Effects of 10 known or suspected spindle poisons in the *in vitro* porcine brain tubulin assembly assay

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We tested the 10 known or suspected spindle poisons (colchicine, econazole nitrate, chloral hydrate, hydroquinone, diazepam, thiabendazole, cadmium chloride, thimerosal, pyrimethamine and vinblastine) of the coordinated EEC programme for induction of aneuploidy with the *in vitro* porcine brain tubulin assembly assay. The influence of the compounds on different parameters [lag-phase, polymerization velocity, endabsorption (steady-state level), reversibility, influence on disassembly at 4°C] was evaluated. Colchicine [IC_{30} (30% inhibition concentration): 0.002 mM), vinblastine (IC_{30}: 0.002 mM), thimerosal (IC_{30}: 0.03 mM), thiabendazole (IC_{30}: 0.5 mM) and chloral hydrate (IC_{30}: 60 mM) led to an inhibition of tubulin assembly *in vitro*. No influence on the steady-state level was obtained with econazole nitrate (up to 0.1 mM), diazepam (up to 2.5 mM), cadmium chloride (up to 1 mM), pyrimethamine (up to 1 mM) and hydroquinone (up to 25 mM), the highest dose tested being limited either by precipitation or by reaching the maximal solubility of the compound in the solvent used. Diazepam enhanced the lag-phase and slightly reduced the polymerization velocity dose-dependently; however, all the treated test mixtures reached the same end absorption levels as the control. The influence on the disassembly process was studied at 4°C. Microtubules treated with colchicine, econazole nitrate, diazepam, thiabendazole, cadmium chloride, thimerosal and pyrimethamine reached the same end absorption level after disassembly as the untreated control. Chloral hydrate reduced the disassembly rate but the end absorption of the control was not reached, the 30% reduction concentration being 0.25 mM. Hydroquinone at very high doses (>10 mM) stimulated the disassembly process. The *in vitro* tubulin assembly assay is, on the basis of this small database, an indicative pre-screening test for aneuploidy inducing chemicals which act mechanistically via interaction with tubulin and/or microtubule associated proteins, both components of the spindle apparatus.

Introduction

Aneuploidy is a particularly pressing problem in humans. Man appears unique among all investigated species in showing a particularly high level of aneuploidy. No other single type of chromosome abnormality contributes more to human ill health and suffering. Human aneuploidy has multiple consequences. These include miscarriage (abortion), infertility, congenital malformations, mental retardation and cancer. The frequency of aneuploidy in newborn humans is in the range of 0.4−0.8% (Hook and Hammerton, 1977) and 35% of all spontaneous abortions turned out to be aneuploid (Sankaranarayanan, 1979; Jacobs and Hassold, 1980). Chromosome malsegregation may also be involved in the formation of malignant tumors (Hook, 1983). In somatic tissues, aneuploidy can play a role in tumorigenesis, i.e. by a possible participation during initiation and/or promotion/progression of malignancy or by expression of oncogenes (Tsutsui *et al.*, 1983; Kondo *et al.*, 1984; Evans *et al.*, 1985).

The mechanisms and the cellular targets leading to the induction of aneuploidy are to a large extent different from those leading to gene and chromosomal mutations where DNA is involved as the principal target.

The mechanisms leading to chromosomal malsegregation are little understood. Furthermore, there are relatively few chemicals that have been tested in a comprehensive battery of aneuploidy assays (Waters *et al.*, 1986). The European Economic Communities (EEC) initiated a coordinated programme with the aim of developing a database on aneuploidy (Parry, 1987; Parry *et al.*, 1990). The assay systems included in the programme range from the polymerization of tubulin *in vitro* to the production of aneuploid gametes in intact animals.

Tubulin and microtubule associated proteins (MAPS and Tau; for review, see Dustin, 1984) are the main constituents of the spindle. In spite of the biochemical characterization of the spindle structures, the molecular mechanisms of mitosis are still under investigation. Mammalian brain tubulin has been studied in great detail, and much is known about its behaviour *in vitro* (Timasheff, 1979; Gaskin, 1982). Different studies compared the chemical induction of aneuploidy in a cellular system with the influence of the same chemicals on an *in vitro* mammalian brain tubulin system (Albertini *et al.*, 1985, 1988a,b, 1989; Gröschel-Stewart *et al.*, 1985; Hartley-Asp *et al.*, 1985; Sharp and Parry, 1985; Zimmermann *et al.*, 1985a,b, 1986).

In the present study, results were obtained with the 10 core chemicals of the coordinated EEC Programme, previously tested in *Saccharomyces cerevisiae* D61.M for chromosome malsegregation (Albertini, 1990), by using the *in vitro* porcine brain tubulin assembly. To investigate the influence on the disassembly process a new experimental approach was undertaken. The temperature-controlled compartment of the spectrophotometer was cooled down from 37°C to 4°C within 2 min, thereby inducing disassembly of microtubules which were formed at 37°C in presence of the chemical.

Materials and methods

Chemicals

Thimerosal (Thiomersal; CASRN 54-64-8) was obtained from Serva, Heidelberg (FRG); pyrimethamine (58-14-0), vinblastine (865-21-4) and econazole nitrate (27220-47-9) from Sigma, St Louis, MO (USA); colchicine (64-86-8), cadmium chloride (10108-64-2), hydroquinone (123-31-9) and chloral hydrate (302-17-0) from Fluka Chemie AG, Buchs (Switzerland); diazepam (439-14-5) and thiabendazole (148-79-8) from F.Hoffmann-La Roche Ltd, Basel (Switzerland); dimethylsulfoxide (DMSO, 67-68-5) from Merck AG, Darmstadt (FRG).

Tubulin isolation

Porcine brain tubulin was prepared according to Williams and Lee (1982). For the purification, two cycles of assembly and disassembly were used. The protein content of the final preparations was determined by the method of Bradford (1976). Purity of each tubulin preparation was evaluated by a slightly modified SDS−PAGE method (Laemmli, 1970; Laemmli and Favre, 1973). The preparations were stored in ice overnight and used the following day for the assembly and disassembly test.

Tubulin assembly assay

Tubulin assembly was followed photometrically by measuring the increase in absorbance at 350 nm. PIPES buffer (100 mM, pH 6.9) with 2 mM EGTA [ethylene glycol-O,O'-bis(2-aminoethyl-N,N,N',N'-tetraacetic acid], 1 mM $MgSO_4$, 2 mM dithioerythritol and 4 M glycerol were mixed in an ice bath with 5 μl DMSO or buffer containing different amounts of the test compound and 10 μl of a 50 mM solution of GTP [guanosine-5'-triphosphate (dilithium salt)] in 10 mM PIPES buffer. Immediately before the start of the experiment 250−350 μl of the tubulin preparation were added. The final volume of the reaction mixture was adjusted to 1.0 ml with buffer, thoroughly mixed and poured into a cuvette placed in the temperature-controlled compartment (37°C) of a recording spectrophotometer. The relative change of absorbance was followed simultaneously in five cuvettes in a five-position cuvette holder and absorbance recorded with 12 s intervals between each cuvette so that a given cuvette was read at 1 min intervals.

Tubulin disassembly assay

To investigate the influence of chemical compounds on the disassembly process of microtubules the following new experimental approach was conducted. After the assembly test at 37°C was completed, the temperature-controlled compartment was cooled down to 4°C within 2 min. During the following 30−40 min the influence of the test compounds on the process of disassembly in the cold was followed by monitoring the change in absorbance at 350 nm.

In vitro tubulin assay

The influence of the different compounds on the in vitro assembly kinetics of porcine brain tubulin can be studied by comparing different parameters (Fig. 1). (i)lag-phase: time elapsed until the polymerization reaches 10% of end absorption. (ii) polymerization velocity (dA/dt) in the range between 10 and 90% of end absorption. (iii) end absorption (steady-state level): Absorbance at the end of the assembly; the 30% inhibition concentration (IC_{30}) of compounds inhibiting the assembly process can be calculated. (iv) depolymerization velocity ($dA*/dt*$). (v) end absorption after disassembly process at 4°C.

Results

The results obtained with the ten chemicals evaluated are summarized in Table I. The highest dose tested was either limited by the solubility of the test compound or by precipitation of the compound in the aqueous medium.

Out of the 10 chemicals evaluated, five led to an inhibition of the in vitro polymerization of porcine brain tubulin (Figure 2): colchicine, chloral hydrate, thiabendazole, thimerosal and vinblastine. The concentrations necessary to reduce the steady-state level by 30% covered a wide range: 0.002 mM (colchicine and vinblastine), 0.03 mM (thimerosal), 0.5 mM (thiabendazole) and 60 mM (chloral hydrate). Five compounds (econazole nitrate, hydroquinone, diazepam, pyrimethamine and cadmium chloride) had no effect on the steady-state level reached at the end of the assembly process (Figure 2). The highest 'no effect' doses for the five compounds on the steady-state level were: 0.1 mM for econazole nitrate, 25 mM for hydroquinone, 2.5 mM for diazepam, 1 mM for pyrimethamine and 1 mM for cadmium chloride.

An enhanced lag-phase (time elapsed until the onset of tubulin polymerization) was observed after treatment with diazepam (Figure 3), thimerosal (Figure 4) and vinblastine. The other compounds had no influence on this parameter. A dose-dependent reduction of the polymerization velocity was induced by colchicine, enconazole nitrate, diazepam (Figure 3), thimerosal (Figure 4), thiabendazole (Figure 5) and vinblastine. With regard to the reversibility of the assembly process all compounds, except chloral hydrate, did not affect tubulin disassembly at 4°C (same end absorption after disassembly at 4°C as control).
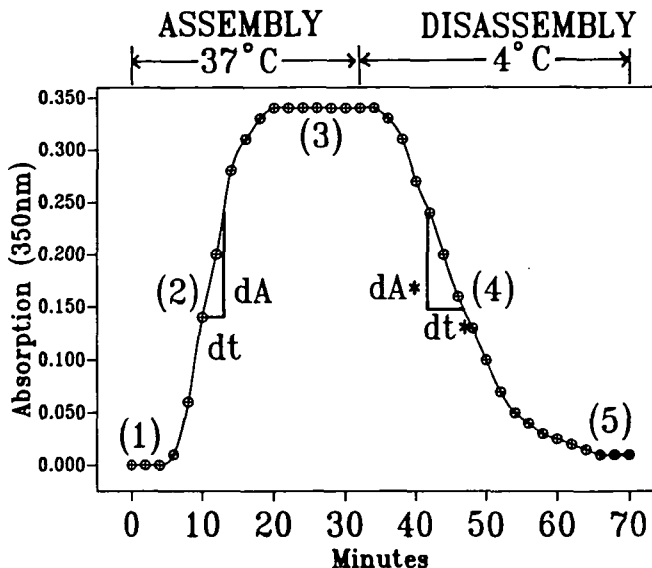

Fig. 1. Scheme of in vitro tubulin assay.

The influence of the 10 compounds on the disassembly process at 4°C was investigated. Chloral hydrate showed a reduction in the disassembly rate (depolymerization velocity as well as the end absorption reached). The concentrations needed for reduction were much lower than those needed for a reduction of the assembly steady-state level (IC_{30} assembly: 70 mM; IC_{30} disassembly: 0.25 mM). Hydroquinone appears to lead to an enhancement of the depolymerization velocity at high concentrations (>10 mM). All other compounds showed no interference with the disassembly process.

Discussion

Since there is unequivocal evidence that frequency of aneuploidy can be increased in experimental organisms by a variety of treatments, there is naturally a concern that human populations, if exposed to aneuploidy inducers, might suffer increased rates of aneuploidy. Extrapolation of findings from experimental organisms to man, however, is hampered by extensive differences of the cell division processes.

Difficulties may arise because the direct target of a treatment is specific to the test organism or because a treatment has an effect via secondary physiological processes which are confined to the test system.

Little is known about the mechanisms leading to chromosomal malsegregation. One specific major target is the spindle, particularly the spindle microtubules. The in vitro tubulin assembly assay could, therefore, be employed as a test system for differentiating aneuploidy inducing chemicals acording to their action mechanisms.

In the present study, the 10 core chemicals which were selected for an EEC coordinated programme on aneuploidy systems on the basis of their known ability to affect specific target organelles were tested in the in vitro porcine brain tubulin asembly assay.

As summarized in Table II all compounds previously proposed to act via spindle/tubulin were positive in our in vitro system, but showed considerable differences in their mode of action. Compounds suggested to be acting via other mechanisms were negative.

Induction of aneuploidy in different test systems by the 10 compounds of the EEC study is discussed in several recent

Table I. Summary of the effects of the 10 EEC aneuploidy programme compounds on tubulin assembly

Compound	Tubulin assembly test[a]						Disassembly assay[b]
	Lag-phase	Polymerization velocity	End absorption	Reversibility	IC_{30} (mM)	Dose range tested (mM)	
Colchicine	0	wR	R	Y	0.002	0.001−0.01	N
Econazole nitrate	0	wR	0	Y	—	0.001−0.1	N
Chloral hydrate	R (>20 mM)	wE (>20 mM)	R (>20 mM)	N	60	0.01−100	R (IC_{30}:0.25 mM)
Hydroquinone	0	0	0	Y	—	0.005−25	prob. E (>10 mM)
Diazepam	E	R	0	Y	—	0.001−2.5	N
Thiabendazole	0	R	R	Y	0.5	0.001−1	N
Cadmium chloride	0	0	0	Y	—	0.005−1	N
Thimerosal	E	R	R	Y	0.03	0.005−0.5	N
Pyrimethamine	0	0	0	Y	—	0.001−1	N
Vinblastine	E	R	R	N	0.002	0.001−0.1	ND

[a]0, no effect; R, reduction; wR, weak reduction; E, enhancement; Y, yes; N, no; ND, not determined
[b]Effect on depolymerization velocity or end absorption after disassembly.

publications (Miller and Adler, 1989; Albertini, 1990; Xu and Adler, 1990).

Colchicine

Our *in vitro* results obtained with colchicine, the best known of all microtubule poisons, are in agreement with published results (Kilmartin, 1981; Dustin, 1984; Wallin *et al.*, 1988). Colchicine inhibits the formation of microtubules by binding to tubulin dimers. Once microtubules have formed there does not seem to be an interaction and the disassembly process is not affected. The observed disappearance of microtubules could, therefore, result from a continued disassembly and disability to form microtubules or from slowing down the process of assembly (Dustin, 1984). Our results obtained with the disassembly assay support this hypothesis. Surprisingly, the number of studies (up to 1986) providing results acceptable to the Committee for Aneuploidy (Gene-Tox programme) for colchicine is extremely meager (for details see special issue *Mutation Research* **167** (1986) 1−188). Furthermore, conflicting data in plants are reported. More recently, Miller and Adler (1989) reported mitotic arrest and C-mitotic effects in mouse bone marrow cells after treatment with colchicine. In *S.cerevisiae* no induction of chromosomal malsegregation was reported by Albertini (1990) and Parry *et al.* (1990). These negative findings in yeast are not surprising, because the binding of colchicine to yeast tubulin is > 1000-fold weaker than the binding to mammalian tubulin (Kilmartin, 1981).

Chloral hydrate

Chloral hydrate, a metabolite of trichloro- and tetrachloroethylene, both potential human carcinogens, causes an inhibition of the assembly as well as the disassembly process. The disassembly process at 4°C was much more sensitive than the assembly process at 37°C: the 30% reduction concentrations were 70 mM and 0.25 mM for assembly and disassembly, respectively, indicating a different mode of action.

Diazepam

Diazepam, a benzodiazepine, prolongs the lag-phase and has only little influence on the polymerization velocity and no effect on the steady state level, i.e. there is no reduction of the final amount of microtubules. Depending on the coordination of cellular processes a delay of the onset of microtubule formation might not only lead to a cell cycle delay but could also lead to serious disturbances of chromosome distribution.

Studies with diazepam in various aneuploidy test systems *in vitro* yielded equivocal results: chromosome maldistribution
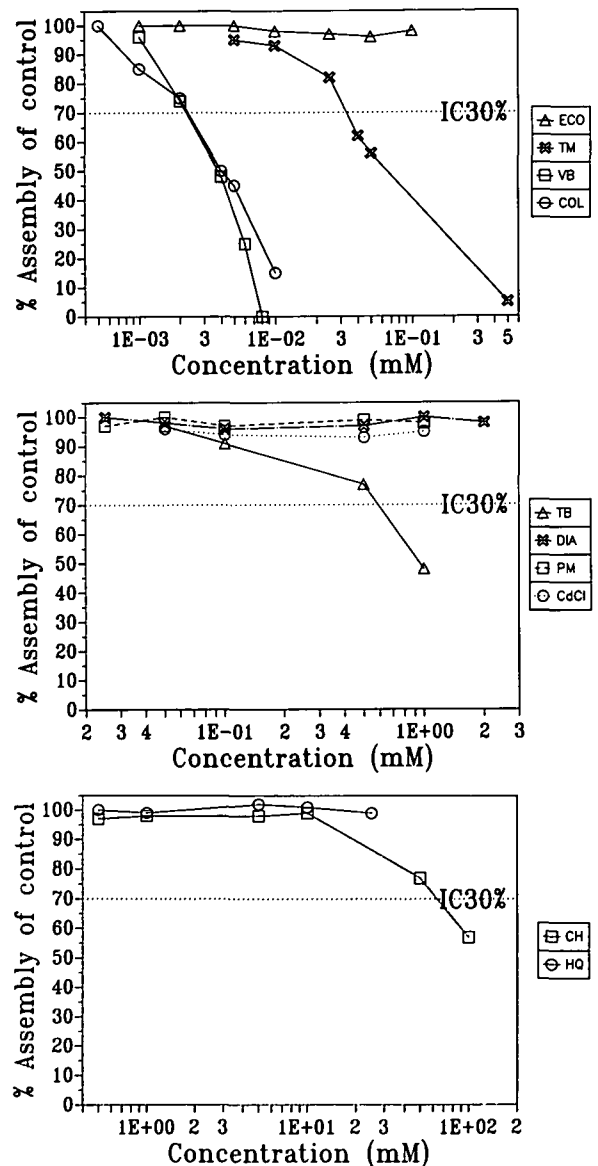

Fig. 2. Dose-dependent inhibition of the *in vitro* tubulin assembly by the 10 EEC aneuploidy core-chemicals. The percentage in comparison to the control (steady-state level = 100%) is given. (a) ECO: econazole nitrate; TM: thimerosal; VB: vinblastine; COL: colchicine. (b) TB: thiabendazole; DIA: diazepam; PM: pyrimethamine; CdCl: cadmium chloride. (c) CH: chloralhydrate; HQ: hyroquinone.
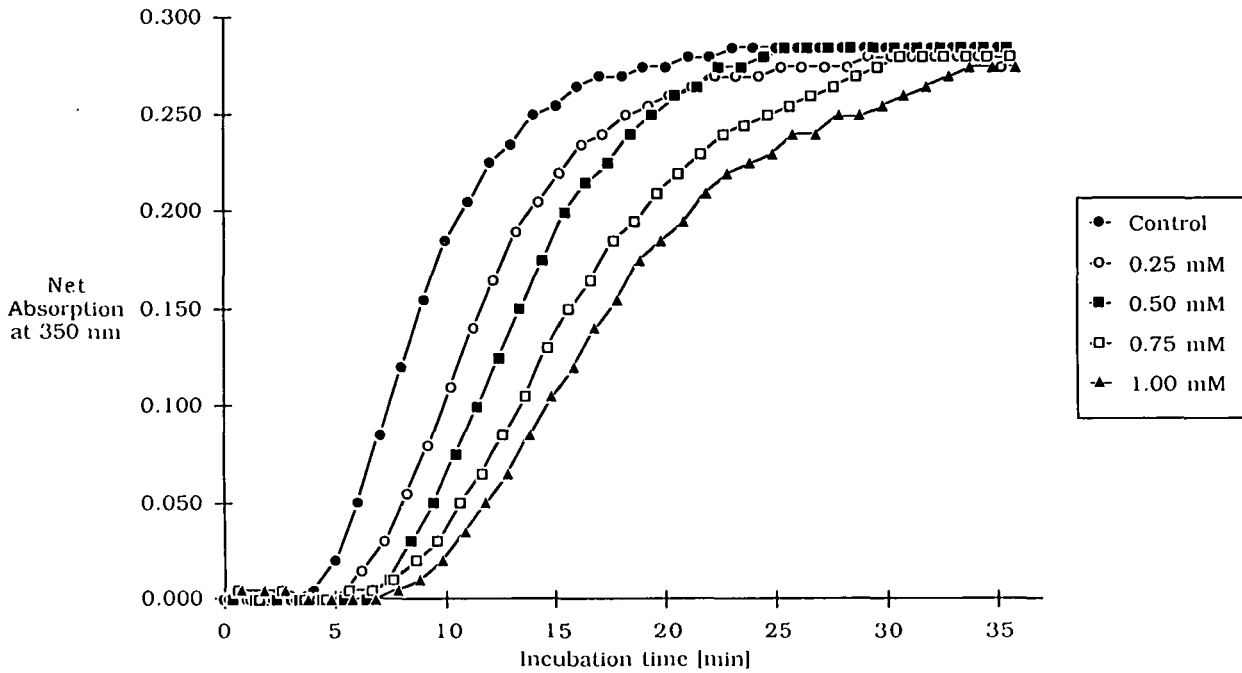
Fig. 3. Porcine brain tubulin assembly test with diazepam, Tubulin was incubated with different concentrations of diazepam.
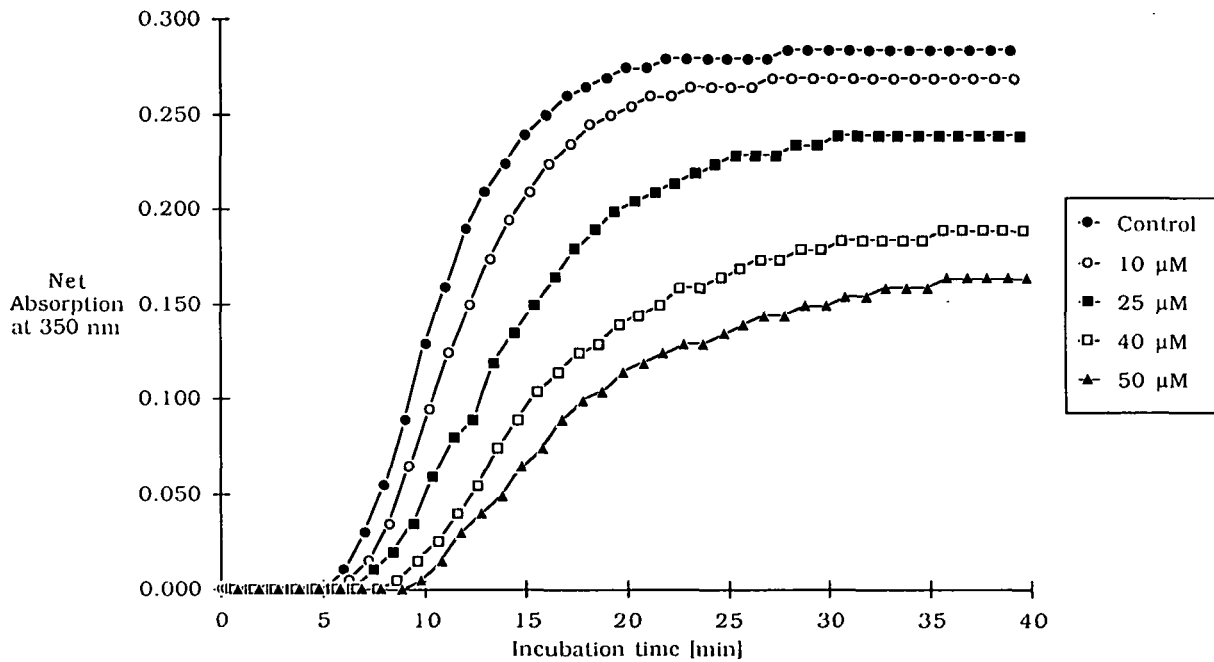

Fig. 4. Porcine brain tubulin assembly test with thimerosal. Tubulin was incubated with different concentrations of thimerosal.

and mitotic profile disturbances have been reported for various established cell lines (Anderson *et al.*, 1981; Hsu *et al.*, 1983a,b; Lafi *et al.*, 1987; Lafi and Parry, 1988) whereas tests in *S.cerevisiae* D61.M were negative (Albertini, 1990). *In vivo*, in mouse bone marrow, no mitotic arrest or c-mitotic effects were observed at doses which clearly reduced bone marrow cell proliferation (Miller and Adler, 1989).

Hydroquinone

Hydroquinone (metabolite of benzene) was reported to inhibit the assembly of rat brain tubulin (Irons and Neptun, 1980; Irons *et al.*, 1981). In contrast to these findings no effect on the assembly of porcine brain tubulin was observed by us. This

discrepancy might be attributable to the ue of glycerol, which is believed to modify the native properties (Dustin, 1984).

Thiabendazole

Thiabendazole belongs to an interesting group of microtubule poisons, the benzimidazole derivatives. The biochemical basis of action is still poorly understood for benzimidazole compounds. With regard to susceptibility for mammalian and yeast tubulin, large variations in the binding properties were observed (Kilmartin, 1981). Nocodazole showed nearly identical affinity for mammalian and yeast tubulin, while bavistan showed ~ 3000-fold stronger binding to yeast tubulin. The IC_{30} of thiabendazole (0.5 mM) for porcine brain tubulin is intermediate
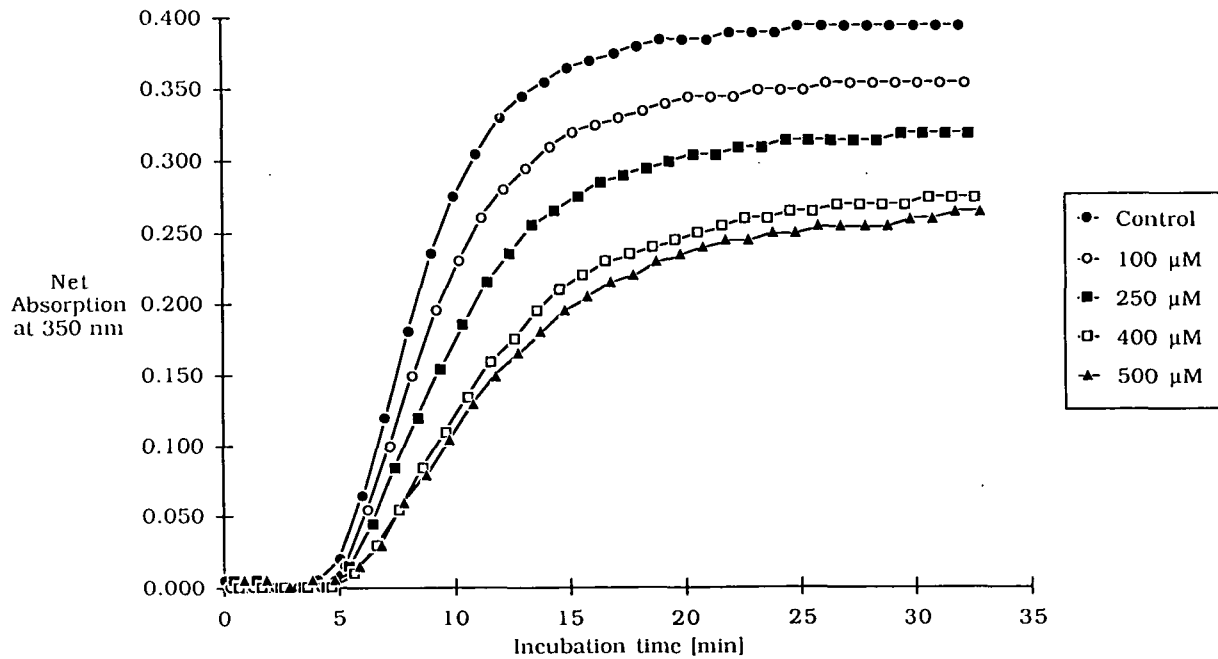
Fig. 5. Porcine brain tubulin assembly test with thiabendazole. Tubulin was incubated with different concentrations of thiabendazole.

to those of noncodazole (0.007 mM) and bavistan (> 1.3 mM). The affiity of thiabendazole to yeast tubulin has not been reported, but since all three compounds are active in *S. cerevisiae* D61.M in the dose range 2−25 μg/ml it is likely that—like bavistan—thiabendazole shows a preferential binding to yeast tubulin.

Thimerosal

Thimerosal, a organic mercury compound shows a relatively low IC_{30} of 0.03 mM. Organic mercury compounds are known to bind to sulfhydryl residues on tubulins and prevent polymerization. Thimerosal affects all three parameters investigated during the assembly process resembling the properties of well known microtubule poisons (colchicine, vinblastine).

Conclusion

We were able to show that microtubules are fairly easy to prepare and can be used as a simple screening object to study the effects of chemicals on assembly−disassembly processes.

Although no conclusive evidence of aneuploidy induction can be obtained from such *in vitro* tests alone, the *in vitro* tubulin assay can be used to pinpoint those chemicals which lead to cytoxicity and/or aneuploidy by inhibiting microtubule assembly.

A specific determination of the mode of action will become possible after detailed characterization of the interactions between chemicals and the individual protein components of the tubulin assembly system. This knowledge may allow predictions as to the *in vitro* effects of the test chemicals. In the work presented here apparently three basically different modes of actions can be distinguished: Diazepam enhances predominantly the lag-phase, thimerosal all three parameters (lag-phase, polymerization velocity and steady state) and thiabendazole the polymerization velocity and the steady-state level but not the lag-phase.

Although only a small database is available at present, the *in vitro* tubulin assay seems well capable of determining those chemicals inducing aneuploidy via interactions with spindle proteins, and thereby allowing the differentiation of aneuploidy inducing agents acting on spindle components and those acting via other mechanisms.

Table II. Posible mechanisms (Liang and Brinkley, 1985), *in vitro* tubulin assembly assay results and results obtained in *S. cerevisiae* D61.M (Albertini, 1990).

Chemical	Possible mechanism	Tubulin assay	Yeast assay
Colchicine	Tubulin polumerization	+	−
Econazole nitrate	Membrane effects	−	−
Chloral hydrate	Spindle elongation	+	+
Hydroquinone (benzene metabolite)	−		−
Diazepam	Centrioles/centromeres	−	−
Thiabendazole	Tubulin polymerization	+	+
Cadmium chloride	Enzyme systems	−	−
Thimerosal	Microtubule assembly	+	+
Pyrimethamine (pyrimidine base analogue)	−	−	−
Vinblastine	Tubulin crystallization	+	NT

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