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Genotoxicity assessment of new synthesized acridine derivative — 3,6-diamino-10-methyl-9,10-dihydroacridine

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Abstract

A new synthesized acridine derivative, 3,6-diamino-10-methyl-9,10-dihydroacridine (AcrH), was tested for in vitro reverse mutations with *Salmonella* TA strains, chromosome aberrations and sister chromatid exchanges (SCE) in human lymphocytes, and for in vivo chromosome aberrations in bone marrow of mice. Using the classic plate incorporation method, mutagenicity of AcrH in bacterial cells (TA97a, TA98, TA100 and TA102) was observed in the experiments performed with, and without, rat liver S9 metabolic activation. The reverse mutation assay showed no difference in mutagenic activity between AcrH and acriflavine (Acr^+) in the test with TA97. The results of in vitro chromosome aberrations assay revealed potential clastogenicity. The test using macroculture of human lymphocytes induced mainly chromatid gaps. The experiments with human lymphocytes revealed SCE-inducing effect of AcrH and Acr^+. In an in vivo study, AcrH given intraperitoneally to *Balb/c* mice did not cause any significant increase in the percentage of cells with aberrations compared to the negative control.

Keywords: 3,6-Diamino-10-methyl-9,10-dihydroacridine; *Salmonella typhimurium*; Chromosome aberrations; Sister chromatid exchange; Human lymphocytes; Mouse bone marrow

1. Introduction

Commercial mixture of proflavine (3,6-diaminoacridine) and its *N*-methyl quaternary salt, euflavine, is known as acriflavine (Acr^+) (Ferguson and Denny [1]). Acr^+ representing a group of cationic DNA intercalators plays an important role due to its outstanding antibacterial properties. It is assumed that Acr^+ binds rapidly to saturate the surface of the membrane cell, which is negatively charged due to presence of phosphate anions in the outer leaf. Therefore, only very small fractions of Acr^+ molecules can penetrate into the bacterial cells to interact with the genetic material. To overcome transportation barriers through the plasma mem-

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branes, a strategy of 'Trojan horse' can be envisaged. This strategy may involve application of an uncharged, hydrophobic Acr^+ precursor, which can release Acr^+ inside bacterial cells. The preparation of a stable Acr^+ precursor of unique properties, namely 3,6-diamino-10-methyl-9,10-dihydroacridine (AcrH) and its antibacterial, antiviral and antifungal activity was described earlier by Adamus et al. [2]. It was shown that, upon dissolving AcrH in aerated water or other polar solvent, spontaneous reoxidation takes place and Acr^+ is reformed quantitatively. Reoxidation takes place during several hours in H_2O, and it can be much longer in less polar solvents or in solvents characterized by poor oxygen solubility. The time-dependent experiments (0.5–24 h) showed that Acr^+ and $O_2^{\cdot-}$ generated from AcrH inactivated bacteria of *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* about five times faster and *Staphylococcus aureus* over 15 times faster than Acr^+ in reference solution of similar concentration. The mechanism of AcrH reoxidation to Acr^+ involves a sequential electron–proton–electron transfer after Marcinek et al. [3], and Gębicki et al. [4] (see Fig. 1).

The aim of this study was to evaluate the genotoxic activity of AcrH using in vitro and in vivo screening tests and additionally, to compare the genotoxic activity of AcrH and Acr^+ under the experimental conditions.

2. Materials and methods

2.1. Chemicals

Acr^+ neutral (CAS No. 8048-52-0, product of Aldrich Chemical) was used to synthesize AcrH by $NaBH_4$ reduction of 3,6-diamino-10-methyl-acridinium chloride. The synthesis was performed according to the method described by Gilliot [5]; Adamus et al. [2] describe the characteristics and properties of AcrH. The chemical structure is shown in Fig. 1. In all tests, AcrH was assayed after it had been solved in 50% ethanol or 1,2-propanediol (propylene glycol; POCH, Gliwice) on the day of the assay.

2.2. Reverse mutations assay with Salmonella TA strains

The Salmonella/microsome plate-incorporation assay was employed according to Ames et al. [6] as revised by Maron and Ames [7]. The *S. typhimurium* tester strains TA97a, TA98, TA100 and TA102 were supplied by B.N. Ames, University of California, Berkeley, CA. The cultures were grown in 'Oxoid' nutrient broth No. 2 for 10 h at 37°C with shaking. The test was performed both with, and without, S9 fraction (50 μl/plate) prepared from liver of male outbred Imp:WIST rats. The inducing agent for S9

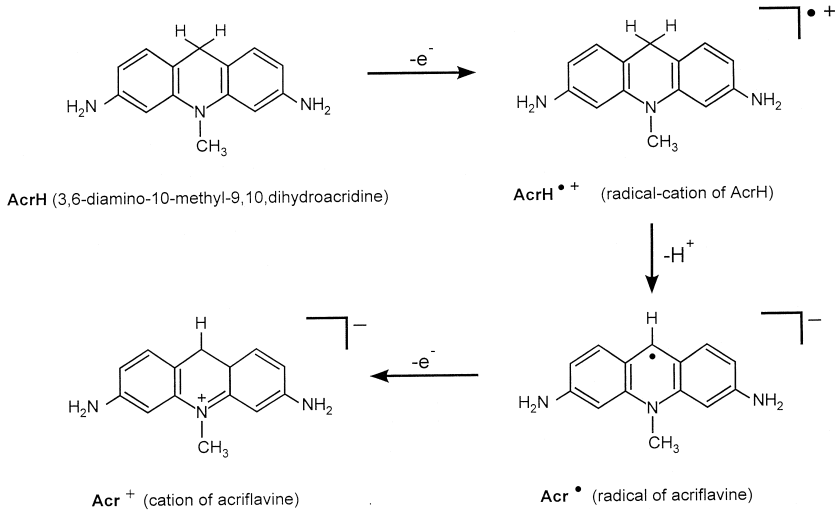

Fig. 1. The mechanism of AcrH reoxidation to Acr^+ involves a sequential electron–proton–electron transfer.

preparation was Aroclor 1254. The protein concentration of S9 fraction, determined by the Lowry method [8], was 38.3 mg/ml. The test was conducted up to the soluble concentration limit of AcrH (100 μg/100 μl, ca. 50% ethanol). Each dose level was tested in duplicate on three separate experiments. Revertant colonies were counted 48 h after incubation with automated bacterial colony counter Biotran II from New Brunswick Scientific, NJ. A mutagenic test was positive if the compound induced a concentration-dependent increase in the number of revertants per plate as compared to the number of revertants per control plates, and the increase had to be at least twice the controls rate.

2.3. Chromosome aberrations assay without S9-mix in human lymphocytes

Venous blood from 10 healthy adult male non-smoker donors without a known drug or medical history was used in the experiment. The necessary metaphases were obtained in the conventional manner from the series of 48 h macroculture of lymphocytes. The culture medium was Eagle's fluid 1959 (MEM). Colcemid (Serva, 0.15 μg/ml) was added 2 h before termination to arrest cells in the metaphase. Chlormethine hydrochloride (Nitrogranulogen, Polfa; 0.1 μg/ml) was used as the positive control. Two doses (0.1 and 0.5 μg/ml) of AcrH and Acr^+ prepared and diluted in MEM (negative control) were added to the culture of lymphocytes (protected from exposure to visible light). The basic experiment in this study was when a compound was added to 24 h culture of lymphocytes (S phase). Experiment when a compound was added at the beginning of incubation (stage G_0 of the cell cycle) was used for cytogenetic testing of S-independent compounds which can produce aberrations at all stages of the cell cycle. In a pilot experiment, 0.5 μg/ml was the highest dose which made it possible to obtain metaphases for analysis. Preparations were made by the air-drying method and were stained by Giemsa. Slides were coded for data entry and observed according to the standard procedure after Preston et al. [9]. Analysis for chromatid and chromosome type aberrations was performed in all satisfactorily spreading and stained metaphases. The classification of gaps and breaks for non-banded preparations was in accordance with the ISCN [10].

2.4. Sister chromatid exchanges (SCE) assay without S9-mix in human lymphocytes

Peripheral blood was obtained from five male donors. The lymphocyte cultures were incubated for 72 h in the presence of bromodeoxyuridine (BrdU, Sigma, 10 μg/ml). AcrH and Acr^+ (0.1 and 0.5 μg/ml) were added to the cultures at the beginning of the incubation or 24 h afterwards. Colcemid was added to the culture 2 h prior to harvesting. The slides were coded and stained for sister chromatid differentiation according to Perry and Wolff [11]. Analysis of SCE was performed in M2 metaphases with a minimum of 25 mitoses per point.

2.5. In vivo chromosome aberrations assay in bone marrow of mice

The assays were performed on bone marrow cells from male *Balb/c* mice (weighing about 25 g, 8 weeks old) according to the standard procedures as described by Brusick [12]. Based on preliminary experiments, AcrH was given by intraperitoneal injection (i.p.) at 28 mg/kg (50% of LD_{50}). The genotoxicity of Acr^+ in this test was not examined. Colchicine (Sigma, 3.3 mg/kg i.p.) was given 2 h before the mice were sacrificed to inhibit cell mitosis. 1,2-Propanediol and cyclophosphamide (Endoxan, Asta Medica, the negative and positive control, respectively) were injected i.p. to four mice at 48 h before the end of the experiment. Bone marrow samples were collected in the 30th, 48th and 72nd h after the injection of AcrH. The chromosome preparations from hipotonized and fixed bone marrow cells (both femurs of each mouse) were air-dried and stained by Giemsa. The analysis of chromosome aberrations was performed in average of 50 metaphases per mouse.

3. Statistical analysis

The recorded results were statistically analyzed with the help of: Fisher's exact test (chromosome aberrations in vitro and in vivo) and Mann–Whitney's test (SCE), with the level of significance set at $p \leq 0.05$ or higher. The numerical data in Table 2 compared with the historical control were analyzed by Chi-square test with Yates' correction.

4. Results

4.1. Induction of reverse mutation in bacterial cells

The results of mutation tests, without and with the supplementation of S9 rat liver supernatant are summarized in Table 1. AcrH was detectably mutagenic at 50 μg/plate in the absence of S9 activation (TA97a, TA98 and TA102). At this dose applied to the plates, AcrH induced an approx. 2.7-, 4.0- and 2.3-fold increase in the number of revertants. With S9 activation, the lowest doses at which the plate counts exceeded two times the control revertant yield were: 1 μg/plate (TA98 3.4-fold), and 10 μg/plate (TA97a 2.2-fold, TA100 2.1-fold). The presence of S9 metabolic activation resulted also in a reduction of toxic effect of AcrH on tester cells. The effect of AcrH and Acr^+ in TA97a, where the yields of revertants were higher than the control number, was similar (about 2.7- and 2.3-fold, at 50 μg/plate, −S9; and about 2.2- and 2.3-fold at 10 μg/plate, +S9, respectively). In the absence of S9 fraction, the induced revertant numbers decreased due to the toxic effect of AcrH as evidenced by reduced growth of the his^- background lawn.

4.2. Analysis of chromosomal aberrations: in vitro tests

The frequency of aberrations for the tested compounds are presented in Table 2. No aberrations were found in the cells analyzed in the control cultures. However, for both compounds at 0.1 μg/ml, the percentages of cells with aberrations were similar to the control frequency of aberrations found in other reports (about 2.0%). For AcrH at 0.5 μg/ml and Acr^+ at 0.1 and 0.5 μg/ml, a significant increase of cells with aberrations as compared to the negative control was observed for both lymphocyte treatment times. In another experiment, the mean number of chromosome aberrations per cell (including gaps) of lymphocyte culture containing only culture fluid was 3.0%. When the percentages of the aberrated cells were compared for both chemicals given at two time intervals vs. the historical control, a significant increase in the percentage of the aberrated cells was

Table 1
Induction of reverse his^- mutation in *S. typhimurium* strains

Compound/dose per plate, μg or μl		Mean number of revertants per plate and standard deviation							
		TA97a		TA98		TA100		TA102	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Spontaneous reversion		114 ± 8	124 ± 7	24 ± 2	29 ± 3	116 ± 7	104 ± 9	244 ± 14	344 ± 23
Negative control (50% ethanol)		119 ± 8	130 ± 7	24 ± 3	32 ± 5	119 ± 7	114 ± 10	249 ± 19	345 ± 28
	1	123 ± 13 (120 ± 7)[a]	159 ± 21 (180 ± 23)	30 ± 5	109 ± 17	127 ± 14	130 ± 14	279 ± 32	382 ± 32
	10	214 ± 22 (161 ± 22)	289 ± 25 (310 ± 27)	44 ± 7	425 ± 48	157 ± 21	242 ± 15	442 ± 58	522 ± 67
	50	325 ± 24 (279 ± 28)	427 ± 47 (454 ± 40)	98 ± 9	643 ± 48	189 ± 19	361 ± 18	576 ± 80	700 ± 58
	100	187 ± 31 (383 ± 19)	599 ± 51 (638 ± 60)	35 ± 7	618 ± 79	126 ± 22	254 ± 29	536 ± 63	686 ± 73
Positive controls: 4-nitroquinoline-*N*-oxide	0.5							524 ± 32	
4-nitro-*o*-phenylenediamine	3.0	271 ± 15		357 ± 26					
Sodium azide	1.5					787 ± 53			
2-aminofluorene	5.0	123 ± 10	575 ± 30			116 ± 11	663 ± 35	235 ± 32	516 ± 40
Benzo(*a*)pyrene	5.0			27 ± 2	328 ± 27				

[a] The values in parentheses represent the number of revertants in terms of mean ± S.D. for Acr^+.

Table 2
Chromosome aberrations frequency — in vitro tests in culture of human lymphocytes

Compounds/dose (μg/ml culture)	Number of cells scored	Number of aberrations							Percent of cells with aberrations
		G′	G″	B′	B″	DIC	EF	AF	
Negative control	450 (240)[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.00 (0.00)
3,6-diamino-10-methyl-9,10-dihydroacridine									
0.1	349 (140)	0 (2)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.29[f] (1.43)
0.5	230 (70)	5 (2)	0 (0)	1 (0)	1 (0)	0 (0)	0 (0)	0 (0)	3.04[d] (2.86)[b]
Acriflavine									
0.1	300 (140)	5 (3)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	2.33[c] (2.86)[b]
0.5	160 (80)	6 (3)	0 (0)	1 (5)	1 (1)	0 (0)	0 (0)	1 (0)	5.63[d] (11.25)[d,f]
Positive control (chloromethine hydrochloride)									
0.1	110 (140)	9 (3)	5 (0)	9 (1)	4 (0)	0 (2)	2 (0)	5 (0)	30.91[d,f] (4.29)[c]
Historical control[e]	1900	55	1	1	0	0	0	0	3.00

[a] The table gives the results of the effects observed for the test compounds, when added at the beginning of incubation (data in parentheses) and 24 h before harvesting of cells (data without parentheses); G′ — chromatid gap, G″ — chromosome gap, B′ — chromatid break, B″ — chromosome break, DIC — dicentric, EF — exchange figures, AF — acentric fragment.
[b] Significant differences vs. negative control at ($p \leq 0.05$).
[c] Significant differences vs. negative control at ($p \leq 0.01$).
[d] Significant differences vs. negative control at ($p \leq 0.001$).
[e] Relevant literature: Ferenc et al. [18].
[f] Significant differences vs. historical control.

noted only for Acr^+ added at 0.5 μg/ml at the beginning of the incubation period. However, the percentage of the aberrated cells for AcrH at 0.1 μg/ml was significantly lower compared to the historical control. The recorded percentages of cells with aberrations were compared between Acr^+ and AcrH for the doses 0.1 μg/ml and 0.5 μg/ml. In experiments when the compounds were added 24 h before harvesting, the percentage increase of cells with aberrations was observed, but the difference between the compared numerical values was statistically insignificant. In experiments when the compounds were added at the beginning of incubation, a significant percentage increase of cells with aberra-

Table 3
SCE frequency — in vitro tests in culture of human lymphocytes

Compounds	Dose (μg/ml)	Number of cells scored	SCE/cell (mean ± S.D.)
Negative control	0.0	162 (138)	8.56 ± 2.71 (7.93 ± 2.60)
3,6-diamino-10-methyl-9,10-dihydroacridine	0.1	126 (118)[a]	10.77 ± 3.85[b] (10.98 ± 4.46)[c]
	0.5	–[d] (–)[d]	
Acriflavine	0.1	149 (126)	15.56 ± 4.89[c] (12.63 ± 4.74)[c]
	0.5	–[d] (–)[d]	

[a] The table gives the results of the effects observed for the test compounds added to culture of lymphocytes 48 h (data without parentheses) and 72 h (data in parentheses) before harvesting.
[b] Significant at $p \leq 0.05$ (vs. negative control).
[c] Significant at $p \leq 0.001$ (vs. negative control).
[d] No M2 cells or a few mitoses.

Table 4
Chromosome aberrations frequency — in vivo tests in bone marrow of mice

Compounds/dose (mg/kg body weight)	Duration of action (h)	Number of cells scored	Number of aberrations							Percent of of cells with aberrations
			G′	G″	B′	B″	DIC	AF	POL	
Negative control (1,2-propanediol) v/v 0.1 ml	72	200	1	0	0	0	0	0	0	0.5
3,6-diamino-10-methyl-	30	200	1	0	0	0	0	0	0	0.5
9,10-dihydroacridine	48	100	0	0	0	0	0	0	1	1.0
28.0	72	140	0	0	0	0	0	0	0	0.0
Positive control (cyclophosphamide) 40.0	48	80	0	0	0	0	0	12	1	16.2[a]

G′ — chromatid gap, G″ — chromosome gap, B′ — chromatid break, B″ — chromosome break, DIC — dicentric, AF — acentric fragment, POL — polyploid.
[a] Significant at $p \leq 0.001$ (as compared to the negative control).

tions was observed for Acr^+ at 0.5 μg/ml ($p \leq 0.05$).

4.3. Analysis of SCE: in vitro tests

Mean SCE frequency per cell for the tested compounds are presented in Table 3. For both compounds at 0.5 μg/ml, insufficient numbers of metaphases with differentially stained chromatids were observed. Therefore, the SCE values presented above were recorded for one dose only of the compounds in the two time intervals. For AcrH and Acr^+ at 0.1 μg/ml, in both intervals, a significant increase of number SCE/cell as compared to the negative control was observed. The comparison of mean SCE/cell count between Acr^+ and AcrH for the dose of 0.1 μg/ml showed a significant increase in SCE frequencies for Acr^+ added to culture at 72 h before the termination of the experiment ($p \leq 0.05$) and a higher effect for Acr^+ added at 48 h before the termination ($p \leq 0.001$).

4.4. Analysis of chromosome aberrations: in vivo tests

In the cells from bone marrow of mice exposed to AcrH, only two cells with chromosome aberrations were observed (Table 4). The recorded percentage of cells with the aberrations did not differ significantly as compared to the negative control. The observed percentage of cyclophosphamide-exposed cells with aberrations was significantly higher as compared to AcrH and negative control ($p \leq 0.001$) and displayed clastogenicity.

5. Discussion

The genetic toxicity of proflavine and related compounds was studied by many authors (Ferguson and Denny [1]). In a bacterial model with *S. typhimurium* TA series, mutagenic activity of proflavine was observed in the presence and absence of metabolic activation mainly in the strains which detect frameshift mutagens, but also in basepair substitution mutagens (Speck and Rosenkranz [13]). In our studies, indication that AcrH is a mutagenic compound was observed in both series of experiments (with and without the metabolic system). The mutagenic effect was found in all four tester strains. However, AcrH was somewhat more active in inducing frameshift mutations, especially in TA98. An increase of the mutagenic activity was observed in the presence of S9-mix. The mutagenic potential of AcrH was the same as that of Acr^+ in TA97a at comparable doses (50 μg/plate, without S9 and 10 μg/plate, with S9).

DeMarini et al. [14] showed that proflavine had strong clastogenic activity in mouse lymphoma cells. Nishi et al. [15] observed that proflavine induced SCE in Chinese hamster V79 cells. Speit and Vogel [16] tested intercalating dyes (acridine orange, proflavine and methylene blue) and drugs (chlorpromazine, promazine and chlorprothixene) for their ability to induce SCEs with and without photoactiva-

tion by visible light. They found that while in the dark, all these substances increased the frequency of SCEs; they also observed a superimposed effect of visible light on SCE formation for the proflavine and acridine orange, but not for the phenothiazine derivatives, methylene blue and chlorpromazine. In our study, the chromatid type aberrations were mainly observed in the cells exposed in vitro to AcrH and Acr^+. However, a very low number of mitoses with chromosome gaps, chromosome breaks and acentric fragments was observed. A significant increase in the aberration frequencies vs. the negative control was observed at 0.5 μg/ml of AcrH and at both doses (0.1 and 0.5 μg/ml) of Acr^+ for both lymphocyte exposure times. The frequency of aberrations was higher with Acr^+. When using the Preston et al. [9] criteria, the results point to the clastogenic activity of both chemicals. However, clastogenic activity was not observed when the aberration frequency was compared vs. the historical control. A significant increase in the aberration frequency as compared to the historical control was observed at 0.5 μg/ml of Acr^+ when added at the beginning of the incubation. In our study, the mean SCE frequency per cell for AcrH and Acr^+ was significantly higher as compared to the negative control at 0.1 μg/ml for 48- and 72-h exposures. However, it is not clear why Acr^+ induced a higher number of SCEs than AcrH.

Data from in vivo experiments for proflavine and related compounds in mammalian cells are rare. Nasim and Brychcy [17] tested the ability of euflavine to induce dominant lethal mutations in mice, with positive results in males and negative in females. Euflavine also increased the frequency of univalents found in metaphase 1 of male mice. In our study, chromosome aberrations in bone marrow cells of mice exposed to AcrH, analyzed after 30, 48 and 72 h from the moment of the exposure, were not observed.

6. Conclusions

The results of this study show that:

-AcrH is a mutagen which induced reversion of mutations in *Salmonella* TA strains (TA97a, TA98, TA100 and TA102); in TA97a, the mutagenic effect of AcrH is quantitatively similar to that of Acr^+;

-the results of in vitro chromosome aberrations assay compared vs. the negative control point to a potential clastogenicity of AcrH.

-AcrH induced a significantly higher number of SCEs in human lymphocytes, which indicates that AcrH is genotoxic.

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