

Evaluation of the mutagenic potential of the active principle of the herbicide Ametrine in *in vivo* and *in vitro* systems

Wagner José Martins Paiva^{1,3} and Catarina Satie Takahashi^{1,2}

ABSTRACT

The active principle of the herbicide Ametrine was tested to determine its clastogenic potential in human lymphocyte cultures and in Wistar rat bone marrow cells. The drug was administered to the rats by gavage at doses of 10.94, 43.75, 87.50 and 175 mg/kg body weight and the animals were sacrificed 12 and 24 h after treatment. Lymphocytes were treated with doses of 0.00425, 0.0172 and 0.068 mg/ml culture medium. The results showed that Ametrine is not clastogenic, though it seems to be an inducer of SCEs. In the lymphocyte system, the mitotic and proliferation indices underwent a dose-dependent reduction. However, the reduction in mitotic index in rat bone marrow cells was not significant.

INTRODUCTION

The herbicide Ametrine is an s-triazine widely used in world agriculture. This agent is absorbed by plant leaves and/or roots, as is the case for all other s-triazines. These herbicides present acropetal translocation, accumulating in the chloroplasts of apical meristems and inhibiting the Hill reaction in photosynthesis, leading to plant death due to the lack of energy compounds (Jordan *et al.*, 1966; Moreland, 1980). s-Triazines may affect cell respiration by inhibiting ATP synthesis in mitochondria through a mechanism similar to that observed in chloroplasts (Thompson *et al.*, 1974). These herbicides also may affect

RNA synthesis as well as protein synthesis and kinetics (Moreland *et al.*, 1969; Scarponi and Perucci, 1986; Ochoa-Alejo and Crocomo, 1987).

Studies on the mutagenicity of s-triazine herbicides have been carried out by many investigators using different assays. Several authors have reported mutagenicity of different s-triazine herbicides in various test systems, and lack of mutagenicity of others (Lee *et al.*, 1983; Klopman *et al.*, 1985; Garrett *et al.*, 1986).

The objective of the present study was to determine the possible clastogenic effect of the active principle of the herbicide Ametrine on mammalian test systems *in vitro* and *in vivo*.

MATERIAL AND METHODS

Chemical agent

The experiments were carried out using the active principle of the herbicide Ametrine (2-

¹ Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, SP, Brasil.

² Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14049-901 Ribeirão Preto, SP, Brasil. Send correspondence to C.S.T.

³ Departamento de Biologia Geral, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86055-900 Londrina, PR, Brasil.

ethylamino-4-isopropylamino-6-methylthio-s-triazine), kindly provided by the manufacturer, Herbitécnica Defensivos Agrícolas Ltda., Londrina, PR.

***In vivo* system using Wistar rat bone marrow cells**

Wistar rats (*Rattus norvegicus*) from the Animal Rearing Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (FMRP-USP), were used in the *in vivo* experiments. The animals weighed 100 g. Bone marrow cells were obtained by the technique of Ford and Hamerton (1956), modified in the Departamento de Genética e Matemática Aplicada à Biologia (DGMAB), FMRP-USP. For metaphase analysis, the animals were treated with a 0.5 ml solution of 0.16% colchicine (Sigma) for one hour and 30 min before sacrifice.

The active principle of Ametrin was administered by gavage at doses of 175.00, 87.50, 43.75 and 10.94 mg emulsified in 2 ml corn oil per kg of body weight. Six rats (three males and three females) were used per treatment. A group treated with distilled water was used as a negative control; a group treated with "Mazola" corn oil was used as a solvent control, and a group treated by gavage with 30 mg cyclophosphamide/kg body weight was used as a positive control. The animals were treated for a period of 12 h (treatment 1) or 24 h (treatment 2) before being sacrificed by excess ether inhalation. One hundred metaphases per animal were analyzed and 5000 cells were counted to determine the mitotic index (MI).

***In vitro* system using human lymphocytes**

The human peripheral blood lymphocytes used in the *in vivo* experiments were collected from five healthy non-smokers (two men and three women) aged 20 to 30 years. Selection criteria were absence of clinically diagnosed diseases, no use of medications or drugs and no exposure to radiation or to mutagenic and/or carcinogenic toxic products during the preceding six months.

The cytological preparations for the analysis of metaphase cells from human peripheral blood lymphocyte cultures were obtained by the technique of Moorhead *et al.* (1960), modified by the DGMAB of FMRP-USP. For lymphocyte culture, approximately 3 ml plasma was added to a flask containing 60 ml complete culture medium (80% RPMI 1640 medium (GIBCO) + 20% normal human serum + 0.01 mg/ml streptomycin (CEME) + 0.005 mg/ml penicillin (Fontoura Wyeth S/A) + 4% phytohemagglutinin

(PHA) prepared by the DGMAB, FMRP-USP). After homogenization, the culture medium was divided into six 10.5-ml aliquots and the flasks were covered for protection from light; 100 μ l 5-bromo-2'-deoxyuridine (BrdU) was then added (0.1 mg/ml, Sigma) and the flasks were incubated at 37°C for six hours. The drug treatments were carried out after this period. For metaphase preparation, 0.25 ml of a colchicine solution (16 μ g/ml, Sigma) was added to each culture two hours before harvesting. The active principle of Ametrin used in the experiments was dissolved in 50 μ l acetone (Merck) at concentrations of 0.068 mg (a mean dose similar to that to which farm workers are exposed), 0.017 mg and 0.00425 mg/ml culture medium per treatment. Three additional control cultures were prepared: a solvent control treated with 50 μ l acetone only, a positive control treated with 1- β -D-arabinofuranosylcytosine (Ara-C) at a final concentration of 5×10^{-7} M, and a negative control with no other substance added. The material was stained by the fluorescence plus Giemsa method (FPG) of Perry and Wolf (1974) in combination with the method of Korenberg and Freedlender (1974), modified by the DGMAB of FMRP-USP.

One hundred first-cycle metaphases were analyzed per treatment/individual to determine the clastogenic effect of the drugs and 50 second-cycle metaphases were analyzed for sister chromatid exchange (SCE) frequency. The MI was determined by counting 2000 cells per treatment/individual. The proliferation index (PI) was calculated on the basis of metaphases that were stimulated with PHA, according to Preston *et al.* (1987). To rule out the possibility of an alteration of the chromosome aberration data caused by BrdU, a test with 72-h cultures treated with 0.068 mg Ametrine and containing BrdU was performed and compared to cultures incubated for 48 h with the same dose of Ametrine but in the absence of BrdU.

Statistical analysis

The rat data were analyzed statistically by the nonparametric Kruskal-Wallis test, to analyze the equality of averages, providing a unique variable by ranks for these data. The human lymphocyte data were analyzed using Friedman's test to analyze the equality of averages, getting to two variables by ranks for these data. The data were also analyzed by a conditional statistical test for the detection of rare events, using Fisher's test for the clastogenicity data and the χ^2 test for MI, total SCEs and PI, according to Pereira (1991) to compare the proportional data of each group with the

control. The level of significance was set at $P < 0.05$ for all tests.

RESULTS

In vivo Wistar rat bone marrow cell system

Chromosome analysis and analysis of the total number of cells with aberrations demonstrated the absence of clastogenicity for both treatment periods (Tables I and II). The variations in the frequency of chromosome aberrations were small and random for all treatments. Small differences in MI were detected, but were significant (χ^2) only for some concentrations. The MI data for all treatments were significantly different from water. When the effects of the different concentrations were compared with that of diluent only the 87.50 mg and 175.00 mg concentrations were not significantly different. Comparisons between the two periods of treatment for the same drug concentrations showed significant differences for the concentrations 10.95 mg and 87.50 mg.

In vitro human lymphocyte culture system

A comparison of chromosome aberrations in cells in the first division in cultures containing BrdU (MI) did not show differences between the negative control and the control treated with the solvent (Table III). Analysis of cultures containing no BrdU demonstrated that only the negative control presented a chromatid-type break in chromosome 2. These data

rule out the possibility of an effect of BrdU on the induction of chromosome aberrations. Thus, the procedure was used in the present study.

An increase in SCEs and a decrease in PI occurred with increasing Ametrin concentrations (concentrations higher than those utilized here were too toxic for the cultures) (Table IV). Analysis of the clastogenic effects and the total number of cells with aberrations (TCA) showed that these effects were not significant (Table III). The MI (Table III) for the drug at all concentrations were significant when compared with the solvent (χ^2). When the drug plus the solvent was compared to the control, the concentrations of 0.0017 and 0.068 mg/ml were significantly different as was the control vs. solvent (χ^2). When the PI data were compared, only the 0.068 mg/ml concentration was significantly different from the solvent control (χ^2).

DISCUSSION AND CONCLUSIONS

Ametrine is a herbicide belonging to the s-triazine group, which is extensively used in world agriculture. Studies on the mutagenic potential of these herbicides, and of Ametrine in particular, are only incipient. In the analysis of human lymphocyte cultures, we did not test the active principle of Ametrine in the presence of the S-9 microsomal fraction because our objective was to evaluate the direct effect of the drug on the chromosomes, to determine its ability to induce clastogenicity and SCEs and its effect on the cell cycle since, according to Perry (1980), these assays permit the

Table I - Chromosome aberrations, total number of cells with aberrations (100 cells/animal) and mitotic index (5000 cells/animal) in the bone marrow of Wistar rats (three males and three females per treatment) treated by gavage with the active principle of Ametrine emulsified in 2 ml corn oil (diluent) and sacrificed 12 h after treatment.

Treatment (mg/kg body weight)	Aberrations				TCA		MI%
	Gaps		Breaks		With gaps	Without gaps	
	C	IC	C	IC			
Oil	2	0	4	0	6	4	1.66 ^c
10.95	8	0	8	3	19	11	1.44 ^{a,b,c}
43.75	7	0	4	0	11	4	1.08 ^{a,b}
87.50	9	0	6	4	19	10	0.86 ^{a,b,c}
175.00	10	0	8	0	16	8	1.26 ^{a,b}

C = Chromatid; IC = isochromatid; TCA = total number of cells with aberrations; MI = mitotic index.

^aSignificantly lower than the control (water for 24 h) (Table II, χ^2 test).

^bSignificantly lower than oil (diluent) (χ^2 test).

^cSignificantly different from the 24-h treatment (Table II, χ^2 test).

Table II - Chromosome aberrations, total number of cells with aberrations (100 cells/animal) and mitotic index (5000 cells/animal) in the bone marrow of Wistar rats (three males and three females per treatment) treated by gavage with the active principle of Ametrine emulsified in 2 ml corn oil (diluent) and sacrificed 24 h after treatment.

Treatment (mg/kg body weight)	Aberrations				TCA		MI%
	Gaps		Breaks		With gaps	Without gaps	
	C	IC	C	IC			
Water	5	0	6	3	14	9	2.41 ^c
Oil	2	0	4	0	6	6	1.36 ^a
10.94	8	0	3	0	10	3	1.12 ^{a,b,c}
43.75	5	0	5	0	10	5	1.02 ^{a,b}
87.50	5	0	14	1	20	15	1.33 ^c
175.00	3	0	5	4	11	9	1.38
30.00 CP	20	3	121	48	109	97	0.80

C = Chromatid; IC = isochromatid; TCA = total number of cells with aberrations; MI = mitotic index; CP = cyclophosphamide.

^aSignificantly lower than the control (water, χ^2 test).

^bSignificantly lower than oil (diluent) (χ^2 test).

^cSignificantly different from the 12-h treatment (Table I, χ^2 test).

Note: the data obtained with CP were used as a positive control to determine the sensitivity of the test and therefore were not included in the statistical analysis.

Table III - Chromosome aberrations, total number of cells with aberrations (100 cells/individual) and mitotic index (2000 cells/individual) in 72-h human lymphocyte cultures (five individuals) treated with the active principle of the herbicide Ametrine and in control cultures.

Treatment (mg/kg body weight)	Aberrations				TCA		MI%
	Gaps		Breaks		With gaps	Without gaps	
	C	IC	C	IC			
Control	1	0	1	0	2	1	3.54 ^b
Solvent	0	0	1	0	1	1	4.13 ^a
0.00425 mg	0	0	1	0	1	1	3.24 ^b
0.017 mg	0	0	0	2	2	2	2.59 ^{a,b}
0.068 mg	0	0	3	0	3	3	2.18 ^{a,b}
Ara-C 5.10 ⁻⁷ M	6	0	35	12	21	20	3.12

C = Chromatid; IC = isochromatid; TCA = total number of cells with aberrations; MI = mitotic index; Ara-C = 1- β -D-arabinofuranosyl-cytosine.

^aSignificantly different from the control (water, χ^2 test).

^bSignificantly different from the solvent (χ^2 test).

Note: the data obtained with Ara-C were used as a positive control to determine the sensitivity of the test and therefore were not included in the statistical analysis.

determination of the action of the active principle of Ametrine on human chromosomes even if intracellular metabolism occurs in lymphocytes *in vitro*. The bone marrow system was used in order to investigate the mutagenic effect of the active principle of Ametrine *in vivo* and thus determine its interaction with the animal's

organism as a whole. In general, this assay permits the drugs to undergo hepatic metabolic activation in addition to gastric metabolism, as is the case for cyclophosphamide (positive control), a drug that needs to be metabolized by the hepatic route in order to cause chromosome damage.

Table IV - Total number of SCEs and mean SCE number per cell (50 metaphases per individual/treatment) and proliferation index (100 cells) in 72-h human lymphocyte cultures (five individuals) treated with different doses of the active principle of Ametrine and controls.

Treatment mg/ml	Total SCE	SCE \pm SEM per cell	PI
Control	1884	7.54 \pm 1.99	2.06
Solvent	1921	7.68 \pm 2.28	2.10
0.00425 mg	2033	8.13 \pm 1.31	1.85
0.0172 mg	2080	8.32 \pm 1.53	1.78
0.068 mg	2211	8.84 \pm 2.15	1.68 ^{a,b}
Ara-C 5.10 ⁻⁷ M	2516	10.06 \pm 2.13	1.65

PI = Proliferation index; Ara-C = 1- β -D-arabinofuranosyl-cytosine.

^aSignificantly different from the control (χ^2 test).

^bSignificantly different from the solvent (χ^2 test).

Note: the data obtained with Ara-C were used as a positive control to determine the sensitivity of the test and therefore were not included in the statistical analysis.

The results obtained in the present study demonstrate that there was no clastogenic effect either *in vivo* or *in vitro* (Tables I, II and III). Other s-triazines such as Atrazine and Simazine also induced no significant chromosome aberrations when tested in CHO and V-79 cells in the presence or absence of the S-9 microsome fraction and lymphocyte assays (Adler, 1980; Loprieno and Adler, 1980; Donna *et al.*, 1981). These results indicate that s-triazine herbicides have no clastogenicity activity on *in vitro* systems, with the exception of the studies of Meisner *et al.* (1992) who found a higher frequency of chromosome aberrations when they applied Atrazine to human lymphocytes. Although these results differ from those obtained by us with Ametrine, they are not conclusive for s-triazine herbicides in general due to the small number of tests carried out with these herbicides.

Studies of the clastogenicity of other s-triazine herbicides on plants indicate that these compounds induce mitotic and meiotic alterations in these systems (Stroev, 1970; Liang and Liang, 1972; Grant, 1972; Lee *et al.*, 1974; Badr, 1986). Only Sawamura (1965) and Müller *et al.* (1972) obtained contrasting results indicating no clastogenicity of these herbicides.

The *in vivo* MI (Table I and II) was reduced by the treatments, except for the 10.94 mg and 43.75 mg concentrations of ametrine in the 24-h treatments. Meisner *et al.* (1992) found elevated MI in all mice treated with 20 ppm of Atrazine, after 90 days of treatment. These results showed probable action by s-triazines on MI.

Cultures with BrdU permitted the determination of PI, a parameter that, according to Preston *et al.* (1987), permits the determination of the cytotoxic potential of certain chemical agents. Only the 0.068 mg/ml concentration showed a decrease in PI when compared to the solvent and to the control. These results demonstrate a toxic effect of the active principle of Ametrine, in agreement with data reported by Mallison and Cannon (1984) who studied the effect of 16 pesticides on the cyanobacterium *Plectonema boryanum* and the cyanophage LPP-1 and concluded that the herbicides Ametrine and Atrazine are toxic for these microorganisms.

Other triazines such as Atrazine and Simazine did not induce SCEs in human lymphocytes in culture (Donna *et al.*, 1981). In contrast, Ghiazza *et al.* (1984) reported that Simazine induced SCEs in human lymphocytes, a fact not observed with Atrazine. Atrazine also induced no increase in SCE frequency in CHO and V-79 cells (Loprieno and Adler, 1980). Chou and Weber (1981) detected an increased number of SCEs induced by Atrazine in *Zea mays* roots, a fact that led them to suggest an effect at the gene recombination level on somatic plant cells. These results demonstrate that Ametrine induces a dose-dependent increase in SCEs in human lymphocytes, whereas other triazines present contradictory results. More complete studies using this type of assay are needed to determine the capacity for SCE induction of these drugs.

S-triazine herbicides have proved to induce point mutations in several plants (Plewa and Wagner, 1981; Radke and Grau, 1986). Also the microbiological assays have clearly demonstrated that s-triazines and their metabolites are not mutagenic or promutagenic when activated by mammalian microsome suspensions (Bartsch *et al.*, 1980; Loprieno and Adler, 1980; Moriya *et al.*, 1983). However, the possibility of the s-triazine herbicides inducing point mutations is greater in the presence of metabolic activation by plants as demonstrated by several investigators who observed the mutagenicity of these herbicides through these microbiological assays (Plewa and Gentile, 1982; Gentile *et al.*, 1982; Plewa *et al.*, 1984; Veleminsky and Guichner, 1988).

Other *in vitro* systems, with different parameters from those utilized in this research, showed negative and positive results for s-triazines herbicides, depending on the type of assay used (Loprieno and Adler, 1980; Walters *et al.*, 1982). On the basis of the present results, we suggest that the active principle of the herbicide Ametrine may affect the cell cycle and be toxic to the cells of the systems tested, by being a mild inducer of SCEs. However, Ametrine

had no clastogenic effect on the two test systems utilized.

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RESUMO

O princípio ativo do herbicida Ametrina foi testado para a verificação do seu potencial clastogênico em culturas de linfócitos humanos e células de medula óssea em ratos Wistar. Em ratos, a droga foi administrada por gavagem nas doses de 10,94, 43,75, 87,50 e 175 mg/kg de peso corporal, sendo estes sacrificados 12 e 24 h após o tratamento. Os linfócitos foram tratados com as doses de 0,00425, 0,0172 e 0,068 mg/ml de meio de cultura. Os resultados demonstraram que a Ametrina não apresenta potencial clastogênico embora pareça ser um indutor de SCEs. No sistema de linfócitos os índices mitótico e de proliferação sofreram uma redução que foi dose dependente. Já a redução no índice mitótico nas células da medula óssea de ratos não foi significante.

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