JPET Fast Forward. Published on May 21, 2018 as DOI: 10.1124/jpet.118.248989
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Title Page

Ibuprofen decreases spontaneous activity and enhances nerve evoked contractions to minimize mitomycin C induced bladder dysfunction

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Running Title Page

a) **Ibuprofen minimizes voiding dysfunction caused by MMC**

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c) Number of pages: 28

Number of Figures: 5

Number of references: 31

Abstract wordcount: 241

Introduction wordcount: 569

Discussion wordcount: 1399

d) **Abbreviations:** Acetylcholine – ACh; Adenosine triphostphate – ATP; alpha,beta-methylene-ATP – αβ-mATP; Bacillus Calmette–Guérin – BCG; Electrical field stimulation – EFS; Ibuprofen – IBU; L-N-Nitroarginine - LNNA; Mitomycin C – MMC; Non-steroidal anti-inflammatory drug – NSAID; Prostaglandin E_2 – PGE_2; Voiding patter analysis – VPA

e) Recommended section: Chemotherapy, Antibiotics and Gene Therapy

Abstract

Inflammation may play a causal role in urological side effects reported following intravesical mitomycin C (MMC). Our aim was to investigate the effects of the non-steroidal anti-inflammatory drug ibuprofen (IBU) on the cytotoxic potency of MMC and the potential for IBU to protect against bladder dysfunction. Malignant (RT4, T24) and normal (UROtsa) urothelial lines were treated with MMC followed by ibuprofen, with cell viability and caspase-3 activity assessed. Female C57BL/6JArc mice (Saline/Control, MMC, Saline + IBU and MMC + IBU), received intravesical treatment (1hr) with saline or MMC (2mg/mL), with IBU (1mg/mL) delivered in drinking water (for 7-days). Voiding pattern analysis was conducted prior to and following (1,3,7 days) treatment. A whole bladder preparation was used to assess compliance, contractile responses and urothelial mediator release. Ibuprofen selectively increased the cytotoxic potency of MMC and caspase-3 activity in both malignant cells lines but not in UROtsa. MMC significantly increased voiding frequency at 24 hours and 3 days, while administration of ibuprofen significantly reduced this effect. MMC significantly increased the frequency of spontaneous contractions from 2.3±0.5 contractions/min in saline controls to 4.8 ±0.16 contractions/min, with ibuprofen protecting against this change. Interestingly, while nerve evoked responses were not altered by MMC, they were increased in both IBU groups. Ibuprofen improved voiding dysfunction following MMC treatment through reducing spontaneous phasic activity and enhancing nerve mediated contractions. Ibuprofen use in bladder cancer patients may help to minimize the urological adverse effects associated with intravesical MMC.

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Introduction

Bladder cancer is a common malignancy worldwide, with most patients being diagnosed with the non-muscle invasive form of the disease. Treatment generally begins with transurethral resection of the tumour, followed by intravesical instillation of a chemotherapeutic agent (Babjuk et al., 2011). Mitomycin C is the most common cytotoxic drug used to treat bladder cancer and has been shown to reduce the risk of recurrence (Bosschieter et al., 2018). While an effective intravesical treatment, MMC is known to cause urological side-effects in 34.5-41% of patients who undergo treatment, the most common being increased urinary frequency and urgency, and painful urination (Thrasher and Crawford, 1992; Filson et al., 2014). Other side effects include pelvic pain and haematuria. In some cases, patients will not continue treatment due to the side effects that diminish their quality of life, which in turn has a substantial impact on treatment outcomes for patients.

There is currently no clinically proven management strategy to help control the local adverse effects associated with intravesical treatment for bladder cancer. The muscarinic receptor antagonist oxybutynin has been reported to worsen the urological side effects reported by bladder cancer patients following intravesical immunotherapy with Bacillus Calmette–Guérin (BCG) (Johnson et al., 2013). Recently, Luckenbaugh et al proposed the first management alogorithm with an escalating pharmacological approach for managing MMC induced cystitis in bladder cancer patients (2017). This method included behavioural therapy initially followed by histamine antagonists, combined cholinergic and alpha-adrenoceptor antagonist treatment, intensifying to prednisone combined with anti-histamines in more severe cases. The authors acknowledge that this treatment approach has yet to be assessed in a formal clinical trial.

In vivo studies in rodents have reported alterations in bladder function that persist for several weeks following repeated intravesical treatment with MMC, epirubicin or doxorubicin (Post et al., 1993; Michielsen et al., 2005). These changes include increased urinary frequency, and decreased bladder compliance. In addition, MMC caused significantly more histological damage to the mouse bladder compared to doxorubicin at clinically relevant doses (Post et al., 1993). Farr, etal., recently reported that unlike gemcitabine which is selective for malignant over non-malignant urothelial cells, MMC exhibited no such selectivity, with equivalent cytotoxic potency in normal and malignant bladder urothelial cultures (Farr et al., 2017). This may contribute to the more favourable efficacy and toxicity profile for gemcitabine compared to MMC (Shelley et al., 2012). Experimental studies have reported that doxorubicin, MMC and gemcitabine which are used for intravesical bladder cancer treatment cause release of inflammatory cytokines, an effect that was still evident 1 week following treatment in *in vitro* urothelial models (Kang et al., 2013; Kang et al., 2015a; Farr et al., 2017). The findings of these studies suggest that inflammation may play a causal role in the urological side effects associated with intravesical chemotherapy and is therefore a potential target for treatment.

It is important to consider the impact that an adjunct anti-inflammatory agent may have on the cytotoxic potency of intravesical chemotherapeutic agents such as MMC, and its efficacy as a bladder cancer treatment. Therefore, the first aim of this study was to investigate the effects of non-steroidal anti-inflammatory drug ibuprofen on the cytotoxic potency of MMC in bladder cancer and non-malignant urothelial cells lines. Secondly, we aimed to assess the potential for ibuprofen to protect against bladder dysfunction in mice following intravesical MMC treatment.

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Methods

Materials

Mitomycin C was purchased from Cayman Chemicals. Ibuprofen oral suspension (200 mg/mL) was prepared by National Custom Compounding, Australia. All chemicals used to prepare Krebs bicarbonate solution and saline, as well as carbachol, isoprenaline, adenosine 5'-triphosphate (ATP), alpha,beta-methylene-ATP, atropine and L-N-Nitroarginine (LNNA) were purchased from Sigma Aldrich. The ATP Determination Kit and Amplex Red Acetylcholine Assay Kit were purchase from Molecular Probes.

Cell culture

Human RT4 and T24 bladder urothelial cancer cells (European Collection of Cell Cultures) were cultured in McCoy's 5A culture medium, supplemented with 10% foetal bovine serum and L-glutamine and gentamicin. UROtsa cells (a gift from Professor Scott Garrett, University of North Dakota) were cultured and maintained in low-glucose Dulbecco's modified Eagle's medium, supplemented with 5% FBS and gentamicin. Fresh culture medium was added every 2 days and cells were maintained at 37ºC in a 5% CO2 incubator. Cells were split when they reached approximately 90% confluence. Trypan blue exclusion was used to assess cell viability prior to plating for experiments. Only cell cultures with $\geqslant$95% viability were used.

Cell treatment

Ninety-six well plates were seeded at a density of 50,000 cells/well and incubated overnight at 37°C in a 5% CO2 incubator to allow cells to attach. Following incubation, culture medium was removed and replaced with fresh culture medium containing MMC (0.13nM to 1.3mM). Untreated and vehicle treated controls were included in each experiment. Cells were incubated for 2 hours with MMC to mimic maximum duration of intravesical treatment,

at which time treatment medium was removed. Cells were washed twice with phosphate buffered saline and fresh culture medium added to each of the wells. Cells were maintained in culture for a further 24 or 72 hours, at which time apoptosis and cell viability was assessed. To determine the effects of the anti-inflammatory drug ibuprofen on the cytotoxic potency of MMC, cells were treated as described above, but following treatment, medium containing ibuprofen (5 µM) was added to the cells and then incubated for a 24- or 72-hour recovery period in the presence of the anti-inflammatory drug. Appropriate vehicle controls were included in each experiment. Each condition was tested in triplicate wells, with a minimum of n=5 repeat experiments conducted.

Measurement of cell viability and caspase-3 activity

The resazurin reduction assay, whereby resazurin is reduced to resorufin by viable cells, was used to assess cell viability (O'Brien et al., 2000). Seventy-two hours after MMC treatment, the medium was removed from the 96-well plate and fresh medium containing 44 µM reszurin was added. After 2 hr incubation, reduction of resazurin to resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a Modulus microplate reader. Under all conditions tested, the extent of resazurin reduction was directly proportional to viable cell counts (data not shown).

Caspase-3 activity was assessed in cells treated with MMC alone or in combination with the anti-inflammatory drug as a marker of early apoptosis. Caspase-3 activity was assessed at 24-hours post-treatment using a Caspase-3 Fluorescence Assay Kit (Cayman Chemical). All steps followed the manufacturer instructions. Caspase-3 data was normalised according to cell number using corresponding resazurin reduction data.

Animal model

All procedures were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and with the approval of the University of Queensland Animal Ethics Committee. Adult female C57BL/6JArc mice (32-weeks in age) were used for this study and housed under environmentally-controlled conditions, with 12 hour light-dark cycles and access to food and water *ab libitum*. Animals were randomly allocated into four experimental groups: Saline, MMC, Saline + Ibuprofen or MMC + Ibuprofen.

Intravesical treatment

Mice were anaesthetised with 1-3% isoflurane gas and micturition was induced by mild abdominal massage to prevent dilution of solutions. Bladder catheterisation was then performed with a sterile intravenous 22x1 gauge catheter (Terumo, Japan) through the urethra. Thirty µL of solution (saline or 1 mg/mL MMC, clinical dose) was instilled through the catheter, before removal. Mice were allowed to run freely in their cage with access to food, but not water, for 1 hour on 225 hardened ashless 2µm filter paper (Filtech Pty Ltd, Woolongong, Australia), ensuring they did not void during this treatment period. Micturition was induced 1 hour post-intravesical instillation to expel the drug or saline solution.

Ibuprofen treatment

Ibuprofen was chosen based on initial anti-inflammtory screening in the malignant and non-malignant urothelial culture models (see results and discussion). To determine the effects of ibuprofen on bladder dysfunction caused by intravesical MMC treatment, mice received 1 mg/ml ibuprofen (Bosgraaf. C.A.J., 2006) in their drinking water for 7 days following intravescical treatment with saline or MMC. These animals were compared with the saline or MMC only groups.

Voiding Pattern Analysis

Voiding analysis was performed on the mice before intravesical treatment and was repeated 24hrs, 3 and 7 days after treatment, before euthanasia. All voiding pattern analysis was performed in the morning, beginning at the change-over of the light/dark cycle. Mice were housed singly in cages lined with Filtech® Hardened Ashless Filter Paper #225 for 4 hours with free access to food and water. The filter paper was collected and urine spots detected using a Molecular Imager ChemiDoc XRS ultraviolet transilluminator (#720BR1293 BioRad, California USA). The papers were photographed, digitised, and analysed using Image J software. Voided volume was determined using a standard curve created by measuring the area of urine patches of known volume (1-200 µL) and analysed using linear regression analysis (r^2= 0.997). This protocol was based upon previously reported studies into the voiding patterns in mice (Boudes et al., 2011; Uvin et al., 2013).

Isolated whole bladder preparation

Mice were euthanized by cervical dislocation 7-days post-intravesical treatment. The bladder was then isolated and a three-way cannula inserted via the urethra, to enable recording of intravesical pressure in addition to bladder filling and emptying, and mounted into a modified tissue bath (8ml), containing gassed (95% O_2/5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 118, NaHCO3 24.9, CaCl2 1.9, MgSO4 1.15, KCl 4.7, KH2PO4 1.15, and D-glucose 11.7) at 37°C. Intravesical pressure was measured using a pressure transducer (obtained from GlobalTown® Microtech) connected to a PC via a PowerLab data acquisition system (AD Instruments), using LabChart 7 software (AD Instruments).

Following equilibration, bladder distensions were performed by intravesical infusion of isotonic saline at a rate of 30 µl/min to 40 mmHg to check viability and compliance, with all further distensions for experimental purposes to 20mmHg.

The urothelium is known to play a signalling role, releasing several chemical mediators in response to distension during bladder filling, these include ATP and acetylcholine (ACh). Following distension to 20 mmHg the bladder was drained via the two-way cannula and intraluminal contents collected for measurement of the urothelial mediators ATP and ACh. Samples of the serosal fluid were also collected and all samples were immediately frozen on dry ice and stored at -80°C for later assay of ATP and ACh, using the ATP Determination Kit (Molecular Probes), and the Acetylcholine Amplex (®) Red Assay Kit (Molecular Probes). The assays were performed according to manufacturer instructions and luminescence, fluorescence (Ex. 540/Em. 590 nm) or absorbance measured, using a Modulus micro-plate reader (Promega).

Following distension to 20mmHg, bladders were allowed to equilibrate. During this period of accommodation spontaneous activity was measured as frequency of spontaneous contractions, recorded as number of contractions per minute, and amplitude measured from the peak to the trough of the contraction.

Electrical field stimulation (EFS) was undertaken in bladders after 1 hour equilibration following distension to 20 mmHg. The bladder was electrically stimulated (50V), every 100 seconds at 1, 5, 10, and 20 Hz and contractions were measured as pressure change from baseline. EFS was then repeated at 20Hz in the presence of L-NNA (100uM), atropine (1 uM) and αβ-methylene ATP (10uM). Neurogenic origins of the pressure response to EFS were confirmed using tetrodotoxin (0.1µM), which abolished responses at all frequencies.

Pressure responses to pharmacological agents was also assessed by addition of ATP (10mM), cumulative carbachol concentrations, and relaxation to cumulative isoprenaline concentrations. Finally, 60mM KCl solution was added, to measure non-receptor mediated contractile response in the whole bladder. All contractions and relaxation responses were measured as a change in pressure from baseline.

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Data and statistical analysis

All experiments were randomized, with 5 mice per experimental group and each experimental protocol started on a different day. For cell culture experiments 5 independent experiments were conducted in triplicate as technical replicates to ensure reliability of single values. The data presented for cell culture experiments represent the mean of n=5 single values. Results are expressed as mean ± standard error of the mean (SEM). Data were analysed using a unpaired Student *t*-test, ordinary one-ANOVA or repeated measures two-way ANOVA with Bonferroni's multiple comparisons test, using Graphpad Prism version 6 software (GraphPad, SanDiego, CA). Significance levels were defined as $P<0.05$ (*).

Results

Effect of MMC on malignant and non-malignant urothelial cell lines

Two-hour treatment with MMC resulted in a concentration-dependent decrease in cell viability as measured by resazurin reduction in RT4, T24 and UROtsa (Fig. 1A-C), assessed 72-hours following treatment. MMC was not selective for malignant cell lines (RT4 and T24) over the non-malignant UROtsa, with equivalent cytotoxic potency observed in all of the urothelial cell lines tested (pIC_{50}: RT4 5.66 ± 0.05; T24 5.67± 0.03; UROtsa 5.67 ± 0.05). Ibuprofen did not reduce the cytotoxic potency of MMC but did selectively and significantly increased the cytotoxic potency of MMC in both malignant urothelial cell lines (pIC_{50} RT4: 5.66 ± 0.05 MMC alone vs 5.99 ± 0.06 MMC + IBU $p<0.01$; pIC_{50} T24: 5.67 ± 0.03 MMC alone vs 5.89 ± 0.12 MMC + IBU $p<0.05$), with no effect on the potency in the non-malignant UROtsa cell line (Fig. 1A-C). The increase in cytotoxic potency of MMC when combined with ibuprofen was associated with a significant increase in activity of the apoptotic marker caspase-3 in both RT4 ($p<0.01$) and T24 ($p<0.01$) cells (Fig. 1D).

Effects of MMC and ibuprofen on animal parameters and voiding pattern

Animal body weight and water consumption were measured prior to and at 1, 3 and 7 days following intravesical instillation. These parameters were not significantly affected by MMC or ibuprofen treatment (data not shown).

Voiding pattern data were collected from animals in all four experimental groups, but statistical analysis revealed no significant difference in voiding pattern between the Saline and Saline + IBU groups indicating ibuprofen did not affect voiding behaviour. As a result, the voiding pattern analysis data was combined for Saline and Saline + IBU groups and this combined data is presented graphically as the Saline group (Fig. 2 A-C). Overall there was no

change in total urine volume voided over time or between experimental groups indicating that rate of urine production was not affected by treatment with MMC or ibuprofen (Fig. 2A). Intravesical MMC significantly increased the number of voiding events compared to saline, 24 hours ($p<0.001$) and three days ($p<0.01$) following treatment, (Fig. 2B) indicating increased frequency of urination. While the number of voiding events was still elevated at 7-days following MMC treatment, the increase was not statistically significantly. Ibuprofen attenuated the change in voiding frequency caused by intravesical MMC treatment, with significantly fewer voiding events ($p<0.05$) at 3 days compared to the MMC only group (Fig. 2B). The number of voiding events in the MMC + Ibuprofen group was then not significantly different from the saline group at any time point.

The increase in voiding frequency observed with MMC was accompanied by a decrease in the average volume of each void (spot size) in the MMC group 1- ($p>0.05$) and 3-days ($p<0.05$) following treatment, indicating a decreased volume of urine expelled with each void. While the MMC + Ibuprofen treatment group showed the same trend of decreasing the average volume voided 24 hours after treatment, unlike the MMC only group, the MMC + Ibuprofen group began to recover at 3 days (Fig. 2C).

Effects of MMC and ibuprofen on compliance and contractile responses of isolated whole bladders

No significant difference in bladder compliance curves (data not shown) or bladder capacity (Table 1) was seen between treatment groups. Similarly, intravesical pressure responses to carbachol, ATP, KCl and isoprenaline were not significantly affected by intravesical MMC or ibuprofen administration (Table 1).

Spontaneous phasic contractions were observed in bladders from all treatment groups with the amplitude and frequency of spontaneous contractions quantified during accommodation

following distension to 20mmHg. The amplitude of the spontaneous contractions in the saline group was 0.18 ±0.04 mmHg and increased approximately 2-fold in the MMC treatment group, although the change was not statistically significant ($p>0.05$) (Fig. 3). There was a significant 2-fold increase ($p<0.001$) in the frequency of spontaneous contractions in the MMC group (4.8 ± 0.16 contractions per minute) compared to the saline control (2.3 ±0.5 contractions per minute) (Fig. 3B). Ibuprofen alone did not alter the frequency of spontaneous contractions, with no significant difference observed between the Saline and Saline + Ibuprofen groups. However, ibuprofen protected against the MMC induced changes in frequency of non-voiding contractions with a significant decreased in frequency observed in the MMC + Ibuprofen treatment group when compared to MMC alone ($p<0.05$), maintaining the frequency of spontaneous contraction at the rate observed in the Saline control group (Fig. 3B).

Electrical field stimulation was performed to assess the effect of treatment on efferent nerve-mediated contraction of murine bladders. A frequency-dependent increase in intravesical pressure was observed in bladders from all animal groups (Fig. 4A). Efferent evoked pressure responses were not altered in bladders from MMC treated animals when compared to the saline group (Fig. 4A). Interestingly, the pressure response to EFS was consistently increased at all frequencies in both the Saline + Ibuprofen and MMC + Ibuprofen groups when compared to Saline or MMC alone. The pressure response at 20 Hz in the MMC + Ibuprofen group was significantly greater than in the MMC group ($p<0.05$) (Fig. 4A). Pharmacological agents were added to the bath to determine the relative contribution of nitric oxide, muscarinic and P2X receptors to nerve evoked responses in the murine bladder. Desensitization of P2X receptors with αβmATP, significantly decreased nerve evoked responses in all experimental groups, indicating that ATP is the main neurotransmitter

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involved in efferent responses in the murine bladder (Fig 4B – Saline group), and this is not altered by intravesical treatment with MMC or treatment with ibuprofen (data not shown).

Effects of intravesical MMC and ibuprofen on intraluminal and serosal release of ATP and acetylcholine from isolated whole bladders

The urothelium plays a sensory and signalling role through release of chemical mediators in response to stretch during bladder filling. Samples of intraluminal and serosal fluid were collected from bladders distended to 20mmHg and analysed for the release of urothelial mediators ATP and Ach to determine if changes in their release contributed to MMC induced voiding dysfunction in mice. Overall, there was greater release of both ATP and ACh into the serosal fluid than there was into the intraluminal fluid in all groups. Levels of intraluminal and serosal ATP and intraluminal ACh were not affected by MMC or ibuprofen treatment ($p>0.05$) (Fig. 5). However, serosal ACh was significantly increased in the MMC treatment group ($p<0.05$), an effect that was also evident in the MMC + IBU group ($p<0.001$) (Fig. 5D). The serosal ACh release was not significantly different between the MMC and the MMC + IBU, indicating that ibuprofen did not alter the MMC induced changes in serosal ACh release. Additionally, ibuprofen did not alter serosal ACh in saline treated mice (Fig. 5D).

Discussion

There is abundant clinical evidence that bladder cancer patients experience urological side-effects following intravesical treatment with MMC and other cytotoxic and immune therapies. There is currently no recommended approach to manage these adverse effects that has been supported by evidence from clinical trials. Our research group has previously investigated the effects of intravesical agents, including doxorubicin, gemcitabine and MMC, on various aspects of bladder function, and the results of these studies indicate that inflammation may play a causal role in the urological adverse effects experienced by patients following treatment (Kang et al., 2015a; Farr et al., 2017). Therefore, the aim of this study was firstly to determine if an anti-inflammatory drug affected the cytotoxic potency of MMC and if administration of the NSAID ibuprofen for 7 days following intravesical MMC treatment would minimise the effects of MMC on voiding behaviour and bladder function.

An important concern when considering adjunct therapies for cancer treatment is whether the drug combination will reduce the efficacy of the cancer treatment. Ibuprofen did not decrease the cytotoxicity of MMC in malignant or non-malignant urothelial cultures. However, ibuprofen selectively and significantly enhanced the reduction in cell viability observed in MMC treated bladder cancer cells, but not in normal cell cultures. In addition, the activity of the apoptotic marker caspase-3 was also enhanced in the cancer cell lines only. This suggests that ibuprofen either enhances the cytotoxicity of MMC in malignant cells or has anti-cancer properties itself. A study previously examined the anti-cancer effects of NSAIDs on T24 and RT4 bladder cancer cell lines (Khwaja et al., 2004), including ibuprofen and indomethacin and postulated that NSAIDs induce the expression of a tumour suppression gene, p75NTR. This gene was recently identified as a tumour suppressor and metastasis suppressor in bladder cancer cell lines (Krygier and Djakiew, 2001). Khwaja et al. (2004) found that ibuprofen treatment significantly caused a dose-dependent expression of the p75NTR gene in both RT4

and T24 bladder cancer cell lines. Even though ibuprofen and indomethacin are both non-selective COX inhibitors and both induced a significant expression of the p75NTR gene, ibuprofen was more effective than indomethacin. This may explain why ibuprofen but not indomethacin (unpublished data) enhanced cytotoxicity and apoptosis in RT4 and T24 cells. Ibuprofen was chosen as the anti-inflammatory agent to use in the following animal study due to its selective ability to increase the cytotoxic potency of MMC in bladder cancer cells.

The frequency of urination increased in MMC treated mice, indicating development of an overactive bladder phenotype. This result was expected as urinary frequency is one of the adverse effects reported by bladder cancer patients following intravesical chemotherapy (Witjes, 2008). Ibuprofen reduced the effect of MMC on urinary frequency, and while frequency was slightly increased ($p>0.05$) at 24 hours, it returned to control levels at 3 days post-treatment, unlike the MMC group which did not show a significant decrease in the number of voiding events until 7-days. Overall, the increase in urinary frequency in MMC and MMC + Ibuprofen mice was associated with a decrease in average voided volume. Previously, our research group observed the effects of MMC (2mg/mL) on urothelial mediator release and found that prostaglandin E2 was significantly increased 24 hours after treatment with MMC (Kang et al., 2015a). Prostaglandins are mediators of inflammation (Ricciotti and FitzGerald, 2011) and would be reduced by ibuprofen. It could then be concluded that by decreasing inflammation of the bladder, the increased urinary frequency caused by intravesical MMC was attenuated.

Many studies have observed spontaneous contractions in a number of different animal bladder models. Spontaneous activity can be characterized by either phasic or tonic contractions of the bladder. Contractions of the mouse urinary bladder occur mostly during the accommodation stage or bladder filling. These types of spontaneous contractions are called micromotions (Coolsaet, 1985). Here we observed that intravesical MMC resulted in

an increase in the frequency and amplitude of spontaneous non-voiding contractions measured 7-days following treatment. This increase in spontaneous activity may play a causal role in the increased urinary frequency observed in mice following MMC treatment. It has previously been reported that spontaneous contractions can initiate afferent nerve firing in a murine model of bladder overactivity (McCarthy et al., 2009). Early afferent activation due to increased spontaneous activity could contribute to bladder overactivity in mice following MMC treatment. Prostaglandins rise in response to infection of the bladder, or chemical cystitis (Grover et al., 2011) and have been shown to increase phasic contractions of detrusor smooth muscle (Kobayter, 2011). PGE_2 has the ability to increase the frequency of whole-cell Ca^{2+} transients and spontaneous depolarizations, thereby increasing spontaneous contractions. Kang et al. (2015a) found that MMC causes prostaglandin levels to rise 24 hours following treatment, therefore, the effect that MMC has on the bladder likely causes an inflammatory response.

Given the link between increased spontaneous activity and elevated prostaglandin release, use of cyclooxygenase inhibitors, may minimize or eliminate changes in spontaneous activity caused by PGE_2. Collins et al. (2009) hypothesized that prostaglandins may be released by Interstitial cells of Cajal in the bladder and found that non-selective COX inhibitors reduced spontaneous contractions significantly. In the current study the frequency of spontaneous contractions was significantly reduced by ibuprofen. Thus development of an overactive bladder phenotype in mice treated with MMC may be explained by an increase in spontaneous activity via prostaglandin production.

Any dysfunction in afferent or efferent innervation, is likely to result in inadequate emptying of the bladder on urination. Therefore, the increased urinary frequency in mice may be as a result of sensitization in bladder sensory nerves or due to changes in efferent nerve activity. Bladder dysfunction may also arise from alterations in detrusor contractile responses or

urothelial function. The ex vivo whole bladder preparation was used to assess how MMC treatment affects bladder function. Responses to the pharmacological agents carbachol, ATP and isoprenaline were not affected by treatment with MMC, nor were nerve evoked responses. However, bladder responses to EFS were increased at all frequencies in both the Saline and MMC groups treated with ibuprofen. This indicates that ibuprofen increases the intravesical pressure generated during voiding contractions and likely contributed to the smaller change in urinary frequency observed in the MMC + IBU group compared with MMC alone. The reason for this increased contraction is not entirely clear however, studies have found that prostaglandins may be key modulators in the release of urothelial mediators during cystitis (Tessier et al., 1991; Senbel, 2017). Consistent with this idea, PGE_2 has been reported to inhibit EFS-evoked contraction in rat bladders by activating cAMP-dependent K+ channels (Ruan et al., 2008). As MMC is known to cause cystitis involving production of PGE_2, it may be concluded that ibuprofen acts to minimise PGE_2 synthesis, thereby increasing EFS contractions, thus enhancing voiding contractions and reducing residual volume, and therefore voiding frequency (Pessina et al., 2001). Residual urine volume is linked to urinary frequency and urinary incontinence (Khandelwal and Kistler, 2013), which is one of many adverse effects associated with MMC treatment (Witjes, 2008). While MMC alone did not alter contractile responses to EFS, this may be due to numerous other inflammatory mediators that are not cyclo-oxygenase products also released following intravesical MMC. One such example is the inflammatory mediator bradykinin which has been shown to enhance electrically evoked contractions in the mouse bladder (Fabiyi and Brading, 2006).

Previous studies have reported histological changes to the urothelium following intravesical chemotherapy, this is not surprising given the intimate contact between the urothelium and high concentrations of cytotoxic agents (Becopoulos et al., 1991; Hou et al., 2011; Castillo et al., 2012). Here we assessed changes in urothelial function through measurement of the

urothelial mediators ATP and ACh which are known to regulate bladder function. There was no significant change in release of ATP across the treatment groups at 7-days, indicating that changes in ATP do not contribute to the voiding dysfunction evident in mice treated with MMC. Previously, we have shown that the intravesical agent doxorubicin increased release of ATP in treated porcine bladder immediately following treatment (Kang et al., 2015b). The changes in urothelial mediator release, however, may be transient, as seen in urothelial cultures treated with MMC (Kang et al., 2015a). Serosal release of ACh was significantly increased in the MMC and MMC + ibuprofen treatment groups, compared to the control group. Since ACh remained elevated in the MMC + ibuprofen group it is unlikely that this plays a substantial role in the voiding dysfunction observed in MMC treated mice. While the actions of urothelial derived Ach are not fully understood, it is known that ACh can act in two ways; to contract the detrusor muscle by acting on muscarinic receptors or by acting on afferent nerve terminals altering bladder sensation (Daly, 2007).

Animal behaviour was monitored over the 7 days following treatment, in line with ethical practice. While not an intended outcome measure, it was observed that MMC treatment did cause lethargy in the mice 24 hours after treatment with reduced locomotor activity noted, however, this recovered by 3 days following treatment. Reduced locomotor activity levels is indicative of pain in mice (Burkholder et al., 2012), which may explain the lethargy observed in the mice from the MMC treatment group is likely due to the pain experienced following MMC treatment (Elmamoun et al., 2014). Animals treated with MMC and given ibuprofen in their drinking water also experienced this effect, although to a lesser degree and were fully recovered three days' post-treatment, suggesting ibuprofen reduced the pain caused by intravesical MMC treatment.

The results presented here indicate that MMC has a negative effect on bladder function inducing increased urinary frequency in mice following treatment. MMC increased the

frequency of voiding, an effect that was associated with increased spontaneous contractile. Furthermore, this study has shown the benefits of ibuprofen in conjunction with intravesical treatment, in decreasing the spontaneous activity and voiding frequency, and enhancing nerve evoked response. In conclusion, the urological adverse effect caused by intravesical MMC can be reduced with the use of ibuprofen in standard treatment. This may bring relief to patients who undergo intravesical treatment and in turn improve their quality of life and ability to tolerate treatment.

Authorship contributions:

Project conception: Sellers, Chess-Williams, McDermott

Participated in research design: Sellers, Chess-Williams, McDermott

Conducted experiments: West, Lang

Ethics application: Sellers, Chess-Williams, McDermott

Performed data analysis: West, Lang, McDermott

Wrote or contributed to writing of the manuscript: West, Lang, Sellers, Chess-Williams, McDermott

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Legends for Figures

Figure 1: Effect of MMC and ibuprofen on cell proliferation in (A) RT4, (B) T24 and (C) UROtsa cells measured as resazurin reduction 72 hours following 2-hour MMC treatment, and (D) caspase-3 activity at 24 hours. Data represents mean ± SEM (n=5). Data analysed using one-way ANOVA with Tukey-Kramer multiple comparisons test (*** vs Control; ^^ vs MMC).

Figure 2: Voiding pattern analysis conducted in mice from Control/Saline, MMC and MMC + Ibuprofen groups measuring (A) total voided area (cm^2), (B) number of voiding events, (C) average urine spot size. Data is presented as mean ±SEM (n=6) and analysed using repeated measures two-way ANOVA with significant values indicated (*p<0.05, **p<0.01, ***p<0.001, MMC vs. Saline) (^p<0.01, MMC vs. MMC + Ibuprofen).

Figure 3: Effect of MMC and ibuprofen treatments on spontaneous phasic contractions in the isolated murine bladder. (A) Amplitude of spontaneous contractions and (B) Frequency of contractions. Data represented as mean ±SEM (n=6), analysed using one-way ANOVA with Bonferroni's multiple comparisons test (***p<0.001, MMC vs. Saline) (^p<0.05, MMC vs. Saline + IBU and MMC + IBU).

Figure 4: (A)Nerve evoked pressure responses in isolated bladders, from Saline, MMC, Saline + Ibuprofen and MMC + Ibuprofen groups to EFS at 1, 5, 10 and 20Hz. (B) Nerve mediated contractions to EFS at 20Hz in isolated bladders from Saline control group in the presence of L-NNA (100μM), atropine (1uM) and αβmATP (10μM). Responses were recorded as a change in pressure from baseline. Data are presented as the mean ±SEM (n=6). Data analysed using a (A) repeated measures two-way ANOVA and non-linear regression or (B) one-way ANOVA with Dunnet's multiple comparisons test.

Figure 5: Effect of intravesical MMC treatment and the effects of ibuprofen on the release of ATP and ACh, from isolated bladders in response to distension with saline to 20mmHg. (A and C) intraluminal release and (B and D) release into the serosal fluid. Data represented as mean ±SEM (n=6), and analysed using one-way ANOVA with Bonferroni's multiple comparisons test (* vs Saline, ^ vs Saline + IBU).

Tables

Table 1: Whole bladder responses from Saline, MMC, Saline + Ibuprofen and MMC + Ibuprofen treated mice: filling volume at 40mmHg pressure, pressure response to ATP (10mM) and KCl (60mM), pEC_{50} and maximal responses (mmHg) to carbachol and pIC_{50} and maximal responses to isoprenaline. Data presented as mean ± SEM (n=6) and analysed using One-way ANOVA with Bonferroni's multiple comparisons test.

	Saline	**MMC**	**Saline + IBU**	**MMC + IBU**
Filling volume (µL) at 40mmHg	481±74.6	513±68.1	487±40.6	513±28.1
		Pressure Response		
ATP (1mM)	1.11±0.29	2.39±0.67	2.14±0.67	1.87±0.37
KCl (60mM)	16.8±4.79	10.04±3.55	13.58±1.52	13.32±4.03
		Carbachol		
pEC_{50}	5.85 ±0.22	5.85 ±0.27	6.05 ±0.12	6.03 ±0.16
Maximal Response (mmHg)	28.44 ±6.36	23.69 ±6.09	29.45 ±3.32	28.82 ±4.56
		Isoprenaline		
pIC_{50}	7.03 ±0.33	7.46 ±0.56	7.29 ±0.22	7.05 ±0.35
Maximal Response (mmHg)	2.56 ±0.39	1.92 ±0.74	2.65 ±0.35	2.26 ±0.38

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Figures

Figure 1

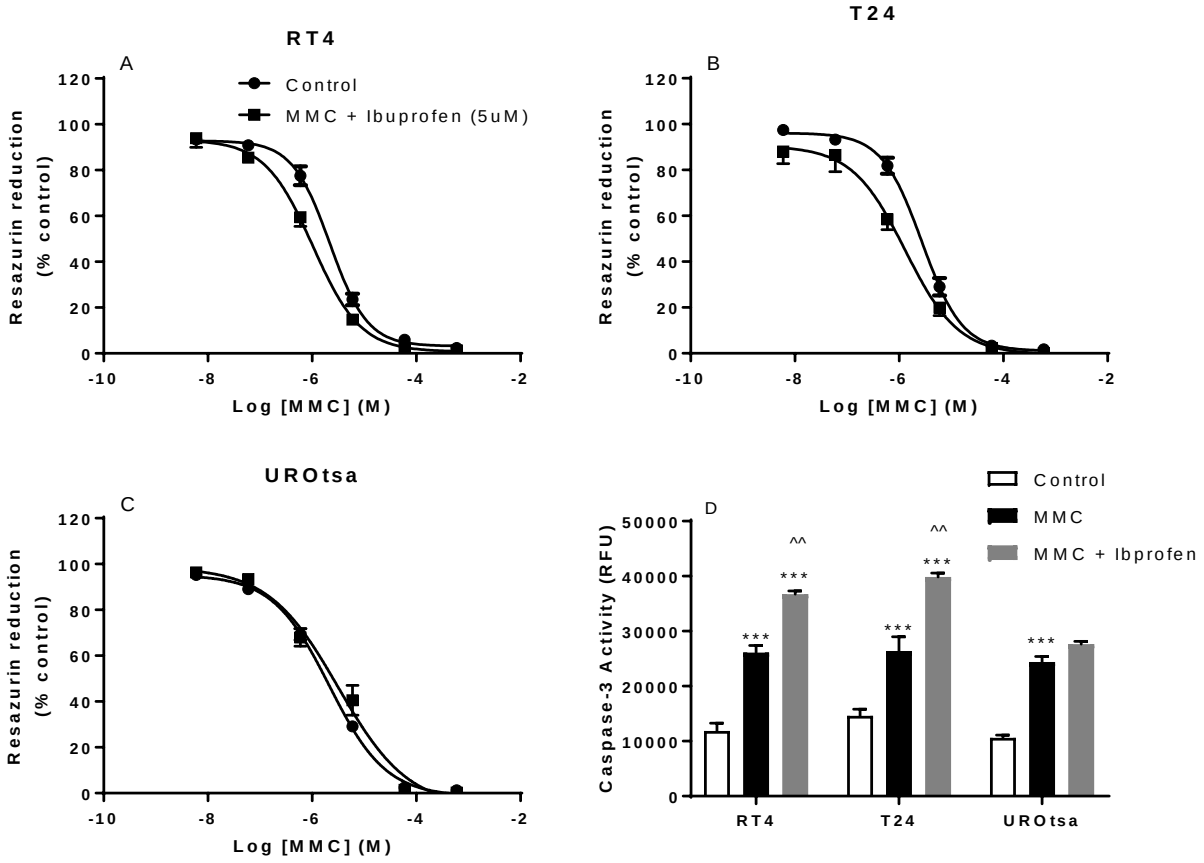

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Figure 2

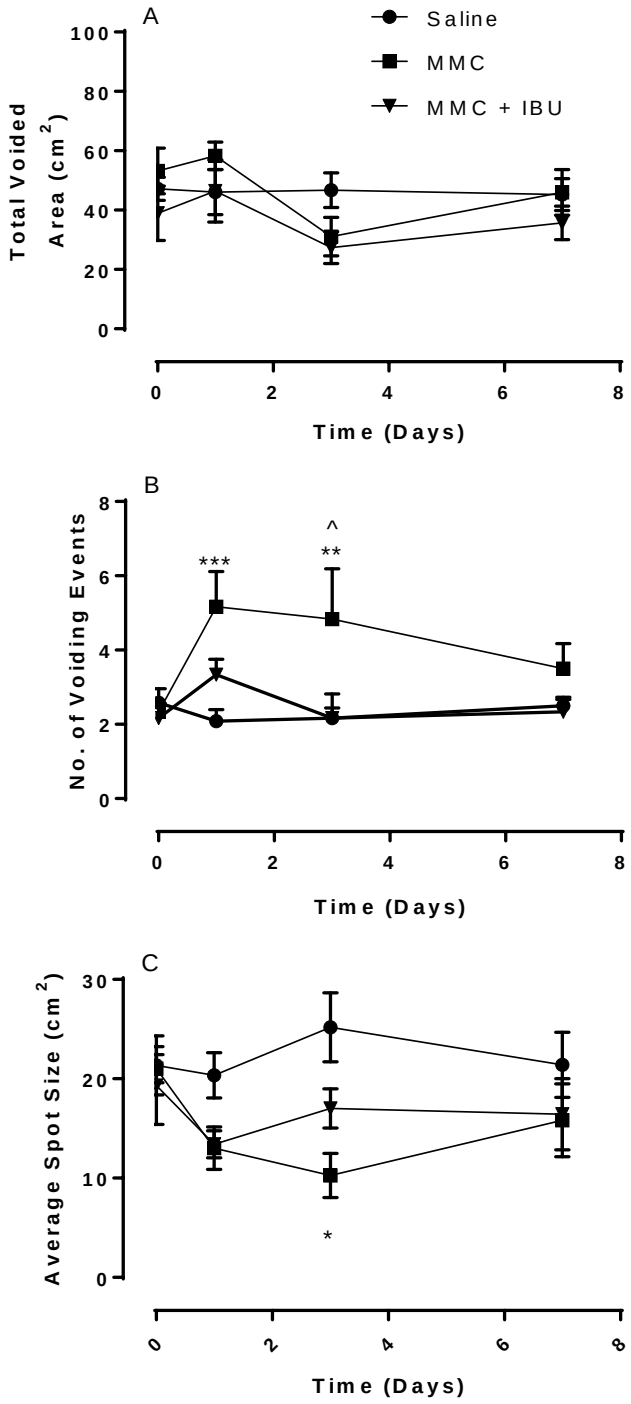

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Figure 3

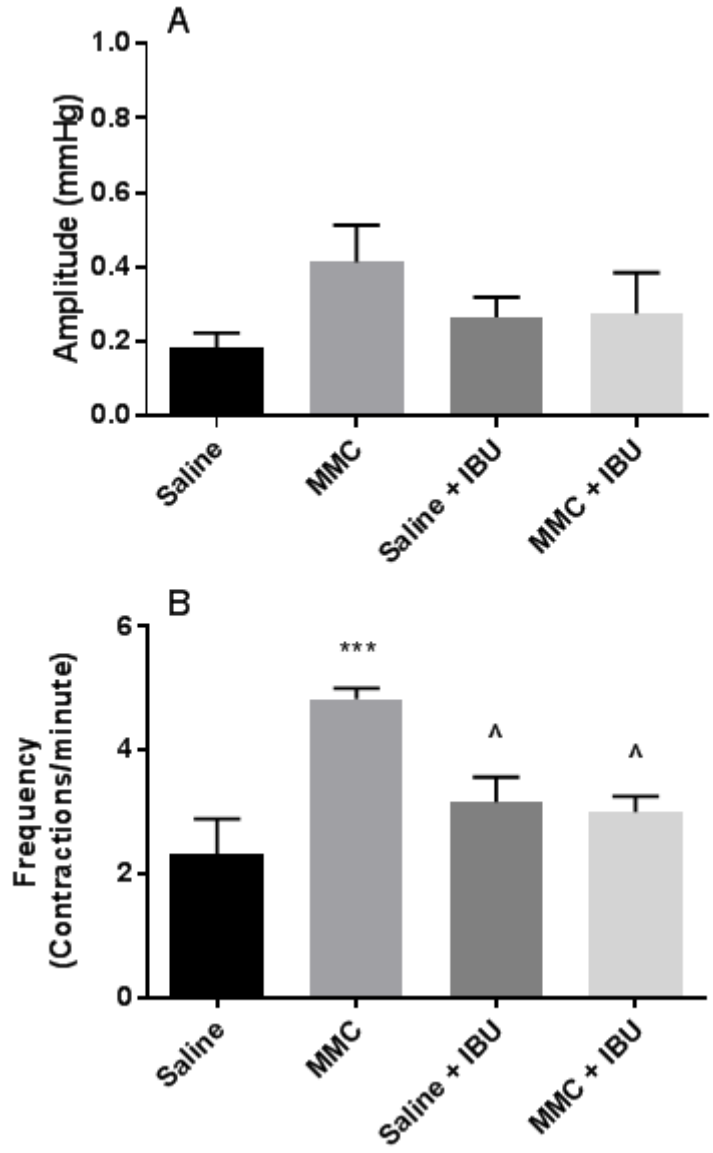

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Figure 4

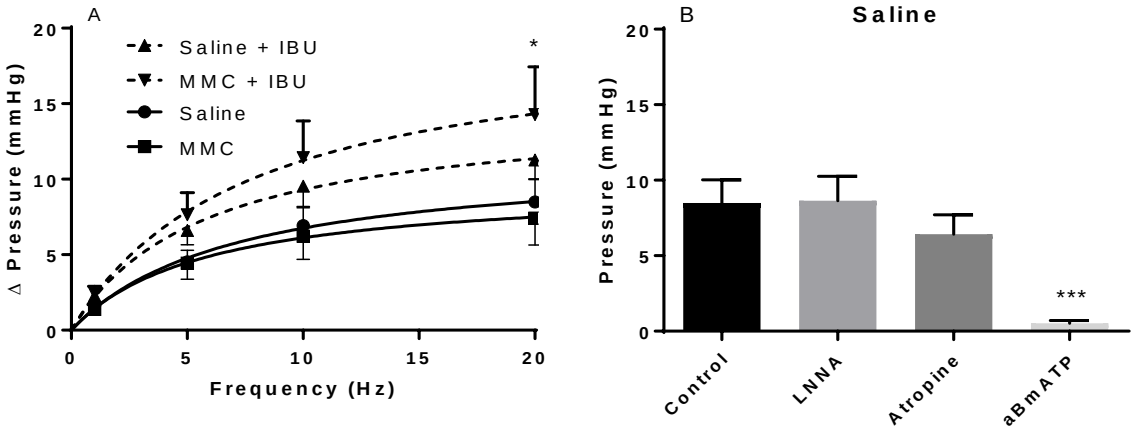

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Figure 5