

## HAMLET Treatment Delays Bladder Cancer Development

Ann-Kristin Mossberg, Yuchuan Hou, Majlis Svensson, Bo Holmqvist and Catharina Svanborg\*

From the Institute of Laboratory Medicine, Department of Microbiology, Immunology and Glycobiology (AKM, YH, MS, CS) and Institute for Clinical Sciences, Department of Oncology, Lund University (BH), Lund, Sweden, Department of Urology, First Hospital of Jilin University (YH), Changchun, Republic of China, and Singapore Immunology Network, A\*STAR (CS), Republic of Singapore

### Abbreviations and Acronyms

BCG = bacillus Calmette-Guerin  
ELISA = enzyme-linked immunosorbent assay  
FCS = fetal calf serum  
HAMLET = human  $\alpha$ -lactalbumin made lethal to tumor cells  
HRTEC = human renal tubular epithelial cells  
mCXCL = macrophage inflammatory protein  
PBS = phosphate buffered saline  
PFA = paraformaldehyde  
TUNEL = terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

**Purpose:** HAMLET is a protein-lipid complex that kills different types of cancer cells. Recently we observed a rapid reduction in human bladder cancer size after intravesical HAMLET treatment. In this study we evaluated the therapeutic effect of HAMLET in the mouse MB49 bladder carcinoma model.

**Materials and Methods:** Bladder tumors were established by intravesical injection of MB49 cells into poly L-lysine treated bladders of C57BL/6 mice. Treatment groups received repeat intravesical HAMLET instillations and controls received  $\alpha$ -lactalbumin or phosphate buffer. Effects of HAMLET on tumor size and putative apoptotic effects were analyzed in bladder tissue sections. Whole body imaging was used to study HAMLET distribution in tumor bearing mice compared to healthy bladder tissue.

**Results:** HAMLET caused a dose dependent decrease in MB49 cell viability in vitro. Five intravesical HAMLET instillations significantly decreased tumor size and delayed development in vivo compared to controls. TUNEL staining revealed selective apoptotic effects in tumor areas but not in adjacent healthy bladder tissue. On in vivo imaging Alexa-HAMLET was retained for more than 24 hours in the bladder of tumor bearing mice but not in tumor-free bladders or in tumor bearing mice that received Alexa- $\alpha$ -lactalbumin.

**Conclusions:** Results show that HAMLET is active as a tumoricidal agent and suggest that topical HAMLET administration may delay bladder cancer development.

**Key Words:** urinary bladder; carcinoma; HAMLET complex, human; lactalbumin; mice

TRANSITIONAL cell carcinoma is a common urological malignancy whose growth may be restricted to the mucosa or that may invade locally into submucosal tissues. In rare cases tumors spread beyond the urinary tract and metastasize, and these invasive tumors require cystectomy and chemotherapy.<sup>1,2</sup> Tumors confined to the mucosa are treated topically with BCG bacteria intravesical instillation or surgery followed by cytostatic drugs. BCG treatment results in prolonged tumor-free periods and delayed tumor progression but is also associated

with severe side effects, especially in immunocompromised individuals.<sup>3,4</sup> Thus, less toxic drugs with more tumor specific properties are needed.

HAMLET is a complex of  $\alpha$ -lactalbumin and oleic acid that kills a wide range of tumor cell lines in in vitro conditions. Embryonic cells are intermediately sensitive to HAMLET but healthy differentiated cells tested to date remained viable.<sup>5,6</sup> The therapeutic potential of HAMLET was established in vivo in patients with skin papilloma. Topically applied HAMLET caused a

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\* Correspondence: Singapore Immunology Network, A\*STAR, 8A Biomedical Grove, 04-06, Republic of Singapore 138648 (telephone: +65-64070072; FAX: +65-64642057; e-mail: catharina\_svanborg@immunol.a-star.edu.sg).

significant reduction in lesion size.<sup>7</sup> Local infusion of HAMLET into rat brains with invasively growing human glioblastoma xenografts delayed tumor development and prolonged survival.<sup>8</sup> Apoptosis was mainly confined to the tumor area, although HAMLET diffused throughout the infused hemisphere.

In patients with superficial bladder cancer HAMLET instillations recently reduced tumor size and caused apoptosis-like death of tumor cells but the study was not designed to examine a therapeutic effect.<sup>9</sup> Thus, we evaluated the therapeutic effects of HAMLET for bladder carcinoma in a murine bladder cancer model.<sup>10,11</sup> Results suggest that HAMLET delays tumor growth and cell death mainly occurs in tumor tissue.

## MATERIALS AND METHODS

HAMLET was produced as previously described,<sup>6,12</sup> lyophilized and frozen in aliquots (1.7 mM in PBS) for in vivo instillation. For imaging HAMLET and native  $\alpha$ -lactalbumin were labeled with Alexa Fluor® 568. We used RPMI 1640 medium, FCS, nonessential amino acids, sodium pyruvate (PAA Laboratories, Pasching, Austria), poly L-lysine, oleic acid (Sigma®), Phenyl-Sepharose® chromatography matrix and DEAE TrisAcryl-M (BioSeptra, Villeneuve la Garenne Cedex, France). Versen was prepared by adding 0.5 mM ethylenediaminetetraacetic acid to PBS (pH 7.2).

### Cell Lines and Primary Cells

We used MB49 cells and HRTEC from renal biopsies.<sup>13</sup> Biopsy was done according to the Helsinki Declaration after obtaining written informed consent. The protocol was approved by the Lund University medical ethics committee, Lund, Sweden.

### In Vitro Cell Death Assay

MB49 cells cultured in RPMI 1640 supplemented with nonessential amino acids, sodium pyruvate and 10% FCS were harvested, suspended in RPMI 1640 without FCS (1 ml,  $1 \times 10^6$ /ml), seeded into 24-well plates and exposed to HAMLET or native  $\alpha$ -lactalbumin (7 to 42  $\mu$ M) for 1 hour at 37°C in 5% CO<sub>2</sub> before FCS (10%) was added. After an additional 2-hour incubation cell viability was determined by trypan blue exclusion. Apoptotic cells were identified using the TUNEL assay (Roche, Basel, Switzerland) by fluorescence microscopy using LSM META 510 software (Carl Zeiss, Jena, Germany). For morphological studies MB49 cells were cultured overnight on poly-L-lysine coated slides (Nalge® Nunc™), washed, exposed to HAMLET or native  $\alpha$ -lactalbumin as described and fixed in PFA.

### Bladder Cancer Model

C57BL/6 female mice weighing approximately 20 gm were bred at the department of laboratory medicine and used at ages 6 to 12 weeks. For intravesical instillation mice were anesthetized by intraperitoneal injection of pentobarbital (40 to 60 mg/kg) supported by isoflurane inhalation. MB49 tumors were established as described previously.<sup>10</sup> Briefly, on day 0 the bladder was emptied and preconditioned by intravesical instillation of 100  $\mu$ l poly-L-lysine solution (0.1 mg/ml) through a soft polyethylene catheter (Clay Adams, Parsippany, New Jersey) with an outer di-

ameter of 0.61 mm for 30 minutes before MB49 tumor cells ( $1 \times 10^5$  in 100  $\mu$ l PBS) were instilled. Five HAMLET or PBS instillations (100  $\mu$ l each) were done at indicated intervals. Mice remained under anesthesia on preheated blocks with the catheter in place to prolong tumor exposure to HAMLET (approximately 1 hour). Groups of 5 mice each were sacrificed at each time point, and bladders were weighed and processed for histology. Mice were weighed at the start of the experiment and after sacrifice. The study was approved by the animal ethics committee, Lund District Court, Lund, Sweden (M30-06).

Urine samples for cytokine analysis were obtained on day 0 and before each instillation. A previously described mCXCL2/3 response to mucosal challenge in the urinary tract<sup>14</sup> was selected to monitor the mucosal response to catheterization mechanics and HAMLET instillation. Urine mCXCL2/3 was quantified by the Quantikine® ELISA kit. Urine was collected by gentle pressure on the mouse abdomen and kept frozen at  $-20^\circ\text{C}$  until analyzed. Urine was used undiluted when possible, or diluted 1/5 or 1/10 depending on collected volume. Concentrations in pg/ml were calculated from ELISA absorbance values using a standard curve and adjusted by the dilution factor before statistical analysis. Approximately half of the 96 samples were run twice and mean values were used for analysis.

### Tissue Staining and Histology

For cryosectioning PFA fixed bladders were immersed in 10%, 15% and 25% sucrose until saturation, embedded in TissueTek® and snap frozen. Cryosections (10  $\mu$ m) made with a Microm HM500M (Microm Microtech, Francheville, France) were mounted on poly-L-lysine coated glass slides. Tissues and glass slides were stored at  $-80^\circ\text{C}$  until use. For paraffin sectioning PFA fixed tissues were dehydrated in alcohol-xylene and paraffin embedded. Serial sections (4 to 5  $\mu$ m) made with a Microm HM 355 (Microm International, Thermo Scientific, Walldorf, Germany) were stained with hematoxylin and eosin or processed for TUNEL staining.

To determine tumor size bladders were serially sectioned. In sections from the area with the largest tumor the tumor size was calculated as the percent of estimated total bladder area, rated as no or tumor less than 10% (+), 10% to 40% (++) , or 40% or greater (+++). Three investigators scored the sections independently and the median was used for statistical analysis.

### Whole Body Imaging

The distribution of Alexa Fluor 568 labeled HAMLET or  $\alpha$ -lactalbumin was monitored in vivo by fluorescence imaging using a CRi Maestro™ GNIR system for 500 to 950 nm imaging. Fluor 568 emission was defined by spectral unmixing using CRi Maestro software. Animals were then sacrificed. Bladders were surgically exposed and the Alexa signal was recorded before the bladders were harvested and processed for histological examination of tissue fluorescence. Histological overviews were obtained with a fully motorized DotSlide slide scanner system equipped for epifluorescence with a UPlan Super Apochromat 20 $\times$  lens with a numerical aperture of 0.75, a CC12 charge coupled device color camera and OlyVIA software (Olympus®). For detailed analysis we used epifluorescence with an AX60, DP 70

charge coupled device camera (Olympus). The same filter sets with a theoretical excitation maximum at 580 nm and an emission maximum at 620 nm were used in each microscope.

### Statistical Analysis

Treatment groups were compared using the unpaired *t* and chi-square tests. We compared mCXCL2/3 concentrations with Dunn's multiple comparison test (nonparametric ANOVA). Other parameters were compared with InStat® 3 and Excel®.

## RESULTS

### HAMLET Killed MB49 Cells In Vitro

To ensure that MB49 cells were sensitive to HAMLET the cell death response was examined in vitro (fig. 1). Cells incubated with HAMLET died with an LD<sub>50</sub> of 14  $\mu$ M at 3 hours (fig. 1, A). Cells changed morphology and rounded with a decrease in size (fig. 1, B). Dying cells showed evidence of DNA damage on TUNEL staining (fig. 1, C). In contrast, differentiated HRTEC were not killed by HAMLET and showed no positive TUNEL staining (fig. 1, D). Native  $\alpha$ -lactalbumin had no tumoricidal effect (fig. 1, A).

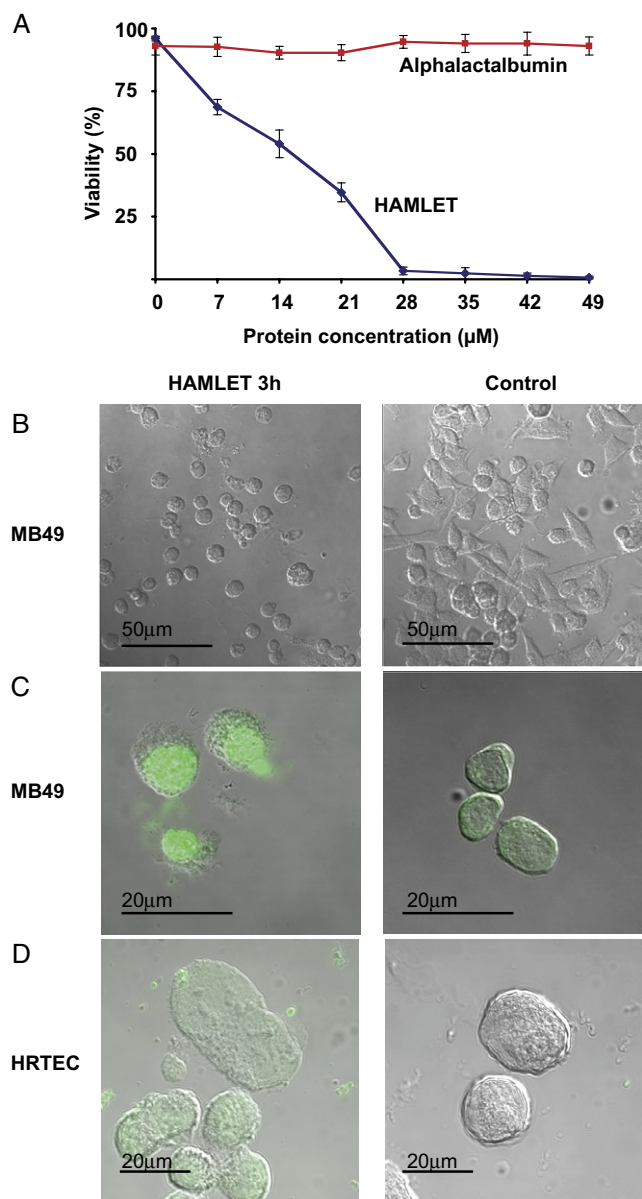
### Tumor Development

Tumors were established in mice by intravesical instillation of MB49 cells and mice were sacrificed in groups of 3 at regular intervals to follow tumor progression. Small tumors were detected after 3 days and volume increased further until day 14 (fig. 2, A). In most animals tumors grew with a distinct border to surrounding healthy tissue but with a characteristic lack of organization, and heterogeneity in size and chromatin structure (fig. 2, B). Tumors were identified by histology and tumor size was estimated as a percent of total bladder area (fig. 2, C).

There was no significant change in whole animal weight with tumor progression (data not shown). Due to substantial variation bladder weight was not considered a useful end point (fig. 2, D). Hematuria was more common in tumor bearing mice but did not correlate with tumor size ( $p > 0.001$ , for the entire data set, data not shown).

### HAMLET Effect on Tumor Development

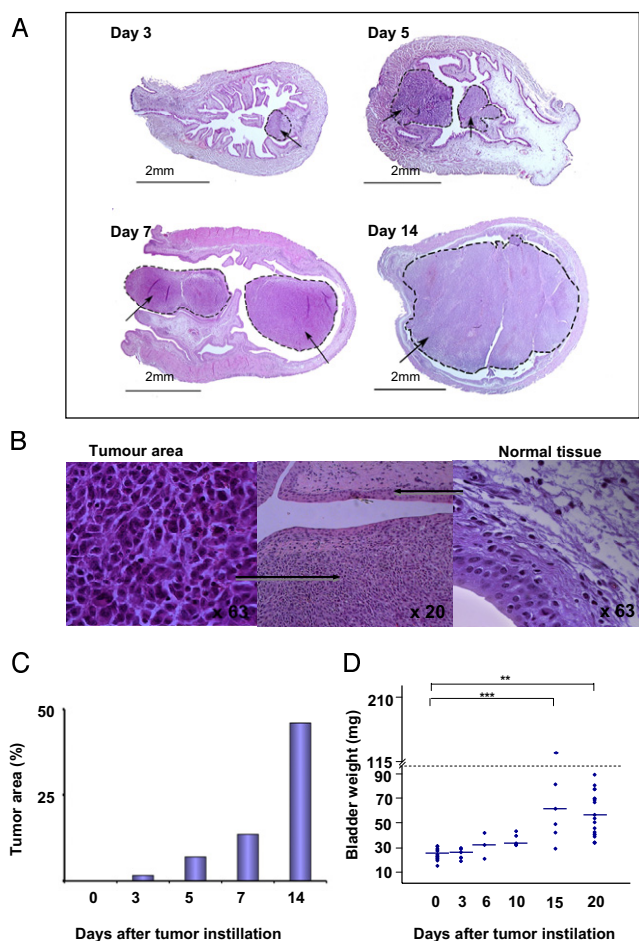
To examine the HAMLET therapeutic effect tumor cells were installed on day 0, followed by 5 intravesical instillations of HAMLET or PBS (fig. 3, A). In a pilot study groups of 5 mice each were given instillations on days 1, 3, 5, 7 and 9, and groups of 3 to 5 mice each were sacrificed on days 6, 10 and 16. This HAMLET treatment protocol significantly delayed tumor development ( $p < 0.05$ , fig. 3, A). Figure 3, A shows examples of the difference in tumor size between HAMLET treated mice and controls in hematoxylin and eosin stained sections with tumor marked by a dotted line.



**Figure 1.** Effect of HAMLET on MB49 cells in vitro. *A*, dose dependent MB49 cell death in response to 3-hour (3h) HAMLET instillation, quantified by trypan blue exclusion. Same concentrations of native  $\alpha$ -lactalbumin did not induce cell death. *B*, MB49 on poly-L-lysine coated slides shows morphological changes and detachment after treatment with 14  $\mu$ M HAMLET compared to control cells. MB49 cells rounded up and detached, showing changed morphology, but healthy cells did not respond. *C*, TUNEL staining of MB49 tumor cells after 3-hour 14  $\mu$ M HAMLET treatment. Native  $\alpha$ -lactalbumin served as negative control. *D*, lack of TUNEL staining in HAMLET treated healthy HRTEC.

To increase HAMLET treatment efficiency the protocol was adjusted (fig. 3, B). To increase exposure time we used the 0.7  $\times$  19 mm Neoflon™ catheter, which remained inside the bladder to prevent voiding and HAMLET loss. Groups of 5 mice each were given instillations on days 1, 2, 3, 5 and 7, and





**Figure 2.** MB49 tumor development. *A*, tumor size increase from tumor cell instillation to sacrifice on day 14. Dotted line indicates tumor area. *B*, bladder sections show examples of tumor and healthy tissue. H & E. *C*, tumor size quantification using H & E stained bladders sections at different time points. Size was estimated as percent of total bladder area in same section. *D*, bladder weight was examined as tool to measure tumor progression.

sacrificed on day 8. The therapeutic effect of HAMLET was confirmed with a more pronounced difference in tumor development ( $p < 0.02$ ). HAMLET treated mice lacked detectable tumors ( $\pm$ ) more often than controls (33% vs 0%,  $p < 0.02$ , *fig. 3, B*) and tumors were significantly reduced (mean score 1.9 vs 2.5,  $p < 0.02$ ) vs controls. Results suggest that HAMLET significantly delays bladder cancer progression by restricting tumor growth.

#### HAMLET Bladder Tumor Uptake in Vivo

HAMLET tumor uptake and retention were investigated in tumor bearing and tumor-free mice by intravesical instillation of Alexa Fluor labeled HAMLET or  $\alpha$ -lactalbumin 8 days after tumor cell instillation. Whole body fluorescence imaging showed that Alexa-HAMLET was retained in the bladder of tumor bearing mice for at least 2 days (*fig. 4, A*). Retention was

tumor specific since there was no fluorescence signal in controls that received Alexa-HAMLET (*fig. 4, A*). Tumor bearing mice receiving Alexa- $\alpha$ -lactalbumin did not show a positive fluorescence signal after 2 days (*fig. 4, A*).

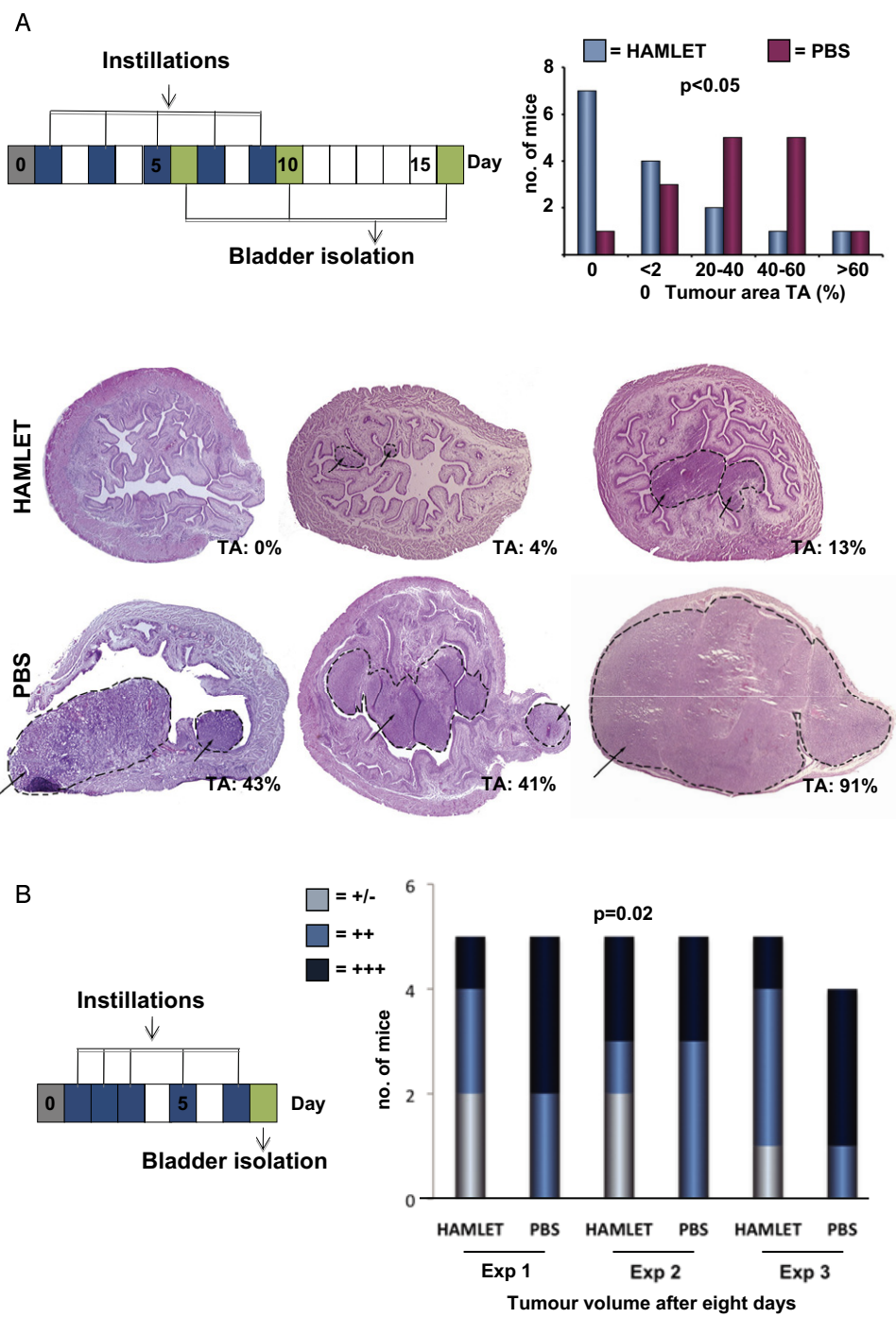
To further localize Alexa-HAMLET tumor bearing mice were sacrificed 4 hours after Alexa-HAMLET instillation. Bladders were surgically exposed and fluorescence signals were recorded by whole body imaging. There was a strong HAMLET signal in tumor bearing mice but no significant fluorescence signal in controls that received Alexa-HAMLET (*fig. 4, B*). To study the distribution of Alexa Fluor labeled HAMLET bladder tissue sections were obtained and scanned by fluorescence microscopy (*fig. 4, C*). A strong Alexa Fluor signal was detected just under the epithelium and distributed throughout the bladder (*fig. 4, D*). In contrast, staining was low or undetectable in sections from the nontumor bearing mouse exposed to Alexa-HAMLET (*fig. 4, C*). Results suggest that bladder tissue with MB49 tumor cells took up and retained HAMLET more efficiently than healthy bladder tissue.

#### Cell Death Evidence in HAMLET Treated Tumors

A human bladder cancer study showed evidence of DNA damage in tumor sections but not in healthy tissue.<sup>9</sup> To examine whether HAMLET caused a similar response in the murine model frozen bladder sections from tumor and nontumor bearing mice 1 day after HAMLET instillation were subjected to TUNEL staining (*fig. 5*). TUNEL positive cells were abundant in 4 of 5 mice and confined to the tumor area (*fig. 5, A*). In contrast, there was no evidence of TUNEL staining in healthy mice treated with HAMLET (*fig. 5, C*). *Figure 5, B* shows TUNEL positive tumor tissue adjacent to areas containing healthy TUNEL negative mucosal tissue.

#### Innate Immune Response to HAMLET Instillation

The human study showed a perivascular TUNEL positive neutrophil infiltrate, suggesting that HAMLET instillation may trigger an innate immune response.<sup>9</sup> To examine whether the instillation process, the HAMLET complex or tumor growth may trigger mucosal inflammation mCXCL2/3 was quantified by ELISA in urine samples from tumor bearing mice and controls inoculated with HAMLET or PBS (*fig. 6*). mCXCL2/3 concentrations were significantly higher in tumor bearing than in healthy mice ( $p < 0.0001$ ) but there was no response to the catheterization procedure. There was a higher mCXCR2/3 response to HAMLET than PBS in controls ( $p < 0.04$ ) but not in tumor bearing mice ( $p$  not significant).



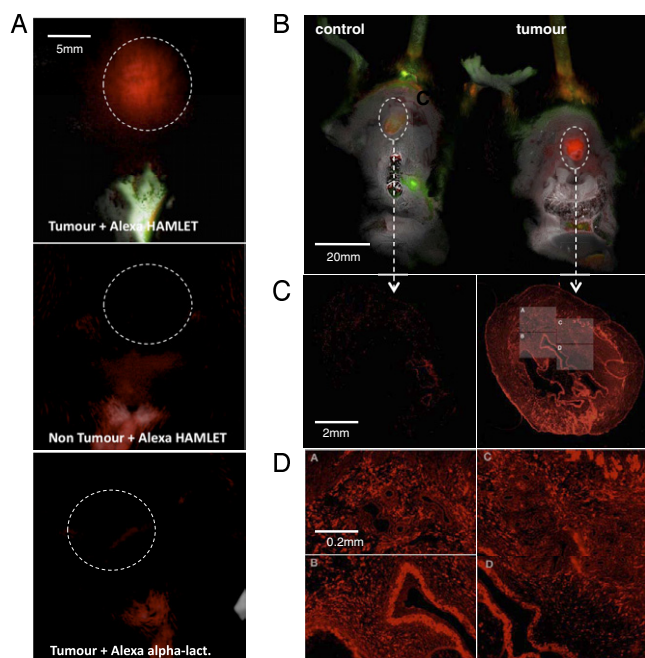
**Figure 3.** Delayed tumor growth after HAMLET treatment. *A*, pilot study experimental design shows timing of tumor cell inoculation and repeat intravesical HAMLET instillations. Gray box indicates tumor cell instillation. Blue boxes indicate day of HAMLET or PBS instillation. Green boxes indicate day of sacrifice. Tissue sections reveal difference in tumor development in mice receiving HAMLET or PBS. Tumor area (TA) is shown as percent of total bladder area. *B*, modified experimental design and difference in tumor progression between HAMLET treated mice and controls. *Exp*, experiment.

**DISCUSSION**

Superficial papillary tumors are often removed by transurethral resection but the recurrence rate is high.<sup>2</sup> Intravesical instillation of antitumor agents is common in patients with bladder cancer using various topical treatments, including BCG, thiotepa,

epirubicin and mitomycin. BCG may result in a recurrence-free interval of at least 2 years in about 70% of treated patients but side effects are common.<sup>15,16</sup> Our results show in a mouse tumor model that HAMLET is retained in tumor tissue, killing tumor cells and delaying tumor growth. Together





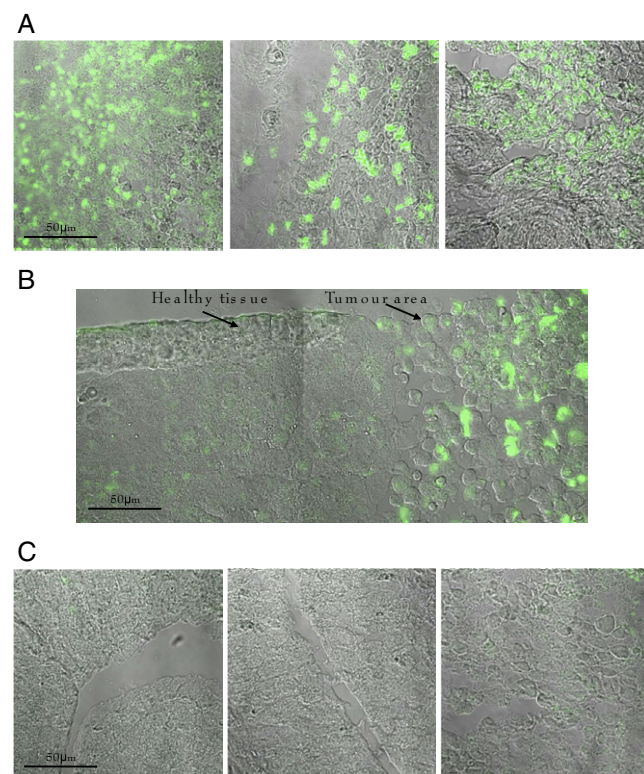
**Figure 4.** Bladder tumor HAMLET retention. *A*, Alexa signal over bladder area detected by whole body imaging (dotted circle) in tumor bearing mice instilled with Alexa-HAMLET. No signal was detected in tumor-free mouse or in tumor bearing mouse instilled with Alexa- $\alpha$ -lactalbumin (*alpha-lact.*). Images were captured 2 days after instillation. *B*, whole body imaging shows Alexa-HAMLET retention in surgically exposed bladder (dotted circle) of tumor bearing mouse but not in healthy mouse bladder. Mice were sacrificed 4 hours after instillation. *C*, Alexa-HAMLET in tissue sections of tumor bearing and healthy mice. *D*, different bladder areas show HAMLET uptake and spread to different parts of tumor bearing bladder.

with absent side effects these findings suggest that HAMLET may have potential as a topical agent for bladder cancer.

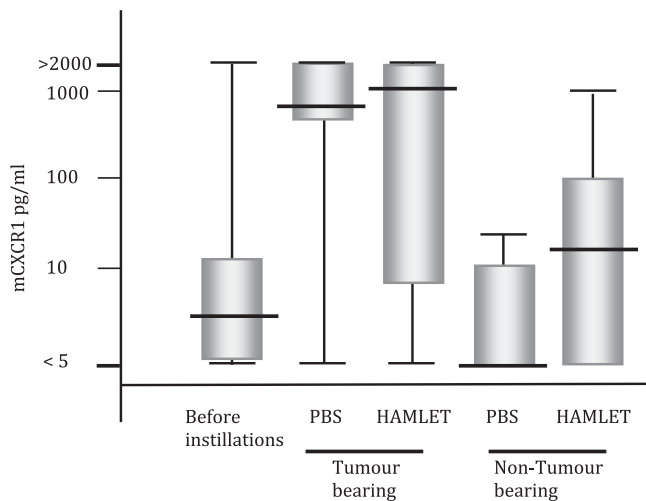
Mouse models are essential to assess the efficacy of bladder cancer therapy and the MB49 model is a useful candidate.<sup>17,18</sup> The MB49 bladder carcinoma cell line was originally isolated from a carcinogen induced bladder tumor in C57BL/6 mice.<sup>11</sup> Cells grow rapidly and establish a local mass within a week. MB49 tumors mimic the growth of human infiltrating urothelial carcinoma that invades beyond the lamina propria.<sup>19</sup> We only addressed local tumor growth and the window for topical treatment was narrow since MB49 tumors were visible after 3 days. Tumor size estimates are difficult since specific markers are lacking and previous study in this model has usually relied on bladder weight or survival.<sup>18,20</sup> We increased the accuracy of tumor size estimates by measuring visible tumor relative to the entire bladder area in serial sections. Since disseminating tumor cells are not visible, this technique probably underestimates tumor size. Based on these measurements HAMLET instillation reduced tumor

growth but the effect was partial and more than half of the treated animals had some remaining tumor. This may reflect partial inactivation of the instilled HAMLET complex by serum since the tumor and frequent catheterization may cause leakage of serum components into the bladder.

In vitro studies show that many carcinoma cell lines are sensitive to HAMLET and undergo a rapid death response. Leukemia cells are most sensitive but cells of different solid tumors as well as bladder tumors are killed by HAMLET in vitro.<sup>5</sup> In our study the MB49 cell line died in response to HAMLET with kinetics and morphological changes similar to those of other cancer cell lines tested. However, healthy differentiated pediatric kidney cells were resistant to the HAMLET challenge. This difference in sensitivity was also observed in vivo since bladder sections from the tumor area were positive for TUNEL staining while healthy tissue did not respond. These results confirm observations previously made in the clinical study of a marked difference in TUNEL positive cells between biopsies of healthy tissue and tumor sections.<sup>9</sup>



**Figure 5.** TUNEL staining (green) of tissue sections of HAMLET treated, tumor bearing mice and controls. *A*, sections from 3 tumor bearing mice show TUNEL staining in tumor areas after 5 HAMLET instillations in 10 days. *B*, combined sections from tumor bearing mouse reveal TUNEL staining in tumor adjacent to lumen but no staining in adjacent, healthy tissue from same animal. *C*, sections from 3 tumor-free mice showed no TUNEL staining after HAMLET instillation.



**Figure 6.** Urine mCXCL2/3 was determined on day 0 and before each HAMLET or PBS instillation. There was significant difference between mice with vs without tumor burden ( $p < 0.0001$ ) and HAMLET instilled mice vs controls ( $p < 0.04$ ).

The broad antitumor activity of HAMLET is unusual and suggests that highly conserved cell death pathways are activated.<sup>21</sup> However, the cell death mechanisms are not fully understood. HAMLET interacts with several cellular targets, including histones, proteasome subunits and endoplasmic reticulum stress sensors (Storm et al, unpublished data).<sup>21,22</sup> Thus, HAMLET differs from many small molecule agonists that activate single death response pathways.<sup>23</sup> Healthy differentiated cells do not internalize HAMLET to the same extent in vitro and transcriptomic studies suggest that healthy cells respond to HAMLET with innate immunity, including interleukin-6 and CXCL8 (Storm et al, unpublished data). Consistent with these observations in

our study HAMLET instillation appeared to cause inflammation based on urine mCXCR2/3 levels. Inflammation reflected tumor growth, confirming previous studies.<sup>14</sup>

Alexa-HAMLET was retained by bladder tumors but not by healthy bladder tissue. Similar enrichment of HAMLET in tumors coupled to diffusion was previously noted in the rat glioblastoma xenograft model, in which diffusion throughout the entire hemisphere was observed after convection enhanced delivery of radio labeled HAMLET into the striatum.<sup>8</sup> These observations suggest that HAMLET may also trace and kill invasive tumor cells, which has considerable therapeutic interest. The properties of the complex in complex tissues, and the mechanisms of HAMLET diffusion and apparent selectivity must be further investigated.

## CONCLUSIONS

HAMLET delayed bladder cancer progression and there is evidence for tumor selectivity. While HAMLET is not 100% specific for malignant cells, our results mark another step toward further targeting with decreased negative effects in healthy tissue. Results also confirm previous suggestions that HAMLET is a useful tool to better understand conserved death pathways in tumor cells.

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