Comparative sensitivity of tumor and non-tumor cell lines as a new 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 new 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.

Keywords: lysine-based surfactants; cytotoxicity; cell sensitivity; MTT; NRU; counterions

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 regards 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 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 (Fischer 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 widely 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, 2006b). 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^{α},N^{ε} –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 (Sánchez et al., 2006a; Vives et al., 1999).

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 ºC 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 x 10^5 for MCF-7, 8.5 x 10^4 for 3T3 and HeLa, 5 x 10^4 for 3T6, HaCaT and NCTC 2544. Cells were incubated for 24 h under 5% CO_2 at 37 ºC 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 Mossmann (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. Plates were then placed in a microtitre-plate shaker for 10 min at room temperature and the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of tetrazolium salt reduction by viable cells against the untreated cell control (cells with medium only).

2.5.2. NRU assay

Based on the protocol described by Borenfreund and Puerner (1985), the NRU assay determines the accumulation of the NR dye in the lysosomes of viable, undamaged cells. Following exposure to the surfactants solutions, cells were incubated for 3 h with NR dye solution (50 µg/ml) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, followed by the addition of 100 µl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured the absorbance of

the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

2.6. Statistical analyses

The cytotoxicity of each surfactant was expressed as percentage of viability with regard to untreated control wells (the mean optical density of untreated cells was set at 100% viability) in terms of its IC_{50} (concentration causing 50% death of the cell population), calculated from concentration-response curves. Results are expressed as mean ± standard error of the mean (S.E.M.) of at least three independent experiments.

Statistical analyses of the individual IC_{50} -values were performed using Student's *t*-test or one-way analysis of variance (ANOVA) to determine the differences between the sets of data. Tukey's *posthoc* multiple comparison test was also used to further identify significant differences between cell lines, as indicated, using the SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$, $p < 0.01$, $p < 0.005$ or $p < 0.001$ were considered to denote significance. Pearson's correlation coefficients between the IC_{50} -values obtained from the cytotoxic assays and cell lines were also calculated by linear regression analysis.

3. Results

3.1. In vitro cytotoxicity of the compounds

Dose-response curves for each surfactant obtained from NRU and MTT assays in all six cell lines are given in Fig. 2 and 3, for the surfactants with organic and inorganic counterions, respectively. These cytotoxicity assays revealed that a 24 h-exposure of the tumor and non-tumor cell lines to different concentrations (ranging from 7.8 to 500 µg/ml) of the five anionic lysine-based surfactants produced a dose-dependent reduction in the number of viable cells. Differences between cell lines as well as various sensitivity to the surfactants were demonstrated by calculating the half maximal inhibitory concentrations (IC_{50}) (Fig. 4). IC_{50} -values ranged from 103.67 µg/ml (77KL with 3T6 cells) to 468.53 µg/ml (77KL with MCF-7 cells), highlighting the wide cell sensitivity to these compounds.

Observation of IC_{50} -values shows that some cells lines were markedly less sensitive to the cytotoxic effects of the surfactants. In general, the tumor cell lines HeLa

and MCF-7 (Fig. 4e and f, respectively) and 3T3 fibroblasts (Fig. 4a) were more resistant to the deleterious effects of the surfactants, while 3T6 and NCTC 2544 cell lines (Fig. 4b and d, respectively) showed greater sensitivity, with IC_{50} -values lower than 200 µg/ml. By scoring the sensitivity of the cells to the five surfactants, we can ranked the cell lines from the most resistant to the most sensitive as follows: MCF-7 > 3T3 > HeLa > HaCaT > NCTC 2544 > 3T6 by the NRU assay, and 3T3 > MCF-7 > HeLa > HaCaT > NCTC 2544 > 3T6 by the MTT assay.

The levels of toxicity varied depending not only on cell line and substance tested, but also on the viability assay used. Regardeless of the compound or cell line, cytotoxicity was detected and observed earlier by the MTT assay. In contrast, the NRU assay was less sensitive and discriminative. The MTT assay (Fig. 2b and d, Fig. 3b, d and f) showed significant decrease in the metabolic activity of cells in a concentration-dependent manner and, in general, the lowest concentration of each surfactant that significantly reduced cell viability in all six cell lines ranged from 31.25 to 125 µg/ml. In contrast, no apparent cytotoxicity was detected when the same concentration range was assessed by the NRU assay (Fig. 2a and c, Fig. 3a, c and e). In this assay, significant loss of cell viability was detected for all the surfactants only at the highest concentrations tested (250 and 500 µg/ml), except for 3T6 and NCTC 2544 cell lines, which showed greater sensitivity to the surfactants in both assays.

The surfactants differed only in the type of counterion, and the overall results showed that those with organic counterions (77KK and 77KT) were less cytotoxic than those with inorganic counterions (77KL, 77KP and 77KS). The IC_{50} -values for 77KK and 77KT ranged from 129.07 to 404.37 µg/ml, thereby indicating that they were less cytotoxic than the surfactants with inorganic counterions. The relatively low IC_{50} -values for 77KS and 77KP, ranging from 113.97 µg/ml to 298.67 µg/ml, indicate higher cytotoxicity. In contrast, the surfactant 77KL with an inorganic lithium counterion was the most cytotoxic to 3T6 and NCTC 2544 cells (IC_{50} -values ranging from 103.67 to 146.23 µg/ml), while it had only mild toxic effects on 3T3 and HaCaT cells, with IC_{50} -values ranging from 143.07 to 316.97 µg/ml. Moreover, 77KL was the least cytotoxic surfactant on the tumor cell lines, showing a cytotoxic effect (IC_{50}) only at 468.53 and 350.07 µg/ml for MCF-7 cells, and 351.23 and 244.10 µg/ml for HeLa cells, by the NRU and MTT assays, respectively. On the basis of the mean cytotoxicity of the surfactants on each cell line, as established by the MTT and NRU assays, we ranked the compounds from the least to the most cytotoxic as follows: 77KK < 77KT < 77KL < 77KP < 77KS.

3.2. Comparative analyses between cell lines and cytotoxicity assays

The IC_{50} -values of the six cell lines obtained from the MTT and NRU assays were analyzed by one-way ANOVA followed by Tukey's *posthoc* test to identify significant differences (Table 1). Of note the response of 3T3 cells to the five surfactants differed significantly from almost all the other cell lines, as determined by the MTT assay. The NRU assay showed that the sensitivity of 3T6 and NCTC 2544 cells to the surfactants did not differ significantly from each other, but showed significant differences from all the other cell lines, except in response to 77KS, as this surfactant produced fewer differences between the cell lines.

Correlation analyses were performed to examine the relationship between the cell types. In general, poor correlations were obtained between the cell lines, even when the comparisons were performed only with the IC_{50} -values derived from the same cytotoxicity assay. Nevertheless, significant correlations were obtained for some combinations of cells and endpoints (Table 2).

Furthermore, correlation analyses between the MTT and NRU assays (IC_{50} -values) for each surfactant in the six cell lines was performed by mean square root linear regression analysis. The relationship between the assays was expressed by their equations of linear regression and by Pearson's correlation coeficient (r). Significant correlations were obtained for 77KK ($p < 0.05$), 77KL ($p < 0.05$) and 77KS ($p < 0.05$), and also when the responses for all five surfactants were correlated simultaneously ($p < 0.01$). These observations indicate that, despite the individual characteristics of each cell line and compound tested, in general relatively good agreement was obtained between the two cytotoxicity assays (Fig. 5).

4. Discussion

In vitro cytotoxicity assays with established cell lines are useful tools for the general screening of chemicals in toxicological studies (Crespi, 1995). Surfactants are among the most versatile and widely used excipients in pharmaceutical products, and thus research on their toxic effects are required before they are considered for potential applications in topical drugs, cosmetic products (Martinez et al., 2006; Sanchez et al., 2004, 2006a), and drug delivery systems (Nogueira et al., 2011; Wang et al., 2007). It has been reported that the type of (co) surfactant in topical pharmaceuticals and

nanoparticulate devices has great impact in their potential cytotoxic effects (Schöler et al., 2001; Weyenberg et al., 2007).

Here we studied the sensitivity of several tumor and non-tumor cell lines to the toxic effects of five anionic lysine-based surfactants that differ in the nature of their counterions. For this purpose, we compared the performance of the MTT and NRU assays, two widely used and relatively simple *in vitro* bioassay methods that provide information on cell metabolic activity and membrane integrity (particularly in the lysosomal compartment), respectively. A comparative study based on a range of different cell lines and *in vitro* endpoints are highly suitable during toxicity screening of chemicals with potential interest in the pharmaceutical industry. The information derived from a more complete study can raise the knowledge concerning to the safety profile of bioactive compounds. Data on HaCaT, 3T6 and NCTC 2544 cell lines have been previously reported by our group (Sanchez et al., 2004, 2006a); however, in this work we used the raw data to recalculate the IC_{50} -values using a properly fitted regression model.

The cytotoxicity assays indicated significant differences between the cell lines (Table 1) in their sensitivity to the five compounds tested. Our observations are consistent with earlier studies that reported significant differences in the cytotoxic effects of chemicals depending on the cell type tested (Backorová et al., 2011; Burlando et al., 2008; Lestari et al., 2005; Schröterová et al., 2009; Tan et al., 2008; Wang et al., 2002). Although the effect of the surfactants was concentration-dependent, the cellular attributes of each particular tumor or non-tumor cell line also contributed to the overall outcome. The greater resistance of the tumor cell lines could be partly explained by the fact that healthy and cancer cells show differences in their structure and function, such as differences in metabolic activity and molecular composition (Frey et al., 2007). The particular characteristics of each cell type could lead to varied mechanisms of defense, and consequently distinct sensitivity to the toxic effect of a chemical compound. The overall variability in cell sensitivity was supported by the general poor or moderate correlation between cell lines ($r < 0.85$, correlation not significant). Nevertheless, some significant correlations were obtained. The two tumor cell lines (HeLa and MCF-7) presented a relatively good relationship with each other and also with the 3T3 non-tumor cell line. HeLa and MCF-7 cells have similar phenotypic characteristics (Leporatti et al., 2009), which could explain their similar sensitivity to surfactants. In contrast, no significant correlations were found between the fibroblasts (3T3 and 3T6) or keratinocytes (HaCaT and NCTC 2544). The increased sensitivity of 3T6 and NCTC 2544 cells to the

surfactants may explain this lack of correlation. Hayat and Friedberg (1986) reported stronger cytotoxicity in transformed 3T6 cells compared to their untransformed counterparts 3T3 cells, which is consistent with our findings. The high resistence of 3T3 cells could be partly explained by their sensitivity to growth contact inhibition in contrast to 3T6 cells (Todaro and Green, 1963). The contact inhibition property could favor a more differentiated phenotype for the 3T3 cells, in which the cytotoxic effect of the surfactant may be less potent. Moreover, cytotoxicity and phototoxicity studies have shown that HaCaT keratinocytes are more resistant than NCTC 2544 (Burlando et al., 2008; Leccia et al., 1998). The lower sensitivity of HaCaT cells could be attributed to their high degree of differentiation, which involves more developed keratinization, whereas NCTC 2544 cells are scarcely differentiated (Boukamp et al., 1988). Based on the variability of cell responses, we recommended a combination of cell lines of different origins for a reliable primary screening for the potential toxicity of chemical compounds with potential biomedical applications. Cell -culture systems with established banked cell lines are reproducible and high throughput approaches, and thus can provide a forecast of some adverse effects prior to human exposure.

Our results show that the MTT assay was more sensitive in detecting cell damage than NRU assay, regardless of the cell line or compound assessed. MTT assay revealed loss of viability at concentrations at which no significant cytotoxic effect was observed with the NRU assay. These results were not unexpected, since it has been previously reported that responses vary depending on the cytotoxicity assay used (Burlando et al., 2008; Fotakis and Timbrell, 2006; Jondeau et al., 2006; Schröterová et al., 2009; Weyermann et al., 2005). Originally, it was assumed that the reduction of MTT occurred exclusively in the mitochondrial compartment; however, later studies showed that MTT is also reduced by oxido-reductase- type enzymes in microsomal and cytosolic fractions (Berridge et al., 2005; Liu et al., 1997). The NRU assay is based on the capacity of viable cells with intact plasma membranes to incorporate and bind the supravital NR dye in lysosomes. This process is dependent on the capacity of the cell to maintain pH gradients through the production of ATP (Repetto et al., 2008). The NRU endpoint is assumed to be universal among cell types, independently of their nature, while tetrazolium-based assays are more specific, as they measure the activity of intracellular enzymes whose expression, localization and activity depend on the cell type (Schröterová et al., 2009). Therefore, differences in cytotoxic responses could be related to specific toxicological mechanism of the surfactants tested as well as the characteristics of the cell lines. Moreover, the finding

that the NRU assay detected lower cytotoxicity than the MTT assay suggests that the mechanism of toxicity exerted by these surfactants involves an early effect on the metabolic activity of the cells, while plasma membrane and lysosomal compartments could be affected at a later stage. Although significant differences were found between the IC_{50} -values in some cell lines, significant correlations were obtained between the MTT and NRU assays for the surfactants 77KK, 77KL, 77KS, and also when all compounds were correlated simultaneously. This generally good relationship between the two endpoints even when individual differences were observed within experimental data is in agreement with previous studies (Borenfreund et al., 1988). On the basis of our results, we recommend that the inter-correlation data from these two assays be carefully analyzed before considering similar tools for the overall evaluation of cytotoxicity. The combination of several endpoints might be recommendable in order to distinguish between the effects on specific organelles or general cytotoxicity.

It is worth mentioning that compounds containing counterions differ in their capacity to interact with biological membranes, and the type of counterion is one of the factors that determine the efficiency of this interaction (Kleszczynska et al, 1998; Nogueira et al., 2011). Therefore, given that the class of lysine-based surfactants studied here had the same chemical structure, differing only in the type of counterion, the characteristics of the counterion may be crucial to the cytotoxic effect. Surfactants bound to heavy counterions, lysine (77KK) and tris (77KT), showed low toxicity. On the basis of this observation, we suggest that there is a relationship between the size of the counterion and the cytotoxic properties of these compounds: the heavier the counterion they are bound to, the lower the cytotoxicity they induce. This finding could be explained by the influence of the volume of surfactant polar head on cell membrane penetration. The larger the volume and radius of the polar head, the lower the penetration, which would explain why high concentrations of these surfactants were required to membrane penetration (Maugras et al., 2001; Sarapuk et al., 1997; Selve et al., 1991). In contrast, surfactant 77KL was the least cytotoxic to tumor cells, indicating a specific interaction of the inorganic lithium counterion with these cells. Moreover, surfactant 77KS was one of the most cytotoxic compounds regardless of the assay or cell line used. This observation suggests non-specific toxicity of the inorganic sodium counterion in the assays and cell lines assessed.

5. Conclusions

Our results show significant differences in the cytotoxicity of the surfactants. These differences are attributed to differences in the sensitivity of model cell lines to these compounds and the characteristics of the two assays used. The two tumor cells and 3T3 fibroblasts were more resistant to the surfactants, while 3T6 and NCTC 2544 cells were the most sensitive. The MTT assay was more sensitive in detecting cell damage, regardless of the cell line or compound tested. We conclude that the type of counterion in these compounds determines the degree of surfactant interaction with the cell: in general the compounds with organic counterions are the least cytotoxic. The outcome of this study enhances knowledge about the potential toxic effects of novel biocompatible lysine-based surfactants prior to preformulation trials of pharmaceutical devices. Altogether, our findings highlight the relevance of an appropriate choice and combination of endpoints and cell -culture systems in toxicity studies, which may raise the information output related to all major toxic effects of bioactive compounds. A complete and detailed toxicological evaluation *in vitro* may increase the reliability of results, and also prevent overestimation or underestimation of cytotoxicity. Furthermore, this comparative study, performed with six different cell types and two endpoints, may also serve as a basis for further toxicological screenings of different chemical compounds.

Conflict of interest statement

The authors have declared no conflict of interest.

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Figure captions:

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.

Fig. 2. Representative concentration-response curves from 24-h exposure of the two tumor and four non-tumor cell lines to surfactants with organic counterions: 77KK (panels a and b) and 77KT (panels c and d). Values were obtained from NRU (panels a and c) and MTT (panels b and d) assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate.

Fig. 3. Representative concentration-response curves from 24-h exposure of the two tumor and four non-tumor cell lines to surfactants with inorganic counterions: 77KL (panels a and b), 77KP (panels c and d) and 77KS (panels e and f). Values were obtained from NRU (panels a, c and e) and MTT (panels b, d and f) assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate.

Fig. 4. Cytotoxicity of the lysine-based surfactants expressed as IC_{50} -values (μg/ml) on (a) 3T3, (b) 3T6, (c) HaCaT, (d) NCTC 2544, (e) HeLa and (f) MCF-7 cell lines, and measured by NRU (dark bars) and MTT (white bars) assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate. NRU and MTT assays were compared by the Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ denote significant differences.

Fig. 5. Correlation of IC_{50}-values (μg/ml) between the NRU and MTT assays. (a) 77KK, (b) 77KT, (c) 77KL, (d) 77KP, (e) 77KS and (f) all surfactants. (1) 3T3, (2) 3T6, (3) HaCaT, (4) NCTC 2544, (5) HeLa and (6) MCF-7. r = *Pearson's* correlation coefficient. * $p < 0.05$, ** $p < 0.01$ denote significant correlation.

Table 1. One-way ANOVA results and multiple comparison of cell types [a]

	NRU					MTT				
	77KK	77KT	77KL	77KP	77KS	77KK	77KT	77KL	77KP	77KS
One-way ANOVA	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
3T3 *vs* 3T6	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
3T3 *vs* NCTC	p<0.001	p<0.001	p<0.001	p<0.001	p<0.005	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
3T3 *vs* HaCaT	NS	NS	NS	NS	p<0.005	p<0.005	p<0.001	p<0.001	p<0.001	p<0.001
3T3 *vs* HeLa	NS	NS	NS	NS	p<0.05	p<0.001	p<0.001	NS	NS	p<0.001
3T3 *vs* MCF-7	NS	NS	p<0.001	NS	NS	NS	NS	NS	p<0.001	p<0.001
3T6 *vs* NCTC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3T6 *vs* HaCaT	p<0.001	p<0.001	p<0.001	p<0.05	NS	p<0.001	NS	NS	NS	NS
3T6 *vs* HeLa	p<0.001	p<0.001	p<0.001	p<0.05	NS	p<0.005	p<0.05	p<0.001	p<0.005	NS
3T6 *vs* MCF-7	p<0.001	p<0.001	p<0.001	p<0.005	p<0.005	p<0.001	p<0.001	p<0.001	NS	NS
NCTC *vs* HaCaT	p<0.001	p<0.001	p<0.001	p<0.05	NS	p<0.05	NS	NS	NS	NS
NCTC *vs* HeLa	p<0.001	p<0.001	p<0.001	p<0.05	NS	NS	NS	p<0.001	p<0.005	NS
NCTC *vs* MCF-7	p<0.001	p<0.001	p<0.001	p<0.005	NS	p<0.001	p<0.001	p<0.001	NS	NS
HaCaT *vs* HeLa	NS	NS	NS	NS	NS	NS	NS	p<0.005	p<0.005	NS
HaCaT *vs* MCF-7	NS	NS	p<0.001	NS	NS	p<0.005	p<0.001	p<0.001	NS	NS
HeLa *vs* MCF-7	NS	NS	p<0.001	NS	NS	p<0.001	p<0.001	p<0.005	p<0.005	NS

NS = not statistically significant

[a] One-way analysis of variance; *posthoc* multiple comparison by the Tukey test.

Table 2. Significant correlations of IC_{50} -values between the cell types studied by using NRU and MTT cytotoxicity assays

Cell line – Cytotoxicity assay	Pearson's correlation coeficient	p value
3T3 NRU vs HaCaT NRU	0.930	$p < 0.05$
3T3 MTT vs HaCaT NRU	0.899	$p < 0.05$
3T3 MTT vs MCF-7 NRU	0.983	$p < 0.01$
3T3 MTT vs MCF-7 MTT	0.994	$p < 0.01$
HeLa NRU vs HaCaT NRU	0.959	$p < 0.05$
HeLa NRU vs MCF-7 NRU	0.961	$p < 0.01$
HeLa NRU vs MCF-7 MTT	0.986	$p < 0.01$
HeLa NRU vs 3T3 MTT	0.982	$p < 0.01$
HeLa MTT vs NCTC 2544 NRU	0.953	$p < 0.05$
HaCaT MTT vs 3T6 NRU	0.918	$p < 0.05$
MCF-7 MTT vs HaCaT NRU	0.910	$p < 0.05$
MCF-7 MTT vs MCF-7 NRU	0.989	$p < 0.01$

Figure(s)

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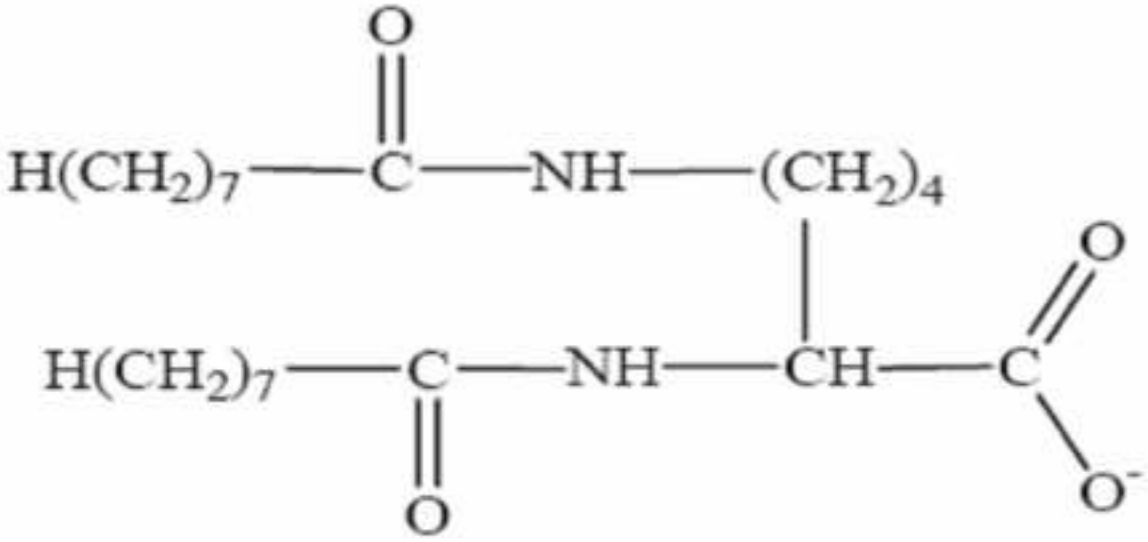
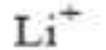
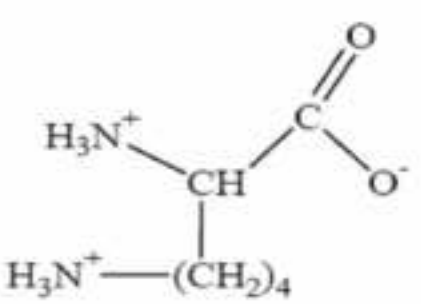


(77KP)



(77KL)

(77KS)



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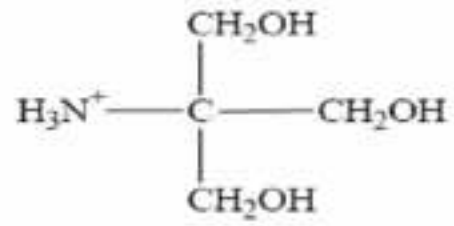
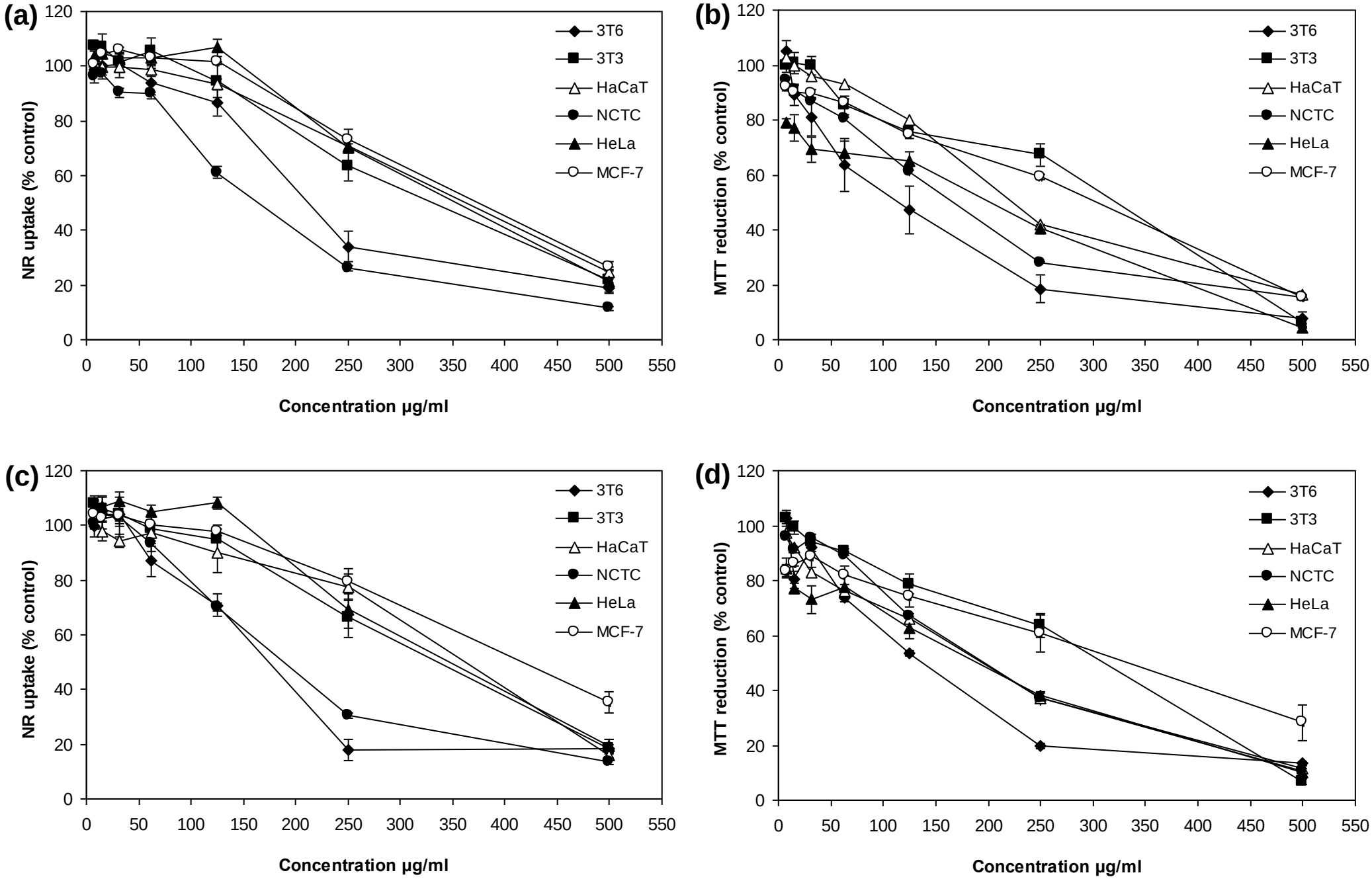
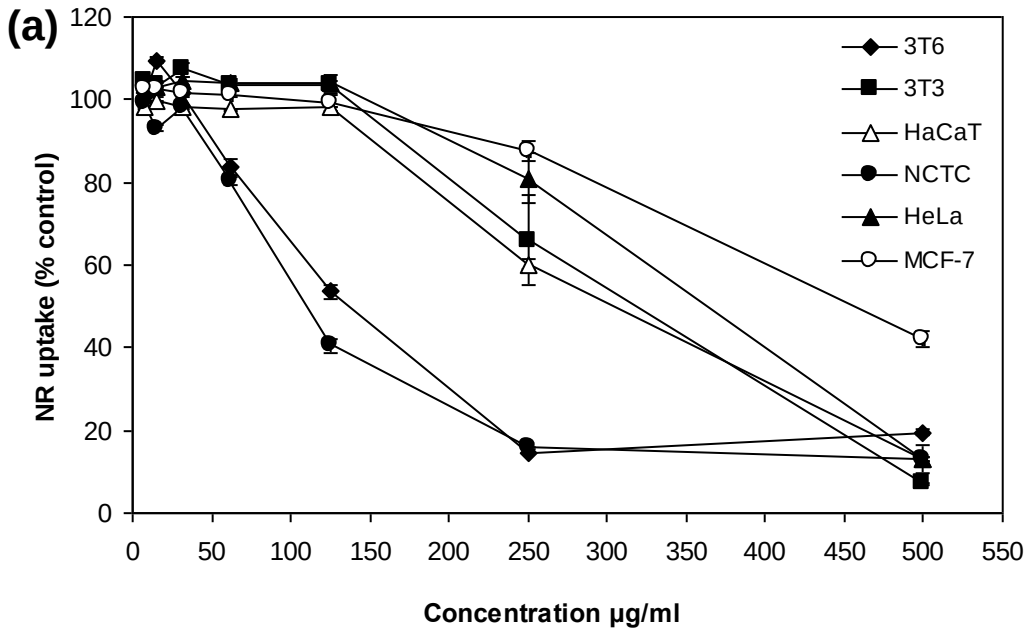


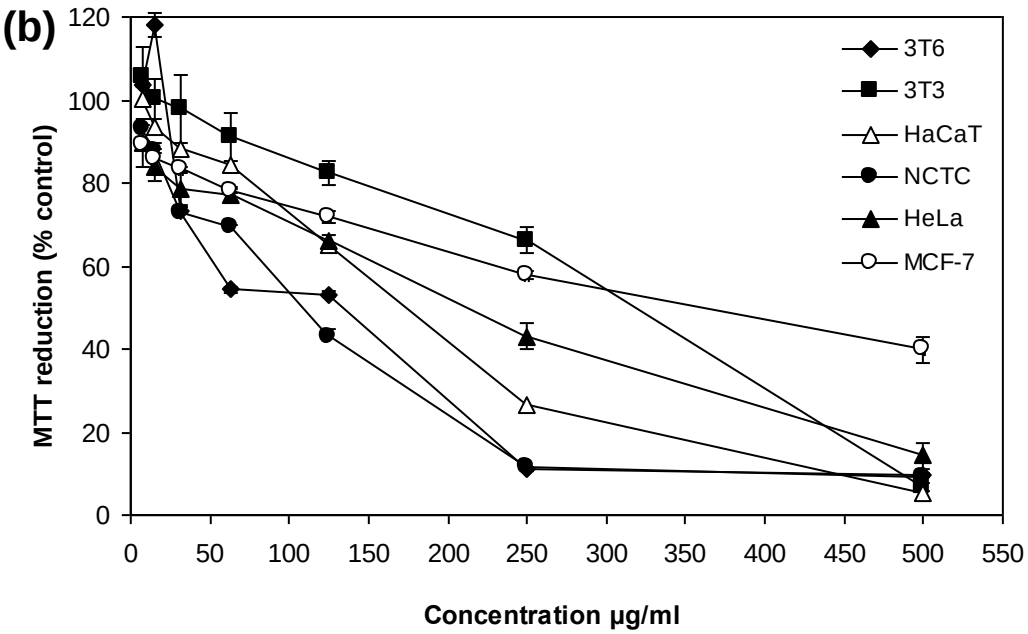
(77KT)

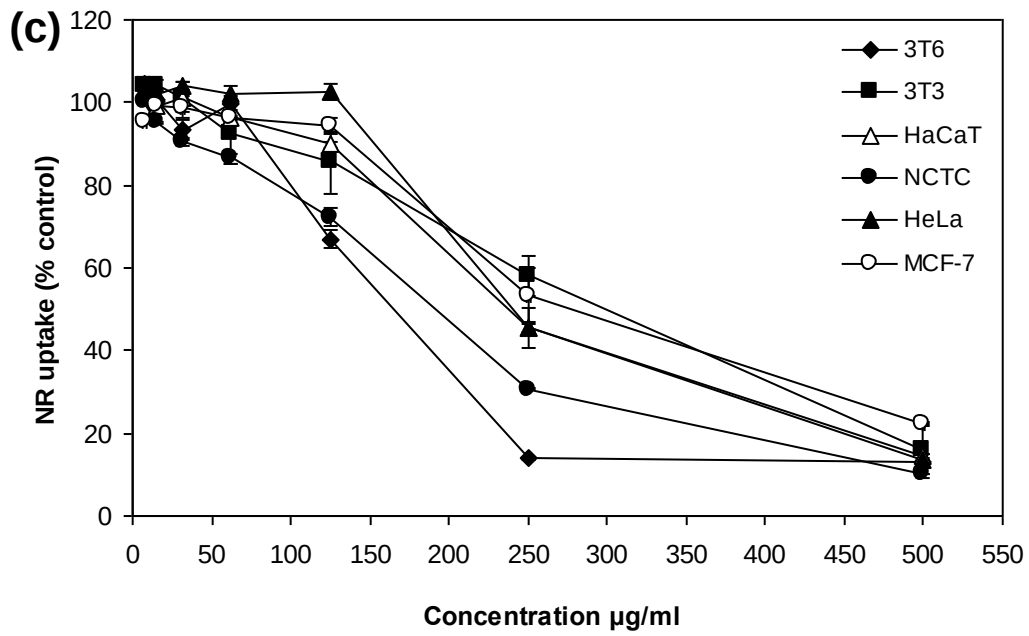
Figure(s)


(a)
NR uptake (% control)
Concentration µg/ml
(b)
MTT reduction (% control)
Concentration µg/ml
(c)
NR uptake (% control)
Concentration µg/ml
(d)
MTT reduction (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7

Figure(s)


(a)
NR uptake (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7


(b)
MTT reduction (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7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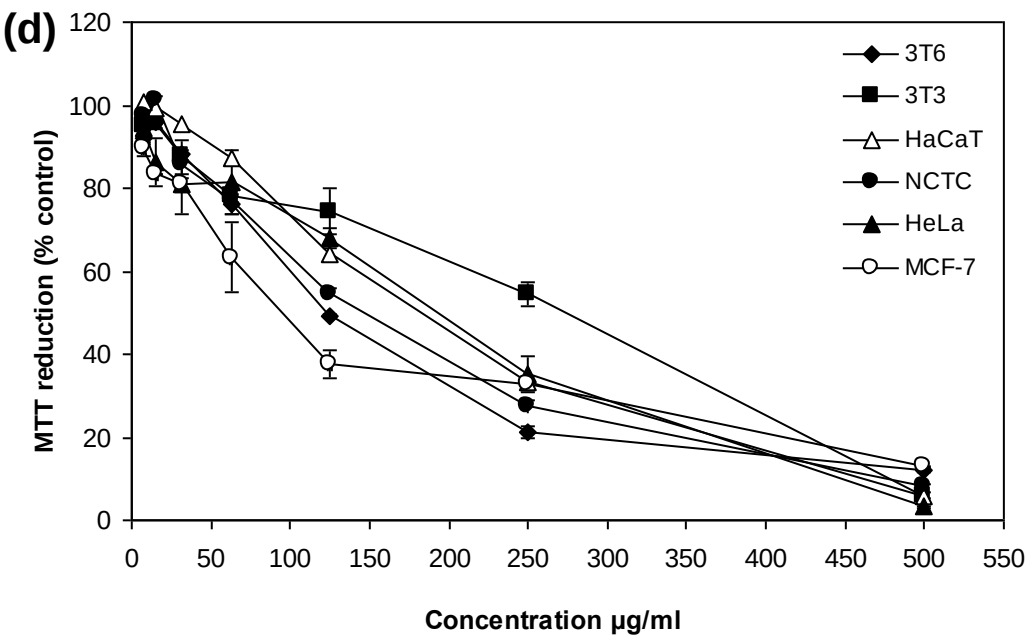
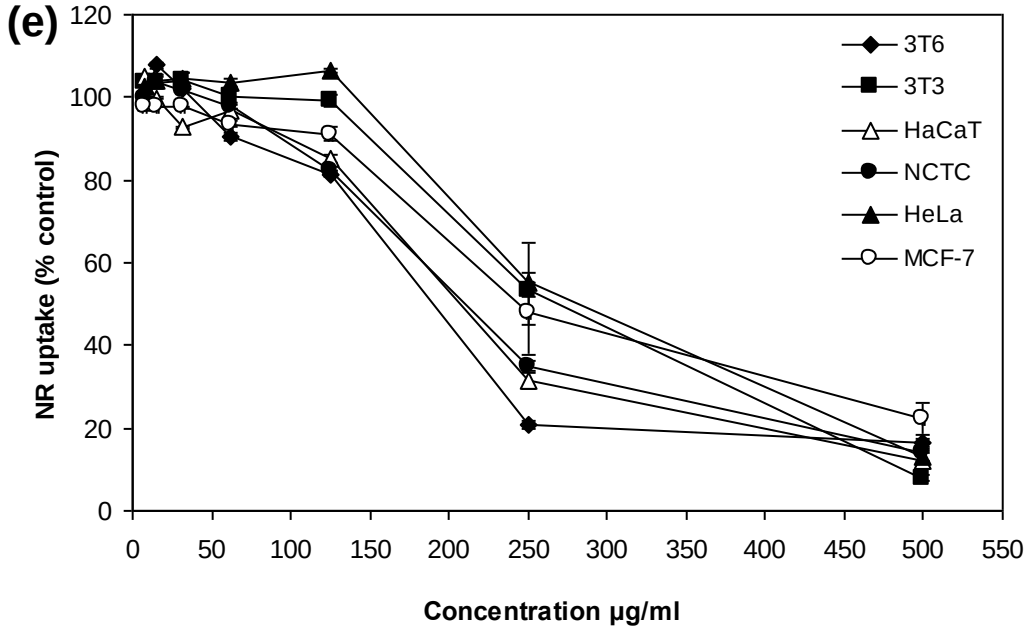
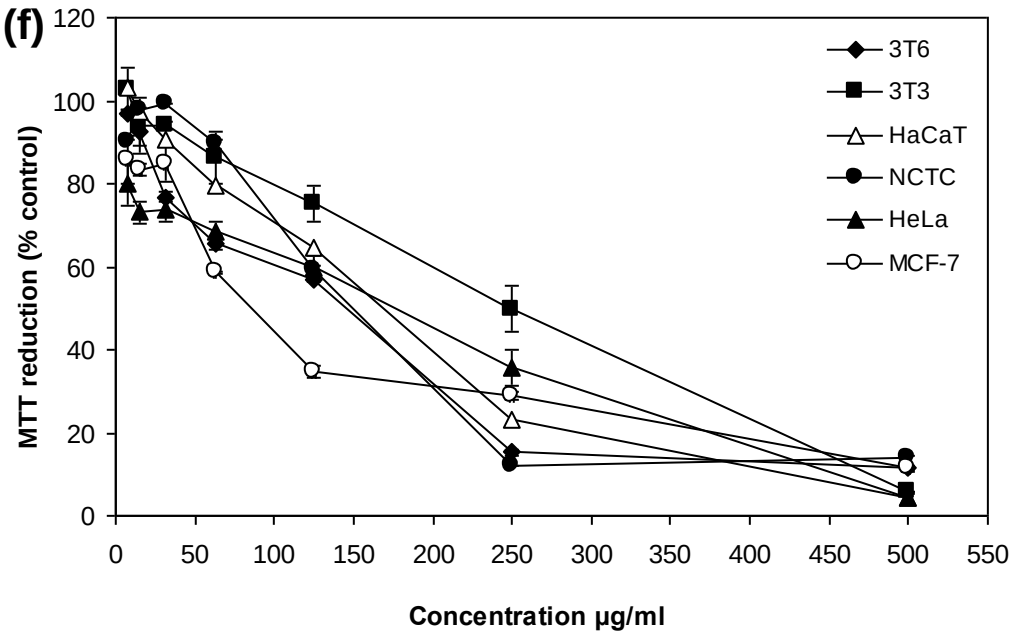
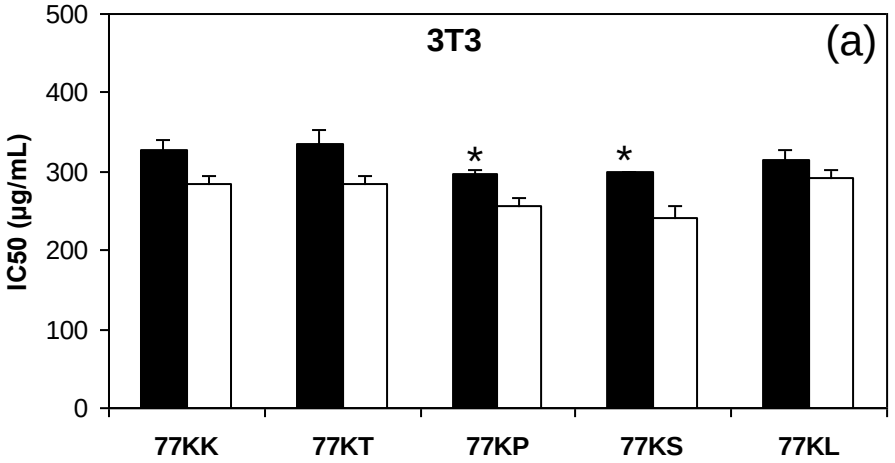
NR uptake (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7

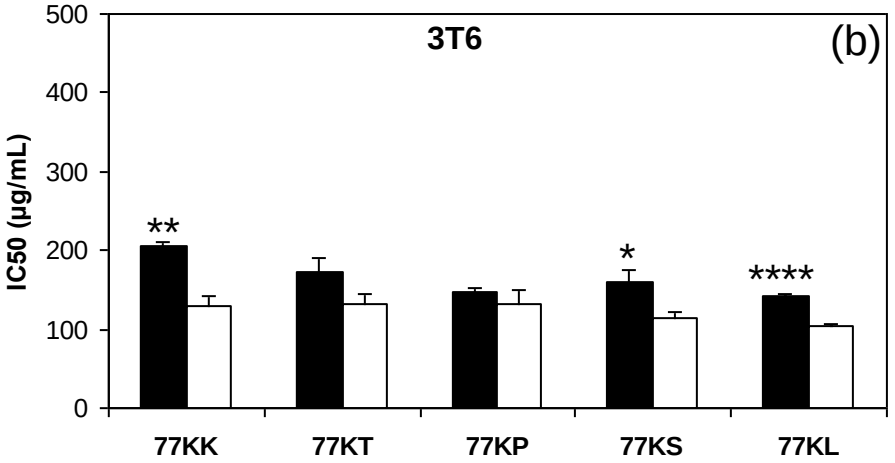

(d)
MTT reduction (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7

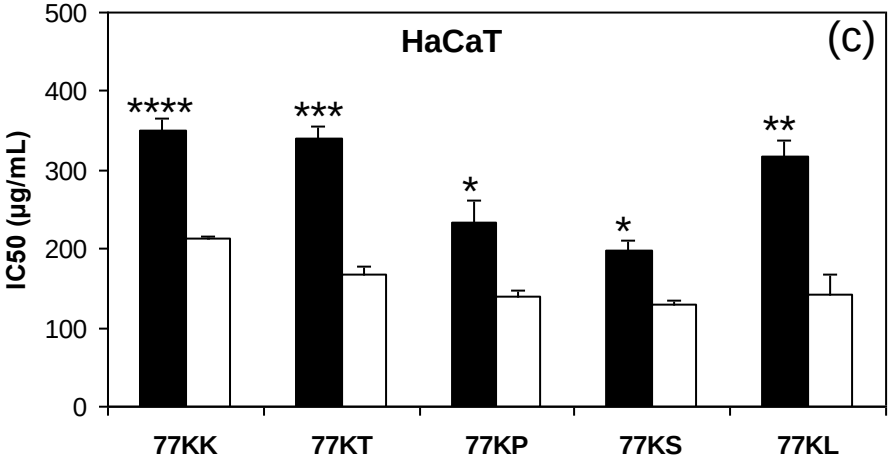

(e)
NR uptake (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7

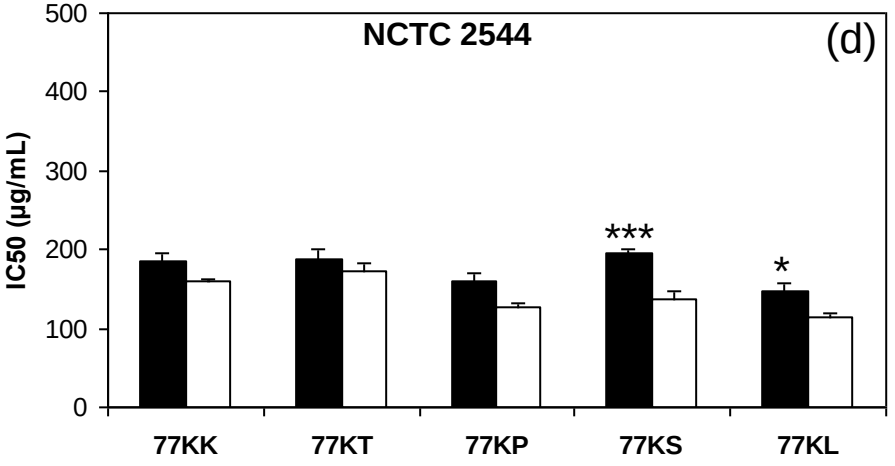

(f)
MTT reduction (% control)
Concentration µg/ml
3T6
3T3
HaCaT
NCTC
HeLa
MCF-7

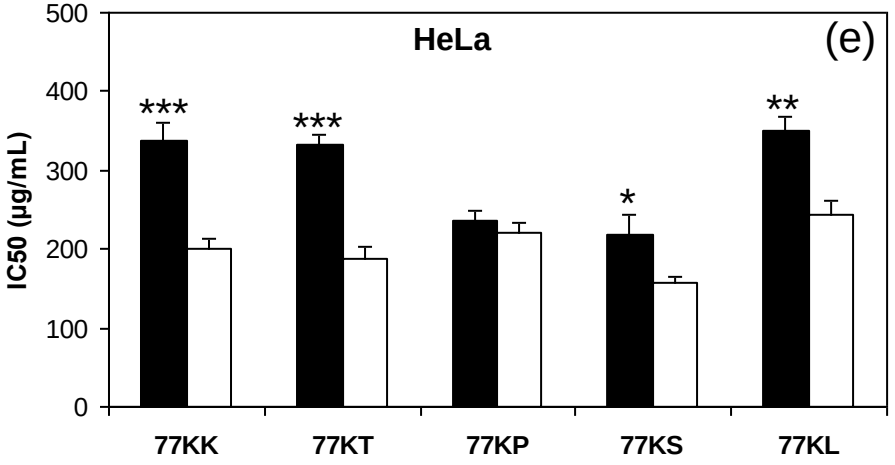
Figure(s)

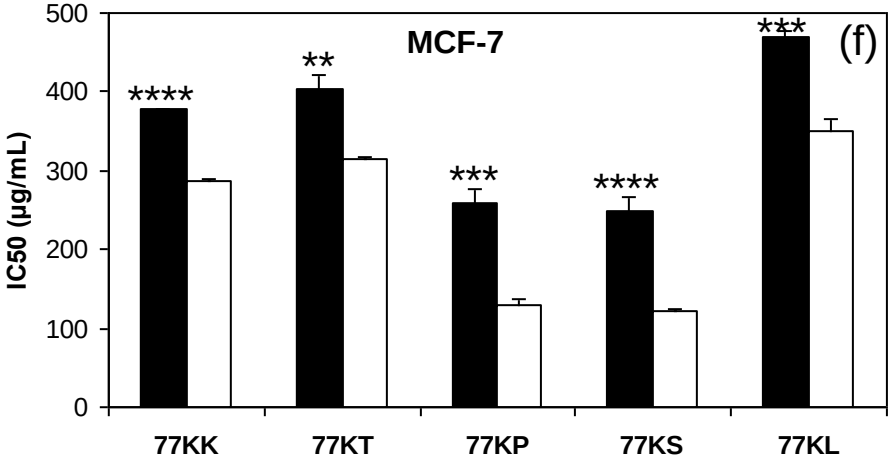

3T3
(a)
IC50 (µg/mL)
500
400
300
200
100
0
*
*
77KK
77KT
77KP
77KS
77KL


3T6
(b)
IC50 (µg/mL)
500
400
300
200
100
0
**
*
****
77KK
77KT
77KP
77KS
77KL

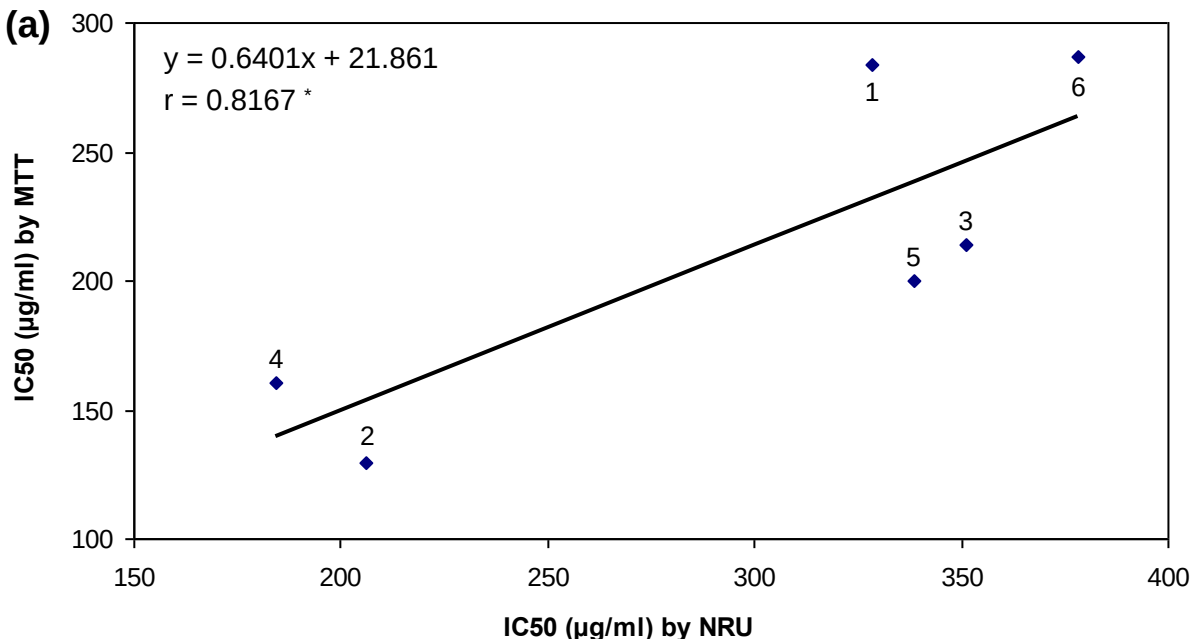

HaCaT
(c)
IC50 (µg/mL)
500
400
300
200
100
0
****
***
*
*
**
77KK
77KT
77KP
77KS
77KL

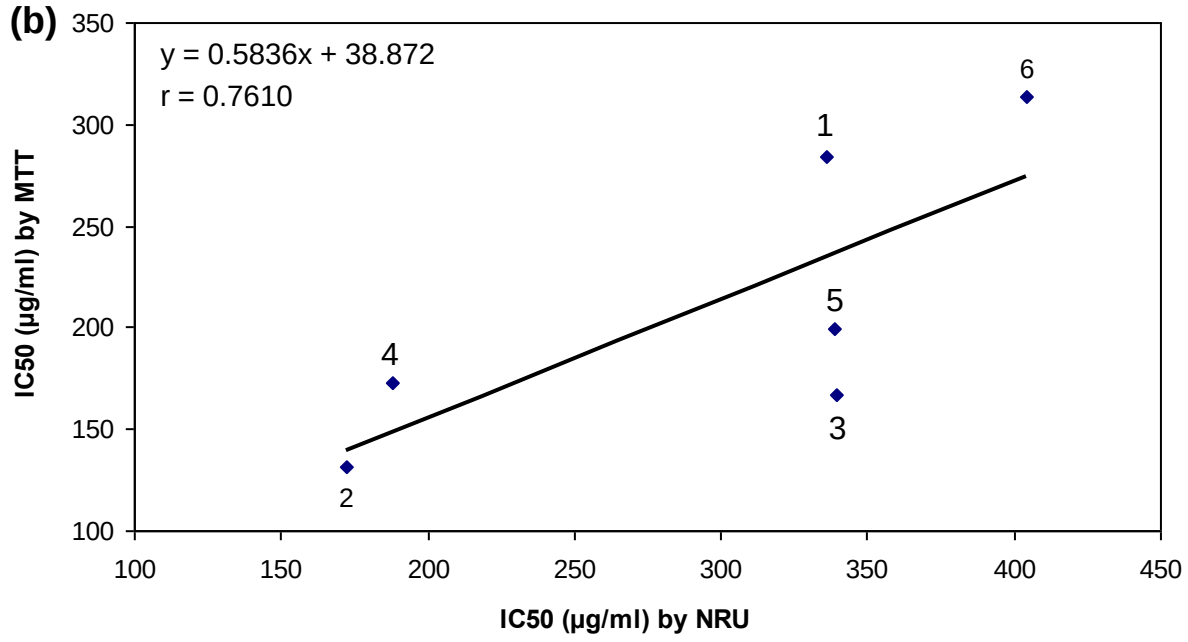

NCTC 2544
(d)
IC50 (µg/mL)
500
400
300
200
100
0
***
*
77KK
77KT
77KP
77KS
77KL

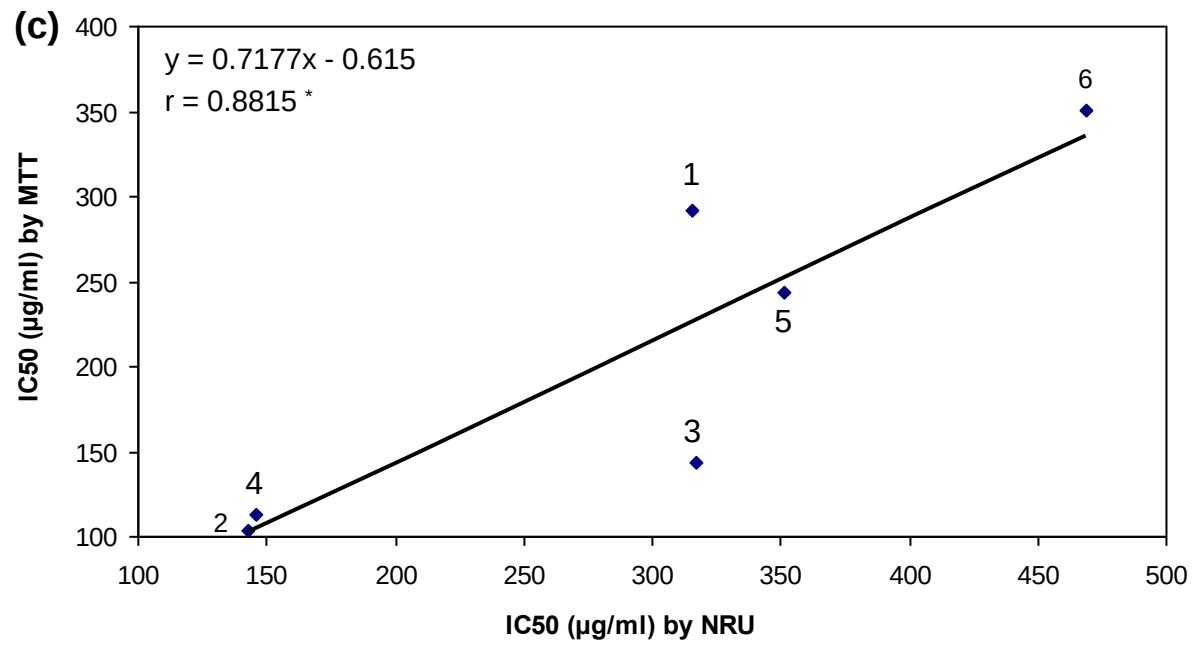

HeLa
(e)
IC50 (µg/mL)
500
400
300
200
100
0
***
***
*
**
77KK
77KT
77KP
77KS
77KL

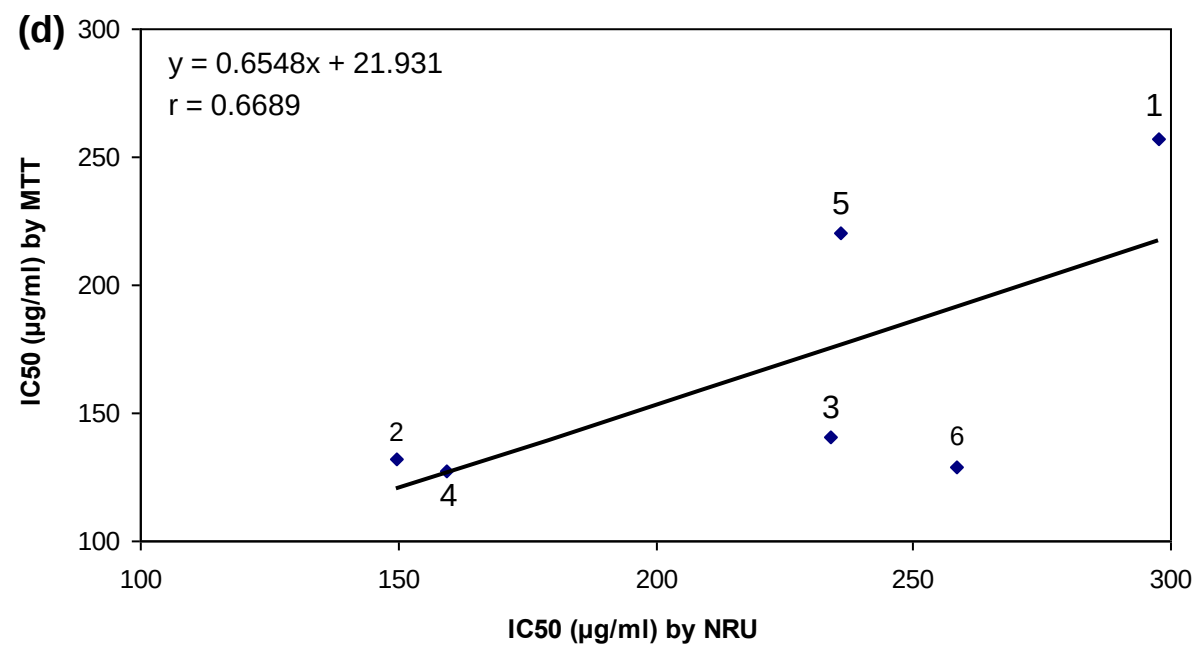

MCF-7
(f)
IC50 (µg/mL)
500
400
300
200
100
0
****
**
***
****
***
77KK
77KT
77KP
77KS
77KL

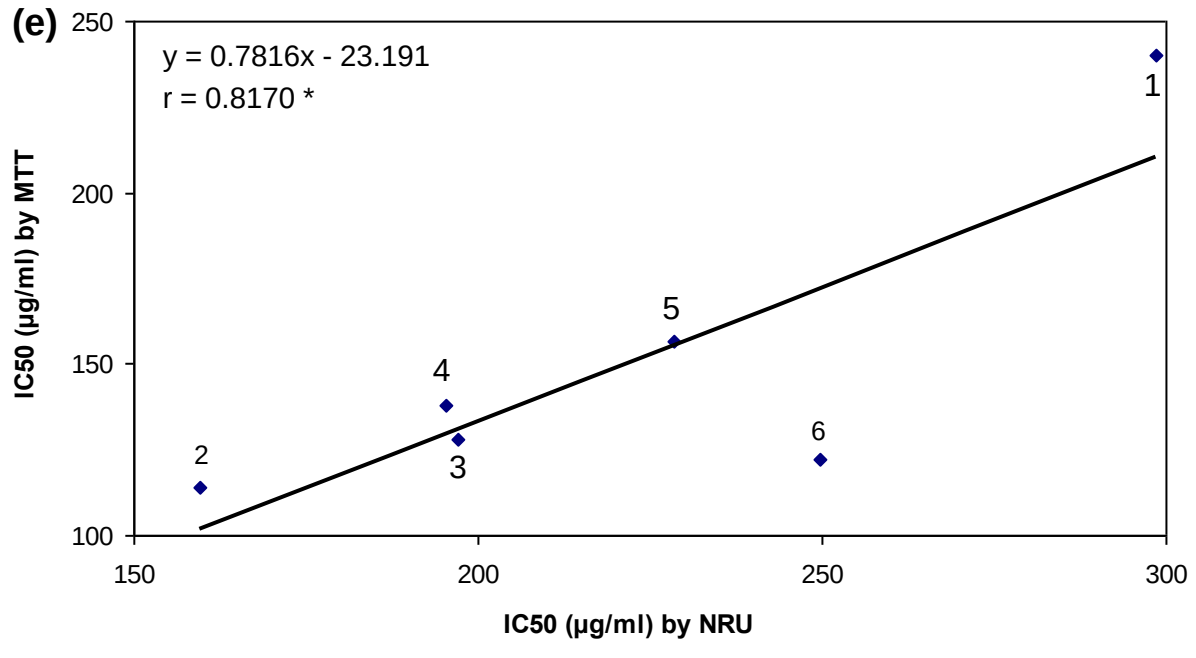
Figure(s)

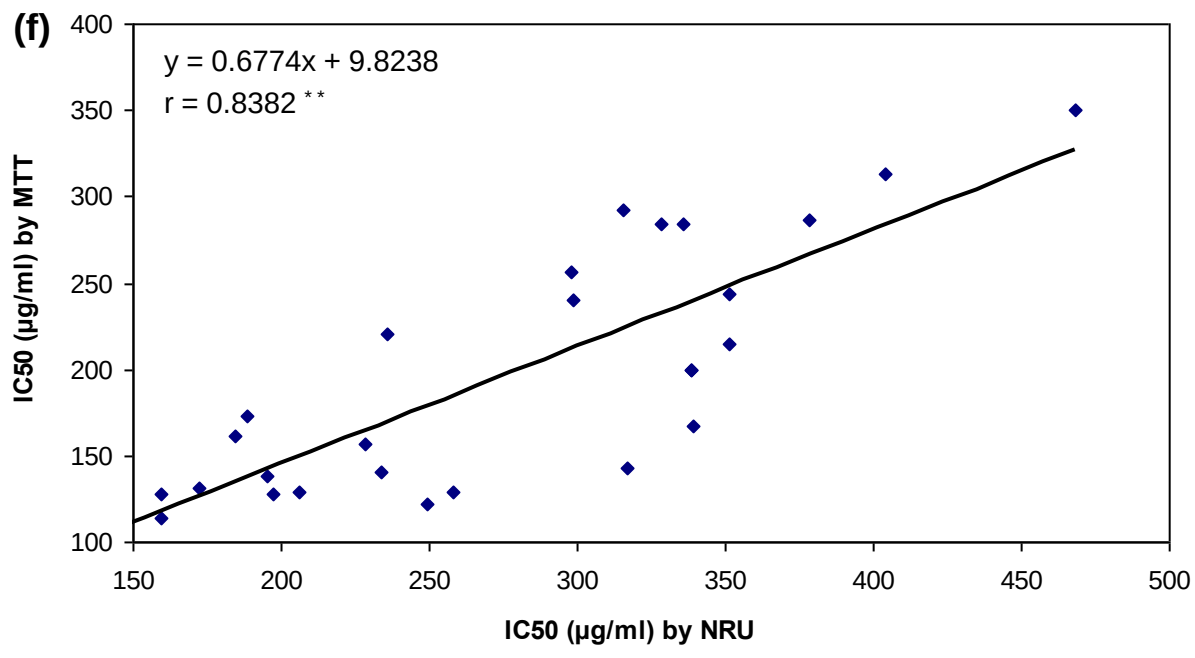

(a)
y = 0.6401x + 21.861
r = 0.8167 *
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU
1
2
3
4
5
6


(b)
y = 0.5836x + 38.872
r = 0.7610
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU
1
2
3
4
5
6


(c)
y = 0.7177x - 0.615
r = 0.8815 *
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU
1
2
3
4
5
6


(d)
y = 0.6548x + 21.931
r = 0.6689
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU
1
2
3
4
5
6


(e)
y = 0.7816x - 23.191
r = 0.8170 *
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU
1
2
3
4
5
6


(f)
y = 0.6774x + 9.8238
r = 0.8382 **
IC50 (µg/ml) by MTT
IC50 (µg/ml) by NRU