Local anesthetics: New insights into risks and benefits

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Lidocaine demethylates DNA in breast cancer cells

Chapter 4.1

Lidocaine demethylates DNA in breast cancer cells in vitro

Based on

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In vitro, lidocaine demethylates DNA in breast cancer cells

**Introduction**

Surgical tumour removal remains a highly relevant treatment option for cancer patients, despite concerns that the perioperative period may facilitate progression of the underlying disease due to immunosuppression, surgical stress, and administration of drugs suspected of promoting tumour spread.\(^1\) Regional anaesthetic procedures, or the intravenous administration of local anaesthetics, have both been demonstrated to reduce perioperative surgical stress.\(^2\)\(^3\)

The ultimate clinical relevance of these effects is unclear, and currently available evidence is undecided as to whether potential positive effects of regional anaesthesia are relevant enough to influence patient outcome after cancer surgery.\(^4\)\(^-\)\(^8\) Current large-scale multicenter randomized controlled trials are projected to last until the end of the decade.\(^1\)

In the meantime, research is focused on potential mechanisms of local anaesthetic-induced tumour suppression. Several pathways have been described in literature. On one hand, local anaesthetics, at high doses, are cytotoxic in vitro,\(^9\)\(^10\) and on the other hand, they may induce sensitization of tumour cells to chemotherapeutics\(^11\) and heat.\(^12\)

Another potential mechanism whereby local anaesthetics may influence tumour growth is by interaction with the tumour epigenome.\(^13\) In malignancy, increased methylation frequently leads to down-regulation of tumour suppressor genes, favouring tumour progression.\(^14\)

The prototype ester-type local anaesthetic, procaine, has been shown to demethylate DNA and inhibit tumour growth and in the MCF-7 breast cancer cell line,\(^15\) and similar results were later obtained in hepatoma\(^9\) and leukaemia cell lines.\(^16\) Epigenetic effects of amide-type local anaesthetics, such as lidocaine, have not been examined. Given the structural differences between these two types of local anaesthetics, differential effects on epigenetic features are possible. In addition, lidocaine is very widely used in contemporary regional anesthesia, and increasingly employed intravenously in the context of multimodal treatment regimens.\(^3\)

We sought to examine the effects of the prototype amide-type local anaesthetic, lidocaine, on DNA methylation in prototypical breast cancer cell line
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cultures. Our working hypothesis was that exposure of cancer cells to lidocaine would decrease DNA methylation.

Methods

Cell culture

Human breast cancer cell lines BT-20 (estrogen receptor-negative) and MCF-7 (estrogen receptor-positive) were obtained from the American Type Culture Collection (ATCC) and were cultured according to company 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 to authenticate the cell lines, using 10 ng of template DNA applying the Geneprint PowerPlex 16 System (Promega) following the manufacturer’s recommendations as previously described.17

Drug treatments

The following drugs were purchased from Sigma Aldrich (Vienna, Austria): lidocaine n-ethyl bromide (L5783) and procaine hydrochloride, (P9879), both dissolved in distilled water. We treated BT-20 and MCF-7 breast cancer cell lines with a final varying concentration lidocaine, procaine and the positive control substance, 5 µM 5-aza-2'-deoxycytidine (DAC) for 72 and 96 hours respectively. 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. Lidocaine and procaine were dissolved in water to a final concentration of 1 M and 0.5 M respectively, aliquoted, and stored at -20°C. Whenever needed, a fresh aliquot was diluted to the desired final concentration.

Effect of lidocaine and procaine on cell proliferation.

We analyzed the effects of lidocaine and procaine on cell proliferation in the human breast cancer cell lines BT-20 and MCF-7 during 72 hours and 96 hours incubation by counting the cell number. To this end, Breast cancer cells were seeded into medium size cell culture bottles in MEM medium with 10% FCS (MCF-7: 2.2
Mio cells, BT-20: 1 Mio cells) and treated 24 hours later. After the indicated incubation time, 72 hours or 96 hours respectively, the cells were trypsinized and counted (Beckman coulter). Biotin-labeled POD TUNEL Apoptosis detection kit for adherent cell was used according to manufacturer’s protocol (GenScript, Piscataway, NJ). Cells were visualized using an Olympus 1X70 inverted Microscope in conjunction with Kappa ImageBase software V2.7.2 (Gleichen, Germany).

**Global Genomic DNA Hypermethylation**

Global genomic 5-methylcytosine content was determined by quantitative MethyLight assay specific for Chromosome 1 SAT2 repeat sequences. We analyzed the effects of 1mM procaine, different lidocaine concentrations (0.01mM, 0.1mM, 1mM) and 5µM DAC on the global DNA methylation status in MCF-7 and BT-20 breast cancer cells after 72, 96 and 120 hours (Fig. 2). Genomic DNA from treated cells was extracted using the DNeasy tissue kid (Qiagen) method. Sodium bisulfite conversion of genomic DNA and MethyLight was performed as described previously.

**DNA methylation and RNA expression of several Tumour Suppressor Genes**

We decided to test the effects of lidocaine in comparison to procaine on particular hypermethylated loci in both cell lines. RASSF1A, GSTP1 and MYOD1 are known epigenetically inactivated genes in breast cancer. Primers and Probes for COL2A1 (reference gene), RASSF1A, MYOD1 and GSTP1, and Chromosome 1 juxtacentromeric satellite 2 (SAT2) DNA sequences have recently been published. We compared the effects of 1mM lidocaine and procaine. Total cellular RNA was extracted by the standard acid guanidium thiocyanate-phenol-chloroform method and reverse transcription of RNA was performed as previously described. Primers and probe for Real time quantitative PCR analysis (qPCR) for RASSF1A, MYOD1 and GSTP1 were purchased from Applied Biosystems (AB Assay ID: Hs00200394_m1, Hs00159528_m1 and Hs00168310_m1). Primers and probes for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIIID; was used as endogenous RNA control) were used according to Bieche et al. Real-time PCR was performed using an ABI Prism 7900HT.
Detection System (Applied Biosystems, Foster City, CA). The standard curves were generated using serially diluted solutions of standard cDNA derived from the Hs578T carcinoma cell-line.

Statistics

Results are expressed as mean ± standard deviation (SD). The unpaired Student's t-test was used for the comparison of the various effects after the different treatments in normally distributed data, and the Mann-Whitney U test for non-normally distributed data. P-values less than 0.05 were considered as statistically significant. SPSS 17.0 was used for the statistical analyses.

Results

Effect of lidocaine and procaine on cell proliferation.

Treatment with 1mM procaine or 1mM lidocaine resulted in significant reductions in cell number, while lower concentrations of local anaesthetics did not lead to significant cell number reduction (Fig. 1A). In both cell lines no increase in the apoptosis rate was observed upon lidocaine (1mM, 0.1mM, 0.01mM) or procaine (1mM) treatment respectively (Fig. 1B).

Global Genomic DNA Hypermethylation

At baseline, methylation was 100-fold higher in BT-20 than in MCF-7 cells. At equal dose, lidocaine was a stronger demethylating agent than procaine (Fig. 2). In MCF-7 cells, we observed demethylation after 96, but not 72, hours after treatment with 1mM procaine(Fig. 2A; p=0.004). Treatment with 1 mM lidocaine resulted in a statistically significant increase of global Sat2 DNA methylation after 72 hours, whereas the treatment with 0.1mM and 0.01mM lidocaine revealed a significant demethylation after 72 and 96 hours (Fig. 2A; p<0.001, respectively). In comparison to procaine, treatment with 0.1mM or 0.01mM lidocaine had a stronger demethylating effect (Fig. 2A). Even in comparison to the demethylating agent, DAC, the treatment with 0.1 or 0.01mM lidocaine revealed a more significant decrease of DNA methylation (Fig. 2A).
In BT-20 cells, the treatment with 1mM procaine revealed a significant decrease of DNA methylation after 72 and 96 hours (Fig. 2B; $p<0.001$ or $p=0.019$, respectively). We observed a dose-dependent decrease in DNA methylation in response to lidocaine (1mM, 0.01mM, 0.01 mM) after 72 hours (Fig 2B; $p<0.001$, $p<0.001$ or $p=0.004$, respectively) and 96 hours (Fig. 2B; $p<0.001$, $p=0.034$ or $p=0.38$, respectively), similar to effects noted upon incubation with DAC.

Additionally we treated the breast cancer cells with 0.01mM, the clinically relevant dose of lidocaine, for 48, 72, 96 and 120 hours. We observed a statistical significant demethylating effect in MCF-7 after 72 to 120 hours, whereas in BT-20 cells the demethylating effect was seen only between 72 and 96 hours (Fig. 3).

**DNA methylation and RNA expression of specific Tumour Suppressor Genes**

In MCF-7 cells, after 72 hours or 96 hours respectively, neither DNA-methylation nor mRNA expression changes in our three exemplary tumour suppressor genes were observed (Fig. 4). Only for MYOD1 we observed a statistical significant increase in DNA methylation after 72 hours in lidocaine treated MCF-7 cells (Fig. 4A; $p=0.006$), without effecting the MYOD1 mRNA expression. In procaine treated cells we identified an increase in MYOD1 mRNA expression after 72 hours (Fig. 4B; $p=0.036$). DAC as control substance decreased methylation of the three tumour suppressor genes (Fig. 4A), and resulted in an increased mRNA expression only for MYOD1 after 72 hours (Fig. 4B).

In BT-20 cells we identified no decrease in methylation or mRNA expression upon procaine or lidocaine treatment after 72 or 96 hours. The treatment with DAC gave rise to a decreased RASSF1A, GSTP1 and MYOD1 DNA methylation, and increased the mRNA expression of these three tumour suppressor genes (Fig 4B).
Discussion

The present study demonstrates that lidocaine time- and dose-dependently demethylates DNA in BT-20 breast cancer cells (estrogen receptor-negative cell line), whereas in MCF-7 cells (estrogen-receptor-positive cell line) this demethylating effect was only observed to a smaller, albeit statistically significant, extent. This effect was noted at concentrations corresponding to those reached after systemic application of local anaesthetics, or after systemic absorption from epidural or paravertebral anaesthesia. These concentrations are, however, insufficient to cause direct cytotoxicity. Elicitation of cytotoxic effects would necessitate concentrations of local anesthetic that can only be reached after direct application of local anesthetic on the tumour intraoperatively. Finally, epigenetic modification appears to be transient, since demethylation was returned to non-significant levels after 120 hours in BT-20 cells. While global methylation status was profoundly influenced, the expression status of three exemplary tumour suppressor genes (RASSF1A, GSTP1 and MYOD1) was not significantly changed, such that further studies will need to elucidate which genes are affected by the demethylating effects of lidocaine.

The potential clinical implication of these results is that a potent pathway leading to malignancy seems to be influenced by local anaesthetics at clinically relevant doses. This may, in part, explain beneficial effects of regional anaesthesia on cancer recurrence observed in some studies. Furthermore, the magnitude of this effect is dependent upon epigenetic features of the tumour type. Whereas we observed statistically significant alterations in methylation status in both exemplary cell lines, effects were much more pronounced in an ER-negative cell line, which is associated with high methylation levels at baseline. Thus, beneficial effects relating to epigenetic modulation of cancer biology may be limited to certain types of tumours. The ultimate clinical relevance of our findings remains to be determined.

The issue whether the mode of anaesthesia influences outcome after cancer surgery has been a topic of intense debate. In a retrospective study on melanoma patients, Schlagenhauff et al. found that patients who had undergone melanoma excision under general anaesthesia had a decreased survival as compared to those
receiving local (infiltration) anaesthesia only. It should be noted that anaesthetic infiltration of peri-neoplastic tissues leads to very high regional concentrations of local anaesthetic in the milimolar range. These concentrations of local anaesthetics are known to be cytotoxic when applied to tumour cell lines. In contrast, when local anaesthetics are administered during epidural or paravertebral anaesthesia, low micromolar concentrations are attained in the systemic circulation. In breast cancer patients, a retrospective study revealed a substantial benefit with regards to metastatic spread in patients in whom paravertebral anaesthesia had been used during mastectomy. Corresponding data on abdominal cancer patients could, however, not replicate these promising findings, except for a subgroup of older patients in one study. In another study, all-cause mortality was reported to be reduced after resection of rectal, but not colonic, malignancies when epidural anaesthesia was part of anaesthetic technique. Results regarding prostate cancer patients remain equivocal. Two studies in patients suffering from ovarian carcinoma have linked intraoperative epidural analgesia to increased 3- and 5-year survival, and increased recurrence-free interval, while epidural anesthesia during brachytherapy had no beneficial survival effect in patients with cervical cancer. All these equivocal results may indicate that either, regional anaesthesia has no effect, or, they may be explained by the biological heterogeneity of tumours. Our results indicate that lidocaine influences cell lines with different biological properties differently, exerting strong demethylating effects on ER-negative, and less pronounced effects on ER-positive, cell lines. Thus, the question of whether regional anaesthesia or, for that sake, other components of the perioperative care process, modulate cancer recurrence is perhaps not one that can be answered unequivocally. Rather, our findings would indicate that tumour-suppressive effects of local anaesthetics may only detectable in specific types of cancer.

The role of local anaesthetics in tumour progression may be indirect or direct. Indirect actions of regional anaesthesia include attenuation of the neuroendocrine response to surgery followed by improved preservation of
immunocompetence, and effects on the administration of drugs suspected of modulating tumour growth, e.g., opioid-sparing effect. Direct actions are caused by direct effects of local anaesthetic on tumour cells, or sensitizing effects towards other therapeutic measures such as thermo- and chemotherapy. Local anaesthetics have been shown to protect against tumour cell invasion in concentrations attained clinically after local infiltration and suppress the proliferation of a number of tumour cell lines. Our results suggest that next to classic cytotoxic effects seen at doses exceeding those attained clinically, local anaesthetics are also able to subtly modulate tumour biology. Modulation of tumour epigenome was observed at far lower doses than those needed to elicit overt cytotoxicity. Cancer treatment strategies based upon epigenetics are being developed, and the ester-type local anaesthetic, procaine, had been suggested as a candidate substance. Our results suggest that lidocaine is an even more potent demethylating agent than procaine.

The stability and expression of the human genome is controlled by methylation of specific regions of desoxyribonucleic acid (DNA). Next to the classic four nucleotides adenine, thymine, guanine, and cytosine, a fifth base, 5-methylcytosine, confers epigenetic features to control a variety of physiologic and pathologic processes. Increasing evidence suggests epigenetic aberrations as a major pathogenic factor in the development of malignancy. Modulation of tumour suppressor or promoter genes has been described as a key event in cancer. In short, increased methylation can lead to silencing of tumour suppressor genes, thereby promoting tumour progression. In these cases, decreased methylation may be of therapeutic benefit. Catalyzation of methylation is achieved by the family of DNA methyltransferases (DNMT). DNMT1 seems to be the main pathogenic enzyme in human cancer, while its suppression can significantly inhibit tumour growth. We hypothesized that similar to procaine, lidocaine leads to demethylation by inhibiting DNMT. In an early landmark study, Kennedy et al. postulated that sensitizing effects of lidocaine for the chemotherapeutic, bleomycin, were potentially caused by interference with DNA integrity. We show that in vitro, lidocaine is an even more potent DNA-demethylating agent than procaine.
The differential effect of lidocaine at a concentration of 1mM in BT-20 and MCF-7 cell lines is a novel finding, but not unexpected given the heterogeneity of human cancer types. In part, it confirms previous *ex vivo* experiments, in which inhibitory effects of serum taken from patients undergoing regional anaesthesia impeded ER-negative cells in vitro.\(^{35}\) For example, breast cancer, which we chose to serve as the tumour paradigm for this study, has been subdivided into at least five distinct subtypes based upon gene expression profiling, and immunohistochemical markers.\(^{38}\) These are characterized by differential sensitivity to treatment, and clinical outcome, and biological heterogeneity is only beginning to be fully understood.\(^{38}\) In our case, BT-20 cells showed a much higher index of methylation than MCF-7 cells, with PMR values 100-fold higher in BT-20 cells (Fig. 2). Demethylating effects of local anaesthetics may depend on a high level of pre-existing methylation to show effect. Conversely, in cancer types in which DNA methylation is not a key pathogenic factor, the potential therapeutic effect seems negligible.

Finally, some potential limitations of our study should be briefly mentioned. First, we used lidocaine as a prototypical amide-type local anaesthetic, whereas in clinical routine, long-acting local anaesthetics are more frequently used for epidural anaesthesia and analgesia. We chose to investigate lidocaine as the first prototypical substance since it constitutes the most widely investigated local anaesthetic, and is the only local anaesthetic that is routinely administered locally for infiltration, for epidural or paravertebral anaesthesia, or intravenously as part of multimodal anaesthetic and analgesic regimens.\(^3\) Extrapolating from the fact that both procaine and lidocaine exert demethylating effects, the same should be anticipated for long-acting compounds such as bupivacaine and ropivacaine, even if testing this hypothesis was beyond the scope of the present study. These results were obtained in two cell lines representative of estrogen-positive and estrogen-negative breast cancer, respectively. These cell lines have been extensively researched and validated. Yet, they may feature genotypic and phenotypic drift over time. We sought to minimize this potential bias, by authenticating cell lines using amplification of several STR loci. Local anaesthetics have been demonstrated to
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sensitize tumour cells towards cytotoxic effects of chemotherapeutics, and it is thus possible that such sensitization may also take place towards demethylating effects of novel chemotherapeutics.

Our findings suggest that demethylating tumour-suppressive effects of anaesthetic interventions may only be detectable in specific types of cancer due to differential methylation profiles. Lidocaine dose-dependently demethylates DNA of breast cancer cell lines in vitro.

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Figure Legends

Figure 1: Effect of lidocaine and procaine on cell proliferation of MCF-7 and BT-20 human breast cancer cells. (A) Cell numbers after lidocaine and procaine treatment. Results of 8 independent experiments are shown. (B) TUNEL assay. Results of 1 representative experiment out of 4 independent experiments are shown. Results are expressed as mean ±SD. Statistical significance between control and treated samples was assessed by the unpaired Student's t-test (*, p < 0.05; ***, p < 0.001).

Figure 2: Global Genomic SAT2 DNA methylation analysis. (A) SAT2 DNA methylation levels in MCF-7 and (B) BT-20 breast cancer cells treated with 1 mM procaine, 1mM lidocaine, 0.1mM lidocaine, 0.01mM lidocaine or 5 µM 5-aza-2’-deoxycytidine (DAC) respectively. Results of 8 independent experiments are shown. Results are expressed as mean ±SD. Statistical significance between control and treated samples was assessed by the unpaired Student's t-test (*, p < 0.05; ***, p < 0.001).

Figure 3: Global Genomic SAT2 DNA methylation analysis: time series. (A) SAT2 DNA methylation levels in MCF-7 and (B) BT-20 breast cancer cells treated with 0.01mM lidocaine for 48, 72, 96 and 120 hours. Results are expressed as mean ±SD. Results of at least 3 independent experiments are shown. Statistical significance between control and treated samples was assessed by the unpaired Student's t-test (*, p < 0.05; ***, p < 0.001).

Figure 4: DNA methylation and RNA expression analysis of Tumour Suppressor Genes. (A) DNA methylation (results of 8 independent experiments are shown) and (B) mRNA expression (results of 5 independent experiments are shown) of RASSF1A, GSTP1 and MYOD1 after a treatment with 1mM procaine, 1mM lidocaine or 5µM DAC for 72 or 96 hours respectively. Results are expressed as mean ±SD. Statistical significance between control and treated samples was assessed
by the unpaired Student's t-test or Mann-Whitney U test (for GSTP1 and MYOD1 mRNA expression) (*, \( p < 0.05 \); ***, \( p < 0.001 \)).
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Figure 1
Figure 2

![Graph A](image1)

![Graph B](image2)
Figure 3

A. MCF-7

B. BT-20

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Figure 4