Targeting Folate Receptors to Treat Invasive Urinary Bladder Cancer

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Abstract

Folate receptors (FR) may be of use for targeted delivery of cytotoxic drugs in invasive urothelial carcinoma (iUC), for which improved therapy is needed. FR expression and function in iUC were explored and the antitumor activity and toxicity of a folate-targeted vinblastine conjugate were evaluated in dogs with naturally occurring iUC, an excellent model for human iUC. FR immunohistochemistry was carried out on iUC and normal human and dog bladder tissues together with nuclear scintigraphy in dogs to monitor iUC folate uptake. Dose escalation of a folate-targeted vinblastine compound, EC0905, was conducted in dogs with biopsy-confirmed, FR-positive iUC. FRs were detected by immunohistochemistry (PU17) in most primary iUC and many nodal and lung metastases from dogs, and scintigraphy confirmed folate uptake in both primary and metastatic lesions. The maximum tolerated dose of EC0905 in dogs was 0.25 mg/kg IV weekly, with neutropenia at higher doses. Tumor responses included partial remission (>50% reduction in tumor volume) in five dogs and stable disease (<50% change in tumor volume) in four dogs. Immunoreactivity to PU17 was similar in humans (78% of primary iUC, 80% of nodal metastases). Less immunoreactivity to mab343 (22% of cases) occurred. FR-β was noted in 21% of human iUC cases. Our findings suggest folate-targeted therapy holds considerable promise for treating iUC, where FR-β may be important in addition to FR-α. Cancer Res; 73(2); 875–84. ©2012 AACR.

Introduction

Invasive urinary bladder cancer (invasive urothelial carcinoma, iUC) that has metastasized causes more than 14,000 deaths each year in the United States (1, 2). Nonspecific cytotoxic drugs used to treat iUC have failed to eradicate the cancer, and have caused substantial toxicity in many patients (2, 3). Targeted therapy for iUC may offer the opportunity to increase treatment efficacy and reduce toxicity.

An emerging approach to direct drugs to cancer cells while limiting damage to normal cells has been to exploit folic acid (folate, vitamin B9) uptake in cancer cells (4, 5). Folate receptor (FR) expression in normal tissues is limited, while upregulation of FRs has been noted in several forms of human cancer (5, 6). Folate has been conjugated to a variety of cancer treatment agents including traditional chemotherapy drugs and other agents (7–12). In drug delivery, the folate–drug conjugate binds to FRs and is internalized by endocytosis (4). Within the mildly acidic environment of the endosome, FRs change conformation, thus releasing the conjugate. A self-immolative linker contained with the conjugate breaks inside the endosome to release the active drug, which subsequently diffuses across the endosomal membrane and exerts its pharmacologic activity within the cell. The FRs then recycle to the cell surface for another round of folate conjugate uptake (4).

Of the 4 isoforms of the FR, the α- and β-isoforms are glycosylphosphatidylinositol-anchored to the cell membrane, are functional for high-affinity folate binding, and can be exploited to deliver cancer treatment agents into the cancer cells (4–12). The expression of FR-α is minimal in normal tissues and is typically limited to the apical surfaces of a few polarized epithelia, where it is inaccessible to intravenously administered folate-conjugated drugs (4, 6). Human cancers that overexpress FR-α include those arising from epithelial cells in the ovary, lung, breast, kidney, and colon (4–6, 12). FR-β is largely expressed on activated macrophages and monocytes, although it is also expressed in some hematopoietic cancers and other cancers (4–6).

Although the overexpression of FRs has been documented in several forms of cancer in humans, FR expression in iUC has not been defined. This work was carried out to determine FR expression in human iUC and to study folate-targeted therapy in a highly relevant animal model of iUC, that is, naturally occurring iUC in dogs. Naturally occurring iUC (also referred to
as invasive transitional cell carcinoma) in dogs and humans is very similar in histopathology, molecular features, biologic behavior including local invasion and distant metastasis, and chemotherapy response (13). Dogs with iUC offer an ideal model to evaluate new cancer therapies. Positive findings from dog studies can be translated into human studies, as well as improve the outlook for dogs with cancer. Vinblastine, a vinca alkaloid chemotherapeutic agent (14), was selected for the pilot study of folate-targeted therapy in dogs. Vinblastine has activity against iUC in humans (15, 16) and dogs (17), and it has been successfully conjugated to folate for therapy in humans (ref. 9, 18; ongoing phase III clinical trial, Endocyte Inc., West Lafayette, IN). The objectives of the work were to determine FR expression in iUC in humans and dogs with comparison to the normal bladder, and to conduct a pilot study of folate-targeted therapy in dogs with iUC.

Materials and Methods

FR expression was detected by immunohistochemistry (IHC) in human and canine iUC samples, with comparison to normal bladder tissues. Folate uptake and binding were determined by ex vivo folate-binding assay on iUC tissues and by in vivo nuclear scintigraphy in dogs with iUC. A pilot study was conducted in dogs with naturally occurring iUC to investigate the potential antitumor activity and toxicity of folate-targeted vinblastine treatment. The work was conducted with the approval of the Institutional Review Board and Animal Care and Use Committee.

Tissues

Human iUC tissues (primary tumor and lymph node metastases), bladder tissues adjacent to the cancer, and samples from normal bladders were obtained from the Indiana University Simon Cancer Center Tissue Bank, Indianapolis, Indiana and the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio. Canine iUC (primary tumor, lymph node, and lung metastases) and normal bladder from normal dogs were obtained from the Indiana Animal Disease Diagnostic Laboratory and the Purdue Comparative Oncology Program, Purdue University (West Lafayette, IN).

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on formalin-fixed tissues similarly as previously described (19–22). IHC on human tissues included 3 primary antibodies: a rabbit polyclonal antibody (PU17), a monoclonal antibody to FR-α (mab343), and a monoclonal antibody to FR-β (mab909) kindly provided by Dr. Philip Low, Purdue University, West Lafayette, Indiana, and by Endocyte. IHC studies focused on FR-α, which is expressed in other epithelial cancers. Folate-binding assay and IHC (FR-α and FR-β) were conducted on a subset of samples. Immunostaining with mab343 and PU17 was carried out at Purdue University; IHC with mab909 was conducted at the Mayo Clinic, Rochester, Minnesota. PU17 was selected for canine tissues, as there was consistent and specific immunoreactivity in the apical (luminal) membrane and cytoplasm of renal proximal tubular epithelial cells (positive control; Fig. 1).

There was no immunoreactivity to the monoclonal antibodies in the canine tissues. With the polyclonal antibody used in the canine studies, paired negative reagent control slides were included using the Universal Negative control serum (Biocare Medical). Slides were reviewed independently by 3 investigators (L. Cheng and D. Dhawan for human specimens and J.A. Ramos-Vara and D. Dhawan for canine specimens). Any discrepancies between reviewers were resolved by screening those cases concurrently to reach a consensus. The percentage of positively immunostained tumor cells was categorized as follows: 0, 0% to 9% of cells; 1, 10% to 19% of cells; 2, 20% to 49% of cells; 3, 50% to 79% of cells; and 4, 80% to 100% of tumor cells staining. Staining intensity (0, no staining; 1, equivocal staining; 2, moderate to intense staining; and 3, highest intensity staining) was multiplied by category for percentage of positive cells to determine the IHC score (23, 24). The FR location (membrane

![Image](image-url)
or cytoplasm) was recorded. Positive cases were those with immunoreactivity in 10% or more of tumor cells (IHC score ≥ 1).

**Folate-binding assay**

Assessment of folate binding in snap-frozen iUC tissues and control tissues was conducted as previously described (6).

**Scintigraphy**

Following pet owner consent, privately owned dogs with naturally occurring iUC were imaged with a technetium-folate conjugate (99mTc-EC20; ref. 4, 22). Briefly, 5 mCi 99mTc was added to EC20 solution (Endocyte) and injected intravenously 2 hours before imaging. For imaging, dogs were placed under general anesthesia. Full-body static images were acquired in right and left lateral, ventrodorsal, and dorsoventral recumbencies over a 90-second per view time using a single-head gamma camera (MiE Equine Scanner H.R.; Scintron VI). Various positional acquisitions were also obtained according to tumor location.

**Synthesis of folate–vinblastine conjugate (EC0905) and confirmation of specific activity**

In preparation for subsequent dog studies, EC0905 (Fig. 2A) was synthesized (25), and specific antitumor activity evaluated in vitro and in vivo in rodents. Desacetylvinblastine hydrazide was converted to a pyridinyldisulfanyl-activated derivative while reacting with the heretobifunctional cross-linker, 2-[benzotriazole-1-yl-(oxycarbonyloxy)-ethyldisulfanyl]-pyridine. The product was reacted with the peptide-based folate-spacer unit Pte-γGlu-[[1-amino-1-deoxy-D-glucitol]-Glu]-Glu]-[[1-amino-1-deoxy-D-glucitol]-Glu]-Cys-OH, and the resulting conjugate purified by preparative high-performance liquid chromatography (HPLC). Clean product fractions were combined, lyophilized, and characterized by 1H-NMR and liquid chromatography/mass spectrometry (LC/MS).

Dose-dependent FR-specific activity of EC0905 was determined as described previously (Fig. 2B; ref. 26). KB cell (American Type Culture Collection; ATCC) integrity was confirmed (Genetica DNA Labs, Cincinnati, Ohio) with cells being 100% identical to reference cells from the ATCC. KB cells from the ATCC do contain HeLa markers.

The in vivo activity and toxicity of EC0905 were determined in nu/nu mice (Balb/c-derived; Charles River, Wilmington, MA) fed a folate-free diet (Harlan diet #TD00434, Harlan Teklad, Madison, WI) to achieve serum folate concentrations similar to those in humans (26, 27). EC0905 (2 μmol/kg) or vehicle control was administered intravenously 3 times per week for 2 weeks to mice with FR-positive KB xenografts, and tumor size and animal performance were monitored.

**Dose-escalation study of EC0905 in dogs with iUC**

A pilot study of EC0905 was conducted in privately owned dogs with naturally occurring iUC at the Purdue University...
Veterinary Teaching Hospital (PUVTH). Dogs lived at home with their families and came into the PUVTH for evaluation and treatment. Inclusion criteria included: measurable, histologically confirmed iUC, positive folate uptake detected by scintigraphy or FR expression observed in the tumor via IHC, expected survival of 6 weeks or longer, and written dog owner consent. The pilot study was open to dogs that had failed or who were not eligible for standard therapy.

EC0905 was administered by rapid injection through an intravenous catheter once weekly (starting dose 0.2 mg/kg on the basis of laboratory animal studies and interspecies scaling; unpublished data, Endocyte). Dose escalation was carried out within dogs (after 2 doses with no toxicity) and between dogs (minimum 3 dogs per dose group and 6 dogs treated at the maximum tolerated dose, MTD). The dose was increased to 0.225 mg/kg, then to 0.25 mg/kg, and later increased by 0.02 mg/kg for each subsequent dose group. Toxicity was assessed by complete blood counts (CBC), serum chemical profiles, urinalyses, physical exams, and owner observations, and was classified by the Veterinary Cooperative Oncology Group (VCOG) criteria (28). Urologic toxicity was categorized as previously described (29). The MTD was defined as the highest dose that resulted in 0 of 6 dogs having grade 4 toxicity and 0 or 1 of 6 dogs having grade 3 toxicity.

The EC0905 dose was reduced by 10% if grade 2 toxicity was noted, and by 20% if grade 3 or higher toxicity occurred. Treatment was delayed if the neutrophil count was less than 3,000/μm³ or platelet count less than 100,000/μm³ the day treatment was due. Treatment was scheduled to continue until 8 weeks beyond complete remission, until cancer progression, or until unacceptable toxicity (considered unacceptable by the dog owner or attending veterinarian) was noted. Dogs that failed EC0905 due to cancer progression or unacceptable toxicity were eligible to receive other therapies off study.

Physical exam, medical history, and CBC were carried out weekly. Monthly evaluation included CBC, serum biochemical profile, urinalysis, urinary tract ultrasound, and ultrasound mapping of bladder masses. Urinary tumors were measured by a single ultrasound operator following a standardized mapping procedure (30, 31), and estimated tumor volume was recorded. Ultrasound was selected because it is standardized mapping procedure (30, 31), and estimated tumor volume was recorded. Ultrasound was selected because it is a non-invasive imaging modality that allows for repeated imaging at the same site.

Statistical analysis
Statistical analyses included Wilcoxon—Mann–Whitney test, Spearman’s rho (r), χ² analysis, and Fisher exact test. P < 0.05 was considered significant for all analyses.

Results
Folate receptor expression and folate uptake in canine iUC
Samples were studied from 74 dogs with muscle invasive iUC. Using the WHO classification for canine iUC (32), there were 49 T2 tumors (muscle invasive) and 25 T3 tumors (tumor invading neighboring organs). There were 40 spayed female, 32 neutered male, and 2 intact male dogs (median age, 11 years; range, 4—17 years), with several breeds represented. Nodal metastases were present in 23 dogs (31%), and distant metastases in 28 dogs (38%). FR expression was detected in 56 of 74 (76%) primary tumors (78% of T2, 72% of T3 tumors), in 7 of 12 (58%) nodal metastases, and in 10 of 21 (48%) of lung metastases (Table 1, Fig. 1). In 67% of cases, the FR expression in the primary tumor and lung metastases were similar (either positive in both sites, or negative in both sites); in 33% of cases, FR expression was detected in the primary tumor but not in metastases. The FR expression in the primary tumor did not differ between dogs with metastases and dogs without metastases. Immunoreactivity was noted in the epithelial cells in 8 of 8 normal bladders from dogs (>80% cells positive, 2–3+ staining intensity, and membrane and cytoplasmic in location).

Folate binding was detected ex vivo in iUC samples from 10 dogs (range, 0.17–3.10; median, 1.31 pmol FR/mg protein). All 10 cases had positive immunoreactivity in tumor cells on IHC. No differences were observed in IHC findings (percentage of positive cells, staining intensity, and stain location) between samples with the lowest versus the highest folate binding.

Folate binding in normal full-thickness bladder sections (median, 0.13 pmol FR/mg protein; range, 0.05–0.35 pmol FR/mg protein; n = 7) was significantly less (P < 0.0062) than that in the iUC tissues. The binding in normal bladder mucosal sections (median, 0.39 pmol FR/mg protein; range, 0.09–1.22 pmol FR/mg protein; n = 5) was not different (P = 0.098) than in iUC.

Scintigraphy was carried out in 13 dogs, with shipment of the 99mTc scheduled to allow scanning with approximately 5 mCi. The actual mean activity of the injected conjugate was 6.2 mCi per dog (range, 3.8–10.2 mCi per dog). EC20 uptake was detected in the iUC (primary and/or metastases) in 12 of 13 dogs (Fig. 3). The 12 dogs with positive scans also had FR expression in tumor cells detected by IHC. One dog with bulky spread of the cancer had a negative scan and negative IHC. With the EC20 being eliminated through the urine, special steps were required to observe uptake in bladder masses. This included catheterization, removing urine from the bladder, and distending the bladder with sterile saline as well as individual dog positioning based on tumor location within the bladder. Uptake in the cancer in the urethra and prostate was observed, especially when the bladder was shielded (Fig. 3). Six of 13 dogs scanned had histopathologically confirmed distant metastases of iUC at necropsy. Two additional dogs...
had radiographic evidence of lung metastases not confirmed by biopsy (owners declined necropsy). One of the 2 dogs had fine needle aspiration cytology consistent with carcinoma; in the other dog, the pulmonary lesions were considered too small and deep for safe aspiration. EC20 uptake was observed in 4 of 6 dogs with biopsy-confirmed metastases as well as in 2 of 2 dogs with radiographic evidence of metastases (Fig. 3). In 2 dogs, the metastatic lesions were obscured by nonspecific EC20 uptake in the liver, as has been previously reported (4). Shielding the liver facilitated detection of radioactivity in lung metastases (Fig. 3), although lesions very caudal in the lung field were still overshadowed by the liver.

Folate receptor expression and folate uptake in human iUC

IHC was conducted on tumor samples from 37 humans with iUC including 8 T2, 1 T2a, 3 T2b, 2 T3, 7 T3a, 7 T3b, 3 T4, and 6 T4a tumors (WHO 2010 TNM staging system; ref. 33). Nodal metastases were reported in 23 of the patients. Distant metastases were not detected. The median patient age was 65 years (range, 39–82 years), and there were 25 male and 11 female patients (gender was not recorded in 1 patient).

Immunoreactivity to PU17 was noted in tumor cells in 29 of 37 (78%) primary tumors including 83% of T2, 75% of T3, and 78% of T4 tumors as well as in 12 of 15 (80%) nodal metastases (Table 1, Fig. 4). The epithelium adjacent to the tumor was negative in all 5 cases with mab343, and the urothelium in the normal bladders was negative in 2 of 2 cases.

In the folate-binding assay, binding ranged from 0.06 to 10.12 pmol FR/mg protein (median, 1.71 pmol FR/mg protein, n = 17; Table 2). There was a significant positive association between the level of folate binding and IHC score for mab343 (rₚ = 0.55; P = 0.023), but not between the level of folate binding and PU17 IHC score (rₚ = 0.45; P = 0.067) or mab909 IHC score (rₚ = 0.09; P = 0.75; n = 14). In 2 cases (Table 2, case # 11 and 12), IHC with PU17 revealed immunoreactivity in inflammatory cells, but not tumor cells. FR-β was expressed in tumor cells in 3

### Table 1. Results of IHC to detect folate receptor expression in canine and human invasive urothelial carcinoma

<table>
<thead>
<tr>
<th></th>
<th>IHC, Canine iUC, PU17</th>
<th>IHC, Human iUC PU17</th>
<th>IHC, Human iUC Mab343</th>
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</thead>
<tbody>
<tr>
<td><strong>Primary tumor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number cases</td>
<td>74</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Number positive</td>
<td>56 (76%)</td>
<td>29 (78%)</td>
<td>8 (22%)</td>
</tr>
<tr>
<td>FR location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>6 (11%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>18 (32%)</td>
<td>19 (66%)</td>
<td>3 (37%)</td>
</tr>
<tr>
<td>Both</td>
<td>32 (57%)</td>
<td>10 (54%)</td>
<td>5 (63%)</td>
</tr>
<tr>
<td>% Positive tumor cells per section</td>
<td>70.4 ± 25.5</td>
<td>56.3 ± 27.0</td>
<td>31.9 ± 26.0</td>
</tr>
<tr>
<td>Stain intensity</td>
<td>2.1 ± 0.7</td>
<td>1.5 ± 0.7</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td><strong>Lymph node metastases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number cases</td>
<td>12</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Number positive</td>
<td>7 (58%)</td>
<td>12 (80%)</td>
<td>6 (35%)</td>
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<tr>
<td>FR location</td>
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<tr>
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<td>1 (14%)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Cytoplasm</td>
<td>3 (43%)</td>
<td>8 (75%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Both</td>
<td>3 (43%)</td>
<td>4 (25%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>% Positive tumor cells per section</td>
<td>71.0 ± 26.5</td>
<td>49.2 ± 11.6</td>
<td>53.3 ± 30.1</td>
</tr>
<tr>
<td>Stain intensity</td>
<td>1.8 ± 0.8</td>
<td>1.3 ± 0.5</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td><strong>Lung metastases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number cases</td>
<td>21</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>Number positive</td>
<td>10 (48%)</td>
<td>Not available</td>
<td>Not available</td>
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<td>FR location</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>0</td>
<td>Not available</td>
<td>Not available</td>
</tr>
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<td>Cytoplasm</td>
<td>7 (70%)</td>
<td>Not available</td>
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</tr>
<tr>
<td>Both</td>
<td>3 (30%)</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>% Positive tumor cells per section</td>
<td>85.0 ± 13.9</td>
<td>Not available</td>
<td>Not available</td>
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<tr>
<td>Stain intensity</td>
<td>1.8 ± 0.8</td>
<td>Not available</td>
<td>Not available</td>
</tr>
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</table>

Table 1. Results of IHC to detect folate receptor expression in canine and human invasive urothelial carcinoma

Folate-Targeted Therapy in Bladder Cancer

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879

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of 14 cases (21%). Folate binding in 1 sample available from a normal bladder was 0.48 pmol FR/mg protein. Folate binding was noted in bladder tissues adjacent to the cancer (median, 2.32 pmol FR/mg protein; range, 0.04–3.09 pmol FR/mg protein; n, 5).

In vitro and in vivo activity of EC0905 in mice

In in vitro experiments, FR-positive KB xenografts were highly sensitive to EC0905 with an IC<sub>50</sub> of 2 nmol/L; cytotoxicity was dependent on FR expression (Fig. 2B). EC0905 caused tumor regression in mice, while tumors in untreated mice grew rapidly (Fig. 2C). EC0905 was well tolerated with no weight loss in treated mice (Fig. 2D).

Pilot study of folate–vinblastine conjugate in dogs with iUC

Characteristics of dogs enrolled in the clinical trial of EC0905 and a summary of the doses given are provided in Table 3. Ten dogs received 106 doses of EC0905 (range, 1–46 doses per dog) with no injection reactions. The dog receiving 1 dose died unexpectedly of a non–cancer related condition.

The EC0905 dose was escalated to 0.27 mg/kg. Unacceptable gastrointestinal (GI) toxicity (marked anorexia of several days duration) and lethargy occurred in 2 of 3 dogs receiving this dose. The 0.25 mg/kg dose group was then expanded to 6 dogs, and toxicities included grade 1 gastrointestinal toxicity in 3 dogs, grade 1 lethargy in 1 dog, and grade 2 lethargy in 1 dog. The MTD of EC0905 was defined as 0.25 mg/kg once weekly. No urologic toxicity occurred. In dogs receiving several weeks of treatment, dose reduction did become necessary due to GI toxicity and neutropenia. Neutropenia occurred in 4 dogs receiving a lower dose of EC0905 (grade 1 in 3 dogs and grade 2 in 1 dog).

Tumor response was assessed in 9 of the 10 dogs. Tumor response could not be assessed in 1 dog who died following surgery for acute gastric dilatation and volvulus a few days after treatment. The dog had no evidence of EC0905 toxicity and had been eating well; there was no indication that the gastric dilatation was due to the cancer or treatment. Tumor response in 4 dogs included partial remission in 5 (56%) dogs and stable disease in 4 (44%) dogs. Median time to treatment failure was...
FR expression was still present in the cancer in all 6 dogs. Treatment failure was due to cancer progression in 4 dogs, other life-threatening illness unrelated to cancer or treatment in 3 dogs, other concurrent cancers in 2 dogs, and discontinuation of EC0905 in 1 dog due to the dog owner’s inability to return the dog for follow-up. Five cancers in 2 dogs, and discontinuation of EC0905 in 1 dog due to unrelated to cancer or treatment in 3 dogs, other concurrent cancer progression in 4 dogs, other life-threatening illness. Five dogs were expressing FR (13, 17, 34, 35). Another positive finding was that EC0905 was generally well tolerated. As in humans (4, 6, 36), FR expression was detected in the normal bladder and kidneys in dogs, but no toxicity to these organs was noted with EC0905. Lack of renal and urologic toxicity has also been observed with folate-targeted therapy in humans (18, 37).

58 days (range, 3–428 days). Treatment failure was due to cancer progression in 4 dogs, other life-threatening illness unrelated to cancer or treatment in 3 dogs, other concurrent cancers in 2 dogs, and discontinuation of EC0905 in 1 dog due to the dog owner’s inability to return the dog for follow-up. Five of 9 dogs went on to receive other therapies after discontinuing EC0905. The median survival in all 10 dogs was 115 days (range, 3–428 days). Necropsy was carried out on 6 dogs, and marked FR expression was still present in the cancer in all 6 dogs.

**Discussion**

The study results provide strong evidence for the potential benefit of targeting FRs to treat iUC. In the canine studies, FRs were expressed in the majority of iUC cases studied, and folate uptake was confirmed by ex vivo folate-binding assay and by in vivo scintigraphy. Specific antitumor activity and safety of EC0905 was observed in mice, and the drug had impressive antitumor activity in dogs with iUC. Canine iUC is very similar to iUC in humans in regard to cellular and molecular features, sites and frequency of metastases, and response to traditional chemotherapy drugs (13). Although the pilot study only included 9 dogs with posttreatment follow up, the finding of partial remission in 5 dogs (including durable remission of up to 47 weeks) and stable disease in 4 dogs was considered very positive. In comparison, standard chemotherapy regimens for iUC in dogs (carboplatin, vinblastine, or mitoxantrone with or without a cyclooxygenase inhibitor) result in remission (mostly partial) in approximately 35% of dogs and stable disease in 45% to 50% of dogs (13, 17, 34, 35). Another positive finding was that EC0905 was generally well tolerated. As in humans (4, 6, 36), FR expression was detected in the normal bladder and kidneys in dogs, but no toxicity to these organs was noted with EC0905. Lack of renal and urologic toxicity has also been observed with folate-targeted therapy in humans (18, 37).

The findings in dogs together with the results of the IHC in human tissues provide justification to pursue studies leading to folate-targeted therapy in humans with iUC. Defining the percentage of humans that could benefit will require further study. Using monoclonal antibodies (mab343 and mab909) for IHC, FR expression in iUC cells was detected in 43% of human cases. The results of the PU17 IHC suggest that a higher percentage of human iUCs express FRs, with 78% of samples having immunoreactivity in the tumor cells. The immunoreactivity in the iUC sections in dogs and humans suggest that this polyclonal antibody could bind to FR-β, as well as FR-α in the cancer cells. It is possible that the findings with mab343 and mab909 underestimate the number of patients with FR-positive cancer. Mutations in the FR, other changes in receptor confirmation, and loss of epitopes over time could preclude mabs from binding to the FR, leading to an underestimation of the frequency of FR expression. In addition, subjectively, it appeared that immunoreactivity was more prevalent in recent collected iUC specimens than from older archival specimens. A next step would be to conduct scintigraphy to detect folate uptake in iUC in humans.

### Table 2. Results of folate-binding assay and IHC in 17 cases of human invasive urothelial carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Binding, pmol FR/mg protein</th>
<th>IHC PU17 Pos/neg, IHC score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IHC mab343 Pos/neg, IHC score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IHC mab909 Pos/neg, IHC score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.06</td>
<td>+, 3</td>
<td>−, 0</td>
<td>−, 0</td>
</tr>
<tr>
<td>2</td>
<td>0.60</td>
<td>+, 3</td>
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<td>−, 0</td>
</tr>
<tr>
<td>3</td>
<td>0.74</td>
<td>+, 4</td>
<td>−, 0</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>0.76</td>
<td>+, 3</td>
<td>−, 0</td>
<td>−, 0</td>
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<tr>
<td>5</td>
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</tr>
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<td>1.49</td>
<td>+, 6</td>
<td>−, 0</td>
<td>+, 6</td>
</tr>
<tr>
<td>9</td>
<td>1.71</td>
<td>−, 0</td>
<td>−, 0</td>
<td>−, 0</td>
</tr>
<tr>
<td>10</td>
<td>2.27</td>
<td>+, 3</td>
<td>+, 1</td>
<td>−, 0</td>
</tr>
<tr>
<td>11</td>
<td>2.77</td>
<td>−, 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−, 0</td>
<td>−, 0</td>
</tr>
<tr>
<td>12</td>
<td>3.17</td>
<td>−, 0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−, 0</td>
<td>+, 4</td>
</tr>
<tr>
<td>13</td>
<td>3.67</td>
<td>+, 8</td>
<td>+, 9</td>
<td>−, 0</td>
</tr>
<tr>
<td>14</td>
<td>5.13</td>
<td>+, 12</td>
<td>−, 0</td>
<td>−, 0</td>
</tr>
<tr>
<td>15</td>
<td>5.75</td>
<td>+, 4</td>
<td>+, 3</td>
<td>−, 0</td>
</tr>
<tr>
<td>16</td>
<td>9.16</td>
<td>+, 8</td>
<td>−, 0</td>
<td>−, 0</td>
</tr>
<tr>
<td>17</td>
<td>10.12</td>
<td>+, 12</td>
<td>+, 3</td>
<td>NA</td>
</tr>
</tbody>
</table>

**NOTE:** Positive IHC results indicate immunoreactivity detected in ≥10% of the tumor cells in the sections.

<sup>a</sup>The IHC score was determined by multiplying the category for percent positive cells (1 = 10–19% positive, 2 = 20–49% positive, 3 = 50–79% positive, and 4 = 80–100% positive) by the stain intensity (1–3 with 3 being the most intense stain intensity).

<sup>b</sup>In cases 11 and 12, immunoreactivity was noted in inflammatory cells using PU17, but not in tumor cells. Immunoreactivity was also noted in inflammatory cells in these cases with mab909.
The scintigraphy studies in dogs provided insight into how to best observe folate uptake in primary and metastatic urothelial carcinoma (iUC). Observing uptake in metastases is especially important. The majority of deaths from iUC in humans are due to chemotherapy-resistant metastases (2, 3), and this may be an area where folate-targeted therapy could be most important. To observe metastases in close proximity to the liver, it was necessary to shield the radioactivity in the liver. As previously observed (4, 38), there can be nonspecific uptake of 99mTc-EC20 in the liver of humans and animals. The administration of antifolate compounds before the administration of folate radioconjugates has been reported to reduce uptake in nonmalignant tissues in humans (38). This approach was not used in the dog study; thus, more background may have been present. For patients who pursue bladder-sparing treatment options for iUC (39), being able to detect folate uptake in the primary tumor in the bladder would also be important. In the dog studies, using a catheter to remove the radioactive urine from the bladder and redistending the bladder with saline was useful in detecting folate uptake in tumor masses in the bladder. Shielding the liver, kidneys, and bladder facilitated the detection of the 99mTc-EC20 uptake in nearby organs (urethra, ureters, prostate, and lymph nodes).

The analysis of tissues obtained following EC0905 treatment in dogs offered the opportunity to help rule out 1 of the possible mechanisms that could lead to failure of folate-targeted therapy over time, that is the potential loss of FRs. It was important that marked FR expression was still present in iUC cells in tissues from 6 of 6 cases examined post-treatment. This indicates that patients that have failed folate–vinblastine could still benefit from other folate–drug conjugates.

In conclusion, this study provides strong evidence for the potential benefit of folate-targeted therapy in iUC, and support for subsequent studies in humans with iUC. FR-β, as well as FR-α, could be important in iUC. Nuclear scintigraphy studies could further define the percentage of humans who could benefit from folate-targeted therapy.

**Disclosure of Potential Conflicts of Interest**

D. Dhawan and D.W. Knapp have ownership interest in a patent application file by Purdue University to use folate-targeted therapy for canine bladder cancer. P.S. Low has employment as Chief Science Officer, a commercial research grant from, and ownership interest (including patents) in Endocyte, Inc. C.P. Leamon has ownership interest (including patents) in Endocyte, Inc. No potential conflicts of interest were disclosed by the other authors.

---

### Table 3. Characteristics of the dogs enrolled in the trial of EC0905 and summary of EC0905 doses given

<table>
<thead>
<tr>
<th>Dog characteristics</th>
<th>Number</th>
<th>Age, median (range in years)</th>
<th>Gender</th>
<th>Neutered male</th>
<th>Spayed female</th>
<th>Breed</th>
<th>Body weight, mean ± SD (kg)</th>
<th>Tumor invading bladder wall</th>
<th>Tumor invading neighboring organs</th>
<th>Nodal metastases present</th>
<th>Distant metastases present</th>
<th>Prior therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dogs</td>
<td>10 dogs</td>
<td>12.6 (10.3–13.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.1 ± 9.8</td>
<td>10 dogs</td>
<td>4 dogs</td>
<td>1 dog</td>
<td>4 dogs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Number dogs</th>
<th>Number doses</th>
<th>Median doses/dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>3</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>0.225</td>
<td>5</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>0.25</td>
<td>6</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>0.27</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Reduced dose &lt;0.2a</td>
<td>2</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

*The starting dose in the dose-escalation plan was 0.2 mg/kg per week. In 2 dogs, the dose was reduced to less than 2 mg/kg (0.15–0.18 mg/kg) due to myelosuppression.*
Conception and design: D. Dhawan, L. Cheng, C.P. Leamon, D.W. Knapp
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Dhawan, J.A. Ramos-Vara, J.F. Naughton, L. Cheng, P.S. Low, C.P. Leamon, P.J. Klein, M.O. Koch, D.W. Knapp
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References


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Folate-Targeted Therapy in Bladder Cancer

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