Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants

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Lipophilicity but not stereospecificity is a major determinant for local anaesthetic-induced cytotoxicity in human T-lymphoma cells

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Background and objectives: Local neurotoxicity of local anaesthetics (LA) is a well-known phenomenon that is determined by lipophilicity. Recent reports have indicated relevance of LA-induced cytotoxicity also in non-neuronal tissues. Thus, this study re-evaluates the role of lipophilicity for LA-induced cytotoxicity in non-neuronal cells. Furthermore, the toxicities of pipercoloxylidine S(-) enantiomers were investigated.

Methods: LA-induced cytotoxicity was investigated in vitro in T-lymphoma cells (Jurkat). Cells were incubated with eight different LA, two of the ester- and six of the amide-type. Annexin V – FITC and 7-AAD double staining followed by flowcytometry were used to investigate the fraction of early apoptotic cells as well as the overall cell death. For the eight LA the concentrations leading to 50% cell death (LC50) were calculated and compared. In a second step we compared the toxicities of S(-) bupivacaine and its racemate as well as R(+) and S(-) ropivacaine.

Results: Concentration-dependent cytotoxicity was observed for all investigated LA. Apoptosis was seen at low concentrations while necrosis was the cause for cytotoxicity at higher concentrations. LC50-values of the different LA yielded the following decreasing order of toxicity: Tetracaine, bupivacaine, ropivacaine, prilocaine, procaine, lidocaine, articaine, mepivacaine. The toxicity correlated with octanol/buffer partition coefficients, but was independent of the chemical type (ester- or amide-type). There was no effect of stereoisomerism on apoptosis and cell death.

Conclusions: Moderate correlations for cytotoxicity with lipophilicity and clinical potency of LA can be found in non-neuronal cells that are lower than those previously reported with neuronal cells. Structural factors like ester- or amide-type or stereospecificity do not have any influence on cytotoxicity. Although S(-) enantiomers may be advantageous in regard to systemic toxicity, they have no advantage in regard to local cytotoxicity in vitro.

Keywords: Toxicity, local anaesthetics, apoptosis, enantiomerism, stereospecificity
Introduction

Cytotoxicity and apoptosis induction have been reported for several local anaesthetics (LA) in different tissues of varying origin in vitro and in vivo [1-7]. While the focus traditionally has been on neurotoxicity, cytotoxicity in other tissues like muscle and cartilage has recently received more attention [3-6]. Thus, cytotoxicity of LA seems to be a universal phenomenon. We demonstrated recently in neuronal cells that lipophilicity and potency of LA are factors determining neurotoxicity [8]. In this study we tested whether these results could be transferred to non-neuronal cells by using a T-lymphoma cell culture model that had been evaluated for lidocaine-induced cytotoxicity previously [1]. Furthermore, we wanted to determine if stereoisomerism can influence cytotoxicity of LA.

The mechanism of neural toxicity is unrelated to blockade of the voltage-gated sodium channel or electrical inactivation of the neurons [9-11]. Therefore, it is not surprising that LA are not only toxic in excitable tissues like neurons [1, 2] or muscle [3, 4] but also in non-excitatable tissues [5-7, 12-15]. Specific properties of LA, like the chemical structure, might determine its toxicity. Ester-type LA have been considered to be more neurotoxic in comparison to amides [16-18]. There are conflicting results whether stereoisomerism of LA affects their cytotoxic effects. The more recently introduced S(-) enantiomers have been shown to be less cardiotoxic after systemic administration in comparison to the R(+) enantiomers [19, 20]. Likewise, S(-) bupivacaine seemed to be more beneficial in a mice model of central excitotoxicity [21], whereas the bupivacaine enantiomers were equally neurotoxic when applied intrathecally in rats [22]. Thus, the question whether toxicity induced by LA on the cellular level is influenced by stereospecific properties remains unanswered. Beyond the possible clinical relevance of such a finding, a stereospecific effect would suggest a specific binding structure for LA by which toxicity is induced.

Therefore, we determined the concentration-toxicity curves of the LA articaine, bupivacaine, lidocaine, mepivacaine, prilocaine, procaine, ropivacaine and tetracaine in human T-lymphoma cells to re-evaluate our previous results in a neuroblastoma cell line [8]. Furthermore, we compared the cytotoxicity of ropivacaine enantiomers as well as of the commercially available drug formulations S(-) bupivacaine (levobupivacaine) and racemic bupivacaine, which contains both enantiomers by the same amount.
Methods

Reagents

All LA were investigated as their hydrochloride salts. Bupivacaine, lidocaine, mepivacaine and prilocaine were obtained from AstraZeneca (London, United Kingdom). Procaine was purchased from Jenapharm (Jena, Germany), while articaine and tetracaine were obtained from Sanofi Aventis (Paris, France). S(-) bupivacaine (levobupivacaine) was purchased from Abbott (Wiesbaden, Germany). Differences in expressed formulation of S(-) bupivacaine were taken into account by using the molecular weight of the base instead of the hydrochloride salt for calculation of all dilutions [23]. Ropivacaine and its enantiomers were kindly provided by AstraZeneca, Research and Development (Södertälje, Sweden). Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from Gibco, Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

The origin and characteristics of human Jurkat T-lymphoma cells have been described before [1, 24, 25]. The cell line was grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated foetal calf serum and 50 µg ml\(^{-1}\) each of penicillin and streptomycin. Cells were cultured in a humidified atmosphere containing 5% carbon dioxide at 37°C.

Exposure to LA and Experimental Protocol

To produce logarithmic growth, cells were cultured for 12 to 24 h in complete medium at a concentration of 4 x 10\(^5\) cells ml\(^{-1}\) prior to the experiment. Subsequently, samples were prepared each with 4 ml of cell suspension and a density of 1 x 10\(^5\) cells ml\(^{-1}\). Cells were treated for 24 h at 37°C with medium alone as negative control, or indicated concentrations of LA diluted in the cell culture medium. In a second set of experiments the enantiomers of ropivacaine and bupivacaine were evaluated according to the same protocol, but three concentrations around the LC\(_{50}\) were chosen. The pH value of the medium (7.40, range 7.35-7.43) was not influenced by the addition of LA.

Apoptosis Detection Assay

Staining with fluorescein isothiocyanate (FITC) conjugated annexin V (Molecular Devices, Franklin Lake, NJ, USA) and 7-aminoactinomycin D (7-AAD) was used.
Positive staining for annexin V indicates the translocation of phosphatidylserine to the outer surface of the plasma membrane in presence of early apoptosis [26]. In contrast, cells in a later state of apoptosis or primarily necrotic cells stain positive for 7-AAD, because the cells are not able to exclude 7-AAD via the disrupted membrane [27]. Overall cell death is defined as the sum of percentage of early apoptotic cells (annexin V positive) and necrotic cells (annexin V + 7AAD positive) in absence of cells solely stained with 7-AAD.

For annexin V / 7AAD staining, cell culture medium was transferred from sample wells to analyzing tubes. Cells were then washed twice with cold PBS and resuspended in 97 µl annexin binding buffer (10 mM N-[2-hydroxyethyl]piperazin-N'-3[propanesulfonicacid]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$) at a concentration of 1 $\times$ 10$^6$ cells ml$^{-1}$. Next, the samples were incubated with 5 µl of annexin V – FITC and 2 µl 7-AAD (50 µg ml$^{-1}$) for 15 min protected from light at room temperature. 150 µl of annexin binding buffer were added, cells resuspended and analyzed immediately.

All flowcytometric analyses were performed using a FACScalibur flowcytometer and CellQuest analysis software (BD Biosciences). A minimum of 10,000 cells were analyzed for each probe.

**Statistical Analysis**

All experiments were performed in triplicate. SPSS (Statistical Package for Social Sciences) program version 16.0 (SPSS Inc. Chicago, IL, USA) was used for calculations. Results are expressed as means (standard deviation). Under the guidance of our statistics department the concentration-response curves of the different LA were calculated by probit regression. Comparison of the LC$_{50}$, which were obtained from probit analysis as means (95% confidence interval) was achieved by ANOVA with Tukey’s post hoc test. Spearman rank correlations were estimated for LC$_{50}$ values with lipid solubility, clinical blocking potency, ionisation constant (pKa), unionised fraction at pH 7.4, protein binding and molecular weight. In order to evenly weight results from all 8 investigated LA, only LC$_{50}$ values for racemic bupivacaine and ropivacaine were included in correlation analysis. Comparisons between percentages of cell death induced by different enantiomers were tested with Wilcoxon rank-sum test. P < 0.05 was considered significant.
Results
All LA induced early and late apoptosis (or primary necrosis) as indicated by a 7-AAD and annexin V double-staining. LA concentrations below the half maximal toxic concentration (LC50) led to an increase of cells in an early stage of apoptosis (annexin V positive only) of 69.7 ± 18.9% of overall cell death. In contrast, higher LA concentrations (above LC50) induced double-staining for annexin V and 7-AAD in 78.6 ± 13.1% of detected cell deaths, indicating a later state of apoptosis or primary necrosis.

Investigated LA induced a reduction of cell viability (annexin V positive cells irrespective of 7AAD staining) in a concentration-dependent manner (fig.1). The concentrations decreasing the cell viability varied between 0.2 mM and more than 10 mM for the different LA (fig. 1).

Figure 1

![Fig. 1 Concentration-dependent viability of cells (percentages of cells staining negative for annexin V and 7AAD) after 24 h exposure to one of 8 different local anaesthetics. Sigmoid shaped curves for concentration-viability relationship were calculated by probit regression analysis using the results from triplicate experiments with at least six different concentrations of each local anaesthetic. Single symbols represent results from single experiments.](image)
Concentration-toxicity functions were analysed by means of probit regression. Cell death was defined as the percentage of cells staining positive for annexin V only and the percentage of cells staining positive for annexin V and 7-AAD. The concentration that induced 50% cell death (LC\textsubscript{50}) was derived from the regression analysis. All LC\textsubscript{50}-values are displayed in figure 2. ANOVA revealed significant differences between the LA yielded the following decreasing order of toxicity (mean; CI): Tetracaine (0.23 mM; 0.21-0.24), bupivacaine (1.3 mM; 1.2-1.5), ropivacaine (2.3 mM; 2.1-2.5), prilocaine (2.7 mM; 2.5-3.0), procaine (4.7 mM; 3.9-5.5), lidocaine (4.7 mM; 4.4-5.0), articaine (5.3 mM; 4.8-5.9), mepivacaine (6.1 mM; 5.8-6.3). However, in contrast to all other comparisons, there were no significant differences between the toxicities of procaine and lidocaine as well as between articaine and mepivacaine according to the applied Tukey’s post-hoc-test.

**Figure 2:**

![Graph showing LC\textsubscript{50} values for different LA](image)

**Fig. 2** Half-maximal toxic concentrations (LC\textsubscript{50}) of investigated local anaesthetics. LC\textsubscript{50}-values (mean, 95% confidence interval) were calculated from probit analysis and tested by ANOVA with Tukey’s post-hoc test; * = P < 0.05. Please note that apart from indicated differences, all comparisons of local anaesthetics on either sides of a significance sign revealed significantly different LC\textsubscript{50} values.

In a separate set of experiments, we focused on the role of enantiomerism for LA-induced cytotoxicity. At the selected 3 concentrations S(-) bupivacaine and its racemate induced equal cytotoxicity (fig. 3). Accordingly, calculated LC\textsubscript{50} for S(-) bupivacaine (1.2 mM; 1.1-1.3) did not differ significantly from racemic bupivacaine. Likewise, similar percentages of cell death were detected after incubation with ropivacaine enantiomers (fig. 3). Furthermore, we found no difference in calculated
LC$_{50}$ values, which were 2.9 mM (2.6-3.3) for S(-) ropivacaine and 2.7 mM (2.2-3.3) for R(+) ropivacaine. Thus, these data confirm, that the cytotoxicities of both bupivacaine and ropivacaine are not dependent of stereospecific properties.

**Figure 3:**

![Figure 3](image_url)

Fig. 3 Comparisons of apoptosis/cell death induction between different enantiomers of bupivacaine and ropivacaine. Cells in early apoptosis, staining positive for annexin V and negative for 7-AAD, are delineated in the hatched areas, whereas cells in late apoptosis or necrosis are delineated in black areas. There was no significant difference between S(-) bupivacaine (S-Bupi) and racemic bupivacaine (Bupi) or between S(-) ropivacaine (S-Ropi) and R(+) ropivacaine (R-Ropi), respectively.

The LC$_{50}$-values correlated moderately with octanol-buffer distribution coefficients and the clinical potencies of the LA as known from the literature [28-30], indicated by correlation coefficients of $r = -0.738$ and $r = 0.741$, respectively. Correlation scatter plots and regression line with 95% confidence intervals are displayed in figure 4. Both factors correlated significantly with the measured toxicity of LA (P<0.05), while all other physicochemical properties (pKa, unionized fraction at pH 7.4, protein binding and molecular weight) [28] did not. Thus, the more lipophilic and the more potent a LA is, the more cytotoxic it is.
Figure 4:

Fig. 4 Scatter plots of correlation between toxicity (concentration of 50% cell death = LC\textsubscript{50}) and octanol-buffer partition coefficient (A) and relative experimentally effective anaesthetic concentrations (B). Each circle represents one local anaesthetic. Triangles indicate the values for S(-) enantiomers of bupivacaine and ropivacaine, while squares mark the values for R(+) ropivacaine. The straight line represents the linear regression, whereas the curved lines symbolise the 95% confidence interval. R delineates correlation coefficient according to Spearman’s rank sum test. In order not to bias results, only LC\textsubscript{50} values from racemic ropivacaine and bupivacaine were included in correlation analysis. The measured toxicity correlated significantly with lipophilicity [28] and potency [29] of the local anaesthetic, although the variation was considerable.

Discussion
The investigated LA were cytotoxic at concentrations well below those injected clinically. In our \textit{in vitro} model bupivacaine induced toxicity at concentrations as measured in cerebrospinal fluid of humans within 45 min after induction of spinal anaesthesia [31]. As demonstrated in neuronal cells before, the toxicities of LA correlated with their octanol-buffer partition coefficients and their relative clinical potencies in this non-neuronal cell culture. Furthermore, the cytotoxicity of ropivacaine and bupivacaine was not stereospecific.

It has been demonstrated before that LA can induce apoptosis [2, 12-15, 32-34]. Many studies investigated only one single substance [12-14, 32-34]. Therefore, it is difficult to compare the toxic potencies of various LA. Boselli et al. compared the
toxicities of lidocaine and ropivacaine in human lymphocytes [15]. Unfortunately, in that study only one concentration of each drug was investigated. The authors admitted this methodological disadvantage and recommended studies investigating broader ranges of concentrations and LA.

Recently, Lee and colleagues found that only tetracaine, but not lidocaine, S(-) ropivacaine, racemic bupivacaine or S(-) bupivacaine, induced apoptosis in a primary astrocyte cell culture model [18]. Unfortunately, these authors only investigated concentrations below 1 mM. At these concentrations only tetracaine caused significant cell death in our model. Since the authors did not investigate higher concentrations, their results do not contradict our findings. Perez-Castro et al. and our group compared the toxic effects of procaine, mepivacaine, lidocaine, chloroprocaine, ropivacaine and bupivacaine in human neuroblastoma cells and found a similar order of toxicity as presented here [8, 35]. In comparison to these results in neuroblastoma cells the Jurkat lymphoma cells used here were about 30-50% more sensitive to LA-induced toxicity. Thus, counter-intuitively, non-neuronal cells seem to be more sensitive to LA-induced toxicity than neuronal cells. However, mepivacaine was comparatively less toxic in lymphoma cells than in neuronal cell for unknown reasons. Although the clinical potencies of articaine, mepivacaine, lidocaine and procaine are roughly the same, they vary in cytotoxicity almost 3-fold (fig. 4). Nevertheless, lipophilicity - and therewith potency – determines the toxicity of LA also in non-neuronal cells, although to a lesser extent as in neurons ($R^2=0.54$ compared to 0.77, respectively) [8]. Therefore, the moderate correlation found in this study suggests that lipophilicity is not the only determinant of toxicity in non-neuronal cells, but other structural properties might also influence toxicity. As in neuronal cells before [8], no other investigated physicochemical property like ionization constant or protein binding correlated significantly with observed cytotoxicity. This observation was therefore one of the reasons to explore further on structural factors like enantiomerism.

Lirk and colleagues compared the neurotoxic effects of lidocaine, bupivacaine and ropivacaine in rodent primary dorsal root ganglia cell cultures [2]. In equipotent concentrations the percentages of cell death did not differ between these LA. Although they compared only one concentration of each of three LA, their results are in line with our findings.
The LC$_{50}$ of bupivacaine in our model is in the concentration range as measured in cerebrospinal fluid of patients within 45 min after single-shot spinal anaesthesia [31]. Unfortunately, there are no data measuring concentrations in the cerebrospinal fluid over a longer period. However, the calculated half-life of bupivacaine in the cerebrospinal fluid was 50.8 min in patients after intrathecal injection [31]. In our model cells were exposed for 24 h to a constant concentration of LA. That may explain why toxicity is very rarely observed, clinically. Thus, beyond concentration the time of exposure to a LA is important in regard to cytotoxicity.

Probably the most interesting finding from our study is that cytotoxicity of LA is not stereospecific. Research suggesting that the more recently introduced S(-) enantiomers induced less systemic neuro- and cardiototoxicity ensured their quick adoption into clinical practice [36]. After peripheral nerve blocks, bupivacaine seemed to be associated with an increased percentage of nerve injuries in comparison to S(-) ropivacaine in one retrospective study [37], but these results were not confirmed by more recent evidence [38]. In an animal model of myotoxicity, bupivacaine enantiomers equally decreased ATP levels in muscles by mitochondrial enzyme inhibition, but only S(-) bupivacaine interfered significantly with calcium homeostasis [39]. After intrathecal application in rats, nerve injury scores were not significantly different after injection of R(+) or S(-) bupivacaine [40]. Although this animal model is much closer to the clinical situation, it can quantify the effects only semi-quantitatively, while our model has the advantage of quantifying the effects of stereoisomerism more precisely. Our results are in accordance with the above-mentioned in vitro studies in muscle and neuronal tissues. Thus, the local cytotoxic effect of LA seems to be not stereospecific in any tissues.

In isolated guinea pig hearts, the S(-) enantiomer of bupivacaine blocked the atroventricular conduction to a smaller extent than the R(+) enantiomers [20], whereas the inhibition of mitochondrial bioenergetics in the rat heart was not stereoselective [41]. Therefore, one might speculate that similar to the effects of lidocaine [1, 33], the induction of mitochondrial dysfunction and subsequent release of apoptotic substances may be the relevant and not stereospecific action of all investigated LA regarding their toxicity.

Cell culture models like the one used in the present investigation have several limitations in transferring results to the in vivo situation: Jurkat lymphoma cells are dividing in an artificial environment during exposure to LA. Furthermore, Jurkat cells
are not of ectodermal origin, but apoptosis induction and cytotoxicity have been shown in cells of meso, ento- and ectodermal origin [1-7]. As cytotoxicity of LA is common to all tissues, Jurkat cells have been used previously to investigate the mechanism of LA-induced cytotoxicity [1]. Jurkat cells are even more sensitive to LA-induced toxicity than primary cell lines and thus maybe a more sensitive model [2, 42].

We observed a correlation between toxicity and octanol-buffer partition coefficient as well as clinical potency. Thus, the therapeutic margin seems to be approximately the same for all LA, irrespective whether they are of the ester or amide-type. In contrast to other findings the ester-type LA were not more toxic than the amide-type LA [17, 18].

In conclusion, all LA induce concentration-dependent apoptosis and thus cytotoxicity. The cytotoxicity of LA correlates moderately with lipophilicity and clinical potency in non-neuronal cells, but less pronounced compared to neuronal cells. Furthermore, LA-induced cytotoxicity is independent of the chemical type of LA (i.e. ester- or amide-type). Finally, the more recently introduced S(-) enantiomers of LA offer no advantage in regard to local cytotoxicity.

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