Purpose: Imidazoquinolines (Toll-like receptor-7 agonists) are a class of synthetic immune modulating agents. Imiquimod, a member of this drug family, is currently used as first line topical therapy for genital condyloma. It recently showed clinical efficacy against several benign and malignant skin lesions, including actinic keratosis and basal cell carcinoma. Working primarily through the stimulation of a proinflammatory immune response, the mechanism of action of imiquimod may be similar to that through which bacillus Calmette-Guerin is thought to act. We hypothesized that imidazoquinolines have therapeutic potential against bladder cancer. We determined the in vitro and in vivo effects of imidazoquinolines against bladder cancer cells.

Materials and Methods: The human and murine J82, T24, TCC-SUP (American Tissue Culture Collection, Manassas, Virginia) and MBT-2 bladder cancer cell lines were cultured in normal culture medium or medium supplemented with imidazoquinoline. Effects on cell viability, apoptosis induction and cytokine production were evaluated. In addition, the effects of imidazoquinoline on in vivo bladder tumor growth were determined via intravesical instillation in an orthotopic bladder tumor model in the mouse.

Results: A dose dependent decrease in cell viability was observed in all tumor cell lines treated with imidazoquinoline. In addition, imidazoquinoline significantly induced apoptosis and cytokine production. In in vivo experiments most mice treated with imidazoquinoline showed only an intense inflammatory response with no evidence of tumor, while control mice showed tumor growth.

Conclusions: Imidazoquinolines have potent direct activity against bladder cancer cells by decreasing cell viability and inducing apoptosis and cytokine production. In addition, in vivo data suggest antitumor effects in an orthotopic bladder cancer mouse model. Therefore, imidazoquinolines may have therapeutic potential as a synthetic intravesical agent against bladder cancer.

Key Words: bladder; carcinoma, transitional cell; mice; immunotherapy

Intravesical BCG immunotherapy combined with transurethral bladder tumor resection is standard treatment for high grade, superficial urothelial carcinoma of the bladder. Instillation of live attenuated BCG into the bladder results in a local immune response characterized by the maturation of dendritic cells and secretion of proinflammatory cytokines. This immune stimulation leads to a cell mediated immune response that is tumor specific. However, BCG remains only partially effective and serious side effects may occur, including high fever, pneumonitis, hepatitis and sepsis. Efforts continue to develop safer and more effective therapy for bladder cancer.

Recent investigations into TLR signaling suggest a potential role in antitumor immunity. The TLR family (TLR-1 to TLR-10) is a highly conserved group of cell membrane proteins that have a critical role in antimicrobial immunity. The net result of TLR signaling is a coordinated immune response leading to efficient antigen presentation by mature dendritic cells and enhanced production of antigen specific T cells. Evidence also suggests that this TLR pathway may be crucial in antitumor immunity.

The synthetic TLR-7 agonist imiquimod is a member of the imidazoquinoline family and it is currently used as first line topical treatment for genital condyloma. It is also effective against several benign and malignant skin lesions, and it is Food and Drug Administration approved for the treatment of basal cell carcinoma. Activation of TLR-7 by imiquimod leads to an intracellular signaling cascade, causing a potent antiviral and potentially antitumor immune response.

Based on these molecular mechanisms as well as proven efficacy for skin malignancies we hypothesized that imidazoquinolines may have a therapeutic benefit in bladder cancer. We determined if imidazoquinolines have direct in vitro and in vivo biological activity against urothelial cell carcinoma.

MATERIALS AND METHODS

Cells and Culture Conditions
The high grade human bladder cancer cell lines J82, T24 and TCC-SUP (American Tissue Culture Collection, Manass-
sas, Virginia) were maintained under standard sterile conditions in RPMI-1640, and McCoy’s and modified Eagle’s medium, respectively. The murine urothelial cell carcinoma cell line MBT-2 was maintained in modified Eagle’s medium and used for in vitro and in vivo experiments.

**Cell Viability**
The effects of the imidazoquinoline 3M-004 (3M Pharmaceuticals, St. Paul, Minnesota) on bladder cancer cell viability was evaluated in vitro using the MTT assay (Promega, Madison, Wisconsin). In this experiment the bladder cancer cell lines J82, T24 and TCC-SUP were maintained in 96-well plates and treated with increasing doses (0 to 200 μg/ml) of imidazoquinoline. MBT-2 cells were treated with imidazoquinoline (100 μg/ml). After 24 hours the MTT assay was performed according to manufacturer instructions. Results were quantitated using an ELISA plate reader at 490 nm and compared to those in untreated controls.

**Apoptosis Assay**
Bladder cancer cells were incubated with normal culture medium or medium supplemented with imidazoquinoline (100 μg/ml). After 24 hours the supernatant and cells were collected and centrifuged. After removing the supernatant the cells were resuspended, fixed and cytospun at 800 × gravity for 5 minutes onto silanized microscope slides. DNA fragmentation consistent with apoptosis was then detected using an ApopTag® peroxidase in situ apoptosis detection kit. Cells were counterstained with 10% hematoxylin and evaluated microscopically. The percent of apoptotic cells were quantitated by assessing the ratio of positive staining cells to total cells.

**Cytotoxicity Assays**
To assess whether imidazoquinoline directly induced significant cellular necrosis LDH was measured in the supernatant of treated and untreated bladder cancer cell lines using an LDH release detection kit according to manufacturer instructions. The bladder cancer cell lines were placed in 96-well plates and treated with increasing doses (0 to 200 μg/ml) of imidazoquinoline. The supernatant was then transferred onto nitrocellulose membranes. Membranes were blocked with 5% dry milk in PBS/Tween-20 and were then transferred onto nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin primary antibodies for IL-6, TNF-α, IFN-α and IFN-γ were applied to the wells and incubated for 60 minutes. Secondary antibody was then applied for 60 minutes, followed by the addition of diethanolamine buffered substrate. Optical density reading was performed using an ELISA plate reader at 405 nm.

**Detection of TLR-7 Expression**
For Western blotting 40 μg cellular protein samples were heated at 95C with Laemmli sample buffer containing 2% β-mercaptoethanol and run through 8% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Separated proteins were then transferred onto nitrocellulose membranes. Membranes were blocked with 5% dry milk in PBS/Tween-20 and incubated with IMG-581 polyclonal anti-TLR-7 primary antibody (Imgenex, San Diego, California) overnight and with the appropriate peroxidase conjugated secondary antibody (1:5,000). Bands were detected using Western blotting luminol reagent (sc-2048, Santa Cruz Biotechnology, Santa Cruz, California). For TLR-7 quantitation the membrane was stripped and reprobed with anti-β-actin antibody (c-11, Santa Cruz Biotechnology). TLR-7 bands were scanned with ImageJ software and corrected with β-actin bands.

**Pharmacokinetic Analysis of Imiquimod**
Female C3H mice (Charles River Laboratories, Wilmington, Massachusetts) weighing 20 to 25 gm were anesthetized and a 3Fr to 1.5Fr FunnelCath® was inserted into the bladder. Subsequently 100 μl 0.03M citrate buffered saline (pH 6.0) or imidazoquinoline at 0.5 mg/ml in 0.03M citrate buffered saline (pH 6.0) were instilled into the bladder. At 3 hours blood and bladder tissue were collected, and drug and cytokine levels were measured with ELISA.

**Cytokine Production**
Cytokine protein levels were determined in the supernatant of cultured cells using an ELISA protocol. After 24 hours the supernatants of treated and untreated cells were incubated overnight at 4C. After blocking with 3% bovine serum albumin primary antibodies for IL-6, TNF-α, IFN-α and IFN-γ were applied to the wells and incubated for 60 minutes. Secondary antibody was then applied for 60 minutes, followed by the addition of diethanolamine buffered substrate. Optical density reading was performed using an ELISA plate reader at 405 nm.

**Orthotopic Mouse Model**
A previously described orthotopic bladder cancer mouse model was used for in vivo experiments. This model was reproduced at our laboratory by inducing tumor in 20 C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine), as described. Invasive bladder tumors developed in 95% of the mice, as determined microscopically after harvesting bladders. We next established a treatment protocol with imiquimod or PBS as the control. A total of 30 mice were used for this experiment. On the day following tumor instillation (day 1) groups of 15 mice were again anesthetized and catheterized, as described. Subsequently 100 μl imidazoquinoline (100 μg) or control (PBS) were instilled into the bladder for 2 hours. The mice were treated a second time on day 8. On day 15 the mice were sacrificed and the bladders were harvested for pathological evaluation.

**RESULTS**

**In Vitro Study**
The effects on cell viability, apoptosis and cytokine production were characterized, as reported.

**Imidazoquinoline decreased cell viability in bladder cancer cell lines.** MBT-2 cells treated with 100 μg/ml for 24 hours showed a 37% decrease in cell viability compared with controls (fig. 1, A). A dose dependent decrease in cell viability occurred in all cell lines at concentrations up to 200 μg/ml compared with that in untreated controls.
viability was observed in all human tumor cell lines at concentrations up to 200 μg/ml after 24 hours (fig. 1, B). Treatment with imidazoquinoline decreased the total number of cells capable of proliferation by 23% to 91% compared to that in nontreated controls with the most significant effects seen in the TCC-SUP cell line. Similar findings were observed at 8 and 48 hours (data not shown).

**Imidazoquinoline induced apoptosis in bladder cancer cell lines.** After 24 hours 17.1% (95% CI 10.7 to 23.5) of cells treated with imidazoquinoline stained positive for apoptosis compared to only 0.8% (95% CI 0.2 to 1.4) of untreated cells (fig. 2). The role of cellular necrosis was also assessed using an LDH detection assay. Consistent with prior studies, no evidence of significant necrosis was detected in the supernatant of cells supplemented with imidazoquinoline at concentrations of 0 to 100 μg/ml. However, at the highest concentration of 200 μg/ml the range of cytotoxicity was 55% to 80%. Similar findings were observed at 8 and 48 hours (data not shown).

**Imidazoquinoline induced cytokine production in bladder cancer cell lines.** After 24 hours IL-6 and TNF-α significantly increased 2 to 3-fold in the supernatant of cells treated with imidazoquinoline (100 μg/ml) (fig. 3). IL-6 increased 2.8-fold (95% CI 2.3 to 3.5), while TNF-α increased 2.2-fold (95% CI 1.2 to 3.2) (fig. 3). In contrast, IFN-α and IFN-γ did not show a statistically significant increase.

**TLR-7 expression.** All cell lines were shown to express TLR-7 with MBT-2 expressing the highest level (fig. 4).

**Pharmacokinetic analysis of imiquimod.** The average serum imidazoquinoline level in mice was 77 ng/ml (0.28 μM). It did not appear to be a systemic drug level that induced a serum or bladder tissue TNF, MCP-1 or IL-12 response, although there might have been a slight in-

![Fig. 2. Imidazoquinoline induced apoptosis in vitro in TCC-SUP cells cultured in presence or absence of 100 μg/ml imidazoquinoline for 24 hours. Apoptosis was detected using ApopTag peroxidase in situ apoptosis detection kit. A, untreated controls showed no significant apoptosis. Reduced from ×XXX. B, imidazoquinoline treated group showed scattered stained nuclei (brown areas), consistent with apoptosis. Reduced from ×XXX. Inset, high power view of apoptotic and nonapoptotic cell. Reduced from ×100. C, percent of apoptotic cells in treated and untreated groups. Imidazoquinoline induced apoptosis in 17/1% of cells compared to 0.8% in controls. Data are shown as mean and 95% CI (p ≤0.001). A to C, reduced from ×100.](image)

![Fig. 3. Imidazoquinoline induced cytokinet production in vitro in TCC-SUP cells cultured in presence or absence of 100 μg/ml imidazoquinoline for 24 hours. Cytokine protein concentration was detected using ELISA. A, IL-6 secretion increased 2.8-fold after treatment. B, TNF-α decreased 2.2-fold after treatment. Data are shown as mean and 95% CI (p ≤0.02).](image)

**In Vivo Study** We found that in the control group 11 of 13 mice (85%) showed invasive, high grade bladder tumors (fig. 5, A and B). Two of the original 15 mice died immediately after tumor instillation, presumably of sepsis or anesthetic complications. However, in the imidazoquinoline treated group only 3 of 14 mice had tumors (1 of 15 died immediately after tumor instillation). The remainder showed only an intense inflammatory response in the bladder wall with no evidence of tumor (fig. 5, C, D).

**DISCUSSION**

The antitumor effects of imiquimod were clearly demonstrated in various benign and malignant skin lesions. We provide experimental evidence that imidazoquinolines may also have a therapeutic benefit for bladder cancer. We report that TLR-7 is expressed in murine and human bladder cancer cell lines. In addition, we noted that imidazoquinoline has direct biological effects on transitional cell carcinoma cell lines by decreasing cell viability and inducing apoptosis and cytokine production. In addition, initial results in an immune competent, orthotopic mouse model suggest antitumor effects in vivo.

Urothelial carcinoma of the bladder is an ideal target for imidazoquinoline immunotherapy. For 30 years superficial transitional cell carcinoma has been successfully treated with the immune stimulant BCG, indicating susceptibility to immunotherapy. In addition, imidazoquinolines can be instilled transurethrally into the bladder, allowing direct...
contact with affected urothelium. This delivery system, which is similar to topical treatment of skin lesions, allows a localized reaction, resulting in a directed and efficient immune response. Any direct cytotoxicity or apoptosis induced by imidazoquinolines independent of an immune response would also be maximized with this approach. Furthermore, the safety profile of topical imidazoquinolines is favorable compared to that of current therapies such as BCG. In placebo controlled trials local skin reactions were induced by imidazoquinolines independent of an immune response. Any direct cytotoxicity or apoptosis induced by imidazoquinolines independent of an immune response would also be maximized with this approach. Furthermore, the safety profile of topical imidazoquinolines is favorable compared to that of current therapies such as BCG. In placebo controlled trials local skin reactions were the most common side effect of topically applied imiquimod.

### Table 1. Tissue and bladder cytokine analysis

<table>
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<tr>
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<th>Mean ± SD Vehicle (pg)</th>
<th>Mean ± SD Imiquimod (pg)</th>
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<tbody>
<tr>
<td>IL-12p40:</td>
<td></td>
<td></td>
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<tr>
<td>Serum</td>
<td>298 ± 59</td>
<td>432 ± 73</td>
</tr>
<tr>
<td>Tissue</td>
<td>45 ± 8</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>TNF-α:</td>
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<td></td>
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<tr>
<td>Serum</td>
<td>17 ± 3</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Tissue</td>
<td>145 ± 55</td>
<td>60 ± 30</td>
</tr>
<tr>
<td>MCP-1:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>108 ± 12</td>
<td>143 ± 17</td>
</tr>
<tr>
<td>Tissue</td>
<td>142 ± 18</td>
<td>136 ± 37</td>
</tr>
</tbody>
</table>

Bladder tissue cytokine results normalized to pg/30 mg tissue in 6 preparations per group.

### Table 2. Imidazoquinoline tissue and bladder levels

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Serum (ng/ml)</th>
<th>Tissue (µg/gm)</th>
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<tbody>
<tr>
<td>1</td>
<td>191</td>
<td>16.8</td>
</tr>
<tr>
<td>2</td>
<td>51.8</td>
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<tr>
<td>3</td>
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<td>5</td>
<td>40.7</td>
<td>9.52</td>
</tr>
<tr>
<td>6</td>
<td>142</td>
<td>Greater than 28</td>
</tr>
</tbody>
</table>

Bladder tissue drug levels normalized to 1 gm of tissue.

### FIG. 5. Antitumor effects of imidazoquinoline in orthotopic, immune competent mouse model. After instillation of MBT-2 bladder tumor cells mice were treated intravesically with imidazoquinoline or PBS as control. After 2 weeks mice were sacrificed and bladders were harvested for pathological evaluation. A, low power view demonstrates control bladder with multiple tumors. B, high power view of control shows high grade, invasive bladder tumor. C, low power view of imidazoquinoline treated bladder reveals no evidence of tumor. D, high power view of imidazoquinoline treated bladder shows dense inflammatory response with no evidence of tumor. Indeed, generation of a durable cell mediated, tumor specific response may partially depend on apoptosis. Analogous to viral infections, dendritic cells acquire tumor antigen by engulfing apoptotic cells. Effector cytotoxic T lymphocytes are then marshaled for a selective and durable response against tumor cells. In the current study 17.1% of tumor cells treated with imidazoquinoline showed evidence of apoptosis compared to only 0.8% of untreated cells, indicating that this important mechanism may contribute to antitumor effects in bladder cancer.

The in vitro biological effects of imidazoquinolines observed in this and other studies suggest a role for apoptosis induction and direct cytotoxicity. However, these effects occur at much higher concentrations (50 to 200 µg/ml) than what is required for cytokine induction in cells that are known to be TLR-7 responsive. Imidazoquinolines induce cytokines such as TNF-α, IFN and IL-6 in peripheral blood mononuclear cells at concentrations of 0.2 to 5 µg/ml. We also observed that bladder cancer cells increase the secretion of the proinflammatory cytokines IL-6 and TNF-α in response to imidazoquinolines, consistent with prior studies in human blood cell and keratinocyte cell lines. This observation points to a potential dual effect of imidazoquinolines toward bladder tumor cells with 1 being immune mediated and 1 being directly cytotoxic. We do not promote 1 mechanism of action over the other because we present evidence that each in fact may have a role. Further studies are necessary to prove and delineate these effects as well as the concentration for optimal efficacy against bladder cancer.

Because imidazoquinolines likely exert their effects partially through stimulation of the immune system, we examined the antitumor effects in immune competent mice using an orthotopic murine tumor model. Somewhat surprisingly we found that almost 80% of mice showed a complete response to intravesical imidazoquinoline. In mice that responded a significant inflammatory response was seen
throughout the bladder, consistent with an intense immune reaction. While most placebo treated mice demonstrated invasive tumor growth, 15% also had no evidence of tumor. However, these mice did not show a significant inflammatory response in the bladder, suggesting that the absence of tumor was likely due to inefficient tumor implantation. An important limitation of this in vivo study is that intravesical imidazoquinoline treatment was given approximately 24 hours after the instillation of bladder tumor cells. Further investigation is needed to determine if imidazoquinolines can treat more established bladder tumors.

**CONCLUSIONS**

Successful strategies for immunotherapy ultimately require durable tumor specific immune responses without significant toxicity. Based on emerging data and recently elucidated molecular mechanisms imidazoquinolines are an exciting synthetic class of agents with possible therapeutic benefit for various cancers. Based on the findings of this study imidazoquinolines have antitumor effects against bladder cancer cell lines in vitro and in vivo. Therefore, they may be a potential new intravesical therapy for bladder cancer. Our preliminary pharmacokinetic data indicate that with intravesical instillation there is little systemic absorption and, thus, we do not anticipate significant systemic toxicity. Further in vitro and in vivo studies are ongoing. We currently plan to explore the use of imidazoquinolines in a phase I/II trial in patients with high grade superficial transitional cell carcinoma of the bladder. In addition, we are currently performing gain of function and loss of function studies with TLR-7 using RNA interference as well as over expression assays to more clearly delineate the relative contribution of the immune mediated effects and the direct cytotoxic components involved in the therapeutic efficacy of imidazoquinolines.

### Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviations and Acronyms</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCG</td>
<td>bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoassay</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
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</table>

### REFERENCES


