Bull. Natl. Inst. Health Sci., **128**, 65-70 (2010) **Note**

Needle fibers of an azo-dye mixture induce polyploidy in a Chinese hamster cell line CHL

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In a routine safety evaluation of chemicals included in household products, we found a mixture of azo dyes (CMBA, main component: *N*-[5-[(2-cyanoethyl)ethylamino]-4-methoxy-2-[(5-nitro-2,1-benzisothiazol-3-yl)azo]phenyl]acetamide) that precipitated in the culture medium in a characteristic fiber form (around 2 - 33 μm in length) similar to that of asbestos. We compared CMBA with an asbestos, chrysotile B, in a cytotoxicity, chromosome aberration (CA), and micronucleus (MN) test in a Chinese hamster lung cell line (CHL). In the cytotoxicity test, the 50% growth inhibition concentration was 11.0 μg/ml for CMBA and 0.398 μg/ml for chrysotile B asbestos. CMBA and chrysotile B both induced polyploidy in the CA test and equal-sized binucleated and polynuclear cells in the MN test. CMBA differs from chrysotile B chemically. The former is an organic chemical and the latter is a mineral. Although CMBA is soluble in methanol and can be safely disposed by burning, it should be handled carefully when manufactured in a factory.

Keywords: aneugen, azo dye, chrysotile B, fibers

1. Introduction

In a routine safety evaluation, we examined a mixture of azo dyes (CMBA) that has been manufactured for textile dyeing. In the chromosome aberration test, CMBA precipitated in the culture medium as a fiber and the shape and size looked like those of asbestos. Concerned that CMBA might show the same activity as asbestos, we studied it further comparing with the activity of chrysotile B asbestos.

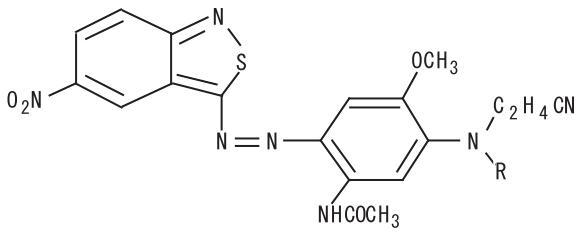
2. Materials and methods

2－1. Chemicals

A mixture (CMBA) of *N*-[5-[(2-cyanoethyl)ethylamino]-4-methoxy-2-[(5-nitro-2,1-benzisothiazol-3-yl)azo]phenyl]acetamide (A: 96.98%; CAS No. 172463-55-7) and *N*-[5-[(2-cyanoethyl)amino]-4-methoxy-2-[(5-nitro-2,1-benzisothiazol-3-yl)azo]phenyl]acetamide (B: 1.26%) with 1.76% unknown chemicals was synthesized by the ordinary azo-coupling method. The chemical structures of two main components, A and B, are shown in Fig. 1. CMBA was dissolved in dimethyl sulfoxide (DMSO). Chrysotile B (UICC) as a reference material was suspended in the culture medium.

2－2. Cells

CHL cells were established from the lung of a female newborn Chinese hamster by Koyama et al.[1] and cloned by Ishidate and Odashima[2]. They were maintained in Eagle's minimum essential medium (MEM; GIBCO 11095-080) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μg/ml) (GIBCO 15140-122) in 5% CO_2 in air at 37°C. The doubling time was around 13 h, and the modal chromosome number was 25.



A: R=C_2H_5, $C_{21}H_{21}N_7O_4S$=467.50

B: R=H, $C_{19}H_{17}N_7O_4S$=439.45

Fig. 1 Chemical structures of A and B, the two main components of CMBA

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2－3．Cytotoxicity test（Colony formation assay）

CHL cells were seeded at 50/well in 24-well plates. After 24-h incubation, they were treated with a test chemical or vehicle control for six days. The colonies formed were fixed with methanol and stained with 3% Giemsa solution. The number of colonies on each well was counted, and the relative survival was calculated based on comparison with the control colonies. The cytotoxic potential of the chemical was expressed as the concentration at which the relative survival was 50% of control (IC_{50}). The IC_{50} value was calculated by the probit method.

2－4．Chromosome aberration（CA）test

Cells were seeded at 1.5 x 10^5/plate（60 mm in diameter）and incubated at 37°C for 17 h. They were then treated with a test chemical for 24 or 48 h, and colcemid（0.2 μg/ml）was added for the final 2 h. For metabolic activation, cells were treated with a test chemical for 6 h in the presence or absence of S9 mix[3]（Kikkoman, Noda, Japan）and cultured with fresh medium for another 18 h with colcemid added for the last 2 h. The S9 fraction was prepared from the livers of Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone. The final concentration of the S9 fraction was 5 v/v%. Chromosome preparations were made as reported previously[4]. All slides were coded, and the number of cells with structural CAs was counted for 100 well-spread metaphases with a modal chromosome number of 25 ± 2. The number of cells with numerical CAs was counted on 100 metaphases. In our historical database[4], the frequency of CHL cells with structural CAs or polyploidy in both untreated and solvent-treated negative controls did not exceed 4%. The experimental groups were judged as negative if the total CA frequency was less than 5.0%, inconclusive if it was 5.0 to up to 10.0%, and positive if it was 10.0% or more. The number of mitotic cells was counted for 1000 cells. Relative mitotic index was calculated based on comparison with the number of mitotic cells in controls to show the concurrent cytotoxicity. Solvent－treated cells served as the negative control. Experiments were performed twice, and representative data are shown.

2－5．Micronucleus（MN）test

CHL cells were seeded and incubated as they were in the CA test, treated with a chemical for 24 or 48 h in the absence of S9 mix（because there was no effect in its presence in the CA test）, and harvested immediately. MN preparations were made as reported previously[5]. The cells were stained by mounting in 40 μg/ml acridine orange in phosphate-buffered saline（PBS）and were immediately observed at 400 x magnification by fluorescence microscopy with a model Nikon Eclipse E600 and a B-2A filter block. All slides were coded, and they were observed and judged as reported previously[6]. Briefly, the number of MN cells among 1000 intact interphase cells was counted. Cells with a main nucleus and a single MN were categorized into two groups: those with a MN whose diameter was less than one-third of the main nucleus and those with a MN whose diameter was one-third to one half the diameter of the main nucleus. A cell with two or more MN was recorded as a multi MN cell. In addition, we examined 1000 total cells and scored polynuclear（PN）cells, including polynuclear cells, karyorrhectic cells, and binucleates, and mitotic（M）cells. Experiments were performed twice and representative data are shown. We analyzed the data using a χ^2-test for treated versus control groups.

3．Results

Fibers of CMBA precipitated in the culture medium are shown in Fig. 2. Single and bundled fibers are shown ranging from around 2 to 33 μm in length.

Chrysotile B, with an IC_{50} of 0.398 μg/ml was much more cytotoxic than CMBA, with an IC_{50} of 11.0 μg/ml

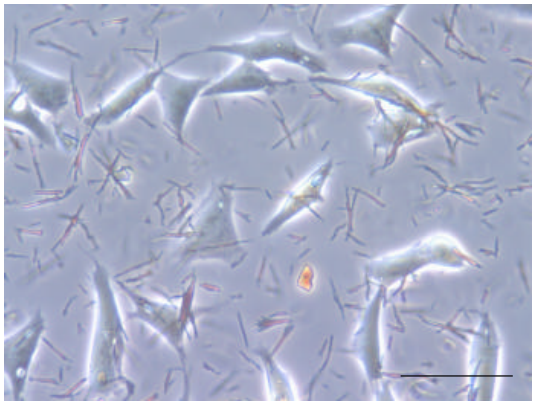

Fig. 2 Phase contrast micrograph of fibers of CMBA precipitated in the culture medium after its DMSO solution was added
Single and bundled fibers are shown ranging the length from around 2 to 33 μm. Bar indicates 50 μm.

(Fig. 3). As fiber concentrations are typically given in $\mu g/cm^2$, those in $\mu g/cm^2$ are added in parentheses for chrysotile B in graphs and a table.

To show the fiber length of CMBA and chrysotile B quantitatively, we measured them as follows: their suspension in the culture medium was placed on a glass microscope slide, covered with a cover slip, and then photographed under a light microscope. Length of fibers was measured on the enlarged photo prints. The fiber length distribution of CMBA and chrysotile B is shown in Fig. 4. CMBA and chrysotile B showed different distribution patterns. The fiber length distribution of CMBA spread broadly mainly from 3 to 15 μm. 77% of the fibers was shorter than or equal to 15 μm. On the other hand, chrysotile B showed a distribution with a peak at 1 μm and 85% of the fibers was shorter than or equal to 5 μm. In this connection, Timbrell reported that 97% of the fibers of the UICC chrysotile B was shorter than or equal to 5 μm[7].

In the CA test, CMBA induced polyploidy after 6-, 24-, and 48-h treatment without S9 mix in a concentration-dependent manner (Table 1). The concurrent cytotoxicity under the conditions was not strong as shown by the relative mitotic index. CMBA did not induce structural CAs under any experimental conditions. CMBA did not induce CAs at concentrations lower than 6.25 μg/ml.

Chrysotile B, on the other hand, induced polyploidy

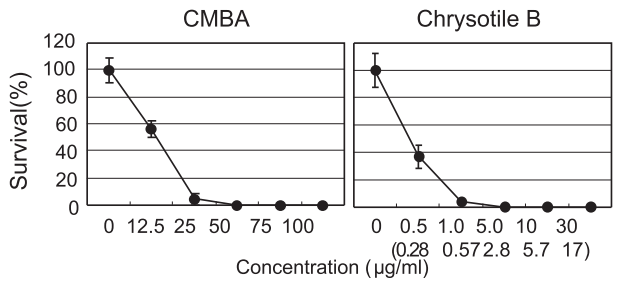

Fig. 3 Cytotoxicity of CMBA and chrysotile B
Values are expressed as mean ± SD for four wells. Figures in a parenthesis indicate concentrations in $\mu g/cm^2$.

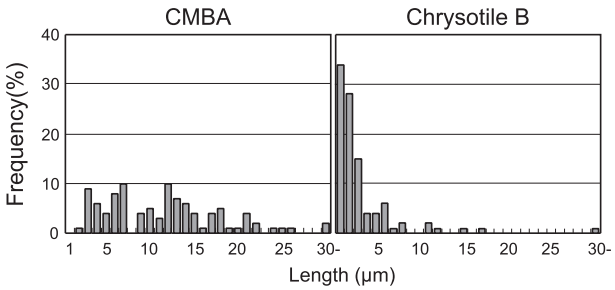

Fig. 4 Fiber length distribution of CMBA and chrysotile B
The average length of CMBA fibers was 10.95 μm.

Table 1 Chromosome aberration test of CMBA

T-R[a] (h)	S9 mix	Concentraton (μg/ml)	polyploid cells (%)	Cells with chromosome aberrtion[b] (%)							Relative mitotic index (%)
				ctg	ctb	cte	f	osb	cse	Total	
6 - 18	−	0	2	1	0	0	0	0	0	1	100
		20	12	0	0	0	0	1	0	1	127
		40	15	0	1	2	0	1	0	4	104
		60	25	2	1	0	0	0	0	3	80.0
		80	10	0	1	1	0	0	0	2	92.0
6 - 18	+	0	0	1	0	0	0	1	0	2	100
		20	1	1	0	1	0	0	0	2	85.6
		40	4	1	0	0	0	0	1	2	97.5
		60	3	1	0	1	0	0	1	3	99.2
		80	3	1	1	1	0	0	0	3	105
24 - 0	−	0	0	0	1	0	0	0	1	2	100
		6.25	1	0	0	0	0	0	0	0	137
		12.5	12	2	1	1	0	0	0	4	159
		25	26	0	0	0	0	0	0	0	123
		50	23	0	1	0	0	0	0	1	105
48 - 0	−	0	2	0	0	0	0	0	0	0	100
		6.25	1	0	0	0	0	0	0	0	71.2
		12.5	16	1	0	0	0	0	0	1	48.1
		25	46	0	0	0	0	0	0	0	65.4
		50	40	0	0	0	0	0	0	0	59.6

[a]Treatment and recovery time. [b]ctg, chromatid and chromosome gaps; ctb, chromatid breaks; cte, chromatid exchanges; f, fragmentation; csb, chromosome breaks; cse, chromosome exchanges.

under all the experimental conditions (Table 2). The lowest concentrations that induced polyploidy were ten times higher in the presence of S9 mix than its absence. Chrysotile B induced structural CAs, but at a low frequency.

We observed bi-, tri-, tetra-, and hexa-nuclear cells in MN preparations after treatment with CMBA (Fig. 5) and after treatment with chrysotile B (data not shown).

CMBA induced a small but statistically significant increase in the frequency of MN cells after 24- and 48-h treatments (Fig. 6). Surprisingly, the frequency of PN cells induced was about 50 times the control value. Binucleates were prominent at both 24- and 48-h, after which the ratio of polynuclear cells among the PN cells increased. CMBA did not significantly increase the frequency of M cells.

Chrysotile B induced the similar pattern of MN, PN, and M cells as CMBA (Fig. 7). The only difference from CMBA in the MN test was the concentrations tested.

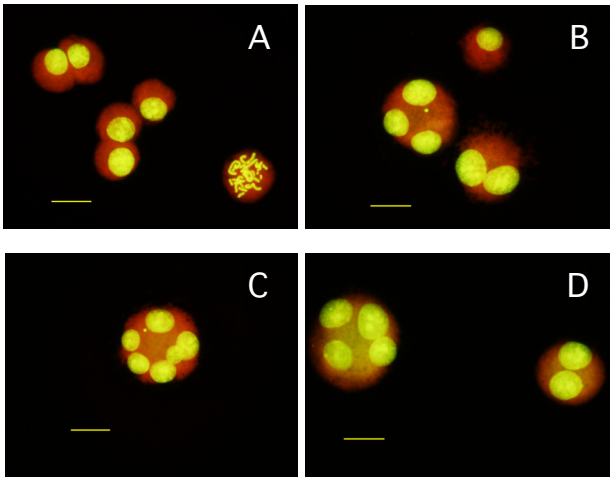

Fig. 5 CHL cells treated with CMBA in MN preparations
A, intact interphase cells and a mitotic cell observed in control. B and C, A binucleate, a tri-nuclear cell with an MN, and a hexa-nuclear cell with a MN observed after treatment with CMBA at 20 μg/ml for 48 h. D, a binucleate and a tetra-nuclear cell observed after treatment with CMBA at 40 μg/ml for 48 h. MN preparations were stained with acridine orange. Yellow indicates nuclei and chromosomes and red indicates cytoplasm. Bar represents 20 μm.

Table 2 Chromosome aberration test of chrysotile B

T-R[a] (h)	S9 mix	Concentraton (μg/ml)	Concentraton (μg/cm^2)	polyploid cells (%)	Cells with chromosome aberrtion[b] (%) ctg	ctb	cte	f	osb	cse	Total	Relative mitotic index (%)
6 - 18	−	0	0	1	0	0	0	0	0	0	0	100
		0.5	0.12	7	0	0	0	0	0	0	0	88.9
		1	0.25	13	1	0	0	0	0	0	1	87.8
		5	1.2	28	2	2	1	0	1	1	7	82.2
		10	2.5	44	2	2	2	0	3	0	6	62.2
		30	7.4	29	0	2	0	0	0	0	2	51.1
		50	12	20	0	0	0	0	2	0	2	42.2
6 - 18	+	0	0	1	1	1	2	0	0	0	3	100
		0.5	0.12	0	0	0	0	0	0	0	0	101
		1	0.25	2	1	0	0	0	0	1	2	120
		5	1.2	4	0	0	0	0	0	0	0	117
		10	2.5	10	1	1	0	0	0	1	3	108
		30	7.4	11	1	1	2	0	0	0	3	93.5
		50	12	15	0	0	1	0	1	0	2	79.2
24 - 0	−	0	0	2	1	0	0	0	0	0	1	100
		0.5	0.12	7	2	0	0	0	1	0	3	138
		1	0.25	10	1	2	2	0	2	0	5	117
		5	1.2	25	0	0	0	0	0	0	0	72.1
		10	2.5	32	0	0	0	0	2	0	2	74.4
		30	7.4								Tox	
48 - 0	−	0	0	1	0	0	0	0	0	0	0	100
		0.5	0.12	9	0	1	0	0	0	0	1	93.0
		1	0.25	15	5	2	1	0	2	1	7	144
		5	1.2	39	0	1	0	1	0	2	3	126
		10	2.5	30	3	7	4	0	4	1	11	86.0
		30	7.4								Tox	

[a, b] See the footnote in Table 1. Tox; cells were killed.

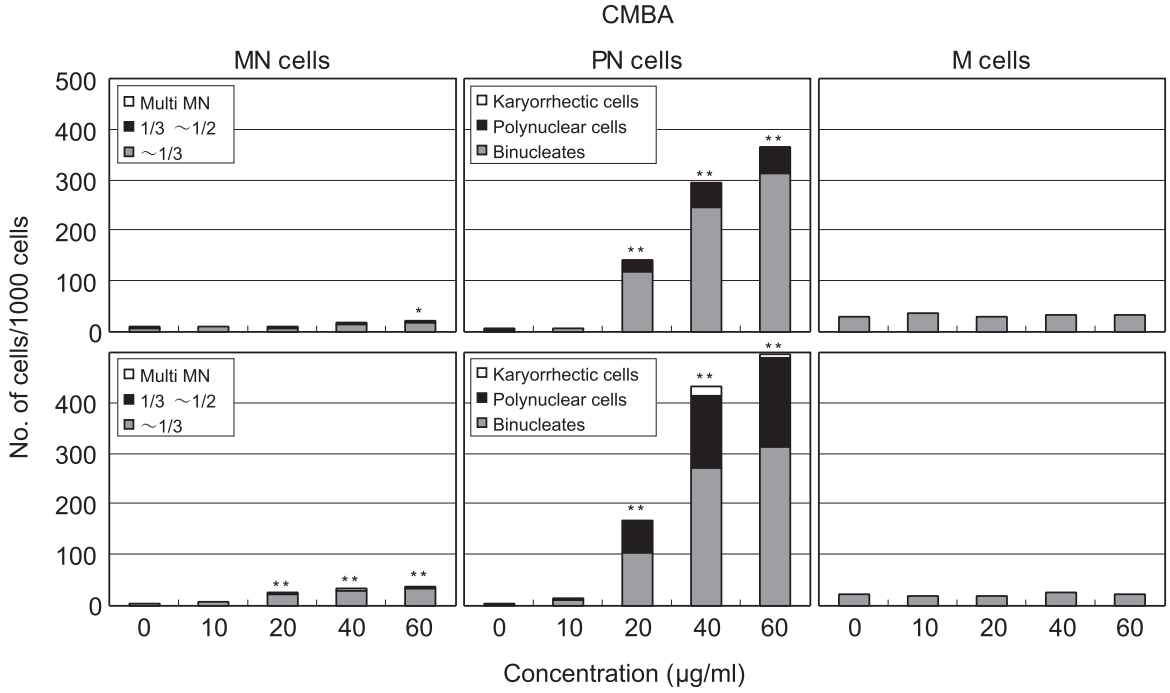

Fig. 6 Results of the *in vitro* MN test with CMBA for 24 h (top) and 48 h (bottom)
The left graphs show the number of MN cells/1000 intact interphase cells. The middle graphs show the number of PN cells/1000 total cells, including binucleates, polynuclear cells, and karyorrhectic cells. The right graphs show the number of M cells/1000 total cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

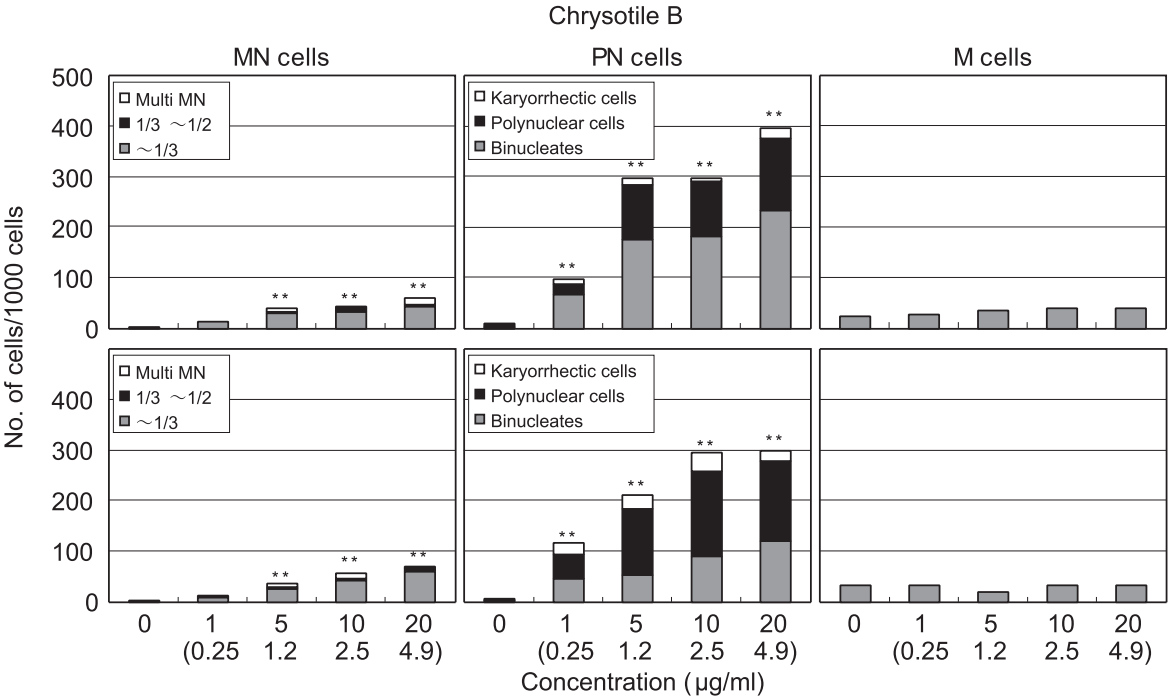

Fig. 7 Results of the *in vitro* MN test of chrysotile B
See legend to Fig. 6. Figures in parentheses indicate concentrations in $\mu g/cm^2$.

4. Discussion

We found that CMBA and chrysotile B showed a similar induction pattern of MN, PN, and M cells in the MN test, and both induced polyploidy in a similar manner in the CA test, although their fiber length distribution was different from each other strictly speaking. Koshi et al.[8] reported that a size-selected sample (81% of fibers was less than 5 μm long) of

UICC chrysotile B induced 34% polyploidy at 10 μg/ml after 46-h treatment. Our result was consistent well with theirs.

In a previous study[6], we showed that clastogens can be distinguished from aneugens in the MN test according to their induction pattern of MN, PN, and M cells. Aneugens were always accompanied by a high frequency of M cells. Vincristine induced a high frequency of MN and M cells in addition to the induction of PN cells. CMBA and chrysotile B, however, were clearly aneugens, but they did not induce M cells. We can estimate that the mechanism of polyploidy induction by CMBA may be different from that of vincristine.

We experienced a similar response of binucleate induction by 2-phenylbenzotriazole－type water pollutant (PBTA-2), which has cytochalasin B (Cyt B) -mimetic activity[9]. At first CMBA seemed to be similar to PBTA-2 in its induction pattern of MN, PN, and M cells in the MN test. But, CMBA was different from PBTA-2 in polyploidy induction in the CA test. PBTA-2 induced 12% polyploidy at most only at a highest concentration of 100 μg/ml. On the other hand, CMBA induced polyploidy in a concentration-dependent manner with an example of 46% at 25 μg/ml. Chrysotile B also induced polyploidy in a concentration-dependent manner with an example of 44% at 10 μg/ml.

One mechanism for the formation of bi- and multinucleated cells is attributed to hydrophobicity[10]. Chrysotile B is a mineral, so that was not the case. Inhibition of actin polymerization could be a mechanism. If so, formation of contractile rings would be inhibited resulting in no cleavage furrows at telophase, but we could observe cleavage furrows after CMBA treatment, even at 40 μg/ml (data not shown). Crocidolite fibers block cytokinesis but not cleavage furrows[11] and that could lead to binucleates. That might also be the case for CMBA. Needle crystals of vitamin B_2 induce polyploidy (32% at 150 μg/ml) in CHL/IU cells[12], apparently by physically fixing the cell shape and thereby preventing normal mitosis. This mechanism seems to be similar to that of the paper above[11].

In the present study, the uptake of fibers into cells was not investigated. Internalization of fibers is considered to play an important role in their cytotoxic and genotoxic effects. Further studies are needed to substantiate the findings in the present study and to understand the mechanism of polyploidy and binucleates induction mentioned above.

In the practical use of CMBA, it is commercially available as a water solution for textile dyeing. That suggests that there may be a very low possibility for us to be exposed to fibers of CMBA in the dyeing industry. CMBA should be handled carefully when manufactured in a factory.

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