Local anesthetics: New insights into risks and benefits
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Chapter 4.2

*Lidocaine and ropivacaine, but not bupivacaine, demethylate deoxyribonucleic acid in cancer cells in vitro*

Based on


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Introduction

More than 100 years ago, it was recognized that tumour surgery makes metastasis more likely.\textsuperscript{1} The perioperative period of tumour surgery may well be a crucial time when patient outcome can be decisively influenced.\textsuperscript{2} Numerous perioperative factors which may explain tumour progression have now been identified. For example, resecting a primary tumour may promote the progression of distant metastases, or even induce tumour self-seeding, where circulating tumour cells re-infiltrate the original site of a resected tumour.\textsuperscript{3} This concept is supported by recent evidence that the number of circulating tumour cells increases dramatically in the perioperative period.\textsuperscript{4} One very important determinant of metastatic potential is the epigenetic signature of tumour cells.\textsuperscript{5}

In a healthy body, epigenetic mechanisms are responsible for the stability and expression of human deoxyribonucleic acid (DNA) as they regulate the methylation of specific DNA regions.\textsuperscript{6} Epigenetic mechanisms are increasingly recognized as pathogenic factors in several forms of cancer.\textsuperscript{6} Specifically, increases in methylation levels can deactivate tumour suppressor genes and lead to the progression of cancer.\textsuperscript{6,7} In these cases, decreasing methylation levels may be of therapeutic benefit. So a new class of chemotherapeutics designed to demethylate tumour DNA has been introduced into clinical practice.\textsuperscript{8}

We have recently shown that the prototype local anaesthetic, lignocaine, can reduce methylation of breast cancer cells at clinically relevant concentrations \textit{in vitro}.\textsuperscript{9} This study sought to determine whether similar demethylating effects could also be observed for two prototype long-acting local anaesthetics typically used for perioperative neuraxial anaesthesia: bupivacaine and ropivacaine. In addition, local anaesthetics have been described as enhancing the tumoricidal effects of conventional chemotherapeutics,\textsuperscript{10} and we wanted to investigate whether local anaesthetics would also increase the demethylating effect of a prototypical demethylating chemotherapeutic, 5-aza-2′-deoxycytidine (DAC).

The aim of this study was therefore to test two hypotheses: 1) that the local anaesthetics lignocaine, bupivacaine, and ropivacaine decrease methylation levels in tumour cells, and 2) that local anaesthetics enhance the demethylating effects of DAC.
Methods

Cell culture

Human breast cancer cell lines BT-20 (estrogen receptor [ER]-negative) and MCF-7 (ER-positive) were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and were cultured according to ATCC recommendations. Amplification of 15 short tandem repeat (STR) loci and the gender-specific locus amelogenin was carried out in the Institute of Legal Medicine of the Medical University Innsbruck, Austria, to authenticate the cell lines. This was done using 10 ng of template DNA, applying the Geneprint PowerPlex 16 System (Promega, Madison, USA) according to the Manufacturer’s recommendations, as previously described.11

Drug treatments

The following drugs were purchased from Sigma Aldrich (Vienna, Austria): lignocaine N-ethyl bromide (L5783), bupivacaine hydrochloride monohydrate (B5274) and ropivacaine hydrochloride monohydrate (R0283), all dissolved in distilled water. We treated BT-20 and MCF-7 breast cancer cell lines with these local anaesthetics first alone and then in combination with varying concentrations of DAC for 72 hours. The following concentrations were used: 10 µM and 100 µM lignocaine, 2 µM and 20 µM bupivacaine, 3 µM and 30 µM ropivacaine; 0.001 µM, 0.02 µM, 0.1 µM, 0.2 µM 0.5 µM and 1 µM DAC. Twenty-four hours after seeding, the medium was removed and replaced with medium containing the drug solutions at the desired final concentration. DAC was dissolved in DMSO to a final concentration of 10 mM, aliquoted, and stored at -20°C. Lignocaine, bupivacaine and ropivacaine were dissolved in water to a final concentration of 1 M and 100 mM respectively, aliquoted, and stored at -20°C. Whenever needed, a fresh aliquot was diluted to the desired final concentration.

Effect of local anaesthetics on cell viability.

We analysed the effects of lignocaine in combination with DAC and bupivacaine and ropivacaine on cell viability in the human breast cancer cell lines BT-20 and MCF-7 during 72 hours’ incubation by the colorimetric MTT assay.
The tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was dissolved in RPMI-1640 without phenol red. The assay was performed according to Manufacturers’ instructions. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630–690 nm.\(^{12}\)

**Global Genomic DNA Hypermethylation**

Global genomic 5-methylcytosine content was determined by quantitative MethylLight assay, specific for Chromosome 1 SAT2 repeat sequences.\(^{13}\) We analysed the effects of 10 µM and 100 µM lignocaine, 2 µM and 20 µM bupivacaine and 3 µM and 30 µM ropivacaine alone or in combination with 0.001 µM, 0.02 µM, 0.1 µM, 0.2 µM, 0.5 µM or 1 µM DAC respectively on the global DNA methylation status in BT-20 and MCF-7 breast cancer cells after 72 hours. Genomic DNA from treated cells was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany). Sodium bisulfite conversion of genomic DNA and MethylLight was performed as described previously.\(^{14}\)

**Additivity**

We sought to determine whether the interaction between DAC and lignocaine as the prototype local anaesthetic was supra-additive. Our calculations were based on the Loewe isobolographic additivity model, which has been described as particularly useful when investigating the interplay between two toxic substances in vitro.\(^{15}\) The half maximal DNA demethylation concentration (EC\(_{50}\)) was calculated in BT-20 cells for DAC and lignocaine from at least 7 independent experiments at different concentrations of DAC and lignocaine, resulting in a preliminary EC\(_{50}\) of 0.08 µM for DAC, and 77.3 µM for lignocaine (line of additivity, Figure 4). We assumed supra-additive effects if 50% of demethylation were achieved with a combination of concentrations significantly lower than those representing the line of additivity.
Demethylating properties of lidocaine, bupivacaine and ropivacaine

Statistics

Results are expressed as mean ± standard deviation (SD). The Mann-Whitney U test was used for the comparison of the various effects after the different treatments. \( P \)-values less than 0.05 were considered statistically significant. SPSS 17.0 (IBM, Vienna, The Austria) was used for statistical analyses.

Results

Effect of lignocaine, bupivacaine and ropivacaine on cell viability

Treatment with 10 µM or 100 µM lignocaine alone had no cytotoxic effect. Lignocaine at concentrations of 10 µM and 100 µM did not increase cytotoxicity of DAC in either BT-20 (Fig. 1A) or MCF-7 (Fig. 1B) breast cancer cell lines. Similarly, treatment with bupivacaine or ropivacaine at doses equipotent to lignocaine showed no cytotoxic effect in either breast cancer cell line (Fig. 1C, 1D).

Effect of bupivacaine and ropivacaine on global genomic DNA-methylation

Treatment with bupivacaine at 2 µM and 20 µM revealed no significant demethylating effect on global genomic DNA methylation in either breast cancer cell line BT-20 (Fig. 2A) or MCF-7 (Fig. 2B).

Treatment with ropivacaine for 72 hours at concentrations of 3 µM or 30 µM decreased methylation in BT-20 cells (Fig. 2A; \( P = 0.003 \) or \( P = 0.023 \), respectively). In MCF-7 cells, no demethylation was observed (Fig. 2B).

Effect of lignocaine, bupivacaine and ropivacaine in combination with DAC on global genomic DNA-methylation.

To determine whether local anaesthetics could increase the demethylating effect of DAC, we treated BT-20 and MCF-7 breast cancer cells for 72 hours with 10 µM and 100 µM lignocaine, 2 µM bupivacaine and 3 µM ropivacaine, and combined these treatments with DAC at several concentrations. We observed increased demethylation after the combined treatment of BT-20 cells with 0.1 µM DAC and 10 µM or 100 µM lignocaine respectively (Fig. 3A; \( P = 0.014 \) or \( P = 0.001 \) respectively) and 0.5 µM DAC with 100 µM lignocaine (Fig. 3A; \( P = 0.008 \)), in comparison to the DAC treatment alone. In MCF-7 cells, only the
combined treatment with 0.5 µM DAC and 10 µM lignocaine revealed a stronger demethylation in comparison to the mono-treatment with 0.5 µM DAC alone (Fig. 3B; \( P=0.006 \)). All other combined treatments of bupivacaine and ropivacaine with various concentrations of DAC revealed no increased demethylating effect in BT-20 or MCF-7 cells, as compared to treatment with DAC alone (Fig. 3).

**Additivity**

Since lignocaine was the most potent local anaesthetic agent as regards demethylating properties in this study, additivity experiments were based upon calculated EC50 in methylation for lignocaine and DAC. These theoretical results were compared to demethylation using several concentrations of DAC (0.02, 0.04, 0.08, 0.16, and 0.32 µM), combined with lignocaine (19.3, 38.7, 77.3, 154.6, 309.2 µM, respectively, Table 1). We then compared demethylation levels with the theoretical line of additivity (Figure 4), and found no supra-additivity (Figure 4, Table 1).

**Discussion**

This study was designed to test the hypotheses that 1) the local anaesthetics lignocaine, bupivacaine, and ropivacaine can decrease methylation levels in tumour cells, and that 2) lignocaine as the strongest demethylating agent would enhance the demethylating effects of DAC – the prototype epigenetic chemotherapeutic.

We found that 1) lignocaine and ropivacaine, but not bupivacaine, induce DNA demethylation, and that 2) lignocaine showed no supra-additive effects when combined with DAC.

The concentrations of local anaesthetics employed in the present investigation are in the range of concentrations reached during epidural infusion of local anaesthetics.\(^\text{16, 17}\) Equipotent concentrations of lignocaine, ropivacaine, and bupivacaine were calculated based on a previous electrophysiological study.\(^\text{18}\) Comparable doses of lignocaine (i.e. up to 10 µM) are observed after typical regimens of perioperative intravenous lignocaine infusion.\(^\text{19}\)
Epigenetic effects of local anaesthetics

Our results build upon and confirm previous results which indicated that at clinically relevant doses, lignocaine demethylates DNA in breast cancer cells in vitro. In addition, our results from individual drug treatments (Fig. 3), and Loewe additivity experiments (Fig. 4) suggest that the methylating effects of lignocaine and DAC are additive. We did not find evidence of supra-additivity. The mechanism by which local anaesthetics may influence methylation was not directly investigated in the present study, but procaine has previously been shown to inhibit DNA methyltransferase-1, the driving force behind the methylation of cytosine.

While local anaesthetics have been shown to make conventional chemotherapeutics work better in selected experimental settings, no-one has yet tested their effects on the performance of demethylating chemotherapeutics. Further investigations are needed to determine the biological consequences of systemic lignocaine on tumour progression in the perioperative setting. In contrast to lignocaine, bupivacaine and ropivacaine seem less potent in inducing demethylation in tumour cells. The reasons for this differential effect can only be speculated upon. The equipotent doses chosen were based on sodium channel blockade, and so we surmise that the demethylating effect of these substances is not related to sodium channel blockade. This is not surprising since at least three alternative effects of local anaesthetics are mediated by pathways independent of sodium channel blockade. Firstly, different local anaesthetics show differential effects on G-protein-mediated priming of human neutrophils \textit{ex vivo}, which are not correlated with their potency in blocking sodium channels. Secondly, given equipotent doses, lignocaine is much more effective than bupivacaine in preventing thrombus formation. And thirdly, Piegeler et al. demonstrated that the effects of lidocaine and ropivacaine on the phosphorylation of Src, a key molecule conjectured in tumour metastasis, were not related to potency at the sodium channel, and that ester-type local anaesthetics had no effect. In contrast to the latter study on Src phosphorylation however, the epigenetic effects of local anaesthetics are discernible in both amide-type and ester-type compounds. In a landmark manuscript, Villar-Garea showed that the prototype ester-type local anaesthetic, procaine, demethylated DNA and inhibited tumour growth in MCF-7 breast cancer cells, and others found
similar effects when investigating solid organ and haematopoietic tumour cell lines.28 20

**Relevance of perioperative epigenetic modulation**

The most important epigenetic alterations are methylation of cytosine residues in DNA to produce 5-methyl-cytosine, resulting in a change in the spatial configuration of histones.29 Most frequently, the pathologic epigenetic changes in malignancy involve increased methylation, which leads to the silencing of tumour suppressor genes.6 29 Modifications in the epigenetic signature of tumour cells are nowadays considered as important as genetic mutations themselves.29 In addition to this increasingly appreciated role in primary oncogenesis, epigenetic alterations are also increasingly understood to influence the prognosis and response to treatment of malignancy, including the probability of metastasis, in many tumours.5 Current treatments targeting the epigenome are associated with considerable side-effects. For example, the paradigmatic anti-epigenetic drug, decitabine (DAC), a potent inhibitor of DNA methylation, is associated with significant adverse effects such as myelosuppression and organ toxicity.30 We have previously shown that lignocaine, given at clinically relevant concentrations, acts as a demethylating agent. Here, we show that lignocaine shows additive demethylating effects when combined with DAC. Given the very good safety profile of systemic or regional administration of lignocaine, this drug offers potentially beneficial epigenetic effects in the perioperative period of tumour surgery.

**Limitations**

In larger concentrations, local anaesthetics can have direct cytotoxic effects on tumour cells, and this may explain protective effects against tumour recurrence observed after local anaesthesia for local superficial tumour excision.31 However, the concentrations used in the present study were found insufficient to cause direct cytotoxicity (Figure 1). Also, we note that the two tumour cell lines that we used have different baseline methylation properties. The effects of demethylation are largest in BT-20 cells, which have a high baseline methylation level.9 In the same way, biological heterogeneity may explain why specific anaesthetic interventions
Demethylating properties of lidocaine, bupivacaine and ropivacaine

 seem to affect outcome in some types of cancer \(^{32,33}\) while no effect was found in other studies,\(^{34}\) and some studies found effects only in defined subpopulations.\(^{35}\)

**Conclusions**

Assessing our study alongside previous evidence, we conclude that, at clinically relevant doses, lignocaine and ropivacaine exert demethylating effects on breast cancer cells in vitro, but bupivacaine does not. When combined, lignocaine and DAC exhibit additive demethylating effects.

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Table 1: Global Genomic SAT2 DNA methylation in BT-20 breast cancer cell line after treatment with various concentrations of DAC, Lignocaine and a combined treatment.

DNA-methylation is indicated as percentage of a fully methylated reference (PMR). Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DAC [µM]</th>
<th>Lignocaine [µM]</th>
<th>SAT2 PMR value (± SD) after DAC treatment alone</th>
<th>SAT2 PMR value (± SD) after Lignocaine treatment alone</th>
<th>SAT2 PMR value (± SD) after combined treatment</th>
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<td>30 (± 7)</td>
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<td>38 (± 7)</td>
<td>14 (± 1)</td>
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<tr>
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<td>39 (± 26)</td>
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<tr>
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<td>154.6</td>
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<td>24 (± 1)</td>
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<tr>
<td>0.32</td>
<td>309.2</td>
<td>14 (± 3)</td>
<td>34 (± 1)</td>
<td>9 (± 0)</td>
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</tbody>
</table>
Figure Legends

**Figure 1:** Effect of lignocaine in combination with DAC, and effects of bupivacaine and ropivacaine on MCF-7 and BT-20 human breast cancer cells. The cell viability was analyzed by MTT analysis after being cultured for 72 h with the indicated drugs. (A) BT-20 cells and (B) MCF-7 cells treated with lignocaine and various concentrations of DAC. (C) BT-20 cells and (D) MCF-7 cells treated with bupivacaine and ropivacaine. Data are represented as the mean ± SD from three independent experiments.

**Figure 2:** Global Genomic SAT2 DNA methylation analysis in breast cancer cell lines after treatment with bupivacaine and ropivacaine. (A) SAT2 DNA methylation levels in BT-20 breast cancer cells treated with 2 µM and 20 µM bupivacaine, 3 µM and 30 µM ropivacaine or 1 µM DAC and (B) MCF-7 breast cancer cells treated with 2 µM bupivacaine, 3 µM and 30 µM ropivacaine or 1 µM DAC. Results from an average of 6 independent experiments are shown. Results are expressed as mean ± SD. Statistical significance between control and treated samples was assessed by Mann-Whitney U test (*, p < 0.05; **, p < 0.01;***, p < 0.001).

**Figure 3:** Global Genomic SAT2 DNA methylation analysis in breast cancer cell lines after combined treatment of lignocaine, bupivacaine and ropivacaine with various concentrations of DAC. (A) SAT2 DNA methylation levels in BT-20 breast cancer cells and (B) MCF-7 breast cancer cells. Results from an average of 6 independent experiments are shown. Results are expressed as mean ±SD. Statistical significance between control and treated samples was assessed by Mann-Whitney U test (*, p < 0.05; **, p < 0.01).

**Figure 4:** Line of additivity based on the preliminary calculated ED50 for DAC and Lignocaine (0.08 µM and 77.3 µM, respectively). The marker denotes the first concentration which leads to a near-50% demethylation (actual ED50).
Figure 1

Demethylating properties of lidocaine, bupivacaine and ropivacaine
Figure 2

A

Sat2 DNA methylation (PMR values)

Control
2 μM bupivacaine
20 μM bupivacaine
3 μM ropivacaine
30 μM ropivacaine
1.0 μM DMC

B

Sat2 DNA methylation (PMR values)

Control
2 μM bupivacaine
3 μM ropivacaine
30 μM ropivacaine
10 μM DMC
Figure 4