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Clinical report

Drug retention following intravesical delivery of fluorouracil therapeutic adhesive in C3H mouse bladder

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We have developed a fibrinogen-based, sustained-retention drug delivery system, therapeutic adhesive (TA), for application to resected tumor beds to reduce local tumor recurrences. In this study we evaluated the feasibility, safety and retention of the TA formulated with 5-fluorouracil (5-FU TA) after intravesical administration in a mouse bladder model. Radiolabeled [^{14}C]5-FU TA or [^{14}C]5-FU solution was delivered intravesically to C3H/He female mice. After drug administration, retention of ^{14}C in the bladder was quantified by storage-phosphor autoradiography. A 2.6-fold increase in retention was observed with 5-FU TA when compared with 5-FU solution. The $AUC_{(2\ min–5\ h)}$ for 5-FU TA was 685 nmol h/mm^3 compared with 260 nmol h/mm^3 for 5-FU solution. No signs of toxicity in the bladder tissue or treatment-associated adverse affects were observed in the mice.

Key words: Bladder cancer, drug delivery, 5-fluorouracil, intravesical therapy, storage-phosphor autoradiography, therapeutic adhesive.

Introduction

Approximately 50 000 new cases of bladder cancer are diagnosed each year; 75% of these are superficial tumors confined to the epithelium of the inner bladder wall. The recurrence rates are high for these superficial tumors (i.e. 50–80%). Moreover, 10–25% of the superficial tumors progress to become muscle-invading cancer.[1]

Treatments for bladder cancer include surgery, radiation and systemic chemotherapy using drugs such as cisplatin, doxorubicin or 5-fluorouracil (5-FU), either as a single agent or in combination.[2] Intravesical drug therapy is provided after transurethral endoscopic resection to delay or prevent recurrence of bladder cancer. Agents that have been used in intravesical therapy include bacillus Calmette-Guérin,[3–6] mitomycin C,[7,8] thiotepa[9,10] and interferon-α.[7] Recently, cytokine gene-modified tumor vaccines have been evaluated preclinically in a mouse MBT-2 bladder tumor.[11] In many cases, using intravesical drug therapy significantly reduces recurrence rates; however, adverse effects such as cystitis, fever and myelosuppression often occur and may limit the ultimate success of this approach.[3,4,10]

Intravesical therapy is performed by instilling a drug solution into an empty bladder and retaining the solution in the bladder for up to 2 h. In an effort to increase retention time in the bladder, we have adapted a fibrinogen-based drug delivery system (developed earlier for other indications) to treat superficial bladder tumors. This delivery system, known as therapeutic adhesive (TA), is a viscous composite of highly purified bovine collagen, human fibrinogen and a chemotherapeutic agent such as 5-FU. It was originally designed for application at surgical sites after tumor resection.[12,13] The objective of the TA delivery system is to increase exposure of the diseased tissue to the chemotherapeutic agent. This is accomplished by providing therapeutic concentrations of drugs at the target site for extended periods while minimizing systemic toxicity. Previous studies with 5-FU containing TA (5-FU TA) demonstrated the sustained drug-retention property of the TA formulation *in vitro* and the delay of tumor development *in vivo*.[12–14] Also, application of 5-FU TA to dermal biopsy sites caused no apparent inhibition in the normal wound healing processes, which were assessed both morphometrically and histologically in a rabbit ear wound-healing model.[15]

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The objective of this study was to evaluate the feasibility, safety and drug retention of a 5-FU TA formulation when compared with a 5-FU solution after intravesical instillation in the mouse bladder. ^{14}C-radiolabeled 5-FU was used both in the TA formulation and in the aqueous 5-FU solution as a marker to assess local drug retention. Storage-phosphor autoradiography was used to visualize and quantify the radiolableled drug within the bladder.

Materials and methods

5-FU TA

5-FU TA was prepared from 5-FU solution (Lyphomed, Deerfield, IL), an aqueous gel containing purified bovine collagen (Matrix Pharmaceutical, Fremont, CA) and human fibrinogen (American Red Cross, Rockville, MD). The three components were thoroughly mixed immediately before use by means of a syringe-to-syringe transfer back and forth through a connecting mixing adapter to yield the following component concentrations: 5-FU, 30 mg/ml; collagen, 18 mg/ml; and fibrinogen, 3 mg/ml. In the autoradiography studies, drug concentration was assessed by adding [2-^{14}C]5-FU (56 mCi/mmol; American Radiolabeled Chemicals, St Louis, MO) to both the TA formulation and a 30 mg/ml 5-FU solution as the control. The resultant specific activity in each of the radiolabeled test agents was approximately 8.7 μCi/mmol and the concentration was approximately 2.0 μCi/ml.

Mice

Inbred C3H/He female mice (Charles River, Hollister, CA) were used for these experiments. The mice were approximately 4 months old with an average body weight of 25 g. The mice were maintained in isolator cages with a diurnal cycle of a 12 h day and 12 h night. Food and water were available *ad libitum*

Intravesical instillation

Mice were anesthetized intramuscularly with 20 μl of a 4:1 mixture of ketamine–HCl solution (100 mg/ml; Fort Dodge, Fort Dodge, IA) and xylazine solution (10 mg/ml; Mobay, Animal Health Division, Shawnee, KS). Dosing of the test drug formulation was performed by transferring material to a 250 μl Hamilton syringe and then attaching a 30 gauge needle covered with surgical microtubing. Under a magnification lamp, the needle was carefully inserted through the urethra into the mouse bladder. Then 50 μl of either [^{14}C]5-FU TA or [^{14}C]5-FU solution was slowly injected into the bladder (equivalent to approximately 60 mg/kg of 5-FU or 0.1 μCi of ^{14}C).

Cryosectioning and storage-phosphor autoradiography

Two mice were used for each of the five monitored time points in the radiography studies: 2 min and 0.5, 2, 3 and 5 h. At the designated time points, the mice were sacrificed, and their bladders were quickly ligated, excised and immediately sprayed with Histofreeze (Fisher Scientific, Millsburg, PA); they were then stored at −20°C until cryosectioning.

Frozen tissue samples were mounted onto cryostat chucks with tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) then sectioned sagittally at a thickness of 20 μm. Bladder sections were collected at about 100 μm increments using a cryostat (Microm HM 505E; Microm, Walldorf, Germany) at −25°C. Approximately 15–27 sections per bladder were obtained. Tissue sections were gently manipulated with a fine brush into microscopic slides; these were allowed to dehydrate at room temperature before exposure to the imaging plates.

The slides containing bladder tissue sections were placed in autoradiography cassettes and exposed onto phosphor-coated imaging plates (BADIIIs, Fuji Photo Film, Japan) for 63 h.[16,17] A strip of authentic ^{14}C standards (American Radiolabeled Chemicals) was exposed along with the tissue sections. After exposure, the plates were scanned and the autoradiograms analyzed and quantified using a FUJI BAS 1000 Mac Bio-Imaging Analyzer (Fuji Medical Systems, Stamford, CT) with MacBas Version 1.0 software.

Quantification of autoradiograms

Individual autoradiograms of each series of bladder cross-sections (obtained at each time point) were read as digital data at a resolution of 200 μm per pixel and 8-bit pixel depth (256 gradations of intensity and color) using Canvas 3.5.1 software (Deneba Software, Miami, FL). The pseudo-color gradation (brightness/contrast scale) was kept con-

stant for all exposures. Image analysis was performed as previously described[17] by selecting a region of interest (ROI) around the visible autoradiogram or the entire area of the section. Instrument response was expressed as photostimulated luminescence (PSL) corrected for background radiation (PSL − BG). Instrument response can also be expressed as a function of the surface area of a selected ROI (PSL − BG/mm^2). The relationship between instrument response (PSL − BG or PSL − BG/mm^2) and radioactivity (d.p.m. or d.p.m./mm^2) is linear over several orders of magnitude ($r = 0.99$) and is defined by the following equation for a 63 h exposure:

$$\text{PSL} = 23.04 \cdot (\text{d.p.m.})^{0.7066}$$

Next, d.p.m. or d.p.m./mm^2 was converted to drug concentration in nmol/mm^3 based on the specific activity of the injected material and the thickness of the tissue section. Previous studies have shown no attenuation of radioactivity with this section thickness.[17] Data from the 15–27 sections obtained per bladder (i.e. per time point) were integrated and the concentration–time profiles thus obtained were plotted using KaleidaGraph 3.0.2 software (Abelbeck Software, Reading, PA). Areas under the concentration–time curves (AUCs) were calculated using the trapezoidal rule. Each curve was fitted for a biexponential decay and the drug half-life ($t_{1/2}$) was estimated from the terminal portion of the curve. The autoradiograms were also transferred to Spyglass Transform, version 3.0 (Spyglass, Savoy, IL), to construct the three-dimensional, framed, color surface plots.

Toxicity

In the toxicity screen, five mice were used in each group; the mice were observed for 4 weeks after a single treatment with unlabeled 5-FU TA or 5-FU solution for signs of weight loss, hair loss, lethargy or urinary tract obstruction. In addition, one mouse per group and an untreated control were sacrificed at day 5 for histologic analysis of bladder tissue.

Results

The direct intravesical instillation of 50 µl of either 5-FU TA or 5-FU solution into a mouse bladder was typically performed without complication. No obstruction of the urethra was observed and mice continued to urinate normally after treatment with the viscous TA formulation, perhaps indicative of the adherence of TA to the bladder wall.

The representative autoradiograms (Figure 1) indicate the amount of the radioactivity remaining in the mouse bladder sections after intravesical instillation of either [^{14}C]5-FU TA or [^{14}C]5-FU solution. An authentic strip of ^{14}C standards is shown for comparison. Tissue sections from the mouse bladders treated with the TA formulation showed higher levels and longer retention of radioactivity (detectable for up to 5 h) compared with bladders treated with 5-FU solution (radioactivity detectable for only 3 h).

The color surface plots (Figure 2) represent a three-dimensional view of the spread and intensity of radioactivity at various time points after intravesical instillation of radiolabeled 5-FU TA or 5-FU solution. Immediately (2 min) after the treatment, the distribution of the radioactivity, measured along the x- and y-axes, was broader for bladders receiving 5-FU solution. However, the intensity measured on the z-axis (representing 5-FU concentration) appeared to be higher in the central cavity in the mice treated with 5-FU TA. In the mice treated with 5-FU solution, the most intense radioactivity was detected along the bladder wall. Two hours after administration, a wider distribution of radioactivity was again measured in bladders treated with 5-FU solution than observed in those treated with 5-FU TA. The higher radiointensity and the decreased spread in bladders treated with 5-FU TA suggest a sustained, local retention of 5-FU when administered in the TA formulation.

Fluorouracil concentrations obtained by converting d.p.m./mm^3 to nmol/mm^3 were plotted as a function of time (Figure 3). The resultant pharmacokinetic data are summarized in Table 1. The $AUC_{(2\ min-5\ h)}$ values for bladders treated with 5-FU TA and 5-FU solution were 685 and 260 nmol h/mm^3, respectively. This 2.6-fold difference may indicate increased drug retention (and concomitant exposure of bladder tissue to 5-FU) using the TA formulation versus simple drug solution. The apparent clearance rates of drug from mouse bladders treated with 5-FU TA and 5-FU solution were 16.8 and 44.2 mm^3/h, and the estimated half-lives were 26 and 19 min, respectively.

In the 4-week toxicity screen, mice treated with either 5-FU TA or 5-FU solution showed no signs of weight loss, hair loss, lethargy or urinary tract obstruction. Histologic evaluation of bladder tissue sections 5 days after the intravesical instillation of either 5-FU TA or 5-FU solution showed no damage

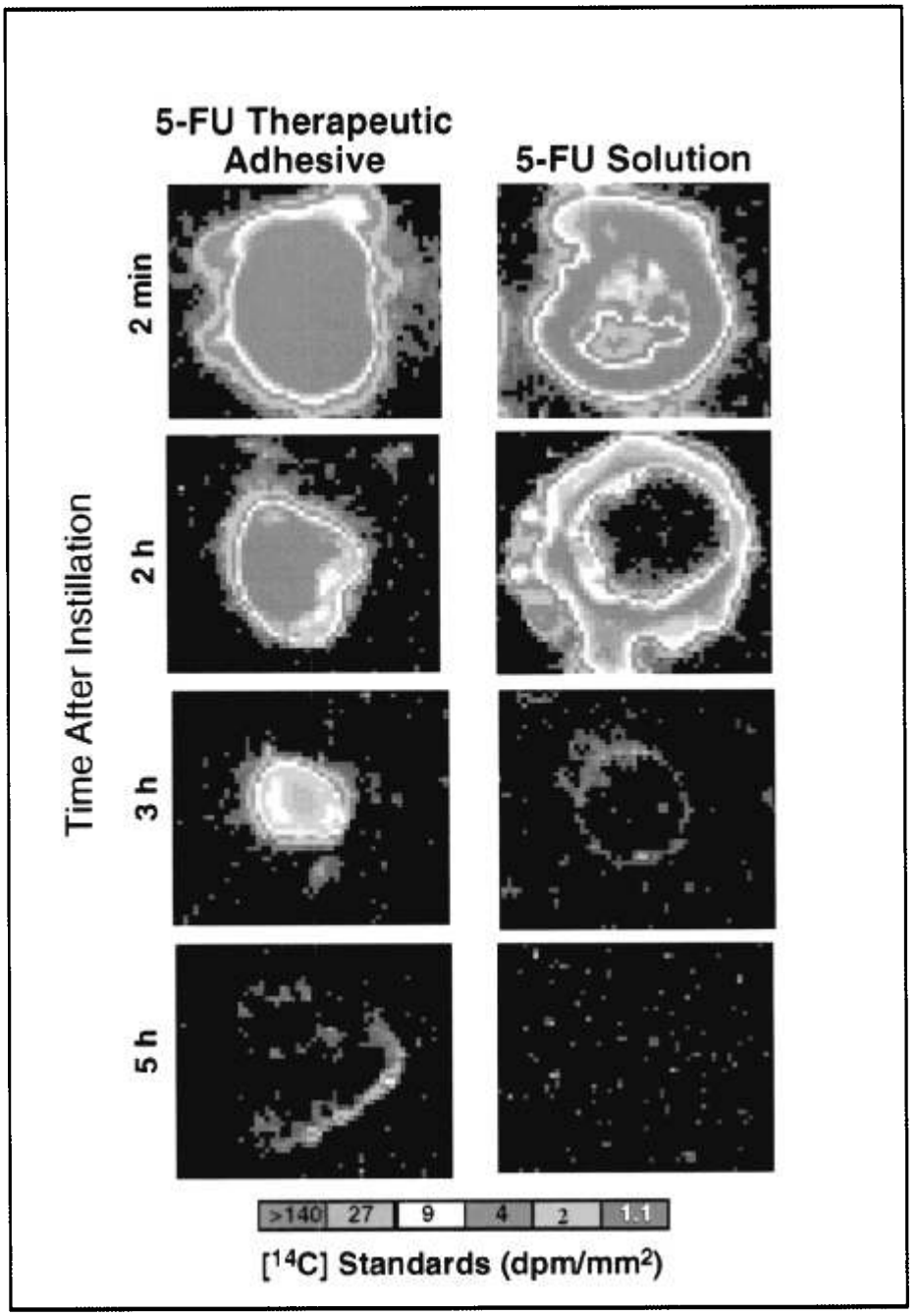

Figure 1. Autoradiograms showing ^{24}C retention in mouse bladder after treatment with [^{14}C]5-FU TA. The highest concentration of ^{14}C is indicated by intense red, the least in blue.

to the bladder urothelium or to the epithelial cells lining the bladder lumen (data not shown).

Discussion

Delivery of either 5-FU TA or 5-FU solution by inserting a needle coated with microtubing through the urethra into a mouse bladder was found to be feasible and safe. No obstruction of the urethra was observed and mice continued to urinate normally for the duration of the study after administration of 50 µl of the viscous TA formulation. The 2.6-fold increase in AUC value and the slower clearance rate observed in the TA-treated bladders indicate a sustained drug retention in the hydrodynamically

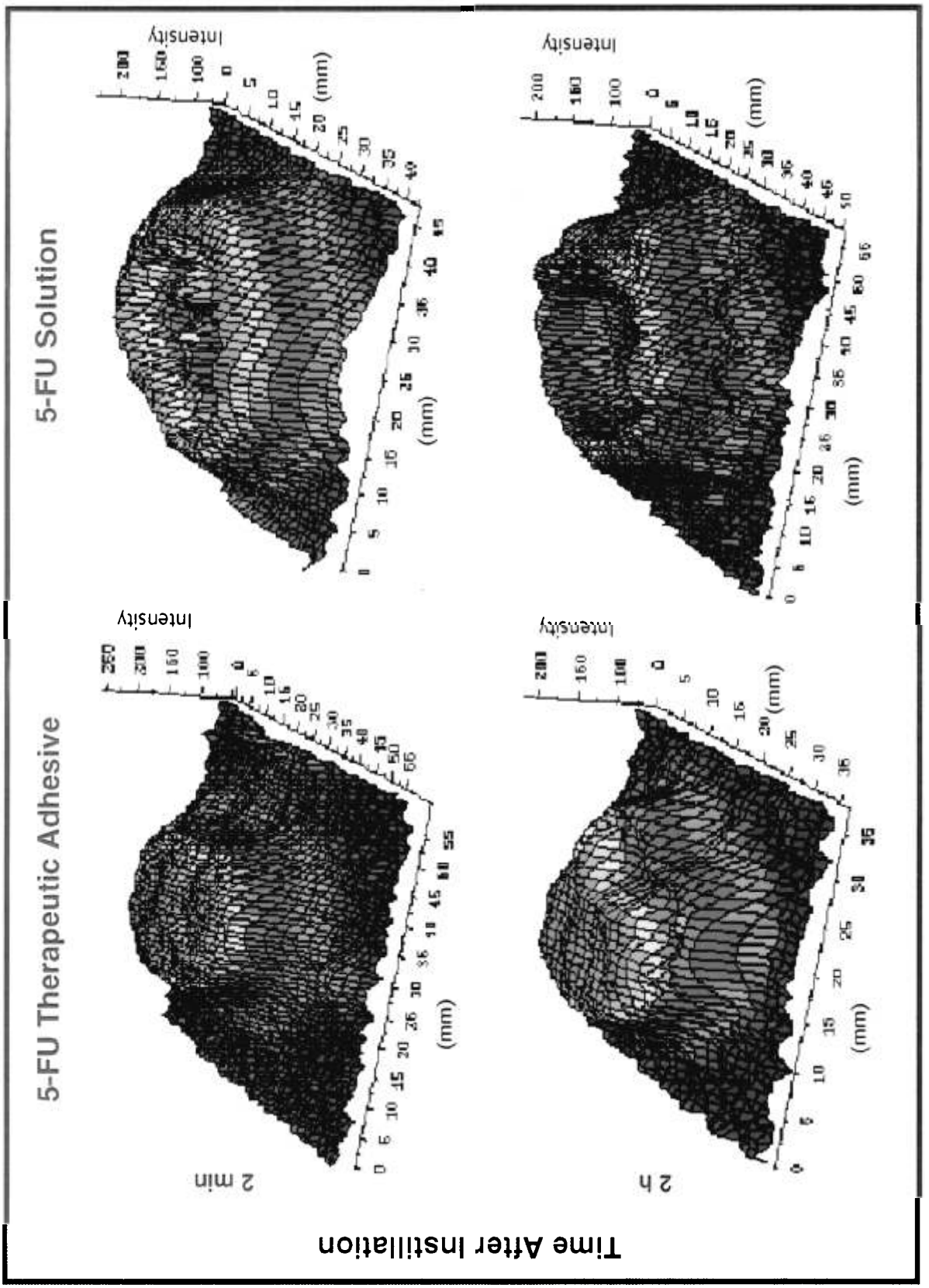

Figure 2. Three-dimensional surface plots showig intensity and distribution of radioactivity after a single dose or 5-FU TA of 5-FU solution delivered intravesically.

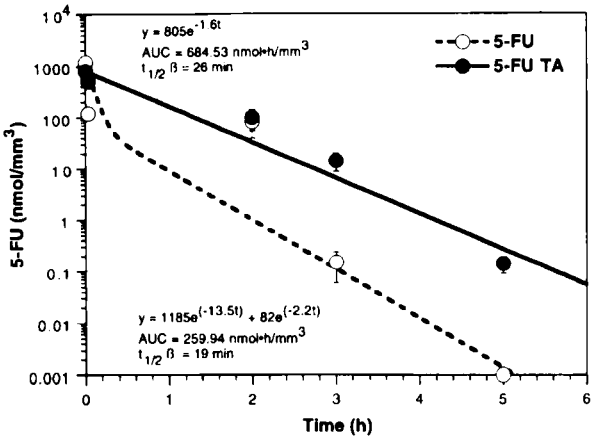

Figure 3. Time course of retention in mouse bladder after intravesical therapy.

Table 1. Summary of ^{14}C retention in mouse bladder after intravesical instillation of 5-FU TA or 5-FU solution

	$AUC_{(2\ min\text{-}5\ h)}$ (nmol h/mm^3)	Clearance[a] (mm^3/h)	$t_{1/2}$ (min)
5-FU TA	685	16.8	26
5-FU solution	260	44.2	19
Ratio (5-FU TA: 5-FU solution)	2.63	—	—

[a]Clearance = dose/AUC.

active environment of a urinary bladder. No signs of weight loss, hair loss or lethargy were observed.

Managing superficial bladder tumors often involves transurethral endscopic resection of the tumor followed by intravesical drug therapy.[18] Pharmacokinetic studies in patients with superficial bladder cancer have shown target site specificity with intravesical mitomycin C therapy following transurethral resection.[19] A preclinical study in dogs supports the advantage of intravesical therapy in maximizing local drug exposure while minimizing systemic exposure.[20] This pharmacokinetic advantage has also been demonstrated following intravesical therapy in patients in which drug connections in tumor-bearing bladder tissues were found to be at least 250-fold higher than those found in systemic host tissues.[21] Such data have been used to develop a model to predict drug exposure in tumors of the bladder wall and to correlate drug exposure with the potential antitumor effect. Is has been hypothesized that increasing the tissue exposure to drug would result in a 20% improvement that would increase the recurrence-free rate from 56 to 76%.[22]

Our intravesical treatment is predicated on earlier studies using a sustained-release injectable gel, locally administered, that increased drug exposure of the diseased tissue. With the use of this injectable gel in mouse solid tumor models, we have found that increased local drug retention correlates with increased antitumor efficacy.[23] The TA formulation is similar to injectable gel but includes fibrinogen to provide adhesive properties. We have demonstrated both enhanced 5-FU retention and antitumor efficacy in several mouse tumor models when 5-FU was delivered in the TA formulation rather than in a simple solution.[13,14]

5-FU has been used systemically to treat a murine carcinogen-induced bladder cancer without significant antitumor effect.[2] However, if the hypothesis that increasing drug exposure to the urothelium will lead to greater drug penetration and/or availability to superficial tumors is correct, improved efficacy rates should be feasible.[22] This preliminary study has shown that a TA drug delivery system can improve drug exposure to bladder tissue by enhancing local dose retention. Thus, the possibility exists that enhanced retention of 5-FU and other chemotherapeutic drugs in a therapeutic adhesive delivery system could result in increased efficacy.

Conclusion

Our current retention studies indicate that drug exposure to the bladder urothelium can be enhanced up to 2.6-fold using therapeutic adhesive drug delivery technology relative to that achieved by drug solution alone.

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