimed at assessing the effects of a series of cyclodextrins on ciliary beating at a cellular level using a cell suspension culture system. Cyclodextrins were chosen as representative absorption enhancers because of their prospects as useful pharmaceutical excipients for nasal drug delivery. The usefulness of these compounds

stems from the fact that cyclodextrins offer several advantages for drug delivery which include improved drug solubilization, protection against physicochemical and enzymatic degradation, as well as the potential for improved absorption (Merkus et al., 1999).

2. Materials and methods

2.1. Chemicals

Dimethyl-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, anionic polymeric soluble-β-cyclodextrin, α-cyclodextrin and γ-cyclodextrin were obtained from AVEBE (Veendam, The Netherlands), Fluka (Buchs, Germany), Cyclolab (Budapest, Hungary), Sigma (St. Louis, MO), and Across (Geel, Belgium), respectively.

2.2. Cell culture procedure

The cells were cultured as described previously (Jorissen and Bessems, 1995a). Human nasal epithelial tissues harvested during elective surgery from healthy patients were washed three times in physiological saline supplemented with 50 μg/ml streptomycin and 50 IU/ml penicillin (Boehringer Mannheim, GmbH, Germany). Subsequently, the cells were dissociated enzymatically overnight under continuous rotation at 4°C using 0.1% pronase (Sigma) in DMEM-F12 1/1 (Life Technologies, Paisley, UK). Deactivation of pronase was achieved using 10% NU-serum before washing the cells with the monolayer culture medium [DMEM-F12 1/1 supplemented with 2% Ultroser G (Life Technologies, Paisley, UK), 10 ng/ml choleratoxin (Sigma), 50 μg/ml streptomycin, and 50 IU/ml penicillin]. The cells were washed three times with the monolayer medium before recovery by centrifugation at 800 rpm for 5 min. Seeding the cells on plastic for 1 h at 37°C and 5% CO_2 reduced the level of fibroblast contamination by selective attachment of fibroblasts to plastic.

Subsequently, cells were plated at a density of 5×10^5 cell/cm^2 in T 75 tissue culture flasks (Falcon, Oxnard, CA) coated with 0.2% collagen gel (extracted from rat tails) and containing 15 ml monolayer culture medium. The medium was changed one day after plating and subsequently three times a week. After 3 weeks of cell growth and de-differentiation in the monolayer culture, the cells were released from the collagen gel with 200 IU/ml-collagenase type IV (Worthington Biochemical Corporation, Freehold, NJ). The resulting epithelial clusters of cells were washed three times in the monolayer medium followed by centrifugation at 800 rpm for 5 min and were further cultured in several T 25-tissue flasks. In order to avoid cell attachment to plastic, the cells were maintained on a gyrotory shaker at 80 rpm for 1 week. In subsequent weeks, the medium consisted of DMEM-F12 1/1 (Life Laboratories, Paisley, UK) supplemented with 10% NU-serum. This medium was changed daily during the first week of suspension culture and three times a week after this period. Regeneration of cilia started after 10 days in the suspension culture. Throughout the cell culture duration, the cells were incubated at 37°C in a 5% CO_2 atmosphere.

2.3. Scanning electron microscopy

The cells were fixed in glutaraldehyde 3.0% in 0.1 M sodium cacodylate buffer at pH 7.4 for 2 h. This was followed by dehydration in a graded ethanol series. The dehydration process was completed by a critical point drying (E300-polaron) with CO_2.

Subsequently, specimens were mounted on aluminum stubs, sputter coated with gold (E5100-polaron), and viewed with a scanning electron microscope (Philips XL20, The Netherlands).

The cell aggregates in suspension culture before and after in vitro ciliogenesis are shown in Fig. 1.

2.4. Preparation of solutions

Solutions of cyclodextrins were prepared by dissolving the required amount of the compound in Ham's DMEM-F12 1/1, pH 7.3 or by spiking with a stock solution of the substance. The final concentrations were expressed in percentage weight-volume (% w/v). DMEM-F12 1/1 served as a control solution in all CBF measurements.

2.5. Ciliary beat frequency (CBF) measurements

Ciliary beat frequency of the cells was determined in T25 tissue culture flasks by computerized microscope photometry (Jorissen et al., 1992). In order to perform the measurements, the control and cyclodextrin solutions were removed just before the measurement leaving the cells adhered to the tissue flask. The CBF of different cells were measured before and after exposure to the cyclodextrin solution at room temperature. Reversibility of cilio-inhibition was determined by washing the cells with DMEM-F12 1/1 for 10 min after exposure to cyclodextrin solution.

Using a sampling frequency of 500 Hz a signal was recorded for a period of 1 min. The signal was analyzed using Fast Fourier Transform. This analysis involved splitting of the signal into periods of 5.12 s, which was then analyzed in ten consecutive periods (a total of 300 data points were obtained for the three batches of each condition investigated). The first harmonic within the time fractions, which represented the mean beat frequency of the cilia, was taken as the ciliary beat frequency. The exposure periods ranged from 30 to 45 min. The degree of CBF change caused by the different concentrations of the tested substances/conditions was classified as follows:

- no effect: $< 0\%$ or statistically insignificant
- mild: 10–20% cilio-inhibition and statistically significant
- moderate: 20–50% cilio-inhibition and statistically significant
- severe: $> 50\%$ cilio-inhibition and statistically significant.

Reversibility of effect (the degree of CBF change) after washing out the test substance was classified as irreversible ($> 50\%$ and statistically significant), partially reversible (10–50% and statistically significant) and reversible ($< 10\%$ and statistically insignificant).

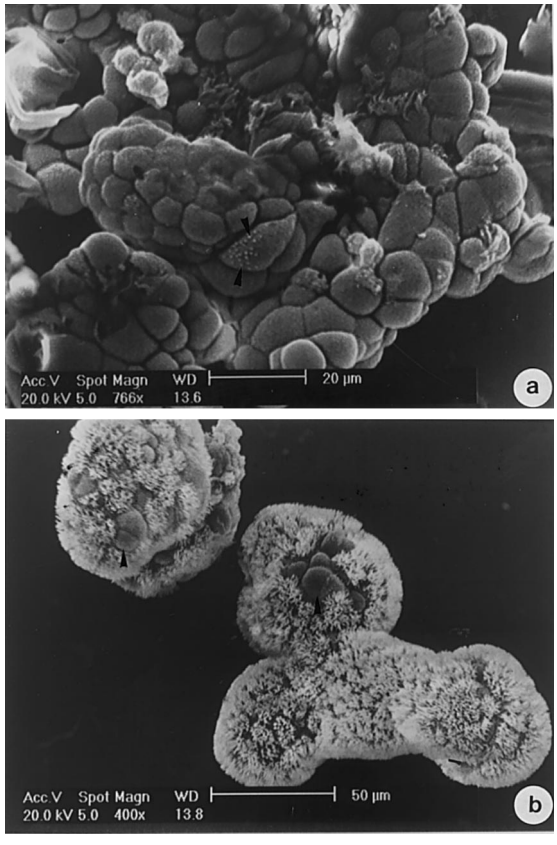

Fig. 1. Monolayers of human nasal epithelial cell aggregates: (a) 1 week in suspension culture, the cells are non-ciliated; (b) 3 weeks in suspension culture, most of the cells are ciliated.

2.6. Statistical analysis and data presentation

For all investigations, the CBF of 30 individual cells was measured (in three batches of 10 cells per batch) before and after exposure to cyclodextrin solution. The mean CBF change after compound exposure was expressed as a percentage $\pm$ SEM ($n = 30$ cells) relative to CBF of the cells before cyclodextrin exposure. Differences between control and treated cells before and after compound exposure was determined by Mann-Whitney U test for unpaired data, and $p < 0.05$ was considered significant.

3. Results

The effect of various cyclodextrins on the ciliary beating of cells in the suspension culture is summarized in Table 1. Exposure to 1–10% w/v γ-cyclodextrin and hydroxypropyl-β-cyclodextrin for 30–45 min did not result in a significant reduction in CBF ($P > 0.5$). γ-cyclodextrin was

Table 1
Effect of cyclodexterins on CBF of human nasal epithelial cells.

Compound	Concentration (% w/v)	% CBF change relative to control (± SEM, $n = 30$ cells)		
		After 30 min exposure	After 45 min exposure	After washing
γ-Cyclodextrin	2	105 ± 4	105 ± 6	121 ± 8
	4	103 ± 2	100 ± 2	119 ± 7
	5	101 ± 2	102 ± 3	116 ± 7
	8	103 ± 4	100 ± 2	115 ± 10
Hydroxypropyl-β-cyclodextrin	1	102 ± 1	103 ± 2	107 ± 1
	2	103 ± 5	95 ± 5	106 ± 4
	3	104 ± 1	103 ± 4	114 ± 3
	4	94 ± 2	90 ± 3	100 ± 3
	5	100 ± 5	98 ± 9	113 ± 6
	10	91 ± 8	90 ± 6	113 ± 7
Anionic-β-cyclodextrin polymer	0.5	103 ± 5	101 ± 5	112 ± 10
	1	97 ± 3	95 ± 9	100 ± 9
	2	97 ± 6	92 ± 4	106 ± 6
	4	101 ± 7	101 ± 4	124 ± 7
	8	93 ± 2	86 ± 4	106 ± 1§
Dimethyl-β-cyclodextrin	1	99 ± 5	97 ± 7	117 ± 7
	2	87 ± 5*	86 ± 4*	102 ± 5§
	3	88 ± 1*	83 ± 4*	95 ± 6§
	4	85 ± 5*	88 ± 3*	103 ± 5§
	5	84 ± 2*	86 ± 1*	90 ± 2§
	10	79 ± 2**	64 ± 4**	55 ± 1‡
α-Cyclodexterin	1	107 ± 6	116 ± 3	127 ± 4
	2	97 ± 9	91 ± 10	102 ± 8
	3	75 ± 1**	77 ± 5**	85 ± 6§
	4	77 ± 9**	73 ± 6**	83 ± 6§
	5	46 ± 4***	46 ± 3***	65 ± 4†

* Mild.
** Moderate.
*** Severe.
§ Reversible.
‡ Irreversible.
† Partially reversible.

found to be the least cilio-inhibitory of the cyclodextrins investigated: ciliary beating maximally decreased from basal CBF of 100% to 97 ± 5% after exposure to 10.0% w/v γ-cyclodextrin for 30 min. Hydroxypropyl-β-cyclodextrin (10.0% w/v) reduced CBF to 90 ± 6% after 45 min exposure. Anionic-β-cyclodextrin polymer (8.0% w/v) had a mild effect on CBF only after 45 min exposure: CBF reversibly decreased to 86 ± 4% within this period.

In contrast to CBF stability observed with the above mentioned compounds, α-cyclodextrin and dimethyl-β-cyclodextrin moderately inhibited CBF in a concentration dependent way. No effect was observed after exposure to 1.0%w/v dimethyl-β-cyclodextrin, while 2–5.0% w/v resulted in mild cilio-inhibition; at a concentration of 10.0% w/v, 21 ± 2% and 36 ± 4% decrease in CBF was observed after 30 and 45 min incubation, respectively. At this concentration (10.0%w/v), it was observed that cilio-stasis occurred in some cells while other cells remained vibrant with high CBF. The reversibility of cilio-inhibition caused by dimethyl-β-cyclodextrin was concentration depen-

dent; the effect of concentrations lower than 10% w/v was reversible after washing with DME F12 1/1.

The effect of α-cyclodextrin on CBF ranged from no effect up to 2.0% w/v to a severe effect at 5.0% w/v. Up to a concentration of 2.0% w/v, no effect was seen after 45 min exposure ($P > 0.05$). Increasing the concentration resulted in significant CBF decrease; exposure to 3.0% w/v decreased CBF by $23 \pm 5\%$, while 5.0% w/v caused $54 \pm 4\%$ cilio-inhibition after 45 min exposure. The inhibition because of α-cyclodextrin (5.0%w/v) was partially reversible.

4. Discussion

The toxicity of absorption enhancers has been the subject of various investigations, dealing especially with the possibility of membrane lesion and the effect on ciliary movement (Ennis et al., 1990; Hermens et al., 1990; Duchêne and Ponchel, 1993; Marttin et al., 1995). In the present study, a human nasal epithelial cell culture system showing ciliogenesis in vitro was used to screen the cilio-inhibitory effect of a series of cyclodextrins. The choice of this culture system was based on the morphological and functional resemblance with nasal epithelium in vivo and the results of an earlier validation study (Agu et al., 1999).

In our culture system, the cilio-inhibitory effect of cyclodextrins, though similar in trend, was not as pronounced as reported in literature for the chicken trachea and human adenoid tissue models. A maximum of $36 \pm 5\%$ decrease in CBF was observed on exposing the cells to 10% w/v dimethyl-β-cyclodextrin for 45 min. It is important to note that at this concentration, the CBF of more than half of the cells was arrested within this period, while the remaining cells maintained relatively high CBF. According to Schipper et al. (1992), 2% dimethyl-β-cyclodextrin resulted in 60% decrease in CBF of both human and chicken embryo trachea cilia after 60 min exposure, while a higher concentration (5%) induced reversible cilio-stasis within 30–60 min exposure. The authors reported that human adenoid tissue was more resistant to the inhibitory effect of this compound than chicken embryo trachea. Similarly, CBF reduction of approximately 50% occurred upon exposing the cultured human nasal epithelial cells to α-cyclodextrin (5%) for 45 min. Cilio-stasis was reported for the compound (5%) in chicken trachea within 50 min incubation period (Merkus et al., 1993). Hydroxypropyl-β-cyclodextrin (10%) caused approximately 10% decrease in CBF within 45 min exposure in cultured cells; a maximum of 22% decrease in CBF of chicken embryo was reported for 10% hydroxypropyl-β-cyclodextrin (Merkus et al., 1993). The absence of cilio-inhibition observed for γ-cyclodextrin using the suspension culture system also concurred with the results of Merkus et al. (1993). No information exists in literature on the effect of soluble-β-cyclodextrin polymer on CBF.

The effect of the cyclodextrins on CBF should be interpreted on an individual basis, and not in comparison to other cyclodextrins as they have completely different solubilizing and absorption enhancing properties.

The lower degree of cilio-inhibition observed in the sequential monolayer-suspension culture in comparison to intact tissues in vitro may be explained by possible species differences and differences in permeability of intact and cultured cells. It has been shown that opening of cellular tight junctions without a significant impairment of cell membrane is also a mechanism of absorption enhancement by cyclodextrins (Haeberlin et al., 1996); so differences in cell architecture in the various systems may contribute to a difference in effect of the compounds. As it is known that the tight junctions between goblet cells or between goblet cells and ciliated columnar cells are less tight than the junctions between ciliated columnar cells (Inagaki et al., 1985; Schmidt et al., 1998), and as it has been shown that in the sequential monolayer-suspension culture system the cell architecture consists of a monolayer of ciliated and non-ciliated columnar cells, which form the wall of the cell aggregates on the lateral membrane (Jorissen et al., 1989), it is likely that the 'tighter junctions' formed between these cells may account for lower cilio-inhibition observed in this culture system. It is also important to mention that surgical trauma may predispose intact tissues to higher

cilio-inhibition upon exposure to chemical compounds.

The trend in cilio-inhibitory effects of cyclodextrins in the suspension culture system are also congruent with in vivo studies. In an in vivo model using rat nasal epithelium, Marttin et al. (1995) estimated the level of epithelial toxicity on exposure to cyclodextrins by estimating the release of marker compounds; the effects of hydroxypropyl-β-cyclodextrin (5.0%) and dimethyl-β-cyclodextrin (2.0%) were not significantly different from control (physiological saline); only dimethyl-β-cyclodextrin (5.0%) was found to release more marker compounds. Also, in acute cilio-toxicity studies using rotifers described by Adriaens et al., (1997), relatively low cilio-inhibition was exhibited by cyclodextrins.

Comparing the histological toxicity of absorption enhancers and their inhibitory effect on CBF, it appears that enhancers with a mild effect on tissue morphology also show minor influences on CBF (Merkus et al., 1991). This is an indication that in vitro CBF studies are a useful tool for screening cilio-toxicity of drugs and excipients.

It is important when considering the cilio-inhibitory effect of cyclodextrins and other excipients to relate the concentrations at which cilio-inhibition is observed to concentrations of these used in pharmaceutical formulations. This is pertinent given the fact that some of these compounds are effective nasal absorption enhancers at low concentrations that do not significantly impair ciliary activity. Cilio-inhibition that may occur at low concentrations of these compounds is usually reversible. For instance, dimethyl-β-cyclodextrin, one of the most effective methylated-β-cyclodextrin derivatives is used at concentrations ranging from 2 to 5%, while hydroxypropyl-β-cyclodextrin may be employed at concentrations as high as 10% without cilio-inhibition (Merkus et al., 1999).

5. Conclusions

The present study demonstrates the pharmaceutical application of a cell culture model to investigate the effect of absorption enhancers (cyclodextrins) on ciliary activity. Given the fact that:

1. irreversible cilio-inhibition observed in this study occurred only at concentrations exceeding those used in pharmaceutical formulations and/or at an unusual exposure time (45 min)
2. in an in vivo situation, dilution by mucus layer and mucociliary clearance contribute to further decrease in local concentrations of the applied compound, this study confirms, from a mucociliary point of view, the safety of the cyclodextrins investigated as nasal absorption enhancers.

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