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Multivariate toxicity screening of liposomal formulations on a human buccal cell line

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Abstract

The influence of various formulation factors on the *in vitro* cellular toxicity of liposomes on human buccal cells (TR146), were studied by using the concept of statistical experimental design and multivariate evaluation. The factors investigated were the type of main phospholipid (egg-PC, DMPC, DPPC), lipid concentration, the type of charge, liposome size, and amount and nature of the charged component (diacyl-PA, diacyl-PG, diacyl-PS, stearylamine (SA), diacyl-TAP) in the liposomes. Both full factorial design and D-optimal designs were created. Several significant main factors and interactions were revealed. Positively charged liposomes were shown to be toxic. The toxicity of negatively charged liposomes was relatively low. Diacyl-TAP was less toxic than SA, and DPPC was less toxic than DMPC. Low level of positively charged component was favourable and essential when using egg-PC as the main lipid. The amount of negatively charged component, the liposome size, and the total lipid concentration did not affect the toxicity within the experimental room. DPPC appeared to be a good candidate when formulating both positively and negatively charged liposomes with low cellular toxicity. The concept of statistical experimental design and multivariate evaluation was shown to be a useful approach in cell toxicity screening studies.

Keywords: Liposomes; Cellular toxicity; Buccal cell line; Multivariate data analysis

1. Introduction

The buccal region appears to be an attractive site for administration of drugs due to the good accessibility, the smooth and relatively immobile surface, the avoidance of possible degradation in the gastrointestinal tract, and avoidance of the first-pass metabolism in the liver. However, continuous saliva excretion and swallowing may lead to a very short residence time in the oral cavity (Rathbone et al., 1994). To overcome this problem novel bioadhesive dosage forms have been developed, such as bioadhesive tablets, bioadhesive patches, bioadhesive gels and ointments, and medicated chewing gums (Gandhi and Robinson, 1994; Hao and Heng, 2003; Birudaraj et al., 2005). Also, liposomes have been investigated as a delivery system in the oral cavity (Harsanyi et al., 1986; Sveinsson and Holbrook, 1993; Farshi et al., 1996; Petelin et al., 1998; Yang et al., 2002; Erjavec et al., 2006). Liposomes may be expected to protect the active ingredient from degradation in the oral cavity, they may act as a depot, and they may be designed to be bioadhesive. A bioadhesive formulation is intended to stay in the mouth for hours; hence, the toxicity of the formulation on the cells coming into close contact with the formulation is an important issue. Toxicity studies of liposomes on various cell lines have been reported in the literature (Layton et al., 1980; Mayhew et al., 1987; Filion and Phillips, 1997, 1998; Berrocal et al., 2000); the sensitivities of various cell lines to the same liposome formulation have been reported to vary. To our knowledge the toxicity to the cells in the oral cavity by liposomal formulations has not previously been addressed.

In this paper we study the toxicity of liposomal formulations using the human buccal cell line TR146. The TR146 cell line has been used as a model for the buccal epithelium in several studies on other substances in the literature (Burgalassi et al., 1996; Jacobsen et al., 1996, 1999; Pedersen et al., 1998; Eirheim et al.,

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2004; Boulmedarat et al., 2005a). All the liposomal formulation variables, e.g. type of the main lipid, the nature and amount of the charged component, and the liposome size, may be expected to influence the toxicity.

Toxicity studies on cell lines are usually carried out as univariate experiments, i.e. one factor is varied at a time. Evaluating the effect of a high number of variables and possible interactions will require a lot of experiments. In this paper, however, we have used the concept of statistical experimental design and multivariate evaluation. In such designs many factors are varied simultaneously in a systematic way, e.g. the influence of a high number of formulation variables can be studied at the same time and interactions between the variables can be detected. By this approach more information can be gained from a smaller number of experiments.

Thus, the objective of this paper was two-fold: (1) to identify important liposomal formulation factors influencing the toxicity on cells in the buccal region of the oral cavity using the TR146 buccal cell line a model, and identify significant interactions between the formulation variables, and (2) to investigate the potential of statistical experimental design and multivariate evaluation in cell toxicity studies. The factors investigated in this study were the type of the main phospholipid, the total lipid concentration, the type of the charge (positive, negative), the liposome size, the nature of the charged component, and the amount of the charged component in the liposomes. Both a full factorial screening design and D-optimal designs were created.

2. Materials and methods

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylglycerol (DMPG) were kindly provided by Nattermann Phospholipids GmbH (Köln, Germany), egg-phosphatidylcholine (egg-PC) and egg-phosphatidylglycerol (egg-PG) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany), dimyristoyl-trimethylammonium-propane (DMTAP), dipalmitoyl-trimethylammonium-propane (DPTAP), dioleyl-trimethylammonium-propane (DOTAP), dimyristoylphosphatidic acid (DMPA), egg-phosphatidic acid (egg-PA), dipalmitoylphosphatidylserine (DPPS) and dioleylphosphatidylserine (DOPS) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), dimyristoylphosphatidylserine (DMPS) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidic acid (DPPA), stearylamine (SA), and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium) was from Promega Corp. (Madison, WI, USA), Hank's balanced salt solution (HBSS) with and without calcium and magnesium were obtained from BioWhittaker Europe (Verviers, Belgium), and *ortho*-phosphoric acid and copper sulphate pentahydrate were from Merck (Darmstadt, Germany). Chloroform and methanol used for liposome preparation were of analytical grade.

The TR146 cell line is derived from a metastasis of a buccal carcinoma (Rupniak et al., 1985) and was provided by Cancer Research Technology Ltd. (London, UK).

2.2. Liposome preparation

Liposomes were prepared by the film-method as follows: the phospholipids were dissolved in chloroform:methanol (2:1), glass beads were added to the flask and the solution was evaporated to dryness in a rotary evaporator. The films were further dried in vacuum (<3 mbar) in a Christ Alpha 2–4 freeze drier (Christ, Osterode am Hatz, Germany) for 20 h. The thin film obtained was hydrated and gently shaken for 2 h at a temperature above the gel to liquid-crystalline phase transition temperature (T_c) with the aqueous solution (Hanks balanced salt solution (HBSS) with or without Ca and Mg) and kept in the refrigerator overnight. The samples were extruded at a temperature above T_c (Lipex extruder, Biomembranes Inc., Vancouver, Canada) using two stacked 800, 200, 100 or 50 nm polycarbonate membranes (Nucleopore®, Costar Corp., Cambridge, USA).

2.3. Liposome characterisation

The intensity mean diameter of the liposomes and the polydispersity index (PI) of the distribution were determined by photon correlation spectroscopy (PCS) at a 90° angle (25 °C) using Zetasizer 1000 (Malvern Instruments, Great Britain). The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured five times using the unimodal model for size distribution. All samples were diluted with HBSS to an appropriate counting rate prior to analysis.

The zeta potential was measured by micro-electrophoresis at 25 °C (Zetasizer 2000 HS, Malvern Instruments, Great Britain). The viscosity and dielectric constant of pure water were used as calculation parameters. All samples were diluted with HBSS to an appropriate counting rate prior to analysis, and all samples were analysed in triplicate.

The lipid concentration in the samples was determined by high-performance thin-layer chromatography (HPTLC) as described elsewhere (Stensrud et al., 1996). In short, the samples were transferred to separate vials followed by freezing and lyophilisation in a Christ Alpha 2–4 freeze drier (Christ, Osterode am Hatz, Germany). The dry samples were dissolved in chloroform, applied to silica gel $60F_{254}$ HPTLC plates (E. Merck, Darmstadt, Germany) using Linomat IV sample applicator (CAMAG, Muttenz, Switzerland), developed in a horizontal developing chamber (CAMAG, Muttenz, Switzerland) using a mixture of chloroform:methanol:distilled water (32.5:12.5:2, v/v) as the mobile phase, and dried. The spots were visualised by immersing into a solution of 8% *ortho*-phosphoric acid and 10% copper sulphate pentahydrate. The plates were scanned at 510 nm using a dual-wavelength flying-spot scanner CS-9000 (Shimadzu, Kyoto, Japan). All samples were analysed in duplicate.