Anti-Cancer Drugs 1997, **8**, pp. 379–385

Effects of prolonged versus short-term exposure paclitaxel (Taxol®) on human tumor colony-forming units

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Paclitaxel shows a broad clinical activity in ovarian, breast and non-small cell lung cancers. However, controversy remains about the respective effects of doses and schedules in paclitaxel cytotoxicity. This study was conducted to compare the cytotoxic activity of short-term (1 h) versus prolonged exposure (14 days) to paclitaxel in human cancer cells. A soft-agar cloning system assay was used to determine the *in vitro* effects of 0.025–25.0 μg/ml paclitaxel against cancer cells taken directly from patients. A decrease in tumor colony formation resulting from drug exposure was considered an *in vitro* response if survival of colonies was up to 50% of that in positive controls. Among 11 evaluable patients' biopsies, both short- and long-term exposure to paclitaxel had significant concentration-dependent effects on the growth inhibition of human cancer cells. With the 1 h exposure schedule, *in vitro* responses were observed in 9, 18 and 64% of evaluable tumor specimens at final concentrations of 0.25, 2.5 and 25.0 μg/ml, respectively. With the prolonged exposure schedule, concentrations of 0.25, 2.5 and 25.0 μg/ml induced 27, 45 and 91 *in vitro* response rates, respectively. In those patients' biopsies prolonged exposure to paclitaxel induced significantly more *in vitro* tumor responses than 1 h administration ($p < 0.01$). Similar trends were observed in ovarian, breast and non-small cell lung cancers. Our data indicate that the duration of exposure to paclitaxel is an important factor in paclitaxel cytotoxicity in human tumors and suggest that long-term exposure may improve the antitumor activity of paclitaxel.

Key words: Cell proliferation, chemotherapy, continuous infusion, docetaxel, taxanes.

Introduction

The diterpenoid paclitaxel promotes the assembly of microtubules and stabilizes them against depolymerization.[1–4] Although those effects primarily affect cell cycle traverse in mitosis,[5,6] paclitaxel also prevents transition from G_0 to S phase,[7] blocks cellular response to protein growth factors like epidermal growth factor, down-regulates cell receptors for binding and secretion of tumor necrosis factor,[8,9] induces apoptotic cell death,[10] and demonstrates angiogenesis inhibition.[1] Previous reports using the human tumor cloning assay have indicated that short-term exposure to paclitaxel yielded activity against a broad spectrum of human tumors including ovarian, breast, non-small cell lung, and head and neck cancers.[8,11]

Several schedules of paclitaxel have been evaluated in efforts to increase the activity and reduce the toxicity of paclitaxel, thereby optimizing its therapeutic index.[12–14] The pharmacokinetic behavior and toxicological profile of paclitaxel have been demonstrated to depend on the duration of administration (e.g. one group of investigators demonstrated that the maximum tolerated dose of paclitaxel was 180 and 240 mg/m^2 for patients receiving the drug over 24 and 3 h schedules, respectively).[15] Hematologic toxicity was much more pronounced in patients treated with paclitaxel on the 24 h schedule, whereas peripheral neuropathy, myalgia and hypotension were more frequent on the 3 h schedule. Considering the logistical convenience, the pharmacokinetic parameters and the toxicity profile of paclitaxel, shorter administration schedules have been incorporated into general clinical practice.[16] Nevertheless, longer exposure duration may in theory portend greater antitumor activity for cell cycle-specific and phase-specific drugs such as paclitaxel. Therefore, the respective effects of treatment durations on the cytotoxic activity of paclitaxel against human tumors warranted further evaluations.

In this study, the relative antiproliferative effects of short-term versus prolonged exposure to pacli-

This work was supported by the Cancer Therapy and Research Foundation of South Texas, San Antonio, TX.

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taxel were evaluated in tumor biopsies taken directly from patients using an *in vitro* soft-agar cloning assay.

Materials and methods

Tumor sample processing

After written informed consent was obtained according to federal and institutional guidelines, histologically and cytologically proven human tumor specimens including direct biopsies, bone marrow aspirates and biopsies, malignant pleural effusions, and malignant ascites were collected by sterile standard techniques as part of routine diagnostic or therapeutic procedures. Biopsies of solid tumors were stored in McCoy's 5A medium (Gibco, Grand Island, NY) containing 10% newborn calf serum, 10 mM HEPES, 90 U/ml penicillin and 90 μg/ml streptomycin (all Gibco) for transport to the laboratory. Preservative-free heparin (10 U/ml; O'Neill, Johns and Feldman, St Louis, MO) was added immediately after collection of fluids to prevent coagulation. Solid specimens were minced and repeatedly passed through metal sieves with a 40 μm mesh (EC Apparatus, St Petersburg, FL) to obtain a single-cell suspension. When necessary, effusions were centrifuged at 150 *g* for 5–7 min and passed through 25 gauge needles to obtain single-cell suspensions. All specimens were washed twice in McCoy's 5A medium containing 5% horse serum (Sigma), 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 2 mM sodium pyruvate, 2 mM glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin and 35 μg/ml L-serine (all Gibco). The viability of cells (ranging from 40 to 100%) was determined on a hemocytometer with Trypan blue. Only viable cells determined the final concentration of plated cells.

Drugs

Paclitaxel was kindly provided by the National Cancer Institute (Bethesda, MD). Paclitaxel was dissolved in a mixture of polyethoxylated castor oil and ethanol to an initial concentration of 6 mg/ml. The stock solution of paclitaxel was prepared in sterile enriched Connaught Medical Research Laboratories medium 1066 (Irvine Scientific, Irvine, CA) and stored at −80°C in sufficient aliquots for individual assays. Tumor cells were exposed to paclitaxel at final concentrations of 0.025, 0.25, 2.5 and 25 μg/ml in the same media for 1 h or 1-4 days. These concentrations were selected because they corresponded to achievable plasma levels in humans.[17]

Human tumor cloning assay

The human tumor cloning assay was performed using the two-layer system described by Hamburger and Salmon[18] with several modifications.[19–21] Base layers contained 0.5% agar (Difco, Detroit, MI) in a mixture of McCoy's 5A medium, 0.6% soy broth (Difco) and 100 μg/ml asparagine (Difco). Cells were plated at a density of 5×10^5/dish in 35 mm Petri dishes (Corning) in a mixture of 0.3% agar, 15% horse serum, 2% fetal calf serum, 5 mg% vitamin C (Gibco), 90 U/ml penicillin, 90 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM glutamine (all Gibco), 2 U/ml insulin (Iletin I; Eli Lilly, Indianapolis, IN), 2 μg/ml transferrin and 4 ng/ml hydrocortisone (both Sigma). Immediately prior to plating, HEPES, pH 7.3 (Gibco, 10 mM final concentration), and sodium pyruvate (2 mM final concentration) were added. All determinations were done in triplicate.

Each experiment included a positive control with orthosodiumvanadate (10^{-3} M; Sigma) which should inhibit all clonogenic cells. The use of a positive control has been shown to greatly increase the reproducibility of the human tumor cloning assay.[19,22] Plates were incubated at 37°C, 5% CO_2, 100% humidity. After 14 days, colonies were counted with an inverted microscope. An experiment was considered evaluable when the water control had 20 colonies/plate or more and the positive control showed 30% or less colony formation compared to the solvent control. An increase in tumor colony formation was considered significant if survival of colonies was 1.5-fold or more compared to the control. A decrease in tumor colony formation was considered significant if survival of colonies was 0.5-fold or less compared to the control.

Statistical analyses

The results were expressed as the percentage of survival of tumor colony-forming units for a particular drug relative to its control. This quantity was calculated as the ratio between the mean number of colonies surviving in the drug-treated dishes and the mean number of colonies growing in the controls. Data were expressed as means and SDs. A significant

inhibition of colony-forming units formations was defined as colony formation of 0.5 × control or less. Statistical comparisons were performed using the χ^2 test for linear trend.

Results

The effects of paclitaxel on colony formation were studied in a total of 22 patients of which 11 were evaluable and allowed a direct comparison of the antiproliferative effects of prolonged versus short-term exposures in the soft-agar cloning assay. The most common tumor types were ovarian, breast and non-small cell lung cancers.

As shown in Table 1, both prolonged and short-term exposure of paclitaxel had a concentration-dependent effect on the growth inhibition of human tumors. The *in vitro* response rates to paclitaxel administered for 1 h increased from 18 to 64% as the paclitaxel concentrations were increased from 2.5 to 25.0 μg/ml. Similarly, *in vitro* response rates to paclitaxel administered for 14 days increased from 27 to 91% as the concentrations were increased from 0.25 to 25.0 μg/ml, respectively. In this study, the *in vitro* response rates were significantly higher with the 24 h exposure ($p < 0.01$). Although the number of any given tumor type was low, similar trends in tumors from patients with ovarian, breast and non-small cell lung cancers were noted. Those data indicate that at isomolar concentrations, continuous exposure to paclitaxel is more potent than short-term exposure against human tumors in cloning assays.

Interestingly, considering the area under the curve (AUC = concentration × duration of exposure) of 1 h and 14 day exposures, the 336-fold increase of the AUC of paclitaxel after 14 day exposure led to 10 and 28% increase in terms of cytotoxic effects at 2.5 and 25.0 μg/ml, respectively (Figure 1). Furthermore, while the increase of concentrations showed limited increase of paclitaxel cytotoxicity, the duration of exposure was effective to maximize the cytotoxicity of paclitaxel (e.g. the cytotoxic effects of 1 h exposure paclitaxel were roughly linear leading to a maximal cytotoxic effect of 53% growth inhibition at 25 μg/ml). Remarkably, the inhibition of cancer cell proliferation remained exponential when paclitaxel was given for 14 days with an 85% cell growth inhibition at 25 μg/ml. Combined together, these data suggest that concentration and the duration of exposure play important but probably different roles in paclitaxel cytotoxicity. High concentrations of paclitaxel seem very efficient to obtain significant cytotoxicity against a large number of cancer cells but remained limited in the ability to reverse resistance in several cell subpopulations. Conversely, the proportion of cancer cells that was able to survive protracted exposure to paclitaxel was more limited in our experiments.

In an attempt to evaluate the effect of prolonged versus short-term exposure to docetaxel, we have examined 15 human tumor samples from which unfortunately only eight samples were evaluable in the tumor cloning assay (Table 1). Although the number of samples is limited, little differences between short-term and protracted exposure were observed with docetaxel (Figure 1).

Discussion

In this study designed to evaluate paclitaxel dose–response relationships and scheduling, we showed that the duration of exposure appears to be a critical parameter in paclitaxel cytotoxicity in human cancer cells taken directly from patients. Previous *in vitro* studies using cancer cell lines have shown that prolonging the duration of paclitaxel exposure generally produces higher cytotoxicity than increasing the drug concentration.[12] Interestingly, several reports suggest that there is a critical 'plateau' concentration above which the antiproliferative effects of paclitaxel do not increase.[1–6,10,12,14] The threshold concentration of this plateau was shown to be inversely related to the duration of treatment.[23] In our study, while the effects of paclitaxel in the human tumor cloning assay did not show a plateau, the cytotoxicity profile of paclitaxel was dependent upon the specific treatment schedule used. We observed that the antiproliferative activity of high concentrations of paclitaxel was more limited with a 1 h schedule than with continuous exposure. The limited dose–response relationship associated with the 1 h schedule may be related to the presence within the tumor biopsy of cancer cells that express high tolerance to the drug. On the other hand, resistance to protracted exposure to paclitaxel may be more difficult to achieve in cancer cells. Similarly, a retrospective analysis of the previous *in vitro* studies shows that the benefit of long-term exposure was more pronounced in taxane-resistant cell lines.[24–26] Moreover, in human tumor biopsy, the presence of cancer cells that are in quiescent phases and therefore unaffected by cytotoxic drugs may give benefit to protracted exposure to cycle- and phase-specific drugs such as paclitaxel.[5,6,13,26]

Table 1. *In vitro* antiproliferative activity of paclitaxel and docetaxel: prolonged versus short-term exposure

Tumor types/origin	Survival (%) of tumor colony-forming units with paclitaxel								Survival (%) of tumor colony-forming units with docetaxel							
	14 day exposure (μg/ml)				1 h exposure (μg/ml)				14 day exposure (μg/ml)				1 h exposure (μg/ml)			
	0.025	0.25	2.5	25.0	0.025	0.25	2.5	25.0	0.025	0.25	2.5	25.0	0.025	0.25	2.5	25.0
Breast cancer/ascites	70	52	26	7	67	73	54	41	78	73	69	46	70	69	53	54
Breast cancer/pleural effusion	33	25	20	18	57	43	37	32	–	–	–	–	–	–	–	–
Breast cancer/pleural effusion	84	83	61	17	86	68	65	25	–	–	–	–	–	–	–	–
Breast cancer/pleural effusion	153	121	96	2	108	102	84	71	–	–	–	–	–	–	–	–
Ovarian cancer/pleural effusion	67	68	52	6	88	74	58	54	90	89	86	67	75	84	56	74
Ovarian cancer/pleural effusion	74	59	41	0	91	85	92	65	78	73	69	46	70	69	53	54
Ovarian cancer/primary	125	106	126	54	87	94	76	87	–	–	–	–	–	–	–	–
Ovarian cancer/primary	85	60	53	19	94	62	59	35	–	–	–	–	–	–	–	–
Lung adenocarcinoma/pleural effusion	55	32	12	0	80	56	46	36	30	23	10	2	59	53	38	25
Lung adenocarcinoma/pleural effusion	94	83	95	34	104	73	82	37	125	118	114	135	96	109	101	103
Lung adenocarcinoma/pleural effusion	60	36	21	7	87	76	54	35	–	–	–	–	–	–	–	–
Lung adenocarcinoma/pleural effusion	–	–	–	–	–	–	–	–	100	93	83	70	–	–	–	–
Lung adenocarcinoma/pleural effusion	–	–	–	–	–	–	–	–	86	97	81	47	–	–	–	–
Lung adenocarcinoma/primary	–	–	–	–	–	–	–	–	82	75	72	41	58	53	58	55
Mean % survival	82	66	55	15	86	73	64	47	84	80	73	57	71	73	60	61
Overall *in vitro* response rate[a]	1/11	3/11	5/11	10/11	0/11	1/11	2/11	7/11	1/8	1/8	1/8	5/8	0/8	0/8	1/8	1/8
(%)	(9)	(27)	(45)	(91)	(0)	(9)	(18)	(64)	(12)	(12)	(12)	(62)	(0)	(0)	(12)	(12)

[a]Inhibition is given as the ratio of the number of specimens inhibited (colony formation 0.5 or less times that of the control) by the number of assessable specimens (%).

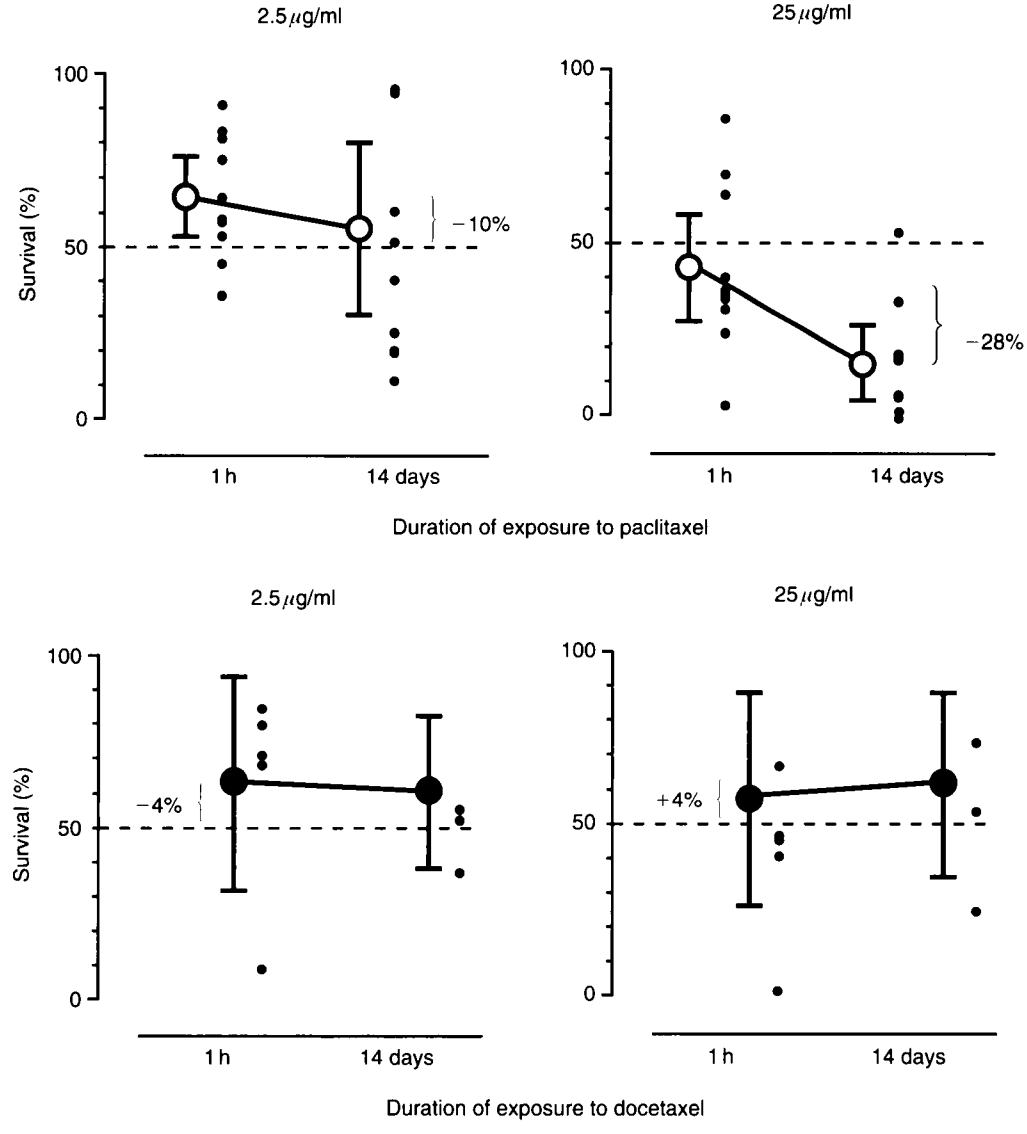

Figure 1. Variation of the cytotoxic effects of paclitaxel and docetaxel with the duration of exposure. The cytotoxic activity of 2.5 and 25 μg/ml paclitaxel (○) and docetaxel (●) was evaluated against freshly expanded human cancer cells taken from patients' biopsies using an *in vitro* soft-agar cloning assay.[19] Each single spot represents the mean of triplicate determinations from an individual evaluable human cancer cell specimen. Curves represent the average variations of the cytotoxic effects of drugs (± 95% confidence interval) in all the tumor specimens after 1 h and 14 day exposures.

Whether the favorable cytotoxic effects of prolonged exposure to paclitaxel *in vitro* may be translated into benefits in clinical trials seems to be more questionable. Controversy was due to the pharmacokinetic parameters and the toxicity profile of paclitaxel in patients.[12–14,27] Pharmacokinetic analyses have shown that biologically relevant plasma concentrations can be achieved by almost any paclitaxel schedule.[16,28,29] Further, studies have shown that the pharmacokinetic behavior of a high dose bolus of paclitaxel appeared to be non-linear while, conversely, roughly linear pharmacokinetics could be obtained when paclitaxel was administered in continuous infusion that yielded low plasma concentrations. In bolus injection, the pharmacokinetic parameters of paclitaxel were associated with a very large volume of distribution in humans and with substantial sequestration of radiolabeled paclitaxel in peripheral tissues.[28–32] Moreover, animal studies have shown that active concentrations of

paclitaxel are maintained in implanted human tumor xenografts for relatively long periods.[33] Therefore, based on pharmacokinetic parameters, the theoretical cytotoxic advantages of administering paclitaxel over a prolonged period of time may not be obvious in clinical trials. Moreover, the toxicity profile of paclitaxel in clinical trials has also been demonstrated to depend on the duration of administration, higher hematologic toxicity being observed with prolonged schedules. To date, short-term injection remains the standard in paclitaxel chemotherapy. However, recent clinical trials have demonstrated that manageable toxicity may be expected with protracted infusion over 96 and 120 h, and with multiple fractionated daily bolus injection for 3 and 5 days.[34–36] Therefore, comparative studies evaluating the antitumor effects of such prolonged administrations of paclitaxel versus conventional schedules are warranted.[37]

Another potentially interesting application of prolonged exposure to paclitaxel is locoregional therapy. Remarkably, paclitaxel possesses a bulky chemical structure and pharmacological characteristics that lead to an extremely slow peritoneal clearance, induce high i.p. concentrations and therefore make it a very attractive drug for i.p. delivery considering its high activity in ovarian cancer.[7–9,11,24,37] Markman and colleagues[38] showed that at the recommended i.p. dose of 125 mg/m^2, the regional concentrations of paclitaxel were several orders of magnitude higher than concentrations required to induce cytotoxic effects *in vitro* and that those concentrations were maintained for several days. Our data showed that prolonged exposure to paclitaxel at concentrations above 2.5 μg/ml (about 3 μM) induced a high antitumor activity. Protracted exposure at such a concentration may easily be achieved by i.p. infusion.

Conclusion

In summary, our data indicate that the duration of exposure to paclitaxel is an important factor in paclitaxel cytotoxicity in human cancer cells, and support the evaluation of prolonged i.v. and i.p. administration of paclitaxel in clinical trials.

Acknowledgments

The authors thank Peggy Durack, Laida Garcia and Alice Louise Goodwin for their excellent assistance in collecting the data and preparing this manuscript for publication.

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(Received 6 February 1997; accepted 14 February 1997)