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

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Editor’s key points

• Lidocaine demethylates deoxyribonucleic acid (DNA) in breast cancer cells, which may have therapeutic potential.
• This cell culture study evaluated the use of bupivacaine and ropivacaine for demethylation in breast cancer cells and also whether lidocaine and the chemotherapy agent 5-aza-2′-deoxycytidine (DAC) have additive demethylating effects.
• Ropivacaine but not bupivacaine demethylates DNA in breast cancer cells and lidocaine and DAC have additive demethylating effects.

Background. Lidocaine demethylates deoxyribonucleic acid (DNA) in breast cancer cells. This modification of epigenetic information may be of therapeutic relevance in the perioperative period, because a decrease in methylation can reactivate tumour suppressor genes and inhibit tumour growth. The objectives of this study were to determine the effect of two amide local anaesthetics, ropivacaine and bupivacaine, on methylation in two breast cancer cell lines and to detect whether the combination of lidocaine with the chemotherapy agent 5-aza-2′-deoxycytidine (DAC) would result in additive demethylating effects.

Methods. Breast cancer cell lines BT-20 (oestrogen receptor (ER)-negative) and MCF-7 (ER-positive) were incubated with lidocaine, bupivacaine, and ropivacaine to assess demethylating properties. Then, we tested varying concentrations of lidocaine and DAC to assess whether their demethylating effects were additive. Cell numbers and global methylation status were analysed.

Results. Lidocaine decreased methylation in BT-20 and MCF-7 cells, ropivacaine decreased methylation in BT-20 cells, and bupivacaine had no demethylating effect. When combined, lidocaine and DAC had additive demethylating effects.

Conclusions. At clinically relevant doses, lidocaine and ropivacaine exert demethylating effects on specific breast cancer cell lines, but bupivacaine does not. The demethylating effects of lidocaine and DAC are indeed additive.

Keywords: cancer; demethylation; epigenetics; local anaesthetic, bupivacaine; local anaesthetic, lidocaine; local anaesthetic, ropivacaine

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More than 100 yr ago, it was recognized that tumour surgery makes metastasis more likely.1 The perioperative period of tumour surgery may well be a crucial time when patient outcomes can be decisively influenced.2 Numerous perioperative factors that 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.4 This concept is supported by recent evidence that the number of circulating tumour cells increases dramatically during the perioperative period.5 One very important determinant of metastatic potential is the epigenetic signature of tumour cells.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.6 However, epigenetic mechanisms are also increasingly recognized as pathogenic factors in several forms of cancer.7,8 Specifically, increases in methylation levels can deactivate tumour suppressor genes and lead to the progression of cancer.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.9,8

We have recently shown that the prototype local anaesthetic, lidocaine, can reduce methylation of breast cancer cells at clinically relevant concentrations in vitro.9 This study sought to determine whether similar demethylating effects could also be observed with two prototype long-acting local anaesthetics such as bupivacaine and ropivacaine which are typically used for perioperative neuraxial anaesthesia. In addition, local anaesthetics have been described as enhancing the tumoral effects of conventional chemotherapeutics10 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: (i) the local anaesthetics lidocaine, bupivacaine, and ropivacaine decrease methylation levels in tumour cells and (ii) local anaesthetics enhance the demethylating effects of DAC.

**Methods**

**Cell culture**

Human breast cancer cell lines BT-20 (oestrogen receptor (ER)-negative) and MCF-7 (ER-positive) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to ATCC recommendations. Amplification of 15 short tandem repeat 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, WI, USA) according to the manufacturer’s recommendations, as previously described.11

**Drug treatments**

The following drugs were purchased from Sigma-Aldrich (Vienna, Austria): lidocaine N-ethyl bromide (LS783), bupivacaine hydrochloride monohydrate (BS274), and ropivacaine hydrochloride monohydrate (RO283), 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 h. The following concentrations were used: 10 and 100 μM lidocaine, 2 and 20 μM bupivacaine, 3 and 30 μM ropivacaine; 0.001, 0.02, 0.1, 0.2, 0.5, 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 dimethyl sulfoxide to a final concentration of 10 mM, aliquoted, and stored at −20°C. Lidocaine, 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 lidocaine 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 h incubation by a colorimetric assay (M5655; Sigma, Vienna, Austria). The tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was dissolved in RPMI-1640 without phenol red. The assay was performed according to the manufacturer’s 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 MethyLight assay, specific for Chromosome 1 SAT2 repeat sequences.13 We analysed the effects of 10 and 100 μM lidocaine, 2 and 20 μM bupivacaine, and 3 and 30 μM ropivacaine alone or in combination with 0.001, 0.02, 0.1, 0.2, 0.5, or 1 μM DAC, respectively, on the global DNA methylation status in BT-20 and MCF-7 breast cancer cells after 72 h. Genomic DNA from treated cells was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany). Sodium bisulphite conversion of genomic DNA and MethyLight was performed as described previously.14

**Additivity**

We sought to determine whether the interaction between DAC and lidocaine 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 (EC50) was calculated in BT-20 cells for DAC and lidocaine from at least seven independent experiments at different concentrations of DAC and lidocaine, resulting in a preliminary EC50 of 0.08 μM for DAC, and 77.3 μM for lidocaine (line of additivity). 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.

**Statistics**

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

**Results**

**Effect of lidocaine, bupivacaine, and ropivacaine on cell viability**

Treatment with 10 or 100 μM lidocaine alone had no cytotoxic effect. Lidocaine at concentrations of 10 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 lidocaine showed no cytotoxic effect in either breast cancer cell line (Fig. 1c and d).

**Effect of bupivacaine and ropivacaine on global genomic DNA methylation**

Treatment with bupivacaine at 2 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. 2a). Treatment with ropivacaine for 72 h at concentrations of 3 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. 2a).

**Effect of lidocaine, 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 h with 10 and 100 \( \mu M \) lidocaine, 2 \( \mu M \) bupivacaine, and 3 \( \mu M \) 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 \( \mu M \) DAC and 10 or 100 \( \mu M \) lidocaine, respectively (Fig. 3A; \( P=0.014 \) or \( P=0.001 \), respectively) and 0.5 \( \mu M \) DAC with 100 \( \mu M \) lidocaine (Fig. 3A; \( P=0.008 \)), in comparison with the DAC treatment alone. In MCF-7 cells, only the combined treatment with 0.5 \( \mu M \) DAC and 10 \( \mu M \) lidocaine revealed a stronger demethylation in comparison with the mono-treatment with 0.5 \( \mu M \) DAC alone (Fig. 3A; \( P=0.006 \)). 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, when compared with treatment with DAC alone (Fig. 3).

**Additivity**

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

**Discussion**

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

We found that (i) lidocaine and ropivacaine, but not bupivacaine, induce DNA demethylation and (ii) lidocaine showed no supra-additive effects when combined with DAC.

The concentrations of local anaesthetics used in the present investigation are in the range of concentrations reached during epidural infusion of local anaesthetics.\(^{16}\) Equipotent concentrations of lidocaine, ropivacaine, and bupivacaine were calculated based on a previous electrophysiological study.\(^{17}\) Comparable doses of lidocaine (i.e. up to 10 \( \mu M \)) are observed after typical regimens of perioperative i.v. lidocaine infusion.\(^{18}\)

![Fig 1](https://academic.oup.com/bja/article-abstract/113/suppl_1/i32/2920007/1329202007) by guest on 13 April 2019

**Fig 1** Effect of lidocaine in combination with DAC, and effects of bupivacaine and ropivacaine on MCF-7 and BT-20 human breast cancer cells. The cell viability was analysed by colorimetric assay after being cultured for 72 h with the indicated drugs. (a) BT-20 cells and (b) MCF-7 cells treated with lidocaine 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 (\( \pm \)S.D) from three independent experiments.
Epigenetic effects of local anaesthetics

Our results build upon and confirm previous results which indicated that at clinically relevant doses, lidocaine 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 demethylating effects of lidocaine 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 lidocaine on tumour progression in the perioperative setting. In contrast to lidocaine, 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 block, and so we surmise that the demethylating effect of these substances is not related to sodium-channel block. This is not surprising as at least three alternative effects of local anaesthetics are mediated by pathways independent of sodium-channel block. First, different local anaesthetics show differential effects on G-protein-mediated priming of

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**Fig 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 and 20 μM bupivacaine, 3 and 30 μM ropivacaine, or 1 μM DAC and (b) MCF-7 breast cancer cells treated with 2 μM bupivacaine, 3 and 30 μM ropivacaine, or 1 μM DAC. Results from an average of six independent experiments are shown. Results are expressed as mean (±SD). Statistical significance between control and treated samples was assessed by the Mann–Whitney U-test (***P<0.01; **P<0.001).
human neutrophils ex vivo, which are not correlated with their potency in blocking sodium channels.21 Secondly, given equipotent doses, lidocaine is much more effective than bupivacaine in preventing thrombus formation.22 23 And thirdly, Piegeler and colleagues 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.24 In contrast to the latter study on Src phosphorylation, however, the epigenetic effects of local anaesthetics are discernible in both amide- and ester-type compounds. In a landmark paper, Villar-Garea showed that the prototype ester-type local anaesthetic, procaine, demethylated DNA and inhibited tumour growth in MCF-7 breast cancer cells,25 and others found similar effects when investigating solid organ and haematopoietic tumour cell lines.26

Relevance of perioperative epigenetic modulation

The most important epigenetic alterations are methylation of cytosine residues in DNA to produce 5-methylcytosine,
resulting in a change in the spatial configuration of histones. Most frequently, the pathological epigenetic changes in malignancy involve increased methylation, which leads to the silencing of tumour suppressor genes. Modifications in the epigenetic signature of tumour cells are nowadays considered as important as genetic mutations themselves. In addition to this increasingly appreciated role in primary oncogenesis, epigenetic alterations are also increasingly understood to influence the prognosis and response to the treatment of malignancy, including the probability of metastasis, in many tumours. 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. We have previously shown that lidocaine, given at clinically relevant concentrations, acts as a demethylating agent. Here, we show that lidocaine shows additive demethylating effects when combined with DAC. Given the very good safety profile of systemic or regional administration of lidocaine, 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. However, the concentrations used in the present study were found insufficient to cause direct cytotoxicity (Fig. 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. In the same way, biological heterogeneity may explain why specific anaesthetic interventions seem to affect outcome in some types of cancer while no effect was found in other studies and some studies found effects only in defined subpopulations.

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

Authors’ contributions
P.L. was responsible for study design, wrote the manuscript, and oversaw interpretation of the data. M.W.H. aided with study design and writing the manuscript. M.F. performed sample preparation and data analysis. N.C.W. aided with study design and writing the manuscript. H.F. was responsible for study design, performed data analysis and interpretation of results, and wrote the manuscript. All authors critically revised the manuscript and have read and approved the manuscript for publication.

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Declaration of interest
None declared.

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