Expression of DT-Diaphorase and Cytochrome P450 Reductase Correlates with Mitomycin C Activity in Human Bladder Tumors

Yuebo Gan, Yiqun Mo, John E. Kahns, Jie Lu, Katherine Danenberg, Peter Danenberg, M. Guill Wientjes, and Jessie L-S. Au

College of Pharmacy [Y. G., Y. M., J. E. K., J. L., M. G. W., J. L.-S. A.] and James Cancer Hospital and Solove Research Institute [M. G. W., J. L-S. A.]. The Ohio State University, Columbus, Ohio 43210, and University of Southern California, Los Angeles, California 90033 [K. D., P. D.]

ABSTRACT
Mitomycin C (MMC) is activated by DT-diaphorase (DTD) and cytochrome P450 reductase (P450R). In cancer cell lines, MMC cytotoxicity is correlated with DTD and P450R expression levels. The present study investigated the relationship between enzyme expression/activity and MMC cytotoxicity in patient bladder tumors. DTD and P450R expression was detected by competitive reverse transcription-PCR and their activity was measured by bioreductive assays. The expression of DTD and P450R in patient tumors (n = 29), as ratios to β-actin levels, varied from 0 to 90% and 0 to 29%, respectively. The DTD expression was significantly correlated with P450R expression (r², 0.32; P < 0.01), whereas the average DTD level was 2-fold higher than that of P450R (P < 0.01). Among the 29 tumors, 21 provided sufficient materials to evaluate tumor sensitivity to MMC. The concentration of MMC required to produce 50% inhibition (IC₅₀) of DNA precursor incorporation for a 2-h treatment ranged from 0.17 to 18.1 μg/ml, indicating a 110-fold intertumor variation, with the high-grade and more invasive tumors being less chemosensitive compared with the low-grade and less invasive tumors. Tumor sensitivity to MMC, as indicated by the inverse of IC₅₀ values, was positively correlated with the expression of DTD (r², 0.28; P < 0.05) and P450R (r², 0.26; P < 0.05). Multivariate analysis indicates DTD expression and P450R expression as better determinants of MMC activity compared with other pathological factors (e.g., tumor grade, stage, and labeling index) that have been shown to significantly correlate with MMC activity. Eleven tumors were studied for the relationship between gene expression level and enzyme activity of DTD and P450R. The DTD activity was significantly correlated with the gene expression level (r², 0.84; P < 0.001). For P450R, there is a trend of a correlation between enzyme activity and its mRNA level, but the correlation was not statistically significant (r², 0.28; P = 0.09). These data indicate that the sensitivity of patient bladder tumors to MMC is correlated with the expression of DTD and P450R in tumors and suggest that the lower expression of these enzymes in the high-grade and more invasive tumors is a cause of the lower efficacy of intravesical MMC in these tumors.

INTRODUCTION
Of the 50,000 new cases of bladder cancer each year in the United States, 80% present as superficial disease and 20% as invasive disease (1). The standard therapy for superficial bladder cancer is transurethral tumor resection. Tumor recurrence after surgery is about 60% (1). Intravesical chemotherapy after transurethral tumor resection reduces the tumor recurrence. The response rate of superficial cancer to intravesical therapy is highly variable, ranging from 2 to 43% for MMC (2). Our laboratory has shown that the major causes of the variable response to MMC treatment are the variability in drug delivery to tumor cells and chemosensitivity of the tumor cells (3–6). For example, histocultures of patient bladder tumors showed a 120-fold interindividual sensitivity to MMC (3), with the higher-grade and more invasive tumors being less sensitive (7). The molecular determinant for the variability in the sensitivity of patient tumors to MMC has not been established.

MMC is activated by one-electron reduction to a semiquinone radical and/or two-electron reduction to a hydroquinone. The one-electron reduction is mainly catalyzed by P450R (8), and the two-electron reductase is mainly mediated by DTD, also known as NAD(P)H:quinone oxidoreductase (9). Multiple studies have indicated a correlation between DTD and tumor sensitivity to MMC: (a) the expression of DTD gene and its enzyme activity correlate with MMC sensitivity in multiple cancer cell lines and lung tumor xenografts (10–15); (b) transfection of the DTD gene into the DTD-deficient and MMC-resistant gastric cancer cell line St-4 enhances the MMC activity by 5- to 10-fold (10); and (c) induction of MMC-resistance in non-small cell
lung cancer cells (PC-9 and LU99) and bladder cancer cells (J82) by continuous exposure to MMC or its analogues is accompanied by decreased DTD activities (12–14). The relationship between DTD and MMC activation is complex. The expression of DTD is induced by multiple drugs including MMC, possibly involving the AP-1 promoter (16) and the nuclear factor xB response element (17). On the other hand, high levels of DTD protect cells from MMC toxicity (reviewed in Ref. 18). Single nucleotide polymorphism in the DTD gene results in altered enzyme function. For example, a C to T change at the position 609 of DTD cDNA results in a greatly diminished DTD protein level and a >95% reduction of DTD activity (19, 20).

The role of P450R in the activation of MMC is less clear. Some studies indicate a relationship between P450R and MMC activity, including the findings that (a) transient transfection of monkey kidney COS1 cells with the P450R gene resulted in activation of MMC and detection of specific MMC-DNA adducts and interstrand DNA cross-linking (21); (b) resistance of Chinese hamster cells to MMC is related to decreased P450R activity (22); and (c) addition of exogenous P450R in the culture medium enhances the MMC activity in cultured cells (23). However, other studies have shown a lack of correlation between P450R activity and tumor sensitivity to MMC and MMC analogues (10, 13, 24).

Despite the rather extensive knowledge on the relationship between quinone reductases and MMC sensitivity in human cancer cells, it is not known whether the expression of these enzymes correlates with the MMC sensitivity in human solid tumors. The goal of the present study was to establish the extent of intertumor variability in the expression of DTD and P450R and whether the variability in the enzyme expression contributes to the variability of MMC sensitivity in human bladder tumors. We used three-dimensional histocultures of human bladder tumors to study the MMC effect. DTD and P450R expression in tumors was determined by competitive RT-PCR, and their activities were measured by bioreductive assays. The major advantages of the histoculture system are the maintenance of tissue architecture, cell-cell interaction, and inter- and intratumoral heterogeneity (25). The clinical relevance of the human tumor histoculture system has been demonstrated by Hoffman and colleagues (Robbins et al., Kubota et al., and Furukawa et al.; 26–28). These investigators have shown in retrospective and semiprospective preclinical and clinical studies that drug response in human solid tumor histocultures correlates with the sensitivity, resistance and survival of cancer patients to chemotherapy (26–28).

**MATERIALS AND METHODS**

**Chemicals and Supplies.** MMC was a gift from Bristol-Myers Squibb Inc. (Wallingford, CT). Sterile pig skin collagen (Spongostan standard) was purchased from Health Designs Industries (Rochester, NY), and [3H]thymidine (specific activity, 65 Ci/mmol) from Moravek Biochemicals Inc. (Brea, CA); BrdUrd, NADH, NADPH, dicumarol, cytochrome C, cytochrome C reductase, DCPIP, and Bicinchoninic Acid kit for Protein Determination from Sigma Chemical Co. (St. Louis, MO); cefotaxime sodium from Hoechst-Roussel (Somerville, NJ); gentamicin from Solo Pak Laboratories (Franklin Park, IL); and MEM from Life Technologies, Inc. (Grand Island, NY). Monoclonal antibodies against BrdUrd were obtained from BioGenex (San Ramon, CA), and the Labeled Streptavidin-Biotin Detection kit from Dako (Carpinteria, CA). The High Pure RNA Isolation kit and First Strand cDNA Synthesis kit from Boehringer Mannheim (Indianapolis, IN), the pGEM-T Easy Vector system and Riboprobe in vitro Transcription system from Promega (Madison, WI), and the Advantage CDNA Polymerase mix from Clontech (Palo Alto, CA). All of the chemicals and reagents were used as received.

**Tumor Specimens and Histocultures.** Specimens of human bladder tumors were placed in MEM within 10–30 min after surgery. Tumors from our institute was obtained from the Tumor Procurement Service at The Ohio State University Comprehensive Cancer Center and tumors from off-campus sources were shipped by overnight delivery services. Tumor grade and stage were determined by pathologists at individual institutions. All of the tumor specimens used in this study were from chemotherapy naive patients.

Histoculture of tumors was performed as described previously (29, 30). In brief, tumor specimens were cut to about 1 mm3. Four to six tumor pieces were placed on a 1-cm2 pre-soaked collagen gel and were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The medium was MEM supplemented with 9% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 100 mg/ml gentamicin, and 95 mg/ml cefotaxime, and had a pH of 7.4.

**Pharmacodynamic Studies.** The antiproliferative effect of MMC was measured by the inhibition of DNA precursor incorporation in tumor cells. Initial experiments used [3H]thymidine as the DNA precursor. On the basis of the finding that the two DNA precursors, BrdUrd and [3H] thymidine, labeled the same cells, which resulted in identical labeling indices in human bladder tumors (31), the nonradioactive BrdUrd was used in subsequent experiments.

Tumor histocultures were exposed to various concentrations of MMC ranging from 0.01 to 100 μg/ml 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 of drug-free medium. Tumors were labeled with [3H] thymidine (1 μCi/ml) or BrdUrd (40 μM) for 96 h, then fixed in 10% neutralized formalin and embedded in paraffin. The embedded tissues were cut into 5-μm sections using a microtome and deparaffinized, and then were processed for autoradiography for 4 days or were stained for BrdUrd incorporation by immunohistochemistry (29, 30). Controls were processed similarly, with the exception of drug treatment. Tissue sections were examined microscopically, the [3H]thymidine- or BrdUrd-labeled tumor cells were scored, and LI was determined.

The relationship of MMC-induced inhibition of proliferation and drug concentration was analyzed by computer-fitting the following equation to the experimental data.

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

(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 of $E_0$, and $n$ is a curve-shape parameter. Values for $IC_{50}$ and $IC_{90}$ were determined.

**Preparation of Internal Standard.** In competitive RT-PCR, internal standards are used to correct for the differences in the amplification efficiency. An ideal internal standard should have the same primer sites and consist of nearly identical DNA sequence as the target gene, with the exception of a small size difference to enable separation from the target gene product. Several methods have been used to develop internal standards, including site-directed mutation to create a restriction enzyme recognition site, introducing a spacer gene into the target sequence, and artificially synthesizing a short internal standard sequence, and the ideal difference to enable separation from the target gene product. Several methods have been used to develop internal standards, including site-directed mutation to create a restriction enzyme recognition site, introducing a spacer gene into the target sequence, and artificially synthesizing a short internal standard sequence.

The amplified sequences were cut with appropriate restriction enzymes, and the two ends were then ligated with ligase to yield the internal standards, which shared the same sequences as the target genes with the exception of the deleted sequences. The detailed procedures were as follows. For *DTD*, the two primers were DTDa, 5'-AGGCTGGTTGGAAGCTGTCG-3' and DTDb, 5'-ATTTGATGGAGTTGAAGGTAGTTTCGT-3'; these two primers amplified a 270-bp cDNA fragment that contains RSAl and *HaeIII* recognition sites separated by 44 bp. For *P450R*, the two primers were P450Ra, 5'-GAAGAGCTACGAGAACCAG-3' and P450Rb, 5'-ATCCAGGTTTCGCAAAGG-3'; these primers amplified a 259-bp cDNA fragment that contains two *HaeIII* recognition sites that were separated by 72 bp. For *β-actin*, the two primers were actina, 5'-GGCGGAAATCTGCTGGACTT-3' and actinb, 5'-GATGGAGTTGAAGGTAGTTTGCG-3', which amplified the 232-bp cDNA fragment that contains two *HaeIII* recognition sites, separated by 52 bp. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes. The amplified PCR products using the above-described primers were cut by the corresponding restriction enzymes.

**Measurement of Enzyme Activity.** Protein concentrations were determined using the Bicinchoninic Acid Kit. The activity of DTD and P450R was measured according to the methods of Benson et al. (33) and Smitskamp-Wilms et al. (13), respectively, with the following modifications. Briefly, aliquots of the supernatant fractions of tumor homogenates containing 0–50 μg of protein (for P450R) or 0–20 μg of protein (for DTD) in a volume of 100 μl were added in duplicate to the wells of a 96-well plate kept on ice. Blank samples without tumor extracts were used to correct for nonenzymatic reduction of substrates. The enzyme reaction was followed by monitoring the absorbance at 600 nm using a microplate reader. Enzyme activity was expressed as the rate of reduction of substrate per mg of protein for both DTD and P450R.

For DTD determination, the standard curve was constructed using different concentrations of DCPIP (0, 2.5, 5, 10, 20, 40, 80, and 160 μM) dissolved in a Tris/NADPH solution [final concentration of 25 mM Tris-Cl (pH 7.4) and 0.2 mM NADH], with and without 75 μM dicumarol, a DTD inhibitor. DCPIP is an electron acceptor for DTD, and its absorbance at 600 nm decreases on reduction by DTD and by nonenzymatic processes. To the tumor protein extract, 200 μl of the Tris/NADPH solution containing 60 μM DCPIP with or without 75 μM dicumarol was added. The enzymatic reaction was initiated by incubating the plate on a water bath at room temperature and followed by measuring the absorbance at 600 nm at 2.5 min. The amount of DCPIP remaining in the samples with and without dicumarol was determined using the corresponding standard curves. DTD activity was calculated as the dicumarol-inhibited fraction of the DCPIP reduction.

The amount of the internal standard was 7.5 × 10^{-14} g for DTD, 3 × 10^{-14} g for P450R, and 3 × 10^{-13} g for β-actin. We then mixed a fixed amount of internal standard with different amounts of target gene template to identify the linear range of the standard curve. Two μl of sample cDNA was used in PCR amplification (25-μl total volume). PCR was performed on the GeneAmp PCR system 2400 (Perkin-Elmer) and was initiated by 1 cycle at 94°C for 3 min, at 55°C for 2 min, and at 68°C for 2 min; then 30 rounds at 94°C for 30 s, at 55°C for 30 s, and at 68°C for 30 s; followed by 68°C for 10 min. The PCR products (10 μl) were electrophoresed on 1.8% agarose and visualized by ethidium bromide staining. The image was captured by a gel documentation system (Gel Print 2000 i, Biophotanics) and analyzed using the GPTools software package. The ratios of the intensities of the bands corresponding to DTD, P450R, and β-actin cDNAs. The expression of DTD and P450R was normalized to the β-actin expression.
at 550 nm, at 2.5 min. Samples containing only oxidized cytochrome C were used as blanks.

**Statistical Analysis.** Differences in mean values between two or more groups were analyzed using Student’s *t* test and the one-way ANOVA, respectively. Predictive relationships between tumor pathobiological parameters and tumor chemosensitivity were evaluated by linear regression analysis using the maximal *r*² selection method and the REG software routine of SAS (Cary, NC). This analysis identifies the model with the highest coefficient of determination for combinations of predictors. An accepted principle of development of a predictive model is to select the simplest model that gives a good description of the data (34). Generally, an increase in model complexity or number of predictors increases the goodness of fit or *r*². We used the Akaike Information Criterion (AIC) to balance model simplicity and goodness of fit (34).

**RESULTS**

**RT-PCR Determination of DTD and P450R Expression.** Fig. 1 shows the RT-PCR products of DTD, P450R, β-actin, and their corresponding internal standards. The use of the internal standards designed to have the same primer sites and nearly identical DNA sequences as the target genes resulted in linearity of the standard curves over a wide concentration range (100-fold for DTD, 1500-fold for P450R, and 1000-fold for β-actin).

**Expression of DTD and P450R in Patient Bladder Tumors.** The DTD expression relative to β-actin expression in the 29 patient tumors ranged from 0 to 89.9%, and the relative P450R expression ranged from 0 to 28.5% (Table 1). The expression of DTD was significantly correlated with the expression of P450R (*r*², 0.32; *P* < 0.01). The expression of both enzymes was inversely correlated with tumor grades (*r*², 0.15, and *P* < 0.05 for DTD; *r*², 0.18, and *P* < 0.05 for P450R) and was lower in invasive (T 2 and T 3 ) tumors compared with superficial (T 0 and T 1 ) tumors (*P* < 0.05 for both DTD and P450R).

**Pharmacodynamics of MMC.** Table 1 summarized the pathobiological data of the 21 patient tumors used to study MMC activity. MMC produced a sigmoidal concentration-dependent inhibition of DNA precursor incorporation; the IC₅₀ and IC₉₀ values show a 110-fold intertumor variability.

**Relationship between DTD/P450R Expression and Tumor Sensitivity to MMC.** In the 21 patient bladder tumors, the expression of DTD and P450R was correlated with tumor sensitivity to MMC; the IC₅₀ and IC₉₀ of MMC were inversely correlated with DTD expression (*r*², 0.28, and *P* = 0.01 for IC₅₀; *r*², 0.25, and *P* < 0.05 for IC₉₀) and with P450R expression (*r*², 0.26 for IC₅₀; *r*², 0.19 for IC₉₀; for both, *P* < 0.05). Multivariate analysis identified DTD expression and P450 expression as the first and second most important single-
parameter determinants of MMC activity, and the combination of expression of DTD and P450R as the most important two-parameter determinant (Table 2).

**Relationship between Gene Expression and Enzyme Activity.** Eleven bladder tumors were studied for the relationship between gene expression and enzyme activity. Only 3 of these 11 tumors were studied for MMC activity. Hence, the relationship between DTD and P450R activities and MMC activity was not determined. In the 11 tumors, there was a significant correlation between enzyme activity and gene expression of DTD (r², 0.84; P < 0.001), whereas a trend but not a statistically significant relationship was found between the activity and gene expression of P450R (r², 0.28; P = 0.09).

**DISCUSSION**

The present finding of a 110-fold intertumor variation in the IC values of MMC among 21 patient bladder tumors is consistent with our previous finding of a 120-fold variation in 60 patient bladder tumors (3, 7). Our results indicate for human bladder tumors: (a) a considerable intertumor variation in the expression and activity of DTD and P450R, with higher enzyme expression in lower-grade and superficial tumors compared with high-grade and invasive tumors; (b) a significant correlation between the expression of DTD and P450R and the sensitivity of tumor histocultures to MMC; and (c) DTD expression and P450R expression as the first and second most important single-parameter determinants of MMC activity, and the combination of expression of DTD and P450R as the most important two-parameter determinant. These results support a role for DTD and P450R in the tumor response to MMC and indicate the lower expression of these enzymes in the high-grade and invasive tumors as a cause of the lower efficacy of intravesical MMC in these tumors. It is noted that the correlation between the gene expression and the enzyme activity for P450R did not reach statistical significance (P = 0.09). This may be in part attributable to the limited sample size and/or attributable to the non-specificity of the enzyme activity assay. The assay for P450R activity measured the reduction of cytochrome C using NADPH as a cofactor. The activity may also come from some other one-electron reductases.

In cultured cells, DTD is considered the more important enzyme in the activation of MMC, as compared with P450R.

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**Table 1**  Patient and tumor characteristics

IC₅₀ and IC₉₀ are the MMC concentrations required for 50 and 90% inhibition of DNA precursor incorporation. The expression of DTD and P450R was measured by competitive RT-PCR and expressed as percentage of β-actin expression. Enzyme activity was determined as described in “Materials and Methods” and expressed as the rate of reduction of substrate per mg of protein. Among the 29 tumors studied, 21 tumors provided sufficient materials for the evaluation of drug activity, and 11 tumors provided sufficient materials for the evaluation of enzyme activity.

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NA, not applicable or not available.
For example, DTD is the only enzyme the activity of which correlates with the sensitivity of 69 cell lines to MMC (35), whereas the activity of P450R is not correlated with MMC sensitivity in multiple cancer cell lines (10, 13, 24, 35). On the other hand, it is well established that the relative contribution of DTD and P450R to the activation of MMC depends on the microenvironment; P450R is the key enzyme under hypoxic conditions, whereas DTD activates MMC under both aerobic and hypoxic conditions (reviewed in Refs. 18, 36). The bioactivation of MMC by DTD is also pH dependent. For example, DTD is active and capable of reducing MMC at or below pH 7.4 but not above pH 7.8 (36). The present study was performed using histocultures of patient tumors. Histocultures are three-dimensional systems that retain some of the features of solid tumors including cell-to-cell interaction and intratumoral microenvironmental heterogeneity. Hence, our findings suggest that both DTD and P450R are important in the activation of MMC in solid tumors. Our laboratory is currently investigating the relationship between the expression of these enzymes and the response to intravesical MMC treatment in individual patients, as a part of a Phase III clinical study.

REFERENCES

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Expression of DT-Diaphorase and Cytochrome P450 Reductase Correlates with Mitomycin C Activity in Human Bladder Tumors

Yuebo Gan, Yiqun Mo, John E. Kalns, et al.


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