Mitigation of Direct Neurotoxic Effects of Lidocaine and Amitriptyline by Inhibition of p38 Mitogen-activated Protein Kinase In Vitro and In Vivo

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Background: Local anesthetic–induced direct neurotoxicity (paresthesia, failure to regain normal sensory and motor function) is a potentially devastating complication of regional anesthesia. Local anesthetics activate the p38 mitogen-activated protein kinase (MAPK) system, which is involved in apoptotic cell death. The authors therefore investigated in vitro (cultured primary sensory neurons) and in vivo (sciatic nerve block model) the potential neuroprotective effect of the p38 MAPK inhibitor SB203580 administered together with a clinical (lidocaine) or investigational (amitriptyline) local anesthetic.

Methods: Cell survival and mitochondrial depolarization as markers of apoptotic cell death was assessed in rat dorsal root ganglia incubated with lidocaine or amitriptyline either with or without the addition of SB203580. Similarly, in a sciatic nerve block model, the authors assessed wallerian degeneration by light microscopy to detect a potential mitigating effect of MAPK inhibition.

Results: Lidocaine at 40 μM/approximately 1% and amitriptyline at 100 μM reduce neuron count, but coinoculation with the p38 MAPK inhibitor SB203580 at 10 μM significantly reduces cytotoxicity and the number of neurons exhibiting mitochondrial depolarization. Also, wallerian degeneration and demyelination induced by lidocaine (600 μM/approximately 15%) and amitriptyline (10 μM/approximately 0.3%) seem to be mitigated by SB203580.

Conclusions: The cytotoxic effect of lidocaine and amitriptyline in cultured dorsal root ganglia cells and the neurodegeneration in the rat sciatic nerve model seem, at least in part, to be mediated by apoptosis but seem efficiently blocked by an inhibitor of p38 MAPK, making it conceivable that coinjection might be useful in preventing local anesthetic-induced neurotoxicity.

Development of new local anesthetics (LAs) is seriously hampered by neurotoxicity. Recently, several new promising LAs under development by our group1 and other investigators2–5 had reached clinical trials that had to be halted because of presumed neurotoxicity. In addition, LAs in current clinical use are well known to cause direct neuronal toxicity, i.e., transient neurologic injury,4–8 cauda equina syndrome,9–11 and even permanent loss of spinal cord function.12 Other than using the lowest possible concentration/dosage of LAs, no other option is currently available to prevent LA-induced direct neurotoxicity.

Although severe LA-induced injury kills neuronal cells via necrosis, increasing evidence suggests that, as severity decreases, apoptosis becomes the main mechanism of cell death.13,14 Interestingly, it recently was shown that LAs, even at clinical concentrations, induce activation of the p38 mitogen-activated protein kinase (MAPK) system,15,16 a specific intracellular signaling pathway also involved in apoptosis.

Mitogen-activated protein kinases are a family of protein kinases that phosphorylate specific serines and threonines of target protein substrates and regulate wide-ranging cellular activities such as gene expression, mitosis, movement, and metabolism, as well as apoptosis. The subfamily of p38 MAPKs is activated by many stimuli, including inflammatory cytokines (e.g., tumor necrosis factor α17–19 and Ca2+20–22 both of which are involved in LA toxicity. Although apoptosis is brought about by an extremely complex system, many antiapoptotic drugs are available (some of which are in phase II clinical trials23–25), raising the possibility that coinjection of LAs with drugs inhibiting apoptosis might be one approach to decreasing neurotoxicity. We selected lidocaine26 as a representative LA in clinical use and amitriptyline as a representative investigational LA, both of which have a high potential for neurotoxicity, particularly amitriptyline.27

We tested the hypothesis that inhibition of the p38 MAPK reduces neurotoxicity caused by the LAs lidocaine and amitriptyline by inhibiting apoptosis. Specifically, we (1) incubated cultivated dorsal root ganglia cells with (a) lidocaine alone or in combination with the p38 MAPK inhibitor SB203580 and (b) amitriptyline alone or in combination with SB203580, assessing cell survival and apoptosis (via detection of mitochondrial depolarization), and (2) injected, in a sciatic nerve block model, (a) lidocaine alone or in combination with SB203580 and (b) amitriptyline alone or in combination with the SB203580 and assessed the extent of wallerian degeneration.

Materials and Methods

We used the model of dissociated rat dorsal root ganglion (DRG) culture for evaluation of cytotoxicity by...
lidoceaine or amitriptyline and the potential alleviation thereof by a p38 MAPK inhibitor. To assess the mechanism of toxicity, we used an early marker of apoptosis, *i.e.*, mitochondrial depolarization. We evaluated the extent of sciatic nerve degeneration by light microscopy after perineural drug injection. We selected the subfacial sciatic nerve injection approach, because this allows application of drug directly onto the nerve with visual confirmation and therefore yields the most reproducible results. The concentrations of test drugs were chosen on the basis of preliminary studies showing that this concentration reduces the cell count by approximately 50% or elicits substantial neural damage, such that a protective effect of p38 MAPK inhibition would be most clearly visible.

**Drugs**

Unless stated otherwise, drugs were purchased from Sigma Aldrich (Vienna, Austria, or St. Louis, MO). For the *in vitro* experiments, the pH of the stock solutions of 1 mM lidocaine was 4.65, and that for 1 mM amitriptyline was 4.46 (in dimethyl sulfoxide [DMSO]). Because a larger volume of amitriptyline stock solution had to be added to the medium (pH of 7.4), the pH of this final solution was lower for amitriptyline than for lidocaine (pH 7.1 for 100 μM amitriptyline and pH 7.38 for 40 mM lidocaine). The osmolality of the medium incubated with lidocaine or amitriptyline was not significantly higher than that of control cultures incubated with vehicle only. The concentration of DMSO was approximately 0.1%, which is known to be the threshold for neurotoxicity. Because a larger volume of amitriptyline than lidocaine stock solution (which contains the potentially neurotoxic substance DMSO) had to be added to the medium to yield the desired final concentration, we increased the DMSO content of the lidocaine solution to obtain identical DMSO concentrations. Similarly, for control cultures with vehicle only (medium), the same concentration of DMSO was used. Although the concentration of DMSO was approximately 0.1%, we thus could obtain identical conditions for all treatment groups to avoid a potentially confounding variable.

For the *in vivo* experiments, 15% lidocaine and 10 mM amitriptyline were freshly prepared by dissolving them in normal saline, resulting in pH values of 4.8 and 6.20, respectively. The addition of SB253080 did not change the pH significantly. We did not adjust the pH, because buffering by the tissue fluid, which has a pH of 7.4, is rapid.

**In Vitro Experiments**

**Neuron Culture.** Dorsal root ganglion cultures were obtained in a manner similar to that described previously. Briefly, neurons were acutely harvested from adult (8–9 weeks) female Sprague-Dawley rats, which were killed by carbon dioxide narcosis according to the institutional protocol (Animal Committee of the Austrian Federal Ministry of Education, Science and Culture, Vienna, Austria). DRG were desheathed and incubated in 5,000 U/ml collagenase for 90 min at 37°C, followed by 15 min in 0.25% trypsin–EDTA. After dissociation in Roswell Park Memorial Institute (RPMI) medium containing 10% horse–5% fetal bovine serum, neurons were plated in RPMI medium supplemented with nitrogen additives (1:100) and antibiotics (penicillin, 1,000 U/ml; streptomycin, 1,000 μg/ml; and amphotericin B, 25 μg/ml in 0.85% saline), all purchased from Invitrogen (Vienna, Austria).

Neurons were allowed for 24 h to adhere to the glass floor of dishes coated with poly-D-lysine/laminin. Poly-D-lysine was applied at a concentration of 0.1 mg/ml in distilled water and laminin at 7 μg/ml in RPMI solution. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO₂.

**Assessment of Cell Survival.** To confirm dose-dependent toxicity of lidocaine and amitriptyline and to assess whether inhibition of p38 MAPK attenuated cytotoxicity, we incubated neurons with 50, 100, and 150 μM amitriptyline or 20, 40, and 60 μM lidocaine, either with or without the addition of 1 or 10 μM SB203580, a selective pyridinyl imidazole inhibitor of p38 MAPK. After 24 h, cultures were fixed with paraformaldehyde (4%) and evaluated by the same experimenter with regard to survival by determining total cell counts. Surviving neurons were defined as cells with round cell bodies, a clearly visible nucleus, and neurofilament-positive staining distinguishing them from glial cells. To confirm that a decreased number of cells is indeed due to cell death (*vs.* reduced adherence to the glass cover slip), we performed pilot studies examining all cells in several culture dishes indicating that cells not counted as “survived” actually had no visible nucleus and deformed cell bodies. In each dish, neurons were counted in 50 visual fields (20× magnification). No differentiation of cell sizes was made.

**Immunohistochemistry.** In a different experiment, we determined the incidence of mitochondrial depolarization, an early hallmark of apoptosis, after exposure to lidocaine, amitriptyline, or control medium. In addition, we assessed whether inhibition of apoptosis is the mechanism by which the p38 MAPK inhibitor SB203580 mitigates LA-induced neurotoxicity. Therefore, we used the fluorescent cell-permeant dye JC-1, which exists either as an aggregate at “physiologic” membrane potentials (∆Ψm less than −100 mV, red fluorescence, 590 nm) or as a monomer at depolarized membrane potentials greater than −100 mV (green fluorescence, 527 nm). JC-1 is selectively incorporated into the mitochondrial membrane and changes its fluorescent color from red to green upon mitochondrial depolarization, suggesting that the ratio of green/red (527/590 nm) fluores-
ence can be used as an indicator of mitochondrial membrane potential $\Delta \Psi m$.\textsuperscript{31,32}

We compared alterations in $\Delta \Psi m$ of control neurons incubated with vehicle only with cultures treated with 100 $\mu$m amitriptyline or 40 $\mu$m lidocaine. In addition, cultures were incubated with the inhibitor of p38 MAPK, SB203580, at 10 $\mu$m. After 4 h of incubation, neurons were incubated with 15 $\mu$m JC-1 for 50 min at 37°C. For that purpose, the JC-1 dye was freshly prepared by diluting a stock solution (1 mg/ml) 1:100 in RPMI medium. Then the cultures were washed twice with RPMI medium supplemented with nitrogen additives to remove excess dye. Cell cultures were evaluated for the number of neurons exhibiting mitochondrial depolarization and the average ratio of green and red fluorescence.

In Vivo Experiments

Subfascial Sciatic Nerve Injection. The protocol for animal experimentation was reviewed and approved by the Harvard Medical Area Standing Committee on Animals (Boston, Massachusetts). For a pilot study, we selected 0.2 ml lidocaine, at a concentration of 15%/approximately 600 $\mu$m, alone or in combination with 10 $\mu$m SB203580 (n = 6/group), to be injected directly beneath the clear fascia surrounding the nerve but outside the perineurium, proximal to the sciatic bifurcation. Amitriptyline at 10 $\mu$m/approximately 0.3% alone or in combination with 10 $\mu$m SB203580 (n = 8/group) was used for comparison. Potential toxicity of either the MAPK inhibitor or normal saline alone was assessed by injecting four rats with SB203580 or four with normal saline alone.

With animals under 1-2% isoflurane inhalation anesthesia, the sciatic nerves of adult female Sprague-Dawley rats weighing approximately 200 g were exposed by lateral incision of the thighs and division of the superficial fascia and muscle as described previously.\textsuperscript{33} The superficial muscle layer was sutured with 4-0 silk, and the wound was closed with metal clips. After the animals recovered from general anesthesia, their sciatic nerve function was evaluated grossly by reaction to pinch of the fifth toe with a forceps and observed for flaccid paralysis due to motor blockade. The contralateral side was used as the control. Animals were then tested in the same manner daily until killed on day 7 after injection.

Pathologic Evaluation. We excised the sciatic nerves following the anesthesia protocol used for surgery 7 days after administering the test dose; the rats were then killed with an intraperitoneal injection of sodium pentobarbital (70 mg/kg). For fixation, the nerves, measuring approximately 2 cm long with the injection site in the middle, were placed on a wooden stick and immersed in 2.5% phosphate-buffered glutaraldehyde for 24 h. These were rinsed three times with phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in serial concentrations of alcohol, and embedded in araldite according to the recommended procedure for neurotoxicologic tissue evaluation.\textsuperscript{34} Twenty 1-um-thick semithin sections from the central 2-mm block of each 6-mm-long segment were prepared for light microscopy and stained with methylene blue, azure II. An observer unaware of the experimental groupings evaluated the tissue sections. A value was assigned for each histologic slide based on a scale with 1-unit increments from 0 to 8 in which 0 represented a normal nerve and 8 represented a finding of wallerian degeneration extending throughout the nerve bundle.

Statistical Analyses

Analysis of variance was used to compare cell culture survival data between treatment groups (20/40/60 $\mu$m lidocaine, lidocaine + 1 $\mu$m SB203580, lidocaine + 10 $\mu$m SB203580; and 50/100/150 $\mu$m amitriptyline, amitriptyline + 1 $\mu$m SB203580, amitriptyline + 10 $\mu$m SB203580) relative to control (vehicle), with the post hoc Dunnett t test method applied to evaluate group differences and to account for multiple comparisons. Analysis of variance with the Bonferroni post hoc correction was used to compare results from mitochondrial depolarization experiments. Means $\pm$ SD were used to summarize the results, because the cell counts followed a normal distribution as tested by the Wilk-Shapiro test. Two-tailed values of $P < 0.05$ were considered statistically significant. A power analysis was performed for the cell culture experiments and indicated that the sample size of five cultures per treatment group would provide 80% power ($\beta = 0.20$) for detecting a difference of 30% or more in an average cell count assuming an SD of 10% within a group (version 5.0, nQuery Advisor; Statistical Solutions, Cork, Ireland). The rationale for choosing a 10% SD is based primarily on the analysis of preliminary data, which indicated that the within-group variability was tight and suggested less variability in the cell culture experiments for a given treatment (lidocaine or amitriptyline, with or without SB203580) as compared with control, specifically with respect to data on cell survival. We chose to specify a 10% SD for the power analyses to yield a relevant effect size (albeit a large effect size of 3.0) estimate for detecting group mean differences by analysis of variance. Furthermore, this SD of 10% was considered a reasonable, scientific estimate of variability.

Injury scores were tested by the Mann-Whitney U test, because skewness was detected, and results are therefore given as the median (range). Because the in vivo experiments were intended as a preliminary study only, we did not perform any power analysis.
Results

In Vitro Experiments

Lidocaine Reduces Cell Count, and Coincubation with SB203580 Significantly Reduces Lidocaine Cytotoxicity. Incubation of DRG cultures with lidocaine at 40 mM decreased the average neuron number in cultures from 506 ± 20 to 228 ± 50 (survival of 45%), and coincubation with SB203580 at 1 μM did not significantly prevent neuronal death (cell count of 246 ± 50). However, at a concentration of 10 μM SB203580, the neuron count was 411 ± 48 (survival rate of 81%; P < 0.001) and, as with amitriptyline, not significantly lower (P > 0.05) than that of controls (medium only) (fig. 1A). The salvaging effect of 10 μM SB203580 was also apparent for 20 and 60 mM lidocaine. At these concentrations, cell survival was increased from 47 ± 5 to 57 ± 20% (not significant) and 30 ± 12 to 58 ± 3% (P < 0.01), respectively. Incubation with SB203580 alone had no detectable effect.

Amitriptyline Reduces Cell Count, and Coincubation with the p38 MAPK Inhibitor SB203580 Significantly Reduces Amitriptyline Cytotoxicity. We confirmed a cytotoxic effect in neurons exposed to amitriptyline at a concentration of 100 μM; the average neuron count as compared with controls (444 ± 42) significantly decreased in cultures incubated with amitriptyline at 100 μM (survival of 38%, 169 ± 20; P < 0.001). Addition of 1 μM SB203580 did not significantly attenuate this effect (average cell count 213 ± 37). In contrast, coincubation with 10 μM SB203580 alleviated the decrease in neuron count, with the survival rate reaching 82% (average cell count 365 ± 30) (fig. 1B). The latter survival rate was significantly higher than that of cultures incubated with 100 μM amitriptyline alone (P < 0.001), although not significantly lower than that of controls (P > 0.05). Improved survival in cultures coincubated with 10 μM SB203580 was also detectable at 50 μM and 150 μM amitriptyline. At these concentrations, cell survival was increased from 43 ± 6 to 64 ± 4% (P < 0.01) and 52 ± 10 to 62 ± 18% (P < 0.01), respectively.

Lidocaine and Amitriptyline Cause Mitochondrial Depolarization, and Coincubation with SB203580 Leads to Reduction Thereof via Inhibition of p38 MAPK. Figure 2 displays representative cells demonstrating loss of membrane potential after exposure to lidocaine or amitriptyline and the prevention of this loss by coincubation with 10 μM SB203580. The percentage of control neurons incubated only with medium (n = 56) displaying depolarized ΔΨm was 7.2 ± 3.0%, indistinguishable from the percentage in neurons coincubated with only SB203580. Incubation with 40 mM lidocaine (fig. 3A) resulted in 57.1 ± 8.6% of neurons with depolarized ΔΨm (n = 53; P < 0.001), and addition of SB203580 decreased this to 34.6 ± 9.4% (n = 52; P < 0.05 compared with controls; P < 0.05 compared with lidocaine group). Similarly, incubation with amitriptyline (fig. 3B) resulted in 20.9 ± 3.8% of neurons with depolarized ΔΨm (n = 48; P < 0.01), whereas coincubation with SB203580 reduced this to 7.8 ± 3.5% (n = 52; not significant compared with controls; P < 0.05 compared with the amitriptyline group).

The average ratio of fluorescent green/red neurons in control cultures was 0.31 ± 0.42, indicating a physiologic mitochondrial membrane potential. Incubation with lidocaine (fig. 3C) increased this ratio to 1.73 ± 1.58 (P < 0.001), whereas coadministration of SB203580 resulted in a ratio of 0.75 ± 0.67 (not significant compared with controls; P < 0.001 compared with the lidocaine group). Treatment with amitriptyline (fig. 3D) increased the fluorescent ratio to 0.60 ± 0.70 (P < 0.001), and coincubation with...
SB203580 resulted in a ratio of 0.20 ± 0.29 (not significant compared with controls; \( P < 0.01 \) compared with amitriptyline group).

In Vivo Experiments

Sciatic Nerve Degeneration Is Induced by Lidocaine and Mitigated by SB203580. Two rats from the lidocaine group were excluded, because they had wound dehiscence and gross infection and were killed per protocol. All of the rats remaining for histologic analyses (15% lidocaine: \( n = 5 \); 15% lidocaine combined with SB203580: \( n = 5 \)) demonstrated complete block of motor and sensory sciatic nerve functions but recovered completely by the next day. Rats treated with 15% lidocaine alone demonstrated severe histologic changes (i.e., wallerian degeneration), with an injury score of 6 (5–8) (representative sample in fig. 4A).

Addition of SB203580 reduced these findings of wallerian degeneration characteristic of LA-induced neurotoxicity, with an injury score of 3 (1–6), \( P < 0.15 \) (representative sample in fig. 4B). Histologic findings were normal in rats treated with 10 \( \mu \)M SB203580 or normal saline alone (\( n = 4 \)/group) (representative sample not shown).

Sciatic Nerve Degeneration Is Induced by Amitriptyline and Mitigated by SB203580. Similar to the lidocaine-treated animals, all rats treated with amitriptyline developed complete sciatic nerve block, as indicated by failure to respond to pinch of the fifth toe as well as by flaccid paralysis. No overt neurologic deficit was seen after 24 h, i.e., pinch to the fifth toe was followed by a brisk withdrawal reaction and vocalization identical to that of the control (contralateral limb). Similarly, posture, gait, and walking pattern were unaffected. Nevertheless, upon histologic analyses, degenerative changes of sciatic nerves treated with amitriptyline (fig. 5A) and

Fig. 2. Attenuation of mitochondrial membrane depolarization by coincubation with the p38 mitogen-activated protein kinase inhibitor SB203580. (A) Control neuron, showing fluorescence at 590 (red) but not at 527 (green) nm, indicating “physiologic” mitochondrial membrane potential. Incubation with (B) 10 \( \mu \)M amitriptyline induced mitochondrial depolarization (increase in green fluorescence), whereas neuron coincubated with (C) 10 \( \mu \)M SB203580 maintained a largely physiologic membrane potential. (D) Neuron with depolarized mitochondria (green fluorescence) upon incubation with 40 \( \mu \)M lidocaine and (E) maintenance of normal membrane potential with the addition of SB203580.

Fig. 3. Coadministration of the p38 mitogen-activated protein kinase inhibitor SB203580 attenuates the depolarization of mitochondria in response to application of the local anesthetics (A and C) lidocaine and (B and D) amitriptyline. This is evident both as a decrease in the number of cells exhibiting (A and B) depolarized mitochondria and (C and D) the average green/red fluorescent ratio of neurons in culture. Low ratios indicate a predominance of red fluorescence and thus “physiologic” membrane potentials. Data are reported as mean ± SD. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \). n.s. = not significant, \( P > 0.05 \); \( n = 5 \) cultures/group.
effective reduction of those changes in nerves treated with amitriptyline combined with MAPK inhibitor (fig. 5B) were observed. However, similar to the group treated with lidocaine, those treated with amitriptyline combined with SB203580 had markedly improved scores, but differences were not statistically significant.

Discussion

We have shown in vitro (cell culture model) and in vivo (sciatic nerve injection model) that a p38 MAPK inhibitor is capable of mitigating clinical (lidocaine) and investigational (amitriptyline) LA neurotoxicity, as suggested by increased neuron survival and improved histologic evaluation (i.e., less wallerian degeneration), respectively. A significant decrease in the percentage of cells demonstrating loss of mitochondrial membrane potential after coincubation with SB203580 compared with that of cells incubated with lidocaine or amitriptyline alone suggests apoptosis as a mechanism of injury induced by these two LA agents.
Apoptosis, probably initiated by LA-induced \textit{p}38 MAPK activation, leads to mitochondrial membrane permeabilization, which causes the release of cytochrome \textit{c} from the mitochondrial intermembrane space, marking the point of no return in the process of cell death. Once in the cytosol, cytochrome \textit{c} triggers the assembly of a caspase activation complex, leading to activation of caspase-3, which in turn triggers oligonucleosomal DNA fragmentation, the hallmark of apoptosis.\textsuperscript{35–42} We demonstrated that both lidocaine and amitriptyline elicit mitochondrial depolarization, an indirect marker of apoptosis. Mitochondrial membrane depolarization is effectively observed during apoptosis; however, it is not specific of apoptosis (some drugs, including LAs at high concentration, uncouple oxidative phosphorylation with a decrease in membrane potential\textsuperscript{43}). Nevertheless, our results corroborate previous investigations in neuronal cell lines suggesting that apoptosis is, at least in part, responsible for the phenomenon of LA-induced nerve injury.\textsuperscript{15,44,45}

Because the apoptotic process is to some extent reversible, the use of neuroprotective drugs interacting with apoptotic pathways could be of considerable benefit. In addition, \textit{p}38 MAPK has been described as mediating various neurodegenerative pathways in primary sensory neurons,\textsuperscript{46,47} while its inhibition has been shown to enhance neuronal survival.\textsuperscript{48} Therefore, coapplication of drugs such as \textit{p}38 MAPK inhibitors may be useful for minimizing the neurotoxic effects of both conventional and novel LAs.

For the \textit{in vitro} experiments, we selected drug concentrations to achieve approximately 50\% cell death, so that a potential mitigating effect of cytotoxicity, \textit{i.e.}, increased cell survival, is easily detectable. Although not the aim of this study, the concentrations chosen for lidocaine seem clinically relevant, because other investigators have chosen similar concentrations for cell culture experiments to simulate expected lidocaine concentrations after intrathecal application.\textsuperscript{43} Similarly, we chose the incubation time of 24 h because under clinical conditions, \textit{e.g.}, epidural anesthesia, primary afferent somata may be exposed to LAs for several days.

For the \textit{in vivo} experiments, however, the concentrations used clearly exceeded those used clinically. We selected these high concentrations because we set out to find a concentration at which we would definitely encounter toxicity, and preferably in all animals, to keep the number of animals needed as low as possible. As Eisenach and Yaksh\textsuperscript{49} pointed out in an editorial, studies with lidocaine at 1\% would require many-fold more animals. Because in pilot studies (data not shown), not even 10\% lidocaine produced significant histopathologic lesions (\textit{i.e.}, wallerian degeneration) in all animals, we increased the concentration to 15\%. This approach is commonly undertaken in toxicity studies; otherwise an ethically unjustifiable number of animals would need to be killed.

It was beyond the scope of this study to assess whether administration of \textit{p}38 MAPK inhibitors could also mitigate neurotoxicity after an LA-induced injury occurred. To the best of our knowledge, this has not yet been studied but certainly could be of great clinical relevance. Interestingly, in a rat sciatic nerve crush injury model, the peroral administration of a MAPK inhibitor (SD-169, a proprietary oral inhibitor of \textit{p}38 MAPK activity not used in the current study) was shown to be effective in reducing injury, both neurobehaviorally and histologically.\textsuperscript{50} For example, pinching the SD-169–treated animals revealed significantly better recovery from sensory deficit, and regenerating nerves were morphologically more mature than untreated nerves when observed 28 days after transection. Furthermore, SD-169 significantly reduced tumor necrosis factor-mediated primary Schwann cell death in culture experiments.\textsuperscript{50}

In this study, we initially observed a \textit{P} value of 0.15 for results with \(n = 5\) animals treated with lidocaine combined with SB203580 with respect to reduction of neuronal injury. The sample size was too small to attain statistical significance, although it clearly demonstrated a beneficial trend. There was evidence from these experiments that a larger group of animals is necessary to reach significance and that small \(n\) values result in treatment effects that are almost statistically significant (considered a strong trend) but do not satisfy the \textit{a priori} definition of \(P < 0.05\) because of low statistical power and a consequently high risk of a type II (β) error. This is clear inasmuch as an evaluation of differences in injury among a group of five animals treated with lidocaine combined with SB203580 did not reach significance (\(P = 0.15\)), but combining results from an additional six animals amplified the effect (\(P < 0.001\), Mann–Whitney U test). We applied nonparametric statistical tests because of the distribution of the data and the small sample sizes. Moreover, our results for amitriptyline showed noticeable improvements in injury results; however, again because of the small sample size, we were unable to make a definitive statistical statement. Notwithstanding, we have interpreted our results, although cautiously, as indicative of a protective neuronal advantage, although based on a limited number of animals. However, in moving ahead, we realize that a larger sample size in each LA group (including controls) is necessary (projected to be at least 10 animals/group for 80\% power) to have an acceptable level of statistical power for detecting statistically significant treatment group differences and avoiding false negatives (type II or β errors).

When eliciting peripheral nerve blocks, the primary target of LA action is the axon and not the DRG cell. Although our investigation focused on \textit{in vivo} sciatic nerve toxicity (\textit{i.e.}, axonal toxicity) and \textit{in vitro} DRG cells (\textit{i.e.}, ganglionic toxicity), we believe there is a strong connection, because death of DRG following demyelination and axonal degeneration after peripheral nerve lesions has clearly been demonstrated to be attributable to apoptosis.\textsuperscript{51} Nevertheless, future studies probably should attempt to correlate findings from peripheral nerve block models with Schwann cell cultures and spinal block models with DRG cell cultures.

In conclusion, apoptosis most probably mediates cyto-
toxicity in cultured DRG cells and axonal degeneration and demyelination in a rat sciatric nerve model elicited by amitriptyline and lidocaine. An inhibitor of p38 MAPK, SB203580, seems to block this process, making it conceivable that its co-injection might prevent LA-induced nerve injury. More histologic studies involving larger sample sizes and additional models (e.g., intrathecal application) are needed to confirm our preliminary in vitro findings.

References


