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Jessie L.-S. Au · John Kalns · Yuebo Gan
M. Guillaume Wientjes

Pharmacologic effects of paclitaxel in human bladder tumors

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Abstract *Purpose*: The goal of this study was to determine whether paclitaxel, when given by a 2-h treatment, produces significant cytotoxic effects in human bladder transitional cell carcinoma and hence qualifies as a candidate drug for intravesical treatment. *Methods*: Histocultures of surgical specimens from patients ($n = 16$) were used. *Results*: Paclitaxel produced partial inhibition of DNA precursor incorporation in about 70% of tumors and induced apoptosis in about 90% of tumors, while these effects were minimal or not detectable in the remaining tumors. In the responsive tumors, the average maximal inhibition of DNA synthesis was 60% and the average maximal apoptotic index was 15%. Resistance to antiproliferative and apoptotic effects was not always found in the same individual tumors, and no relationship was found between the magnitude of antiproliferative and apoptotic effects in individual tumors. The maximal apoptotic index correlated with the LI for the untreated control ($r^2 = 0.42$, $P < 0.01$). More than 95% of apoptotic cells were labeled by DNA precursor, whereas not all labeled cells were apoptotic. The pharmacologic effects of paclitaxel in bladder tumors were qualitatively equivalent to those previously found in human head and neck tumors and in human prostate tumors after treatment for longer periods of 24 to 96 h. *Conclusions*: These results indicate that a 2-h paclitaxel treatment was sufficient to produce antiproliferation and apoptosis in 70–90% of human bladder tumors, and the apoptotic effect appeared to be linked to proliferation and occurred after DNA synthesis.

J.L.-S. Au (✉) · J. Kalns · Y. Gan · M.G. Wientjes
Division of Pharmaceutics and Pharmaceutical Chemistry,
College of Pharmacy, The Ohio State University,
500 West 12th Avenue, Columbus, OH 43210, USA
Tel. (614)292-4244; Fax (614)688-3223

J.L.-S. Au · M.G. Wientjes
Comprehensive Cancer Center, The Ohio State University,
Columbus, OH 43210, USA

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Introduction

About 60% of the 53 000 annual newly diagnozed superficial bladder cancers in the US will recur after complete endoscopic removal of the initial tumor and 10–15% will subsequently develop muscle-invading tumors [1, 15]. Adjuvant intravesical chemotherapy using DNA-damaging agents such as mitomycin C, doxorubicin, and thiotepa, reduces the recurrence rate by 30–50% [15]. We have shown, in a series of preclinical and clinical studies, that the variable and incomplete response to intravesical mitomycin C and doxorubicin is in part a consequence of the inability of currently used drugs to penetrate deep muscle layers and in part a consequence of the low sensitivity of the more aggressive tumors to these drugs [2, 4, 7, 18, 24–26]. A logical approach to develop more effective intravesical chemotherapy for superficial and muscle-invading tumors is to identify effective drugs that have favorable physicochemical properties for urothelium penetration, e.g. higher lipophilicity. We hypothesized that paclitaxel, which has the unique action mechanism of stabilizing microtubules [9] and is highly lipophilic, may be a suitable candidate. This hypothesis is supported, first, by our recently completed study showing favorable bladder tissue penetration characteristics for paclitaxel, i.e. the bladder tissue-to-urine drug concentration of paclitaxel was 15–30-fold higher than those of mitomycin C and doxorubicin [22] and, second, by the efficacy of paclitaxel in bladder cancer, as shown by the 42% partial and complete response rate of metastatic bladder cancer to 24-h treatment with paclitaxel by intravenous infusion [17].

Intravesical chemotherapy selectively delivers high drug concentrations to the tumor-bearing bladder, but the treatment duration is limited to 2 h because it is not feasible to maintain the drug solution in the bladder for

longer periods. The goal of the present study was to determine whether 2-h paclitaxel treatment is sufficient to produce significant antitumor effects. This study required the evaluation of drug sensitivity in individual patient tumors, and was performed using histocultures of surgical specimens of bladder tumors. The major advantages of the histoculture system are the maintenance of a three-dimensional tissue structure and organization, coexistence of tumor and stromal cells, cell–cell interaction, and inter- and intratumoral heterogeneity [23]. The use of tumors from individual patients allows evaluation of the relationship between tumor characteristics and chemosensitivity. The clinical relevance of the histoculture system is supported by the findings of Hoffman and coworkers in retrospective and semiprospective preclinical and clinical studies, which show correlations between in vitro chemosensitivity and patient response and resistance to treatment by mitomycin C, doxorubicin, 5-fluorouracil, and cisplatin [6, 12, 16].

Paclitaxel produces G2/M block and it is generally believed that its apoptotic insult is exerted on M phase cells [9]. The antiproliferative effect of paclitaxel due to G2/M block was measured by the inhibition of DNA precursor and the cytotoxic effect was measured by the induction of apoptosis. We have used these methodologies to separately evaluate these two effects in human head and neck, and prostate tumors [3, 8]. In comparison, evaluation of drug activity in the conventional monolayer cell culture system usually measures total cell number or other surrogate markers of cell number, and therefore does not separate cytostatic from cytotoxic effects.

Materials and methods

Chemicals and supplies

Paclitaxel was a gift from Bristol Myers Squibb Co. (Wallingford, Ct.). Sterile pigskin collagen (Spongostan standard) was purchased from Health Designs Industries (Rochester, N.Y.), ^{3}H-thymidine (specific activity, 61 Ci/mmol) from Moravek Biochemicals (Brea, Calif.), NTB-2 nuclear track emulsion from Eastman Kodak Chemicals (Rochester, N.Y.), bromodeoxyuridine (BrdUrd) from Sigma Chemical Co. (St. Louis, Mo.), cefotaxime sodium from Hoechst-Roussel Co. (Somerville, N.J.), gentamicin from Solo Pak Laboratories (Franklin Park, Ill.), Eagle's minimal essential medium (MEM) from Life Technologies (Grand Island, N.Y.), BrdUrd antibody from BioGenex (San Ramon, Calif.), and LSAB detection kit from Dako (Carpiteria, Calif.). All reagents were used as received.

Tumor histocultures

Specimens of human bladder transitional cell carcinoma were obtained via the Ohio State University Comprehensive Cancer Center Tumor Procurement Service. Tumor pathology was determined by the pathology department. Tumor specimens were placed in culture medium within 10 to 30 min of surgery, and maintained at 4 °C until use. Table 1 lists patient and tumor pathology data.

Histoculture of tumors was performed as previously described [19]. In brief, tumor specimens were cut to about 1 mm^3 and placed in six-well plates. Four to six tumor pieces were placed on a 1-cm^2

Table 1 Patient and tumor characteristics, and tumor sensitivity to paclitaxel. All tumors were transitional cell carcinoma. IC_{30} is the concentration of paclitaxel needed to produce a 30% inhibition of the LI

No.	Age (years) and gender	Stage	Grade	Control LI (%)	Antiproliferative effect		Apoptotic index (%)[a]	
					IC_{30} (μM)	Maximal inhibition of LI at 10 μ*M* (%)	Control	Treated (maximal value)
1	79F	T2	III	43	>10	0[b]	0.62	5.30
2	91F	T3b	III	52	>10	0[b]	3.70	33.2
3	57F	T1	II	18	>10	0[b]	0.37	2.80
4	80F	T3b	II	77	>10	0[b]	3.70	14.0
5	75M	T3b	II	53	>10	0[b]	3.94	20.1
6	59M	T1	III	21	0.007	48	1.60	15.4
7	64M	Ta	I	65	0.430	61	1.32	14.0
8	56M	Ta	II	23	0.025	77	0.88	4.60
9	84M	T1	III	32	1.19	33	2.54	8.70
10	44F	Ta	II	58	0.08	74	0.84	16.2
11	73M	T3b	III	48	1.40	39	3.30	12.1
12	45M	Ta	II	28	3.00	52	2.70	4.90[c]
13	73M	Ta	I	50	0.10	98	0.14	16.5
14	55M	Ta	I	25	0.04	97	1.10	3.20[c]
15	70M	T1	I	17	1.00	68	0.28	3.60
16	57F	T1	II	69	1.14	47	0.86	35.8
Mean	NA	NA	NA	43	0.76[d]	63[d]	1.75	14.5[e]
SD	NA	NA	NA	20	0.93	22	1.35	10.1

[a]Corrected for the apoptotic indices in untreated controls (ranging from <1% to 4%)
[b]Not significantly different from control
[c]Not significantly different from control. All other tumors showed significantly higher apoptotic index after taxol treatment
[d]Mean and SD calculated using the tumors with measurable IC_{30} (tumors 6 to 16)
[e]Mean and SD calculated using the tumors that showed significantly increased apoptotic index due to taxol treatment (all except tumors 12 and 14)

presoaked collagen gel, and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium consisted of MEM supplemented with 9% heat-inactivated fetal bovine serum, 0.1 m*M* 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.

Pharmacologic effects of paclitaxel

Paclitaxel stock solution was prepared in ethanol. Sufficient volume of stock solution was added to the culture medium so that the final ethanol concentration was $<0.1\%$.

The antiproliferative effect of paclitaxel was measured by the inhibition of DNA precursor incorporation in tumor cells. A preliminary study in five bladder tumors showed that the two DNA precursors, BrdUrd and ^{3}H-thymidine, labeled the same cells resulting in identical labeling indices. Hence later experiments used BrdUrd to reduce the use of radioisotopes. Note that this method measured all cells that incorporated the precursor, including the apoptotic cells. After drug treatment, the medium was exchanged and the tumors were washed three times with drug-free medium. Tumors were incubated with 40 μ*M* BrdUrd or 1 μ*M* ^{3}H-thymidine 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, deparaffinized and analyzed for BrdUrd labeling using the LSAB kit and immunohistochemical methods, and for ^{3}H-thymidine labeling by autoradiography, as previously described [8, 19]. Controls were processed similarly, except for drug treatment. Tissue sections were examined microscopically, the BrdUrd- or ^{3}H-thymidine-labeled tumor cells were scored, and the fraction of labeled cells (i.e. labeling index, LI) was determined. A typical experiment used a total of 12 to 20 tumor pieces for each drug concentration. A minimum of 200 cells per tumor piece, or >1500 cells, were counted per concentration.

The fraction of apoptotic cells was determined microscopically, using the established apoptotic features of chromatin condensation and margination, disappearance of nucleoli, formation of membrane blebs, apoptotic bodies and/or cell shrinkage [11]. We have shown in human head and neck tumors that morphological evaluation gives results identical to the TdT-mediated dUTP nick end labeling method [8].

Pharmacodynamic data analysis

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

$$E = (E_0 - Re) \cdot \left(1 - \frac{C^n}{K^n + C^n}\right) + Re \qquad \text{(Eq. 1)}$$

where E is the LI of drug-treated tissues, E_0 is the LI of untreated controls, C is the drug concentration, K is the drug concentration at one-half of $(E_0 - Re)$, *n* is a curve shape parameter, and Re is the residual fraction. Equation 1 reduces to the more commonly used equation for a sigmoidal concentration–effect relationship if Re equals zero. Inclusion of the Re term is necessary to describe the less-than-complete effect (see Results). Values for IC_{30} (the drug concentrations needed to produce 30% inhibition) instead of the more commonly used IC_{50} were determined, because 50% inhibition was not achieved in some tumors.

Statistical analysis

Differences in mean or median values between groups were analyzed using the unpaired Student's *t*-test and/or Wilcoxon's nonparametric two-sample test. Differences in frequencies of distribution of resistant tumors were analyzed using Fisher's exact test. Software for statistical analysis was by SAS (Cary, N.C.).

Results

Histocultures of bladder tumors

Of the 25 tumors processed for histocultures, 16 showed sufficient numbers of tumor cells, i.e. >200 cells per fragment, to enable pharmacologic evaluation. The 96-h cumulative LI of these 16 histocultures was 43 ± 20% (mean ± SD, Table 1), which is comparable to our previously reported LI of 40 ± 23% [4, 19].

Paclitaxel-induced inhibition of DNA synthesis

The antiproliferative effect of paclitaxel varied substantially among individual tumors (Fig. 1, Table 1). In five tumors, the fraction of DNA-synthesizing cells was not reduced by paclitaxel treatment, and the maximal inhibition (E_{max}) was indistinguishable from 0%. The remaining 11 tumors showed significant and apparent concentration-dependent inhibition, and E_{max} values between 33 and 98%. In the latter group, the IC_{30} ranged from 0.007 to 3 μ*M*, or a 430-fold variation (Table 1). There was no correlation between LI and IC_{30} ($P = 0.86$), nor between LI and E_{max} ($P = 0.83$).

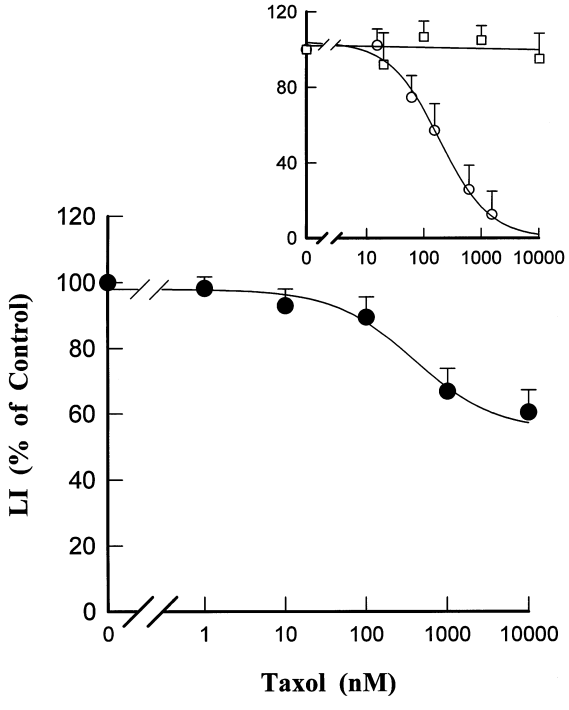

Fig. 1 Relationship between paclitaxel concentration and drug-induced inhibition of DNA precursor incorporation. Human bladder tumors were treated with paclitaxel for 2 h. Inhibition of 96-h cumulative precursor incorporation was expressed as percent of untreated controls. Data-points represent the average of 16 tumors (mean ± SEM). *Inset* The least-sensitive (□, tumor 1) and the most-sensitive (○, tumor 14) tumors. Lines are computer fitted according to Eq. 1

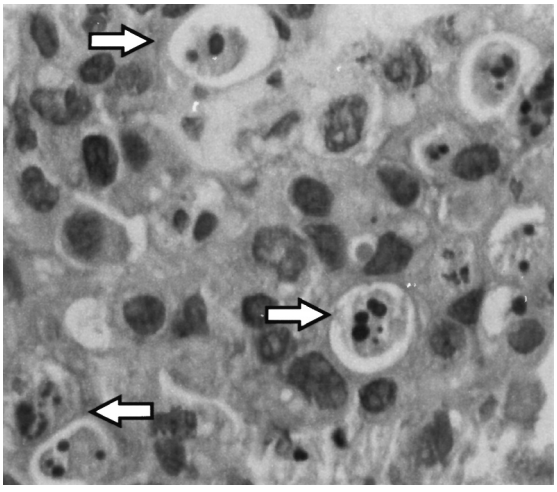

Fig. 2 Paclitaxel-induced apoptotic tumor cells. Apoptotic cells induced by 1 μ*M* paclitaxel (tumor 2; *arrows* condensation of nuclear chromatin with loss of nuclear membrane, disappearance of nucleoli, formation of apoptotic bodies and/or cell shrinkage)

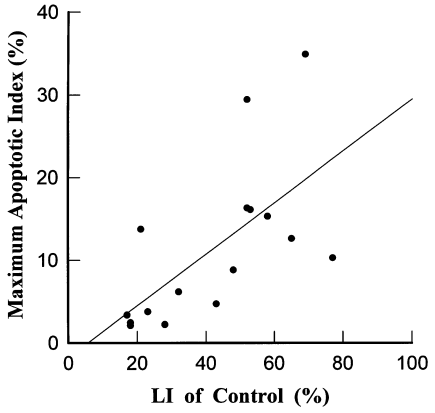

Fig. 3 Relationship between LI and apoptotic index. The correlation between LI of untreated controls and the maximal apoptotic index (corrected for apoptosis in untreated controls) induced by paclitaxel was statistically significant ($r^2 = 0.42$, $P < 0.01$)

Paclitaxel-induced apoptosis

Figure 2 shows the apoptotic morphology of cells after paclitaxel treatment. The maximal fraction of apoptotic cells are listed in Table 1. The untreated controls showed an average apoptotic index of 1.8% (range of <1% to 4%). Of the 16 tumors, 14 showed significant increases in apoptotic index after paclitaxel treatment ($P < 0.05$), and 2 tumors did not show a significant response. For the responsive tumors, the enhancement in apoptotic index became significant at paclitaxel concentrations of 0.1 μ*M* for 3 tumors, 1 μ*M* for 10 tumors, and 10 μ*M* for 1 tumor; and the maximum apoptotic index in individual tumors occurred at 0.1 μ*M* for 1 tumor, 1 μ*M* for 11 tumors, and 10 μ*M* for 4 tumors. Interestingly, there was no apparent relationship between the antiproliferative and apoptotic effects of paclitaxel, i.e. different tumors were resistant to the two effects and there was no correlation between E_{max} and apoptotic index ($P = 0.64$) nor between IC_{30} and apoptotic index ($P = 0.65$).

More than 95% of apoptotic cells were labeled by DNA precursor, but not all labeled cells were apoptotic. The maximum apoptotic index correlated significantly with the LI of untreated controls (Fig. 3).

Tumor pathology and chemosensitivity

Table 2 summarizes the relationships between tumor pathology and tumor sensitivity to paclitaxel. In general, the antiproliferative effect and the apoptotic effect showed different relationships with tumor pathology. The invasive T2 and T3 tumors and the high-grade (i.e. grades II and III) tumors were significantly less sensitive (i.e. higher IC_{30} and/or lower E_{max}) to the antiproliferative effect of paclitaxel than the superficial Ta and T1 tumors and the low-grade (i.e. grade I) tumors. In addition, the fraction of invasive tumors that was resistant to the antiproliferative effect of paclitaxel was significantly higher than the fraction of resistant superficial

Table 2 Relationship between tumor sensitivity to paclitaxel and tumor pathology. IC_{30} is the concentration of paclitaxel needed to produce a 30% inhibition of BrdUrd labeling index

Tumor pathology		*n*	Fraction of tumors resistant to antiproliferative effect[a]	IC_{30} (μ*M*) median (range)[b]	E_{max} mean ± SD[c]	Maximal apoptotic index (%)[c]
Stage	Ta/T1	11	1/11	0.43 (0.01 to >10)	60 ± 28	10 ± 10
	T2/T3	5	4/5	>10 (1.4 to >10)	17 ± 17	14 ± 10
	P-value	NA	0.013	0.008	0.008	0.51
Grade	I	4	0/4	0.24 (0.01 to 1.0)	81 ± 19	9 ± 7
	II/III	12	5/12	2.20 (0.01 to >10)	35 ± 27	12 ± 11
	P-value	NA	0.25	0.07	0.007	0.52

[a]Statistical analysis of frequencies by Fisher's exact test
[b]Median and range rather than mean ± SD are presented because IC_{30} could not be measured in five tumors. Statistical analysis by Wilcoxon's nonparametric two-sample test
[c]Corrected for apoptosis in untreated controls. Statistical analysis by Student's unpaired *t*-test

tumors. On the other hand, the invasive tumors and the high-grade tumors showed a trend of a higher response to the apoptotic effect when compared with the superficial and low-grade tumors, although the differences were not statistically significant.

Interestingly, four of six tumors from female patients were resistant to the antiproliferative effect of paclitaxel. In comparison, the fraction of resistant tumors derived from male patients was lower at 2 of 12 ($P = 0.107$). However, it is noted that there are no known gender differences in responses to paclitaxel.

Discussion

The present study demonstrated that paclitaxel inhibited DNA synthesis in about 70% of human bladder tumors and induced apoptosis in about 90% of tumors. Significant intertumor variations in IC_{30} (430-fold) and apoptotic index (approximately 10-fold) were observed. The lack of correlation between tumor sensitivity to the antiproliferative and apoptotic effects of paclitaxel and the different relationships between tumor pathology and sensitivity for the two effects suggest that the antiproliferative and apoptotic effects of paclitaxel are uncoupled. There are two additional notable aspects of the paclitaxel effects. First, paclitaxel-induced inhibition of DNA synthesis was incomplete even at the highest drug concentration of 10 μM. This is different from the complete inhibition found for other drugs with different action mechanisms, i.e. mitomycin C (DNA alkylator) and doxorubicin (DNA alkylator and topoisomerase II inhibitor), and 5-fluorouridine (antimetabolite) [7, 18, 21]. Second, the finding of nearly all apoptotic cells labeled with DNA precursor and the correlation of the maximum apoptotic index with the LI indicate that paclitaxel-induced apoptosis was linked to proliferation and occurred after DNA synthesis. A similar positive correlation between paclitaxel-induced apoptosis and proliferation has been observed in monolayer cultures of human lung, breast, cervical, colon, ovarian, and astrocytoma cells, and in Chinese hamster ovarian cancer cells [5, 13, 14]. These findings are qualitatively equivalent to those in human head and neck, and prostate tumors [3, 8], suggesting the commonality in the pharmacologic effects of paclitaxel among human solid tumors.

The major difference in the experimental designs of the present study on bladder tumors and our previous studies on human head and neck and prostate tumors is the duration of paclitaxel treatment. The present study used a 2-h exposure, which is the duration of intravesical treatment of superficial bladder cancer, whereas the previous studies used a 24- or 96-h exposure, which is the duration of systemic paclitaxel treatment. In spite of the 12–48-fold differences in treatment durations, there was no difference in the apoptotic effect. The paclitaxel-induced apoptosis in bladder tumors treated for 2 h (14%) was similar to that in head and neck tumors treated for 24 h and in prostate tumors treated for 24 or 96 h (12% in all three cases) [3, 8]. In comparison, the fraction of tumors that responded to the antiproliferative effect appears to increase with increasing treatment time, i.e. 69% (11/16) in bladder tumors after 2-h treatment, 91% (20/22) in head and neck tumors after 24-h treatment, 69% (18/26) in prostate tumors after 24-h treatment and 100% (11/11) after 96-h treatment [3, 8]. The significant antitumor activity of paclitaxel after a relatively short treatment of 2 h may be explained in part by the extensive intracellular accumulation and retention of paclitaxel. We have reported a 140–2300-fold accumulation in human cancer cells and human solid tumor histocultures and a slow release into drug-free medium at a half-life of 5.4 h [10].

The antiproliferative effect of paclitaxel is lower in high-stage tumors compared with low-stage tumors. This is similar to the observations for other drugs currently used for intravesical bladder cancer therapy, i.e. mitomycin C and doxorubicin [7, 20]. On the other hand, both low- and high-stage tumors responded equally to the apoptotic effect of paclitaxel, which suggests that paclitaxel may be useful against invasive as well as superficial diseases because apoptosis is considered an important antitumor effect.

In conclusion, a 2-h paclitaxel treatment produced significant inhibition of DNA synthesis in about 70% of human bladder transitional cell tumors and apoptosis in about 90% of tumors, indicating that intravesical paclitaxel may be useful against bladder cancer in most patients.

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