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Dimethylarsinic acid causes inhibition of microtubule assembly and inhibition of calcium-sensitive disassembly of microtubules via interaction with glutathione†

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The effects of dimethylarsinic acid (DMAA) on microtubule assembly *in vitro* were investigated in terms of the role of glutathione (GSH). Increase in turbidity due to assembly of brain tubulins was monitored in the presence or absence of DMAA. DMAA at concentrations up to 10 mM did not cause inhibition of the increased turbidity. GSH at concentrations over 10 mM enhanced the increase in turbidity. By contrast, the increased turbidity was inhibited markedly when DMAA and GSH were combined in the ratio of 5:1. Moreover, the combination similarly inhibited calcium-induced disassembly of microtubules with a potency equivalent to that of 10 μM taxol. These results suggest that DMAA inhibited not only microtubule assembly, but also calcium-sensitive disassembly of microtubules, via interaction with GSH. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: arsenic compounds; dimethylarsinic acid; glutathione; microtubule assembly

INTRODUCTION

In numerous ecosystems, inorganic arsenicals are reduced and methylated by anaerobes to yield extremely toxic dimethylarsine and trimethylarsine via the formation of methylarsonic acid (MAA) and dimethylarsinic acid (DMAA).[1] By contrast, when inorganic arsenicals were administered to man,[2–4] experimental animals[5–7] and cultured mammalian cells,[8] MAA and DMAA were excreted in urine and culture medium together with unchanged arsenicals. DMAA is a ubiquitous form of arsenic in the environment and is also a major metabolite of inorganic arsenics in man and experimental animals. Furthermore, it has been used as a herbicide or pesticide.[9]

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Toxicological studies *in vitro* showed that DMAA was cytotoxic[10,11] and genotoxic,[12,13] although the potency of the effects was two to three magnitudes lower than that of inorganic arsenicals. Studies have also shown that DMAA causes cytotoxic effects,[10,11] chromosome structural aberrations[13] and apoptosis[11] via interaction with intracellular glutathione (GSH). Moreover, DMAA, when applied to cultured Chinese hamster cells, caused mitotic arrest[14,15] and the subsequent appearance of multinucleated cells.[16] In addition, DMAA caused disruption of the microtubule network in the cells whereas it maintained the organization of actin stress fibers.[15,16] In understanding the mechanism of disruption of the microtubule network by DMAA, an approach to identify the molecular target could be made by investigating the effects of DMAA on the activity of microtubule-organizing centers (MTOCs) in the centrosome[17] and also the effects on the dynamic polymerization–depolymerization cycle of tubulin. For the former, it has been demonstrated that DMAA caused centrosome abnormality specifically in mitotic cells but not interphase cells,[18,19]

suggesting that the abnormality might not be responsible for disruption of the microtubule network in cells exposed to DMAA. Alternatively, studies on the effects of DMAA on microtubule assembly are warranted in order to gain some insight into the mechanism by which DMAA causes disruption of the microtubule network.

In the present study, the inhibitory effects *in vitro* of DMAA on microtubule assembly and calcium-sensitive disassembly of microtubules were investigated, together with the role of GSH in the induction of the effects.

MATERIALS AND METHODS

Chemicals

Taxol and vinblastine were a gift from Dr Kobayashi of the Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan. Colchicine was purchased from Merck (Germany). These compounds were dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.5 mg ml^{-1}. DMAA was obtained from the Tori Chemical Corp. (Uenohara, Kanagawa, Japan). 4-Morpholinoethanesulfonic acid (MES) was obtained from ICN Biomedical Inc., (OH, USA). Ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2-mercaptoethanol (2-ME) and glycerol were obtained from the Kanto Chemical Co., Inc., Japan. GTP was obtained from the Yamasa Corporation, Japan.

Microtubule proteins

Polymerization buffer was composed of 100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM guanosine-5′-triphosphate (GTP) and 1 mM 2-ME. Preparation of microtubule proteins was performed according to the procedure of Kobayashi *et al.*[20] In brief, fresh porcine brains were homogenized at 0 °C in polymerization buffer solution and centrifuged at 50000*g* at 4 °C. Glycerol buffer (8 M glycerol in polymerization buffer) was added to the supernatant, and the mixture was incubated at 37 °C for 30 min and centrifuged at 100000*g* to precipitate the microtubules. The depolymerization and polymerization procedures were repeated twice to purify the microtubule proteins. The concentration of the proteins was quantified using the Coomassie Protein Assay Kit (Pierce). The final concentration of protein for the subsequent bioassay was adjusted to 1.3 mg ml^{-1}.

Microtubule assembly/disassembly assay

Microtubule assembly and disassembly were monitored by the increase and decrease in turbidity at 400 nm in a glass UV cuvette with a Shimadzu model U-3000 spectrophotometer equipped with an electronic temperature controller. Test chemicals were mixed in polymerization buffer with microtubule proteins at 0 °C, then incubated at 37 °C in the spectrophotometer to monitor the turbidity. After the assembly process was completed (20–30 min), $CaCl_2$ solution at a final concentration of 4 mM was added to observe microtubule disassembly. In the cases of the treatment with taxol, colchicines and vinblastine, the concentration of DMSO was adjusted to less than 2%.

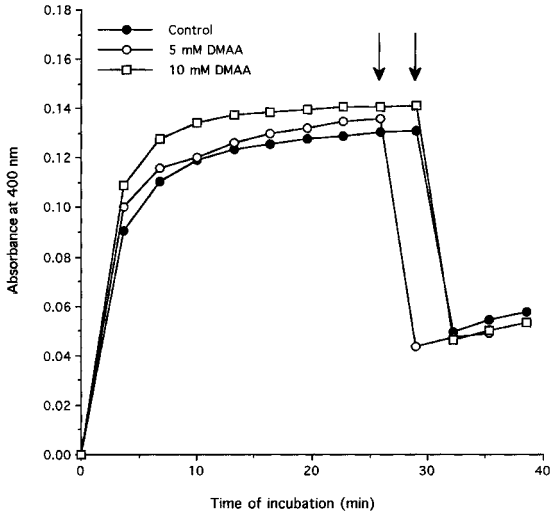

Figure 1. Effects of DMAA on microtubule assembly. Microtubule protein (1.3 mg ml^{-1}) isolated from porcine brain was incubated at 37 °C in assembly buffer in the presence or absence of DMAA. Microtubule assembly was monitored turbidimetrically, as changes in absorbance at 400 nm. At the time points indicated by the arrows, 4 mM $CaCl_2$ was added to cause microtubule disassembly. All the turbidimetrical analyses were repeated serially and the results are expressed as means of results from two separate measurements.

The rates of microtubule assembly and disassembly were determined by the following equation, in which *S* (test experiment) and *C* (control experiment) show the data obtained with and without inhibitors respectively (t_0: initial turbidity; t_{37}: turbidity at 37 °C; t_{ca}: turbidity after addition of $CaCl_2$).

$$\text{Microtubule assembly } (\%) = \frac{S(t_{37} - t_0)}{C(t_{37} - t_0)} \times 100 \quad (1)$$

$$\text{Microtubule disassembly by calcium } (\%) = \frac{S(t_{37} - t_{ca}) \times C(t_{37} - t_0)}{S(t_{37} - t_0) \times C(t_{37} - t_{ca})} \times 100 \quad (2)$$

All the turbidimetrical analyses were repeated serially and the results were expressed as means of results from two separate measurements.

RESULTS

Effects of DMAA on microtubule assembly *in vitro*

Test chemicals were added to the reaction mixtures that contained brain tubulins, EGTA, Mg^{2+} and GTP on ice. Increase in turbidity of the reaction mixture due to

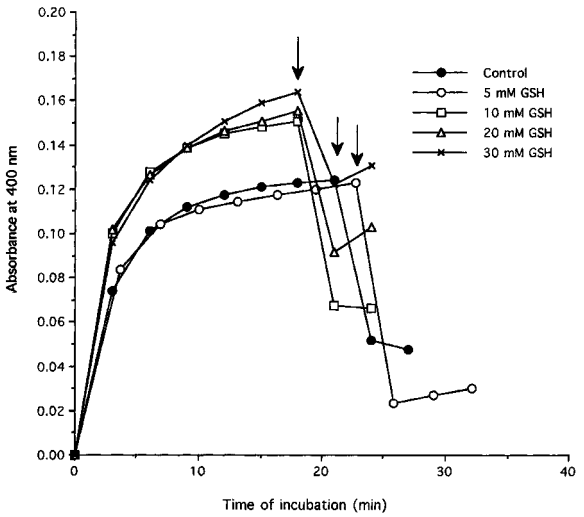

Figure 2. Effects of GSH on microtubule assembly. Microtubule protein (1.3 mg ml^{-1}) isolated from porcine brain was incubated at 37 °C in assembly buffer in the presence or absence of GSH. Microtubule assembly was monitored turbidimetrically, as changes in absorbance at 400 nm. At the time points indicated by arrows, 4 mM $CaCl_2$ was added to cause microtubule disassembly. All the turbidimetrical analyses were repeated serially and the results are expressed as means of results from two separate measurements.

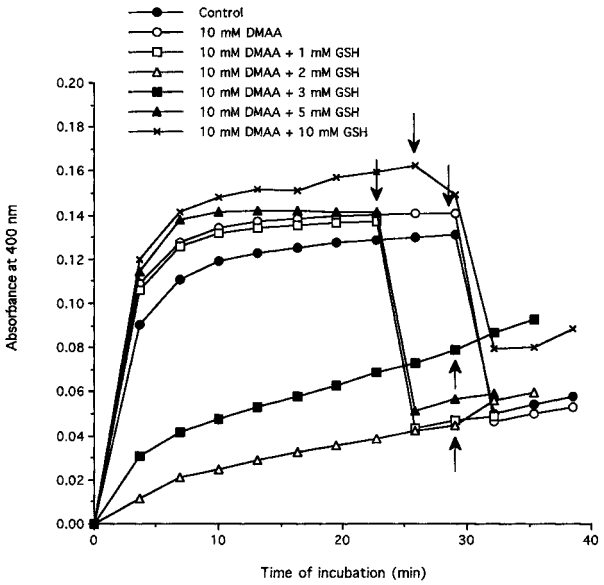

Figure 3. Effects of combination of DMAA and GSH on microtubule assembly. Microtubule protein (1.3 mg ml^{-1}) isolated from porcine brain was incubated at 37 °C in assembly buffer with 10 mM DMAA in the presence or absence of specified concentrations of GSH. Microtubule assembly was monitored turbidimetrically, as changes in absorbance at 400 nm. At the time points indicated by arrows, 4 mM $CaCl_2$ was added to cause microtubule disassembly. All the turbidimetrical analyses were repeated serially and the results are expressed as means of results from two separate measurements.

microtubule assembly was then monitored by incubating at 37 °C. As shown in Fig. 1, the presence of 1 to 5 mM DMAA in the reaction mixture did not cause any changes in the increased turbidity compared with that in the untreated control. However, 10 mM DMAA caused a slight increase in the turbidity compared with the reaction in the control. When 4 mM $CaCl_2$ was added to cause disassembly of the assembled microtubules, no difference in the rate of reduction of the turbidity was observed among reactions with and without DMAA.

Effects of a combination of DMAA and GSH on microtubule assembly *in vitro*

Before investigating the effects of a combination of DMAA and GSH on microtubule assembly, the effects of GSH alone were examined. As shown in Fig. 2, GSH up to 5 mM did not lead to a change in the increased turbidity compared with the control reaction. However, when the reaction was initiated in the presence of 10 to 30 mM GSH, turbidity was already higher than that of the control 3 to 6 min after the reaction was initiated, and at 16 min the turbidity was 1.3 times the control level. By contrast, GSH at concentrations over 5 mM showed resistance to the induction of microtubule disassembly by calcium in a concentration-dependent manner.

Figure 3 demonstrated the effects of combinations of 10 mM DMAA and various concentrations of GSH on microtubule assembly *in vitro*. When 10 mM DMAA was combined with 1 mM GSH, no difference in increased turbidity was observed compared with that caused by 10 mM DMAA alone. However, when combined with 2 mM GSH, DMAA markedly inhibited the increase in turbidity. Moreover, no disassembly of microtubules by calcium was observed in the combination. A combination of 10 mM DMAA with 3 mM GSH also inhibited the increase in turbidity, but was less effective than in combination with 2 mM GSH. This combination also completely inhibited calcium-sensitive microtubule disassembly. By contrast, when 10 mM DMAA was combined with 5 or 10 mM GSH, no decrease in the increased turbidity was observed; in contrast, they enhanced the turbidity over the level caused by DMAA alone.

Figure 3 shows that a combination of DMAA and GSH in the ratio of 5:1 caused both inhibition of microtubule assembly and inhibition of calcium-sensitive disassembly of microtubules. To ascertain the significance of the ratio (5:1) of DMAA to GSH in the inhibition of microtubule assembly, the effects of other 5:1 combinations were investigated. As shown in Fig. 4, a combination of 8 mM DMAA and 1.6 mM GSH caused the same levels of inhibition of the increased turbidity as observed with a combination of 10 mM DMAA and 2 mM GSH. Calcium-sensitive disassembly of microtubules was also inhibited completely by this

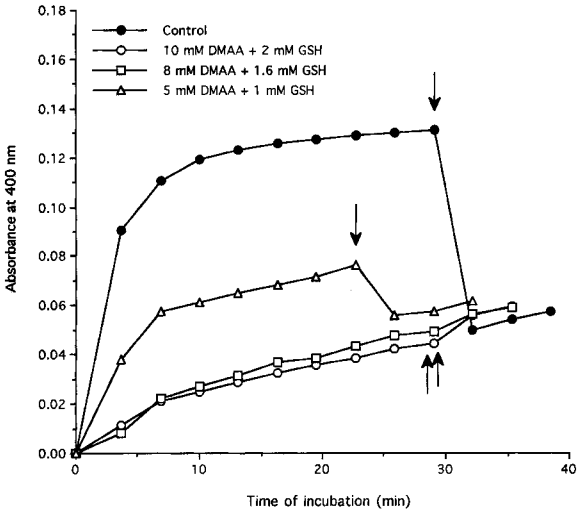

Figure 4. Effects of combination of DMAA and GSH in the ratio of 5:1 on microtubule assembly. Microtubule protein (1.3 mg ml^{-1}) isolated from porcine brain was incubated at 37 °C in assembly buffer with various 5:1 combinations of DMAA and GSH. Microtubule assembly was monitored turbidimetrically, as changes in absorbance at 400 nm. At the time points indicated by arrows, 4 mM $CaCl_2$ was added to cause microtubule disassembly. All the turbidimetrical analyses were repeated serially and the results are expressed as means of results from two separate measurements.

combination. By contrast, the same 5:1 ratio with a combination of 5 mM DMAA and 1 mM GSH, whilst effective in inhibiting microtubule assembly, was less effective than the other 5:1 combinations. Upon disassembly of the microtubules by calcium, this combination demonstrated a little sensitivity.

The effects of DMAA, GSH and their combination on microtubule assembly and calcium-sensitive disassembly of microtubules are summarized in Table 1, together with the effects of arsenite and well-known microtubule drugs, such as colchicine, vinblastine and taxol. The results demonstrated that 5:1 combinations of DMAA and GSH were as potent as 5 μM vinblastine and a little more potent than 10 μM colchicine in the inhibition of microtubule assembly. Moreover, inhibitory action of the combinations on calcium-sensitive disassembly of microtubules was comparable with that of a well-known compound, taxol (10 μM). Arsenite was ineffective even at 200 μM on microtubule assembly, though it was somewhat inhibitory on calcium-sensitive disassembly of microtubules.

DISCUSSION

Earlier studies have shown that DMAA was less cytotoxic, genotoxic and apoptotic in cells depleted of GSH than in cells

Table 1. Effects of arsenic compounds, GSH and their combinations on microtubule assembly and calcium-sensitive disassembly of microtubules[a]

Treatment	Microtubule assembly (% of control)	Microtubule disassembly by calcium (% of control)
Control	100	100
10 μM taxol	113	0
10 μM colchicine	76.7	98.9
5 μM vinblastine	40.8	ND
5 mM DMAA	105	111
10 mM DMAA	102	110
1 mM GSH	106	ND
5 mM GSH	95.3	131
10 mM GSH	117	97.1
20 mM GSH	135	58.8
30 mM GSH	162	35.4
10 mM DMAA + 10 mM GSH	125	83
10 mM DMAA + 20 mM GSH	119	9
10 mM DMAA + 30 mM GSH	161	0
5 mM DMAA + 1 mM GSH	43	38.5
8 mM DMAA + 1.6 mM GSH	36.6	0
10 mM DMAA + 2 mM GSH	32.5	0
20 μM arsenite	108	119
100 μM arsenite	102	118
200 μM arsenite	96.8	72.1

[a] Microtubule assembly and calcium-sensitive disassembly of microtubules were measured as described in Figures 1 to 4. The results are expressed as percentages of the turbidities of the controls, in the case of microtubule assembly. In the case of calcium-sensitive disassembly of microtubules, the results are expressed as percentages of the rate of calcium-induced decrease of turbidity in the treated groups to the rate in the untreated controls.

not so depleted, suggesting that GSH is required for the induction of these effects by DMAA.[10,11,13] Likewise, the present study demonstrates that DMAA causes the inhibition of microtubule assembly *in vitro* only when it is combined with GSH. DMAA alone did not inhibit the microtubule assembly, even at a concentration of 10 mM, and GSH at concentrations over 10 mM rather enhanced the rate of microtubule assembly. The combination of DMAA and GSH in the ratio of 5:1 was highly effective in inhibiting microtubule assembly. However, the ratios of 10:1, 2:1 and 1:1 did not inhibit assembly. These results suggest that the relative concentration of GSH to DMAA is a critical factor for the inhibition of microtubule assembly. In addition, the results, together with earlier studies, prompt the notion that cells or tissues with a variety of levels of GSH may have a different sensitivity to DMAA toxicity.

The mechanism by which the combination of DMAA and GSH inhibits microtubule assembly is unknown. It has been demonstrated that sulfhydryl (SH) groups in tubulin are related to the binding sites of tubulin molecules for polymerization of microtubules.[21] The maintenance of tubulin SH-groups in the reduced form is essential for polymerization.[22] These findings suggest that tubulin SH-groups are the critical target for reagents that form mercaptides. Therefore, it is considered that metabolites generated by the interaction of DMAA with GSH modify tubulin SH-groups, as found for diamide.[22] By contrast, an increase in turbidity over the control reaction, observed when GSH at concentrations over 10 mM was present in the reaction mixture, might be due to the reduction by GSH of cysteine residues oxidized during the reaction.

In an *in vitro* study, interaction of GSH with DMAA in the ratio of 3:1 formed a 1:1 GSH–dimethylarsinite complex with the concomitant formation of oxidized GSH (GSSG).[23] By contrast, the product generated by interaction of DMAA with GSH in the ratio of 5:1 remains unknown. It is also possible that highly toxic trivalent reactive intermediates, such as monomethylarsonous acid and dimethylarsinous acid,[24] were generated by the reduction of DMAA with GSH. By contrast, when 2 mM GSH was incubated *in vitro* with 10 mM DMAA, it was oxidized to GSSG to a 2.5-times higher level than in the absence of DMAA (from 0.47% in the absence of DMAA to 1.18%). Accordingly, it is also considered that oxidized GSH modified cysteine residues in tubulin.

A combination of DMAA and GSH in the ratio of 5:1 not only caused inhibition of microtubule assembly, it also inhibited calcium-sensitive disassembly of microtubules. For the mechanism of calcium-sensitive disassembly of microtubules, it has been shown that microtubule-associated protein 2, τ factor and tubulin were phosphorylated by calcium-dependent or calmodulin-dependent protein kinase.[25,26] The phosphorylation induced inhibition of microtubule assembly, in accordance with the degree of phosphorylation. In this context, targets for DMAA–GSH (5:1) in the inhibition of calcium-sensitive disassembly of microtubules are complicated and remain to be identified.

REFERENCES

1. Wood JM. *Science* 1974; **183**: 1049.
2. Braman RS and Foreback CC. *Science* 1973; **182**: 1247.
3. Buchet JP, Lauwery R and Roels H. *Int. Arch. Occup. Environ. Health* 1981; **48**: 71.
4. Crecelius EA. *Environ. Health Persp.* 1977; **19**: 147.
5. Lakso JU and Peaples SAJ. *J. Agric. Food. Chem.* 1975; **23**: 674.
6. Tam KH, Charbonneau SM, Bryce F and Lacroix G. *Anal. Biochem.* 1978; **86**: 505.
7. Vahter M. *Environ. Res.* 1981; **25**: 286.
8. Fischer AB, Buchet JP and Lauwery RR. *Arch. Toxicol.* 1985; **57**: 168.
9. Wagner SL and Weswig P. *Arch. Environ. Health* 1974; **28**: 77.
10. Ochi T, Kaise T and Oya-Ohta Y. *Experientia* 1994; **50**: 115.
11. Ochi T, Nakajima F, Sakurai T, Kaise T and Oya-Ohta Y. *Arch. Toxicol.* 1996; **70**: 615.
12. Yamanaka K, Hasegawa A, Sawamura R and Okada S. *Biochem. Biophys. Res. Commun.* 1989; **30**: 43.
13. Oya-Ohta Y, Kaise T and Ochi T. *Mutat. Res.* 1996; **357**: 123.
14. Endo G, Kuroda K, Okamoto A and Horiguchi S. *Bull. Environ. Contam. Toxicol.* 1992; **48**: 131.
15. Ochi T, Nakajima F and Fukumori N. *Arch. Toxicol.* 1998; **72**: 566.
16. Ochi T, Nakajima F, Shimizu A and Harada M. *Toxicol. In Vitro* 1999; **13**: 11.
17. Kellog DR, Moritz M and Alberts BM. *Annu. Rev. Biochem.* 1994; **63**: 639.
18. Ochi T, Nakajima F and Nasui M. *Toxicology* 1999; **136**: 79.
19. Ochi T. *Mutat. Res.* 2000; **454**: 21.
20. Kobayashi H, Sunaga R, Furihata K, Morisaki N and Iwasaki S. *J. Antibiot.* 1995; **48**: 42.
21. Kuriyama R and Sakai H. *J. Biochem.* 1974; **76**: 651.
22. Mellon M and Rebhun LI. Studies on the accessible sulfhydryls of polymerizable tubulin. In *Cell Motility*, Goldman R, Pollard T, Rosenbaum J (eds). Cold Spring Harbor Laboratory: New York, 1976; 1146–1164.
23. Delnomdedieu M, Basti MM, Otvos JD and Thomas DJ. *Chemico-Biol. Interact.* 1994; **90**: 139.
24. Stybro M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR and Thomas DJ. *Arch. Toxicol.* 2000; **74**: 289.
25. Yamamoto H, Fukunaga K, Goto S, Tanaka E and Miyamoto E. *J. Neurochem.* 1985; **44**: 759.
26. Ratan RR and Shelanski ML. *Trends Biochem. Sci.* 1986; **11**: 456.

