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Dimethylarsinic acid targets tubulin in mitotic cells to induce abnormal spindles

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Dimethylarsinic acid (DMA) is the most effective inducer of cell-cycle disruption among the arsenic compounds and their metabolites. The present study was conducted to gain further insight into cell-cycle disruption induced by DMA. The inhibition of cell proliferation and the mitotic arrest induced by DMA were significant and dose-dependent in Chinese hamster V79 cells and the two seemed to be closely related. At less than 140 μM the DMA did not inhibit the proliferation of cells, but it significantly induced mitotic arrest. An indirect immunofluorescence assay using anti-α-tubulin antibodies revealed that DMA induced the formation of abnormal spindles in the metaphase cells even at 350 μM with 5 h of treatment. At 1.4 mM DMA no metaphase cells could form a definite spindle structure. The spindle figures were similar to those induced by colchicine (125 nM) or vinblastine (110 nM), major antimitotic agents. In DMA-treated interphase cells, the microtubule networks were indistinguishable from those of normal cells. With the tubulin-assembly assay estimated by turbidity, DMA at less than 200 μM suppressed tubulin assembly in a dose-dependent manner, whereas at more than 700 μM it enhanced tubulin polymerization remarkably with or without addition of excess guanosine-5′-triphosphate. According to the above findings, we discussed the possibility that DMA, a primary metabolite of inorganic arsenic in mammals, is related to arsenic carcinogenicity. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: dimethylarsinic acid; arsenic; tubulin; mitotic arrest; spindle poison; carcinogenicity

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INTRODUCTION

Arsenic is ubiquitously distributed in nature in a variety of chemical forms. It is a human carcinogen, but the chemical forms of the arsenic compounds associated with carcinogenicity remain to be elucidated.[1] Dimethylarsinic acid (DMA) is a major metabolite of inorganic arsenic in mammals, including humans.[2–4] In general, the acute toxicity of organoarsenic compounds is much lower than that of inorganic arsenic.[5] Methylation of arsenic can be considered a mechanism of detoxification.[6] Many *in vivo* and *in vitro* studies indicate that DMA is a potent clastogenic agent, causing mitotic arrest in cultured mammalian cells[7–9] and aneuploidy in mouse bone-marrow cells.[10] Despite much evidence of the clastogenic effect of DMA, the direct target of DMA has been little reported on. Ochi *et al.* suggested that the primary target of DMA is centrosomes to induce multipolar spindles in Chinese hamster V79 cells on indirect immunofluorescence assay using anti-γ-tubulin antibodies.[11] However, Iwami *et al.* reported that DMA induced c-mitosis in human lymphocytes, and that the effect of DMA is similar to those of antimitotic agents (such as colchicine, vinblastine or paclitaxel).[9]

In the cells treated with antimitotic agents, abnormal spindles are often observed with an

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indirect immunofluorescence assay using anti-α- or -β-tubulin antibodies. Tubulin has three iso-proteins, α-, β-, and γ-tubulins; an α- and β-tubulin dimer assembles in the presence of guanosin-5′-triphosphate (GTP) to form microtubules. On the other hand, γ-tubulin is a component of centrosomes. Antimitotic agents interact with the α- and β-tubulin dimers at various sites, and inhibit GTP-induced normal tubulin assembly and disassembly, resulting in disruption of microtubule dynamics.[12–14] Regulation of the dynamics is an important function for many biological processes, including cell division. Therefore, it is important to study the effects of DMA on tubulin to clarify the mechanism of clastogenic action of DMA. Turbidity assay has been a general and useful cell-free method for measurement of tubulin assembly by spectrophotometry.[15] Turbidity is a reliable measure of the mass of tubulin assembled into a higher molecular weight structure.

The present study was conducted to gain further insight into cell cycle disruption induced by DMA. At first, we treated Chinese hamster V79 cells with DMA at various concentrations and examined the relation between DMA-induced cytotoxicity and mitotic arrest. Next, spindle figures were observed in mitotic cells treated with DMA using an indirect immunofluorescence assay, staining by anti-α-tubulin antibodies. In the cells treated with antimitotic agents, the abnormal spindle figures in the metaphase and/or the disruption of microtubule networks in the interphase can be observed. Then, to determine whether DMA interacts directly with tubulin, the effect on the tubulin assembly was examined in a cell-free system at various concentrations of DMA.

MATERIALS AND METHODS

Materials

Mouse monoclonal anti-α-tubulin antibody (T 9026, Lot 087H4818), paclitaxel, and tubulin (T 4925, Lot 87H4024) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Tubulin was purified from bovine brain by assembly–disassembly cycles and contained approximately 15% microtubule-associated proteins. Vinblastine, of analytical grade, and GTP, of biochemical grade, were purchased from Wako Pure Chemical Co., Osaka, Japan. Giemsa's solution was purchased from Merck KGaA, Darmstadt, Germany. Cy3-conjugated anti-mouse immunoglobulin G (IgG) from goat (PA43002) was obtained from Amersham Pharmacia Bioteck Co., Tokyo, Japan. DMA (purity >99.99%) was obtained from Tri-Chemical Lab., Yamanashi, Japan. DMA was dissolved in water and the pH of the solution was adjusted to 6.5. Colchicine and vinblastine were dissolved in dimethyl sulfoxide and were diluted with water. The Bio-Rad protein assay kit was from Bio-Rad Laboratories, Hercules, CA, USA.

Cell culture and reagent treatment

Chinese hamster V79 cells were cultured in Eagle's Minimum Essential Medium (MEM) with 7% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. The cells were seeded at 2×10^4 on a slide coverslip (22×22 mm^2) or at 2×10^5 on a Falcon 12-well plate. One day after seeding, they were treated with DMA (70 μM–70 mM), colchicine (125 nM) or vinblastine (110 nM).

Cell proliferation assay and mitotic analysis

Cell proliferation was evaluated by colorimetric 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) assay[16] on the 12-well plates. Mitotic cells (cells in metaphase) were analyzed after being fixed in ethanol:acetic acid (3:1) and stained with 2% Giemsa's solution. The mitotic index (percent) was determined as the proportion of metaphase cells in 1000 cells.[8–10] Statistical difference was determined by a two-tailed Student's *t*-test.

Indirect immunofluorescence assay

Indirect immunofluorescence staining of cells using anti-α-tubulin antibodies was performed as reported by Masaki *et al.*[17] with minor modification. Cells grown on coverslips were rinsed at 37 °C with phosphate buffer saline (PBS) (+), fixed in 2% paraformaldehyde–PBS (+), and then further treated with 0.1% Triton X-100 (octoxynol). After nonspecific antibody binding was blocked with blocking buffer (0.1% NaN_3–PBS (+) containing 1% bovine serum albumin), the cells were incubated with mouse monoclonal anti-α-tubulin antibodies for 30 min at 37 °C in a 5% CO_2 atmosphere. The cells were then rinsed and incubated with Cy3-conjugated goat anti-mouse IgG for 30 min at 37 °C in 5% CO_2; thereafter, they were rinsed, mounted on glass slides using glycerol and sealed with nail

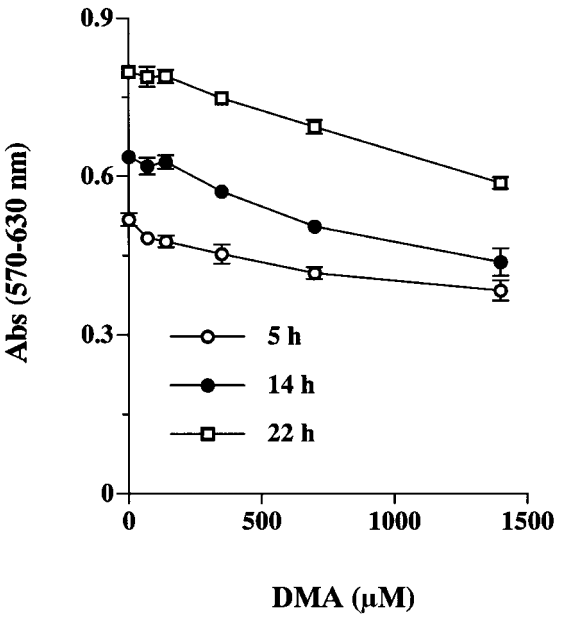

Figure 1 Effects of DMA on cell viability in V79 cells. Cellular proliferation was measured by colorimetric MTT assay, at a test wavelength of 570 nm and a reference wavelength of 630 nm. Data represent means of three or more experiments.

polish. Immunofluorescence images of tubulin were obtained using a fluorescence microscope (Olympus BX50, Japan) with a cooled charge-coupled device camera (Photmetrics, USA) and were pseudocolored using IP Lab Spectrum 3.1.2a (Scanalytics, USA) software.

Tubulin preparation

Tubulin was prepared according to the instructions of the supplier. Briefly, about 7.5 mg of tubulin was dissolved in 1 ml of 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)–NaOH (pH 6.8), 1 mM ethylene glycol-bis (*β*-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA), 0.1 mM GTP, 0.1 mM ethylene diamine tetra-acetic acid (EDTA), 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, and 0.3 mM sucrose, and shaken gently for 5 min at 37 °C. The resolved tubulin was sonicated for 5 min at 0 °C, and centrifuged at 27 000*g* for 40 min at 4 °C. Then, the supernatant was collected and kept at −70 °C until analysis. The frozen supernatant contained only tubulin-dimer. The protein concentration of tubulin was determined by the method of Bradford[18] using an assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Tubulin assembly

Tubulin assembly assay was performed in 100 mM MES buffer, containing 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 2.5 M glycerol (pH 6.5). The concentration of tubulin protein was 0.8 mg ml^{-1}. DMA solution (pH 6.5) was premixed on ice with/without GTP. Tubulin assembly was started by the addition of GTP–DMA or DMA at 37 °C, and stopped by standing on ice. Following the increase in turbidity, optical density was measured at 350 nm[15] with a spectrophotometer at 37 °C. The final concentration of GTP was 1 mM.

RESULTS

Effects of DMA on proliferation and mitosis in V79 cells

DMA inhibited cell proliferation in a dose-dependent manner (140 μM–1.4 mM) at all treatment times (5, 14 and 22 h) (Fig. 1). At concentrations less than 140 μM it hardly inhibited cell proliferation; weak inhibition appeared at concentrations of more than 140 μM. At concentrations above 700 μM the DMA suppressed cell proliferation to 70–80% of the control at all treatment times.

The mitotic index for V79 cells exposed to DMA for 5, 14 and 22 h was calculated. In this experiment, colchicine was not added in order to avoid its mitotic blocking effect and to determine the net index of DMA treatment. DMA (70 μM–1.4 mM) significantly increased the mitotic index in a dose- and time-dependent manner (Fig. 2) compared with the control, i.e. mitotic arrest was induced by DMA. Even treatment with less than 140 μM of DMA resulted in a significant increase in the mitotic index at 5 h treatment ($p < 0.01$). At 350 μM of DMA the mitotic index was markedly increased at 14 h. At more than 700 μM the index was remarkably and steeply increased at 10 h.

Disruption of spindle formation by DMA in metaphase V79 cells

To examine the mechanism of DMA-induced mitotic arrest, the effects of DMA on spindle formation were investigated by indirect fluorescence microscopy. A control metaphase cell is

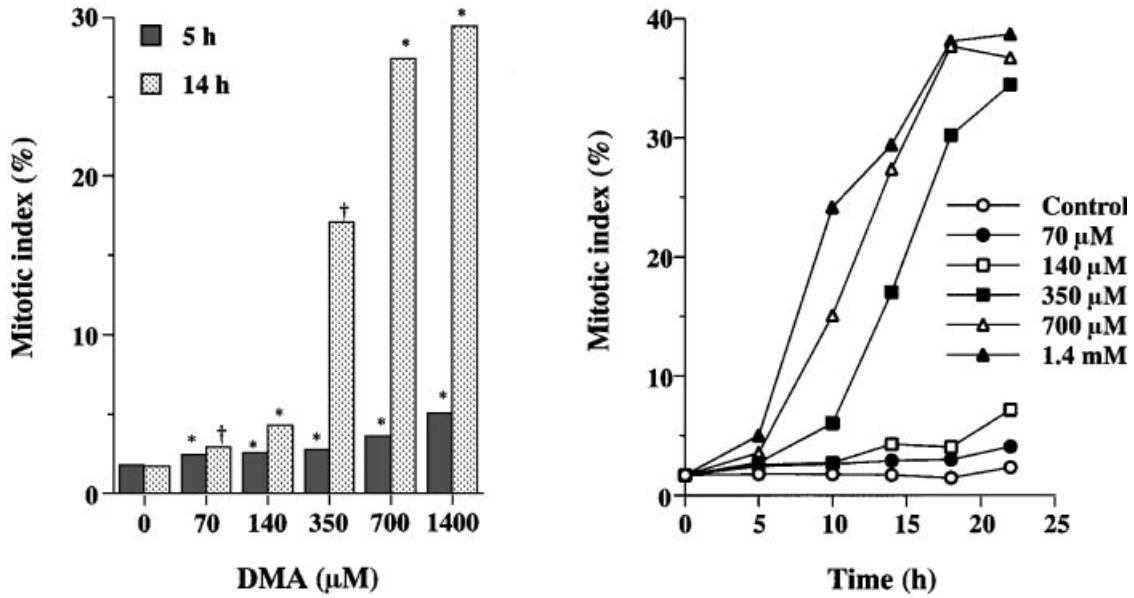

Figure 2 Induction of mitotic arrest in V79 cells treated with DMA. Data represent means of six experiments. *, $p < 0.001$ by Student's *t*-test. †, $p < 0.01$ by Student's *t*-test.

shown in Fig. 3A. DMA changed the morphology of the spindles in metaphase cells at 5 h treatment. At 350 μM, many nearly normal spindles and some types of abnormal spindle (Fig. 3B and C) were observed. At 1.4 mM, no metaphase cells could form a definite spindle structure and normal spindle figures were not observed (Fig. 3D). The spindles and spindle poles were obscure, and spindle-pole-like figures were observed in the centers of cells. The spindle figures induced by DMA were similar to those induced by major antimitotic agents: colchicine (125 nM) or vinblastine (110 nM) (data not shown). The microtubule networks in DMA-treated interphase cells were indistinguishable from those of normal cells. These results show that DMA disrupts spindle formation in mitotic cells like many mitotic agents do.

Effect of DMA on tubulin assembly in a cell-free system

The direct effect of DMA on tubulin was demonstrated by turbidity assay at low (50–200 μM), high (700 μM–3.5 mM), and very high (70 mM) concentrations of DMA. Since tubulin assembly is enhanced in acidic conditions, the pH of DMA solutions was adjusted to 6.5. As shown in Fig. 4, tubulin assembly proceeded rapidly in 5 min. Low concentrations of DMA decreased the turbidity in a concentration-dependent manner (Fig. 4). As shown in Figs 5 and 6, high and very high concentrations of DMA increased the turbidity in a concentration-dependent manner. At the very high concentration of DMA (Fig. 6) the turbidity increase was steep and remarkable. When DMA was added to the control sample after 20 min, the turbidity increased more. Furthermore, without addition of GTP (<5 μM) DMA increased the turbidity steeply, similar to results in the presence of 1 mM GTP. Thus, DMA significantly inhibited normal tubulin assembly at various concentrations in the cell-free system.

DISCUSSION

Our immunofluorescence assay shows that DMA caused serious effects on spindle formation, and that the effects of DMA on spindles differed at low and high concentrations. At a high concentration of DMA (1.4 mM for 5 h) the cells could not form a definite structure, i.e. the spindle itself could not be formed, as observed in V79 cells by Ochi *et al.*[11] DMA at low concentration (350 μM for 5 h) induced some types of abnormal spindle (Fig. 3B and C) not similar to those treated at the high

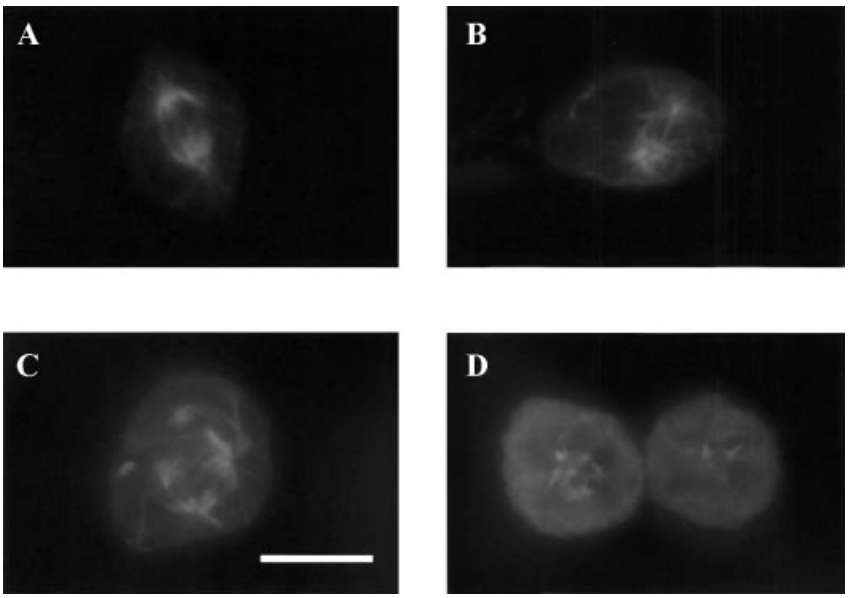

Figure 3 DMA-induced disruption of spindle formation in the metaphase V79 cells following 5 h treatment. The spindle figures of control (A), 350 μM DMA-treated (B, C), and 1.4 mM DMA-treated (D) cells as detected with an indirect immunofluorescence assay using anti-α-tubulin antibody. ×1000. Bar is 10 μm.

concentration (Fig. 3D). DMA did not affect the microtubule network in interphase cells. We suppose that the action of DMA on microtubule assembly may differ at different concentrations of this agent.

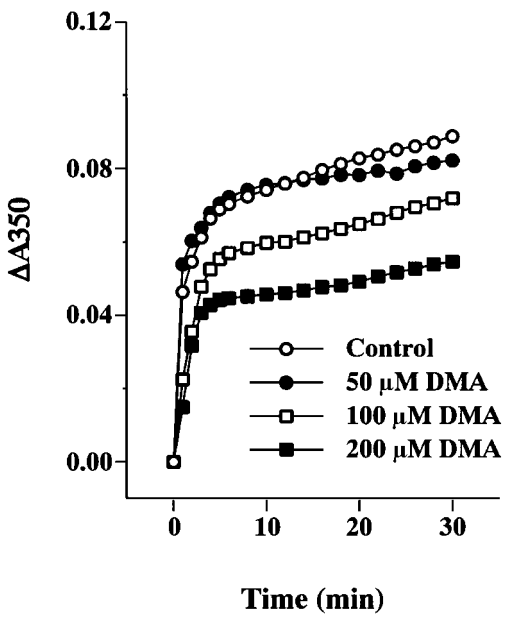

Figure 4 Effects on tubulin assembly of DMA at low concentrations.

Turbidity assay of tubulin assembly under the same concentrations of the immunofluorescence assay gave interesting results. At low concentration, DMA suppressed tubulin assembly in a dose-dependent manner (Fig. 4). However, a high concentration of DMA enhanced tubulin assembly (Figs 5 and 6). Such phenomena have been reported with vinblastine; the agent inhibits tubulin assembly at low concentrations,[19] but it forms some types of non-microtubule tubulin-polymer at high concentrations.[12] Therefore, the tubulin polymer produced by high concentrations of DMA is a non-microtubule polymer. The above findings suggest that DMA-induced suppression of tubulin assembly can form spindles in the cells, but in an abnormal form, and that DMA-produced tubulin-polymers cannot form or hold the spindle structure in the cells because they are of a non-microtubule form. Ochi *et al.* suggested that centrosomes are the primary target of DMA.[11] According to our findings, DMA also targets tubulin and inhibits normal microtubule assembly in metaphase cells. The DMA-induced cell-cycle disruptions may be related with these two effects of the drug.

As shown in Fig. 6, tubulin turbidity increased with or without addition of 1 mM GTP at very high

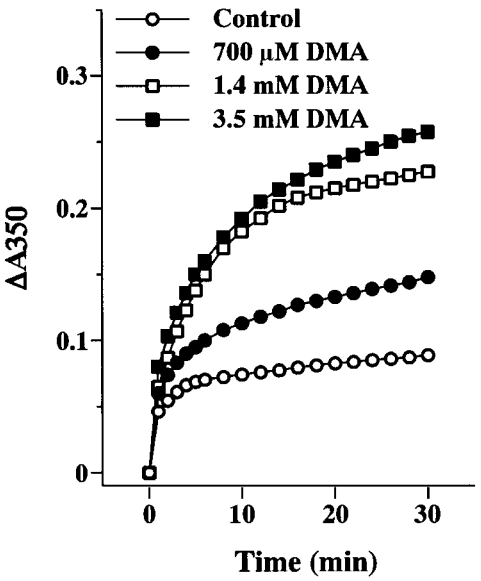

Figure 5 Effects on tubulin assembly of DMA at high concentrations.

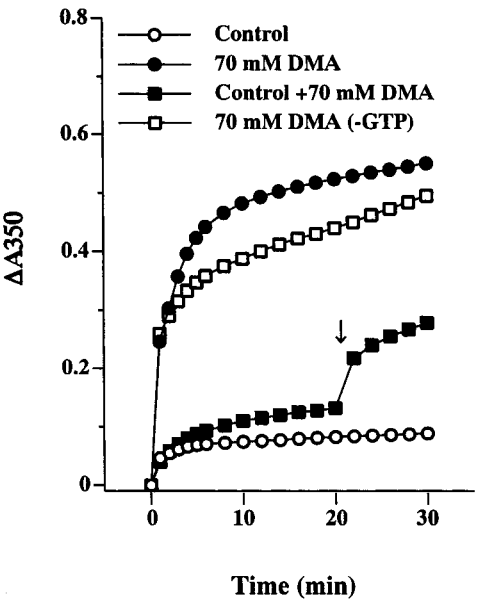

Figure 6 Effects on tubulin assembly of DMA at very high concentrations. Arrow: addition of 70 mM DMA after 20 min on reaction of control tubulin assembly.

concentrations of DMA. The mechanism behind the increase is as follows: DMA induces the formation of tubulin oligomers, as seeds of microtubules, and/or short tubulin-polymers without interposition of GTP, similar to vinblastine or paclitaxel.[12,13] So many seeds and/or polymers are produced in a short time that they aggregate. From our turbidity assay results, DMA may have effects similar to vinblastine, unlike colchicine or paclitaxel. Vinblastine inhibits tubulin assembly in the presence of excess GTP but promotes non-microtubule tubulin-polymerization without GTP.[13,20] Colchicine inhibits tubulin assembly and paclitaxel enhances the polymerization of tubulin, with or without GTP.[13,14] In an additional study (Fig. 7), the combination of DMA and colchicine increased the mitotic indices more than a single treatment of DMA or colchicine. The finding supports a view that DMA does not compete with colchicine and that the effects on tubulins are different from those of colchicine.

DMA suppressed tubulin-GTPase activity, but the inhibition was not complete.[21] Since tubulin-GTPase activity causes tubulin disassembly and instability of microtubule dynamics,[22–24] DMA may suppress tubulin disassembly and disrupt microtubule dynamics.

The cytotoxicity of DMA appeared to be closely related with the effects on tubulin dynamics, since inhibition of cell proliferation was strongly correlated with the mitotic arrest (Figs 1 and 2). This is also supported by our immunofluorescence study,

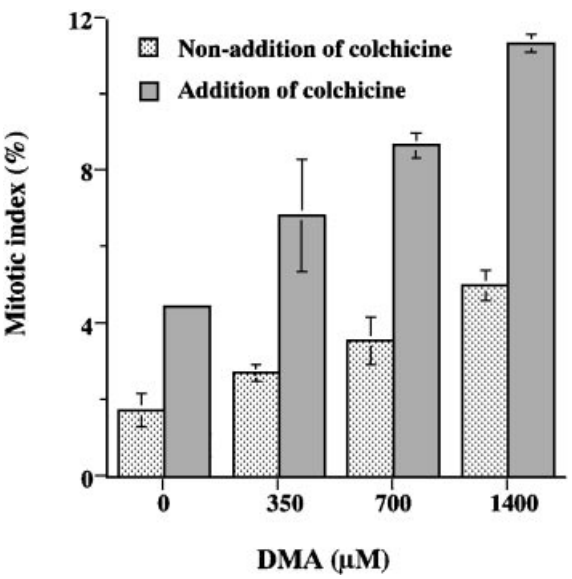

Figure 7 Combined effect of DMA and colchicine in V79 cells. Colchicine was added at 125 nM at the same time as DMA. Error bars show the standard deviation of six data.

which showed that DMA inhibited normal spindle formation. Some carcinogens, such as vinblastine and 17 β-estradiol, exhibit no mutagenicity in the Ames *Salmonella*/microsomal assay,[25] and inhibit normal tubulin assembly.[13] Since DMA is not mutagenic[26] and the agent causes cancer in rats,[27] DMA may belong to a non-mutagenic carcinogen class.

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