Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants
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Benzethonium increases the cytotoxicity of S(+) ketamine in lymphoma, neuronal, and glial cells

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Abstract:

Introduction: Ketamine has been demonstrated to be neurotoxic in animals as well as in patients. Preservatives added to ketamine have been accused to induce this neurotoxicity. Therefore, we investigated whether the most widely used preservative of ketamine—benzethonium chloride—enhances the toxicity of S()-ketamine in vitro in lymphoma, neuroblastoma cells and primary astrocytes.

Methods: Human Jurkat T-lymphoma- and neuroblastoma cells (SHEP) were incubated for 24 hours with commercially available S-ketamine containing benzethonium, pure S-ketamine and pure benzethonium chloride. The rate of early- and late-apoptotic cells was evaluated by flowcytometry. In a second step the combined toxicity of benzethonium and ketamine was investigated in neuroblastoma cells and primary rat astrocytes in a mitochondrial activity assay (XTT). The additivity

Results: In Jurkat T-lymphoma and neuroblastoma cells benzethonium increased the toxicity of ketamine from 32% to 80% and from 64% to 84% cell deaths, respectively. In neuroblastoma cells as well as in primary rat astrocytes the measured combined toxicity was within the confidence interval of the calculated pure additive toxicity as seen in the isobolograms.

Conclusions: We conclude that benzethonium increases the local toxicity of ketamine in cells of hematopoetic, neuronal and glial origin in an additive manner. Therefore, caution is recommended especially when using preservative containing S-ketamine as an additive for long-term neuraxial analgesia.
Introduction

Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, has been administered neuraxially for the treatment of postoperative pain, chronic cancer pain and neuropathic pain, respectively.\textsuperscript{1,2} Although ketamine was shown to induce neurotoxicity when applied intrathecally in animals and patients\textsuperscript{3,4}, there is considerable uncertainty about the possible toxicity of ketamine when applied next to neuronal structures. In the past local neurotoxicity of ketamine has been attributed to added preservatives.\textsuperscript{5,6} Malinovsky et al. demonstrated in rabbits that only animals receiving chlorobutanol (with or without ketamine) developed signs of neurological damage, but not the ones receiving pure ketamine.\textsuperscript{5} Similarly, Errando et al. showed in pigs that ketamine alone induced no neuronal damage, whereas ketamine with benzethonium induced discrete neurotoxic effects.\textsuperscript{6} Surprisingly, benzethonium alone displayed even greater toxicity than the combination of benzethonium with ketamine. Benzethonium itself induces apoptosis in a variety of human epithelial tumors as well as embryonic fibroblasts and has been advocated as an agent with a significant broad-spectrum anticancer activity.\textsuperscript{7} Recently, we demonstrated in human neuroblastoma and T-lymphoma (Jurkat) cells that the preservative-free ketamine racemate as well as the S(\(+\)-enantiomer induce apoptosis in millimolar concentrations via the mitochondrial pathway presumably unrelated to NMDA signaling.\textsuperscript{8} Unfortunately, commercially available preservative-free S(\(+\)-ketamine should not be stored at room temperature for longer than 12 h, which makes administration for a longer time period rather difficult. Although benzethonium containing ketamine has been applied epidurally without signs of neurologic injury\textsuperscript{9}, it would be interesting to know whether its toxicity has a synergetic effect. Therefore, we determined the percentage of apoptotic and necrotic cells induced by S(\(+\)-ketamine, benzethonium, the commercially available combination of both (Ketanest S\textsuperscript{®}, Pfizer, Berlin, Germany) and the combination of the pure substances in human cells of hematogenic and neuronal origin. Subsequently, we tested the synergism of both substances in neuronal cells and primary astrocytes.
Methods

Cell cultures

The origin and characteristics of human Jurkat T-lymphoma cells and human neuroblastoma cells have been described before.\textsuperscript{10-12} Memorial Institute (RPMI) 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 U/ml of penicillin and 50 \( \mu \)g of streptomycin) was used as suspension medium in both cell lines. Culture condition for all cells was a humidified atmosphere containing 5% carbon dioxide at 37°C. Cultures of primary rat astrocytes were prepared from cerebral cortices of P2 Wistar rats. After approval of the local Animal Research Committee, rats were anesthetized using Forene® (Abbott, Abbott Park, IL), decapitated, and the brains quickly removed. To avoid fibroblast or endothelial contamination, cortical tissue was carefully isolated from blood vessels and meninges, rinsed with Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA), dissociated by trypsinization, and suspended in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (80 units/ml), and streptomycin (0.2 mg/ml). Dissociated cells were plated in 75-cm\(^2\) culture flasks (Corning Incorporated Life Sciences, Lowell, MA). After 5 days, cultures were washed with DMEM to remove cellular debris and maintained until subconfluency. Cellular debris, microglia, oligodendrocytes and their early precursor cells were then removed by shaking flasks overnight at 250 rev/min at 37 °C. The resulting cell population consisted of >98% primary rat astrocytes, as determined by immunocytochemical analysis using antibodies against glial fibrillary acidic protein (GFAP; Chemicon International, Temecula, CA). For all following experiments, cells were trypsinated and replated on 6-well cell culture plates coated with poly-D-lysine (PDL; 0.1 mg/ml).

Exposure of cells to benzethonium chloride and S(+) -ketamine

Cells were treated for 24h at 37°C with medium alone as negative control, or indicated concentrations of S(+) -ketamine, benzethonium alone, S(+) -ketamine and benzethonium chloride or Ketanest S® (Pfizer, Berlin, Germany) diluted in cell culture medium.

Flowcytometric analysis

For evaluation of cytotoxic effects cells were stained after complete incubation prior to flowcytometric analysis. The principle of double staining with annexin-V and 7-
aminoactinomycin D (7AAD) has been described before.\textsuperscript{11,12} In brief, staining of cells with annexin-V and 7AAD allows to distinguish between the fraction of cells in an early phase of apoptosis and those already in a late apoptotic or necrotic state. Cells only staining positive for annexin-V but not for 7AAD are defined as early apoptotic, whereas detection of 7AAD staining indicates late apoptosis or necrosis in stained cells. The sum of both fractions indicates the percentage of overall cell death. For staining and flowcytometric analysis, cells were washed twice with cold PBS. Adherent SHEP neuroblastoma cells were first detached with trypsin/EDTA 0.05% for 3 min at 37°C and resuspended at a concentration of $1 \times 10^6$ cells/ml in annexin-V binding buffer (10 mM N-[2-hydroxyethyl] piperazin-N-3[propansulfonic acid]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$). After incubating the cells of 15 min at room temperature with 5 µl annexin-V and 5 µl 7AAD (5 µg/ml) we started measuring with a FACScalibur (Becton Dickinson, Heidelberg, Germany) using CellQuest Pro software. Every measurement includes 10,000 cells.

\textit{Detection of mitochondrial metabolic activity}

For \textit{in vitro} determination of mitochondrial viability we used the tetrazolium hydroxide (XTT) assay. XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye, which can be measured by absorbance. To measure cell viability using XTT, samples were prepared with 100 µl of cell suspension in 96-well cell culture plates and cells were allowed to adhere overnight. Neuroblastoma cells were incubated in a density of 20,000 cells/Well, while primary rat astrocytes were adjusted to 100,000 cells/Well according to their lower metabolic rate. Subsequent to incubation time, 50 µl of XTT assay solution (XTT 1 mg/ml and phenazine methosulfate 50 µM diluted in cell culture medium) were added to each well. Mixing the samples gently for 1 min was followed by incubation for 120 min at 37°C. After additional mixing for 3 min the absorbance was measured spectrophotometrically at a wavelength of 450 nm.

\textit{Statistical analysis}

Each experiment was performed threefold. Results are expressed as mean ± SD. Comparisons between groups were made with Student’s t-test using the SPSS program version 18.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered significant. The calculations of concentration-response relationships and LD$_{50}$ values were performed with non-linear regression analysis using the Graph Pad Prism Software version 5.0 (GraphPad Software Inc., La Jolla, CA) with the log(inhibitor) vs. response mode assuming variable slopes. Resulting equations in the form of
[response=bottomvalue+(topvalue-bottomvalue)/(1+10^((LogLD_{S50}-concentration)\*HillSlope))] were used to draw concentration-response slopes and the isobole (± confidence interval 95%) for benzethonium, S(+)-ketamine and the equitoxic combination of both drugs. An isobologram is a commonly used method to assess combined drug effects. Therefore, a graph of equally effective dose pairs (isoboles) for a single effect level is drawn. A particular effect level is selected, such as 50% of the maximum (LD50) and concentrations of drug A and B (each alone) that give this effect are plotted as axial points in a Cartesian plot. This graph represents the locus of points (concentration pairs) that lead to a simply additive effect. Experimentally observed concentration pairs which produce this specific effect level can be compared with the line of additivity to discriminate between subadditive, additive and superadditive effects. \(^{13}\)

**Results**

In Jurkat T-lymphoma cells S(+)-ketamine (2 mM) and the preservative benzethonium (5 \(\mu\)M) induced 31.8 ± 6.2% and 32.3 ± 5.5% cell death, respectively (Fig. 1A). Addition of benzethonium to the preservative free S(+)-ketamine as well as the commercially available mixture Ketamine S® increased the toxicity to almost threefold (86.3 ± 1.9% and 80.8 ± 6.7%, respectively). All treatments increased the percentage of apoptotic cells.

In neuroblastoma cells the concentrations of all solutions were chosen in the linear part of the concentration-effect curve. In this setting benzethonium alone did not lead to an increased percentage of cell death in human neuroblastoma cells, whereas co-administration of benzethonium to S(+)-ketamine (4 mM) increased its toxicity significantly from 63.7 ± 1.4% to 84.4 ± 6.6% (Fig. 1B). Again, mixture of benzethonium with S(+)-ketamine and the commercially available mixture (Ketanest S®) induced the same amount of apoptosis and cell death (Fig. 1B).

To discriminate the ability of benzethonium and S(+)-ketamine to have a subadditive, additive or superadditive toxic effect, we first investigate the dose response relationship and the calculated LD50 of these substances in human neuroblastoma (SHEP) cells and primary rat astrocytes of the rat using the XTT assay. The dose response curves (Fig. 2) based on increasing doses of S(+)-ketamine and benzethonium allowed to calculate the LD50.
Figure 1:

Fig. 1: Percentage of cell death after 24 h incubation of Jurkat lymphoma cells (A) and neuroblastoma cells (B) with benzethonium, S(+) -ketamine and the mixture of those or the commercially available mixture (Ketanest S®). Black bars delineate late apoptotic or necrotic cell death (Annexin-V pos./7AAD pos.), empty bars delineate early apoptotic cell death (Annexin-V pos./7AAD neg.). Data are given as mean ± standard deviation. * delineates p<0.05, n.s. = not significant. (n + 3)

Figure 2:

Fig. 2: Dose-response relationship of S(+) -ketamine (A) and benzethonium (B) in neuroblastoma cells (SHEP) and primary rat astrocyte cell cultures. Cell viability was measured by XTT-assay evaluating the mitochondrial activity (% of control) after incubation with S(+) -ketamine or benzethonium for 24 h. Black points delineated mitochondrial activity in primary rat astrocytes, grey points delineated mitochondrial activity in human neuroblastoma (SHEP) cells. Each measure point is delineated as mean ± standard deviation. (n = 3)
Subsequently, an isobologram was established to compare the calculated additive effect with the observed effect of the drug combination. The LD50 of S(+) -ketamine was 4.0 mM in human neuroblastoma (SHEP) cells and 4.7 mM in primary rat astrocyte. The LD50 of benzethonium was 10.2 µM in human neuroblastoma (SHEP) cells and 9.5 µM in primary rat astrocytes, showing the differential sensitivity of different cell lines to the LD50 of these substances. To evaluate the LD50 of the combined substances, the cells were exposed to increasing fractions (20-80%) of the LD50 of both substances in combination (Fig. 3).

**Figure 3:**

![Graph A](image)

**Fig. 3:** Combined effect of S(+) -ketamine and benzethonium: Cell viability was measured in human neuroblastoma (SHEP) cells (A) and primary rat astrocytes (B) by XTT-assay evaluating the mitochondrial activity (% of control). S(+) -ketamine and benzethonium were incubated in combination using equitoxic concentrations. Therefore, increasing fractions of the calculated LD50 of each substance were combined to establish a dose-response relationship. For example, 60% of the LD50 (2.09 mM) of S(+) -ketamine and 60% of the LD50 (6.11 µM) of benzethonium in human neuroblastoma (SHEP) cells lead to a reduction of mitochondrial activity by 69±4%. Based on this dose-response relationship, a LD of the combination of S(+) -ketamine and benzethonium was calculated.
The results were used to calculate the combined LD. The combined LD50 was applied to an isobologram of each cell line (Fig. 4). The combined LD50 was within the borders of the 95% confidence interval of the corresponding isobol indicating an additive effect in both cell lines.

**Figure 4:**

**A**

![Isobologram of S(+)-ketamine and benzethonium in human neuroblastoma (SHEP) cells](image)

**B**

![Isobologram of S(+)-ketamine and benzethonium in primary rat astrocytes](image)

**Fig. 4:** Isobolograms of S(+)-ketamine and benzethonium in human neuroblastoma (SHEP) cells (A) and primary rat astrocytes (B). The straight black line connects the LD50 of S(+)-ketamine and benzethonium resulting in locus of points (dose pairs) that will produce this effect in a simply additive combination. The confidence interval (95%) is represented by the dashed lines. The black data point indicates the calculated half-maximal concentrations (LD50) resulting from the experimentally observed dose-response relationship of S(+)-ketamine and benzethonium in combination. Error bars indicate confidence intervals (95%).

**Discussion**

Although preservatives added to ketamine have often been accused to induce neurotoxicity, we showed that the toxic effects of S(+)-ketamine and benzethonium are additive in human lymphoma and neuroblastoma cells and primary rat astrocytes. In a first step of this study, we focused on the apoptosis inducing and therefore cyto- and neurotoxic effects of S(+)-ketamine and benzethonium in a clinically used ratio (e.g. Ketanest S). We demonstrated that the toxic effects of S(+)-ketamine and
benzethonium are at least additive in human lymphoma and neuroblastoma cells. Furthermore, apoptosis was found to be the predominant mode of cell death induced by S(+)ketamine and benzethonium and their mixture. Thus, in cells of mesenchymal and ectodermal origin the toxicity of S(+)ketamine was increased by benzethonium.

In a second step of this study, we investigated the dose-toxicity relationship of S(+)ketamine and benzethonium alone and in combination in neuronal and glial cell cultures to determine whether their toxicities are additive. In both cell types each substance alone displayed an almost identical LD50 but a different slope. Therefore, these substances seem to have similar toxicities in a wide variety of cell types. Finally, these substances displayed additive toxicities in both cell types.

In regard to the toxicity of benzethonium alone in human neuroblastoma cells the sensitivity of our assays (AnnexinV/7AAD and XXT) varied. While the AnnexinV/7AAD assay could identify apoptosis as the predominant mode of cell death, it has shortages in quantifying the total viability of cells by neglecting cells which already disappeared by lysis. Therefore, we instituted the XTT assay, because it quantifies the total viability of all cells and used this assay to determine the additivity of the toxicities. Therefore, the quantitative toxicity of benzethonium alone in neuroblastoma cells in the AnnexinV/7AAD assay is presumably underestimated.

Our results are in contrast to those of Errando et al. who demonstrated that only benzethonium is neurotoxic in vivo and its toxicity is even ameliorated by ketamine. Apart from methodological differences the authors used a benzethonium concentration about 40-times higher than used in the presented investigation leading possibly to an over-exaggeration of benzethonium effects. Neuraxial application of ketamine in man demands a conscientious risk-benefit analysis. Thus, neuraxial application of S(+)ketamine with benzethonium should be even more restricted, since benzethonium increases the toxicity of S(+)ketamine. Unfortunately, preservative free S(+)ketamine solutions have to be stored in a cool environment (4°C) and used at room temperature within 12 h, which limits their application in pain medicine. Although benzethonium containing ketamine has been applied epidurally in the past without neurological sequela, the here demonstrated additive toxicity demands caution applying benzethonium containing ketamine solutions epidurally.
We demonstrated that benzethonium and of S(+)-ketamine display additive toxicity in neuronal and glial cell cultures. Surely this data can not be extrapolated to the clinical situation. But since clinical data on the safety of this drug combination are lacking, epidural application of these drugs should be restricted.

References


