INFLUENCE OF HEXAMIDINE ON REDUCING THE MUTAGENIC ACTION OF DIOXIDINE

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The original domestic preparation of dioxidine (I), developed at the S. Ordzhonikidze All-Union Chemico-Pharmaceutical Institute, is highly effective in suppurative infections, including those with a serious course, which cannot be treated with other antibacterial agents [1]. Substantial advantages of I over preparations studied for comparison have been demonstrated experimentally in colibacillary [2] and suppurative [3] pyelonephritis in rats, as well as suppurative burn infection [4] in mice. And yet, it has been found that I has a mutagenic effect, inducing gene mutations in indicator microorganisms *in vitro* [5] and in experiments with a mammalian carrier [6]; it induces chromosome aberrations in mouse bone marrow cells [6], plant cells and human lymphocyte culture [7], as well as dominant lethal mutations in mouse embryonic cells [6].

A search for methods of reducing the mutagenic activity of I while preserving its antimicrobial properties, in particular, the use of antimutagenic preparations for this purpose, seems important. It was shown earlier that the anticonvulsive preparation hexamidine (II) lowers the level of natural mutation in mice, *Drosophila*, and the Welsh onion [8].

The purpose of the present work was to study the ability of II to lower the level of mutation induced by I. A parallel estimation was made of the influence of II on the antibacterial action of I *in vitro* in experiments on infected animals, as well as on its tolerability including acute toxicity and changes in the activity of the central nervous system induced by I.

EXPERIMENTAL

I and II were used in the form of domestic commercial preparations. The genetic action of I and II was estimated according to the induction of chromosome aberrations in mouse bone marrow cells, in human peripheral blood lymphocyte culture and Welsh onion seedlings, as well as by a determination of gene mutations in indicator strains of mouse typhus bacillus and recessive sex-linked lethal mutations in *Drosophila*.

In an evaluation of chromosome aberrations in the metaphases of the bone marrow of male first-generation CBA × C57Bl/6 mice, two months of age, we used the method described earlier [9]. Both preparations were administered once, 24 h before the animals were sacrificed. A solution of I in distilled water was used in doses of 400, 270, 140, and 10 mg/kg, intraperitoneally, II in doses of 100, 25 mg/kg intraperitoneally, and perorally in a 1% starch suspension. In each variant 4-5 animals were used. Chromosome aberrations in human peripheral blood lymphocyte cultures were determined according to the methodological indications of [10], using the Hungerford method. I, dissolved in the medium, was introduced in concentrations of 100 and 10 µg/ml at 48 h of culturing of the cells, II in a concentration of 10 µg/ml before and at 48 h of culturing. The material was fixed at 72 h of culturing. Chromosome aberrations in anaphases of Welsh onion seedlings were estimated by the method of [7]. The preparations were diluted each individually, and after mixing them, the filter paper on which the seeds were germinated was moistened with the solution. II in tablets was dissolved to a concentration of 0.5 mg/ml in several drops of DMSO and distilled water, I (2.1, 0.5 and 0.1 mg/ml) in distilled water. Gene mutations were determined *in vitro* on strains

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of *Salmonella typhimurium* by the quantitative method of [11]. The frequency of induced mutations was calculated according to the formula [12]:

$$M = \frac{(a - b) \cdot 4}{d},$$

where a and b are the numbers of mutants in the case of inoculation of 0.25 ml of the incubation mixture in the experimental and control variants, respectively; d is the number of viable bacteria in 1 ml of the incubation mixture in the experimental variant.

The frequency of appearance of lethal mutations in *Drosophila* was determined by the Hehler-5 method [13]; food method of treatment; exposure 48 h. II was used in a concentration of 3 mM in a 2% solution of DMSO in a 5% sugar solution, I in a concentration of 5 mg/ml in a 5% sugar solution.

The antibacterial action of I was estimated according to the minimum bacteriostatic concentrations in liquid nutrient medium, the kinetics of the bactericidal action by inoculation onto petri dishes, and also according to chemotherapeutic effectiveness in experimental colibaccillary sepsis and suppurative burn infection in mice. The bacteriostatic and bactericidal action was studied by the usual method [14]. Colibaccillary sepsis was induced with a 24 h agar culture of *E. coli* strain 675. The microbial suspension was prepared in physiological solution, mixed with a 0.25% solution of starvation agar in a 1:4 ratio, and injected intraperitoneally into noninbred mice in a dose of 100 million microbial cells in a volume of 1 ml. In doses of 200 mg/kg and less, I was introduced intragastrically in physiological solution. II in a dose of 200 mg/kg was used analogously in a 2% starch suspension. The preparation was introduced once immediately after infection of the animals. The experimental results were evaluated according to the survival of the animals on the 10th day of observation. The average therapeutic doses (TD_{50}) were calculated by the method of Reed and Muench [15]. The model of suppurative burn infection was described earlier [4]. I in a dose of 100 mg/kg was injected once a day subcutaneously or introduced with a probe into the stomach in 0.2 ml of isotonic sodium chloride solution; II was introduced intragastrically in a dose of 25 mg/kg in 0.4 ml of starch suspension. The time of treatment was 10 days. The therapeutic effects of the preparations were judged according to the survival and the average lifetime of the animals.

The tolerability of I by the animals was judged according to the acute toxicity and the nephropharmacological activity, which were investigated as manifestations of side effects. The acute toxicity of I and its combination with II was studied on noninbred sexually mature male mice 2.5 months of age. The preparations were introduced in the form of an aqueous suspension with Tween-80, intraperitoneally. The state of the experimental animals and their death were recorded over a period of two weeks. LD_{50} was calculated by the method of Litchfield and Wilcoxon [16]. The neuropharmacological action of I was investigated in experiments on noninbred male mice weighing 18-20 g. I was introduced in doses of 100 and 140 mg/kg, II 25 mg/kg. The influence of the substance on the behavior of the animals (estimated according to a five-point system [17]) and on the motor orientating activity (according to the number of movements in 10 min in a DAUR-20 recorder) was determined. The effects of the interaction of I and II with hexenal (60 mg/kg), apomorphine (10 mg/kg), arecoline (25 mg/kg), and corasole (110 mg/kg) were also determined. All the substances were injected intraperitoneally 45 min before the experiment; corasole, subcultaneously.

RESULTS AND DISCUSSION

Influence of II on the Mutagenic Action of I in Mouse Bone Marrow Cells. As can be seen from Table 1, I induces chromosome aberrations in cells when introduced in doses of 400, 270, and 140 mg/kg but is generically inactive when the dose is lowered to 10 mg/kg. These data coincide with those described earlier [6]. II does not change the mutagenic effect of I, introduced in doses of 400 and 270 mg/kg, but it significantly lowers the level of mutation induced by I in a dose of 140 mg/kg. This effect is manifested both in the case of intraperitoneal and in the case of intragastric introduction of II, but in the latter case to a somewhat lesser degree. In doses from 400 to 140 mg/kg, I causes the appearance of cells with multiple chromosome aberrations (up to 10 or more in each cell). The number of such cells in the case of combined use of II and I in a dose of 400 mg/kg is unchanged; when the dose of I is lowered to 270 mg/kg it is decreased by more than 50%; when the dose is further lowered to 140 mg/kg, cells with multiple aberrations are not encountered. In this case, in variants of combined action, cells with one aberration predominate. When I is used, the number of such cells is negligible.

TABLE 1. Influence of II on the Mutagenic Activity of I in Mouse Bone Marrow Cells

Compound	Dose/ mg weight	Method of administration	Number of metaphases analyzed	Aberrant metaphases	
				number	% ± m
Control		Intraperitoneal	312	3	0,96±0,55
		Peroral	320	3	0,93±0,53
II	100	Peroral	254	2	0,78±0,55
		Intraperitoneal	280	2	0,71±0,50
	25	Peroral	212	1	0,47±0,46
		Intraperitoneal	265	2	0,75±0,52
I	400	Intraperitoneal	255	66	25,88±2,73
	270	"	226	51	22,56±2,78
	140	"	362	58	16,02±1,92
	10	"	309	5	1,61±0,71
I+II	400	Peroral	221	62	28,05±3,02
	100	Intraperitoneal	190	60	31,57±3,37
I+II	400	Peroral	146	53	36,30±3,97
	25	Intraperitoneal	267	75	28,08±2,75
I+II	270	Peroral	305	82	26,88±2,54
	100	Intraperitoneal	291	74	25,42±2,55
I+II	270	Peroral	248	58	23,38±2,68
	25	Intraperitoneal	300	62	20,66±2,33
I+II	140	Peroral	334	18	5,38±1,23
	100	Intraperitoneal	291	6	2,06±0,83
I+II	140	Peroral	341	29	8,05±1,4
	25	Intraperitoneal	302	8	2,64±0,9
I+II	10	Peroral	302	0	0
	100	Intraperitoneal	307	2	0,65±0,4
I+II	10	Peroral	289	4	1,38±0,6
	25	Intraperitoneal	309	4	1,29±0,6

Note. For the combined administration of I and II, the table indicates the mode of administration of II; I was injected intraperitoneally.

TABLE 2. Influence of II on the Mutagenic and Bactericidal Action of I with Respect to *Salmonella typhimurium*

Concentration of preparation, μg/ml		Fraction of surviving bacteria			Frequency of induced mutations, $\times 10^{-8}$		
I	II	experiment 1	experiment 2	experiment 3	experiment 1	experiment 2	experiment 3
0	0	—	—	—	2,4	2,1	1,2
0,75	0	$6,7 \cdot 10^{-1}$	$6,3 \cdot 10^{-1}$	$4,2 \cdot 10^{-1}$	5,8	8,8	9,8
1,5	0	$6,1 \cdot 10^{-1}$	$3,4 \cdot 10^{-1}$	$3,2 \cdot 10^{-1}$	8,7	27	14
3,0	0	$3,9 \cdot 10^{-1}$	$1,6 \cdot 10^{-1}$	$1,2 \cdot 10^{-1}$	24	29	27
4,5	0	$1,7 \cdot 10^{-1}$	$1,4 \cdot 10^{-1}$	$7,1 \cdot 10^{-2}$	64	47	34
0,75	100	$8,3 \cdot 10^{-1}$	$7,4 \cdot 10^{-1}$	$4,8 \cdot 10^{-1}$	—	—	4,8
1,5	100	$6,7 \cdot 10^{-1}$	$5,2 \cdot 10^{-1}$	$4,5 \cdot 10^{-1}$	3,0	13	5,4
3,0	100	$4,7 \cdot 10^{-1}$	$6,3 \cdot 10^{-1}$	$3,5 \cdot 10^{-1}$	12	18	7,4
4,5	100	$5,1 \cdot 10^{-1}$	$5,3 \cdot 10^{-1}$	$2,8 \cdot 10^{-1}$	13	28	11

Influence of II on the Mutagenic Action of I in Human Lymphocyte Culture. Just as in previously described experiments [7], dioxidine induced chromosome aberrations in cells when it was used in concentrations of 10 and 100 μg/ml, up to 4.67 ± 1.44 and 11.57 ± 1.89%. The level of mutations in the control was 0.24 ± 0.24%. In the case of combined use of II in a concentration of 10 mg/ml, a tendency for a decrease in the mutagenic action of I to 2.59 ± 0.79 and 8.1 ± 1.52%, respectively, was noted. However, under these conditions the effect was not statistically significant.

Influence of II on the Mutagenic Action of I in Welsh Onion Cells. When I was used in concentrations of 0.5-2 mg/ml, it increased the percentage of chromosome aberrations in the cells to 20-30 (approximately 9% of the control). The addition of II in a concentration of 0.5 mg/ml entirely removed the mutagenic action of I.

Influence of II on the Mutagenic Action of I in *Salmonella typhimurium* Cells. It was established that in the range of concentrations 0.1-10 μg/ml, II does not significantly influence the bactericidal and mutagenic effects of I. When the concentration of II was increased to 100 μg/ml, a decrease in the mutagenic effect was noted, accompanied by a certain

TABLE 3. Chemotherapeutic Effectiveness of I in Isolated and Joint Use with II on the Model of Colibacillary Sepsis in Mice

Preparation	Daily dose, mg/kg	Survival
I	200	9/10 (90)
	100	10/10 (100)
	50	27/30 (90)
	25	25/30 (83)
	12,5	4/20 (20)
	6,25	0/20 (0)
I+II (20 mg/kg)	200	9/10 (90)
	100	10/10 (100)
	50	30/30 (100)
	25	22/30 (66)
	12,5	6/20 (30)
	6,25	0/20 (0)
Control		0/60

Note. In the numerator: number of surviving animals; in the denominator: number of animals in the group; in parentheses: percentage of surviving animals. TD_{50} 19 mg/kg.

TABLE 4. Chemotherapeutic Effectiveness of I in Isolated and Joint Use with II in Suppurative Burn Sepsis in Mice

Group of animals	Survival	Average lifetime, days (M ± m)
First control (burn)	8/10 (80)	9±0,6
Second control (burn + infection)	1/20 (5)	2,8±0,4
Treated with I in a dose of 100 mg/kg	16/20 (80)	8,5±0,8
Treated with II in a dose of 25 mg/kg	0/20 (0)	2,9±0,3
Treated with I (100 mg/kg) + II (25 mg/kg)	16/20 (80)	8,45±0,4

Note. In the numerator: number of surviving animals; in denominator: number of animals in the group; in parentheses: percentage of surviving animals.

increase in the survival of the bacteria (Table 2). The dependence of the bactericidal and mutagenic effects of dioxidine on the dose in variants in which the bacteria were treated with I alone or a combination of I and II shows that at the indicated concentration of I a decrease in the frequency of induced mutations is observed in variants with combined use of the preparations. At the same time, there is a decrease in the bactericidal activity of I — the fraction of surviving bacteria is increased. The mechanisms of the observed effects are as yet not clear. The possibility remains that an excess of II somehow hinders the penetration of I into the bacterial cell.

Influence of II on the Mutagenic Action of I in *Drosophila*. The frequency of appearance of recessive sex-linked lethal mutations in adult individuals of *Drosophila* in variant I was 0.99 ± 0.33%. In the variants with combinations I and II, a tendency was observed for a decrease in the effect (0.36 ± 0.21%). It is important that the level of induced mutagenesis in the variants with I significantly exceeded this index in the control, while in the case of combined use of I and II there were no differences between experiment and control (control level 0.11 ± 0.06%).

Influence of II on the Chemotherapeutic Activity of I. The minimum bacteriostatic concentrations with respect to Gram-negative bacteria were unchanged in the presence of I in a concentration of 1000-1 μg/ml. As seen from Table 3, in colibacillary sepsis in mice, I in doses of 200-25 mg/kg has a strong chemotherapeutic effect (100-83% of the animals survived). When the dose is lowered to 12.5 mg/kg, the preparation gives a weak therapeutic effect. Similar results were obtained in the combined use of I and II. The average therapeutic dose for I, both in the case of isolated use and in joint use with II, was 19 mg/kg.

From Table 4 it follows that in the control group of animals with burn without infection, 20% of the mice die within 10 days, while their average lifetime is 9 ± 0.6 days. In the group of animals with burn and infection with suppurative bacillus, 95% of the mice die during the experiment. The average lifetime of the mice in this group was 2.8 ± 0.4 days. Treatment with I caused survival of the animals in 80% of the cases, and the average lifetimes of the mice was 8.5 ± 0.8 days. II had no therapeutic effect; the death of the animals and average lifetime did not differ from the corresponding indices in the control group. The combined use of I and II produced the same survival of the animals and average lifetimes as in the case of the isolated use of I. The therapeutic effect of I was not reduced by intragastric administration of II in the same dose.

Influence of II on the Neuropharmacological Activity of I. In doses of more than 100 mg/kg, I produces a certain exciting effect: it increases the motor activity of the animals and intensifies the responses to stimuli. II in a dose of 25 mg/kg does not change the be-

TABLE 5. Influence of I and II on the Effects of Hexenal, Apomorphine, Phenamine, and Arecoline (M ± m)

Compound	Dose, mg/kg	Duration of hexenal sleep, min	Duration of stereotypy, min		Duration of arecoline hyperkinesis, min
			apomorphine	phenamine	
Control		35±5,3	51,3±0,5	90,0±2,4	21,3±0,21
I	100	37±0,07	56,5±0,8	98,0±1,9	21,5±0,27
I	140	39±0,4	59,0±1,5	99,0±0,9	21,6±0,09
II	100±25	63±0,27	56,0±1,8	99,0±2,5	20,0±0,08
II	25	65±1,9	50,0±0,08	82,0±1,8	20,0±0,2

havior of the animals. The joint use of II and I produces the same stimulation as the administration of I alone. Both substances did not significantly change the orientative activity of the animals and did not affect the body temperature. I and I in conjunction with II somewhat intensified the response of fear and aggression. I in conjunction with II induces a certain lengthening of the soporific effect of hexenal. The same action is exerted by II alone. Practically no influence on the apomorphine and phenamine stereotype is observed. When I and II are administered jointly, they somewhat lengthen arecoline hyperkinesis (Table 5).

Thus, the joint use of I and II does not significantly change the properties of the two preparations. I has a weak stimulating effect on the central nervous system, both in isolated action and jointly with II.

Influence of II on the Acute Toxicity of I in Mice. As a result of the experiments it was established that LD_{50} of I in the case of intraperitoneal injection is 750 (662-840) mg/kg. LD_{50} of I in combination with II in a dose of 100 mg/kg is equal to 790 (734-849) mg/kg in the case of intraperitoneal injection of II and 770 (693-854) mg/kg in the case of peroral administration of II. The differences in acute toxicity of I and its combination with II are statistically insignificant ($PR < f_{PR}$).

Thus, the potential antimutagenic activity of II with respect to the mutability induced by a chemotherapeutic preparation, I, has been studied on various biological objects. In not one of the tests used did II increase the level of mutation induced by I. A lowering of the level of mutation induced by I was registered on a number of test objects.

On models of colibacillary sepsis and suppurative burn infection in mice it was shown that II does not decrease the therapeutic effectiveness of I. The differences in acute toxicity of I and its combination with II are statistically insignificant. The neuropharmacological activity of I and II is not significantly changed by joint use of the preparations.

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PHOSPHORIC ESTERS OF 3-HYDROXY-20-KETOPREGNANE DERIVATIVES

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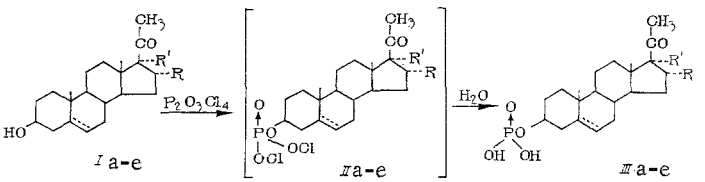
Recently the phosphoric esters of corticosteroid compounds, dexamethasone, hydrocortisone, and prednisolone, have been widely applied in medicinal practice [1]. The phosphates of the antiinflamatory corticosteroids form readily soluble sodium salts, so that they can be used as injection forms for parenteral and intravenous administration, and also as drops in ophthalmology [2].

The preparation of 21-phosphoric esters of corticosteroids in a high yield and in a one-stage instead of a multistage synthesis [3] became possible due to the appearance of a new phosphorylating agent, pyrophosphoryl chloride [4]. Phosphorus oxychloride and phosphorus pentachloride are not suitable for the phosphorylation of a steroid molecule, since they give either disteroid phosphates [5] or dehydration products, especially in the presence of a 17-hydroxyl group [6].

It is known that pregn-5-en-3β-ol-20-one has an antiinflammatory and antiallergic activity, and is used as a drug [1].

It was interesting to prepare 3-phosphoric esters of pregnenolone substituted at the 16- and 17-carbon atoms, and to study their pharmacological activity.

By the action on the molecule of steroid (I) of 2 moles of pyrophosphoryl chloride at low temperature in tetrahydrofuran (THF) solution, the dichlorophosphate (II) is obtained.



I a-e II a-e III a-e

III a) R=CH3, R'=H, Δ5 ; d) R=CH3, R'=H, 5α
b) R=H, R'=OH, Δ5 ; e) RR'= -O-C(CH3)2-O-, Δ5
c) R=CH3, R'=OH, Δ5 ;

Without separation, the dichlorophosphate II is treated with an excess of water, and the monophosphate III is obtained.

The properties of the 3-phosphoric esters of 3-hydroxy-20-hetopregnane derivatives (IIIa-e) obtained by this scheme, and the properties of their disodium IVa, e and dipotassium IVb, c, d salts are listed in Table 1.

All the synthesized compounds are colorless, crystalline materials, which are fairly soluble in alcohols. Their disodium and dipotassium salts are practically insoluble in water, in contrast to 21-phosphoric esters.

In the IR spectra of IIIa, d, e there are absorption bands in the 2300-2380 cm^{-1} region, corresponding to the phosphoric acid hydroxyl. In compounds IIIb, c, besides the absorption bands at 2380 and 2370 cm^{-1}, respectively, there are bands at 3370 and 3420 cm^{-1}, corresponding to the 17α-hydroxyl. In the IR spectra of the disodium and dipotassium salts of 3-phosphoric esters IVa-e, the absorption bands at 2300-2380 cm^{-1} are missing.

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