Intravesical Chemotherapy of High-Grade Bladder Cancer with HTI-286, A Synthetic analogue of the Marine Sponge Product Hemiasterlin

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Abstract

Purpose: HTI-286 is a fully synthetic analogue of the natural tripeptide hemiasterlin that inhibits tubulin polymerization and has strong cytotoxic potential. In this study, we evaluate the inhibitory effects of HTI-286 on human bladder cancer growth, both in vitro and as an intravesical agent in an orthotopic murine model.

Experimental Design: Various bladder cancer cell lines were treated with HTI-286 and mitomycin C (MMC) in vitro. Human KU-7 bladder tumor cells that stably express firefly luciferase were inoculated in female nude mice by intravesical instillation and quantified using bioluminescence imaging. Mice with established KU-7-luc tumors were given HTI-286 or MMC intravesically twice a week for 2 h. Pharmacokinetic data was obtained using high-performance liquid chromatography – mass spectrometry analyses.

Results: In vitro, HTI-286 was a potent inhibitor of proliferation in all tested cell lines and induced marked increases in apoptosis of KU-7-luc cells even after brief exposure. In vivo, HTI-286 significantly delayed cancer growth of bladder tumors in a dose-dependent fashion. HTI-286, at a concentration of 0.2 mg/mL, had comparable strong cytotoxicity as 2.0 mg/mL of MMC. The estimated systemic bioavailability of intravesically given HTI-286 was 1.5% to 2.1% of the initial dose.

Conclusions: Intravesical HTI-286 instillation therapy showed promising antitumor activity and minimal toxicity in an orthotopic mouse model of high-grade bladder cancer. These findings provide preclinical proof-of-principle for HTI-286 as an intravesical therapy for nonmuscle-invasive bladder cancer and warrant further evaluation of efficacy and safety in early-phase clinical trials.
patients eventually progress because of clonal selection of therapy-resistant cells or the development of cells with a drug resistant phenotype (9). Thus, there have been great efforts to identify novel antimicrotubule agents that overcome taxane resistance. Experimental compounds in clinical trials include novel taxanes, epothilones, and peptide-like agents. HTI-286 is a fully synthetic analogue of the naturally occurring tripeptide hemiasterlin (10, 11). HTI-286 binds to the tubulin heterodimer at a unique site that seems to be in the interface of the tubulin subunits and is close to but distinct from the Vinca binding site (12, 13). In contrast to the taxanes, HTI-286 depolymerizes rather than stabilizes existing microtubules at high stoichiometric amounts (14). More importantly, however, at low concentrations all of these compounds interfere with spindle-microtubule dynamics (7, 15). HTI-286 has been described as a poor substrate for P-glycoprotein and, consequently, exhibited reduced multidrug resistance (16, 17). This peptide-like agent is composed of sterically congested amino acids that confer stability to the molecule, thus providing good bioavailability. Because of its excellent systemic efficacy and relatively high molecular weight of 473.7 g/mol and because intravesical instillation allows for optimal delivery of therapeutic agents with minimized systemic toxicity, we hypothesized that HTI-286 has promising potential as intravesical therapy for noninvasive bladder cancer. We found that HTI-286 markedly inhibited tumor growth of all tested bladder cancer cell lines in vitro and orthotopic KU-7-luc tumors in vivo. Moreover, using high-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis, we observed good bladder tissue drug concentrations with only little absorption through the bladder wall into the bloodstream. Directly compared with the current standard intravesical chemotherapy [2 mg/mL of mitomycin C (MMC)], 0.2 mg/mL HTI-286 showed equal efficacy in vivo. These findings provide preclinical proof-of-principle for further evaluation of HTI-286 as an intravesical agent and make it a rational candidate for phase I trials in patients with refractory nonmuscle-invasive bladder carcinoma.

Materials and Methods

Cell lines, vectors, and compounds. The human bladder cancer cell lines RT4 and UM-UC3 were purchased from the American Type Culture Collection. Cells were maintained in McCoy’s medium (Invi
trogen) containing 10% heat-inactivated fetal bovine serum and kept at 37°C in a humidified 5% CO2 atmosphere. MGH-U3 cells were obtained as a generous gift from Dr. Y. Fredet (L’Hôtel-Dieu de Québec) and maintained in MEM supplemented with 10% fetal bovine serum and 2 mmol/L l-glutamine (Invitrogen; ref. 18). The KU-7 cell line was kindly provided by Dr. M. Tachibana (Keio University; ref. 19). These cells were maintained in DMEM containing 5% fetal bovine serum and used for orthotopic model development. KU-7 cells were infected with a lentivirus containing the firefly luciferase gene by Dr. Craig Logsdon (Promega). Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy
dphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was combined with phena
drazine methosulfate in a ratio of 20:1. This mixture was then added to the plates in a 1:5 ratio with media and incubated for 2 h at 37°C. The absorbance was read at 490 nm using a microplate reader (Becton Dickinson Labware), and absorbance values were normalized to DMSO or untreated controls. Each experiment was done with six technical replicates and repeated at least thrice.

Cell cycle analysis. Flow cytometric analysis of propidium iodide–stained cells was done to determine their DNA profile for cell cycle analysis and to quantify apoptotic rates by detection of cells with sub-G0 DNA content as previously reported (21, 22). Briefly, KI-7 cells were plated in 10-cm dishes and treated at 50% confluence for 2 h with different concentrations of HTI-286 or MMC control, then washed and incubated in drug-free medium. One to three days later, cells were trypsinized to produce a single-cell suspension and fixed with 70% ethanol. The next day, cells were washed with phosphate-citrate buffer to elute fragmented low–molecular weight DNA and incubated with RNase A (0.5 mg/mL) at 37°C for 30 min. Subsequently, cells were stained on ice with 1 mL of 50 µg/mL propidium iodide (Sigma), a fluorescent dye that intercalates double-stranded DNA. Relative DNA content was then analyzed on a dual laser flow cytometer (Beckman Coulter Epics Elite; Beckman). Each assay was repeated thrice.

Orthotopic murine model of bladder cancer. The intravesical model was a modification of the techniques described by Ahlering et al. (23) and Watanabe et al. (24). Eight-week-old female nude mice (Harlan) were anesthetized with isoflurane. A superficial 6/0 polypropylene purse-string suture was placed around the urethral meatus before a lubricated 24-gauge felco angiocatheter (Medex Medical) was passed through the urethra into the bladder. After a single irrigation of the bladder with 100 µL PBS, two million KU-7-luc cells were instilled as single-cell suspension in 50 µL, and the purse-string suture was tied down for a 2.5-h period, during which the mice were kept anesthetized. To quantify in vivo tumor burden, animals were then imaged on days 4, 8, 12, 16, and 21 with an IVIS200 Imaging System (Xenogen; see below).

Necropsy was done after 3 weeks. The whole bladders were removed, fixed in 10% buffered formalin, and embedded in paraffin. At least one 5-µm section was obtained from each specimen and stained with H&E using standard techniques (Vector Laboratories). All slides were reviewed by a pathologist (L.F.) and were scanned on a BLISS workstation at magnification of 20× (Bacus Laboratories). Animal procedures were done according to the guidelines of the Canadian Council on Animal Care.

Bioluminescence imaging. Both KU-7-luc cells and murine xenografts were imaged using an IVIS200 camera (Xenogen). Cultured cells were imaged in black, clear-bottomed 96-well plates (BD Falcon) 15 min after addition of D-luciferin to the growth medium (0.15 mg/mL; Xenogen). To measure orthotopic tumors, mice were injected i.p. with 150 mg/kg luciferin, anesthetized with isoflurane 5 min later, and imaged in the supine position exactly 15 min after injection. Data was acquired and analyzed using Living Image software version 2.50 (Xenogen).
In vitro bioluminescence of KU-7-luc cells. Cells were serially diluted 1:2 starting with 2 × 10^6 cells per well. Luciferin (0.15 mg/mL) was added to the wells the next day, and 15 min thereafter, the plate was imaged for 30 s in an IVIS200 system. Experiments were done in quadruplicate. Bioluminescence per well (±SE) was quantified in photons per second and correlated to the cell number per well. Efficacy of HTI-286 (C) and MMC (D) after continuous or short-term exposure. KU-7-luc cells were treated with different concentrations of HTI-286 or MMC for either 72 or 2 h, respectively. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays were done after 72 h. Representative assays of three experiments with six technical replicates. D. Effects of short-term exposure to HTI-286 on the cell cycle were analyzed by flow cytometry of propidium iodide-stained cells harvested after 24 or 72 h of growth. D. KU-7-luc cells at 50% confluence were treated with different concentrations of HTI-286 for 2 h, and the fractional DNA content was assessed 1 d later. The graph shows significant dose-dependent increases in the G2-M cell cycle phase and corresponding decreases in G0-G1 at higher dose levels (*, *P < 0.01). E. KU-7-luc cells were exposed to HTI-286 for 2 h, and flow cytometry was done after 3 d. The graph shows dose-dependent increases in the apoptotic sub-G1 phase (*, *P < 0.01). Data presented are the mean of three independent experiments.

In vivo efficacy studies. Two separate experiments were done on a total of 50 mice. After quantitation of tumor burden 4 days after the initial tumor inoculation, the first set of 25 mice was randomized for treatment with either vehicle control, low dose (0.01 mg/mL), or high dose (0.05-0.125 mg/mL) HTI-286 prepared in PBS, respectively. Levels of bioluminescence were equivalent among the experimental groups. Intravesical chemotherapy was given twice weekly starting on day 4 in a similar fashion to the above-described method for tumor inoculation. The chemotherapy volume instilled was 75 µL per mouse, and the purse-string suture was removed after 2 h. Likewise, the second efficacy study consisted of three groups, which were treated with 50 µL of 2 mg/mL MMC, 0.2 mg/mL HTI-286, or control, respectively.

HPLC-MS analysis. To determine the extent of systemic absorption of intravesically given HTI-286 and bladder tissue drug levels, a total of 10 mice were instilled with 75 µL of 0.01 mg/mL, 0.05 mg/mL, or 0.125 mg/mL HTI-286. To prevent urination, a purse-string suture was removed after 2 h. Likewise, the second efficacy study consisted of three groups, which were treated with 50 µL of 2 mg/mL MMC, 0.2 mg/mL HTI-286, or control, respectively.

Separation was carried out with an Exterra C18 column (3.5 µm, 50 mm × 2.1 mm; Waters) with a 20% to 95% methanol gradient from 0.5 to 5 min at 0.25 mL/min, followed by 100% methanol for 4 min and reequilibration at starting conditions for 5 min. Formic acid (0.05%) was present in the gradient and reequilibration segments, and column temperature was 30°C. Luteolin (Sigma), a flavonoid with similar retention time to HTI-286, was chosen as internal standard to normalize for extraction variances or potential ion suppression (25). Retention times for HTI-286 and luteolin were both ~7.7 min, and multireaction monitoring in ES+ mode was used for the quantitative analysis. M/z 474>162 and 474>303 transitions provided the best sensitivity for HTI-286, and m/z 287>135 and 287>153 were optimal for luteolin. Collision energies were 30, 15, 33, and 33 V, respectively, and cone voltages were 45 V for HTI-286 and 27 V for luteolin. Capillary voltage was set at 3 kV, source and desolvation temperature were 100 and 300°C, respectively; desolvation gas (500 L/h), multiplier voltage (650 V), and data were collected at unit resolution. No interference between HTI-286 and luteolin was observed. Calibration samples were prepared from 1 to 1,000 ng/mL with 200 ng/mL luteolin present in each standard. Peak areas (AUC) for both HTI-286 multireaction monitorings were summed, and the ratio versus summed luteolin AUCs was used to generate calibration curves using QuanLynx (Waters). 1/X weighing was used to fit calibration data resulting in R^2 of >0.98 and deviations of <15%. RMS S/N for 1 ng/mL was >1,000.

A stock solution of 2 µg/mL luteolin was used as an internal standard for sample extractions. Serum samples were extracted by addition of 200 µL acetonitrile to 100 µL serum and 12.5 µL internal standard, vortexing and centrifuging at 15,000 × g for 5 min. Supernatant (250 µL)
was dried (Centrivap Concentrator, Labconco), reconstituted in 100 μL 20% methanol, and centrifuged at 15,000 × g for 5 min, and the clarified extract transferred to LC vials. Bladders were weighed in tared 10-mL round-bottomed tubes, 0.5 mL water were added, and samples were homogenized for 30 s with a PowerGen Model 125 homogenizer (Fisher Scientific). Acetonitrile (1.0 mL) was added to the homogenized bladders; samples were vortexed well, transferred to Eppendorf tubes, and centrifuged at 15,000 × g for 5 min. Supernatant (50 μL) and internal standard (20 μL) were then added to 130 μL water in LC vials. Urine samples were simply diluted by addition of 20 μL of urine and 100 μL of internal standard to 880 μL water. Inject volumes of 50 μL were used for all samples. Recoveries from spiked samples were >90%, and no ion suppression was evident.

Statistical analyses. All results are expressed as means ± SE. The mean values, corresponding SEs, and curve fitting were calculated using Prism 4.03 (GraphPad Software). Data were analyzed by one-way ANOVA and two-sided t-tests using InStat 3.06 (GraphPad). P values of <0.05 were considered statistically significant. Regression plots were used to describe the relationship between bioluminescence and cell number.

Results

In vitro efficacy of HTI-286 and MMC. The inhibitory effects of HTI-286 and MMC on bladder cancer cell growth were examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium proliferation assays. Both low-grade (RT4, MGH-U3) and high-grade (UM-UC3, KU-7) urothelial carcinoma cell lines were used. Cells were grown for 72 h in the presence of either compound, then viability was assessed, and the concentration of drug producing 50% inhibition (IC50) was estimated from the dose-response curves. HTI-286 was a potent inhibitor of proliferation in all tested cell lines at low nanomolar concentrations (Table 1). The mean IC50 combining all cell lines was 4.0 ± 2.1 nmol/L, which is in agreement with previously published results obtained in 18 human tumor cell lines (16). The RT4 cells required slightly higher concentrations of HTI-286, probably related to their multilayered mosaic-like growth pattern. Moreover, this cell line represents the most well-differentiated tumor (26), and it has been shown that primary human hepatocytes were much less sensitive to HTI-286 than hepatic neoplastic cells (27). MMC also showed good efficacy across all cell lines at doses comparable with those reported previously and with higher IC50 values for more rapidly proliferating cells (28, 29).

Recently, we have validated an orthotopic murine model of bladder cancer in which tumor burden is longitudinally quantified using bioluminescence imaging (30). In this model, we use KU-7 bladder cancer cells, because this cell line has high tumor take rates, grows efficiently and reliably, is reproducible, and does not need secondary agents, such as trypsin or electrocautery, that traumatize the urothelium before tumor inoculation. To enable bioluminescent imaging, parental KU-7 cells were infected with a lentivirus containing the firefly luciferase gene (20). The resultant population was expanded, and the level of bioluminescence was determined in vitro using an IVIS200 system. Figure 1A shows a representative experiment demonstrating strong correlation between light emitted from the cells and cell number. Although luciferase activity of the cell line could theoretically decrease with time, during routine in vitro testing over >1 year, there has been no detectable loss of luciferase activity (data not shown).

Next, we sought to evaluate the effects of short-term versus long-term exposure of KU-7-luc cells to HTI-286 or MMC (Fig. 1B and C). A 2-h incubation period was chosen to mimic standard clinically used intravesical treatment protocols, as well as previous studies by Au et al. (31, 32). Compared with 72 h of continuous exposure, the IC50 for 2 h of HTI-286 treatment increased from 2.0 nmol/L to a concentration of 38 ± 4.9 nmol/L, as assessed after 3 days of growth in normal medium. Similarly, the IC50 of MMC rose from 484 nmol/L to 5.8 ± 1.2 μmol/L.

Effects of HTI-286 on cell cycle distribution. Antimicrotubule agents arrest cells in mitosis and induce apoptosis (7, 16). To quantify these effects after brief exposure to HTI-286, KU-7 cells were treated for 2 h with different concentrations of drug or DMSO vehicle control and flow cytometry of propidium iodide–stained cells was done after 24 and 72 h of growth in normal medium. As shown in Fig. 1D, 24 h after a brief 2-h treatment, HTI-286 induced a dose-dependent increase in cells accumulated in mitosis (G2-M). Approximately, 60% of the entire cell population was detected in this phase of the cell cycle when exposed to 2 h of 50 or 100 nmol/L HTI-286. Thereafter, the cells underwent concentration-dependent apoptosis (Fig. 1E).

In vivo efficacy of HTI-286 in orthotopic xenografts. In vivo studies were done in a total of 60 mice to test the efficacy of HTI-286 and MMC as intravesical agents, as well as to characterize HTI-286 uptake in and through the bladder wall. Of the 50 mice in which we transurethrally inoculated KU-7-luc tumor cells, three showed evidence of tumor spread to the kidneys and only one had a urethral but no bladder tumor. Therefore, only 46 mice were randomly selected to receive intravesical treatment with HTI-286, MMC, or vehicle control. While the low-dose HTI-286 treatment in the first experiment was kept constant at 0.01 mg/mL (21 μmol/L), we increased the HTI-286 concentration in the high-dose arm from 0.05 (twice) to 0.125 mg/mL (264 μmol/L) for the final four administrations. The resulting amount of HTI-286 per mouse per treatment was still approximately four times lower than the concentrations currently used in systemic animal studies (1.5 mg/kg). The DMSO control solution was adjusted in correspondence to the high-dose treatment group and contained maximally 1.25% DMSO in PBS. As early as on day 8, the control mice progressed significantly faster than HTI-286–treated mice (P = 0.034 between control and high-dose treatment group; Fig. 2A). After 2 weeks, both treatment groups themselves differed significantly (P = 0.033). At the end of the

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**Table 1.** IC50 of HTI-286 and MMC in bladder cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 ± SE (nmol/L)</th>
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<tbody>
<tr>
<td></td>
<td>HTI-286</td>
</tr>
<tr>
<td>MGH-U3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>RT4</td>
<td>10.2 ± 1.3</td>
</tr>
<tr>
<td>UM-UC3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>KU-7</td>
<td>2.0 ± 0.5</td>
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NOTE: Data shown are the mean of three to five experiments with six technical replicates.
In an IVIS200 camera.* therapy was given biweekly. Tumorgrowth was determined on days 8, 12, 16, and low-dose (0.01 mg/mL), or high-dose (0.05-0.125 mg/mL) HTI-286. Intravesical tumor cells on day 0 and randomized on day 4 to receive either vehicle control, HTI-286 or 2.0 mg/mL MMC or vehicle control. Tumor bioluminescence was quantified on days 8, 12, 16, and 21.* P HTI-286 or 2.0 mg/mL MMC or vehicle control. Twenty-two female nude mice were intravesically inoculated with KU-7-luc tumor cells on day 0 and treated twice weekly starting on day 4 with either 0.2 mg/mL HTI-286 or 2.0 mg/mL MMC or vehicle control. Tumor bioluminescence was quantified on days 8, 12, 16, and 21.* P 0.05 between both treatment arms and the control group, but not between HTI-286 and MMC groups.

FIG. 2. A. effects of HTI-286 on growth of orthotopic bladder cancer xenografts. Twenty-four female nude mice were intravesically inoculated with 2 × 10⁶ KU-7-luc tumor cells on day 0 and randomized on day 4 to receive either vehicle control, low-dose (0.01 mg/mL), or high-dose (0.05-0.125 mg/mL) HTI-286. Intravesical therapy was given biweekly. Tumor growth was determined on days 8, 12, 16, and 21 in an IVIS200 camera.*

Pharmacokinetic HPLC-MS analyses. To elucidate the pharmacokinetic properties of intravesically given HTI-286, we next instilled 10 mice with three different concentrations of the agent. Four mice received 75 μL of the low-dose treatment (0.01 mg/mL), whereas one mouse was given our initial high-dose treatment solution of 0.05 mg/mL and five were given the later dose of 0.125 mg/mL. Two hours after treatment, HTI-286 concentrations in urine, bladder tissue, and serum were measured by an HPLC-MS assay developed in-house and are shown in Table 2. The included estimation of the percentage of the initial HTI-286 dose was based on measured urine volumes, bladder weights, and the assumption of a volume of distribution of 2.0 L/kg (33). Mean drug concentrations in bladder tissues were dependent on the initial dosing solution and were between 35% and 15% of the final urine concentration, indicating that HTI-286 does partition across the urothelium. Serum concentrations of HTI-286 were several orders of magnitude lower than the concentrations in urine and bladder tissues, and the estimated systemic bioavailability was between 1.5% and 2.1%. Overall recovery of HTI-286 was around 75%. This mass balance is slightly lower than previous reports of intravesical paclitaxel in dogs (34), which is mainly caused by loss of drug during experimental procedures, e.g. the six washes of excised mouse bladder tissues that included some residual urine.

In accordance with an estimated half-life of 8.4 h, all serum samples of those mice treated with HTI-286 in the above pharmacodynamic in vivo experiment and sacrificed 3 days after their last instillation had HTI-286 levels below our detection limit of 10 pg/mL.

In vivo comparison of HTI-286 and MMC. Our last experiment consisted of 22 mice which were randomized into three treatment groups with equal tumor burden to receive high-dose HTI-286, MMC, or control. Based on in vivo data evaluating other more toxic drug candidates and the effects of different instillation volumes on systemic toxicity (data not shown), we decided to decrease the intravesical instillation volume from 75 to 50 μL. However, to maintain the amount of HTI-286 given to each mouse, we therefore correspondingly increased its concentration from 0.125 mg/mL (0.26 mmol/L) to 0.2 mg/mL (0.42 mmol/L). For MMC, we chose to apply the currently recommended concentration of 2 mg/mL (35). At these dose levels, both compounds were well tolerated. Compared with control mice, both MMC and HTI-286 significantly inhibited bladder tumor growth (Fig. 2B). There was no statistically significant difference between HTI-286 and MMC except on the very first measurement after beginning therapy (day 8) where HTI-286 showed some superiority over MMC (P = 0.012). This difference might be explained by a more rapid onset of cytotoxic activity of HTI-286. After 3 weeks, tumor growth in both treatment arms was inhibited by 84% (HTI-286) and 99% (MMC). In the MMC treatment arm, there were 38% complete responses, whereas in the HTI-286 group, 29% of mice showed no evidence of disease on final pathology. Compared with the first set of in vivo experiments, the tumors in this experiment were in general slightly smaller, which was most likely related to counting variability on the day of tumor inoculation. In accordance with results from the phase III MMC efficacy trial by Au et al. (35), our HTI-286 data suggest that smaller instillation volumes with higher drug concentration result in greater inhibition of tumor growth (84% in Fig. 2B versus 76% in Fig. 2A). However, this assumption disregards the dose escalation in the high-dose treatment arm of the first experiment, as well as their elevated levels of bioluminescence.

Discussion

We believe that orthotopic implantation of human bladder cancer cells into immunodeficient mice remains the best model available to elucidate human bladder cancer biology and to assess the effects of intravesical anticancer therapy in vivo. Transgenic models are equally important, offering some advantages but with shortcomings as well. Although they allow...
the study of tumor growth after the mutation of a single gene, they are, as with syngeneic models, murine instead of human tumors and require a significant investment of time and resources to establish (36). Using both histologic evaluation and magnetic resonance imaging, we have recently validated an orthotopic mouse model in which luciferase expressing KU-7 bladder cancer cells are transurethrally inoculated (30). KU-7 are high-grade urothelial carcinoma cells whose biological behavior is not necessarily typical of noninvasive disease, but the key characteristic of the model is lack of deeper invasion at early time points, which makes these tumors amenable to intravesical therapy. Bioluminescence has previously been used for monitoring bladder cancer (37–40), but, to our knowledge, we were the first to thoroughly validate bioluminescence imaging in this setting (30), and this study is the first to use it to compare treatment efficacy of novel versus established intravesical chemotherapeutic agents. However, the lack of an immune response in our model has to be acknowledged, and therefore, head-to-head comparisons against BCG as the most potent intravesical therapy to date are not possible. Because bioluminescence imaging allows longitudinal surveillance of single mice, there are two limitations to our murine model of bladder cancer worth mentioning. We have observed that catheter insertion and intravesical pressure induced by the purse-string suture or manual emptying of the bladders can trigger episodes of hematuria and sometimes even tumor loss in some animals. Therefore, proper Sham-treated controls are of vital importance. Secondly, in our chemotherapy-treated groups, a small cohort of animals responded only weakly to intravesical therapy. This is most likely caused by tumor growth within the bladder wall lacking contact to the bladder lumen itself (Fig. 3B). To further optimize bladder tumor models, we, like other groups, continue to search for the “ideal” cell line that combines reliable tumor take with true superficial growth.

BCG is the most effective regimen for bladder cancer prophylaxis (6) but causes irritative voiding symptoms and, less frequently, systemic adverse events, including life-threatening BCG sepsis. In a rapidly aging population, definitive surgical treatment for recurrent high-risk bladder cancer is often not feasible or desirable for the patient. Furthermore, patients who do not tolerate or respond to BCG represent a significant challenge. Therefore, development of novel intravesical therapies for noninvasive bladder cancer is essential. HTI-286 is a fully synthetic analogue of the marine sponge product hemiasterlin that has shown promise in several preclinical studies (10, 16, 17, 27, 41). Similar to docetaxel, which is currently being evaluated as an intravesical agent for refractory superficial bladder cancer (8), HTI-286 targets microtubules and leads to

Fig. 3. Histologic images of KU-7-luc tumors. A, microscopic appearance of a representative control-treated mouse bladder 3 wk after KU-7-luc tumor instillation. After H&E staining of a whole bladder section, this slide shows multifocal and aggressive pT1 disease. Magnification, 1.25× on a BLISS microscope imaging workstation (Bacus Laboratories). B, KU-7-luc tumors grow partially below the urothelial lining. Magnification, 10×. C, zoomed image of the interface between tumor and muscularis propria, excluding muscular invasion at early time points. Magnification, 20×. D, high-power view demonstrating hyperchromasia and mitotic figures within KU-7-luc tumors. Magnification, 20×. E, muscle-invasive neoplasm 1 mo after KU-7-luc tumor cell inoculation in an untreated pilot mouse. Magnification, 10×. F, representative histologic image of normal urothelium of a mouse from the high-dose HTI-286 treatment arm (Figs. 2A and 4). Magnification, 10×.
growth arrest and apoptosis (16). A potential advantage for HTI-286, however, is that it is less susceptible to the development of transport-based treatment-resistance than taxanes (16). In vitro, HTI-286 showed strong antitumor activity at low nanomolar doses across all cell lines tested (Table 1). Whereas MMC required slightly higher doses in rapidly proliferating cell lines (28), HTI-286 was, in contrast, more effective in these than in well-differentiated RT4 cells. Even brief exposure to the drug led to growth arrest and subsequent apoptosis in KU-7 cells (Fig. 1D and E). In vivo, HTI-286 significantly inhibited bladder cancer growth of KU-7-luc xenografts in a dose-dependent fashion (Fig. 2A). At a dose level of 0.2 mg/mL, HTI-286 exhibited a similar antitumor activity to 2 mg/mL MMC (Fig. 2B). After 3 weeks, there was no statistically significant difference between both compounds. We note that bweekly treatment with MMC differs from the established weekly regimen, but within our mouse model, tumors grow very quickly and become muscle-invasive after ~1 month (Fig. 3E), necessitating a more frequent dosing schedule. A precise biologically equivalent dosing of HTI-286 and MMC cannot be determined by our data; however, this was not our goal nor was it our purpose to challenge MMC or BCG as first line treatments. In contrast, MMC exhibited excellent efficacy in our model. Higher doses of HTI-286 were not examined, primarily because even at low doses, HTI-286 exhibited good pharmacokinetic properties with high levels of HTI-286 found in the bladder walls (Table 2). We believe our results provide convincing evidence that HTI-286 should be clinically evaluated for its potential to improve the armamentarium we have against high-risk nonmuscle-invasive bladder cancer.

The urinary bladder is relatively impermeable to toxic substances and easily accessible, making it an ideal environment for the instillation of intravesical agents. Systemic absorption of chemotherapeutic drugs through the bladder wall is thought to be unlikely for agents with a molecular weight over 300 g/mol (42), theoretically minimizing systemic risk for HTI-286 (473.7 g/mol) via this route of administration. However, our HPLC-MS method for HTI-286 still did detect some systemic uptake with an estimated systemic bioavailability around 2% of the instilled dose. This is in accordance with results obtained for intravesical paclitaxel and supports the suggestion that transport across the urothelial barrier is determined not only by molecular size but also by the degree of lipophilicity of the compound (34). Yet, compared with humans, the mouse bladder wall is very thin and with an assumed logarithmical decrease in drug penetration across the tissue (34), lower serum levels of HTI-286 in humans would be likely. Systemic uptake in mice may also be mediated in part by ureteral reflux and absorption at the kidney level. After injecting methylene blue into mouse bladders, reflux was evident with treatment volumes as low as 50 μL. In a previous phase I trial, there were concerns that serum levels as low as 0.5 ng/mL might lead to neutropenia (43); thus, although very low HTI-286 levels would be expected in patients, serum...
HTI-286 levels should be closely monitored in future clinical studies. Nonetheless, during our experiments, we did not observe any weight loss, diarrhea, or persistent hematuria in mice treated intravesically with HTI-286. Although HTI-286 can be diluted in saline and thereby circumvents the side effects that can result from the use of a vehicle (e.g., hypersensitivity to Cremophor, ref. 44), in our hands, some DMSO was required to facilitate its solubility at high concentrations. To what extent this had an influence on our in vivo results remains questionable, because there is conflicting data on the confounding effects of DMSO. Whereas early studies suggested beneficial antitumor effects (45), more recent reports describe that DMSO increases urine production and drug removal by perfusing capillaries (46).

In conclusion, our studies provide strong preclinical proof-of-principle for the use of HTI-286 in high-grade nonmuscle-invasive urothelial carcinoma. The distinctive mechanism of microtubule disruption of HTI-286, in addition to being a poor substrate for P-glycoprotein, suggests a complementary response to existing therapies. Further research is warranted to evaluate its efficacy and safety in early-phase clinical trials and to develop improved formulations of HTI-286, such as mucoadhesive nanoparticles.

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References

Clinical Cancer Research

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