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# Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for *in vitro* cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications

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ABSTRACT

Surfactants are used as additives in topical pharmaceuticals and drug delivery systems. The biocompatibility of amino acid-based surfactants makes them highly suitable for use in these fields, but tests are needed to evaluate their potential toxicity. Here we addressed the sensitivity of tumor (HeLa, MCF-7) and non-tumor (3T3, 3T6, HaCaT, NCTC 2544) cell lines to the toxic effects of lysine-based surfactants by means of two *in vitro* endpoints (MTT and NRU). This comparative assay may serve as a reliable approach for predictive toxicity screening of chemicals prior to pharmaceutical applications. After 24-h of cell exposure to surfactants, differing toxic responses were observed. NCTC 2544 and 3T6 cell lines were the most sensitive, while both tumor cells and 3T3 fibroblasts were more resistant to the cytotoxic effects of surfactants. $IC_{50}$-values revealed that cytotoxicity was detected earlier by MTT assay than by NRU assay, regardless of the compound or cell line. The overall results showed that surfactants with organic counterions were less cytotoxic than those with inorganic counterions. Our findings highlight the relevance of the correct choice and combination of cell lines and bioassays in toxicity studies for a safe and reliable screen of chemicals with potential interest in pharmaceutical industry.



## 1. Introduction

Surfactants represent one of the most widely applied excipients in the pharmaceutical and cosmetic industry due to their surface and interface activities. In order to minimize adverse reactions derived from the toxic potential of surfactants, the type of surfactant and concentration used should be considered when designing products for preformulation trials (Benassi et al., 2003; Paulsson and Edsman, 2001). Our previous research into new surfactants with low toxicity and a wide range of applications led to the development of a range of biocompatible surfactants derived from amino acids (Benavides et al., 2004a,b; Martinez et al., 2006; Mitjans et al., 2003). In this context, amino acid-based surfactants constitute a promising choice for applications in topical pharmaceutical products, as well as in novel biocompatible drug delivery devices (Morán et al., 2010; Nogueira et al., 2011). As the surface properties (hydrophobicity and surface charge) have a major impact on cellular uptake of particulate drug delivery systems, the incorporation of charged surfactants in these carriers may improve the targeting to specific cells and tissues, e.g. in cancer therapy (Schöler et al., 2001). Before this class of compounds can be approved for these purposes, however, accurate information about their toxicity is required. Thus, a complete toxicological evaluation of their effects should be performed by comparing a battery of complementary *in vitro* bioassays (Fisher et al., 2003).

Safety evaluation of new products or ingredients destined for human use is crucial prior to exposure. Therefore, rapid, sensitive and reliable bioassays are required in order to examine the toxicity of these substances. Established cell lines are useful alternative test systems for toxicological studies of this kind (Crespi, 1995); however, they must be chosen with care with regard to their origin (Jondeau et al., 2006). Moreover, cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture (Martinez et al., 2006). Cell damage is manifested in several ways, including mitochondrion and plasma membrane dysfunction and, fluctuating intracellular reduction capacity (Kim et al., 2009). Current standard approaches to gauge the degree of cell damage include assays that measure various aspects of cell viability, such as metabolic activity and plasma membrane integrity. The MTT reduction assay, which

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determines cell metabolic activity, is among the most commonly used endpoints. This method measures the reduction of MTT salt to a colored insoluble formazan in active mitochondria in viable cells and also, in certain cases, outside the mitochondria (Berridge et al., 2005; Liu et al., 1997). The neutral red uptake (NRU) assay, which is also widely used in biomedical applications, measures the uptake of neutral red dye by viable cells with intact plasma membrane, and its concentration in lysosomes (Repetto et al., 2008). Differences in the sensitivity of endpoints, together with the type of cell model and the nature of the chemicals being tested, may explain inconsistencies in the results reported (Schröterová et al., 2009). Therefore, complementary endpoint assays based on various mechanisms, as well as comparative analysis of the sensitivity of several cell types, are strongly recommended to increase the reliability of results (Fisher et al., 2003; Schröterová et al., 2009).

Here we studied the sensitivity of two tumor and four non-tumor cell lines of different origins to the toxic effects of five anionic lysine-based surfactants that differ in the nature of their counterions. The choice of dermal and tumor cells as model systems is based on the wide use of surfactants in topical pharmaceuticals and more recently in drug delivery devices (e.g. in cancer therapy), respectively. The knowledge about the cytotoxicity and potential mechanisms of surfactant interaction with healthy and tumor cells may help on the development of specific and effective devices for cancer therapy. In previous studies, we identified a number of toxic effects of this class of surfactants (Sanchez et al., 2004, 2006a,b). Nevertheless, given that no single *in vitro* assay has the capacity to mimic all events that occur *in vivo*, and in order to complete these toxicological studies, here we performed a comparative evaluation using six cell types and two cytotoxicity assays, MTT and NRU. These two assays evaluate different cell physiological mechanisms and are considered to be the most common methods applied to study cell viability after exposure to toxic substances (Fotakis and Timbrell, 2006). To gain insight into structure-dependent toxicity, we also discuss the influence of the counterions on the cytotoxic effects of the surfactants. This comparative study performed using six cell lines and two *in vitro* endpoints can be considered a suitable approach for toxicological screening of chemical compounds prior to pharmaceutical applications.

## 2. Materials and methods

### 2.1. Chemicals and reagents

L-Lysine monohydrochloride, L-lysine, Tris, and the bases NaOH, LiOH and KOH were purchased from Merck (Darmstadt, Germany). 2,5-Diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye and dimethylsulphoxide (DMSO) were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA) and penicillin–streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

### 2.2. Surfactants tested

Five anionic amino acid-based surfactants derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine and with counterions of distinct chemical nature were studied: two salts with organic counterions – lysine salt (77KK) and Tris (hydroxymethyl) aminomethane salt (77KT); and three salts with inorganic counterions – sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1). These surfactants were synthesized in our laboratory as previously described (Sanchez et al., 2006a; Vives et al., 1999).

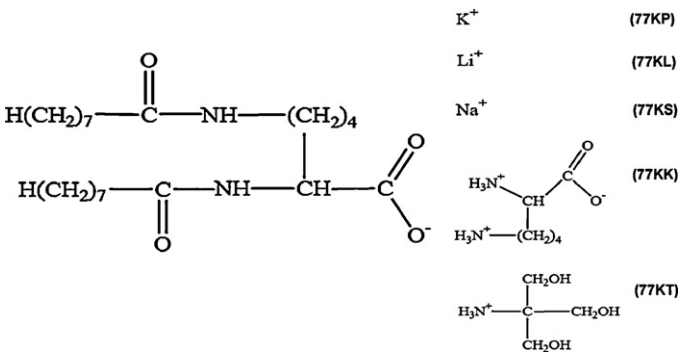


**Fig. 1.** Molecular structure of anionic lysine-based surfactants with distinct counterions. The codes P, L, S, K and T represent potassium, lithium, sodium, lysine and Tris, respectively.

### 2.3. Cell cultures

Two tumor cell lines (HeLa, human epithelial cervical cancer and MCF-7, human breast cancer) and four non-tumor cell lines (3T3, murine Swiss albino fibroblasts; 3T6, spontaneously transformed 3T3 murine Swiss albino fibroblasts; HaCaT, spontaneously immortalized human keratinocytes and NCTC 2544, normal human undifferentiated keratinocytes) were used. The 3T3, HeLa and MCF-7 cell lines were grown in DMEM medium (4.5 g/l glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The HaCaT cell line was cultured under the same conditions as described above, except for supplementation with 10 mM Hepes buffer. The NCTC 2544 and 3T6 cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were routinely grown in 75 $cm^2$ culture flasks and maintained at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were trypsinized using trypsin–EDTA when they reached approximately 80% confluence.

### 2.4. Experimental design

Cells were seeded into the central 60 wells of 96-well cell culture plates in 100 μl of complete culture medium at the following initial densities (cells/ml): $1 \times 10^5$ for MCF-7, $8.5 \times 10^4$ for 3T3 and HeLa, $5 \times 10^4$ for 3T6, HaCaT and NCTC 2544. Cells were incubated for 24 h under 5% $CO_2$ at 37 °C and the medium was then replaced with 100 μl of fresh medium supplemented with 5% FBS containing 0.22-μm filter-sterilized surfactant solution at the required concentration (serial dilutions between 7.8–500 μg/ml). Each concentration was tested in triplicate and control cells were exposed to medium with 5% FBS only.

### 2.5. Cytotoxicity assays

#### 2.5.1. MTT assay

The MTT assay is based on the protocol first described by Mosmann (1983). In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After cell incubation for 24 h, the surfactant-containing medium was removed, and 100 μl of MTT in PBS (5 mg/ml) diluted 1:10 in FBS-free medium without phenol red was then added. Plates were further incubated for 3 h, after which time the medium was removed, and cells were washed in PBS. The purple formazan product was then dissolved by adding 100 μl of DMSO to each well.