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BIOLOGY CONTRIBUTION

PACLITAXEL SENSITIVITY CORRELATES WITH P53 STATUS AND DNA FRAGMENTATION, BUT NOT G2/M ACCUMULATION

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Purpose: The antitumor agent paclitaxel (Taxol®) has been shown to arrest cells in mitosis through microtubule stabilization and to induce apoptosis. The tumor suppressor gene p53 is implicated in the regulation of cell cycle checkpoints and can mediate apoptotic cell death. Although initial studies demonstrated that various DNA-damaging agents can induce p53, more recent studies have also shown p53 induction following nonDNA–damaging agents, including paclitaxel. We investigated the influence of p53 abrogation on paclitaxel-induced cell kill and correlated the extent of mitotic arrest and DNA fragmentation by paclitaxel with the drug's cytotoxic effect.

Materials and Methods: The parental human colorectal carcinoma cell line (RKO) with wild-type p53 alleles, and two transfected RKO cell lines with inactivated p53 (RKO.p53.13 with transfected mutant p53 and RC 10.3 with HPV-16-derived E6 gene) were exposed to graded doses of paclitaxel (1–100 nM) for 24-h intervals. The functional status of p53 in cells was assessed by thymidine and BrdU incorporation following exposure to ionizing radiation (4 Gy). Reproductive integrity following paclitaxel treatment was assessed by clonogenic assay. Immunolabeling and microscopic evaluation were used to assess mitotic accumulation and micronucleation. Apoptosis was assayed using DNA fragmentation analyses.

Results: A 4-fold increase in paclitaxel sensitivity was observed among RKO cells deficient in p53 function compared with wild-type RKO cells (IC 50: 4 nM, 1 nM, 1nM for RKO, RKO.p53.13, RC 10.3, respectively). The increased cytotoxic effect in RKO cells with inactive p53 correlated with an increased propensity towards micronucleation and DNA fragmentation following paclitaxel treatment. However, no significant difference in peak mitotic accumulation was observed among RKO cells with functional or abrogated p53.

Conclusions: RKO cells lacking functional p53 demonstrate significantly enhanced sensitivity to paclitaxel compared with that of wild-type RKO cells. This response corresponded with increased micronucleation and DNA fragmentation in cells deficient in p53 function. Although previous published reports of enhanced paclitaxel sensitivity in p53-deficient cells correlated this finding with increased G2/M arrest, we did not observe any significant correlation between paclitaxel-induced cell kill and the degree of mitotic arrest. Our data suggest that apoptosis is the predominant mechanism of paclitaxel cytotoxicity in RKO cells and is likely mediated by a p53-independent process. **© 1999 Elsevier Science Inc.**

Paclitaxel, p53, Apoptosis, Sensitization.

INTRODUCTION

Paclitaxel is an antitumor agent increasingly being utilized in the treatment of human malignancies (1). Its potential role as a cytotoxic agent evolved from earlier observations of its microtubule-stabilizing properties resulting in the accumulation of cells in the G2/M phase of the cell cycle. (2, 3) In addition, cells exposed to paclitaxel develop multiple micronuclei because the nuclear membrane reforms around unsegregated sister chromatids (2). It has been postulated that paclitaxel's ability to interfere with normal cytokinesis should confer preferential sensitivity to rapidly dividing cells. Published data from both *in vitro* and clinical studies, however, suggest significant variation in cellular sensitivity to paclitaxel (4), suggesting that alternative mechanisms may be responsible for the drug's primary cytotoxic effect.

The p53 tumor-suppressor gene has been implicated in the regulation of many critical cellular functions, including cell cycle arrest and apoptosis (5–8). It has also been reported that the functional status of the p53 protein may be an important determinant of cellular sensitivity to anticancer agents, including paclitaxel (9–12). Although initial studies

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demonstrated p53 induction following cellular exposure to DNA-damaging agents, more recent studies have shown similar results following treatment with nonDNA-damaging agents, including paclitaxel (13, 14).

The cytotoxic effect of paclitaxel has been correlated both with its ability to interfere with normal microtubule function and with its ability to induce apoptosis (2, 12, 15). In this study, we attempted to further elucidate the significance of p53 abrogation on paclitaxel sensitivity and to correlate the extent of mitotic arrest and DNA fragmentation with the drug's cytotoxic effect.

MATERIALS AND METHODS

Cells

The human colorectal carcinoma (RKO) cell line with wild-type p53 alleles and two derivative cell lines that lack p53 function (RC 10.3 and RKO.p53.13) were obtained from Dr. M. B. Kastan, Johns Hopkins University, Baltimore, MD. RC-10.3 cells are RKO cells that contain a pCMV-E6 expression construct, and RKO.p53.13 are transfected with mutant p53 (16). Cells were maintained in RPMI 1640 media supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin-streptomycin, and 1% nonessential amino acids.

Clonogenic assays

Cells in an exponential phase of growth (5×10^5) were plated into 60-mm dishes in 5.0 ml of media, incubated for 24 h, and subsequently exposed to paclitaxel or ionizing radiation. Cells receiving both treatments were exposed to paclitaxel (1–10 nM) for 24 h prior to radiation. Following treatment, cells were washed twice with HBSS to remove the drug, replated as single cells, and incubated for 12 more days at 37°C in 5% CO2/95% air. Cells were fixed with methanol: acetic acid (3:1) and stained by Crystal Violet. Only colonies containing $>$ 50 cells were scored as deriving from viable, clonogenically capable cells.

Radiation treatment

Cells were exposed to graded doses (0–8 Gy) of ^{137}Cesium γ rays at a dose rate of 1.1 Gy/min.

Drug treatment

Paclitaxel was obtained from Dr. Peter Schiff of the Department of Radiation Oncology at Columbia Presbyterian Medical Center. A stock solution (10^{-3} M) was prepared in dimethyl sulfoxide (DMSO) and stored at -40°C until required. In all samples, the final DMSO concentration was below 0.2%.

Immunolabeling

Cells, 5×10^4, growing exponentially were pulse-labeled with 10 μM of bromodeoxyuridine (BrdU) prior to drug treatment. Cells were fixed at increasing time intervals (up to 24 h) in methanol: acetic acid (3:1), stained using a fluorescein-tagged antibody to BrdU (Becton Dickinson San Jose, California), counterstained with propidium iodide, and evaluated using incident fluorescent microscopy.

Apoptotic assay

DNA fragmentation analyses were determined by extracting DNA from 6×10^6 cells. Cells were trypsinized, pelleted, and washed twice with PBS. Cells were resuspended in a cold lysis solution (20 M TrisHCl, 10 mM EDTA, 0.2% Triton X-100) and centrifuged at 12,000 *g* at 5°C for 10 min. Proteinase K (80 μg/ml) was added to the supernatant and incubated overnight in a water bath at 50°C. DNA extraction was carried out with phenol/chloroform followed by precipitation with ammonium acetate and isopropanol. Samples were pelleted, dissolved in TE buffer (1 mM TrisHCl, 0.1 mM EDTA) containing RNase, and fractionated on a 1.5% agarose gel containing ethidium bromide. DNA patterns in agarose gel were detected by ethidium bromide activation by UV light (17). Quantitation of DNA fragmentation was achieved using the NIH Image 1.59 program.

Western blot analysis

Whole-cell lysates were prepared, separated on 4–12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes according to the procedure described previously (18). p53 and p21 proteins were detected using anti-p53 and anti-p21 antibodies (Oncogene Research Products, Cambridge, MA), respectively, and visualized by chemiluminescence (Amersham Corporation, Arlington Heights, IL).

RESULTS

To assess the influence of p53 abrogation on paclitaxel-induced reproductive cell death, colony-forming experiments were performed using escalated doses of the drug for 24-h exposure intervals. As shown in Fig. 1, concentration-dependent cell kill was observed in all cell lines. However, the two cell lines with inactivated p53 (RKO.p53.13 and RC10.3) demonstrated 4-fold greater sensitivity to paclitaxel compared with the parental RKO cell line (IC_{50} values of 1 nM, 1 nM, and 4 nM, respectively).

The observation that cells exposed to paclitaxel accumulate in mitosis has lead to the hypothesis that the drug's primary mechanism of action is via mitotic spindle inhibition (2). Because p53 is implicated in a mitotic-related checkpoint (19, 20), we investigated the effect of p53 inactivation on mitotic arrest following paclitaxel and correlated this response with the drug's cytotoxic effect. Cells were treated with increasing concentrations of paclitaxel (2.5–100 nM) for periods of up to 24 h, and then examined for DNA synthesis (assessed by BrdU incorporation) and mitotic accumulation.

The basal fraction of BrdU-labeled cells was approximately 50% for all cell lines. In the absence of paclitaxel, $\sim$ 90% of cells showed BrdU labeling by 24 h. Exposure of cells to 10 nM paclitaxel resulted in $\sim$15% reduction in the

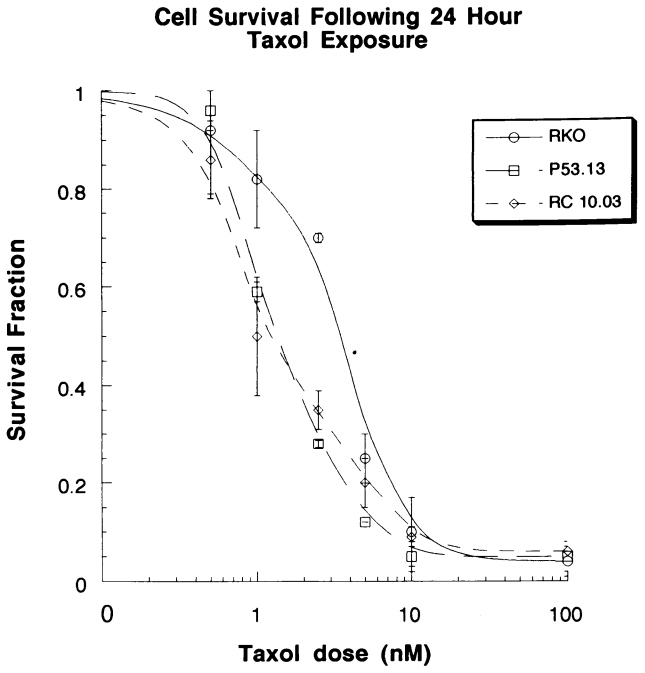

Fig. 1. Cell survival determined by the colony-forming assay following 24-h exposure to graded doses of paclitaxel (0–100 nM). Colonies containing > 50 cells were scored as clonogenically viable. RKO cell lines with inactivated p53 (RKO p53.13 and RC 10.3) demonstrated 4-fold greater sensitivity to paclitaxel than wild-type RKO cells. (IC_{50} values were 1 nM, 1nM, and 4 nM, respectively.)

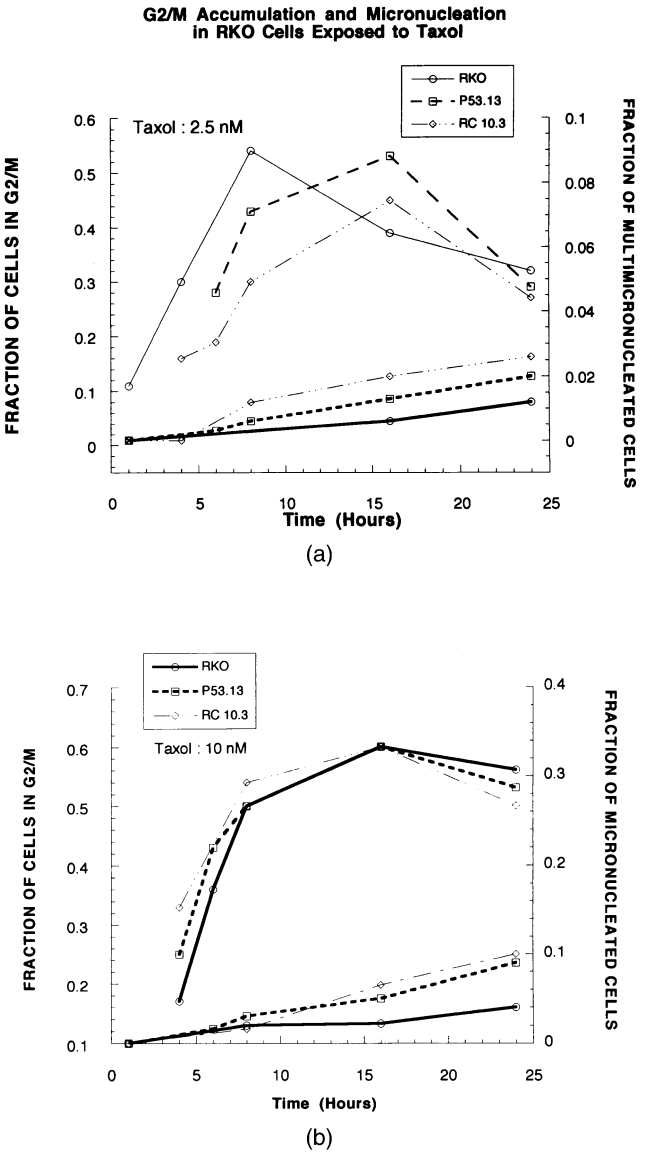

Fig. 2. G2/M accumulation and micronucleation following 24-h exposure to (a) 2.5 nM paclitaxel and (b) 10 nM paclitaxel in cells with functional (RKO) and inactivated p53 (RKO p53.13 and RC 10.3). A significant time- and concentration-dependent increase in both G2/M accumulation (upper curves) and micronucleation (lower curves) was observed in all cell lines. No significant difference in the extent of G2/M accumulation was observed among RKO cells with or without functional p53. In contrast, RKO cells with abrogated p53 (RKO p53.13, RC 10.3) demonstrated significantly increased micronucleation following exposure to (a) 2.5 nM paclitaxel and (b) 10 nM paclitaxel, compared with parental RKO cells. Cell cycle analysis was performed using the immunolabeling technique described in Methods.

labeling index of all 3 cell lines relative to untreated controls, suggesting that paclitaxel did not significantly inhibit DNA synthesis in these cells. In contrast, we observed a significant concentration- and time-dependent increase in the mitotic index of cells exposed to paclitaxel; however, no significant difference was observed in the peak mitotic index among cells with normal or inactive p53 (0.52, 0.50 and 0.42 following 2.5 nM paclitaxel, and 0.60, 0.62, and 0.58 following 10 nM paclitaxel in RKO, RKOp53.13 and RC10.3 cells, respectively) (Fig. 2, upper curves). Although peak mitotic accumulation occurred earlier in cells with functional p53, no significant difference in the kinetics of mitotic accumulation was observed following exposure to 10 nM. (Fig. 2b)

It has been observed that cells exposed to paclitaxel develop multiple micronuclei as a consequence of nuclear membrane reformation around unsegregated chromosomes (2). It has been proposed that this process ultimately results in cell death, possibly due to additional mutations that may occur in genetically unstable polyploid cells. Alternatively, it is possible that the presence of micronucleation may trigger an apoptotic response. We compared the extent of micronucleation and DNA fragmentation following paclitaxel exposure in cells with normal and abrogated p53 and correlated our findings with the drug's cytotoxic effect. RKO cells deficient in p53 function demonstrated significantly greater propensity toward micronucleation following paclitaxel treatment compared with wild-type RKO cells. The proportion of micronucleated cells were 1%, 2%, and 2.5% following 24-h exposure to 2.5 nM paclitaxel, and 3.6%, 10%, 10% following 24-h exposure to 10 nM in RKO, RKOp53.13, and RC 10.3 cells, respectively (Fig. 2, lower curves). Furthermore, the appearance of micronucleated cells corresponded with a decline in the mitotic index, particularly in cells lacking p53.

Figure 3 illustrates our observation of dose-dependent DNA fragmentation following paclitaxel treatment in all

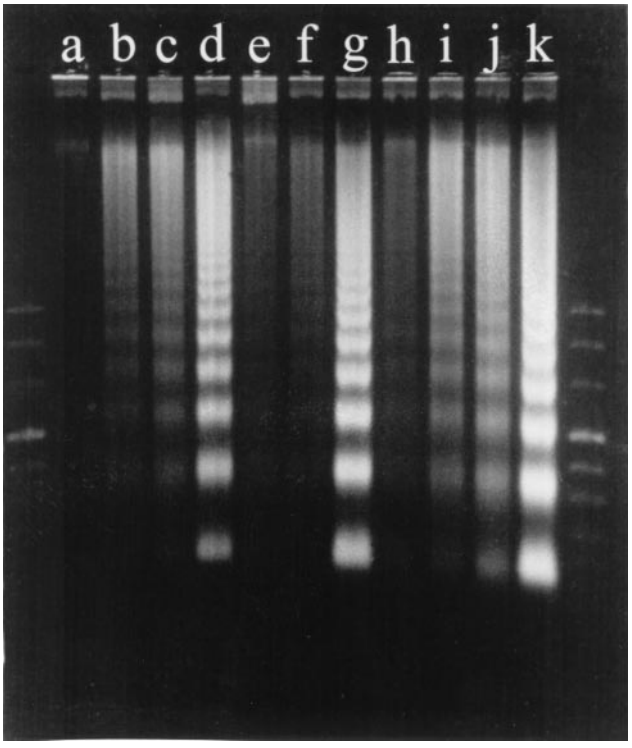

Fig. 3. Induction of DNA fragmentation in response to paclitaxel exposure. A concentration-dependent increase in DNA fragmentation was observed in RKO cells with functional p53 (lanes a–d) and inactivated p53 (RKO p53.13, lanes e–g, and RC 10.3, lanes h–k). Lanes a, e, and h represent untreated controls; lanes b, f, i following 24-h exposure to 5 nM paclitaxel; lanes c, j following 24-h exposure to 10 nM paclitaxel; lanes d, g , k following 24-h exposure to 100 nM paclitaxel in RKO, RKO p53.13, and RC 10.3 cells, respectively.

cell lines; however, RKO cells with inactivated p53 revealed significantly greater DNA fragmentation compared to the parental cell line. These differences have been quantitated using the NIH 1.59 Image program, and the results are shown in Figure 4. Cells deficient in p53 function demonstrated 4-fold enhancement of paclitaxel-induced DNA fragmentation over that in normal RKO cells.

Western blot analyses performed under similar treatment conditions revealed a significant increase in p53 and p21 protein levels in RKO cells, but no induction in RCOp53.13 and RC 10.3 cells (data not shown).

Finally, there have been several studies evaluating the potential role of paclitaxel as a radiosensitizing agent due to its ability to arrest cells in the most radiosensitive phase of the cell cycle, G2/M (4, 21–25). Conflicting results have been reported, and the determinants of supra-additivity of paclitaxel and radiation remain unknown. We treated RKO cells (with active and inactive p53) with paclitaxel and γ radiation to assess if alterations in p53 status affected the interaction between these two agents. Cells were exposed to graded doses of the drug (1–10 nM) for 24 h prior to irradiation (1–8 Gy), replated and analyzed 12 days later for colony formation. The interaction between paclitaxel and ionizing radiation was uniformly additive in RKO cells, irrespective of their p53 status.

DISCUSSION

Paclitaxel continues to have an increasing role in the treatment of human malignancies, particularly ovarian and

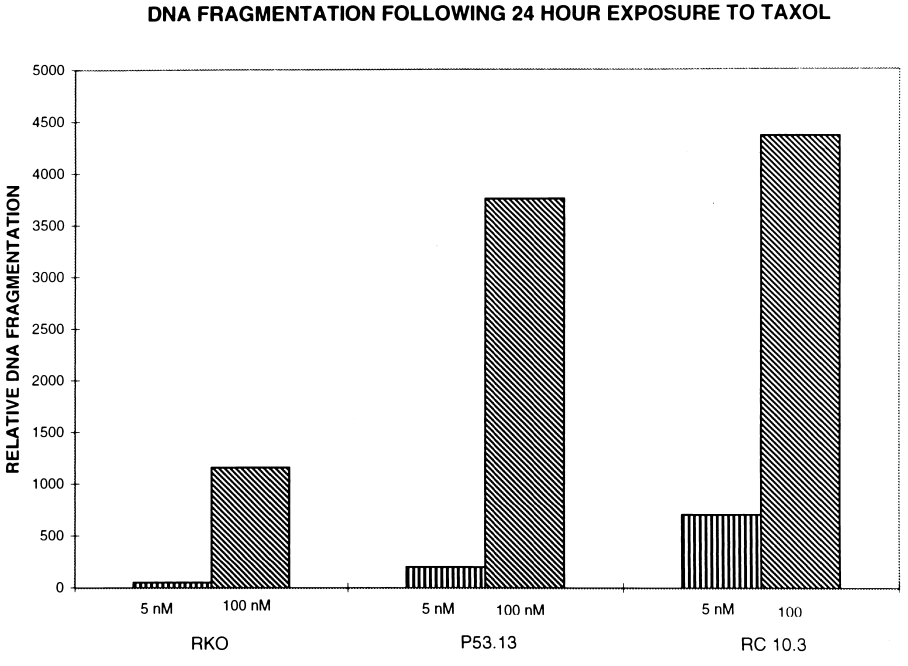

Fig. 4. Increased DNA fragmentation in RKO cells with inactivated p53. Quantitative analysis of paclitaxel-induced apoptosis using the NIH Image 1.59 program demonstrates significantly increased DNA fragmentation in RKO p53.13 and RC 10.3 cells with abrogated p53 compared with wild-type RKO cells.

breast cancer (1). It has been postulated that the main target responsible for its cytotoxic action is the microtubule, based on the following observations. Paclitaxel can lead to the induction of tubulin assembly and cause the formation of microtubules, which remain stable under conditions that would normally cause their disassembly, such as exposure to low temperatures (2). There have been reports suggesting that paclitaxel's cytotoxic effect is directly correlated with its ability to inhibit mitotic progression; however, more recent data reveal other mechanisms may play an important role, including programmed cell death (26–29).

In our study, we observed increased sensitivity to paclitaxel in RKO cells with inactivated p53 compared with parental RKO cells with functional p53. This response correlated with increased micronucleation and DNA fragmentation. No significant difference in mitotic accumulation was observed, suggesting that, in RKO cells, paclitaxel-induced cell kill occurs via (p53-independent) programmed cell death.

These findings corroborate the report of Milross *et al.* who demonstrated a correlation between the *in vivo* antitumor effect of paclitaxel (measured by tumoral growth delay) and paclitaxel-induced apoptosis. No significant correlation was observed between the extent of mitotic accumulation and the drug effect (15).

The increased sensitivity to paclitaxel associated with p53 abrogation has not been consistently observed (30). Debernardis *et al.* (30) did not observe any correlation between p53 status and paclitaxel sensitivity in ovarian cancer cells; however, no cell line studied demonstrated apoptotic cell death following drug exposure. Therefore, it is possible that the increased paclitaxel sensitivity observed with p53 abrogation may be specific to cells capable of inducing programmed cell death.

The molecular mechanism underlying the apoptotic response to paclitaxel remains unclear. Paclitaxel can induce bcl-2 phosphorylation, which may be less capable of forming heterodimers with (the pro-apoptotic) bax protein (27, 28). This may lead to increased formation of bax:bax homodimers, potentially driving the cell toward apoptosis (31). Alternatively, p53-independent paclitaxel-associated apoptosis may be related to the release of the macrophage-derived cytokine, tumor necrosis factor α (29). Lanni *et al.* (29) demonstrated inhibition of paclitaxel-induced apoptosis in cells exposed to anti-TNF antibodies.

There have been many reports exploring the potential role of paclitaxel as a radiosensitizing agent due to its ability to block cells in the most radiosensitive phase of the cell cycle, G2/M (4, 21–25). In our experiments, we observed a simple additive interaction between paclitaxel and ionizing radiation, irrespective of p53 status. This suggests that G2/M accumulation alone is not sufficient for paclitaxel radiosensitization. Cells must be arrested in G2/M, but remain reproductively viable for paclitaxel to be a potential radiosensitizer. Furthermore, the data suggest that supra-additivity may be more dependent on drug concentration than on intrinsic cell factors, including p53; however, additional studies are required.

In conclusion, our data suggest that RKO cells lacking functional p53 are more sensitive to paclitaxel than RKO cells with normal p53 due to increased micronucleation and DNA fragmentation induced by the drug. In contrast to other reports, we did not observe any difference in paclitaxel-induced mitotic accumulation in the more sensitive cells that lacked p53, suggesting that p53-independent apoptosis is likely the predominant mechanism of cytotoxic action of paclitaxel in RKO cells. Additional studies are required to understand the molecular basis for this differential response to enhance the effectiveness of paclitaxel in the treatment of patients with malignant disease.

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