Apoptosis induction by different local anaesthetics in a neuroblastoma cell line

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Background. Local anaesthetics are known to induce apoptosis in clinically relevant concentrations. Hitherto, it is unknown what determines the apoptotic potency of local anaesthetics. Therefore, we compared apoptosis induction by local anaesthetics related to their physico-chemical properties in human neuronal cells.

Methods. Neuroblastoma cells (SHEP) were incubated with eight local anaesthetics, two of the ester and six of the amide types. At least, five concentrations of each local anaesthetic were evaluated. After incubation for 24 h, rates of cells in early apoptotic stages and overall cell death were evaluated by annexin V and 7-amino-actinomycin D double staining by flow cytometry. The concentrations that led to half-maximal neurotoxic effects (LD₅₀) were calculated and compared for all local anaesthetics.

Results. All local anaesthetics were neurotoxic in a concentration-dependent manner. All drugs induced similar rates of early apoptotic cell formation at low concentrations, whereas at high concentrations, late apoptotic or necrotic cell death predominated. Comparison of LD₅₀ values of the different local anaesthetics resulted in the following order of apoptotic potency from high to low toxicity: tetracaine > bupivacaine > prilocaine = mepivacaine = ropivacaine > lidocaine > procaine = articaine. The toxicity correlated with octanol/buffer coefficients and also with experimental potency of the local anaesthetic, but was unrelated to the structure (ester or amide type).

Conclusions. All commonly used local anaesthetics induce neuronal apoptosis in clinically used concentrations. The neurotoxicity correlates with lipid solubility and thus with the conduction blocking potency of the local anaesthetic, but is independent of the chemical class (ester/amide).

Br J Anaesth 2009; 103: 711–18

Keywords: measurement techniques, flowmetry; model, neuroblastoma cells; toxicity, local anaesthetics; toxicity, neurotoxicity

Accepted for publication: July 21, 2009

Local neurotoxicity after neuraxial application of local anaesthetics is rare. Nevertheless, it is a severe problem when it occurs. Clinical profiles of neurotoxicity have been based on the reported incidence of cauda equina syndrome or transient neurologic syndrome (TNS) after spinal anaesthesia.¹–³ Clinically, lidocaine is the local anaesthetic which has most often been linked to local neurotoxicity, although incidental neuronal damage has been described for other local anaesthetics as well. In animal studies, neurotoxicity of many local anaesthetics has been demonstrated.⁴–⁸ Experimental evidence suggests that the mechanism of local anaesthetics-induced neurotoxicity is unrelated to the blockade of the voltage-gated sodium channel or electrical inactivation of a nerve.⁷ ⁹
Comparison of the neurotoxic potency (concentration-dependency) for different local anaesthetics is difficult in animals for biometric reasons.

Local anaesthetics induce elevations of intracellular calcium concentration through external influx or release from intracellular stores. In this process, apoptosis has been shown to be one mechanism of neurotoxicity in vitro, especially in marginally toxic concentrations.

Recent studies have delineated the subcellular mechanism of apoptosis induction by local anaesthetics in neuronal cell cultures. Hence, this model seems suitable for the investigation of the neurotoxic and neuroapoptotic potential of different local anaesthetics.

The investigation of the toxicity of different local anaesthetics may help to evaluate which properties of local anaesthetics are responsible for their toxic effects. Does the chemical structure, that is, ester or amide type, influence their toxicity? Ester-type local anaesthetics have been considered to be somewhat more neurotoxic in comparison with amides. Are there certain physicochemical properties that determine the toxic potential of a local anaesthetic like lipophilicity, pKa value, protein binding, or molecular weight?

Therefore, we compared the concentration-dependent neuroapoptotic and neurotoxic potencies of the amide-type local anaesthetics bupivacaine, lidocaine, mepivacaine, prilocaine, and ropivacaine and also the ester type local anaesthetics procaine and tetracaine in our human neuronal cell culture model.

Methods

Reagents

Unless stated otherwise, reagents were purchased from Sigma Aldrich (St Louis, MO, USA). All local anaesthetics were obtained in the highest commercially available concentration as their hydrochloride salts. Bupivacaine, lidocaine, mepivacaine, and prilocaine were obtained from AstraZeneca (London, UK). Procaine was purchased from Jenapharm (Jena, Germany), and articaine and tetracaine were acquired from Sanofi Aventis (Paris, France). None of the commercially available solutions contained preservatives. Ropivacaine was 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).

Cell culture

Human neuroblastoma cells (SHEP) are a subclone derived from the human neuroblastoma cell line SK-N-SH and have been characterized before. All cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with l-glutamine, supplemented with 10% heat-inactivated fetal calf serum and 50 μg ml⁻¹ each of penicillin and streptomycin. All cells were cultured under equal conditions including a humidified atmosphere containing carbon dioxide 5% at 37°C.

Exposure to local anaesthetics and experimental protocol

Before the experiments, cells were cultured overnight in complete medium at a density of 4×10⁵ cells ml⁻¹ to allow logarithmic growth. Adherent cells were cultured in 3 ml samples with fresh medium alone as negative control, or one added local anaesthetic for 24 h at concentrations as indicated. Addition of local anaesthetics did not alter the pH value of the medium (7.39, range 7.35–7.43).

Apoptosis detection assay

The fraction of cells in an early state of apoptosis was determined by staining cells with fluorescein isothiocyanate-conjugated (FITC) annexin V and counterstaining with 7-amino-actinomycin D (7-AAD). Annexin V binds to phosphatidylserine on the outer leaflet of the plasma membrane. 7-AAD is excluded by cells with intact membranes. Therefore, 7-AAD staining reveals membrane disintegration and is a marker for primary or secondary necrotic cell death. Cells staining with annexin V-FITC (below referred to as annexin V), but not with 7-AAD, are defined as early apoptotic. Briefly, for annexin V/7-AAD staining, cell culture medium including detached cells was transferred from sample wells to analysing tubes. Adherent cells were trypsinized for 3 min with 1.5 ml 0.05% trypsin with 1 mM EDTA per sample. Detached cells were added to corresponding analysing tubes to pool all cells from each sample. Subsequently, cells were washed twice with cold PBS and resuspended in annexin binding buffer 97 μl (10 mM N-[2-hydroxyethyl]piperezine-Ν'-[3-propanesulfonicacid]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 1×10⁶ cells ml⁻¹. Next, annexin V-FITC 5 μl and 7-AAD 2 μl (50 μg ml⁻¹) were added and samples were incubated for 15 min in the dark at room temperature. Subsequently, annexin binding buffer 150 μl was added, cells were resuspended and analysed immediately.

All fluorescence-activated cell sorting analyses were performed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest analysis software (BD Biosciences, Franklin Lake, NJ, USA). For each determination, a minimum of 10 000 cells were analysed.

Statistical analysis

All experiments were performed at least in triplicate. Results are expressed as means (sd). All calculations were made with the SPSS program version 15.0 (SPSS Inc.,
Chicago, IL, USA). Concentration–response curves of the different local anaesthetics were determined by probit regression under the guidance of our statistics department. The LD$_{50}$ was obtained from probit analysis and compared by means of analysis of variance (ANOVA) with Tukey’s post hoc test. Correlations between LD$_{50}$ values with lipid solubility, pKa, blocking potency, non-ionized fraction, protein binding, molecular weight, and experimental blocking potency were made by means of Spearman’s rank correlation test. $P<0.05$ was considered significant.

**Results**

Concentrations of local anaesthetics inducing cell death in $<50\%$ of analysed cells resulted in a considerable fraction of cell positive for annexin V only. This indicates an early stage of apoptosis in at least a fraction of the analysed cells. Nevertheless, also at these concentrations, cells staining positive for annexin V staining and 7-AAD were present (Fig. 1), indicating late apoptosis or necrosis. Higher concentrations (leading to cell death in more than 50% of analysed cells) of all investigated local anaesthetics led to increased fractions of cell staining positive both for annexin V and for 7-AAD. This indicates a late stage of apoptosis or primary necrosis. Thus, all investigated local anaesthetics induced cell death in a concentration-dependent manner (Fig. 2). The toxic concentrations inducing early stages of apoptosis and late stages of cell death varied over a wide range (almost hundred-fold) for the different local anaesthetics.

In order to compare the toxicity of all eight local anaesthetics, the concentration-toxicity functions derived from regression analysis were used to calculate the concentrations that induced $\sim 50\%$ overall cell death (LD$_{50}$). Overall cell death was defined as the sum of cells staining positive for annexin V only and cells staining positive for annexin V and 7-AAD. This indicates a late stage of apoptosis or primary necrosis. Thus, all investigated local anaesthetics induced cell death in a concentration-dependent manner (Fig. 2). The toxic concentrations inducing early stages of apoptosis and late stages of cell death varied over a wide range (almost hundred-fold) for the different local anaesthetics.

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In order to identify factors affecting toxicity, the LD$_{50}$ values were correlated to a number of known physico-chemical properties as displayed in Table 1. LD$_{50}$ values correlated well with octanol/buffer distribution coefficients. Spearman’s rank correlation coefficient was $-0.88$ ($P<0.01$), that is, the higher the local anaesthetic solubility in oil, the lower was the observed toxic concentration. In contrast, LD$_{50}$ values did not correlate with pKa values, fraction of non-ionized local anaesthetic, or protein binding. Finally, LD$_{50}$ values correlated positively with experimental effective anaesthetic concentrations (correlation coefficient: $0.81$, $P<0.05$).\textsuperscript{29–32}

The percentage of early apoptosis (annexin V+, 7-AAD-) of all cells first increased in a concentration-dependent manner, reaching a maximum with local anaesthetic concentrations well below the LD$_{50}$ values (Fig. 4). The highest percentage of cell death by early apoptosis varied between the different local anaesthetics between 14% and 28% after 24 h of incubation. With a further increase in concentration, the percentage of early apoptotic cell formation decreased and returned to baseline for almost all local anaesthetics (Fig. 4).

**Discussion**

All local anaesthetics investigated were neurotoxic at concentrations observed intrathecally after spinal anaesthesia. They induced in a concentration-dependent manner apoptotic and with higher concentrations necrotic cell death. The toxicity of the local anaesthetics correlated with their octanol/buffer partition coefficient and thus their relative clinical potency.

Several publications demonstrated that lidocaine and other local anaesthetics can induce apoptosis in neuronal and non-neuronal cells.\textsuperscript{13, 14, 19–21, 33–37} Most of these studies investigated one drug only.\textsuperscript{13, 20, 21, 33–37} Therefore, toxic potency of various local anaesthetics from different models cannot be compared for methodological reasons. Boselli and colleagues\textsuperscript{10} compared the toxic and apoptotic potential of lidocaine and ropivacaine in human lymphocytes. Unfortunately, only one concentration of lidocaine and a different—not equipotent—concentration of ropivacaine were investigated. These shortcomings are discussed by the authors themselves and they advocate studies with more concentrations and local anaesthetics in human neuronal cell lines, as presented here.

More recently, Perez-Castro and colleagues\textsuperscript{38} compared the cytotoxic effects of short (10 min) exposure to procaine, mepivacaine, lidocaine, chloroprocaine, ropivacaine, or bupivacaine in human SH-SY5Y neuroblastoma cells and found the same order of toxicity as seen in the data presented here. In contrast to the results presented here, they found apoptosis (non-quantitative caspase activation) only after exposure to high concentrations of lidocaine and bupivacaine. Probably, exposure to very high concentrations for a very short time leads to a greater predominance of necrotic cell death, whereas during long-term exposure of neurons, apoptosis is one major mechanism of cell death.

Lirk and colleagues\textsuperscript{14} compared the neurotoxic potentials of lidocaine, bupivacaine, and ropivacaine in equipotent concentrations in primary cell cultures of rat dorsal root ganglia. They evaluated their blocking potential on voltage-gated sodium channels NaV$_{1.1}$–3 and NaV$_{1.6}$ in rat pituitary cells, and applied equipotent concentrations of each of the three local anaesthetics on primary cell cultures of rat dorsal root ganglia for 24 h. At equipotent concentrations, the percentage of cell death did not differ
Fig 1 Flow cytometric analysis of neuroblastoma cells incubated for 24 h with local anaesthetics. Dot plots represent typical results after incubation with medium alone (control), lidocaine, bupivacaine, or tetracaine. Intensity of red fluorescence by 7-AAD-stained cells is indicated on the ordinate, whereas intensity of green fluorescence emerging from cell-bound annexin V-FITC is indicated on the abscissa. Unstained and therefore vital cells lead to a population of dots in the lower left quadrants as in controls. Cells in the lower right quadrants are stained positive for annexin V-FITC only and are therefore in an early apoptotic stage, whereas cells in the upper right quadrants were stained positive for annexin V-FITC and 7-AAD indicating late apoptosis or primary necrosis. Note that vital cells and early apoptotic cells can be found at lower concentrations of all local anaesthetics, whereas at high concentrations, almost all cells show signs of lost cell membrane integrity.
between the three local anaesthetics. Their results are reconfirmed and generalized to more local anaesthetics through the results of the present study.

The concentrations of local anaesthetics that induced apoptosis in our model are within the same range as those observed intrathecally after single-shot spinal anaesthesia.
in primates and in sciatic nerves of rodents during nerve blockade.\textsuperscript{39, 40} Therefore, concentrations inducing neurotoxicity could be reached clinically.\textsuperscript{41} However, after a single-shot spinal anaesthesia or peripheral nerve block, these concentrations are only reached for about 1 h, whereas in our cell culture model, concentrations were kept constant for 24 h. It is well known that beyond the concentration, the time of exposure to a local anaesthetic is important for the development of neurotoxicity; therefore, neurotoxicity after single application is a rare complication clinically. Thus, intraneural injection of lidocaine 2\% (~78 mM) for single-shot sciatic nerve block did not lead to any functional nerve damage, although this concentration is more than eight times the LD\textsubscript{50} concentration observed here.\textsuperscript{42}

Although we used human tumour cells which might generally be resistant to apoptosis induction, they were actually more sensitive to apoptosis induced by local anaesthetics than primary rodent dorsal root cell cultures and hybrid immortalized dorsal root ganglia.\textsuperscript{14, 20} Obviously, the cell culture model used for the presented study has several limitations in translating data to the \textit{in vivo} situation. Human SHEP neuroblastoma cells are growing and dividing during the local anaesthetic exposure rather than being mature terminally differentiated neurones. They are derived from malignant neural crest cells which would ordinarily differentiate into the sympathetic chain, adrenals, or dorsal root ganglia. Nevertheless, despite those limitations, our model of a human neuronal cell line seems even more sensitive in detecting minor differences between different local anaesthetics compared with \textit{in vivo} studies.

Previous animal studies compared the neurotoxic, but not the apoptotic, potency of different local anaesthetics.\textsuperscript{4, 5, 7, 43} No study has compared a wide range of local anaesthetics in varying concentrations, but a few studies compared different local anaesthetics in equipotent concentrations. Myers and colleagues\textsuperscript{4} investigated the effect of 2-chloroprocaine, tetracaine, lidocaine, and bupivacaine in high concentrations on the sciatic nerve of rats. All drugs induced swelling, but 2-chloroprocaine and tetracaine significantly more than lidocaine or bupivacaine. However, the effects of different local anaesthetics were compared at a single concentration, and thus equipotency could not be ensured. The same group investigated the blocking and toxic concentrations of etidocaine, lidocaine, 2-chloroprocaine, and procaine on the sciatic nerve of rats 2 days after a single-shot injection. They found a perfect correlation between nerve blocking concentrations and toxic concentrations.\textsuperscript{44} Unfortunately, they instituted only two commonly used drugs. Nevertheless, their findings regarding procaine and lidocaine are in accordance with the data presented here.

Sakura and colleagues\textsuperscript{7} reported that equipotent concentrations of lidocaine and bupivacaine resulted in an equal sensory deficit after 4 days in a model of spinal anaesthesia in rats. In a subsequent study, Sakura and colleagues\textsuperscript{45} found an increased functional and morphological neurotoxicity of lidocaine in comparison with bupivacaine, probably because this study was more appropriately powered. Yamashita and colleagues\textsuperscript{46} in a similar model found bupivacaine and ropivacaine to be significantly less toxic than lidocaine and tetracaine. Recently, Umbrain and colleagues\textsuperscript{47} demonstrated that intrathecal administration of equipotent doses levobupivacaine and lidocaine led to release of glutamate and prostaglandin E\textsubscript{2}, while on repeated injection, lidocaine but not levobupivacaine led to enhanced prostaglandin release.

To summarize, most \textit{in vivo} animal studies have found rank orders of toxicity in accordance with our results. The only difference is that lidocaine seems to be more toxic in some studies. This varying toxicity of lidocaine might be related to other mechanisms not detected in all models. Thus, lidocaine could release inflammatory substances like CGRP as recently reported.\textsuperscript{48} The release of inflammatory substances only under certain circumstances could explain why the incidence of TNS varies within a wide range between 4\% and 33\%.\textsuperscript{1}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Concentration-dependent induction of early apoptotic cells. The fractions of cells undergoing an early stage of apoptosis were identified by positive annexin V-FITC and negative 7-AAD staining after 24 h incubation with the different investigated local anaesthetics. Note that induction of early apoptosis increases within the first three concentrations of each local anaesthetic, and at higher concentrations, the fraction of cells with signs of early apoptosis decrease again revealing a change in the mechanism of cell death from apoptosis to necrosis.}
\end{figure}
Local anaesthetic induced neuroapoptosis

The high degree of correlation between the toxicity and octanol/buffer coefficient is remarkable. Since the lipophilic properties of local anaesthetics correlate with their potency, a correlation between toxicity and potency at the sodium channel is obvious. That might suggest that the toxicity is mediated via the sodium channel. Studies inducing a long-term blockade with tetrodotoxin\(^7\) or functionally inactivating a nerve\(^9\) have shown that a conduction block \textit{per se} does not lead to nerve fibre degeneration. Furthermore, apoptosis induction and toxicity have also been shown in cells not expressing voltage-gated sodium channels.\(^{22} 33 35 36\) This implies that local anaesthetics developed on the basis of the known structures are predictably neurotoxic depending on the lipophilic properties. Therefore, one could argue that we have to develop local anaesthetics with a completely different structure in order to completely avoid this toxicity, for example, tetrodotoxin which displays no local toxicity.\(^7\) Disappointingly, the structurally different tricyclic antidepressant amitriptyline which also inhibits the voltage-dependent sodium channel induces apoptosis and local neurotoxicity.\(^{50}\) More recently, lipophilic effects of a series of amino-amide local anaesthetics on human potassium channels have been reported.\(^{51}\) This interaction might also be involved in toxic actions of local anaesthetics, and again lipophilic properties seem to play an important role in determining the toxic potential.

Some studies have attributed the cytotoxicity of local anaesthetics and amitriptyline to an unspecific membrane effect as a detergent.\(^{52} 53\) However, these physicochemical effects occur at concentrations approximately 10-fold higher. Furthermore, findings that cells are protected against local anaesthetic induced apoptosis by Bcl-2 overexpression, caspase-9 deficiency, caspase-inhibitors, and p38 mitogen-activated-kinase-inhibitors argue against a detergent-like effect of local anaesthetics as the principal cause of cell death.\(^{14} – 16 22 54\) Nevertheless, it is conceivable that higher concentrations of local anaesthetics, which induce necrosis, might produce a less specific membrane effect.

Since tetracaine is the most toxic of the local anaesthetics investigated here, one might ask whether the chemical structure (amide \textit{vs} ester type) influences the neurotoxicity. In agreement with Tan and colleagues,\(^13\) we found that tetracaine and procaine, as the two ester-type local anaesthetics investigated, are at the two ends of the toxicity scale. Therefore, a possible influence of the ester–amide bond on their neurotoxic effects is highly unlikely.

In conclusion, all local anaesthetics produce neuropaoptosis and neurotoxicity in a concentration-dependent manner. The toxicity correlates with the lipophilicity and therefore with the potency of the local anaesthetic. Esters or amide-type local anaesthetics are equally neurotoxic. Local neurotoxicity seems to be a universal phenomenon of all clinically used local anaesthetics and linked more to physicochemical properties than to their molecular structure.

Acknowledgement
We acknowledge the expert statistical guidance by Reinhart Willers from our institutional statistics department.

Funding
This work was supported by institutional sources only.

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