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GENOTOXIC ACTIVITY OF NEWLY SYNTHESIZED DERIVATIVES OF CYANO-PYRIDONE IN MURINE CELLS *IN VIVO* AND *IN VITRO*

Possible genotoxic activity of two newly synthesized cyano-pyridone compounds [4-(N-methyl-phalimidyl-3)-3-cyano-4-methyl-pyridone-2 (MPhCMP) and 1-(4-hydroxyphenyl)-3-cyano-4-methyl-pyridone-2 (HCMP)] with in vitro antitumor activity was studied both in in vitro and in vivo murine test systems. In L5178Y mouse lymphoma cells, HCMP did not induce micronuclei (MN) at the highest available (because of toxicity) concentration (100 μg/ml), while MPhCMP at dose of 50 μg/ml induced 2.6-fold, and at dose of 100 μg/ml 3.95-fold increase of number of the cells with MN. The concentration of 100 μg/ml is a threshold of toxicity of MPhCMP. In experiments on possible DNA damaging activity (the comet assay) of both substances using the same doses as in in vitro mutagenesis assay, we did not reveal any evidence of DNA damage. The acute toxicity of compounds was studied on male Swiss albino mice. LD_{50} values of MPhCMP and HCMP were 177.5 and 288 mg/kg, respectively. MPhCMP was more potent MN inductor than HCMP (2.5-fold at doses equivalent to ½ of LD_{50}). Both substances possessing in vitro antitumor activity along with weak genotoxicity have a good chance for successful in vivo antitumor studies in rodents.

Introduction. Newly synthesized two derivatives of cyano-pyridone showed antitumor activity *in vitro* on 3 murine tumor cells lines: MG-22A — hepatoma, B-16 — melanoma, Neuro2A — neuroblastoma [1]. Especially they were active against Neuro2A cells. It would be of interest to study the genotoxic activity of these compounds because there are no data on the genotoxicity profile of structurally related substances in the available literature.

U.S. Environmental Protection Agency (EPA) and the United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (UKCOM) recommend that all compounds tested for mutagenicity should be studied using mouse L5178Y lymphoma and bone marrow cells [2]. L5178Y mouse lymphoma cells is a suitable cell system for assessing the clastogenic and aneugenic [by means of the micronucleus (MN) assay], and also genotoxic (DNA damaging, by means of the comet assay) properties of various agents [3]. The MN assay both *in vivo* and *in vitro* is widely used for the assessment of clastogenic and aneugenic effects of compounds, including new drugs [4]. The comet assay (single-cell gel electrophoresis) is a rapid and well-established test system for detecting DNA strand breaks [5].

The aim of the present work was to study the MN-inducing activity of two newly synthesized substances in bone marrow polychromatic erythrocytes (PCEs) of mice and in L5178Y mouse lymphoma cells, and to evaluate DNA-damaging activity of substances in L5178Y mouse lymphoma cells.

Materials and methods. ***Compounds studied.*** 4-(N-methyl-phalimidyl-3)-3-cyano-4-methyl-pyridone-2 (MPhCMP) and 1-(4-hydroxyphenyl)-3-cyano-4-methyl-pyridone-2 (HCMP) were synthesized by Melikyan according to a new method of synthesis of substituted pyrrolinones [6]. The only difference between the two compounds is 4-(N-methyl-phalimidyl-3)- group in MPhCMP instead of 1-(4-hydroxyphenyl)- in HCMP. Mentioned newly synthesized substances dissolved in dimethyl sulfoxide (DMSO) were used in the experiments.

Cell culture. L5178Y $tk^{+/-}$ mouse lymphoma cells were routinely cultured in suspension in RPMI-1640 supplemented with 98 units/ml penicillin, 95 μg/ml streptomycin, 0.25 μg/ml L-glutamine, 107 μg/ml sodium pyruvate, and 10 % heat-inactivated horse serum (all from «Sigma», Germany).

The cell cultures were grown in a humidified aerial atmosphere containing 5 % CO_2 at 37 °C.

In vitro micronucleus assay. In the first experiment, exponentially growing L5178Y cells (10^6 cells in 5 ml medium) were treated with the substances at doses of 500 and 1,000 ng/ml overnight and in the second one at doses of 1 and 10 μg/ml. Since we recorded neither toxic nor MN-inducing effects, another schedule of treatment was applied: incubation of substances with lymphoma cells for 4 h [3]. After removing the chemicals by centrifugation and replacement of the medium, the cells were incubated for 18 h (expression time). Mitomycin C (MMC, 50 μg/ml) and DMSO were used as positive and negative (solvent) controls, respectively. Slide preparation, staining and criterion to distinguish the MN were the same as described earlier [7]. One thousand cells were scored in each slide, 3 slides per each point.

Comet assay. Comet assay was performed as described earlier with the same negative (DMSO, the solvent of the substances) and positive [ethyl methansulphonate (EMS), 300 μg/ml] controls [8]. L5178Y cells (10^6 cells in 5 ml medium) were treated with the substances or the solvent alone for 4 h. The doses of the substances were chosen according to the data obtained in the MN assay (100 μg/ml of both substances). After removing the chemicals by centrifugation and replacement of the medium, the cells were incubated for 18 h. Then the cells (3×10^3/slide) were processed and stained as described earlier [7]. The cells were analyzed at 1250× magnification using computer-aided image analysis. Images of 50 cells (25 from each slide) were evaluated by means of the software program NIH Image 1.54 (NIH, USA). Tail moment was evaluated and presented in arbitrary units (a. u.).

In vivo micronucleus assay. To investigate *in vivo* the MN-inducing activity of the substances, we first studied their acute toxicity using the approach of Lorke [8]. In the experiments on toxicity and mutagenicity, Swiss albino male mice (22–25 g) bred in the Institute of Fine Organic Chemistry (Yerevan, Armenia) were used. Both substances were dissolved in DMSO (Sigma, USA), and were injected intraperitoneally. The MN-inducing activity of the substances was determined according to Kirkhart's protocol [9]. Each experimental group consisted of 5 mice. The substances were administered to mice twice at 0 and 24 h at doses of $^1/_2$ and $^1/_5$ of LD50, and in case of MPhCMP also at 1/10 and 1/20 of LD50. The mice were sacrificed in 48 h. Cyclophosphamide (Mosmedpreparati, Russia) in a dose of 25 mg/kg (dissolved in 0.2 ml saline) and the vehicles (0.2 ml DMSO and 0.2 ml of starch) served as positive and negative controls, respectively. The slides from bone marrow cells were prepared and analyzed as described earlier [7]. Each coded slide was assessed for MN in 2,000 PCEs. The percentage of PCE among the erythrocytes was also calculated.

Statistics. Analysis was performed by means of Student's *t*-test.

Results and discussion. Incubation of lymphoma cells with both substances at doses of 500 and 1,000 ng/ml, and also 1.0 and 10.0 μg/ml overnight did not induce any significant increase of MN frequency. No change of the number of cells was observed in comparison with the controls. Hence, in mentioned doses range both substances proved to be non-toxic and non-mutagenic for the mouse lymphoma cells. Since we recorded neither toxic nor MN-inducing effects, we applied another schedule of treatment: the lymphoma cells were incubated with substances for 4 h, washed from the substances using centrifugation and replacement of cell medium, and then incubated for 18 h [3]. The substances were studied for MN-inducing activity in a concentration range of 10–1000 μg/ml. The results of the experiments are presented in Tables 1 and 2. Since HCMP was substantially toxic for lymphoma cells at concentration of 100 μg/ml after 4 h incubation (decreasing the quantity of cells to 48 %), but not MN inductor, we did not study other concentration of the substance. In contrast, MPhCMP was not toxic, but induced significant increase of MN compared with negative control, and we studied additionally 50, 250 and 500 μg/ml of this substance for MN activity (table 2). In this experiment MPhCMP showed substantial toxicity at dose of 100 μg/ml decreasing the number of cells to 37 % after 4 h incubation. Higher concentrations of the substance were highly toxic for lymphoma cells. MPhCMP at dose of 50 μg/ml induced 2.6-fold, and at dose of 100 μg/ml 3.95-fold increase of number of the cells with MN, although the difference was not statistically significant ($p > 0.05$). MMC used as positive control induced 14.8-fold elevation of cell with MN at dose of 50 μg/ml.

Table 1

Micronucleus-inducing activity of substances in mouse lymphoma cells

Chemical agent	Dose, μg/ml	Number of MN, ‰, mean ± SE	Range per 1000 cells	Relative cell growth after incubation for	
				4 h	overnigh
MPhCMP	10	5.3 ± 2.3	3–7	88	78
	100	18.7 ± 2.9 *	16-21	79	57
	1000	toxic	N/A	12	21
HCMP	10	7.0 ± 1.1	6–8	91	85
	100	9.7 ± 2.9	8–13	48	69
	1000	toxic	N/A	11	44
MMC (positive ontrol)	50	69.7 ± 15.6 *	58–85	88	82
DMSO (negative control)	1.0	4.7 ± 1.7	3–6	100	100

* $p < 0.001$ compared to negative control (Student's t-test).

Table 2

Micronucleus-inducing activity of MPhCMP in mouse lymphoma cells

Chemical agent	Dose, μg/ml	Number of MN, ‰, mean ± SE	Range per 1000 cells	Relative cell growth after incubation for	
				4 h	overnigh
MPhCMP	50	11.3 ± 4.6 *	7–15	89	82
	100	17.0 ± 2.9 *	14–19	37	76
	250	toxic	N/A	11	59
	500	toxic	N/A	5	61
MMC (positive ontrol)	50	60.7 ± 7.6 *	55–69	74	89
DMSO (negative control)	1.0	4.3 ± 2.0	2–5	100	100

* $p < 0.001$ compared to negative control (Student's t-test).

Table 3

DNA-damaging activity of substances in the comet assay

	Dose, μg/ml	Tail moment (arbitrary units)
	Experiment 1	
MPhCMP	100	0.33 ± 0.14
HCMP	100	0.27 ± 0.12 *
EMS	300	11.89 ± 0.68 **
DMSO	1	0.72 ± 0.18
	Experiment 2	
MPhCMP	100	0.89 ± 0.11 *
HCMP	100	1.30 ± 0.17 *
EMS	300	8.49 ± 0.37 **
DMSO	1	2.33 ± 0.16

* $p < 0.01$ and ** $p < 0.001$ compared to negative control (Student's t-test)

Repeated experiments to study possible DNA damaging activity of substances (the comet assay) have shown lack of such activity of both substances (tables 3). Moreover, both substances decreased substantially the levels of DNA damage. In the first experiment the decrease induced by MPhCMP was not statistically significant (table 3), but in all other cases it was. According to these data, it would be of interest to study the possible antigenotoxic action of both substances as well.

All substances were studied for acute toxicity. For MPhCMP the following results were obtained: LD_{50} = 177.5 mg/kg (here and for another substance, the first digit is the number of mice which died after the administration, and the second one is the number of mice to which the substance was administered; 10 mg/kg — 0/3; 100 mg/kg — 0/3; 1000 mg/kg — 3/3; 140 mg/kg — 0/3; 225 mg/kg — 3/3; 370 mg/kg — 3/3; 600 mg/kg — 3/3). The following results were obtained for HCMP: LD_{50} = = 288 mg/kg (10 mg/kg — 0/3; 100 mg/kg — 0/3; 1000 mg/kg — 3/3; 140 mg/kg — 0/3; 225 mg/kg — 0/3; 370 mg/kg — 3/3; 600 mg/kg — 3/3). Accor-

Table 4

Micronucleus-inducing activity of substances in bone marrow polychromatic erythrocytes of Swiss albino mice

Chemical agent(LD50, mg/kg)	Dose in mg/kg (number of administration)	Number of MN, ‰, mean ±SE	Range per 1000 PCE	Percent of PCE
MPhCMP (177.5)	89 × 2	20.6 ± 1.8 **	17–26.5	44.4 ± 1.5 *
	36 × 2	11.4 ± 0.8 **	8.0–13.5	49.6 ± 1.0 *
	18 × 2	7.4 ± 0.8 *	3.5–8	50.2 ± 1.7
	9 × 2	3.0 ± 0.6	1–4	52.0 ± 1.7
HCMP (288)	145 × 2	8.0 ± 1.1 *	6.5–11.5	46.8 ± 1.5 *
	58 × 2	3.2 ± 0.9	1–5	53.4 ± 1.1
Cyclophosphamide (positive control)	25 × 2	22.1 ± 1.7 **	18–26	52.4 ± 1.4
DMSO (negative control)	0.2 ml × 2	2.0 ± 0.6	0–3	53.4 ± 1.0

* $p < 0.01$ and ** $p < 0.001$ compared to negative control (Student's t-test); every group consisted of 5 mice; 2,000 PCEs were studied in each mouse.

ding to the data obtained (table 4), MPhCMP is 1.63-fold more toxic than HCMP for mice. At the same time, HCMP induced significantly increased level of MN at doses range of ½-1/10 of LD_{50}. Maximal increase of MN number (10-fold) was observed after MPhCMP injection at dose equal to ½ of LD_{50}. It is noteworthy that cyclophosphamide at dose of 25 mg/kg induced almost the same level of MN as MPhCMP. This drug permanently used in our laboratory as a positive control, induced 22.1 ‰ PCEs with MN, very close to our recent data [10]. It should be noted that the acute toxicity of MPhCMP for mice is about 1.5-fold less than cyclophosphamide one (about 300 mg/kg) [10]. But, at the same time, cyclophosphamide is much more active MN inductor and induces the same level of PCEs with MN after administration at dose equal to $^1/_{12}$ of LD_{50} than MPhCMP at dose equal to ½ of LD_{50}.

HCMP was active in MN assay only at dose equal to $^1/_2$ of LD_{50} decreasing significantly the number of PCEs and thus being toxic for hemopoietic cells. MPhCMP was toxic for hemopoietic cells decreasing significantly the number of PCEs at doses equal to $^1/_2$ and $^1/_5$ of LD_{50}.

Hence, HCMP is not MN inductor at maximum possible usable dose close to toxic one. MPhCMP is a weak mutagen compared with cyclophosphamide.

It is noteworthy that the only difference in the chemical structure of two compounds is a 4-(N-methyl-phalimidyl-3)- group in MPhCMP instead of a 1-(4-hydroxyphenyl)- in HCMP. The substitution of 1-(4-hydroxyphenyl)- group to 4-(N-methyl-phalimidyl-3)- one led to substantial increase in acute toxicity (1.6-fold) and mutagenicity (MN induction; 2.5-fold in vivo and 2.0 in vitro) in mice. The toxicity of both compounds for mouse lymphoma cells is almost the same.

It is interesting that MPhCMP, mutagenic both in *in vivo* and *in vitro* systems, showed no activity in the comet assay which detects DNA strand breaks. The analysis of the literature data has shown that even some strong mutagens and carcinogens are not active in the comet assay (for example MMC used in the present work as positive control in in vitro MN assay) [12].

In conclusion, both newly synthesized compounds are relatively weak MN-inducing agents *in vivo* and are inactive in the comet assay (they do not damage DNA of mouse lymphoma cells). MPhCMP is more potent MN inductor than HCMP. But both substances are mutagenic at doses close to toxic ones. In *in vitro* assay MPhCMP was again a weak inductor of MN while HCMP was not active. The L5178Y $tk^{+/-}$ mouse lymphoma cell model is a good predictor of mutagenic action of the substances studied *in vivo*. Both substances possessing *in vitro* antitumor activity along with weak genotoxicity have a good chance for successful *in vivo* antitumor studies in rodents. Further investigations in the area of biological activity (both genotoxic and anti-genotoxic ones) of a new class of compounds (cyano-pyridone derivatives) are certainly warranted.

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РЕЗЮМЕ. Изучена генотоксическая активность двух новосинтезированных соединений, содержащих циано-пиридоновую группу (MPhCMP и HCMP) и показавших противоопухолевую активность в системе *in vitro*. В клетках лимфомы мышей L5178Y соединение HCMP не индуцировало микроядер (МЯ) в максимально нетоксичной концентрации, в отличие от соединения MPhCMP, индуцировавшего МЯ в концентрации 50 и 100 мкг/мл. В тесте на ДНК-кометы оба соединения не показали активности, что доказывает неспособность их вызывать повреждения ДНК. HCMP индуцировало повышение уровня эритроцитов с МЯ в костном мозге мышей только в дозе, эквивалентной ½ от $ЛД_{50}$. MPhCMP было активнее в этом тесте, вызывая возрастание числа эритроцитов с МЯ начиная с дозы, эквивалентной $^1/_{10}$ $ЛД_{50}$.

РЕЗЮМЕ. Вивчено генотоксичну активність двох новосинтезованих сполучень, що містять ціано-піридонову групу (MPhCMP і HCMP) та показали протипухлинну активність в системі *in vitro*. В клітинах лімфоми мишей L5178Y сполучення HCMP не індукувало мікроядер (МЯ) у максимально нетоксичній концентрації на відміну від сполучення MPhCMP, що індукувало МЯ у концентрації 50 та 100 мкг/мл. У тесті на ДНК-комети обидва сполучення не показали активності, що свідчить про їх нездатність викликати пошкодження ДНК. HCMP індукувало підвищення рівня еритроцитів з МЯ в кістковому мозку мишей тільки у дозі, що еквівалентна $^1/_2$ $ЛД_{50}$. MPhCMP було активніше в цьому тесті, викликаючи збільшення числа еритроцитів з МЯ починаючи з дози, що еквівалентна $^1/_{10}$ $ЛД_{50}$.

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