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The Effect of Lidocaine and Bosutinib on 4T1 Murine Breast Cancer Cell Behaviour *In Vitro*

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Abstract. *Background/Aim: Systemic lidocaine has recently emerged as a promising agent possessing numerous potentially anti-neoplastic effects. In vitro studies suggest that lidocaine may prevent metastasis by acting on the tyrosine kinase enzyme Src. Intravenous lidocaine has been reported to reduce pulmonary metastasis in vivo in a murine breast cancer model, however the beneficial effect is abolished by the Src inhibitor bosutinib. In this study we examined whether lidocaine and/or bosutinib affects 4T1 breast cancer cell activity in vitro and whether any drug interactions similar to that seen in murine models occur. Materials and Methods: 4T1 murine breast cancer cells were exposed to lidocaine and/or bosutinib. Cell viability after 1 h of exposure was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell migration after 24 h of exposure was measured using the Oris™ migration assay. Results: Lidocaine and bosutinib alone or combined inhibited 4T1 cell viability and migration, but only at supratherapeutic concentrations. Bosutinib did not modulate lidocaine's effect on viability or migration at any concentration tested. Conclusion: Although lidocaine may inhibit 4T1 metastasis in vivo, a direct effect on 4T1 cells is not detectable in vitro at non-toxic concentrations and unlike murine model testing, no unusual interaction with bosutinib was detected. Lidocaine's anti-metastatic properties are likely to be complex and multifactorial and difficult to replicate outside of a biological host.*

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Annually, more women worldwide die from breast cancer than any other malignancy with over 620,000 related deaths in 2018 alone (1). If detected early then surgery is often curative, however, breast cancer frequently recurs post-operatively in the form of metastatic disease which is invariably fatal (2). Tumour manipulation during surgery may dislodge cancer cells into the circulation (forming circulating tumour cells or CTCs) which may then lodge in remote tissues, lying dormant until eventually proliferating to form metastases (3). Pathophysiological processes instigated by the surgical stimulus, including the neurohumoral stress response, inflammation and immune suppression, are known to facilitate the survival and proliferation of residual cancer cells (4). The choice of anesthetic technique and agents used during surgery is postulated to both modulate these adverse physiological changes and directly affect CTCs, thus altering the likelihood of cancer cell survival (5). The perioperative period is now recognised as a critical phase during which the choice of anesthetic agents used and their effects on cancer cells disseminated during surgery could be vitally important in modulating the patient's risk of developing future metastatic disease (6).

The amide local anesthetic lidocaine is frequently administered intravenously in the perioperative period both as an analgesic agent and also to hasten the return of gut function following abdominal surgery (7). The effect of amide local anesthetics, including lidocaine, on cancer biology has been assessed in numerous laboratory and pre-clinical studies. Previous *in vitro* studies have shown that lidocaine possesses inhibitory effects on cellular activity (including viability, migration and proliferation) across a wide variety of cancer cell types, including breast, pancreatic and hepatocellular carcinoma and lung adenocarcinoma cells (8-11). Beneficial anti-neoplastic effects have also been attributed to lidocaine treatment in several *in vivo* studies – lidocaine decreased tumour size when administered intraperitoneally in mouse

models of melanoma and hepatocellular carcinoma, and intravenously in models of melanoma and retinoblastoma (9, 12-14). These laboratory findings are supported by clinical evidence from a recent retrospective cohort study which detected an improvement in early overall survival following pancreatectomy in patients who received an intravenous lidocaine infusion during the procedure (15).

The closest animal model to replicate the perioperative surgical stimulus and intravenous lidocaine's effect on breast cancer recurrence is the murine model initially described by Johnson *et al*. (16). In this model female Balb/C mice are inoculated with syngeneic 4T1 breast cancer cells (closely mimicking highly aggressive human triple-negative breast cancers) in the mammary fat pad (17). The resulting breast cancer is excised under sevoflurane anesthesia with a variety of agents co-administered intravenously. Following recovery, the animals are euthanised 2 weeks post-operatively and the pulmonary and hepatic metastatic burden is quantified. To date, three separate investigations have demonstrated a consistent reduction in post-operative pulmonary metastases in animals treated with intravenous lidocaine during surgery (16, 18, 19).

How lidocaine may inhibit cancer cell metastasis is unclear. Various mechanisms have been suggested including inhibition of the enzyme Src (20-23). Src is an intra-cellular tyrosine kinase involved in regulation of a wide array of cellular processes including survival, migration and proliferation (24). Increased Src expression is associated with progression of breast, colon and pancreatic cancer (25). Activation of Src increases expression of downstream products of its signalling cascade including the matrix metalloproteinases MMP-2 and MMP-9, which enzymatically degrade extracellular matrix and thus facilitate cancer cell migration and invasion (26-29). Dual Src/Bcr-Abl inhibitors such as bosutinib (Src Kinase Inhbitor, SKI-606) are used to treat leukaemia and have also been clinically investigated as potential therapies for breast cancer, although results have been unconvincing (30, 31).

Reduced expression of MMP-2 has previously been associated with lidocaine-induced reduction in pulmonary metastasis in the 4T1 murine breast cancer surgical model, but whether this is directly related to an effect on Src is not known (19). Introduction of the Src-inhibitor bosutinib into this model had no effect on metastasis, but when administered in combination with lidocaine, bosutinib effectively inhibited lidocaine's observed anti-metastatic effect. Such an unusual interaction has been noted previously. Tarpley *et al*. treated human triple-negative breast cancer cells with bosutinib and dasatinib, a similar although less specific Src inhibitor (32). Whereas bosutinib had minimal effects on cell viability and proliferation at therapeutic concentrations, dasatinib had potent inhibitory effects at low doses. When both drugs were used in combination, bosutinib enhanced cell proliferation and blocked the inhibitory effects of dasatinib. The authors hypothesised that bosutinib causes kinome re-programming and activation of alternate signalling pathways that lead to cellular resistance to dasatinib's inhibitory effects.

In this experiment we examined the effect of lidocaine alone and in conjunction with bosutinib on murine 4T1 breast cancer cells in an *in vitro* setting. Our aim was to assess whether lidocaine or bosutinib directly affect viability or migration of 4T1 cells at standard therapeutic concentrations, and whether both combined possess synergistic or antagonistic effects on cancer cell behaviour similar to that observed *in vivo*.

Materials and Methods

Cell culture. Unmodified 4T1 murine breast cancer cells were cultured in Roswell Park Memorial Institute (RPMI) medium with 1% (v/v) penicillin/streptomycin and 15% (v/v) fetal calf serum (FCS) in T25 culture flasks in a 37˚C, 5% CO_2 tissue culture incubator as per protocol (17). Prior to experimental use, cells were removed from their flasks, concentrated by centrifugation, counted, re-suspended in RPMI at a concentration of 1×10^6 cells/ml and viability greater than 80% was confirmed using an automated cell counter (Countess, Invitrogen, Waltham, MA, USA).

Cell viability study. Drug effects on cell viability were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay that enables the quantification of viable cells present in a sample via the conversion of tetrazolium dye MTT to insoluble purple formazan crystals by active enzymes in mitochondria present in living cells. 4T1 cells at a density of 2×10^4 cells/cm^2 were seeded in 96-well plates and allowed to adhere. Stock solutions of 10 mM bosutinib (Wyeth, Madison, NJ, USA) in dimethyl sulfoxide (DMSO) and lidocaine hydrochloride 10 mM (Braun, Melsungen, Germany) in 0.9% sodium chloride were prepared. Using these stock solutions, lidocaine, bosutinib or both were diluted in FCS-containing medium and added to the wells at varying concentrations and subsequently exposed to the 4T1 cells for 1 h. MTT solution was prepared by the addition of 0.5 mg/l MTT to FCS-containing media. The drug-containing media were removed and 100 μl of MTT solution was added to each well, followed by incubation of the plate for 3 h at 37˚C in 5% CO_2. This solution was then removed from the wells and any formazan crystals present were solubilised by adding 100 μl DMSO followed by agitation of the plate for 20 minutes. Well absorbance was measured at 570 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). Viability was expressed as percentage of the non-drug exposed control. Three separate and independent experiments were conducted using different cell passages for each.

Cell migration assay. The effect of lidocaine and bosutinib on 4T1 cell migration on a collagen substrate was determined using the Oris™ Cell Migration Assay (Platypus Technologies, Fitchburg, WI, USA), as per the manufacturer's protocols. Oris™ Cell Migration Assembly Kit-FLEX 96-well plates were prepared by coating each well with 7–8 μg/cm^2 rat tail collagen I (Sigma, Wicklow, Ireland). 4T1 cells (1×10^5 cells in 100 μl medium) were seeded into the collagen-coated wells. An Oris™ cell seeding stopper was placed in each well prior to introducing the cells to restrict cell seeding to

outer regions of the well. Seeded plates were incubated overnight at 37˚C in 5% CO_2 to allow for cell attachment.

Stoppers for experimental wells were then removed to create a detection zone of 2-mm diameter into which cells could migrate. Lidocaine and bosutinib or both (from stock solutions) were added to the wells in varying concentrations and the 96-well plates were incubated for 24 h at 37˚C in 5% CO_2 to allow time for migration. Following incubation, stoppers were removed from negative control wells immediately prior to cell staining, so that no migration could have occurred in these wells.

Staining and fixation was conducted with Coomassie blue (1% w/v Coomassie blue in 40% v/v methanol, 10% v/v acetic acid). The medium was removed and the wells washed with phosphate-buffered saline (PBS), then 100 μl of Coomassie blue was added for 10 minutes. Following decanting of the stain, cells were washed with PBS twice. Cell migration into the 2-mm central detection zone was quantified by measuring absorbance using a bottom-reading colorimetric plate reader (SpectraMax M3, Molecular Devices) using a 570-nm measurement filter. A template mask (Oris™) was used to shield all regions of the wells, other than the 2-mm detection zone. Absorbance values were averaged across eight experimental wells for each passage to obtain a single experimental absorbance. Background absorbance values were averaged across all negative control wells for each cell line and this value was subtracted from the mean experimental absorbance to obtain a single absorbance value, indicative of cell migration, for each passage of cells. Three separate and independent experiments were conducted using different cell passages for each.

Statistical analysis. Use of the D'Agnostino and Pearson omnibus normality test found that the cell viability data were non-normally distributed, therefore data were analyzed using the Kruskal-Wallis test and Dunn's method for correction of multiple comparisons. As migration data were normally distributed, comparisons between groups were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's method for correction of multiple comparisons. Results are given as mean±standard deviation (SD), unless otherwise indicated. Probability values of <0.05 were considered statistically significant. Data analysis was performed using Prism 9.1.0 (GraphPad, San Diego, CA, USA).

Results

Drug effects on 4T1 cell viability. Lidocaine did not significantly reduce 4T1 cell viability after exposure of 1 h (Figure 1A-C) compared to control, except at 3mM, the highest concentration tested: 50.4%±14.6% (mean±SD) *vs*. control, $p<0.001$. Bosutinib alone did not significantly affect cell viability after 1 h compared to control at any concentration tested (maximum 1 μM, $p=0.085$). Likewise, the addition of bosutinib to lidocaine did not significantly reduce cell viability further than lidocaine alone, even at the highest concentrations; 1 μM of bosutinib and 3 mM of lidocaine: 34.9%±13.2% *vs*. 3 mM of lidocaine alone: 50.4%±14.6%, $p=0.452$.

Drug effects on 4T1 cell migration. Lidocaine did not significantly affect 4T1 cell migration following 24 h of exposure (Figure 1D-F) compared to control, except at 3 mM (highest concentration tested): 47.4%±67.0% (mean±SD) *vs*. control, $p<0.001$. Similarly, bosutinib alone did not affect migration except at a very high concentration (10 μM): 8.4%±4.9% *vs*. control, $p<0.001$. The addition of high concentration lidocaine (3 mM) to high concentration bosutinib (10 μM) did not cause a significant reduction in cell migration compared to high concentration of bosutinib alone: 0.8%±1.2% *vs*. 8.4%±4.9%, $p=0.214$.

Discussion

In this study, we investigated whether lidocaine and/or bosutinib possess direct inhibitory effects on murine 4T1 breast cancer cells *in vitro* (as assessed by standard viability and migration assays) and whether any antagonistic effects occur during their use in combination, as has been observed in mouse studies (19). To the best of our knowledge, this is the first study to examine the effects of either lidocaine or bosutinib on 4T1 cells *in vitro*.

The drug concentrations used experimentally were chosen to reflect therapeutic plasma concentrations achieved during animal model testing, and indeed during human clinical use. Lidocaine plasma concentrations achieved during perioperative intravenous infusion are typically in the range of 0.5-5 μg.ml-1 (2-22 μM) (33). Risks of central nervous system and cardiovascular toxicity increase rapidly as plasma concentrations rise above 20 μM (34). As the lidocaine intravenous infusion times used in all *in vivo* studies was limited to 25 minutes, and as the plasma half-life of lidocaine is short, we modelled this brief time period using a 1-h drug exposure for the viability study (16, 18, 19, 35). The concentration ranges of bosutinib tested reflect clinical therapeutic concentrations and were similar to those used in previous studies examining human breast cancer cells (32, 36). As no inhibition of migration was seen after 24 h of drug exposure (except at supratherapeutic concentrations), shorter exposure periods were not tested.

Previous studies examining human breast cancer cells demonstrated that lidocaine inhibited viability and migration only at high concentrations (>1 mM), with no effect at low concentrations (1-20 μM) typically measured in plasma during intravenous infusion (37). We detected similar results with murine breast cancer cells in our experiment; no inhibition of 4T1 viability or migration was observed at clinically relevant lidocaine concentrations. Lidocaine's effect on viability and migration appears to be cancer-specific: some cancer cells are highly sensitive to low concentrations of lidocaine (*e.g.*, 1 μM in lung cancer cells) (38).

Two studies have reported that human breast cancer cell viability was unaffected by bosutinib at any of the

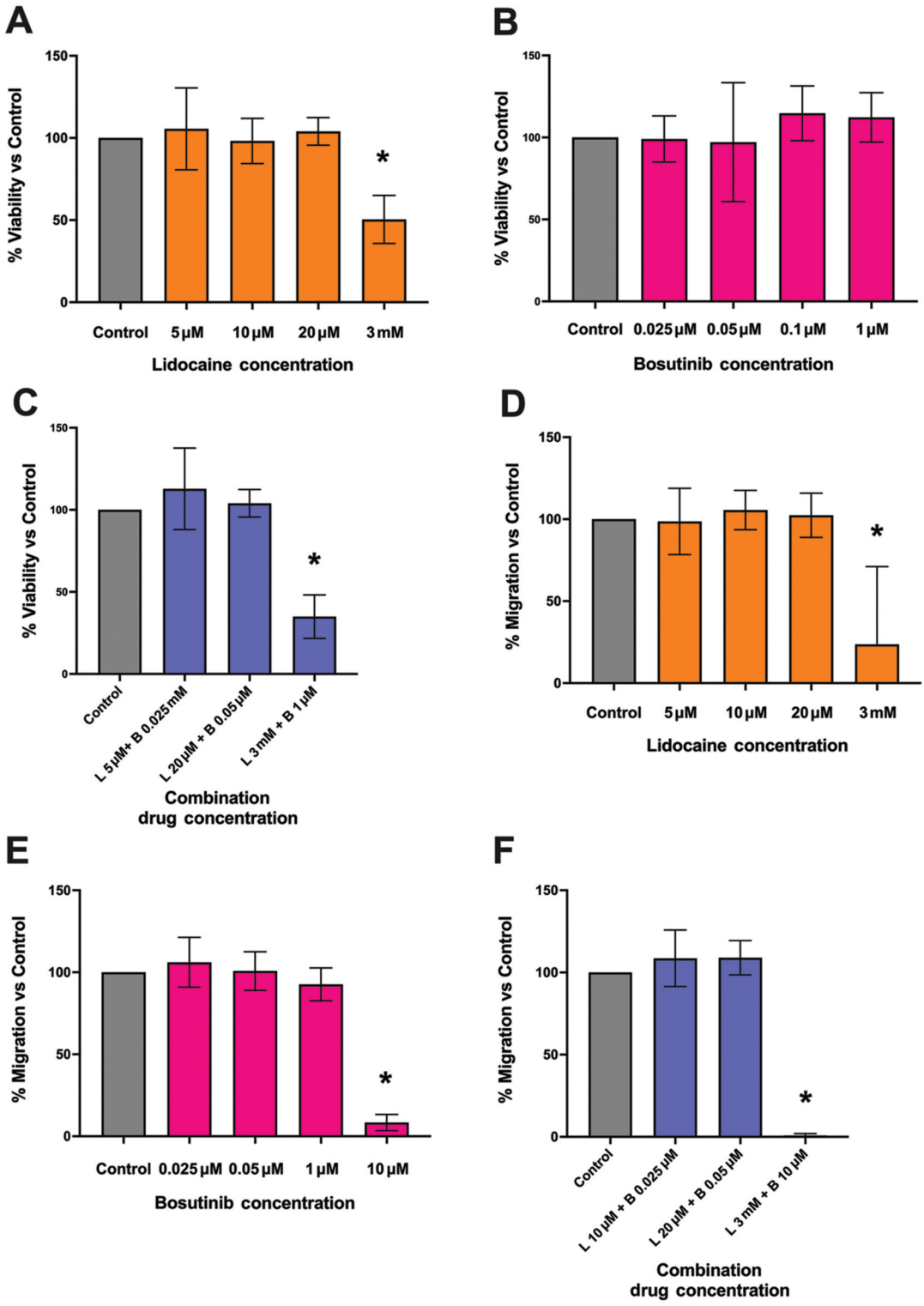

Figure 1. *Lidocaine and bosutinib effects on 4T1 cancer cell viability and migration. A, B, C: In vitro 4T1 murine cancer cell viability after 1-h treatment with lidocaine, bosutinib or both, as measured by MTT assay (mean, SD). n=3 experiments, each experiment had 8 samples per group. *indicates significant differences vs. control ($p<0.05$) using the Kruskal-Wallis test and Dunn's method for multiple comparisons. L, Lidocaine; B, bosutinib; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation. D, E, F: In vitro 4T1 murine cancer cell migration following 24-h treatment with lidocaine, bosutinib or both (mean, SD). n=3 experiments, each experiment had 8 samples per group. *indicates significant difference compared to control ($p<0.05$) using one-way analysis of variance and Dunnett's method for multiple comparisons. L, Lidocaine; B, bosutinib; SD, standard deviation.*

concentrations tested (≤1 μM), similarly to the absence of any effects on 4T1 viability observed in the present study (32, 36). Bosutinib has been shown to inhibit human breast cancer cell migration at low concentrations typically encountered in plasma during clinical therapy (0.1-0.3 μM) (36), however we observed no effect on 4T1 migration except at very high concentrations (10 μM). Our results suggest that 4T1 murine cells are, compared to human cancer cells, relatively resistant to direct inhibition of migration or viability by standard therapeutic concentrations of lidocaine or bosutinib for short durations, and combined treatment has no synergistic or antagonistic effect *in vitro*. This may be due to the absence of Src-activating inflammatory mediators *e.g.*, tumor necrosis factor-alpha (TNF-α) *in vitro* (10), hence the effect of Src inhibitors may be less pronounced than *in vivo*.

In conclusion, lidocaine and bosutinib alone or in combination do not affect 4T1 migration or viability at standard therapeutic concentrations when tested in an *in vitro* setting. The inhibition of pulmonary metastases previously detected in murine 4T1 breast cancer surgery models is unlikely to be due to direct effects of lidocaine on 4T1 cell viability or migration. Unlike previous *in vivo* observations, the addition of the Src inhibitor bosutinib did not have any appreciable influence on lidocaine's effects. Further studies are required to assess lidocaine's influence on a broad range of cellular targets in the 4T1 surgical model to characterise the mechanism underlying lidocaine's beneficial anti-metastatic effects. The planned large randomised clinical trial named Volatile Anesthesia and Perioperative Outcomes Related to Cancer (VAPOR-C, NCT04316013) will help to determine if lidocaine's pre-clinical anti-cancer effects will translate into real patient benefits (39).

Conflicts of Interest

None.

Authors' Contributions

T.P.W., P.D.C. and D.J.B. conceived and designed the experiments; T.P.W. and P.D.C. performed the experiments; T.P.W. P.D.C. and D.J.B. performed the statistical analysis; T.P.W. wrote the manuscript; D.J.B. reviewed the manuscript; All Authors read and approved the final manuscript.

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