Evidence of Non-Uniformity in Urothelium Barrier Function between the Upper Urinary Tract and Bladder

Nicholas A. Williams a, Luke Barnard a, *Chris J. Allender a, Jenna L. Bowen a,
Mark. Gumbleton a, Tim. Harrah b, Aditya Raja c Hrishi B. Joshi c

aSchool of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII Ave., Cardiff, UK
bDepartment of Research and Development, Boston Scientific, Urology and Women’s Health Division, Marlborough, MA 01752, USA
cDepartment of Urology, University Hospital of Wales, Heath Park, Cardiff, UK

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Corresponding author
*Chris J. Allender, PhD
Room 0.45, School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII Ave, Cardiff, UK, CF10 3NB
Tel. +44 (0)29 208 75824
Fax. +44 (0)29 208 74149
Email. allendercj@cf.ac.uk
Abstract

**Purpose:** To compare the relative permeability of the upper urinary tract and bladder urothelium to mitomycin C (MMC).

**Materials and Methods:** *Ex vivo* porcine bladder, ureters and kidneys were dissected out and filled with 1 mg ml⁻¹ MMC. Following 60 min, organs were emptied and excised tissue samples sectioned parallel to the urothelium. Sectioned tissue was homogenised and extracted MMC quantified. Transurothelial permeation across the different urothelia was calculated by normalising the total amount of drug extracted to the surface area of the tissue sample. Average MMC concentrations at different tissue depths (concentration – depth profiles) were calculated by dividing the total amount of drug recovered by the total weight of tissue.

**Results:** MMC permeation across ureteral urothelium (9.07 µg cm⁻²) was significantly greater than across bladder (0.94 µg cm⁻²) or renal pelvis urothelium (3.61 µg cm⁻²). Concentrations of MMC in the ureter and kidney were markedly higher than those achieved in the bladder at all tissue depths and average urothelial MMC concentrations were > 6.5 fold higher in the ureter and renal pelvis than the bladder.

**Conclusions:** For the first time, evidence that the upper urinary tract and bladder exhibit differing permeability to a single drug is reported. *Ex vivo* porcine ureter is significantly more permeable to MMC than bladder urothelium and consequently higher MMC tissue concentrations can be achieved after topical application. The data presented in this study correlates with the theory that mammalian upper tract urothelia represent a different cell lineage to the bladder and that they are innately more permeable to MMC.
Introduction

Urothelial carcinoma is the fourth most common tumour type and can occur in the lower or upper urinary tract. Bladder cancer accounts for 90 - 95% of all urothelial carcinoma, whilst those originating in the upper urinary tract (UTUC) represent 5 - 10%. Although rare, the incidence of UTUC has increased over the last three decades and now stands at ~ 2 cases per 100,000 person-years. Owing to restricted symptomology, disease is commonly advanced at diagnosis. Consequently prognosis is poor with an overall 5-year survival rate of < 50%. Although the urinary tract is lined by one continuous urothelium, UTUC exhibit a different pathology to those of the bladder; most importantly UTUC are significantly more aggressive and invasive.

Regardless of tumour location, European Association of Urology (EAU) guidelines state the gold standard treatment for UTUC is radical nephroureterectomy (RNU). For certain patients, endoscopic management has emerged as a new treatment option and in 2009 it accounted for > 10% of all UTUC surgical interventions in England. This conservative approach allows preservation of the kidney, whilst sparing the patient the complications and morbidity associated with major surgery. Although no randomised controlled trials comparing endoscopic management with RNU have been performed, a systematic review of oncological outcomes suggested that, for specific favourable low-grade UTUC elective cases, endoscopic management can yield effective oncological control and renal preservation. This is supported by comparable 5-year disease specific survival between immediate RNU and endoscopic management. Unfortunately these benefits come at the expense of unfavourable tumour progression, with one study reporting recurrence in 68% of the cohort. In an attempt to reduce recurrence after endoscopic management, the administration of post-operative, adjuvant topical chemotherapy with agents such as mitomycin C (MMC) and immunotherapy with Bacillus Calmette - Guérin (BCG) has been reported. The rationale behind this stems from the established efficacy of these agents in the management of bladder cancer. The efficacy of topical chemotherapy in UTUC however is not proven. The poor quality of the studies (small, retrospective studies with limited follow up and no control arms) prevents results from demonstrating unequivocal benefit. If topical drug delivery is to be of benefit in reducing the
recurrence of UTUC, then efficacious concentrations of drug must be achieved in the target tissue.

Currently, the accepted dogma is that urothelial permeability is consistent throughout the urinary tract. This is largely based on the assumption that histologically the urothelium is unchanged in the upper and lower urinary tract. To date, no study has sought to investigate the relative permeability of the bladder, ureter and renal pelvis urothelium. However evidence suggests that despite apparent histological homology, protein expression on the surface of the urothelial umbrella cells is not consistent. Given the important role the umbrella cells play in maintaining barrier function, we therefore hypothesise that this may give rise to varying transurothelial permeation at these distinct locations. This study aims to compare the relative permeability of the upper urinary tract urothelium and bladder urothelium to MMC.

Materials and Methods

**Topical instillation of MMC to isolated porcine bladder, ureter and kidney**

*En bloc* porcine urinary tracts from pigs weighing 70 - 90 kg were obtained fresh from a local abattoir within five minutes of sacrifice and immediately immersed in cold, oxygenated Krebs buffer. Working in a shallow bed of Krebs, excess perivesical fat was trimmed and the bladder, ureters and kidneys dissected out. Ureters (~10 cm) were dissected out so as to leave ~ 2 cm attached to the bladder and kidney. Organs were rinsed with saline to remove residual urine and filled using an open - ended ureteral catheter (5 Fr, 70 cm, Cook medical Inc, Bloomington, IN, USA) with MMC solution (1 mg ml\(^{-1}\) in normal saline) (mitomycin C 40 mg powder for solution for injection, Prostrakan, UK). The bladder, kidney and ureter were filled through the urethra, ureteral orifice and directly into the ureter respectively. Since the volume of the renal pelvis is variable, pre - experimental test instillations with methylene blue (1 mg ml\(^{-1}\) in normal saline) were carried out to ensure adequate contact with the renal pelvis urothelium was achieved. Post - instillation, entry orifices were sutured and the organs submerged in oxygenated Krebs maintained at 37 °C in a waterbath for 60 min. Four experiments, each representing a different *ex vivo* porcine urinary tract, were performed.
Investigating the distribution of MMC into the bladder, ureter and kidney wall

Following 60 min instillation, organs were removed, emptied and opened with a single vertical incision. To remove surface - adsorbed drug, the urothelium was thoroughly rinsed with saline. Tissue samples from areas of drug contact (observed due to purple staining conferred by MMC) were excised and their surface area measured. Tissue samples were immediately snap frozen between two metal plates using liquid nitrogen, fixed to cork mounts with OCT (Tissue - Tek™, CRYO - OCT Compound, Fisher Scientific UK Ltd, Leicestershire, UK) and the tissue sectioned using a cryostat (Leica CM3050 S, Leica Microsystems, Buckinghamshire, UK). The time between experiment end and freezing was less than 2 min. Samples were serially sectioned parallel to the urothelial surface at 50 µm thickness and sections collected in pre - weighed 1.5 ml eppendorf tubes. Two 50 µm tissue sections between 0 and 100 µm were grouped for analysis, as were the two 50 µm sections between 100 and 200 µm. Groups of six 50 µm tissue sections between 200 and 1,400 µm and groups of twelve 50 µm tissue sections between 1,400 and 7,400 µm were also grouped. For all tissues, sections were weighed, homogenised (Precellys®24, Bertin Technologies Inc, Bordeaux, France) and the drug extracted in 1 ml of mobile phase for 24 h with 10 min sonication per sample. Samples were then centrifuged (7,000 RPM, 2,680 g) and the supernatant isolated for analysis using HPLC. Average drug concentrations at different tissue depths were calculated by dividing the total amount of drug recovered by the total weight of tissue. Transurothelial permeation was calculated by normalising the total amount of drug extracted from all tissue sections to the surface area of the tissue sample.

HPLC analysis of MMC

MMC was analysed by HPLC (Kromasil C18, 5 µm, 250 mm x 4.6 mm i.d column, Supelco Inc). The mobile phase consisted of 80 % 5 mM phosphate buffer (pH 7) : 20 % acetonitrile, with UV detection at 365 nm. The injection volume was 20 µl and flow rate 1ml min⁻¹.
**Validating extraction of MMC**

Deionised water (0.5 ml) was added to the tissue homogenate of previously extracted sectioned urinary tract tissue. Samples were then immediately vortexed, centrifuged and the supernatant discarded. Ethyl acetate (0.25 ml) was added to the tissue homogenate and MMC extracted for 12 h with 10 min sonication per sample. Samples were then centrifuged, the supernatant isolated and any extracted MMC quantified by HPLC.

**Quantifying tissue layer depths of the ureter, bladder and kidney**

Samples of ureter, bladder and kidney (~ 1 cm$^2$) were taken from porcine urinary tracts excised immediately post-sacrifice on site at the abattoir. Samples were fixed, sectioned and stained with Masson’s trichrome prior to examination by light microscopy. The mean depths of the different tissue layers for the ureter, bladder and kidney were measured directly from photomicrographs using NIS ‐ Elements Basic Research imaging software (Nikon Instruments Europe B.V, Amsterdam, Netherlands).

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism version 6.0c (GraphPad Software, Inc, San Diego, California, USA). For all comparisons, one ‐ way ANOVA with Tukey’s post‐hoc test for multiple comparisons was used.

**Results**

**Topical instillation of MMC to isolated porcine bladder, ureter and kidney**

Using a ureteral catheter, 1 mg ml$^{-1}$ MMC was instilled into isolated porcine bladder, ureter and kidney. Owing to natural intra ‐ species variation, urinary organs varied in size and subsequently the volume of MMC instilled varied (Table 1). Pre ‐ experimental test instillations with methylene blue demonstrated complete exposure of the renal pelvis urothelium following filling of the kidney through the ureteral orifice (Supplementary information, Figure S1). Coincidently, MMC stained the urothelium purple making it easy to identify tissue areas exposed to drug solution (Figure 1).
Table 1. Individual organ dimensions and volumes of MMC instilled in each of the four experiments.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Weight (g)</th>
<th>Size (cm)</th>
<th>Instillation (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urinary tract 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>26.32</td>
<td>Whole</td>
<td>16.5</td>
</tr>
<tr>
<td>Ureters</td>
<td>0.87 / 1.13</td>
<td>5 / 6</td>
<td>1 / 1.9</td>
</tr>
<tr>
<td>Kidneys</td>
<td>147.5 / 151.5</td>
<td>Whole</td>
<td>8.5 / 9.5</td>
</tr>
<tr>
<td><strong>Urinary tract 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>29.13</td>
<td>Whole</td>
<td>17</td>
</tr>
<tr>
<td>Ureters</td>
<td>1.64 / 2.22</td>
<td>10 / 10</td>
<td>1.8 / 2.0</td>
</tr>
<tr>
<td>Kidneys</td>
<td>104.94 / 103.86</td>
<td>Whole</td>
<td>7.1 / 10.9</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>40.56</td>
<td>Whole</td>
<td>24.3</td>
</tr>
<tr>
<td>Ureters</td>
<td>2.24 / 2.31</td>
<td>10 / 10</td>
<td>2.25 / 2.75</td>
</tr>
<tr>
<td>Kidneys</td>
<td>159.5 / 143.9</td>
<td>Whole</td>
<td>14 / 14.5</td>
</tr>
<tr>
<td><strong>Urinary tract 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
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<td>Whole</td>
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</tr>
<tr>
<td>Ureters</td>
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<td>10 / 10</td>
<td>2 / 3.3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>145.2 / 144.5</td>
<td>Whole</td>
<td>11 / 16.25</td>
</tr>
</tbody>
</table>

Figure 1. *Ex vivo* porcine bladder (A), kidney (B) and ureter (C) following 60 min instillation of MMC (1 mg ml⁻¹). Purple staining of the tissue surface enables easy identification of drug contact areas.

**HPLC analysis of MMC**

HPLC analysis of MMC produced sharp, near-symmetrical peaks that eluted at a stable retention time (Supplementary information, Figure S2). The lower limit of detection
(LLOD) and quantification (LLOQ) was 0.003 and 0.01 µg ml⁻¹ respectively, with good sensitivity shown in homogenised tissue (Supplementary information, Figure S2 D - E).

Validating extraction of MMC

To validate the MMC extraction protocol, a second extraction in ethyl acetate was carried out on samples from each tissue type (two 50 µm sections between 100 - 200 µm tissue depth for each of the four urinary tracts). Prior to the addition of ethyl acetate, a washing step was included to remove any adsorbed MMC from the surface of the tissue homogenate. MMC is highly soluble in ethyl acetate and the solvent has been used to extract MMC from bladder tissue by other groups¹⁸. For all tissue sections the amount of MMC extracted in the secondary step was below the LLOD, suggesting complete extraction of MMC had been achieved in the analytical extraction.

Quantifying tissue layers depths of the ureter, bladder and kidney

Although the relative thickness of tissue layers within the bladder wall is well established, the upper urinary tract is less well characterised. Sections of tissue were stained with Masson's trichrome (Figure 2A - D) and tissue layer depths measured directly from photomicrographs using NIS-Elements imaging software (Figure 2D). Measurements (calculated as the distance between the top and base of the layer) were taken across the whole of the micrograph and the mean thickness of the tissue layers calculated (Table 2).
**Figure 2.** Representative photomicrographs of bladder (A), kidney (B) and ureter (C - D) sections stained with Masson’s trichrome. All samples taken from a single *ex vivo* porcine urinary tract. Example of ureteral smooth muscle layer measurements calculated with NIS - Elements imaging software (D).

**Table 2.** Tissue layer measurements for *ex vivo* porcine ureter, bladder and kidney. Values represent mean of 20 measurements for each layer from 2 whole porcine urinary tracts.
Investigating the distribution of MMC into the bladder, ureter and kidney wall

Concentration - depth profiles were constructed to examine the relative distribution of MMC in the different urinary tract tissues (Figure 3). Average concentrations of MMC in the ureter and kidney were markedly higher than those achieved in the bladder at all tissue depths investigated (Figure 3A). This was the case in each of the four experiments carried out (Figure 4). Variation in the relative proportion and composition of tissue layers of the upper and lower urinary tract makes comparison of drug concentrations in the lamina propria and detrusor muscle difficult. The urothelium of the upper and lower porcine urinary tract however is of similar size (Table 2). Average urothelial MMC concentrations were > 6.5 fold higher in the ureter and renal pelvis than the bladder (Figure 3B, 48.37, 45.00 and 6.86 µg g⁻¹ respectively).

Considering the large variation in thickness of the bladder, ureter and kidney wall (Table 2), drug recovered from the tissue was normalised to surface area allowing a comparison of permeation to be made (Figure 3C). MMC permeation across ureteral urothelium (9.07 µg cm⁻²) was significantly greater than that across bladder urothelium (0.94 µg cm⁻²) (p < 0.001). Transurothelial permeation across renal pelvis urothelium was markedly greater than bladder urothelium (3.8 fold higher) however this was not statistically significant when assessed at an alpha level of 0.05 (p = 0.08). This is likely owing to the relatively small sample size in relation to the natural variability in permeation of the urinary tracts investigated. Permeation across ureteral urothelium was significantly greater than renal pelvis urothelium (p < 0.01) and at tissue depths beyond 350 µm MMC concentrations in the ureter were greater than those in the kidney (Figure 3A).
Figure 3. A) Average concentration - depth profiles (mass of drug per gram of tissue) of MMC in ex vivo porcine bladder, ureter and kidney wall following 60 min instillation of 1 mg ml⁻¹ MMC. B) Average urothelium concentrations (calculated at 150 µm depth) following 60 min instillation of 1 mg ml⁻¹ MMC (** p < 0.01 for the ureter and kidney versus the bladder, calculated by one - way ANOVA with Tukey’s post – hoc test for multiple comparisons). C) Transurothelial permeation of MMC in ex vivo porcine bladder, ureter and kidney wall following 60 min instillation of 1 mg ml⁻¹ MMC (*** p < 0.001 for the ureter versus the bladder, ** p < 0.01 for the ureter versus the kidney, calculated by one - way ANOVA with Tukey’s post – hoc test for multiple comparisons). (For all figures, n = 4 urinary tracts ± SEM).
**Figure 4.** Concentration – depth profiles (mass of drug per gram of tissue) of MMC in *ex vivo* porcine bladder, ureter and kidney wall following 60 min instillation of 1 mg ml<sup>-1</sup> MMC. Symbols show mean ± SD of all raw data (tissue samples analysed, n = ‘Urinary Tract 1’: 6 bladder, 5 ureter and 5 kidney replicates, ‘Urinary Tract 2’: 5 bladder, 8 ureter and 5 kidney replicates, ‘Urinary Tract 3’: 4 bladder, 8 ureter and 3 kidney replicates and ‘Urinary Tract 4’: 6 bladder, 4 ureter and 5 kidney replicates respectively).

**Discussion**

There have been no reports of studies comparing the relative permeability of the upper and lower urinary tract to topically delivered drugs. MMC is one of the few drugs that is used topically to treat disease of the upper urinary tract<sup>10</sup> and bladder<sup>19</sup> and as such served as a clinically relevant exemplar to investigate transurothelial permeability at these different sites. Transurothelial permeation of MMC across porcine ureteral and renal pelvis urothelium was markedly greater than that of the bladder, although significance was only shown for the ureter. Consequently drug tissue concentrations achieved in the upper urinary tract were significantly greater than in the bladder at all
tissue depths investigated (Figure 3A, Table 3). Bladder wall concentrations of MMC reported here are similar to those reported by other groups\textsuperscript{18} (Supplementary information, Figure S3), although owing to differences in experimental design it is not reasonable to make direct comparisons. Unfortunately, no previous study has sought to investigate MMC concentrations achieved in the upper urinary tract following local delivery and therefore no ureteral or kidney are available with which to draw comparison. Indeed, this is the first report of a study comparing urothelial permeability between the upper urinary tract and bladder for any molecule.

The greater permeability of porcine ureter and renal pelvis urothelium might be explained by the relative uroplakin (UP) content of the different regions of the urinary tract\textsuperscript{16}. The urothelium forms a continual lining of the renal pelvis, ureter, bladder and proximal urethra. It was generally believed that the upper and lower urinary tracts were lined by one homogenous urothelium\textsuperscript{16}. Urothelia at these different regions are morphologically similar in terms of thickness and were presumed to perform a similar barrier function\textsuperscript{15}. Histology of the \textit{ex vivo} porcine urinary tract showed no discernable difference in the thickness of the urothelium of the upper urinary tract or bladder (Figure 4, Table 2). However recently it has been shown that, based on ultrastructure and uroplakin content, the urothelium of the mammalian urinary tract can be divided into at least three different cell lineages: renal pelvis / ureter, bladder / trigone and proximal urethra\textsuperscript{16}. Immunofluorescence and transmission electron microscopy of bovine urothelium indicates urothelial cells of the bladder contain more uroplakins than those of the ureter\textsuperscript{16}. Additionally immunoblot analysis of bovine urothelia cultured \textit{in vitro} showed the bladder to contain $\sim$ 10 times more uroplakin than either the ureter or renal pelvis. When maintained under identical \textit{in vitro} conditions, bovine urothelia from the bladder and ureter exhibited very different proliferative potential and formed morphologically distinct colonies. Conversely, \textit{in vitro} cultured urothelia from the renal pelvis showed indistinguishable growth potential from that of the ureter. Preliminary work by the same group suggested the concept of urothelial heterogeneity also extended to the monkey and human\textsuperscript{16}.

Following on from this work, Riedel \textit{et al} showed that, with respect to uroplakin composition, urothelial heterogeneity was indeed more prominent in umbrella cells of
the human ureter than those of the bladder\textsuperscript{17} (the renal pelvis was not investigated). Immunohistochemical staining revealed that 15 of the 18 ureters investigated possessed a significant subpopulation of ureteral umbrella cells lacking UPIII and UP1b. The authors concluded that the UPIII / UP1b pair may in fact be completely absent from the ureters. In comparison, only 2 of the 10 bladder samples investigated lacked UPIII and UP1B and both of these samples were taken from the ureteral orifice or its immediate surrounding (suggesting the urothelium may have been of ureteral origin). UPIII is integral to the formation of an effective urothelial barrier\textsuperscript{20}. UPIII knockout mice exhibit a more permeable urothelium demonstrated by the increased penetration of methylene blue into umbrella cells and a higher transurothelial permeability to water and urea\textsuperscript{20,21}. Similar to findings in the human ureter, UPIII knockout mice exhibit reduced production of the UPIII partner protein UP1b. It is possible the lack of the UPIII / UP1b pair in the human ureter might render human ureteral urothelium more permeable than that of the bladder. Interestingly the authors also found that umbrella cell-associated cytokeratin 20 (CK20), an additional marker of urothelial differentiation\textsuperscript{22}, showed a more extended expression among umbrella cells of the bladder than among those of the ureter. Evidence therefore suggests that mammalian ureteral urothelium is less differentiated than that of the bladder. Although the UP content of the human renal pelvis was not investigated, bovine data\textsuperscript{16} and evidence that the renal pelvis and ureter are from the same cell lineage suggests it may exhibit a similar heterogeneity and increased permeability.

It should be pointed out that uroplakin expression studies have not been carried out in the pig. Urothelial heterogeneity is suggested to be an explanation for the results observed in this study based on results from other mammalian species in the literature as discussed. Furthermore pigs are established and well characterised models of the human urinary tract\textsuperscript{23–25} exhibiting similar physiology\textsuperscript{26–28}, tissue structure and composition to that of humans\textsuperscript{29–31}. In addition to the presence of UPs, the umbrella cells of the urothelium poses intercellular tight junctions (TJ) and a glycosaminoglycan (GAG) layer both of which are believed to increase the impermeability of the urothelium\textsuperscript{32–34}. Investigating the relative TJ protein expression and GAG density in the lower and upper urinary tract will yield further information regarding the observed difference in permeability and such work is encouraged.
Conclusions
For the first time, evidence that the upper urinary tract and bladder exhibit differing permeability to a single drug is reported. Ex vivo porcine ureter is significantly more permeable to MMC than bladder urothelium and consequently higher MMC tissue concentrations can be achieved after topical application. The renal pelvis was also found to be markedly more permeable, although significance was not achieved. The data presented in this study correlates with the theory that the mammalian renal pelvis and ureter represent a different cell lineage to the bladder and that they are innately more permeable. A less differentiated urothelium may have no major functional consequences for the ureter or renal pelvis as, in comparison to the bladder, upper tract urothelia have less barrier requirements (lower intraluminal pressure, less distension and storage requirements) and therefore the presence of fully functional uroplakins may not be essential\(^\text{17}\). However there may be distinct advantages when considering the topical administration of drug to the upper urinary tract. Increased urothelial permeability to chemotherapeutics such as MMC would potentially allow higher drug concentrations to be achieved in the ureteral and kidney wall. Unfortunately, unlike bladder UC\(^\text{35–38}\), to our knowledge MMC concentrations necessary to effectively treat UTUC have not been established. Nonetheless, if delivered to the upper urinary tract in an effective manner, the increased permeability of the ureter and renal pelvis urothelium could have important ramifications for the conservative treatment of UTUC.

Acknowledgments
The authors would like to thank Derek Scarborough for his excellent technical assistance.
References


Supplementary Information

S.1. Test instillations with methylene blue

S 1. Porcine kidney cross-section following instillation of methylene blue (1 mg ml⁻¹) via the ureteral orifice. The technique results in complete exposure of the renal pelvis urothelium to the instilled solution.
**S.2. HPLC analysis of MMC**

S 2. Example HPLC chromatograms showing the analysis of MMC calibration standards (A-C) and drug recovered from bladder (D) and renal pelvis (E) tissue samples near the LLOQ.
S.3. Comparison of MMC bladder wall concentrations determined in this study and those reported in the literature

S 3. Comparison of MMC concentration - depth profiles in the bladder wall from this study (blue symbols, 60 min instillation of 1 mg ml⁻¹ MMC to ex vivo porcine bladder) and that of Wientjes et al¹ (purple symbols, 60 - 120 min instillation of 0.5 mg ml⁻¹ MMC to in vivo human bladder). Figure A shows mean values ± SD and figure B shows median values from the same data. (n = 4 bladders ± SD and 7 bladders ± SD for this work and that of Wientjes et al respectively).
References