Pharmacodynamics of Doxorubicin in Human Bladder Tumors

Yuebo Gan, M. Guillaume Wientjes, Robert A. Badalament, and Jessie L-S. Au

ABSTRACT

Intravesical doxorubicin treatment delivers high drug concentrations to the bladder wall, yet the treatment produces only a variable and incomplete response in superficial bladder cancer and insignificant activity in muscle-invasive disease. This study evaluated the pharmacological basis for the clinical observations and potential prognostic indicators of tumor sensitivity to doxorubicin. The pharmacodynamics of doxorubicin were studied using histocultures of surgical specimens of seven superficial (Ta and T1) and nine invasive (T2-T4) tumors. After a 2-h exposure, drug treatment caused a concentration-dependent inhibition of proliferation, as measured by bromodeoxyuridine incorporation into DNA. Extensive cell death was observed at high concentrations (>10 μM) in sensitive tumors. The IC50 values ranged from 0.14 to 5.2 μM for superficial tumors and from 1.4 to >100 μM for invasive tumors. A comparison of the IC50 and IC90 values with the drug concentrations previously determined in human bladder walls showed that IC50 was achieved in all Ta tumors, 67-100% of T1 tumors, and one of the nine invasive tumors, whereas IC90 was achieved in all T2 tumors but none of the other tumors.

To determine the biological basis of variable doxorubicin sensitivity among different tumors, we examined the relationship of chemosensitivity with tumor pathology and with expression of multidrug resistance p-glycoprotein (Pgp) and p53 protein. The invasive, high-grade, highly proliferative, p53- and Pgp-positive tumors were more resistant than the superficial, lower-grade, p53- and Pgp-negative tumors. All tumors were negative for bcl-2. The rank order of these factors was p53 expression > tumor stage > grade > bromodeoxyuridine labeling index > Pgp. Statistical analysis using the Akaike Information Criterion indicates that the two-parameter combination of p53 expression with stage further improved the predictive value.

The present study shows that: (a) there was a >700-fold difference in doxorubicin sensitivity among superficial and invasive tumors; (b) the variable and incomplete response of superficial bladder cancer to intravesical doxorubicin therapy is likely due to the 35-fold variability in tumor chemosensitivity and, to a lesser degree, the 4-fold variability in tissue pharmacokinetics; (c) the lack of response of invasive cancer to intravesical doxorubicin therapy is likely the result of the inadequate drug concentrations presented to the deep muscle layers and the low chemosensitivity of the more aggressive tumors; and (d) the combination of p53 expression and high stage was the most significant predictor of doxorubicin sensitivity, whereas Pgp expression was the least important.

INTRODUCTION

Intravesical chemotherapy has been shown to be effective and is used in conjunction with transurethral resection to prevent recurrence and stage progression of superficial bladder cancer located in the urothelium and lamina propria. The three most commonly used agents are mitomycin C, doxorubicin, and thiotepa. The response rate of superficial cancer to intravesical therapy is highly variable, ranging from 2 to 43% for mitomycin C, 17 to 54% for doxorubicin, and 8 to 26% for thiotepa (reviewed in Ref. 1). In contrast, intravesical chemotherapy is ineffective and is not used against the higher-stage cancers, i.e., T2-T4 tumors that are located in the muscle layers.

Successful treatment requires delivery of effective drug concentrations to the target sites and sensitivity of tumor cells to the drug. For bladder cancer, the target tumor cells are the cells dislodged by surgery and implanted elsewhere in the bladder, the nonresected tumor cells, and the initiated cells in the bladder wall. These cells may be located at various depths in the bladder wall, i.e., urothelium, lamina propria, and superficial and deep muscle layers. Tissue pharmacokinetics, or drug concentration-time profiles at the different tissue layers, are expected to be critical determinants of treatment effectiveness.

Our laboratory has shown that the major causes of the variable and incomplete response to mitomycin C treatment are the variabilities in urinary concentration, drug penetration into tumor-bearing bladder tissues, and chemosensitivity of tumor cells (2-5). For doxorubicin, our completed studies established the urine and tissue pharmacokinetics of intravesical doxorubicin in patients and showed that the superficial bladder tissues were exposed to concentrations that were >2000 fold higher than those found in the systemic circulation, whereas the deeper muscular layers received 14-35-fold lower concentrations (6). There are no data to indicate whether the observed tissue concentrations are sufficient to produce an antitumor effect in the superficial and high-stage tumors.

The first goal of the present study was to establish the effective doxorubicin concentrations against superficial and muscle-invasive human bladder tumors and to compare these concentrations with the drug concentrations achieved in bladder tissues to determine the causes of variable and incomplete responses of superficial disease and the different responses of...
the superficial and muscle-invading diseases to intravesical doxorubicin therapy.

The second goal was to examine the biological basis of the variable doxorubicin sensitivity among patient tumors. The following factors were studied.

**Tumor Aggressiveness.** We previously reported an inverse relationship between tumor aggressiveness (i.e., stage, grade, and proliferation rate) and tumor sensitivity to mitomycin C (7).

**Expression of Multidrug Resistance Pgp.** The role of Pgp in bladder tumor sensitivity to doxorubicin is controversial. Native or acquired resistance of human bladder cell lines and human bladder tumors to doxorubicin has been linked to increased Pgp expression (8, 9). In contrast, other reports indicate that Pgp expression alone does not explain the differences in doxorubicin activity in bladder cell lines and human tumors (10, 11), and a recent study showed no correlation between Pgp expression in patient tumor specimens and the subsequent patient response to intravesical doxorubicin therapy (12).

**p53 Status.** A relationship between p53 gene mutation and chemoresistance has been shown in a number of human cancers (reviewed in Ref. 13). Fibrosarcoma cells expressing wild-type p53 were found to be sensitive to doxorubicin, whereas those with p53 deficiency or mutated p53 were resistant (14).

**bcl-2 Expression.** The expression of bcl-2 is known to correlate with chemoresistance (reviewed in Ref. 15). Transfection of lymphocytic leukemia cells, neuroblastoma cells, and malignant glioma cells with the bcl-2 gene confers resistance to chemotherapeutic drugs, including doxorubicin (16–18), whereas antisense-mediated reduction of bcl-2 gene expression in non-Hodgkin’s lymphoma cells increases chemosensitivity (16, 19).

The present studies required the evaluation of drug sensitivity in individual patient tumors. We used histocultures of the surgical bladder tumor specimens for this purpose. The major advantages of the histoculture system are the maintenance of a three-dimensional tissue architecture, cell-cell interaction, and intertumoral and intratumoral heterogeneity (20). The use of tumors from individual patients allows evaluation of the relationship between tumor characteristics and chemosensitivity. Robbins et al. (21), Furukawa et al. (22), and Kubota et al. (23) have shown in retrospective and semiprospective preclinical and clinical studies that a drug response assay in human tumor histocultures, using inhibition of DNA precursor incorporation or inhibition of metabolic reduction of tetratolium dye as an end point, correlates with the sensitivity, resistance, and survival of head and neck, colorectal, and gastric cancer patients who have been treated with mitomycin, doxorubicin, 5-fluorouracil, or cisplatin.

**Materials and Methods**

**Chemicals and Supplies.** Doxorubicin was a gift from Adria Laboratories (Columbus, OH) or was purchased from Sigma Chemical Co. (St. Louis, MO). Sterile pigskin collagen (Spongostan Standard) was purchased from Health Designs Industries (Rochester, NY), BrdUrd from Sigma, cefotaxime sodium from Hoechst-Roussel (Somerville, NJ), gentamicin from Solo Pak Laboratories (Franklin Park, IL), and MEM from Life Technologies, Inc. (Grand Island, NY). Antibodies against BrdUrd, p53 (DO7), and Pgp (JSB-1) were obtained from BioGenex (San Ramon, CA); bcl-2 antibody and a labeled streptavidin-biotin detection kit were from DAKO Corp. (Carpinteria, CA); and Pgp polyclonal antibody (ab-1) was from Oncogene (Cambridge, MA). All chemicals were used as received.

**Tumor Specimens.** Primary human bladder tumors were obtained via transurethral resection or cystectomy. Tissues were provided by the Tumor Procurement Service at The Ohio State University Comprehensive Cancer Center. Ten tumors were from The Ohio State University James Cancer Hospital, and 11 tumors were from the neighboring Riverside Methodist Hospital. The tumor grade and stage were determined by the pathology departments in the two institutions. The university pathology department classified the tumors as defined by Pauli et al. (24), whereas the Riverside pathology department used the WHO classifications (25). Tumor specimens were placed in medium within 10–30 min after surgery and maintained at 4°C until use. Patient and tumor data are listed in Table 1. All patients were chemotherapy naive and did not receive doxorubicin treatment for their disease management.

**Histocultures.** Histoculture of tumors was performed as described previously (2, 7). In brief, tumor specimens were cut to about 1 mm³. Four to six tumor pieces were placed on a 1-cm² presoaked collagen gel and cultured in six-well plates in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The culture medium consisted of MEM supplemented with 9% heat-inactivated fetal bovine serum, 0.1 mm nonessential amino acids, 100 µg/ml gentamicin, and 95 µg/ml cefotaxime. The pH of the medium was 7.4. After culture for 3 or 4 days, the tumors were used for pharmacodynamic studies.

**Pharmacodynamic Studies.** The antiproliferation effect was measured by the inhibition of BrdUrd incorporation in tumor cells. Tumor histocultures were exposed to various concentrations of doxorubicin ranging from 0.01 to 100 µM for 2 h. The exposure time is equivalent to the duration of drug instillation for intravesical therapy in patients. After drug treatment, the medium was exchanged, and the tumors were washed three times with 5 ml drug-free medium each. Tumors were incubated with 40 µM BrdUrd for 48 h then fixed in 10% neutralized formalin and embedded in paraffin. The embedded tissues were cut into 5-µM sections using a microtome, deparaffinized, and stained for BrdUrd incorporation. Controls were processed similarly, with the exception of drug treatment. Tissue sections were examined microscopically, the BrdUrd-labeled tumor cells were scored, and the fractions of labeled cells (LI) were determined. A typical experiment used a total of 12–24 tumor pieces for each drug concentration. Approximately 200 cells/tumor piece, or >1500 cells, were counted per concentration. Eight to 12 pieces for each concentration or 48–108 tumor pieces per...
depths in the bladder wall. A comparison of the IC values of tissue pharmacokinetic data established in our previous studies whether the tumors are likely to receive effective drug concentration to the experimental data: tumors with the bladder tissue pharmacokinetics will indicate tumors of different stages and, therefore, located in different present study provided the IC values for Depth Profile. The theoretical basis for the urothelium (0-200 μm), and Eq. C describes the concentrations for computer simulations to generate the bladder tissue pharmacokinetic models and the experimental procedures for IC50 and IC90 were determined.

For computer simulations are detailed in a previous publication (4).

**Table I** Patient and tumor characteristics and tumor sensitivity to doxorubicin

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender/age</th>
<th>Surgical procedure</th>
<th>Stage</th>
<th>Grade</th>
<th>Control LI (%)</th>
<th>IC50 (μM)</th>
<th>IC90 (μM)</th>
<th>Pgp</th>
<th>p53*</th>
<th>bcl-2</th>
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<tbody>
<tr>
<td>1</td>
<td>M /85b</td>
<td>TURb</td>
<td>T1</td>
<td>I</td>
<td>41.3</td>
<td>1.4</td>
<td>34.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>F /68</td>
<td>TUR</td>
<td>T2</td>
<td>II</td>
<td>51.5</td>
<td>1.6</td>
<td>77.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>3</td>
<td>M /80</td>
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<td>T3b</td>
<td>III</td>
<td>45.6</td>
<td>5.4</td>
<td>72.4</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>M /64</td>
<td>TUR</td>
<td>T3</td>
<td>II</td>
<td>41.9</td>
<td>1.5</td>
<td>58.9</td>
<td>–</td>
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</tr>
<tr>
<td>5</td>
<td>F /S5</td>
<td>Cystectomy</td>
<td>T1a</td>
<td>III/IV</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>6</td>
<td>M /75</td>
<td>TUR</td>
<td>T2a</td>
<td>III/IV</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>7</td>
<td>F /84</td>
<td>TUR</td>
<td>T3</td>
<td>II</td>
<td>39.8</td>
<td>33.6</td>
<td>&gt;100</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>M /61</td>
<td>TUR</td>
<td>T2</td>
<td>III</td>
<td>38.3</td>
<td>3.8</td>
<td>96.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>9</td>
<td>M /76</td>
<td>TUR</td>
<td>T3</td>
<td>II</td>
<td>61.8</td>
<td>5.2</td>
<td>68.2</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>10</td>
<td>M /61</td>
<td>Cystectomy</td>
<td>T3b</td>
<td>III</td>
<td>29.6</td>
<td>3.9</td>
<td>79.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>11</td>
<td>M /60</td>
<td>TUR</td>
<td>T3</td>
<td>I</td>
<td>26.7</td>
<td>0.14</td>
<td>0.88</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>12</td>
<td>M /56</td>
<td>TUR</td>
<td>T3</td>
<td>II</td>
<td>42.5</td>
<td>4.8</td>
<td>40.6</td>
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<td>M /66</td>
<td>TUR</td>
<td>T3</td>
<td>I</td>
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<td>0.57</td>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>M /76</td>
<td>TUR</td>
<td>T3</td>
<td>I</td>
<td>32.7</td>
<td>0.28</td>
<td>9.9</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>15</td>
<td>M /65</td>
<td>TUR</td>
<td>T2</td>
<td>II</td>
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<td>1.4</td>
<td>90.7</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>16</td>
<td>M /63</td>
<td>TUR</td>
<td>T3a</td>
<td>III</td>
<td>56.9</td>
<td>15.3</td>
<td>&gt;100</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- p53 expression in untreated controls.
- M, male; F, female; TUR, transurethral resection; –, negative; +, positive.
- Grade III according to Pan et al. (25).
- Grade III and IV (undifferentiated carcinoma) according to the WHO classifications (25).

**Pharmacodynamic Data Analysis.** The relationship of doxorubicin-induced inhibition of proliferation and drug concentration was analyzed by computer fitting the following equation to the experimental data:

\[ E = E_0 \times \left(1 - \frac{C}{K + C}\right) \]  

(A)

where \( E \) is the LI of drug-treated tissues, \( C \) is the drug concentration, \( E_0 \) is the LI of untreated controls, \( K \) is the drug concentration at one-half \( E_0 \), and \( n \) is a curve shape parameter. Values for IC50 and IC90 were determined.

**Computer Simulation of Drug Concentration-Tissue Depth Profile.** The present study provided the IC values for tumors of different stages and, therefore, located in different depths in the bladder wall. A comparison of the IC values with the bladder tissue pharmacokinetics will indicate whether the tumors are likely to receive effective drug concentrations for successful therapy. We used the urine and bladder tissue pharmacokinetic data established in our previous studies (6, 26) as input for computer simulations to generate the bladder tissue concentration-depth profile. The theoretical basis for the tissue pharmacokinetic models and the experimental procedures for computer simulations are detailed in a previous publication (4).

Eq. B describes the concentration-depth profile in the urothelium (0–200 μm), and Eq. C describes the concentrations in the deeper tissues that are perfused by capillaries.

\[ 0–200 \, \mu m: C_{\text{depth}} = C_s - \frac{C_s - C_{200}}{200} \times \text{depth} \]  

(B)

\[ 200–4000 \, \mu m: C_{\text{depth}} = (C_{200} - C_s) \times e^{-\frac{0.059}{w_{v0}}} \times (\text{depth} - 200) + C_s \]  

(C)

where \( C_s \) is the concentration of unionized drug in the bladder cavity, \( C_{200} \) is the concentration at the interface between the urothelium and the deep tissues, \( C_p \) is the tissue concentration in equilibrium with the perfusing blood, and half-width (\( w_{v0} \)) is the thickness of tissue over which the drug concentration declines by 50%. Within the urothelium, the decline in \( C_{\text{depth}} \) is linear with depth and depends on the drug partition and diffusion (which are dependent on the ionization and lipophilicity of the drug) and the concentration gradient across the urothelium. Note that the tissue concentration-depth profile can be generated with the knowledge of \( C_s \) and \( w_{v0} \), the ratio of \( C_s/C_{200} \), and the ratio of \( C_p/C_s \) varies for individual patients and changes with time (see below). \( w_{v0} \) and the ratios of \( C_s/C_{200} \) and \( C_p/C_s \) are determined by the physicochemical property of the drug and the drug removal by blood flow. These are constant parameters for a given drug in a given system and were determined experimentally for doxorubicin in bladders of 10 patients who received 40 mg/20 ml intravesical doxorubicin for 1–2 h immediately before radical cystectomy (26). The median values were 40 for the \( C_s/C_{200} \) ratio, 534 μM for \( w_{v0} \), and 1010 for the \( C_s/C_p \) ratio, respectively.

Because the urine concentrations and, thus, the tissue concentrations are not constant with time, the initial drug concentration could not be used as measure of drug exposure. A measure of total exposure, i.e., the product of concentration and exposure time \((C \times T)\) was developed. To generalize \( C \times T \) from a situation in which drug concentrations are constant with time (e.g., under culture conditions) to a situation in which concentrations change with time (e.g., dilution by urine), \( C \times T \) is calculated as the time integral of \( C \) over the instillation period, \( T_{\text{inst}} \) (Eq. D). The drug exposures corresponding to 50 or 90% inhibition are denoted as \( C \times T_{50} \) and \( C \times T_{90} \), respectively.

\[ C \times T = \int_{0}^{T_{\text{inst}}} C \times d(time) \]  

(D)
The C_v values are needed to generate the profiles. We used the urine pharmacokinetic data determined in eight superficial bladder cancer patients who received six weekly intravesical treatments of 40 mg/20 ml doxorubicin, starting within days after transurethral tumor resection. Three profiles were generated using the highest, median, and lowest values of the urine concentration-time data.

**Computer Simulations.** The simulations were done using an IBM-compatible, Intel 486 microprocessor-equipped personal computer (Gateway, North Sioux City, SD). Simulations used numerical integration, over 5-min discrete time intervals, programmed in SAS Basic language.

**Immunohistochemistry.** Adjacent sections of tumors were used to simultaneously detect BrdUrd, Pgp, p53, and bcl-2 by immunohistochemical staining. The immunohistochemical methods were performed as described previously (27). Briefly, six continuous sections were mounted in a configuration of two rows and three columns on each slide. After dewaxing and rehydration sequentially in xylene, ethanol, and water, the tissue sections were boiled for 5 min in a 0.01 M sodium citrate solution (pH 6.0) in a microwave oven then cooled and washed in PBS. After wiping off the excess PBS, lines were drawn on the glass slide between the tissue sections using a paraffin marker. The paraffin lines created a barrier that confined the different antibody solutions applied to different tissue sections. The tissue sections were incubated with DAKO blocking solution for 10 min and subsequently with one of the following antibody solutions for 2 h: a mouse anti-BrdUrd monoclonal antibody (1:250 dilution); a rabbit antihuman Pgp antibody (JSB-1, 1:200 dilution); a rabbit antihuman Pgp polyclonal antibody (ab-1, 1:100 dilution); a mouse antihuman p53 antibody (DO7, 1:100 dilution); and a mouse antihuman bcl-2 antibody (1:50 dilution). The incubation was carried in a humidified chamber at room temperature. The antibodies were diluted in PBS containing 5 mg/ml BSA. After washing with PBS, the tissue sections were covered with the linker solution and then with peroxidase-conjugated streptavidin solution. After washing twice with PBS, tissue sections were incubated for 5–7 min with the chromogen diaminobenzidine and counterstained with hematoxylin. The negative controls used mouse IgG as the primary antibody. For positive controls, we used human adrenal gland for Pgp, tumor-infiltrating lymphocytes for bcl-2, and human head and neck FaDu cancer cells, which are known to overexpress mutant p53, for p53. The tumors that showed nuclear staining in more than 10% of tumor cells were considered p53 positive (28).

**Statistical Analysis.** The differences in mean values between groups were analyzed using an unpaired Student’s t test when the SDs were of similar magnitude and by the Wilcoxon two-sample test when otherwise. Software for statistical analysis (NPAR1WAY and TTEST procedures) was by SAS (Cary, NC). Multiple comparisons used one-way ANOVA testing with posthoc t test comparison (GLM procedure). Predictive relationships between tumor pathological parameters and tumor chemosensitivity were evaluated by linear regression analysis using the “maximal r^2” selection method and the REG software routine of SAS. This method determines which model has the highest coefficient of determination for combinations of predictors. An accepted principle of development of a model is to select the simplest model that gives a good description of the data (29). Generally, an increase in model complexity or number of predictors increases the goodness of fit or r^2. We used the AIC to balance model simplicity and goodness of fit (29).

**RESULTS**

**Histocultures of Bladder Tumors.** Twenty-one specimens of bladder carcinoma were evaluated. Sixteen tumors were successfully cultured, yielding sufficient viable tumor cells (>200 cells/fragment) for pharmacodynamic evaluation. The other five tumors either failed to incorporate BrdUrd, were contaminated, or did not contain sufficient tumor cells. Factors that appeared to favor tumor cell proliferation in histoculture were: (a) presence of a high fraction of tumor cells in the tissue; (b) small tumors; and (c) peripheral portions of a tumor. All of the Tp and Tp1 tumors were small, with a high fraction of tumor cells, and were successfully cultured.

The 48-h cumulative BrdUrd LI of the 16 histocultures was 45 ± 18% (mean ± SD). These values are comparable to the LI of 40 ± 23% in 39 bladder tumor histocultures determined using a 96-h labeling by [3H]thymidine, as reported in our previous study (7).

**Pharmacodynamics of Doxorubicin.** Doxorubicin produced a sigmoidal, concentration-dependent inhibition of the BrdUrd LI (Fig. 1). Extensive cell death was observed at the higher drug concentrations, i.e., more than 25-fold greater than the IC_{50} (data not shown). The sensitivity of individual tumors to the antiproliferative effect of doxorubicin varied, with a >700- and >100-fold range for IC_{50} and IC_{90}, respectively. Table 1 summarizes the IC values. At the highest concentration of 100 μM, doxorubicin inhibited proliferation by 90–100% in 10 of 16 tumors, between 70 to 85% in 4 tumors, and between 10 to 30% in 2 tumors.

**Comparison of IC Values with Bladder Tissue Pharamacokinetics.** Tumors of different stages are located in different tissue depths in the bladder wall, i.e., Tp tumors are in the...
urothelium, T1 tumors are in the lamina propria, T2 tumors are in superficial muscle layers, and T3 and T4 tumors are in deep muscle layers. Fig. 2 compares the drug exposure at different tissue depths in human bladders with the $C \times T_{50}$ and $C \times T_{90}$ for tumors of different stages. $C \times T_{50}$ and $C \times T_{90}$ are modifications of IC$_{50}$ and IC$_{90}$ that take into account the duration of drug exposure (see "Materials and Methods"). For T$_{50}$ tumors, the median exposure in the bladder wall exceeded the $C \times T_{50}$ and $C \times T_{90}$, indicating that, on average, all four T$_{50}$ tumors would receive sufficient drug exposure to produce a 50 and 90% inhibition. For T$_{90}$ tumors, 67% (two of three) received $C \times T_{90}$, but none received $C \times T_{90}$. The T$_{2}$-T$_{4}$ tumors received insufficient exposure for a 50 or 90% inhibitory effect. We previously found a 4-fold intersubject variability in urine pharmacokinetics (6). The expected 4-fold range in tissue concentrations is reflected by the highest and lowest concentration-depth profiles in Fig. 2. This pharmacokinetic variability slightly altered the expected treatment efficacy; the fraction of T$_{50}$ and T$_{90}$ tumors that received $C \times T_{50}$ increased from 86% (six of seven) for the lowest profile to 100% (seven of seven) for the highest profile; the fraction of T$_{2}$ tumors increased from 0 to 25% (one of four); and the fractions of T$_{3}$ and T$_{4}$ tumors remained unchanged at 0%.

Relationship of Drug Sensitivity and Tumor Pathology.

The relationship between the doxorubicin-induced antiproliferation effect and tumor grade, stage, and proliferation rate was examined. Clinically, T$_{0}$ and T$_{1}$ tumors are considered superficial disease, whereas T$_{2}$-T$_{4}$ tumors are considered invasive disease. The mean IC$_{50}$ for the superficial tumors was 1.53 ± 1.73 μM, which was significantly lower than the mean IC$_{50}$ of 29.8 ± 41.0 μM in the invasive tumors ($P < 0.01$). Grade III tumors showed a higher IC$_{50}$ of 33.4 ± 42.3 μM compared with the IC$_{50}$ values of 0.61 ± 0.69 and 2.05 ± 1.81 μM for the grades I and II tumors, respectively ($P < 0.01$). The IC$_{50}$ showed a weak correlation with the LI ($r^2 = 0.17; P = 0.12$). IC$_{50}$ data showed similar relationships with tumor grade, stage, and LI as the IC$_{50}$ data (not shown).

Pgp, p53, and bcl-2 Expression. Four of the sixteen tumors stained positive for Pgp by both the monoclonal antibody JSB-1 and the polyclonal antibody ab-1 (Fig. 3, a and b). In general, the JSB-1 antibody gave a more intense staining than the ab-1 antibody. The staining was cytoplasmic and showed a granular pattern in two tumors and a diffuse pattern in the other two tumors. The cytoplasmic Pgp staining is consistent with a previous report (8). All Pgp-positive tumors were of high stage (T$_{2}$-T$_{4}$) and high grade (grade III). The mean IC$_{50}$ was 31.4 ± 46.0 μM for the 4 Pgp-positive tumors and 12.8 ± 29.0 μM for the 12 Pgp-negative tumors. This difference approached statistical significance ($P = 0.052$). The 12 Pgp-negative tumors remained Pgp negative after drug treatment.

p53 was detected in the untreated controls of four tumors, all of which were of high stage (T$_{2}$-T$_{4}$) and high grade (grade III), and included three of the most resistant tumors. The IC$_{50}$ values were 58.8 ± 49.4 μM for the p53-positive tumors and 3.7 ± 4.2 μM for p53-negative tumors ($P = 0.07$). Only one of the p53-positive tumors was also Pgp positive.

Doxorubicin treatment altered the p53 status. All 12 tumors that were p53 negative prior to drug treatment became p53 positive after treatment with doxorubicin. At the highest doxorubicin concentration of 100 μM, the fraction of p53-positive cells in individual tumors ranged from 10 to 100% (mean, 72%). For the four tumors that were p53 positive prior to drug exposure, doxorubicin treatment resulted in an increased fraction of p53-positive cells in three tumors from 37 to 97% and a decreased fraction in one tumor from 70 to 10%. Chemosensitivity did not correlate with the maximal fraction of p53-positive cells or with the fraction of p53-positive cells at IC$_{50}$.

bcl-2 expression was not detected in the 16 tumors, either before or after doxorubicin treatment. This suggests that bcl-2 expression is uncommon in bladder cancer and not inducible by doxorubicin.

Relative Ranking of Predictors of Doxorubicin Sensitivity. Table 2 shows the ranking of prognostic indicators of IC$_{50}$ and maximal inhibition, as determined by multivariate regression. As a single parameter, pretreatment p53 protein expression gave the highest coefficient of determination ($r^2$), indicating p53 as the most important determinant of the doxorubicin resistance. The rank order for single parameters was p53 > stage > grade > LI > Pgp.

We determined the best-fitting linear regression models with two or more fitting parameters. The combination of the two best single predictors, p53 expression and stage, improved the IC$_{50}$ prediction as determined by $r^2$. Other two-parameter combinations resulted in inferior $r^2$ values. Addition of further parameters contributed only marginally to the $r^2$ value and did not improve the model fit.
Doxorubicin Pharmacodynamics in Bladder Tumors

Fig. 3 Immunohistochemical detection of Pgp and p53. Staining of Pgp in human bladder tumors by JSB-1 (a) and ab-1 (b) antibodies. A bladder tumor (patient 14) showed no p53 expression in an untreated control (c) and p53 expression after treatment with 10 μM doxorubicin (d). Chromogen: diaminobenzidine (brown) counterstained with hematoxylin (blue).

Table 2 Correlation of tumor pathology and chemosensitivity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IC50</th>
<th>r²</th>
<th>AIC</th>
<th>P</th>
</tr>
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<tr>
<td>p53</td>
<td>0.5479</td>
<td>102.4</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>0.3760</td>
<td>107.9</td>
<td>0.01</td>
<td></td>
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<tr>
<td>Grade</td>
<td>0.2016</td>
<td>111.5</td>
<td>0.08</td>
<td></td>
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<tr>
<td>LI</td>
<td>0.1665</td>
<td>112.2</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Pgp</td>
<td>0.0624</td>
<td>114.1</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>p53 + stage</td>
<td>0.6318</td>
<td>101.2</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>p53 + stage + LI</td>
<td>0.6376</td>
<td>102.9</td>
<td>0.006</td>
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<tr>
<td>p53 + stage + LI + Pgp</td>
<td>0.6450</td>
<td>104.6</td>
<td>0.02</td>
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<td>0.6466</td>
<td>106.5</td>
<td>0.04</td>
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</tbody>
</table>

DISCUSSION

Pharmacological Basis for Variable and Incomplete Response. The present study showed a >700-fold range in the IC50 of doxorubicin in patient bladder tumors. In comparison, the variability in the IC50 of mitomycin C, another agent commonly used in intravesical therapy, was smaller at 120-fold (7). For both drugs, the chemosensitivity inversely correlated with the tumor aggressiveness, with the high-stage and high-grade tumors being more resistant. These data suggested that biological differences among well-differentiated superficial and undifferentiated invasive tumors contribute to differences in chemosensitivity.

Among the superficial T0 and T1 tumors, the variability in IC50 was about 35-fold, which is much greater than the 4-fold variability in urine and tissue concentrations of doxorubicin in patients shown in our previous study (26). In comparison, the variabilities in drug exposure and chemosensitivity of T0 and T1 tumors to mitomycin C are greater, at 20- and 70-fold, respectively (2-4, 7, 30). Data of the present study suggest that the variable and incomplete response of T0 and T1 tumors to intravesical doxorubicin therapy is mainly the result of the variability not appear to result in a better model. This was confirmed by AIC analysis. Table 2 lists the individual predictors and the two-, three-, four-, and five-parameter combinations with highest r² values.
in chemosensitivity, whereas the variability in urine and tissue concentrations is less important.

Intravesical chemotherapy is not used to treat invasive disease, in part because regional therapy does not offer control over disseminated disease and in part because of anecdotal observations of a low response of locally advanced disease to this treatment modality. Our earlier data on mitomycin C indicate that the latter observation may be due to the relatively low sensitivity of the advanced tumors and the inability of the drug to penetrate the deep tissues (2–4). Similar findings were made for doxorubicin, i.e., 20-fold higher IC values in the muscle-invading tumors than in the superficial tumors shown in the present study and a >10 fold lower drug exposure in the muscle layers compared with the urothelium and lamina propria shown in a previous study (26). Consequently, although most of the superficial tumors received sufficient drug exposure for a significant effect (all T1 tumors and 67% of T2 tumors received \( C \times T_{50} \)), nearly all the invasive tumors did not (0–25% of the T3 tumors and none of the T4 tumors received \( C \times T_{50} \)). These data are consistent with the clinical observation of a poor response of invasive tumors to intravesical doxorubicin therapy.

There are two additional considerations relating to the above conclusions. First, although inhibition of DNA synthesis is generally believed to result in or correspond with a clinical response, the extent of inhibition required for such an effect is not known. Kubota et al. (23) have shown that the cutoff drug-induced inhibition in vitro for correlating with drug sensitivity in patients varies for different drugs. For example, a 90% inhibition by mitomycin C but a 70% inhibition by 5-fluorouracil correlated with survival in gastric cancer patients. Our conclusions were made under the assumption that a 50% inhibition, on six repeated weekly treatments as is commonly used in intravesical therapy, is sufficient to produce a response. If, in fact, a higher inhibition, e.g., 90%, is needed, the data in Fig. 2 would suggest that the T1 tumors cannot be effectively managed by intravesical doxorubicin therapy. Second, our projection of the fraction of responding tumors was based on a comparison of \( C \times T \) values, calculated using the drug concentration in the culture medium for the chemosensitivity study, with the tissue concentration-depth profiles. The \( C \times T \) values were not corrected for tissue partitioning, whereas the tissue concentrations were the actual, measured concentrations that had taken tissue partitioning into account. A correction for a tissue:plasma ratio of 22, as reported in the literature (31), would give much higher \( C \times T \) values and, consequently, lower fractions of responding tumors: i.e., three of four T1 tumors and all T1–T4 tumors would not receive \( C \times T_{50} \).

p53, Pgp Expression, and Chemosensitivity to Doxorubicin. Mutations of p53 and nuclear accumulation of p53 protein are significantly associated with the grades and stages of bladder tumors, with a lower frequency of p53 mutations in superficial, low-grade tumors (<20%) than in invasive, high-grade tumors (~60% Refs. 28, 32–34). In the present study, 44% (4 of 9) of the invasive tumors (stage T2 and above) and none of the 7 superficial tumors stained positive for p53. A separate, larger study in our laboratory found that 13% (3 of 23) of T1, T3, and T1 tumors were p53 positive. Our data show that p53 protein expression in untreated controls and the combination of p53 expression and high stage were the best predictors of doxorubicin sensitivity in human bladder tumors. Hence, p53 expression, in addition to its reported role as a good predictor of poor prognosis (28, 35–39), also may serve as a good predictor of doxorubicin resistance.

A recent report shows that doxorubicin can up-regulate a mutant form of p53 in a human squamous carcinoma A431 cell line (40), whereas other reports show that doxorubicin either did not alter or decreased the p53 expression in the human breast MCF-7 and T-cell leukemia MOLT4 cell lines and in human breast tumors (41, 42). Our finding of a posttreatment increase in p53 staining in 15 of 16 tumors, including the 12 p53-negative tumors before drug treatment, indicates that a short exposure to doxorubicin for 2 h was able to induce p53 protein expression in a high percentage of bladder tumors. We cannot determine from our results whether the induced p53 was of the wild type or mutant gene, because the antibody used for detection cannot distinguish the two forms.

The fraction of Pgp-expressing bladder tumors was 25% overall, 50% among high-grade tumors and 44% among invasive tumors (Table 1). These values are within the reported range of 13–75%, and the observation of a higher value in the high-grade and high-stage tumors is consistent with the data in the literature (8, 12, 43, 44). The Pgp-positive tumors showed higher IC values than Pgp-negative tumors, with the difference approaching statistical significance (\( P = 0.052 \)). Therefore, we suggested Pgp expression to be an indicator of bladder tumor sensitivity to doxorubicin.

Recent reports show significant increases in the fraction of Pgp-positive tumors from bladder cancer patients after multiple systemic and intravesical treatments by methotrexate, vinblastine, doxorubicin, and cisplatin (44, 45). Our results indicate that, unlike p53 expression, Pgp was not induced by a single, short-term treatment with doxorubicin. Hence, the increase in Pgp-positive tumors observed in previous studies may be due to the selective pressure brought on by coadministered agents and/or multiple doxorubicin treatments.

Conclusions. The data of our present and previous studies (2, 4, 7, 30) indicate that the variable and incomplete response of superficial bladder cancer patients to intravesical therapy by doxorubicin and mitomycin C is likely due to variabilities in tumor chemosensitivity and urine and tissue pharmacokinetics, and that the historical finding of a poor and lower response of invasive tumors to intravesical treatment by these drugs, when compared with superficial tumors, is likely due to the differences in chemosensitivity among the two subsets of tumors and the inability of these drugs to reach effective concentrations in invasive tumors. Chemosensitivity of individual tumors is a function of the tumor and the drug, whereas the tissue pharmacokinetics is a function of the physicochemical properties of the drug and the physiological factors that determine drug removal from the tissue. Exploitable pharmacological

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4 J. Kalns, Y. Gan, and J. L-S. Au, unpublished results.
approaches to improve the management of locally advanced disease include: (a) identification of agents that are effective against the more aggressive tumors; (b) identification of agents that readily penetrate the deep bladder tissues; and (c) use of absorption enhancers to decrease the barrier function of the urothelium and therefore to increase the drug exposure in deep tissues. Prognostic indicators such as p53 expression and tumor stage may be used to discriminate likely doxorubicin responders from nonresponders.

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Pharmacodynamics of doxorubicin in human bladder tumors.

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