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Covalent Binding of Reactive Estrogen Metabolites to Microtubular Protein As a Possible Mechanism of Aneuploidy Induction and Neoplastic Cell Transformation

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Neoplastic cell transformation induced by estrogens and some other carcinogens such as benzene appears to involve the induction of mitotic aneuploidy rather than DNA damage and point mutations. As metabolic activation may also play an important role in the mechanism of carcinogenesis of these nongenotoxic compounds, we have studied the interaction of reactive quinone metabolites of various estrogens and of benzene with the major microtubular protein, tubulin, in a cell-free system. Covalent binding of the radioactively labeled metabolites to the α- and β-subunit of tubulin was found to depend on the structure of the metabolite. When the adducted tubulins were tested *in vitro* for their ability to polymerize to microtubules, inhibition of microtubule assembly was observed in every case, although to varying extents. It is proposed that the formation of covalent tubulin adducts may impair the formation of mitotic spindles and thus contribute to chromosomal nondisjunction and aneuploidy induction.

Introduction

It is well established that certain estrogens can induce cancer in experimental animals and are associated with tumor formation in man (*1*). However, the mechanisms underlying estrogen-mediated tumorigenesis are far from clear. There is a general consensus that hormonally active compounds may act as promoters of cancer, in particular in cells responding to the hormones by growth. It is, however, still a matter of debate whether estrogens have the ability to induce cancer independent of their hormonal activity.

A suitable experimental system for studying the nonhormonal mechanism of estrogen carcinogenesis makes use of Syrian hamster embryo (SHE) fibroblasts. These cells are devoid of detectable levels of estrogen receptors, and their growth is not influenced by estrogens (*2*); nevertheless, they are neoplastically transformed by a variety of natural and synthetic estrogens (*2,3*). Most interestingly, transformation has been shown to occur in the absence of detectable point mutations (*3–5*). Instead, the induction of near-diploid aneuploidy (*3,5*) and of micronuclei (*6*) was observed in these cells, and a chromosomal mechanism has been proposed for cell transformation by estrogens (*5*). The transforming capability of a number of estrogens did not correlate with their hormonal potency but rather with structural features, suggesting that metabolic pathways may be of importance for cell transformation. In particular, peroxidase-mediated oxidation appears to be crucial in the metabolic activation of estrogens (*2*).

In order to elucidate the role of peroxidative estrogen metabolites in the biochemical mechanism of aneuploidy induction, we have studied the covalent binding of these metabolites to microtubular proteins *in vitro*, as well as the functional consequences of such adduct formation for microtubule assembly. Hydroquinone, a major metabolite of the human carcinogen benzene, was included in these studies because it also has the ability to undergo peroxidative metabolism.

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Methods

Covalent Tubulin Binding

^{14}C-Diethylstilbestrol (^{14}C-DES) or ^{14}C-hydroquinone (33 μM in 1 mM phosphate buffer, pH 7.5) was incubated with horseradish peroxidase (HRP; 1.9 U/mL) and H_2O_2 (825 μM) for 10 min at 25°C. The oxidation was stopped by addition of catalase (final concentration, 315 U/mL).

Aliquots of the incubation mixture containing quinoid metabolites thus generated or of a solution of ^{14}C-*N*-ethylmaleimide (NEM) in phosphate buffer were added to twice-cycled bovine microtubule protein (3 μM) (*7*) in reassembly buffer [100 mM 2-(*N*-morpholino)-ethanesulfonic acid, 1 mM ethyleneglycol-*bis*-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 mM $MgCl_2$, pH 6.4] and incubated for 30 min at 37°C. Microtubule assembly was initiated by addition of guanosine triphosphate (GTP; final concentration 0.5 mM) and allowed to proceed for another 30 min. Protein was precipitated by addition of trichloroacetic acid and applied to sodium dodecyl sulfate (SDS, 7.5%, polyacrylamide gels for electrophoresis. After Coomassie blue staining, the dried gels were analyzed by autoradiography and by combustion of excised gel slices in a Packard 306 sample oxidizer, followed by liquid scintillation counting.

Microtubule Assembly

Tubulin was incubated with quinoid metabolites or unmetabolized compounds as described above. GTP was added, and the increase in turbidity at 350 nm was determined after 30 min. Values obtained from incubations in which the test compounds were omitted served as reference (100% assembly). Aggregation of tubulin was assumed if the increase in turbidity was not at least 80% reversible on incubation for 30 min at 4°C or if electron microscopy revealed precipitated material other than microtubules.

Results

Covalent Tubulin Binding

The peroxidative metabolism of stilbene-type estrogens, e.g., diethylstilbestrol and indenestrol A, proceeds via semiquinoid and quinoid intermediates (Fig. 1). Similarly, catechol metabolites of steroidal estrogens, e.g., 2-hydroxy-estradiol, and the benzene metabolite hydroquinone yield semiquinones and quinones upon oxidation (Fig. 1). Although the reactivity of these products with DNA *in vitro* is rather low, they can very efficiently bind to proteins in a covalent manner (*8*,*9*). In the absence of semiquinone (phenoxy) radicals (i.e., if the oxidation of the estrogens is stopped prior to the

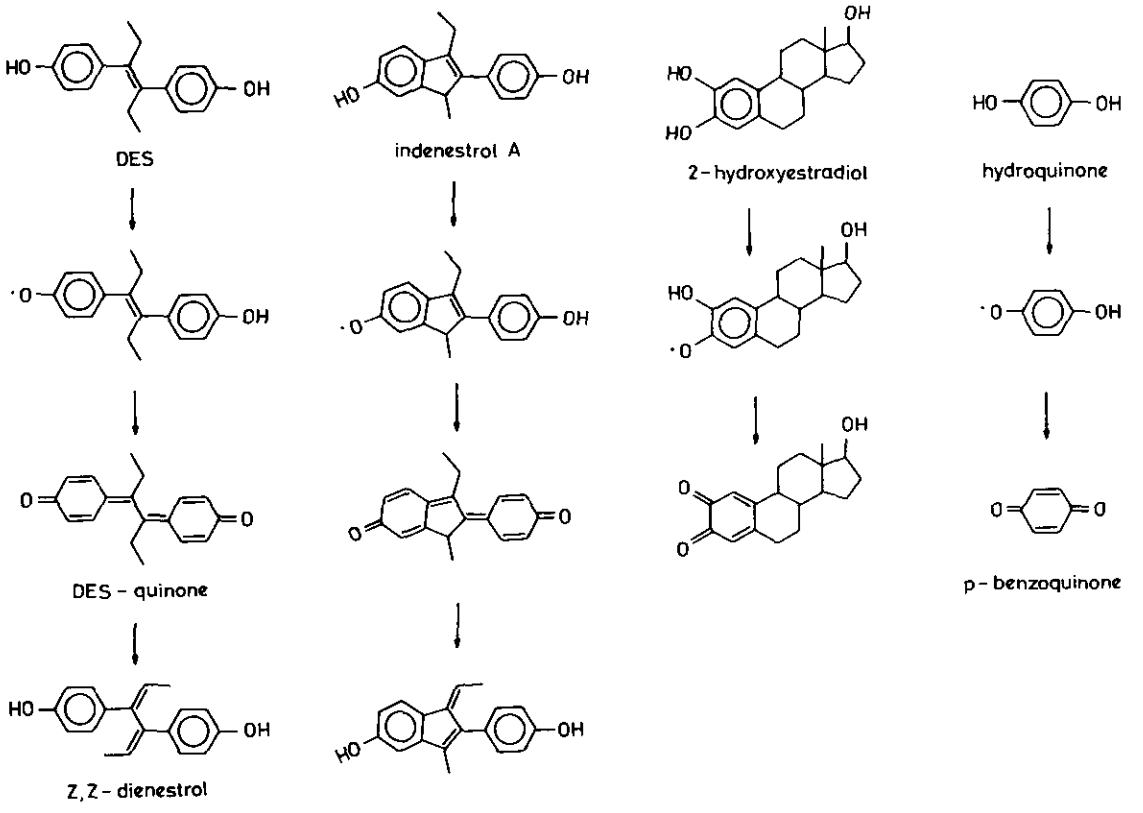

FIGURE 1. Peroxidative metabolism of DES, indenestrol A, 2-hydroxyestradiol, and hydroquinone.

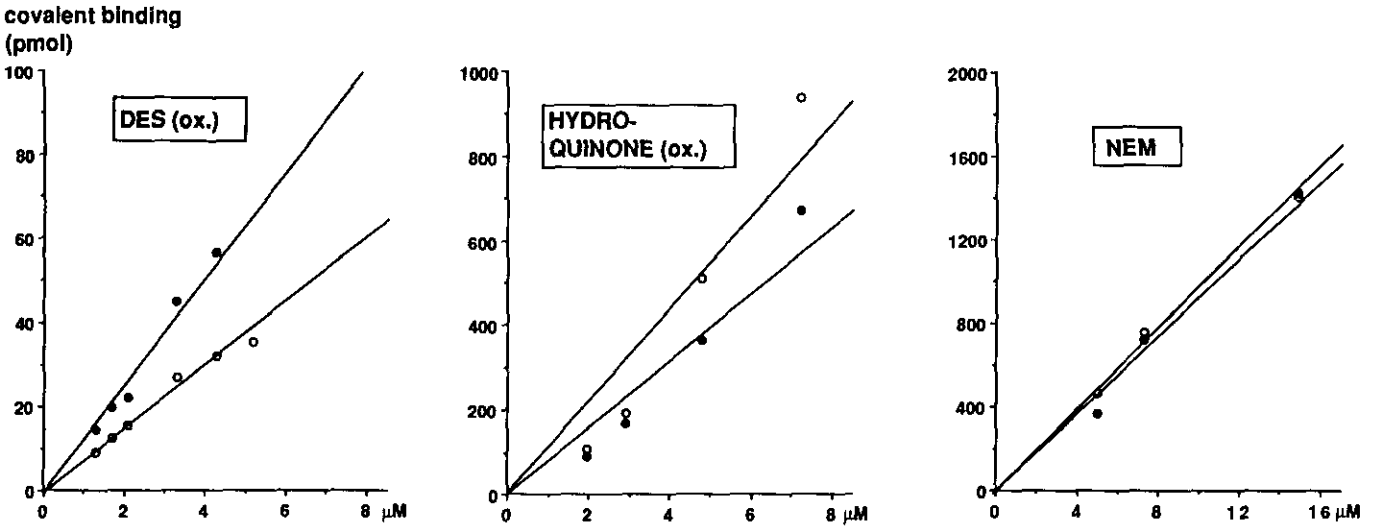

FIGURE 2. Covalent binding of quinoid metabolites and NEM to the α-subunit (○) and β-subunit (●) of tubulin. Tubulin (1.5 nmole) was incubated in 500 μL reassembly buffer with ^{14}C-NEM or quinoid metabolites of ^{14}C-DES or ^{14}C-hydroquinone under the same conditions as used in the tests for microtubule assembly (Fig. 3). Radioactivity bound to the two subunits of the protein was determined after polyacrylamide gel electrophoresis by combustion of excised gel slices.

addition of the protein) the binding species are most probably quinones. For the quinone metabolites of both stilbene-type and steroidal estrogens, a rather selective binding reaction to the major microtubular protein, tubulin, has been observed, which most likely involves the C-terminal domain (*10*).

In Figure 2, the extent of covalent tubulin binding of peroxidative metabolites of ^{14}C-DES, generated *in vitro* by reaction with HRP/H_2O_2, is compared with that of the corresponding oxidation products of ^{14}C-hydroquinone, *p*-benzoquinone. The binding of NEM, a nonspecific sulfhydryl-reactive compound, was studied in parallel.

The data indicate that both NEM and oxidized hydroquinone bind to tubulin to a much higher extent than do the metabolites of DES. This difference is most probably due to the rapid rearrangement of DES to Z,Z-dienestrol (Fig. 1) and does not reflect a lower reactivity of DES-quinone compared to the other quinones. The ratio of labeling of the two subunits of tubulin differs for the different compounds tested. While metabolites of DES and also of other estrogens such as 2-hydroxyestradiol (*10*) bind preferentially to β-tubulin, *p*-benzoquinone causes a higher labeling of the α-subunit. NEM binds to both subunits to about the same extent.

Microtubule Assembly

The relevance of the covalent binding to tubulin for its functional activity was studied by analyzing the GTP-dependent microtubule assembly *in vitro*. In addition to the compounds tested for covalent binding, indenestrol A was included in the assay as another stilbene-type estrogen capable of forming quinoid metabolites (Fig. 1). The inhibition of microtubule assembly by colchicine served as a reference. For each of the estrogens and hydroquinone, the effects of the quinoid metabolites (allowed to bind covalently under the conditions described) were compared with those of the unmetabolized parent compounds.

The results shown in Figure 3 indicate that the metabolites of DES, hydroquinone, and indenestrol A give rise to a dose-dependent inhibition of microtubule assembly. The effect is most probably a consequence of the covalent binding, as it is not observed when the reactive quinones are scavenged from the incubation mixture with glutathione prior to the addition of tubulin (data not shown). High concentrations of metabolites give rise to an irreversible aggregation of tubulin as indicated by its failure to depolymerize at 4°C and by electron microscopy. A similar effect is observed with NEM (Fig. 3). Unmetabolized hydroquinone and indenestrol A do not affect microtubule assembly in the concentration range tested, while DES inhibits significantly (Fig. 3). As DES undergoes considerable autoxidation under the incubation conditions used (data not shown), it cannot be excluded that the inhibition by DES is, at least in part, due to the autoxidation products rather than to the parent compound.

In Table 1, the degree of covalent binding observed at 30% inhibition of microtubule assembly is compared for NEM and the metabolites of DES and hydroquinone. Assuming that the covalent binding is directly responsible for the inhibition, we conclude from the data that the tubulin binding of NEM is much less effective than that of the quinoid metabolites, especially those of DES.

Discussion

The results presented here demonstrate that quinoid metabolites of estrogens can react with tubulin and dis-

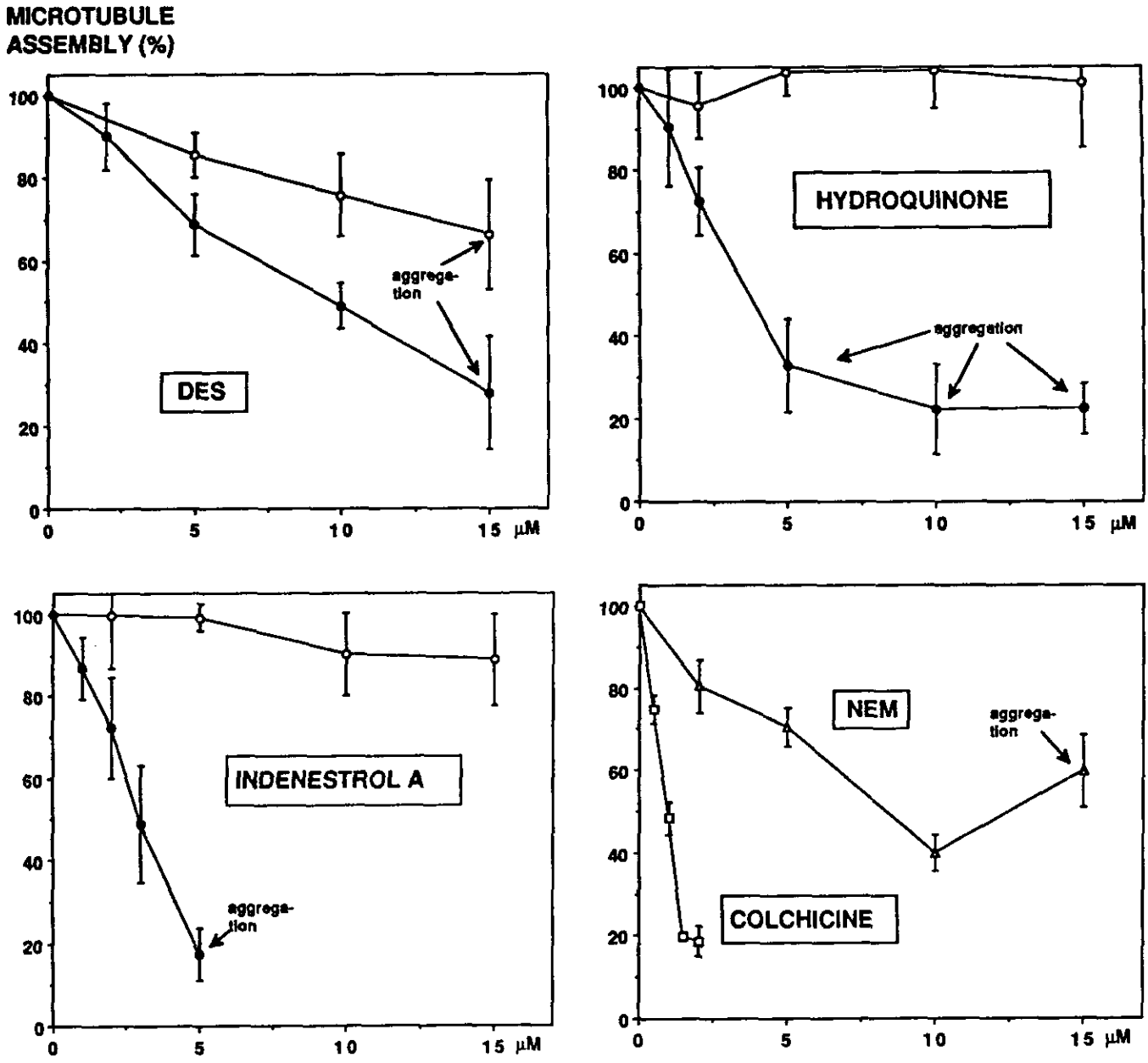

FIGURE 3. Inhibition of microtubule assembly. Tubulin, 1.5 nmole in 500 μL reassembly buffer, was incubated with either unmetabolized compounds (○, □, Δ) or products of peroxidative metabolism (●) for 30 min at 37°C. Microtubule assembly was started by adding GTP, and the increase in turbidity was determined after 30 min. Control incubations containing all components except the test compounds were used as reference (100% assembly). Electron microscopy and depolymerization at 4°C were used to confirm microtubule formation and detect aggregation. Data are means of three independent experiments ± SD.

turb its GTP-dependent assembly into microtubules. The extent of covalent binding associated with a significant inhibition of microtubule assembly is much lower than that observed after a nonspecific modification of sulfhydryl groups by NEM.

These findings and the observation reported here and earlier that DES has an inhibitory effect on tubulin assembly even without covalent binding (*11,12*), might best be explained by postulating a binding site in a functionally important domain of tubulin that has affinity for estrogens and can be irreversibly blocked by the structurally related quinoid metabolites. If a significant covalent modification of tubulin by estrogen metabolites takes place *in vivo*, it may represent the primary lesion for the induction of neoplastic cell transformation by these compounds, giving rise to chromosomal nondisjunction and aneuploidy (Fig. 4).

The similar although apparently less specific inhibi-

Table 1. Covalent binding to tubulin observed at 30% inhibition of microtubule assembly.[a]

Compound	pmole covalent binding/ 1.5 nmole of α-Tubulin	β-Tubulin
Quinoid metabolites of		
DES	42 ± 5	63 ± 6
Hydroquinone	185 ± 55	135 ± 35
NEM	490 ± 50	400 ± 50

[a] Values are calculated from the data shown in Figures 2 and 3.

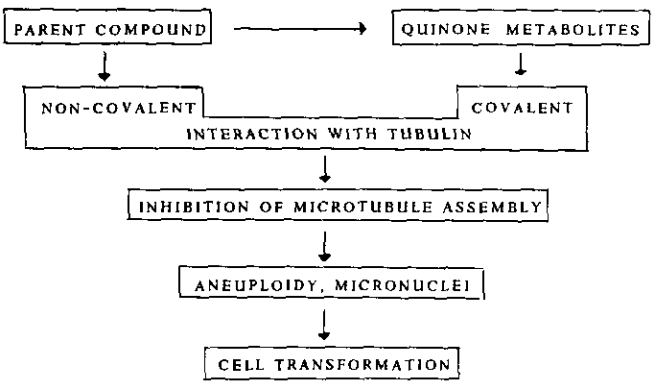

FIGURE 4. Possible mechanism of cell-transformation initiated by an interaction with the cytoskeletal protein tubulin that gives rise to mutations on the chromosomal level.

tion of microtubule assembly by peroxidative metabolites of hydroquinone and, thereby, secondary metabolites of benzene, suggests that the proposed mechanism may be of more general importance for the initiation of carcinogenesis. The carcinogenicity of benzene, like that of DES, is also apparently not induced by point mutations (*13*).

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REFERENCES

1. IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 21, Sex Hormones (II). International Agency for Research on Cancer, Lyon, France, 1979.
2. McLachlan, J. A., Wong, A., Degen, G. H., and Barrett, J. C. Morphological and neoplastic transformation of Syrian hamster embryo fibroblasts by diethylstilbestrol and its analogs. Cancer Res. 42: 3040–3045 (1982).
3. Tsutsui, T., Suzuki, N., Fukuda, S., Sato, M., Maizumi, H., McLachlan, J. A., and Barrett, J. A. 17β-Estradiol-induced cell transformation and aneuploidy of Syrian hamster embryo cells in culture. Carcinogenesis 8: 1715–1719 (1987).
4. Barrett, J. C., Wong, A., and McLachlan, J. A. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. Science 212: 1402–1404 (1981).
5. Tsutsui, T., Maizumi, H., McLachlan, J. A., and Barrett, J. C. Aneuploidy induction and cell transformation by diethylstilbestrol: a possible chromosomal mechanism in carcinogenesis. Cancer Res. 43: 3814–3821 (1983).
6. Schmuck, G., Lieb, G., Wild, D., Schiffmann, D., and Henschler, D. Characterization of an *in vitro* micronucleus assay with Syrian hamster embryo fibroblasts. Mutat. Res. 203: 397–404 (1988).
7. Shelanski, M., Gaskin, F., and Canto, C. R. Microtubule assembly in the absence of added nucleotides. Proc. Natl. Acad. Sci. USA 70: 765–768 (1973).
8. Epe, B., and Metzler, M. Nature of the macromolecular binding of diethylstilbestrol to DNA and protein following oxidation by peroxidase/hydrogen peroxide. Chem.-Biol. Interact. 56: 351–361 (1985).
9. Degen, G. Role of prostaglandin-H synthase in mediating genotoxic and carcinogenic effects of estrogens. Environ. Health Perspect. 88: 217–223 (1990).
10. Epe, B., Hegler, J., and Metzler, M. Site-specific covalent binding of stilbene-type and steroidal estrogens to tubulin following metabolic activation *in vitro*. Carcinogenesis 8: 1271–1275 (1987).
11. Sharp, D. C., and Perry, J. M. Diethylstilbestrol: the binding and effects of diethylstilbestrol upon the polymerisation and depolymerisation of purified microtubule protein *in vitro*. Carcinogenesis 6: 865–871 (1985).
12. Hartley-Asp, B., Deinum, J., and Wallin, M. Diethylstilbestrol induces metaphase arrest and inhibits microtubule assembly. Mutat. Res. 143: 231–235 (1985).
13. Snyder, R., Longacre, S. L., Witmer, C. M., Kocsis, J. J., Andrews, L. S., and Lee, E. W. Biochemical toxicology of benzene. In: Reviews in Biochemical Toxicology, Vol. 3 (E. Hodgson, J. R. Bend, and R. M. Philpot, Eds.), Elsevier/North Holland, New York, 1981, pp 123–153.