

Taxol, Radiation, and Oncogenic Transformation¹

Tom K. Hei² and Eric J. Hall

Center for Radiological Research, College of Physicians & Surgeons, Columbia University, New York, New York 10032

ABSTRACT

The novel antineoplastic drug taxol has been shown to be active clinically against several types of human tumors. With improvement in treatment strategy and the number of long-term survivors increasing, a question that needs to be addressed is the potential carcinogenic effect of the treatment in the induction of second malignancies. We show here that when tested using an *in vitro* assay for oncogenic transforming potential, taxol is ineffective in focus induction at doses significantly higher than those used in the clinic. However, taxol enhances the oncogenic potential of γ -rays in a synergistic fashion. The fact that taxol blocks cells at the G₂/M phases of the cell cycle may account for this interaction since G₂/M are relatively radiosensitive phases of the cell cycle.

INTRODUCTION

Taxol, a novel antineoplastic drug extracted from the bark of *Taxus brevifolia*, has been shown to be active clinically against various human tumors, including advanced ovarian cancer, breast cancer, and malignant melanoma (1-3). The discovery of the possible antitumor effect of taxol can be dated back to the late 1960s, when a crude extract of bark from the western yew tree was shown to be cytotoxic against a range of murine tumors. It had been known since the Middle Ages that the leaves and bark of the yew were toxic to domestic animals. Later studies identified taxol as the active component of the bark extract (4). Schiff *et al.* (5, 6) first demonstrated that taxol functioned through its ability to stabilize microtubules and promoted microtubule assembly. As a result, the drug can selectively block cells in the G₂ and M phases of the cell cycle. Recent studies by Tishler *et al.* (7, 8) showed that taxol enhanced the radiosensitivity of a grade 3 astrocytoma cell line in a dose-dependent manner. Thereby, this drug shows promise as an adjunct to radiotherapy as well as showing activity alone as a chemotherapy agent.

As anticancer therapy improves and the number of long-term survivors increases, a question that always arises for a new treatment agent is its potential oncogenicity. To some extent, the development of a second malignancy is the price of success, since a patient must be a long-term survivor, with the original tumor controlled before a second malignancy can become evident. The incidence of second malignancies induced by radiotherapy and chemotherapy is well documented in patient studies (9, 10). In addition, a variety of *in vitro* assays of oncogenic transformation have been used to compare the oncogenic potential of the many classes of anticancer agents available. Most known chemotherapy agents can induce malignant transformation *in vitro* (11, 12). Due to the long latency for cancer induction in humans by either radiation or chemical agents and the large population base required to compare the carcinogenic potential of these compounds, model systems that give quick and meaningful answers are clearly needed. In the present study, the effects of taxol on cell growth kinetics and oncogenic transforming potency, either alone or in combination with γ -irradiation, were assessed using the mouse C₃H 10T1/2 cell system.

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² To whom requests for reprints should be addressed, at Center for Radiological Research, College of Physicians & Surgeons, Columbia University, VC-11-218, 630 West 168th Street, New York, NY 10032.

MATERIALS AND METHODS

The C₃H 10T1/2 mouse embryo fibroblast cell line was used for these studies (13). Cells were maintained in Eagle's basal medium supplemented with 10% heat-inactivated fetal bovine serum (Biofluid, Rockville, MD) and 25 μ g/ml gentamycin. Cells were routinely cultured in 75-cm² flasks and incubated in 5% CO₂-air at 37°C. Only cells from passages 8 through 12 were used in these studies. The spontaneous transforming frequency for this batch of cells, expressed as the fraction of dishes with foci, ranged from 0.01 to 0.03.

Taxol (NSC 125973) was obtained from the National Cancer Institute drug program by Dr. P. Schiff of the Department of Radiation Oncology at Columbia University, and the drug was made available for the present study through a continuing collaboration. The drug was dissolved in dimethyl sulfoxide at a stock concentration of 1 mM. Working concentrations were prepared fresh from the frozen stock just before use.

To determine cytotoxicity, asynchronous C₃H 10T1/2 cells growing in 25 cm² flasks were treated with graded doses of taxol at 37°C for 24 h under aerated conditions. A 24-h treatment protocol was chosen for the present studies to correlate with the population doubling time of 10T1/2 cells of ~18-20 h. In experiments where radiation was involved, the drug-treated cultures were irradiated with γ -rays from a ¹³⁷Cs irradiator at an absorbed dose rate of 118 cGy/min. After treatment, the cultures were washed twice with buffered salt solution, trypsinized off the dishes, counted with a Coulter electronic counter, and replated in 100-mm-diameter dishes for colony formation. Cultures were incubated for 10-12 days; after that time they were fixed with formaldehyde and stained with Giemsa, and the number of colonies was counted.

To determine the effects of taxol on the growth kinetics of C₃H 10T1/2 cells, exponentially growing cells were plated at 5 × 10⁴ cells/60-mm-diameter Petri dish in 4 ml of growth medium. After overnight incubation, the cultures were treated with graded doses of taxol for a 24-h period. At the end of the treatment, all cultures were washed twice with buffered salt solution and replenished with fresh medium. For each data point, 3 replicate dishes of cells from each treatment group were trypsinized, and the total number of cells per dish was determined.

For the transformation assay, taxol- and/or radiation-treated cultures were trypsinized and replated in 100-mm-diameter dishes at a density such that approximately 200-400 viable cells would survive the treatments (14, 15). All treated and control cultures were maintained for a total of 6 weeks, and the medium was changed every 10 days. The cultures were then fixed, stained, and scored for type II and III transformed foci as described previously (16, 17). All dishes were coded and scored independently twice. Transformation frequencies were expressed as fractions of dishes with foci. Overall, 6 separate experiments involving a total of 2800 dishes were included in these studies.

RESULTS

Taxol stabilizes microtubular structures, the effects of which on cell morphology in the C₃H 10T1/2 cells are shown in Fig. 1. Control cultures of the 10T1/2 cells demonstrate a fibroblastic morphology with a few occasional mitotic figures at any one time during exponential growth (Fig. 1a). In contrast, cultures treated with a 500 nM dose of taxol for 24 h showed substantially more mitotic figures (Fig. 1b). The cells lost their polarity. They were flattened and appeared in some instances to have fragmented nuclei (Fig. 1b, arrows).

In addition to being able to block cells in the G₂/M phases of the cell cycle, taxol is also cytotoxic. Fig. 2 shows the surviving fractions of 10T1/2 cells treated with graded doses of taxol for a 24-h period. Taxol induced a concentration-dependent cytotoxicity in the cells. A 100 nM dose of taxol resulted in 55% of treated cells being clonogenically viable, whereas the corresponding value for human astrocytoma cells reported previously by Tishler *et al.* was ~0.1% (8).

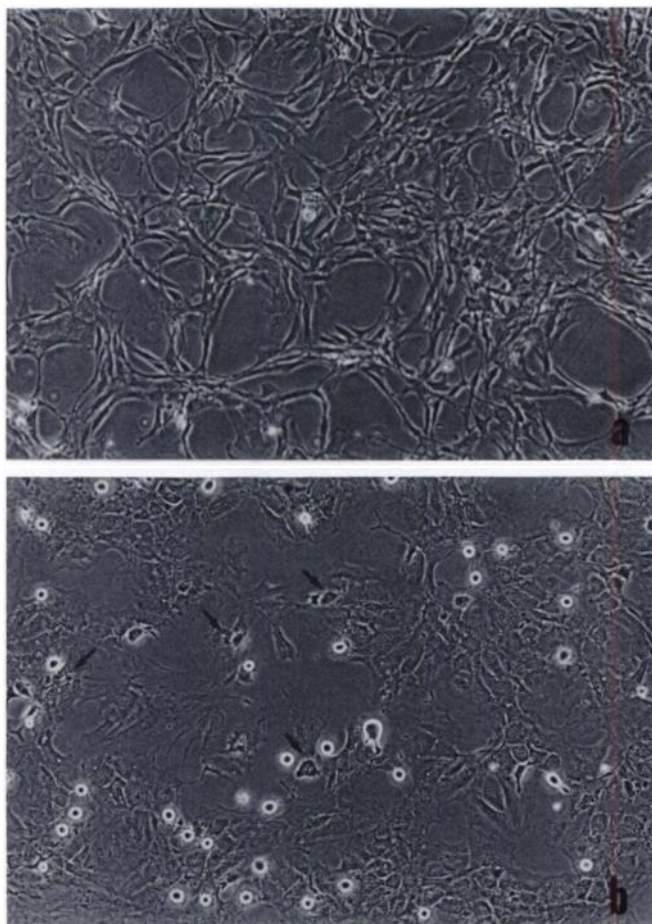


Fig. 1. Phase contrast photomicrograph of control C₃H 10T1/2 cells (a) or 10T1/2 cells treated with a 500 nM dose of taxol for a 24-h period (b). Note the amount of mitotic figures and cellular morphology in the taxol-treated culture relative to the control. Note the appearance of fragmented nuclei in the taxol-treated culture (arrows). $\times 100$.

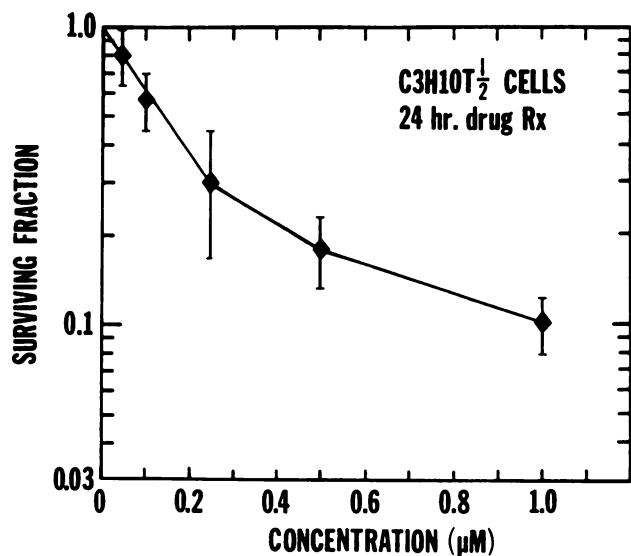


Fig. 2. Surviving fractions of the C₃H 10T1/2 cells treated with graded doses of taxol for a 24-h period. Data are pooled from 4 or 5 experiments. Bars, SEM.

The effect of graded doses of taxol on the growth kinetics of C₃H 10T1/2 cells is shown in Fig. 3. Untreated control cells had a population doubling time of ~ 19 h during the logarithmic phase of growth. Taxol-treated cultures, however, demonstrated a dose-dependent delay in the growth rate of the cells. A 50 nM dose of taxol, which resulted

in a surviving fraction of 0.8, essentially had minimal effect on the growth of the cells once the drug was removed. In contrast, cells treated with a dose of 500 nM of taxol had a population doubling time that was 2½ times longer than that of the control.

The oncogenic transforming potential of graded doses of taxol in C₃H 10T1/2 cells is shown in Fig. 4. Table 1 gives details on the number of dishes used, the number of viable cells at risk, the total

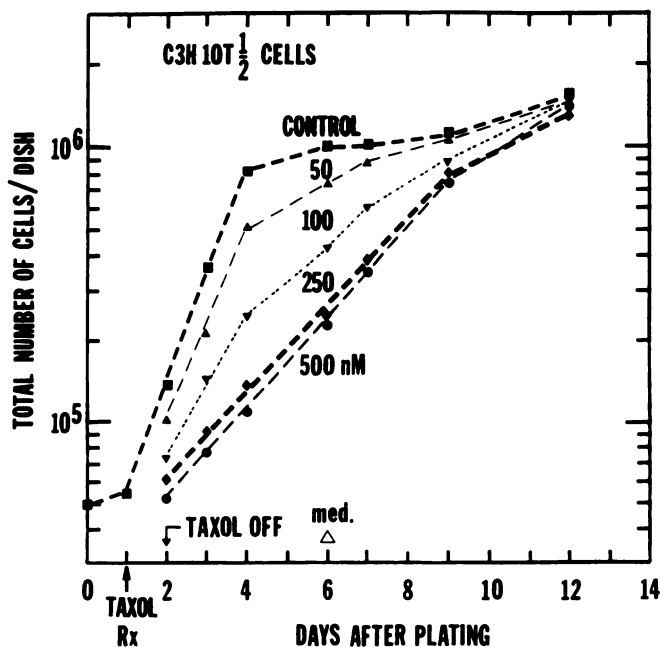


Fig. 3. Effects of graded doses of taxol on growth kinetics of C₃H 10T1/2 cells. Exponentially growing cells were treated with the drug for 24 h in air. After treatment, the cultures were washed and the total number of cells per 60-mm diameter Petri dish was counted. The T_d for the control was ~ 19 h versus 48 h in cells treated with a 0.5 μM dose of taxol. Data were pooled from 2 experiments.

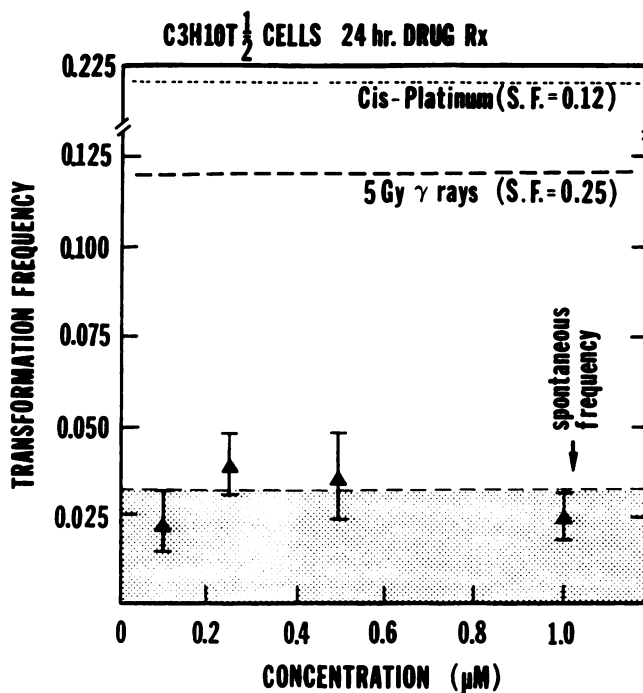


Fig. 4. Oncogenic transforming potential of graded doses of taxol in C₃H 10T1/2 cells. Data are pooled from 3 experiments. Bars, SD. Shaded area, spontaneous transforming frequency. Transformation data from a 4-Gy dose of γ -irradiation and from a 1.0- $\mu\text{g}/\text{ml}$ dose of cis-platinum (replotted using data from Ref. 26) are included for comparison.

Table 1 Transformation incidence produced in C₃H 10T $\frac{1}{2}$ cells by graded doses of taxol

Dose (μ M)	SF ^a	No. of dishes	Total cells at risk ($\times 10^4$)	No. of foci		TF ^b	Pooled TF ^c
				II	III		
Taxol							
0.10	0.62	60	2.42	2	2	0.067	0.022 \pm 0.009
	0.53	61	1.63	0	0		
	0.48	72	1.86	0	0		
	0.57	80	2.31	1	1		
0.25	0.42	77	4.79	2	3	0.064	0.041 \pm 0.013
	0.37	71	2.08	2	0	0.028	
	0.19	70	1.07	1	1	0.028	
0.50	0.19	75	2.76	1	0	0.013	0.036 \pm 0.012
	0.28	71	3.21	1	3	0.056	
	0.10	76	0.95	1	2	0.039	
1.00	0.15	69	3.65	2	0	0.029	0.020 \pm 0.01
	0.08	79	2.11	0	1	0.013	
Control							
	0.47 ^d	28	1.31	1	1	0.071	0.026 \pm 0.01
	0.42	60	2.53	0	0		
	0.34	80	2.68	3	0		
	0.36	62	2.81	0	1		

^a Surviving fraction.

^b Transformation frequency expressed as fraction of dishes with type II and III foci.

^c Pooled transformation frequency \pm SD.

^d Plating efficiency.

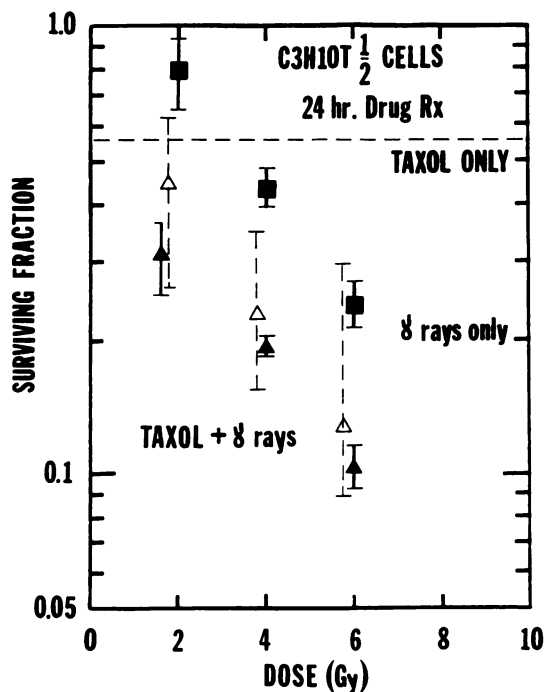


Fig. 5. Effects of a 100 nM dose of taxol (average survival fraction = 0.56) on the radiosensitivity of 10T $\frac{1}{2}$ cells to γ -rays (■). In cells pretreated with taxol followed by radiation, the actual experimental survival data (\blacktriangle) are well within the range of the calculated values (\triangle) assuming an additive interaction of the two agents (mean \pm SD). Data are pooled from 3 to 5 experiments. Bars, SD.

number of type II and III foci scored in each group, and the pooled transformation frequencies. Transformation incidence was expressed as fractions of dishes with foci. Comparable transformation data from cultures either irradiated with a 5-Gy dose of γ -rays or treated with a 1.0- μ g/ml dose of *cis*-platinum for 24 h are included for comparison. Over the range of doses examined, taxol was essentially nononcogenic. At concentrations up to 1 μ M, which resulted in a surviving fraction of \sim 10%, taxol induced a transformation frequency in C₃H 10T $\frac{1}{2}$ cells that was indistinguishable from the spontaneous level.

The fact that taxol blocks cells in phases of the cell cycle that are relatively more radiosensitive suggests a potential interaction between

the two agents. Fig. 5 shows the survival level of 10T $\frac{1}{2}$ cells irradiated with graded doses of γ -rays with or without a 24-h pretreatment with a 100 nM dose of taxol. Taxol treatment resulted in a 44% reduction in the clonogenic capacity of the culture. In combination with γ -irradiation, taxol interacted in a simple additive manner in relation to cell killing. The resultant survival level from a combined exposure fell within the statistical range of the calculated values, assuming the two agents acted in an additive manner. Similarly, in cells pretreated with graded doses of taxol followed by a single, 2-Gy dose of γ -rays (survival fraction = 0.78), the resultant survival level obtained was largely additive (data not shown).

The oncogenic transforming potential of a combined taxol and γ -irradiation treatment for C₃H 10T $\frac{1}{2}$ cells is shown in Fig. 6. Table

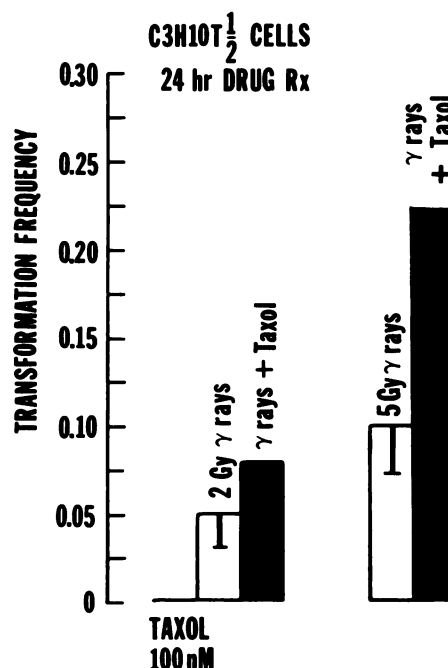


Fig. 6. Oncogenic transforming incidence in C₃H 10T $\frac{1}{2}$ cells pretreated with a 100 nM dose of taxol and subsequently irradiated with graded doses of γ -rays. Transformation frequencies were expressed as a fraction of dishes with foci. Bars, SD.

Table 2 Effects of taxol on modulating the oncogenic transforming potential of γ -irradiation in C₃H 10T $\frac{1}{2}$ cells

Treatment	SF ^a	No. of dishes	Total cells at risk ($\times 10^4$)	No. of foci		TF ^b	Pooled TF ^c
				II	III		
γ -Rays 2 Gy	0.90	57	2.75	1	2	0.053	0.053 \pm 0.02
	0.79	55	1.56	2	1	0.054	
5 Gy	0.23	80	3.99	3	5	0.100	0.106 \pm 0.03
	0.20	43	1.27	1	4	0.116	
Taxol 0.1 μ M	0.53	61	1.63	0	0	0.017	0.0075
	0.61	56	1.23	1	0		
Taxol + 2 Gy	0.30	76	1.92	3	3	0.079	0.082 \pm 0.03
	0.46	34	0.63	1	2	0.088	
+ 5 Gy	0.09	67	2.21	5	12	0.254	0.224 \pm 0.04
	0.11	71	1.53	7	7	0.197	
Control	0.42 ^d	60	2.53	0	0	0.014	0.0076
	0.26	73	3.60	1	0		

^a Surviving fraction.

^b Transformation frequency expressed as fraction of dishes with foci.

^c Pooled transformation incidence \pm SD.

^d Plating efficiency.

2 gives details of the experimental data. The transformation incidence was expressed as fractions of dishes with foci. While taxol at the concentration used (100 nM) demonstrated no transforming effect on its own, it enhanced the oncogenic transforming effect of γ -irradiation. Although the degree of enhancement in oncogenicity by taxol at a 2-Gy dose of γ -rays was not statistically significant given the relatively low yield of transformants, the result at 5 Gy supports a supra-additive interaction between the two agents.

DISCUSSION

As an antineoplastic drug, taxol is distinct from other antimicrotubule cancer drugs such as colchicine and vincristine. Whereas the latter compounds induce microtubule disassembly, taxol stabilizes and promotes microtubule assembly, thereby shifting the equilibrium in favor of the formation of stable, yet nonfunctional microtubules (5, 18). As such, taxol induces abnormal spindle aster formation and is the basis for its ability to block cells in the G₂ and M phases of the cell cycle. In addition, cellular functions that are normally attributed to the cytoskeleton, such as cell morphology, motility, intercellular transport, and cellular attachment, are all drastically affected in taxol-treated cells. The effect of taxol on cell morphology is clearly illustrated in Fig. 1. This morphological change can be correlated with the characteristic formation of disorganized microtubules that are often aligned in parallel bundles, as shown previously using indirect immunofluorescence microscopy with antibodies against tubulin (6).

Apart from blocking cells in mitosis, taxol is also cytotoxic to the C₃H 10T $\frac{1}{2}$ cells in a dose-dependent manner. Compared to earlier published work using a human tumor cell line, taxol is definitely less toxic to rodent cells in general (7, 19). The drug is about 500-fold more toxic to human astrocytoma cells than to comparably treated 10T $\frac{1}{2}$ cells. The mechanism(s) that mediate this huge difference in sensitivity is not known. The uptake or efflux of the drug may differ between the two cell types. In addition, the ability to metabolize taxol may also be different between human and rodent cells. Studies using human leukemia cell lines suggest that the sensitivity to taxol is directly related to the formation of irreversible spindle esters in taxol-treated cells (20).

In contrast to either *cis*-platinum or γ -irradiation, which produce a significant transformation incidence in C₃H 10T $\frac{1}{2}$ cells, taxol induces a transforming frequency that is not much different from the

spontaneous level even at high cytotoxic doses of the drug. These findings are consistent with earlier studies using other antimicrotubule drugs such as vincristine and colcemid (21, 22). Although conflicting transformation results have been reported with Syrian hamster embryo cells (23), such discrepancies have largely been attributed to differences in the cell assay system. Since C₃H 10T $\frac{1}{2}$ cells are already immortalized, the assay tends to measure a later, as opposed to an earlier, stage in oncogenic transformation.

Previous studies using human astrocytoma cells demonstrated that a combination of taxol and radiation treatment kills cells in a synergistic manner (8). Results of the present studies, however, support an additive interaction between the two agents in C₃H 10T $\frac{1}{2}$ cells. Data obtained recently using other human tumor cell lines, such as the cervical carcinoma cells, also suggest an additive interaction for cell killing from a combined radiation and taxol treatment (24). The fact that taxol blocks cells in the G₂/M phase of the cell cycle may form the basis for the observed enhancement by taxol of radiation-induced transformation. Recent studies by Miller *et al.* (25) showed that radiation-induced transformation in C₃H 10T $\frac{1}{2}$ cells is cell cycle dependent, with G₂ being the most sensitive phase for the transforming effect of ionizing radiation (25).

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REFERENCES

- McGuire, W. P., Rowinsky, E. K., Rosenshein, N. B., Grumbine, R. C., Ettinger, D. S., Armstrong, D. K., and Donehower, R. C. Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. *Ann. Intern. Med.*, *111*: 273-279, 1989.
- Holmes, F. A., Frye, D., Theriault, R. L., Walters, R. S., Forman, A. D., Newton, L. K., Buzdar, A. U., and Hortobagyi, G. N. Phase II study of taxol in patients with metastatic breast cancer. *Proc. Am. Soc. Clin. Oncol.*, *10*: 60, 1991.
- Legha, S. S., Ring, S., Papadopoulos, N., Raber, M., and Benjamin, R. A. Phase II study of taxol in metastatic melanoma. *Cancer (Phila.)*, *65*: 2478-2481, 1990.
- Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., and McPhail, A. T. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.*, *93*: 2323-2327, 1971.
- Schiff, P. B., Fant, J., and Horwitz, S. B. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.)*, *277*: 665-667, 1979.
- Schiff, P. B. and Horwitz, S. B. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA*, *77*: 1561-1565, 1980.

7. Tishler, R. B., Schiff, P. B., Geard, C. R., and Hall, E. J. Taxol: a novel radiation sensitizer. *Int. J. Radiat. Oncol. Biol. Phys.*, **22**: 613-617, 1992.
8. Tishler, R. B., Geard, C. R., Hall, E. J., and Schiff, P. B. Taxol sensitizes human astrocytoma cells to radiation. *Cancer Res.*, **52**: 3495-3497, 1992.
9. Coleman, C. N. Secondary neoplasms in patients treated for cancer: etiology and perspective. *Radiat. Res.*, **92**: 188-200, 1982.
10. Storm, H. H. Secondary primary cancer after treatment for cervical cancer: late effects after radiotherapy. *Cancer (Phila.)*, **61**: 679-688, 1988.
11. Hall, E. J., Miller, R. C., Osmak, O., and Zimmermann, M. A comparison of the incidence of oncogenic transformation produced by X-rays, misonidazole and chemotherapeutic agents. *Radiology*, **145**: 521-523, 1982.
12. Marquardt, H., Phillips, F. S. and Sternberg, S. S. Tumorigenicity *in vivo* and induction of malignant transformation and mutagenesis in cell cultures by Adriamycin and daunomycin. *Cancer Res.*, **36**: 2065-2069, 1976.
13. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a cloned line of C₃H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.*, **33**: 3231-3238, 1973.
14. Hei, T. K., Komatsu, K., Hall, E. J. and Zaider, M. J. Oncogenic transformation by charged particles of defined LET. *Carcinogenesis (Lond.)*, **9**: 747-750, 1988.
15. Hei, T. K., He, Z. Y., Piao, C. Q., and Hall, E. J. Studies with bifunctional bioreductive drugs. [I] *In vitro* oncogenic transformation potential. *Radiat. Res.*, **124**: S44-S49, 1990.
16. Reznikoff, C. A., Bertram, J. S., Brankow, D. W., Heidelberger, C. Quantitative and qualitative studies on chemical transformation of cloned C₃H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.*, **33**: 3239-3249, 1973.
17. IARC/NCI/EPA Working Group. Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols. *Cancer Res.*, **45**: 2395-2399, 1985.
18. Rowinsky, E. K., Cazennave, L. A., and Donehower, R. C. Taxol: a novel investigational antimicrotubule agent. *J. Natl. Cancer Inst.*, **82**: 1247-1259, 1990.
19. Lopes, N. M., Adams, E. G., and Bhuyan, B. L. Cell kill kinetics and cell cycle effects of taxol on human and hamster ovarian cell lines. *Proc. Am. Assoc. Cancer Res.*, **32**: 1957, 1991.
20. Rowinsky, E. K., Donehower, R. C., Jones, R. J., and Tucker, R. W. Microtubule changes and cytotoxicity in leukemic cell lines treated with taxol. *Cancer Res.*, **48**: 4093-4100, 1988.
21. Benedict, W. F., Banerjee, A., Gardner, A., and Jones, P. A. Induction of morphological transformation in mouse C₃H 10T1/2 clone 8 cells and chromosomal damage in hamster A[T₁]C1-3 cells by cancer chemotherapeutic agents. *Cancer Res.*, **37**: 2202-2208, 1977.
22. Borek, C., and Hall, E. J. Oncogenic transformation produced by agents and modalities used in cancer therapy and its modulation. *Ann. N.Y. Acad. Sci.*, **397**: 193-210, 1982.
23. Tsutsui, T., Suzuki, N., Maizumi, H., and Barrett, J. C. Vincristine sulfate induced cell transformation, mitotic inhibition and aneuploidy in cultured Syrian hamster embryo cells. *Carcinogenesis (Lond.)*, **7**: 131-135, 1986.
24. Geard, C. R., Jones, J. M., and Schiff, P. B. Taxol and radiation. *J. Natl. Cancer Inst.*, in press, 1993.
25. Miller, R. C., Geard, R. C., Geard, M. J., and Hall, E. J. Cell cycle dependent radiation induced oncogenic transformation of C₃H 10T1/2 cells. *Radiat. Res.*, **130**: 129-133, 1992.
26. Hall, E. J., and Hei, T. K. Modulating factors in the expression of radiation induced oncogenic transformation. *Environ. Health Perspect.*, **88**: 149-155, 1990.