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GENOTOXICITY

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Genotoxicity study of a new tetraalkylammonium derivative of 6-methyluracil (agent No. 547)

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Abstract Agent No. 547 (1,3-bis[ω-(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide), a newly synthesized inhibitor of mammalian-specific acetyltcholinesterase (EC 3.1.1.7) was investigated for genotoxicity using the DNA-repair test, Ames test and in vivo micronucleus test with mouse peripheral blood erythrocytes. Agent No. 547 did not cause significant changes in growth of repair-deficient *Escherichia coli* tester strains. The compound was non-mutagenic in *Salmonella typhimurium* strains TA98 and TA100 with and without rat microsomal activation mixture. However, we observed a marked increase in number of His^+ revertants for both tester strains in preincubation assays. The results obtained in the micronucleus test indicate that agent No. 547 possesses significant clastogenic activity. At the high dose tested (0.5 mg/kg), the compound induced a seven-fold increase in the number of micronuclei over the spontaneous background 48 h after treatment. The results suggest that further work should be promoted to identify the metabolic pathways involved in genotoxicity of agent No. 547 in mammalian cells and to evaluate the real risk of its exposure.

Keywords 1,3-Bis[ω-(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide · Acetyltcholinesterase inhibitor · Genotoxicity

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Introduction

Synthetic inhibitors of cholinesterases are subjected to a considerable number of studies focused on the production of new drugs and pesticides. Recently a new class of highly effective and irreversible type inhibitors of mammalian-specific acetylcholinesterase (AChE; EC 3.1.1.7), which possess unique biochemical and toxic properties has been found among tetraalkylammonium derivatives of 6-methyluracil (Reznik et al. 1998).

1,3-Bis[ω-(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide (agent No. 547), one of the most active derivatives of 6-methyluracil, demonstrates at extremely low doses (0.1–1.0 nM) an irreversible and progressive type of inhibition of AChE from bovine erythrocytes without forming covalent bonds with the enzyme. The process can be characterized by a bimolecular rate constant ($k^0 = 3.5 \times 10^9$ M^{-1} min^{-1}) (Reznik et al. 1998).

Despite effective interaction with AChE in vitro, agent No 547 and some of its analogues show a very low level of acute toxicity; the LD_{50} for rats is 1.0–2.0 mg/kg body weight (Zobov et al. 1998).

Taking into account prospective applications of agent No. 547 in medicine and agriculture, it is necessary to evaluate the possible hazardous genetic endpoints of exposure to this chemical. In this connection, we designed studies to examine the genotoxic potency of the compound using endpoints of DNA-damage (DNA-repair test), mutagenicity (Ames test) and clastogenicity (micronucleus test).

Materials and methods

Chemicals

Agent No. 547, 1,3-bis[ω-(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide, was obtained from Arbuzov Institute of Organic and Physical Chemistry, Russian Academy of Science, Kazan, Russia. The chemical structure of agent No. 547 (Fig. 1) has been confirmed by mass spectrometry,

elemental analysis, IR-spectroscopy, NMR-spectroscopy (Reznik et al. 1998).

1-Methyl-3-nitro-1-nitrosoguanidine (Serva), 2-aminofluorene (Sigma) and benzo[*a*]pyrene (Fluka) were selected as positive controls in Ames test. Ethyl methanesulfonate and colchicine (both from Sigma) were used as known diagnostic chemicals in the DNA-repair test and micronucleus assay, respectively.

Bacterial DNA-repair test

In the present work we used a suspension procedure of the DNA-repair test to evaluate the DNA-damaging potency of the test compound (Leifer et al. 1981; McCarroll et al. 1981). The tester strains of *Escherichia coli* WP2 (*trpE65*; repair-proficient) and strains defective in excision repair (uvrA; *trpE65 uvrA155*) or in recombination capacity (recA; *trpE65 recA*) were obtained from the Scientific Research Institute of Genetics (Moscow, Russia). These strains were used for detecting a sensitive growth inhibition depending on reparable DNA damage produced by the test chemicals. Bacterial growth was estimated by optical density measurement at 580 nm after 6 h incubation in nutrient broth. A survival index was calculated by dividing the percentage survival of the repair-deficient strain by the percentage survival of the repair-proficient strain. Ethyl methanesulfonate, 350 μg/ml, was used as a positive control.

Ames test

The *Salmonella* mutagenicity test has been used to measure the mutagenic potential of agent No. 547. For all experiments we used *Salmonella typhimurium* auxotrophic tester strains TA98 (*hisD3052, rfa, ΔuvrB, pKM101*) and TA100 (*hisG46, rfa, ΔuvrB, pKM101*), which were obtained from the Institute of Biological Testing of Chemical Compounds (Kupavna, Russia). The tester strains can revert to histidine prototrophy (His^+) through specific mutational events. The number of His^+ revertant colonies is a direct measure of the mutagenic potential of test compound. A plate-incorporation test was performed according to the method described by Maron and Ames (1983). The compound was tested with and without microsomal activation mixture (S9 mix). The S9 mix was prepared using lyophilized microsomal fractions of rat liver obtained from the Research Institute of Ecology and Genetics of Microorgansims (Perm, Russia). Use of S9 mix in in vitro assays allows us to incorporate the main metabolic reactions of xenobiotic transformation in mammalian organism and determine the promutagens. In addition, the compound has been examined using the recommendations of Matsushima et al. (1980) and Maron and Ames (1983) for a preincubation procedure. The reaction mixture was as follows: 0.1 ml tester strain culture with density of $1–2\times10^9$cells/ml, 0.1 ml test solution in sterile distilled water, 0.5 ml preincubation medium ($Na_3C_6H_5O_7$ 0.1 g, $KH_2PO_4.3H_2O$ 2.1 g, K_2HPO_4 0.9 g, $(NH_4)_2SO_4$ 0.2 g, 0.4 ml 2% $MgSO_4$ solution, 5 ml 40% glucose solution – all per 100 ml distilled water). After 40 min preincubation at 37°C, 0.1 ml of the mixture was placed on a minimal glucose agar plate and the number of revertant colonies was scored after a further 48-h incubation at 37°C. As diagnostic mutagens for positive controls, we used 1-methyl-3-nitro-1-nitrosoguanidine (5 μg/plate) for both the TA98 and TA100 strains in the absence of S9 mix, 2-aminofluorene (15 μg/plate) for TA98, and benzo[*a*]pyrene (5 μg/plate) for TA100 in the presence of S9 mix.

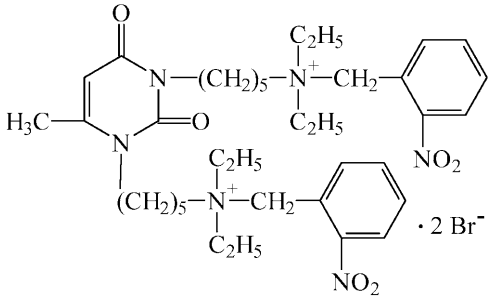

Fig. 1. The chemical structure of agent No. 547 (1,3-bis[ω-(diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide

Micronucleus assay

In order to determine the genotoxic effect of agent No. 547 at the chromosomal level we used an in vivo micronucleus test with mouse peripheral blood erythrocytes. Scoring micronuclei in anucleated erythrocytes is a measure of chromosomal breakages and chromosomal segregation errors. Thus, this measure allows the evaluation of clastogenic activity of the test compound.

Male mice CBAxC5713 L/G (10–16 weeks old, 25–30 g) were used for all studies. Agent No. 547 was dissolved in sterile distilled water and the mice were injected intraperitoneally at dose levels corresponding to 12.5, 25, 50% of the LD_{50}. Control animals received equivalent volumes of distilled water. Colchicine (1.3 mg/kg), a well-known spindle toxin, was used as a positive control.

Peripheral blood from a tail vein (5 μl) was collected 48 h and 72 h after treatment and placed on glass slides. All slides were air-dried, fixed with methanol and stained for 30 min in Giemsa solution (Koch Light Laboratories, Berks., UK) diluted with distilled water 1:3.

Micronuclei were determined microscopically by examination of 2000 erythrocytes per slide for each dose.

Statistical analysis

The data obtained were analysed for statistical significance with Student's *t*-test. The significance level for differences in data ranged from $P < 0.05$ to < 0.001.

Results

DNA-repair test

Results obtained in DNA-repair test are presented in Fig. 2. Agent No. 547 at the concentrations applied had no influence on the viability of the *E. coli* repair-proficient strain WP2 or the repair-deficient strain recA. Under the same conditions, the survival indexes for the

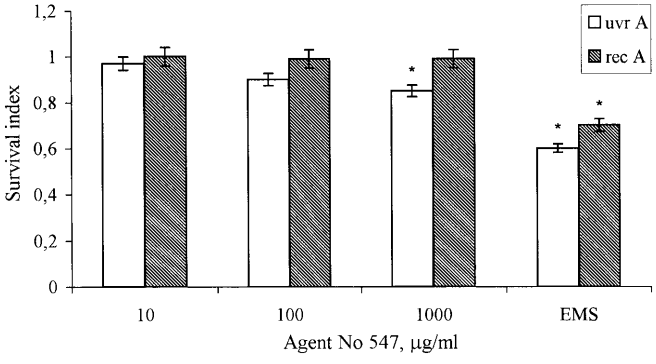

Fig. 2. DNA-damaging effect of agent No. 547 in *Escherichia coli* tester strains, uvrA and recA (*EMS* ethyl methanesulfonate, 350 μg/ml). $*P < 0.05$, significantly different from control

E. coli strain uvrA exposed to agent No. 547 were slightly lower. However, the decrease in viability of the uvrA tester strain was statistically significant only at the highest concentration tested (1000 μg/ml).

Ames test

Table 1 shows the results of the mutagenicity tests on agent No. 547 in the presence and absence of rat liver microsomal activation mixture (S9 mix). The compound did not cause any significant increase in the number of His^+ revertants over the background rate of the *S. typhimurium* strains TA98 or TA100 at any concentration tested.

When S9 mix was included, agent No. 547 gave a negative response in the assay as well. At the same time, 40 min preincubation of the *S. typhimurium* tester strains in the presence of agent No. 547 yielded a compound-dependent change of tester cells' response. This resulted in the number of induced His^+ mutants exceeding the number of spontaneous revertants by about two-fold for TA98 and three-fold for TA100 (Table 2).

Micronucleus test

The mean numbers of micronuclei in erythrocytes 48 and 72 h after injection of agent No. 547are shown in Fig. 3. The spontaneous incidence of micronuclei was approximately 6–8 per 2000 erythrocytes examined. It can be seen from Fig. 3 that the number of micronuclei at all applied doses of the agent No. 547 differed significantly from that of the negative control. The compound caused development of micronuclei in a dose-dependent manner at 48 h after injection. In previous studies, it was emphasized that the peak response of peripheral erythrocytes to many chemicals seems to occur approximately 48 h after treatment (CSGMT 1992). In our work, the greatest number of micronuclei was found 48 h after injection of 0.5 mg/kg agent No. 547 as well. We observed seven-fold increase of induced mi-

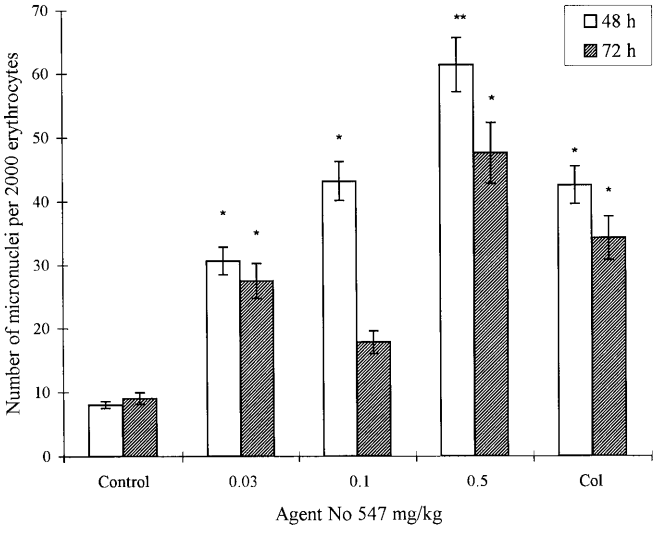

Fig. 3. The results of the in vivo micronucleus assay using erythrocytes of peripheral blood from mice CBA×C5713 L/G sampled 48 and 72 h after injection of agent No. 547 or colchicine (*Col*, 1.3 mg/kg). $*P < 0.05$, $**P < 0.001$, significantly different from control

Table 1. Mutagenicity of agent No. 547 in *Salmonella typhimurium* strains TA98 and TA100 analysed with (+*S9*) and without (–*S9*) metabolic activation using S9 mix

Treatment	Concentration (μg/plate)	Number of His^+ revertants/plate			
		TA98		TA100	
		–S9	+S9	–S9	+S9
Control (H_2O)		38.0 ± 10.3	65.2 ± 23.7	32.1 ± 9.5	39.7 ± 17.2
Agent No. 547	10	33.7 ± 8.9	59.3 ± 19.5	20.4 ± 7.4	37.3 ± 13.2
	100	25.6 ± 9.7	62.7 ± 10.8	26.3 ± 11.7	43.5 ± 16.8
	1000	39.4 ± 14.5	71.2 ± 25.2	27.8 ± 14.2	51.1 ± 18.9
Diagnostic mutagen		297.0 ± 31.3**[a]	672.8 ± 98.3**[b]	288.4 ± 45.2**[a]	429.1 ± 73.3**[c]

[a]1-Methyl-3-nitro-1-nitrosoguanidine (5 μg/plate) as diagnostic mutagen
[b]2-Aminofluorene (15 μg/plate) as diagnostic mutagen
[c]Benzo[*a*]pyrene (5 μg/plate) as diagnostic mutagen
$**P < 0.001$, significantly different from control

Table 2. Mutagenic effect of the agent No. 547 in *S. typhimurium* TA 98 and TA 100 analysed in a preincubation assay

Treatment	Concentration (μg/plate)	Number of His^+ revertants/plate	
		TA 98	TA 100
Control (H_2O)		120.1 ± 42.3	36.8 ± 14.8
Agent No. 547	10	131.7 ± 28.9	45.1 ± 20.3
	100	152.4 ± 47.8*	58.3 ± 22.6*
	1000	220.4 ± 54.5**	116.5 ± 44.1*

$*P < 0.05$,
$**P < 0.001$, significantly different from control

cronuclei values over the spontaneous rate. Interestingly, at 72 h after injection, a reduction in the number of induced micronuclei was already detected.

Discussion

The present investigation was conducted to examine the genotoxic effects of the newly synthesized compound 1,3-bis[ω-diethyl-ortho-nitrobenzylammonio)-pentyl]-6-methyluracil dibromide in different test systems, namely the DNA-repair test, the Ames test (plate-incorporation and preincubation assays) and an in vivo micronucleus test with mouse peripheral erythrocytes.

This compound, being an irreversibly acting inhibitor of AChE, could be used as an effective agent in pharmacology and agriculture.

The biochemical mechanism of AChE inhibition for the majority of the extensively used chemicals, such as organophosphorus compounds or carbamates, is based on covalent binding to the active centre of the enzyme by means of phosphorylation, acylation or alkylation reactions (Wilson et al. 1960; Wallace and Kemp 1991; Blasiak et al. 1999; Anikienko et al. 2001). Unlike this type of action, tetraalkylammonium derivatives of 6-methyluracil appear to exert their activity due to the nitrobenzyl and 6-methyluracil groups in the molecule binding to a large hydrophobic zone near the enzyme's active centre. It was suggested that the simultaneous presence in the molecule of a heterocyclic base (structural analogue of a fragment of some coenzymes) and a tetraalkylammonium group imitating an important functional group of the natural substrate molecule could provide a significant complementary enzyme-inhibitor interaction (Reznik et al. 1998; Anikienko et al. 2001).

The results obtained in this work indicate that, generally, agent No. 547 does not possess significant DNA-damaging potency in *E. coli* repair-proficient (WP2) and repair-deficient tester strains (uvrA, recA).

The compound tested did not induce base-pair substitution as frameshift gene mutations in bacterial cells (*S. typhimurium* TA 98 and TA100). We have used a microsomal fraction from rat liver to incorporate metabolic transformation of the compound in mammalian cells and evaluate a possible mutagenicity endpoint of this process. It was found that biotransformation of the compound by microsomal enzymes in vitro does not lead to the formation of active metabolites that could increase the gene mutation rate in *S. typhimurium* tester strains. Surprisingly, we observed a marked increase in number of His^+ revertants in both tester strains with the preincubation assay. Unique positive response in the preincubation test has been shown for several compounds by other investigators (Gatehouse et al. 1987, 1994). It has been argued that certain classes of chemicals (*N*-nitrozoamines, azo dyes, and some nitrocompounds) are effectively tested for mutagenicity by the preincubation procedure (Prival et al. 1984; Dellacro and Prival 1989). Rosenkranz et al. (1980) and Dillon et al. (1992) demonstrated a preference of the preincubation method for some labile and volatile chemicals. There are a few mechanisms that account for the enhanced positive response in the preincubation assay. The most likely is that a compound and tester cells are in a close contact for the initial period that prevents binding of the compound and its metabolites to agar. In addition, the preincubation method provides conditions for more intensive metabolism of the test compound (Gatehouse et al. 1994). Based on our results, we can propose that the mutagenic effect obtained with agent No. 547 in the preincubation assay is connected with the labile intermediates that could be formed during the biotransformarion of the compound by bacterial enzymes, for example through the reduction of nitrobenzyl fragments of the molecule. It is well known that successive reduction of nitro-groups by bacterial nitroreductases with the formation of reactive intermediates plays a key role in expression of the mutagenicity of nitro-containing chemicals. (Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1985; Wild 1990).

Agent No. 547 was also examined for genotoxicity at the chromosomal level using mouse peripheral blood erythrocytes. At the present time, the micronucleus assay with peripheral erythrocytes is regarded as a good alternative to the conventional bone marrow assay and is widely used for detection of clastogenicity of chemical compounds. The advantages of the test have been discussed in the literature (MacGregor et al. 1980; CSGMT 1992).

Agent No. 547 caused a significant number of micronuclei in mouse peripheral erythrocytes. Moreover, 48 h after treatment the micronuclei response was clearly dose-dependent. We observed a peak value 48 h after injection of the compound at a high dose (0.5 mg/kg). This value was very high and is compatible with that for well-known clastogenic agents. The results presented here are in agreement with the reported activities for some pyrimidine derivatives. Thus, it has been demonstrated that some fluorinated pyrimidine derivatives produced a clear clastogenic effect, inducing chromosomal aberrations in Chinese hamster cells (Yajima et al. 1981; Shibahara et al. 1993; Ohuchida et al. 1996) and micronuclei in mouse bone marrow (Shibahara et al. 1993), and gave a negative response in a reverse mutation test in strains of *S. typhimurium* (Yajima et al. 1981; Shibahara et al. 1993; Ohuchida et al. 1996).

It is obvious that biochemical mechanisms of the biological effects of synthetic pyrimidine derivatives could be compatible with the role of pyrimidines in RNA and DNA metabolism and other vitally important processes. Based on the results obtained in this work and from other data in the literature, we suggest that specific genotoxic effects of agent No. 547 can probably be explained by the alterations in significant biochemical pathways induced by the compound.

Thus, despite insufficient mutagenic potency for bacterial cells, agent No. 547 demonstrated a significant

clastogenic activity in mouse peripheral erythrocytes. Therefore, we consider that further investigations are necessary to identify the mechanisms of biotransformation pathways for the compound in mammalian organisms and to evaluate the real risk of its exposure.

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