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Reversible inhibition of mammalian tubulin assembly *in vitro* and effects in *Saccharomyces cerevisiae* D61.M by mitomycin C

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Gaulden reported a novel and unexpected mitomycin C (MMC) effect, namely a pronounced retardation of very late prophase and loss of chromosome orientation in neuroblasts of the grasshopper *Chortophaga viridifasciate*. Because this effect may be due to interactions of MMC with non-DNA targets, MMC was tested for its interaction with porcine brain tubulin assembly *in vitro* and for the induction of chromosomal malsegregation in the diploid yeast *Saccharomyces cerevisiae* strain D61.M. A reversible dose-dependent inhibition of tubulin assembly was observed. Since no biological activation system was present in the incubation mixture this inhibition seems to result from an interaction of unactivated MMC with the assembly process. The possible chemical activation of MMC by reduction with 1,4-dithioerythritol (DTE) was investigated by omission of this compound during isolation and polymerization of tubulin. The absence of DTE resulted in a strong reduction of the net tubulin assembly. Also under these conditions MMC led to a dose-dependent inhibition of the assembly, indicating that the effect of MMC on tubulin assembly is independent of a reductive chemical modification. In *S.cerevisiae* D61.M, MMC did not induce chromosome loss, but induced other genetic events (possibly mutations, deletions or mitotic recombination) as was detected by an increase of the total number and of the frequency of cycloheximide-resistant colonies. This effect could be observed with and without the addition of rat liver S9 as an exogenous activation system.

Introduction

Mitomycin C (MMC), a well known bifunctional alkylating agent forming cross-links as well as mono-adducts in DNA (Waring, 1968), shows a wide spectrum of genotoxic activities. It induces gene mutations in excision repair proficient, but not in excision repair deficient bacteria (Levin *et al.*, 1984). The induction of chromosome aberrations, mitotic recombination and sister-chromatid exchanges (SCEs) have been reported (for references see Adler, 1981). It has been shown that MMC requires biological reductive activation to produce DNA intrastrand cross-links (Iyer and Szybalski, 1963).

Recently, the research group of M.E.Gaulden (Ferguson *et al.*, 1985; Gaulden *et al.*, 1985) reported a novel, unexpected biological effect induced by MMC. In neuroblasts of the grasshopper *Chortophaga viridifasciata* they observed a pronounced retardation of very late prophase which led to loss of chromosome orientation within the nucleus. This effect on mitosis is reversible and is expressed as an extended duration of very late prophase in relation to MMC concentration and time of exposure (Gaulden *et al.*, 1985). This reversible inhibition of mitosis could be related to an interaction of MMC with a non-DNA target as, for example, the spindle. We therefore included MMC in our testing program for chemicals interacting with mammalian tubulin assembly *in vitro*. At the same time the effect of the omission of the reductive compound dithioerythritol [DTE; used together with glycerol for the protection of the tubulin SH-groups during the *in vitro* polymerization (Dustin, 1984; Roberts and Hyams, 1979)] on the tubulin assembly in presence of MMC was studied to exclude a possible chemical reduction of MMC by DTE. Since MMC effects on the tubulin assembly were found, we tested MMC also for the ability to induce chromosome loss and/or other genetic events (mutation, mitotic recombination, deletion) in the diploid yeast *Saccharomyces cerevisiae* strain D61.M.

Materials and methods

Chemicals

Mitomycin C (MMC, CASRN 50-07-7) was obtained from Sigma (St Louis, MO, USA), colchicine (COL, 64-86-8) from Fluka AG (Buchs, Switzerland), bavistan (carbendazim: methyl benzimidazole-2-yl-carbamate, 10605-21-7) from Riedel-de Haen AG (Seelze, Hanover, FRG), and dimethylsulphoxide (DMSO, 67-68-5) from Merck AG (Darmstadt, FRG).

Tubulin isolation

Porcine brain tubulin was prepared according to Williams and Lee (1982). Two cycles of assembly and disassembly were performed for purification. Two different tubulin preparations were used for the two assays with COL and MMC. The final preparation contained 10.3 mg protein/ml in the preparation used for the COL experiments and 13.6 mg protein/ml for the one used for the MMC tests. The protein content was determined using the method of Bradford (1976). The preparations were stored on ice overnight and used the following day for the assembly test (tubulin was never stored for longer periods).

Tubulin assembly test

Tubulin assembly was followed photometrically by measuring the increase in absorbency at 350 nm (Albertini *et al.*, 1985, 1988b). 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 5–40 μl of 100 mM Pipes buffer containing different amounts of MMC or COL and 10 μl of a 50 mM solution of GTP [guanosine-5'-triphosphate (dilithium salt)] in 100 mM Pipes buffer. Immediately before the start of the experiment the tubulin preparation was added (250 μl in the case of MMC and 400 μl for the tests with COL). 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.

To test the effects of the omission of the reductive compound dithioerythritol, porcine brain tubulin was isolated and tested for its polymerization capacity in the absence of this compound.

Yeast strain

The diploid strain D61.M of *S.cerevisiae* has been described previously (Zimmermann and Scheel, 1984; Zimmermann *et al.*, 1985; Resnick *et al.*, 1986). This strain is heterozygous for *cyh*^{R}*2*, *leu1*, *trp5* and *ade6* on chromosome VII (*cyh*^{R}*2* and *leu1* are positioned on its left arm and *ade6* on its right arm) and homozygous for *ade2-40* on chromosome XV. *Ade6* and *leu1* are located close to the centromere. The loss of the entire chromosome VII containing the *trp5*-marker can be detected by scoring for white, cycloheximide-resistant colonies requiring leucine.

Compounds were judged to be positive if at least two doses showed a reproducible increase in the absolute number of the monosomic colonies and in

Table I. Genotoxic effects of MMC in *S.cerevisiae* D61.M

Concentration (μg/ml)	Activation[a]	CFU[b] (×10⁶)	Survival (%)	No. of resistant colonies[c]	Frequency[d] (×10⁻⁵)	Resistant		Chromosomal malsegregation[g] (×10⁻⁶)
						white[e]	leu⁻[f]	
Experiment 1								
0[h]	–	57.8	100	4302[k]	49.6	9[k]	3	0.35
5	–	70.0	121	10154[k]	96.7	10[k]	2	0.18
10	–	69.4	120	11408[k]	109.6	12[k]	0	–
20	–	62.2	108	26148[k]	280.3	26[k]	1	0.11
40	–	39.4	68	26520[k]	448.7	42[k]	0	–
80	–	44.2	77	26852[k]	405.0	61[k]	4	0.49
160	–	31.9	55	26996[k]	564.2	74[k]	4	0.91
0	+	76.4	100	4952[k]	43.2	25[k]	5	0.43
5	+	135.6	178	6620[k]	32.5	14[k]	3	0.15
10	+	154.4	202	11894[k]	51.4	13[k]	2	0.08
20	+	85.2	112	11372[k]	88.9	20[k]	2	0.15
40	+	93.0	122	22235[k]	159.4	17[k]	1	0.07
80	+	112.8	148	23308[k]	137.8	42[k]	0	–
160	+	126.0	165	24275[k]	128.4	39[k]	2	0.07
Positive control: bavistan								
0[i]	–	88.8	100	4249[k]	31.9	2[k]	1	0.08
20	–	4.5	8	917[l]	41.1	208[l]	204	91.47
Experiment 2								
0[h]	–	84.1	100	1329[n]	63.2	7[p]	5	1.19
2.5	–	82.3	98	1551[n]	75.4	10[p]	4	0.97
5	–	85.2	101	1865[n]	87.6	12[p]	6	1.41
10	–	74.1	88	2400[n]	129.6	6[p]	1	0.27
15	–	74.7	89	2919[n]	156.3	17[p]	5	1.34
20	–	75.5	90	3333[n]	176.6	18[p]	4	1.06
30	–	64.2	76	2614[m]	271.4	17[o]	3	1.56
40	–	56.5	67	3289[m]	388.1	22[o]	2	1.19
60	–	56.5	67	3407[m]	402.0	20[o]	3	1.77
80	–	56.0	66	3472[m]	413.3	23[o]	2	1.20
0[h]	+	94.5	100	1421[n]	60.2	7[p]	3	0.63
2.5	+	97.2	103	1427[n]	58.7	11[p]	8	1.65
5	+	95.3	101	1405[n]	59.0	8[p]	6	1.26
10	+	102.3	108	1633[n]	63.9	6[p]	6	1.17
15	+	103.1	109	1788[n]	69.4	16[p]	7	1.36
20	+	105.4	112	2292[n]	86.9	12[p]	5	0.95
30	+	125.1	132	2818[n]	90.1	18[p]	8	1.28
40	+	121.5	129	3582[n]	117.9	12[p]	4	0.66
60	+	146.0	154	2912[m]	132.9	7[o]	1	0.23
80	+	122.6	130	3061[m]	166.5	12[o]	3	0.82
Positive control: bavistan								
0[i]	–	86.7	100	1102[n]	50.8	5[p,q]	4	1.15
10	–	41.3	48	2812[k]	45.4	67[k]	53	8.62
15	–	14.8	17	3760[l]	50.8	209[l]	177	23.92
Experiment 3								
0[h]	–	82.2	100	428[m]	34.7	7[k,q]	4	0.40
2.5	–	87.9	107	626[m]	47.5	18[k]	8	0.60
5.0	–	83.7	101	849[m]	67.6	22[k]	11	0.87
10.0	–	81.3	98	1114[m]	91.4	18[k]	5	0.41
15.0	–	76.5	92	1375[m]	119.8	27[k]	6	0.52
20.0	–	71.1	86	1627[m]	152.6	28[k]	4	0.38
30.0	–	70.8	86	1848[m]	174.0	28[k]	4	0.38
40.0	–	64.5	78	2628[m]	271.6	51[k]	7	0.78
60.0	–	52.0	63	3212[m]	411.8	62[k]	6	0.85
Positive control: bavistan								
0[i]	–	86.4	100	402[m]	31.0	13[k]	5	0.38
10	–	48.0	56	1884[k]	26.2	62[k]	59	8.21
15	–	11.6	13	902[k]	51.8	94[k]	92	53.10

[a]Activation (−: without S9; +: with S9).
[b]CFU: colony forming units per ml.
[c]Total number of cycloheximide-resistant colonies plated on five plates (red and white colonies).
[d]Frequency of cycloheximide-resistant colonies per 10^5 survivors.
[e]White: Total number of white, cycloheximide-resistant colonies on five plates.
[f]Leu⁻: Total number of white, cycloheximide-resistant, leucine-dependent colonies on five plates.
[g]Frequency of white, leucine auxotrophic cycloheximide-resistant colonies.
[h]Solvent control (H_2O).
[i]Solvent control (DMSO).
[k]Volume plated per plate: 30 μl undiluted.
[l]Volume plated per plate: 100 μl undiluted.
[m]Volume plated per plate: 30 μl of a 10^{-1} dilution.
[n]Volume plated per plate: 50 μl of a 10^{-1} dilution.
[o]Volume plated per plate: 60 μl of a 10^{-1} dilution.
[p]Volume plated per plate: 100 μl of a 10^{-1} dilution.
[q]Only four instead of five plates were counted.

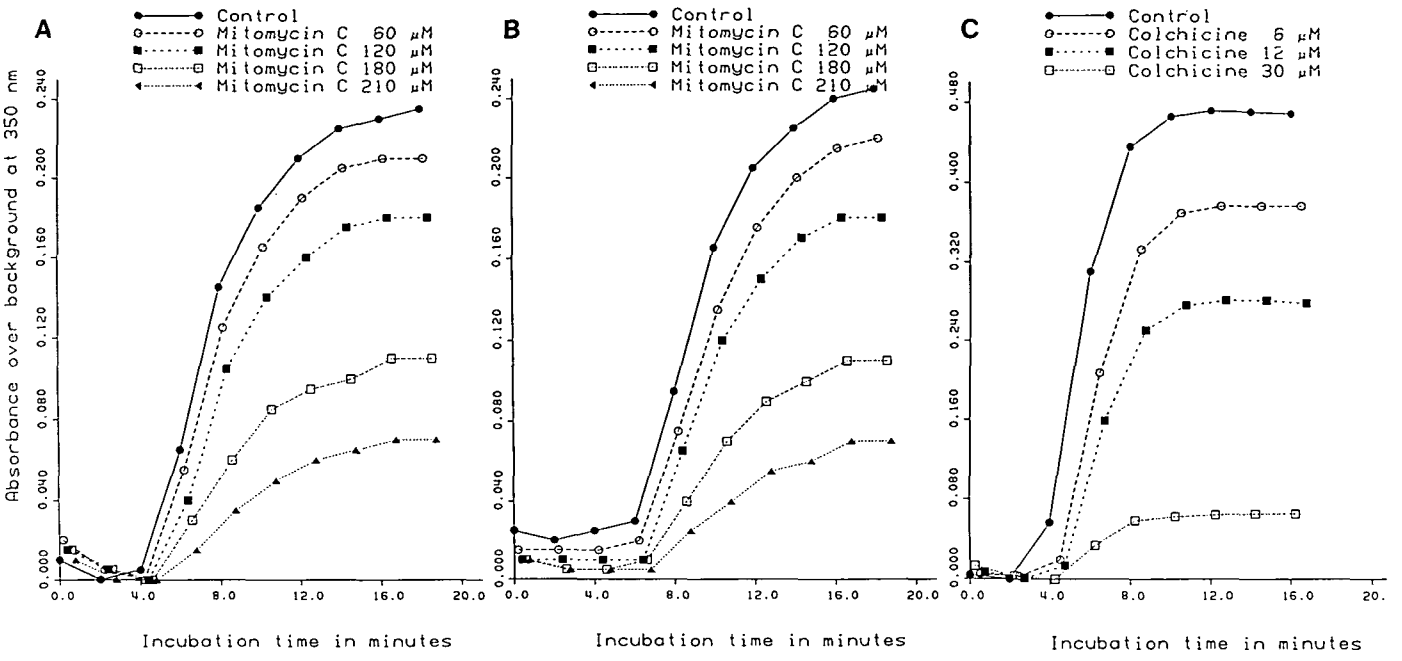

Fig. 1. (**A**) Tubulin assembly test with 3.4 mg/ml porcine brain tubulin and different concentrations of MMC. Incubation at 37°C. (**B**) Second cycle of tubulin assembly. At the end of the first cycle shown in (**A**) the cuvettes were incubated for 30 min at 0°C and then the second cycle was run by an incubation at 37°C without further additions. (**C**) Tubulin assembly test with 4.1 mg/ml porcine brain tubulin and different concentrations of COL. Incubation at 37°C.

the frequency of malsegregants per 10^6 survivors. The induced frequencies must be $>2 \times 10^{-6}$. Other genetic events, such as gene mutation, mitotic recombination or deletion, lead to red cycloheximide-resistant colonies, as well as to white, cycloheximide-resistant, leucine prototrophic colonies (in case of at least two simultaneous events). The results of the total number of resistant colonies (red and white colonies) were designated as positive if there was a dose-related increase of the absolute number besides an increase in the frequency of >2-fold (>50% survivors) or >3-fold (<50% survivors) compared to the control (Albertini *et al.*, 1988a).

Treatment procedures

Cells were grown in a liquid medium (YEP medium) containing yeast extract (1%), peptone (2%) and glucose (2%) to a cell density of $3-5 \times 10^7$ cells/ml. Before treatment, the cultures were diluted 20-fold with fresh medium and allowed to grow for 3–4 h. The initial titre at the beginning of the treatment was $\sim 1 \times 10^6$ cells/ml. The cells were incubated during 18 h (overnight) at 28°C in a water bath with a orbital shaker together with the test compound. MMC was dissolved in H_2O and bavistan in DMSO [the amount of DMSO in the final incubation medium was <2% (v/v) (Zimmermann and Scheel, 1984)]. The total volume of the treatment mixture was 5 ml. Volumes of 100 µl or 30 µl of the undiluted suspension or 30, 50, 60 or 100 µl of a 10^{-1} dilution were plated (as indicated in Table I), without washing, directly on solid complete medium with cycloheximide (2 mg/l) to score either for cycloheximide-resistant colonies (red and white) and/or for white, cycloheximide-resistant colonies, which were subsequently tested for leucine auxotrophy by streaking out on synthetic medium without leucine. White, cycloheximide-resistant colonies not growing on these plates were classified as leu^-. The red colonies indicate the induction of mitotic recombination between the centromere and the cyh locus or other mutational events. The number of surviving cells were determined by plating 0.1 ml aliquots from appropriate dilutions onto solid complete medium (YEP-medium).

Rat liver S9

Rat liver S9 was prepared according to Ames *et al.* (1975). Liver enzymes were induced with phenobarbital (Siegfried, Switzerland; diluted in pyrogen-free aqua bidest.) and β-naphthoflavone (Serva, Heidelberg, FRG; suspended in corn oil) following the method of Matsushima *et al.* (1976, 1980). The protein content, determined according to Lowry *et al.* (1951), was 37.2 mg/ml. The preparation was shown to be sterile. For experiments including an exogenous activation system, 1 ml S9 mix containing 30% (v/v) S9 (Ames *et al.*, 1975) was added to a 4 ml treatment mixture before the beginning of the incubation.

Colony counting

After 3–5 days (for survivors) and 7–10 days (for resistants) the colonies were marked and counted with a Gallenkamp colony counter (Gallenkamp, Loughborough, UK). In Experiment 1 for plates with >400 colonies, characteristic sectors of 1/2 to 1/12, containing between 200 and 400 colonies, were chosen and counted manually. In experiments 2 and 3 the whole surface of the plates were counted.

Results

Tubulin assembly test

MMC in a dose range from 60 to 210 µM inhibited the tubulin assembly *in vitro* (Figure 1A). The concentration for a 50% inhibition of the assembly was ~150 µM. After 30 min at 0°C the absorbency again reached the initial value independently of the addition of MMC. During the following second cycle MMC inhibited tubulin assembly to the same extent as in the first cycle (Figure 1B). There were only two differences between the first and the second cycle: (a) in the second cycle the curves started out at slightly higher values and (b) the lag-phase before the start of the assembly was longer (7 min compared to 4 min in the first cycle). But finally for the different amounts of MMC the same plateau levels were reached as in the first cycle.

The complete disassembly and the fact that the curves of the second cycle resemble those of the first one show that the inhibition of the tubulin assembly by MMC is reversible.

COL was used as a positive control. As expected, tubulin assembly was inhibited by COL in the concentration range from 6 to 30 µM, the concentration for a 50% inhibition being 12 µM (Figure 1C). This concentration is <10 times smaller than the respective value for MMC.

MMC can be activated by reductive processes. Therefore tubulin isolated without addition of dithioerythritol (DTE), was prepared and assayed *in vitro* for effects of MMC in the absence of this reducing agent. The amount of isolated tubulin was only ~14% compared to the standard procedure with the addition of DTE. Accordingly, the GTP-dependent polymerization process was also much weaker than in the normal *in vitro* assembly assay. The plateau value reached without addition of MMC was only

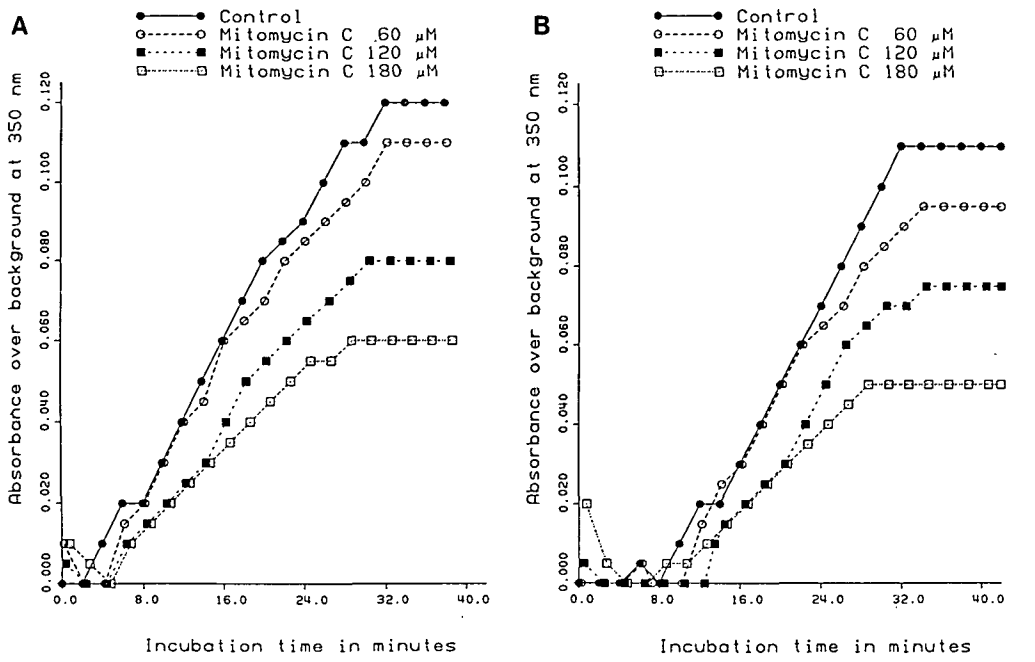

Fig. 2. (**A**) Tubulin assembly test with 3.2 mg/ml porcine brain tubulin without addition of dithioerythritol in the presence of different concentrations of MMC. Incubation at 37°C. (**B**) Second cycle of tubulin assembly without addition of dithioerythritol. At the end of the first cycle shown in (**A**) the cuvettes were incubated for 30 min at 0°C and then the second cycle was run by a incubation at 37°C without further additions.

0.12 U at 350 nm which is ~50% of the maximum absorbency normally obtained (Figure 2). Nevertheless, a dose-dependent reversible inhibition of tubulin polymerization was observed by the addition of MMC (Figure 2A and B). The 50% inhibition concentration was only slightly higher than in presence of DTE (180 µM). The lag time for the second cycle was again longer compared to the first *in vitro* assembly.

Genotoxic effects in yeast

In three independent experiments MMC was tested with and without rat liver S9-mix up to a dose of 160 µg/ml (corresponding to 0.48 mM; Table I, Experiment 1). Only without addition of S9 MMC showed cytotoxic effects [with 40 µg MMC per ml (corresponding to 0.12 mM) 70–80% survivors]. The frequency of the total number of cycloheximide resistant colonies (red and white coloured) increased in a dose-related manner. The effect was stronger without S9 than with S9. With 40 µg MMC/ml without S9 the increase was between 6- and 9-fold compared to the concurrent control, whereas with S9 only a 2- and 4-fold increase was observed. The maximum increase was obtained in the third experiment with 60 µg MMC/ml where a factor of 12 was obtained. The absolute number of white, cycloheximide-resistant colonies also increased dose-dependently in the absence of the activation system, but almost all colonies turned out to be leucine-independent. Therefore they are not the result of chromosomal malsegregation. Correspondingly the frequency of the white, leucin-auxotrophic resistants did not show a dose-dependent increase. An additional test for trypotophan auxotrophy showed that all white colonies were tryptophan prototrophs (data not shown).

These results clearly show that MMC does not induce mitotic chromosomal malsegregation, in the dose range tested, but strongly induces mitotic recombination and/or other genotoxic effects (mutations, deletions).

With 10–20 µg/ml bavistan, used as a positive control for the induction of chromosomal malsegregation (Table I), frequencies for chromosomal malsegregation were obtained in the range usually found (Zimmermann and Scheel, 1984; Mayer and Goin, 1987; Albertini *et al.*, 1985, 1988a). More than 90% of the white, cycloheximide-resistant colonies induced by the bavistan treatment were leucine-dependent indicating malsegregation of chromosomes (Table I).

Discussion

MMC requires activation before it can function as an alkylating agent. The activation process is a NADPH-dependent enzymatic reduction of MMC to its hydroquinone derivative by one of the enzymes belonging to the group of quinone reductases, also referred to as diaphorases. This facilitates a protonation of the aziridine nitrogen thereby promoting intracellular alkylation of nucleophilic centres (Moore, 1977). An additional mechanism for the activity of MMC was found in the formation of oxygen radicals (Pristos and Sartorelli, 1986). Attack by the activated agent on the vital cellular moiety, e.g. DNA, is expected to result in cytotoxicity or mutagenicity; conversely, reaction with non-critical moieties or water will lead to detoxification (Goldberg, 1965). The enzymes (NADPH-cytochrome C reductase, xanthine oxidase) necessary for the activation are ubiquitous among prokaryotic and eukaryotic systems. It was shown by Iyer and Szybalski (1964) that cross-linking of DNA occurs after activation of MMC by cell lysates, e.g. from *Sarcina lutea, Baccilus subtilis* or *Escherichia coli* (for review see Stevens *et al.*, 1965). Therefore, neither bacterial nor yeast mutagenicity test systems need an exogenous metabolizing system for the activation of MMC, as was also found with our yeast experiments. On the contrary, the addition of S9-mix reduced the genotoxic activity of MMC in *S. cerevisiae* D61.M an effect which was also found with bacteria (SOS Chromotest; Quillardet *et al.*, 1985).

In the *in vitro* porcine brain tubulin assembly assay no biological reducing systems (cell lysates, cytochrome C reductase, xanthine oxidase; Pristos and Sartorelli, 1986) were used.

Initially, we intended to use MMC as a negative control. But unexpectedly MMC inhibited the tubulin assembly *in vitro* in a manner comparable to our positive control, colchicine, admittedly in a higher dose range; the 50% inhibition concentrations were 15 μM (Dustin, 1984; Albertini *et al.*, 1988b) and 150 μM for colchicine and MMC, respectively. The omission of the reductive compound DTE in the *in vitro* tubulin assembly was without effect on the inhibitory activity of MMC. These data show that unactivated, i.e. unreduced MMC, is able to interact with tubulin assembly *in vitro*.

In vivo, the reversible inhibition of mitosis in very late prophase (Ferguson *et al.*, 1985; Gaulden *et al.*, 1985) corresponding to the reversible inhibition of tubulin assembly *in vitro* might result either from an interaction of unactivated MMC with the mitotic spindle or from an interaction of MMC with the nuclear envelope leading to a disconnection of centromeres with the nuclear envelope. The reversibility of this *in vivo* effect might be related to the reductive metabolism of MMC resulting in a time-dependent decrease in the concentration of unactivated MMC.

The yeast data of our study can be summarized as follows: MMC induces red and white cycloheximide-resistant colonies [as a result of mutation, mitotic recombination or partial chromosome loss (deletion)], but does not induce chromosomal malsegregation, which would result in an increase of the number of white, cycloheximide resistants showing leucine auxotrophy. Bavistan, used as positive control for the induction of chromosomal malsegregation, on the other hand specifically induced white, leucine auxotrophic, cycloheximide-resistant colonies, but did not increase the frequency of the cycloheximide-resistant colonies. The results with bavistan are in agreement with previously published data (Zimmermann and Scheel, 1984; Albertini *et al.*, 1985, 1988a; Mayer and Goin, 1987).

The MMC effect is not dependent on the presence of an extracellular activation system. It remains to be elucidated whether reductive processes in the yeast cytoplasma can lead to activated, i.e. reduced, forms of MMC which subsequently react with DNA thus inducing recombinational and/or mutational events.

The fact that MMC does not induce chromosome loss in yeast *S.cerevisiae* D61.M may be explained by different hypotheses:

(i) MMC does not interact with yeast tubulin at all due to differences between yeast and porcine brain tubulin.

(ii) The concentration of intranuclear MMC which is not metabolized by reductive processes is too small for the induction of chromosome loss by binding to tubulin, whereas MMC possibly after reductive activation readily interacts with DNA thus leading to different mutational events as detected by the induction of cycloheximide-resistant colonies. The question remains to be answered whether MMC is reduced so effectively inside the yeast cell that not enough of the unmetabolized compound is available for an interaction with tubulin.

The yeast data reported in this paper are in disagreement with those published by Parry (1977). This author found a dose-dependent increase of monosomic colonies in a dose range of 5–40 μg MMC per ml with *S.cerevisiae* D6. The frequency of monosomic colonies/10^6 survivors raised to 400 monosomic colonies per 10^6 survivors with 10 μg MMC/ml. Unfortunately it is not clear whether or not the white, cyh^R colonies were tested for their leucine requirement. In our study we found that almost all white, cycloheximide-resistant colonies are leucine-independent (Table I). The putative aneuploidy induction observed by Parry (1977) therefore seems rather to be due to a high frequency of recombination, mutation or deletion induced by MMC.

In Drosophila, two reports indicate the induction of complete chromosome loss by MMC. One in male germ cells (Graf and Würgler, 1982) and one in immature oocytes (Walker and Williamson, 1975). Unfortunately, the mechanism(s) leading to the elimination of the chromosome from the Drosophila germ cells is (are) not known. Also several other papers deal with the induction of aneuploidy by MMC via non-disjunction in various test systems. Two assays for chromosome gain in Drosophila and one assay with mammalian cells in culture (hyperploidy in human peripheral blood lymphocytes) are negative, whereas all other data (with Aspergillus, Neurospora, Saccharomyces, mammalian somatic cells and mammalian male germ cells) are inconclusive (Dellarco *et al.*, 1986).

With the data available at present it remains open whether MMC actually induces chromosomal malsegregation. Chromosome loss as a consequence of the chromosome breaking activity by activated MMC as well as malsegregation of chromosomes as a consequence of the interaction between unactivated MMC and the tubulin in the mitotic spindle seem to be possible. Further studies, especially with mammalian cells, are needed to establish the potential aneuploidy inducing capacity of MMC and if induction occurs to distinguish between the two possible mechanisms.

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