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The effects of calcitonin nasal preparations and their excipients on mucociliary clearance in an ex-vivo frog palate test

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Abstract—The topical tolerability of the commercial preparation of 1–7 Asu-eel and salmon calcitonin with 2% ammonium glycyrrhyzinate and 0·01% benzalkonium chloride, respectively, and of their excipients mixture in solution with increasing concentrations of ammonium glycyrrhyzinate and benzalkonium chloride, respectively, were assessed by investigating their effects on the mucociliary transport velocity in the ex-vivo frog palate preparation. This preparation provides an integrated biological model readily usable in the laboratory which closely resembles human nasal mucociliary clearance mechanism and can be used for rapid testing and toxicity of agents proposed for topical administration in the upper and lower airways. Frog-Ringer control, 1–7 Asu-eel and salmon calcitonin commercial spray preparations and the excipients plus 2% ammonium glycyrrhyzinate and plus 0·01% benzalkonium chloride did not modify significantly the mucociliary transport velocity, confirming their very good tolerability on ciliated epithelium. Higher concentrations of ammonium glycyrrhyzinate (10 and 20%) caused significant slowing, on average −32 and −55%, respectively. Higher concentrations of benzalkonium chloride (0·05 and 0·1%) also caused significant slowing, on average, −43·5 and −87%, respectively.

Several peptide hormones, such as luteinizing hormone releasing hormone (LHRH), growth hormone, insulin, adrenocorticotropic hormone (ACTH), and calcitonin, are used to treat various pathologies. Because of the peptide structure of these substances, oral administration is generally not feasible since they are broken down in the gastrointestinal tract (Azria 1988). Therefore, these hormones are administered parenterally and because of the long duration of these therapies, a need for daily injection can reduce patient compliance, and increase the incidence of side-effects (Pontiroli et al 1989). For these reasons, other routes of administration have been tried and the intranasal one has recently been shown to be suitable. In recent years, spray preparations that can be administered nasally have been developed. These preparations have received much attention, since absorption of the drug through the nasal mucosa is rapid and patient compliance is improved (Buclin et al 1987). The nasal mucociliary clearance mechanism should be affected as little as possible by nasal medications (Van de Donk 1983), and the effects of the drug itself and of the different additives present in the nasal spray preparations on nasal mucosa should be investigated to evaluate their local tolerability.

The frog palate preparation is an ex-vivo model commonly used to study mucociliary transport. The epithelium of this experimental preparation is ciliated and covered with mucus, thus it is similar to mammalian nasal and tracheobronchial mucosa. In addition, many studies have shown the usefulness of this kind of preparation for evaluating the effects of agents that act on mucus or on ciliary beating (King et al 1974; Puchelle et al 1970; Braga 1981). Moreover, the mucociliary transport velocity (MTV) measured by the frog palate test has recently been proposed as an indication of topical tolerability of different substances (Braga et al 1990). On the basis of the above mentioned considerations, the topical effect of an intranasal spray preparation of Asu-eel calcitonin and of its excipients in solution with increasing concentrations of the ammonium glycyrrhyzinate was assessed. Comparison was also made with an intranasal spray preparation of salmon calcitonin and of its excipients in solution with increasing concentrations of benzalkonium chloride.

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Materials and methods

The MTV of the mucosa of the frog palate was measured as described by Puchelle et al (1970) and Braga (Braga 1981; Braga et al 1990) and modified as described below.

Frogs (*Rana esculenta*) were first pithed and then the palate of the animal was excised and placed in a transparent plexiglass chamber, constructed with a double wall to allow the control of the internal temperature by circulation of hot water. The floor and internal walls were covered with pads of wet gauze to maintain the humidity at 100%.

The experimental conditions were: relative humidity 90–100%, temperature 28 ± 0·5°C. By means of a window at the top of the chamber, the preparation can be observed with a stereomicroscope equipped with a micrometric eyepiece to measure mucociliary clearance. Manipulations are carried out through glove openings on both sides of the chamber. The rate of mucociliary transport was measured with mucus rheologically simulating substance (egg albumin) adjusted to a viscosity of 2·7 ± 0·42 Pa s by means of evaporation obtained by exposure for 4–6 h at a temperature of 35°C with continuous air vacuum pump aspiration.

A small drop of this mucus-simulating agent (5–10 μL) was carefully placed on a selected point of the palate and on this surface a small aluminium disk (0·6 mm diam., 0·07 mg) was placed as a marker. The time for the disk to move 5 mm on the frog palate measured through a micrometer ocular was used to compute the MTV. After 30 min, on average, the preparation was stable and the experiment could be started. There was no depletion of native mucus. Three measurements (one every 5–8 min) were carried out and the mean of these measurements was used as the basal MTV value. The agent under investigation was placed in contact with the palate by dropping a volume of 30 μL using a Hamilton syringe exactly onto the 5 mm of palate under observation and left there for 10 min. The test substance was carefully removed by gravity drainage of the palate followed by lavage with amphibian Ringer solution with subsequent absorption of excess liquid onto peripherally placed filter paper; three additional measurements of MTV were carried out.

Drugs. The materials tested were: 1–7 Asu-eel calcitonin intranasal spray preparation (ECT) (40 000 int. units 1–7 Asu-eel calcitonin, 600 mg sodium chloride, 463 mg sodium citrate, 130 mg methyl-*p*-hydroxybenzoate, 37 mg citric acid, 20 mg propyl-*p*-hydroxybenzoate, 2 g ammonium glycyrrhyzinate, sodium hydroxide as needed to pH 6, distilled water to make up the volume to 100 mL).

The excipients mixture, without 1–7 Asu-eel calcitonin, was also tested in solutions containing 2, 10 and 20 g of ammonium glycyrrhyzinate (2, 10 and 20%, respectively). The other materials tested were: salmon calcitonin intranasal spray preparation (SCT) (55 000 int. units salmon calcitonin, 850 mg sodium chloride, HCl as needed to pH 4·5, 10 mg benzalkonium chloride, distilled water to make up the volume to 100 mL); the

Table 1. Mean values (±s.d.) of mucociliary transport velocity tested on frog palate (n = 6 × 2 for each drug solution) before and after the challenge with the formulation under test.

Preparations	Mucociliary transport velocity (mm s^{-1}) Basal	Final	P
Frog-Ringer	0·25 (±0·05)	0·23 (±0·04)	0·17
ECT	0·26 (±0·03)	0·24 (±0·03)	0·67
Exc. + amm. glyc. (2%)	0·21 (±0·05)	0·21 (±0·05)	0·83
Exc. + amm. glyc. (10%)	0·25 (±0·06)	0·17 (±0·06)	0·0002
Exc. + amm. glyc. (20%)	0·22 (±0·04)	0·10 (±0·02)	0·0007
SCT	0·29 (±0·01)	0·27 (±0·01)	0·09
Exc. + benz. chlor. (0·01%)	0·22 (±0·06)	0·20 (±0·06)	0·06
Exc. + benz. chlor. (0·05%)	0·23 (±0·07)	0·13 (±0·04)	0·003
Exc. + benz. chlor. (0·1%)	0·23 (±0·06)	0·03 (±0·06)	0·0002

ECT = complete formulation of 1-7 Asu-eel calcitonin, Exc. + amm. glyc. = excipients mixture without 1-7 Asu-eel calcitonin plus ammonium glycyrrhyzinate, SCT = complete formulation of salmon calcitonin, Exc. + benz. chlor. = excipient mixture without salmon calcitonin plus benzalkonium chloride.

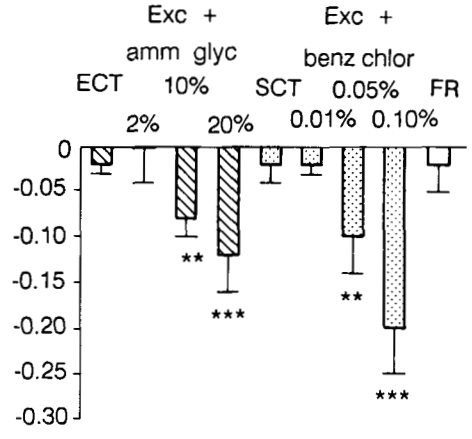

FIG. 1. Mucociliary transport velocity (mean ± s.d.) differences after challenge with the formulation under test vs basal values. $^{**}P < 0{\cdot}05$, $^{***}P < 0{\cdot}001$. For abbreviations, see Table 1.

same excipients as above without salmon calcitonin but with 10, 50 and 100 mg benzalkonium chloride (0·01, 0·05 and 0·1%, respectively).

Frog-Ringer adjusted to the same pH as used in the test solutions was also tested as the control.

The study was performed blind and each drug preparation was randomly tested on six frog palates (in duplicate).

Statistical analysis. Statistical analysis by Student's *t*-test for paired data was used for comparison of two means.

Results

The results are shown in Table 1 and Fig. 1. No statistically significant decrease of MTV was observed with 1-7 Asu-eel calcitonin intranasal spray preparation (−7·7%) or with the formulation containing 2% ammonium glycyrrhyzinate; the same behaviour was observed with salmon calcitonin intranasal spray preparation or with the formulation of benzalkonium chloride 0·01%.

A progressive decrease of MTV induced by increasing concentrations of ammonium glycyrrhyzinate was observed; the formulation of the excipients without Asu-eel calcitonin but with 10 and 20% of ammonium glycyrrhyzinate induced a statistically significant (−32%, $P < 0{\cdot}001$ and −55%, $P < 0{\cdot}001$, respectively) decrease in the MTV.

Statistically significant decreases of MTV were also observed with 0·05 and 0·1% concentrations of benzalkonium chloride (−43·5%, $P < 0{\cdot}05$ and −87%, $P < 0{\cdot}001$, respectively).

The frog-Ringer buffer produced no significant decrease of MTV.

Discussion

The ex-vivo frog palate model is a suitable preparation to study mucociliary transport (Braga 1981; Braga et al 1990). The possibility that the volume of liquid of the preparation might physically dilute mucus lying on ciliated epithelium, thus slowing mucociliary transport and causing incorrect interpretation of the data, was tested by using the same volume of frog Ringer for the challenge and no significant slowing down of rheological properties of mucus-simulating agent was observed, confirming that the volume used is small enough not to interfere with the viscoelasticity of native mucus, the biological variability of which is within 10–15%.

The commercial preparation of 1-7 Asu-eel calcitonin and the formulation with excipients plus 2% ammonium glycyrrhyzinate confirm their very good tolerability in this experimental model, producing no significant variation of MTV.

The same is true for the commercial preparation of salmon calcitonin and the formulation with excipients plus 0·01% benzalkonium chloride. On the other hand, our experimental observations give evidence that increasing concentrations of ammonium glycyrrhyzinate produce depression of ciliary motility in a dose-dependent fashion. Our findings are in agreement also with previous data of Van de Donk et al (1980) on the ciliary beat frequency of chicken embryo trachea, that benzalkonium chloride is safe at a concentration of 0·01% while at higher concentrations a dose-dependent depressing effect on ciliary motility, similarly to ammonium glycyrrhyzinate, is shown. The good correlation between effects on motility and concentrations of both ammonium glycyrrhyzinate and benzalkonium chloride provides further evidence that the excised palate of frog is a suitable preparation to test the topical tolerability of not only drugs, but also excipients, promoters and preservatives contained in nasal formulations. Looking at the results of this study, the importance of the concentration of the excipients must be stressed, in order to avoid any effect on viscous and elastic properties of mucus and on ciliary beating. The data collected with this experimental preparation may be useful as a guide for clinical tests on human nasal mucociliary clearance, both acutely and chronically.

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The binding of the antimalarial arteether to human plasma proteins in-vitro

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Abstract—The binding of the novel antimalarial drug, arteether, to human plasma, pure albumin and α_1-acid glycoprotein has been investigated by ultrafiltration, using [^{14}C]arteether. The protein binding in plasma obtained from 11 healthy male subjects ranged from 73·4 to 81·8% bound, with a mean of 78·7 ± 2·1%. The binding of drug in plasma was mainly accounted for by binding to albumin and α_1-acid glycoprotein. Scatchard analysis of the binding data revealed that the binding affinity of arteether to α_1-acid glycoprotein is much greater (20-fold) than that to albumin. This suggests that α_1-acid glycoprotein is the more important binding protein in plasma. This may have clinical importance due to alterations in plasma protein binding in patients with malaria, as the concentration of α_1-acid glycoprotein is markedly increased during malarial infection.

Qinghaosu, also known as artemisinin, is an antimalarial drug isolated from the Chinese herb qinghao (*Artemisia annua* L.). The herb has been used in malaria therapy in China for over 2000 years. Since the discovery of artemisinin in 1972 by Chinese scientists, several semisynthetic derivatives have been prepared and tested for antimalarial activity. Among these derivatives, arteether and artemether, which are respectively ethyl and methyl ethers of dihydroartemesinin (a reduction product of artemisinin) were found to have greater antimalarial activity than artemisinin itself (Klayman 1985; Trigg 1989). Qinghaosu and its potent derivatives such as arteether and artemether, are effective against both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, and are effective for the treatment of cerebral malaria (Trigg 1989). Malaria parasite resistance to chloroquine and mefloquine has been rapidly increasing in both degree and prevalence throughout the world (Wernsdorfer 1991). As a result, interest in these potent antimalarial derivatives of qinghaosu has increased. The World Health Organization and the Walter Reed Army Institute of Research have selected arteether for clinical development (Melendez et al 1991). This compound is currently in the preclinical phases of development.

As with many other drugs, pharmacokinetic information such as metabolism, clearance, drug distribution and protein binding, is essential for clinical use and dosage optimization. To our knowledge, clinical pharmacokinetic data of arteether are not available since this requires sensitive and reliable analytical methods. In this communication we report in-vitro protein binding of arteether in human plasma and characteristics of its binding to human serum albumin and α_1-acid glycoprotein.

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Materials and methods

Materials. Non-radioactive arteether was supplied by Sapec (Lugano, Switzerland). [^{14}C]Arteether (sp. act. 24·9 μCi mg^{-1}) was synthesized at Research Triangle Institute (Research Triangle Park, NC, USA) and was a gift from the Walter Reed Army Institute of Research (Washington DC, USA). The radiochemical purity of [^{14}C]arteether was 98% by TLC. Human serum albumin (HSA), essentially fatty acid-free; Fraction V HSA and human α_1-acid glycoprotein (AAG) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals were of analytical grade from conventional commercial sources. All the glassware used was silanized with dichlorodimethyl silane–toluene (5:95, v/v) before use.

Plasma samples. Human plasma or serum samples were obtained from 11 healthy male volunteers, aged 22–39 years (mean age = 30). These subjects had taken no medication for at least a week before blood sampling. The plasma for each subject was mixed with [^{14}C]arteether and non-radioactive drug to a final total drug concentration of 100 ng mL^{-1}. The plasma protein binding of arteether in each sample was then determined by ultrafiltration. Blood bank plasma was also used in a number of binding studies. The final total concentration of arteether in these plasma samples was 100 ng mL^{-1}, unless otherwise stated.

Protein solutions. To determine the extent of arteether binding to HSA and AAG, the individual protein was dissolved in 0·1 M isotonic phosphate buffer (pH 7·4). The protein solutions were then mixed with radiolabelled and non-radioactive arteether, and binding determined.

Methods. A commercially available ultrafiltration apparatus MPS-1 (Centrifree with YMT membrane, Amicon Corp., Danvers, MA, USA) was used. The device was loaded with 1 mL plasma or protein solutions and centrifuged at 1500 *g* for 15 min or until the filtrate amounted to about 10% (i.e. 100 μL) of the initial volume. Ultrafiltrate and the initial solution to which the drug had been added were analysed for radioactivity by liquid scintillation counting. All binding experiments were performed at room temperature (22°C), unless otherwise stated. An alternative ultrafiltration device, Millipore Ultrafree MC filter