

ANTI-NEOPLASTIC ACTIVITY OF PACLITAXEL ON EXPERIMENTAL SUPERFICIAL BLADDER CANCER: *IN VIVO* AND *IN VITRO* STUDIES

Ofer NATIV¹, Moshe ARONSON^{3*}, Ora MEDALIA³, Tatiana MOLDAVSKY³, Edmond SABO², Israel RINGEL⁴ and Vladimir KRAVTSOV³

¹Department of Urology, Bnai Zion Medical Center, Haifa, Israel

²Department of Pathology, Bnai Zion Medical Center, Haifa, Israel

³Department of Cell Biology and Histology, Sackler School of Medicine, University of Tel Aviv, Israel

⁴Department of Pharmacology, Faculty of Medicine, The Hebrew University, Jerusalem, Israel

The effects of intravesical administration of paclitaxel (taxol) in a bladder tumor model in mice, as well as the drug's *in vitro* activity on the same tumor cells, have been studied. Two cell lines, derived from MBT-2 cells, were employed in these experiments. The T50 line (obtained by many passages in mice) was much more aggressive *in vivo* than the T5 line. *In vivo* paclitaxel treatment for 3 days after T5 implantation resulted in a considerable retardation of tumor growth, whereas under the same conditions the T50 line was much less, although still significantly, affected. When treatment was started 1 day after tumor implantation, both tumor variants were affected by paclitaxel to the same extent. The *in vitro* experiments utilized the MiCK assay, which allows continuous recording of the kinetics of cell growth. These studies revealed a 39.8% inhibition of cell growth by 2.10⁻⁸M paclitaxel in the T50 line and a 30-fold increase in concentration had only a small additional effect on the degree of inhibition. At 2.10⁻⁸M paclitaxel, growth of T5 was inhibited by 21.7%, which increased to 35.2% at 6.10⁻⁷M. The treated cells displayed bundles of microtubuli, as described for other paclitaxel-treated cells. Int. J. Cancer, 70:297–301, 1997.

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Management of superficial bladder neoplasms (Ta, Tis, T1) consists of transurethral tumor resection followed by intravesical adjuvant treatment (Torti and Lum, 1984; Rubben *et al.*, 1988). The easy accessibility of the cancerous tissue and the possibility of achieving high local drug concentration, have made intracavitary treatment of bladder cancer very attractive. The response observed with the available intravesical agents ranges between 30% (when applied to an existing tumor) to 70% (when applied prophylactically), depending on the drug used (Herr *et al.*, 1987; Huland *et al.*, 1990). Since it was originally presented by Morales *et al.* (1976), Bacillus Calmette-Guérin (BCG) immunotherapy has been considered as the most potent topical agent. However, 30–50% of the patients treated with BCG still experience local recurrence and serious side-effects such as fever, granulomatous prostatitis, hematuria, pneumonitis, sepsis and allergic reactions (Herr *et al.*, 1989; Lamm *et al.*, 1986). Clearly, more effective and less toxic agents are needed. It has been well documented that paclitaxel (taxol), a natural product derived from the bark of *Taxus brevifolia*, displays antineoplastic activity against various human malignancies (McGuire *et al.*, 1989; Murphy *et al.*, 1993; Holmes *et al.*, 1991). We tested the effects of paclitaxel on the growth of MBT-2 cells, both in mice following intravesical administration and in tissue culture. We conducted these experiments with 2 sublines of MBT-2: T5, which was passed only a few times before being successfully implanted into the bladder; and T50 which has undergone many passages in mice, resulting in an aggressive line that no longer requires thermal trauma in the bladder for efficacious implantation.

The *in vitro* studies made use of a new technique, the MiCK assay, which allows the growth kinetics of cells to be continuously recorded. In addition, the morphology of the paclitaxel-treated cells was observed by immunofluorescence microscopy, to verify the appearance of bundles of microtubules.

MATERIAL AND METHODS

In vivo studies

Animals

Inbred 8- to 10-week-old female C3H/eb mice, obtained from the animal facility of the Sackler School of Medicine, Tel Aviv, Israel, were housed at a temperature of 22–24°C in 50–70% humidity with a 10 hr–14 hr dark/light cycle.

Tumor

The most frequently used animal model for studying bladder cancer employs MBT-2 cells. This cell line was obtained from a transitional-cell carcinoma of the bladder induced by oral administration of N-[4-(5-nitro-2-furyl)-2 thiazolyl] formamide (FANFT) (Erturk *et al.*, 1970).

FANFT-induced mouse bladder tumor (MBT-2) was kindly provided by Dr. William R. Fair of the Memorial Sloan-Kettering Cancer Center, New York. The tumor was maintained *in vivo* by serial subcutaneous transplantations into the backs of C3H/eb mice, and in tissue culture in RPMI 1640 medium with 10% FCS supplemented with penicillin (100 units/ml) and L-glutamine (300 mg/l). Following serial transplantations during an 18-month period, the original tumor T5 (which was kept frozen) became more aggressive with an increased growth rate, both *in vivo* and *in vitro*. This highly malignant variant is designated T50 (Nativ *et al.*, 1996).

Tumor-cell implantation

Preparation of single-cell suspensions from subcutaneous tumor was done by mincing the fresh tumor under aseptic conditions and adding RPMI 1640 medium to the minced tissue. The number of viable cells was determined by Trypan-blue exclusion. For implantation of tumor cells, the mice were anesthetized with subcutaneous injections of sodium pentobarbital (0.05 ml/mg body weight). A 24-gauge Teflon IV catheter was inserted into the bladder transurethraly, then a metal wire was introduced into the lumen of the catheter to elicit thermal injury in the bladder mucosa by means of an electro-cautery unit. A total of 5 × 10⁶–5 × 10⁷ viable tumor cells in 0.05–0.1 ml were delivered to the bladder through the cannula. The mice remained anesthetized for another 45–60 min to prevent voiding of tumor cells.

This procedure was applied to the T5 variant. Preliminary experiments with the T50 variant established that there was no substantial difference whether or not heat trauma was inflicted. Hence, all the experiments were conducted without heat trauma.

Drugs

Paclitaxel was kindly provided by Sigma, St. Louis, MO. In the initial *in vivo* experiments, paclitaxel was dissolved in Cremphor

*Correspondence to: Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel. Fax: 972 3 6407432.

EL (Sigma, St. Louis, MO). Due to solvent-related toxicity, Cremophor was replaced by Tween-80 (5%) (Sigma, St. Louis, MO), then the drug solution was sonicated to ensure the solubility of the drug, and later diluted 1:10 in SPF. All the reported experiments were conducted with Tween 80 as a solvent. For *in vitro* studies, paclitaxel was dissolved in dimethyl sulfoxide (DMSO) (Fluka, Buchs, Switzerland).

Experimental design

Three sets of independent experiments were carried out to evaluate the effect of paclitaxel administration on the 2 tumor variants T5 and T50. In each set the animals were divided into 3 treatment groups. Group I received no treatment and served as control. Group II underwent 4–6 intravesical instillations of 0.05–0.1 ml paclitaxel solution (6 mg/ml) 300 µg/g body weight per treatment every other day. This group was further divided in studies employing the T50 variant. In set 2, treatment was started as with the T5 (set 1) tumor 3–4 days after implantation, whereas in set 3 treatment was started 1 day post implantation. Group III was treated according to the schedule of (early) group II but consisting of either 0.1 ml drug solvent or 0.1 ml saline. All the treatments were conducted under light pentobarbital anesthesia.

Evaluation of results and statistical analysis

At the end of each experiment, the animals were killed. Bladders were removed, weighed, fixed in 10% buffered formalin, and then processed for embedding. Slides were stained with H. and E. and examined "blind" to monitor tumor development.

Since mouse bladder weight revealed a non-Gaussian distribution, as demonstrated by the Kolmogorov-Smirnov test, we elected to compare the effect between treatment subgroups by using non-parametric Kruskal-Wallis analysis of variance (ANOVA) of the Mann-Whitney test as indicated.

In vitro studies

Cells

The tumor cells were adapted for work in tissue culture. The cells were grown in RPMI-1640 medium for 2–3 passages until a suitable line developed.

The effect of paclitaxel on cellular microtubules was examined by immunofluorescence techniques according to a published procedure (Ringel and Horwitz, 1987).

MiCK assay, relation between plated cell number and absorbance

The microculture kinetic assay (MiCK assay) for cell-growth kinetics is based upon a linear relation between the number of live cells in a culture and the optical density (OD) of that culture. The MiCK assay was performed as previously described (Kravtsov, 1994) with minor modifications, and the standard curve was determined for the T50 and T5 cells as follows: 250-µl aliquots of cell suspensions in complete RPMI-1640 medium, without phenol red, were plated at various concentrations in duplicate, in a 96-well flat-bottomed microplate (Corning, Corning, NY). The cells were allowed to attach for 6 hr at 37°C and an end-point OD reading was performed at 37°C at a wavelength of 600 nm, using the THERMO-max microplate reader (Molecular Devices, Menlo Park, CA). The data were subjected to regression analysis (Fig. 1) which revealed consistent linearity between the cell concentration and the OD for both types of cells. Incubation of the microplate was continued at 37°C for 24 hr, and an endpoint OD reading was repeated. Regression analysis of the data demonstrated sustained OD/cell number linearity over 24 hr for microcultures of both cell types.

MiCK assay for cell-growth kinetics

Microscopically, T50 and T5 cells show morphological differences, *viz.* the T50 cells are larger than the T5. These differences were expressed by the differing slope of the calibration curves (Fig.

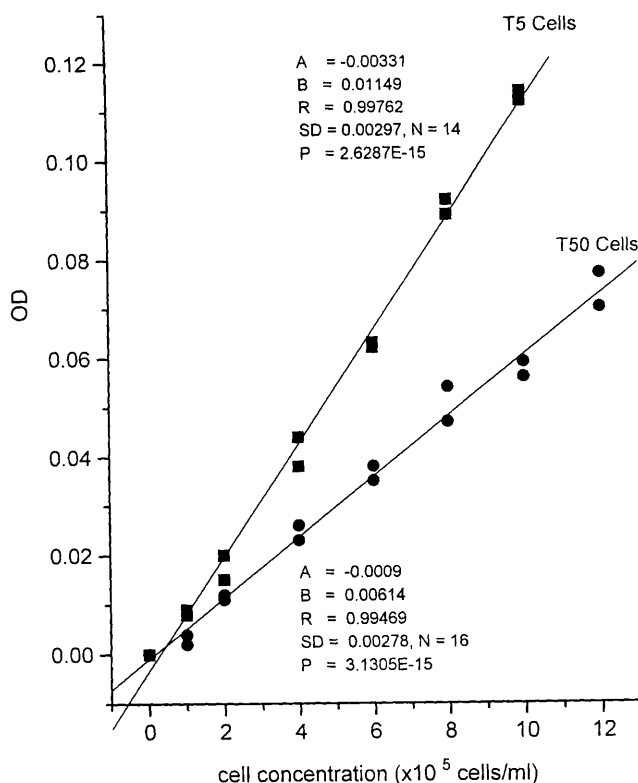


FIGURE 1—Relationship between cell concentration and optical density of adherent T5 and T50 cells.

1); taking these differences into consideration, T50 and T5 cells were seeded in the wells at initial concentrations of 0.75×10^5 or 0.5×10^5 cells/ml respectively. The cells were allowed to accommodate for 24 hr in the CO₂ incubator, after which time paclitaxel was added to the wells in 25-µl aliquots at the indicated concentrations. Control wells received DMSO at a final concentration of 0.1%. The plates were incubated at 37°C, and the OD was read at 600 nm over a 60-hr period. OD readings were taken every 5 min. The data obtained were combined by means of a spreadsheet program (MicroCal Origin, Norhampton, MA) and used to calculate the concentration of cells in the cultures (Fig. 1) as follows: $X = (OD - A)/B$, where X = number of cells per ml, B = slope of the calibration curve, A = OD of the medium without cells (Y intercept). The calculated cell concentrations were plotted against time, thus displaying the results at real time. For a comparison between growth kinetics of the control and drug-treated cultures, values of the best-fit slope of the growth curves were computed and a growth-inhibitory index (GI) was calculated as follows: $GI = (1 - SV_T/SV_C) \times 100$, where SV_T and SV_C represent slope values of the growth curves from the paclitaxel-treated and control cultures, respectively.

RESULTS

In vivo studies

The anti-tumor activity of paclitaxel given intravesically was tested in 281 C3H/eb female mice in 3 sets of independent experiments. Upon completion of the treatment, the animals were killed and their bladders were removed and weighed. Representative H. and E. slides were examined "blind" by a pathologist. Successful tumor-cell implantation was observed in 222 animals (79%) which comprise the study group.

TABLE I - EFFECT OF PACLITAXEL ON MBT-2 TUMOR WEIGHT IN C3H/eb FEMALE MICE FOLLOWING INTRAVESICAL INSTILLATION

Tumor variant	Mean bladder weight (mg) ± SD (combined results) Treatment group			p value*
	I. Control	II. Paclitaxel	III. Solvent	
T5—Set 1 Regular treatment	247 ± 178 (n = 34)	79 ± 106 (n = 31)	204 ± 154 (n = 24)	0.0001
T50—Set 2 Regular treatment	288 ± 179 (n = 21)	194 ± 100 (n = 41)	239 ± 144 (n = 24)	0.05
T50—Set 3 Early treatment	296 ± 211 (n = 14)	94 ± 53 (n = 18)	167 ± 189 (n = 15)	0.001

*Kruskal-Wallis ANOVA.

Studies with T5 (set 1)

Eighty-nine mice transplanted with the T5 variant in 5 independent experiments whose results were combined, were divided into 3 treatment groups. The average bladder weight of the untreated control (group I) reached 247 ± 178 mg, while bladders taken from paclitaxel-treated animals (group II) had significantly lower weights 79 ± 106 mg (p = 0.0001). In the animals treated with the solvent alone (group III), the mean bladder weight was 204 ± 154 mg. While this is significantly higher than the mean weight of the bladders treated with paclitaxel (p = 0.0014) it is definitely lower than that of the control group (p = 0.06). The difference between the untreated animals and those treated by the solvent is attributed to cellular desquamation due to the mechanical effect of bladder irrigation (Table I). The solvent, incidentally, did not show any evidence of toxicity.

Studies with T50 (set 2)

As shown in Table I, when treatment was initiated according to the standard procedure, i.e., 3-4 days after T50 implantation, intravesical administration of paclitaxel was not effective. The average bladder weight of untreated, solvent-treated and paclitaxel-treated animals was 288 ± 179 mg, 239 ± 144 mg, and 194 ± 100 mg, respectively (p = 0.05), (results obtained from 86 mice). In a second cohort of animals (n = 47, 3 independent experiments, set 3), treatment was started 1 or 2 days post tumor transplantation. A significant difference was noted between the control group and the paclitaxel-treated group (average bladder weight 296 ± 211 mg versus 94 ± 53 mg respectively, p = 0.001). The reduction of tumor weight in the solvent-treated animals (mean value 167 ± 189 mg) was mild and did not differ significantly from that of the untreated group (p = 0.2).

In vitro studies

Within the first 24 hr of incubation, the cells were attached to the wells and formed a non-confluent monolayer. As already noted, OD readings were initiated 30 min after addition of paclitaxel to the microcultures. Figure 2 depicts the growth kinetics of both control and paclitaxel-treated T-50 cells. At 24 hr (0 time of the MiCK assay), the cell concentration in the control wells was 1.2 × 10⁵ cells/ml and, after a short initial growth delay, exponential cell growth throughout the entire assay period was obtained. The first doubling was seen at 16 hr of incubation, and a total of 3 doublings could be observed within the 60-hr assay period. In wells containing paclitaxel, a dose-dependent inhibitory effect of the drug on cell growth was evident: however, at the paclitaxel concentrations used, cell growth was only partially inhibited. The first doubling of cells was observed at 20 hr and 24 hr in cultures with 2 × 10⁻⁸M and 6.6 × 10⁻⁷M paclitaxel, respectively (GII values of 39.8% and 54.4%). An intermediate paclitaxel concentration of 2 × 10⁻⁷M caused 40% growth inhibition (data not shown).

Figure 3 shows the growth kinetics of both control and paclitaxel-treated T5 cells during the 60-hr MiCK assay. As before, exponen-

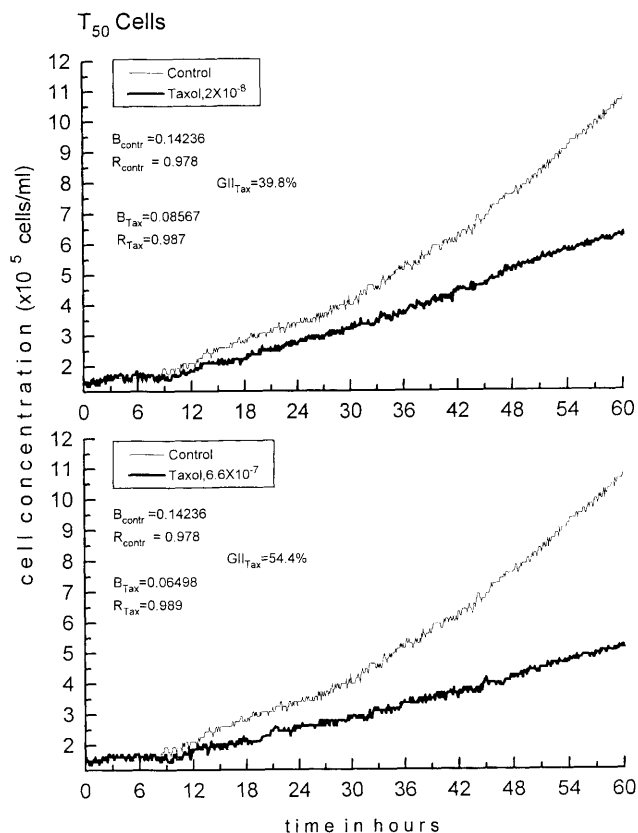


FIGURE 2—Growth kinetics of paclitaxel-treated and untreated (control) T50 cells.

tial cell growth was seen in the control microcultures; however, the T5 cells grew more slowly than the T50. The first cell doubling was reached only after 26 hr, and a total 2.5 doublings occurred within the 60-hr assay. The inhibitory effect of paclitaxel on the growth of T5 cells was less prominent than that observed in cultures of T50 cells. The first doubling in paclitaxel-treated cells was extended to 30 and 36 hr at 2 × 10⁻⁸M and 6.6 × 10⁻⁷M paclitaxel, respectively (GII values of 22% and 35%). In order to achieve more than 50% growth inhibition, 2 × 10⁻⁶M paclitaxel (56% inhibition) was needed (data not shown).

The effect of paclitaxel on the cellular microtubule system of T5 and T50 was examined by immunofluorescence spectroscopy using specific anti-tubulin antibodies. The results indicated the formation of arrays of microtubule bundles, characteristic of the inhibitory action of paclitaxel on cell propagation (data not shown).

DISCUSSION

The toxicity and incomplete efficacy of the commonly used intravesical agents for the treatment of superficial bladder cancer prompted various investigators to search for alternative treatments, including paclitaxel. As already mentioned, in several publications of phase-II studies in which advanced ovarian and breast tumors were treated by paclitaxel, a response rate of more than 30% was reported (McGuire *et al.*, 1989; Holmes *et al.*, 1991). Rangel and Niell (1992) found that 7 human bladder cancer cell lines displayed high sensitivity to paclitaxel. In these studies, the cells were washed and plated after 2 hr exposure to the drug. In the present study, continuous exposure to the drug was employed to explore the possibility of topical application for long durations. Dreicer *et al.* (1994) and associates in the Eastern Cooperative Oncology

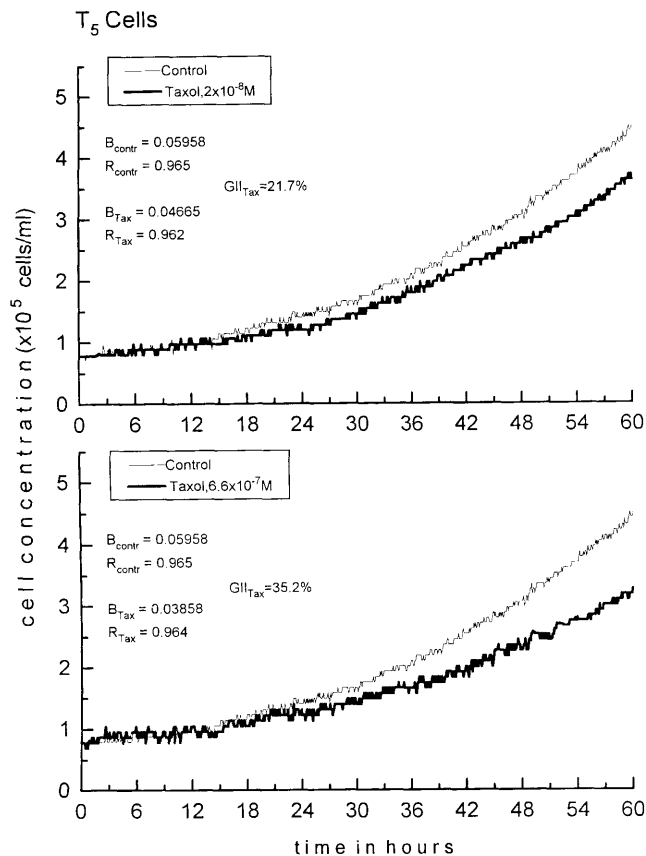


FIGURE 3—Growth kinetics of paclitaxel-treated and untreated (control) T5 cells.

Group (ECOG) treated 26 patients with advanced transitional-cell carcinoma of the urothelium with i.v. paclitaxel given as a single agent. Objective response was noted for 42% (5 with complete response and 6 with a partial response).

Paclitaxel was shown to exert its main anti-tumoral activity by binding to and promoting the assembly of microtubules. This renders them resistant to depolymerization and consequently the cells are arrested at the G2 and M phases of the cell cycle (Schiff *et al.*, 1979; Parness and Horwitz, 1981; Manfredi *et al.*, 1982).

Our *in vitro* experiments have indeed shown that paclitaxel acts on MBT-2 cells via the same mechanism, as the incubation of these cells with paclitaxel results in the formation of bundles of highly ordered arrays of microtubules.

Two points of interest emerge from the *in vitro* studies: (a) Paclitaxel did not stop cell growth but considerably diminished its rate. We speculate that the *in vivo* results comprise 2 mechanisms, namely, growth retardation *per se* and a more effective anti-tumoral host response against partially inhibited cells (due to the effect on the microtubuli which are essential for any movement within the cells). (b) An increase in the drug concentration had only a slight effect.

The MBT-2 model has been used to test the efficacy of various intravesical chemotherapeutic agents since it closely resembles its human counterpart. A good correlation was noted between the activity of various drugs in this animal model and the results of clinical trials in human bladder cancer (Soloway, 1977; Von Hoff, 1980). Since the drug was administered topically, we were able to use high concentrations without untoward side-effects. Thus, we employed 1 mg/kg, which is significantly more than the maximum recommended dose of 135–250 mg/m² which is given to human patients in continuous infusion over 24 hr after heavy premedication. As expected, the only significant side-effects were noted in the subgroup of animals treated with Cremophor EL. This solvent is generally thought to be the causative agent of most of the hypersensitivity reactions associated with paclitaxel administration. Such reactions are principally mediated by the direct release of histamine or other vasoactive substances from mast cells and basophils (Lorenz *et al.*, 1977; Rowinsky *et al.*, 1990). We therefore instilled paclitaxel suspended in Tween 80 into the mouse bladders. In this study, we have shown that intravesical treatment of superficial bladder cancer using paclitaxel as a topical agent resulted in significantly lower tumor weight than in untreated controls. The inhibition of neoplastic growth was more effective in the less aggressive tumor variant (T5). As for the highly malignant clone T50, only early treatment, *i.e.* starting 1 day after tumor-cell implantation, was capable of significantly reducing bladder weight. The less impressive results obtained for T50 when paclitaxel administration was initiated 3–4 days after implantation can most likely be attributed to the fact that the tumor cells had penetrated the bladder mucosa by this time and therefore were out of reach of the drug, and also to the increase in cell numbers.

Our results suggest that topical treatment of bladder cancer with paclitaxel, rather than by i.v. administration, should be considered. This notion is further supported by the above-mentioned findings of Rangel and Niell (1992) concerning the high sensitivity of bladder tumor cells to paclitaxel.

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