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THE STUDY OF GENOTOXICITY OF TWO NEWLY SYNTHESIZED PYRROLINONE DERIVATIVES ON L5178Y MOUSE LYMPHOMA AND BONE MARROW CELLS

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ИЗУЧЕНИЕ ГЕНОТОКСИЧНОСТИ ДВУХ НОВЫХ СИНТЕЗИРОВАННЫХ ПРОИЗВОДНЫХ ПИРРОЛИНОНА НА КЛЕТКАХ ЛИМФОМЫ L5178Y И КОСТНОГО МОЗГА МЫШЕЙ

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Genotoxic activity of two newly synthesized pyrrolinone derivatives (lactone (PTB) and lactame (PTP)) was studied both *in vitro* and *in vivo*. The substances were incubated with L5178Y mouse lymphoma cells for 4 h, then washed from the substances, and incubated for 18 h. PTP induced significantly increased number of micronuclei (MN) only at the highest dose used (1000 μg/ml). The 20-fold increase of the concentration of PTP led to 2.4-fold increase of the number of MN. PTP wasn't toxic for lymphoma cells in the applied concentration range. In contrast, PTB induced significantly increased number of MN in lymphoma cells beginning from the concentration of 50 μg/ml and was toxic for lymphoma cells at concentrations of 500 μg/ml and higher. In the comet assay with L5178Y mouse lymphoma cells both substances were active at all concentrations used (PTP at concentrations of 50 μg/ml and 100 μg/ml, and PTB at concentrations of 100 μg/ml and 250 μg/ml). Acute toxicity and MN-inducing activity of compounds were studied on Swiss albino mice. PTB was substantially more toxic for mice than PTP (LD_{50} was 200 mg/kg and 370 mg/kg respectively). In mouse bone marrow polychromatic erythrocytes PTP induced significantly increased number of MN only at a dose equal to $^1/_2$ of LD_{50}. In contrast, PTB was mutagenic at all doses used – $^1/_2$, $^1/_5$ and $^1/_{10}$ of LD_{50}. At equitoxic doses PTB induced more than 2-fold and 3.5-fold increased levels of MN compared with PTP ($^1/_2$ and $^1/_5$ of LD_{50} respectively). The replacement of =NH group to =O group in chemical structure of pyrrolinone derivatives leads to substantial increase in acute toxicity for mice (1.85-fold) and MN induction activity both *in vivo* and *in vitro*.
***Key Words*: pyrrolin-2-one derivatives, micronuclei, the comet assay, L5178Y mouse lymphoma cells, mouse bone marrow cells.**

Изучена генотоксическая активность двух новых синтезированных производных пирролинона (лактона и лактама) в системах *in vitro* и *in vivo*. Клетки лимфомы мышей L5178Y инкубировали 4 ч с соединениями, отмывали и вновь инкубировали в течение 18 ч. Лактам индуцировал статистически достоверное увеличение числа микроядер (МЯ) только при действии высшей примененной дозы (1000 μг/мл). Повышение концентрации лактама в 20 раз привело к увеличению количества МЯ только в 2,4 раза. Ни одна из использованных в эксперименте концентраций лактама не была токсичной для клеток лимфомы. Лактон, в отличие от лактама, индуцировал появление МЯ в клетках лимфомы начиная с концентрации 50 μг/мл, и был токсичен для клеток лимфомы в концентрации 500 μг/мл и выше. В тесте на ДНК-кометы оба соединения были активны (лактам в концентрации 50 μг/мл и 100 μг/мл, а лактон в концентрации 100 μг/мл и 250 μг/мл). Острая токсичность и способность индуцировать МЯ в костном мозге была изучена на мышах Swiss. Лактон был значительно токсичнее лактама ($ЛД_{50}$ 200 и 370 мг/кг, соответственно). Лактам индуцировал достоверное увеличение количества МЯ только при действии дозы, равной 0,5 от $ЛД_{50}$, в то время как лактон индуцировал МЯ даже при действии дозы, равной 0,1 от $ЛД_{50}$. При действии доз, эквивалентных 0,5 и 0,2 от $ЛД_{50}$, лактон индуцировал в 2 и 3,5 раза больше МЯ, чем лактам. Замена =NH группы на =O в химической структуре производных пирролинона приводит к значительному усилению мутагенности *in vitro* и *in vivo* и острой токсичности для мышей.
***Ключевые слова:* производные пирролинона, микроядра, ДНК-кометы, клетки мышиной лимфомы L5178Y, клетки костного мозга мыши.**

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Abbreviations used: MN — micronuclei; PCE — polychromatic erythrocytes; PTB (lactone) — [3–piperonylamide–4,5,5–trimethyl–4,5,5–trimethyl–Δ^3–butenolid]; PTP (lactame) — [3–piperonylamide–4,5,5–trimethyl–pyrrolin–2–on].

New aryl (heteryl) lactone [3–piperonylamide–4,5,5–trimethyl–4,5,5–trimethyl– Δ^3–butenolid] (PTB) and lactame [3–piperonylamide–4,5,5–trimethyl–pyrrolin–2–on] (PTP) were synthesized recently [1, 2]. The study of possible antitumor activity of PTB has shown its substantial activity *in vitro* on three murine hepatoma MG–22A, melanoma B–16, neuroblastoma Neuro2A cells lines [1, 2].

Both substances are structurally related, and the only difference between them is =O in PTB and =NH in PTP. Genotoxic activity of these compounds is not known.

It would be of interest to study the genotoxic potency of mentioned new compounds *in vivo* and *in vitro* systems, and the relationship between chemical structure and genotoxicity as well.

L5178Y mouse lymphoma cells is a cell system suitable for assessing the mutagenic, clastogenic, aneugenic, and genotoxic (DNA damaging activity) properties of various agents [3, 4]. The micronucleus (MN) assay *in vivo* is used by the pharmaceutical industry in Europe, Japan and the USA in evaluating the mutagenic potential of proposed new drugs [5].

The aim of the present work was to study MN–inducing activity of two 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.

MATHERIALS AND METHODS

Chemicals. Both substances were synthesized by G. Melikyan according to a new method of synthesis of substituted pyrrolinones proposed by Melikyan et al [6]. New derivative of pyrrolin–2–one was synthesized according to proposed method (lactame), and its oxygen structural analogue (lactone) as well. The only difference between two compounds is NH= group in lactame (PTP) instead of O= in lactone (PTB). All chemicals and reagents used for *in vitro* studies were purchased from Sigma, Germany. Cyclophospamide was produced in Russia (Mosmedpreparati).

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 (Sigma, Germany). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37 °C.

In vitro micronucleus assay. In the first experiment exponentially growing L5178Y cells ($1 \cdot 10^6$ cells in 5 ml medium) were treated with substances at doses of 500 and 1,000 ng/ml overnight, and in second one at doses of 1 and 10 μg/ml. Since we did not register either toxic or MN–inducing effect, we used another treatment schedule — incubation of substances with lymphoma cells for 4 h [4]. After removing of the chemicals by centrifugation and medium replacement, the cells were incubated for 18 h (expression time). As positive control mitomycin C (50 μg/ml) and as negative (solvent) control DMSO was used. The cells were then brought onto slides by cytospin centrifugation and were fixed with methanol (–20 °C, overnight). To stain nuclei and MN, the slides were incubated with acridine orange (0.00525% (w/v) in Sorensen buffer, pH 6.8) for 5 min. Slides were washed twice with buffer and mounted for microscopy. Numbers of nuclei and MN were scored at a magnification of x 500. 1000 cells were scored from each slide, 3 slides per each point. Objects were classified as MN if they were clearly separated from the nuclei, were round or oval, were less than $^1/_4$ of nucleus, and showed staining similar to main nucleus.

Comet assay. Comet assays were performed according to Singh et al [7] with modifications. Exponentially growing L5178Y cells ($1 \cdot 10^6$ cells in 5 ml medium) were treated with substances or solvent control (DMSO) for 4 h. Doses range of substances were 50–250 μg/m. As positive control ethyl methansulphonate was used (300 μg/ml), and as negative control — solvent of substances (DMSO). After removing of the chemicals by centrifugation and medium replacement, the cells were incubated for 18 h (expression time). Then cells ($3 \cdot 10^3$/slide) were embedded in low melting agarose (0.5%) which was layered onto fully frosted slides that had been coated with a layer of 0.75% normal agarose (diluted in Ca– and Mg–free PBS buffer). A final layer of 0.5% low melting agarose was added on top. Slides were immersed in a jar containing cold lysing solution (1% Triton X–100, 10% DMSO, and 89% of 10 mM Tris/1% Na laurylsarcosine/2.5 M NaCl/100 mM Na_2EDTA (pH 10) for lysis at 4 °C (1 h). The slides then were pretreated for 20 min in electrophoresis buffer (300 mM NaOH/1 mM Na_2EDTA (pH 13) and after that exposed to 25 V/300 mA for 20 min. Preincubation and electrophoresis were performed in an ice bath. Slides were neutralized for 3 x 5 min in 0.4 M Tris, pH 7.5 and DNA was stained by adding 50 μl of ethidium bromide (20 μg/ml) onto each slide. Cells were analyzed with a 1250 x magnification and using computer–aided image analysis. Images of 50 cells (25 from each slide) were evaluated by the use of the software program NIH Image 1.54 (NIH, USA). Tail moment was evaluated and presented in arbitrary units.

In vivo micronucleus assay. To study the MN–inducing activity of substances *in vivo,* firstly we studied the acute toxicity using the approach of Lorke (1983) [8]. Swiss albino male mice (22–25 g) obtained from the vivarium of Institute of Fine Organic Chemistry (Yerevan, Armenia) were used in experiments on toxicity and mutagenicity. All animal procedures were carried out according to the rules of Ethic Committee.

Substances were dissolved in DMSO (Sigma, USA), and were injected intraperitoneally into mice. For substances PTP and PTB we received the following data: LD_{50} is 370 mg/kg (here and for another substance the first digit is number of mice which died after the administration, and the second one is the number of administered mice; 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 — 1/3; 600 mg/kg — 3/3) and 200 mg/kg (10 mg/kg — 0/3; 100 mg/kg — 0/3; 1000 mg/kg — 3/3; 140 mg/kg — 1/3; 225 mg/kg — 1/3; 370 mg/kg — 3/3; 600 mg/kg — 3/3) respectively.

To study MN–inducing activity of substances we used the protocol described by Kirkhart [9]. Each experimental group consisted of 5 animals. The substances were administered to mice twice with 24 h period at doses equal to $^1/_2$ and $^1/_5$ of LD_{50} (PTB also at dose of $^1/_{10}$ of LD_{50}), and the rodents were sacrificed at 48 h. The substances were dissolved in DMSO and administered intraperitoneally. As positive control, cyclophosphamide (Mosmedpreparati, Russia) at a dose of 25 mg/kg (dis–

solved in 0.2 ml saline) was used according to the schedule and route of administration of substances. As negative control, the vehicle of substances (0.2 ml DMSO) was used. Bone marrow was flushed by means of newborn calf serum (0.15 ml; Sigma, USA) onto slides and smears were prepared. The slides were fixed with methanol (–4 °C) for 20 min, 24 h after the slides preparation. Slides were stained with May — Grunewald and Giemsa (Sigma, USA) buffered at pH 6.2 and 6.8 respectively. After being stained, the slides were coded so that the reader was unaware of the identity of slides being scored. Each slide was assessed for MN in 2,000 PCEs. In addition, the percent content of PCE was calculated among erythrocytes.

Statistical analysis was performed by means of Student's *t*–test.

RESULTS AND DISCUSSION

Genotoxic activity of compounds *in vitro* was studied at Institute of Pharmacology and Toxicology (Wuerzburg, Germany) on mouse L5178Y lymphoma cells.

Incubation of lymphoma cells with both substances at the doses of 500 and 1,000 ng/ml overnight did not induce a significant increase of MN frequency (data are not shown). No change of the number of cells was observed in comparison with controls. The increase of doses of substances in the same experimental protocol to 1.0 and 10 μg/ml also did not induce any significant increase in MN frequency in lymphoma cells (data are not shown). In this case also no significant change of number of cells was registered, and, hence, both substances were not toxic for cells at mentioned concentrations. As both substances were not active in mentioned experimental protocol, we applied another one used in routine mutagenesis studies at Institute of Pharmacology and Toxicology (Wuerzburg, Germany). The data are presented in Table 1. In this case substances were incubated with lymphoma cells for 4 h, then washed from the substances using centrifugation and replacement of cell medium, and incubated for 18 h (expression time). This time PTP induced significantly increased number of MN only at the highest dose used (1000 μg/ml). The 20–fold increase of the concentration of PTP led only to 2.4–fold increase of the number of MN. PTP wasn't toxic for lymphoma cells in the applied concentration range. In the 1st experiment (Table2) PTB significantly increased the number of MN at concentration of 100 μg/ml. In the 2nd experiment (Table 3) PTB was mutagenic beginning from the concentration of 50 μg/ml and was toxic for mouse lymphoma cells at concentrations of 500 μg/ml and higher.

In the comet assay both substances were active at all concentrations used (PTP at concentrations of 50 μg/ml and 100 μg/ml, and PTB at concentrations of 100 μg/ml and 250 μg/ml) (Table 4).

Our study on acute toxicity of substances on Swiss albino mice has shown that PTB was 1.85–fold more toxic than PTP. It is noteworthy that *in vitro* for mouse lymphoma cells PTB showed toxic action at concentration of 500 μg/ml whereas PTP was not toxic for lymphoma cells at concentration of 1000 μg/ml. So in this case mouse lymphoma cells test system was a good predictor of the acute toxicity of substances *in vivo*. In mouse bone marrow PCEs PTP induced significantly increased number of MN only at dose equal to $^1/_2$ of LD_{50} (Table 5). In contrast, PTB was mutagenic at all

Table 1. Micronucleus inducing activity of PTP in mouse lymphoma cells

Chemical agent	Dose (μg/ml)	Number of MN (‰, mean ±SE)	Range per 1000 cells	Relative cells growth after incubation for	
				4 h	overnight
PTP	50	4.3 ± 2.3	2–6	78	81
	100	6.7 ± 1.2	6–8	76	53
	500	9.0 ± 4.0	5–12	81	45
	1000	10.3 ± 1.7*	9–12	91	89
MMC (positive control)	50	50.0 ± 9.8*	44–61	79	47
DMSO (negative control)	1.0	4.2 ± 2.0	2–7	100	100

*$p < 0.001$ compared to negative control.

Table 2. Micronucleus inducing activity of PTB in mouse lymphoma cells (experiment 1)

Chemical agent	Dose (μg/ml)	Number of MN (‰, mean ±SE)	Range per 1000 cells	Relative cells growth after incubation for	
				4 h	overnight
PTB	10	5.7 ± 3.3	2–8	100	72.2
	100	12.3 ± 5.8*	8–18	70.9	68.1
	1000	toxic	N/A	8	25.7
MMC (positive control)	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.

Table 3. Micronucleus inducing activity of PTB in mouse lymphoma cells (experiment 2)

Chemical agent	Dose (μg/ml)	Number of MN (‰, mean ±SE)	Range per 1000 cells	Relative cells growth after incubation for	
				4 h	overnight
PTB	50	11.0 ± 1.7*	8–14	84	86
	100	17.7 ± 4.0*	14–21	69	69
	250	20.3 ± 3.5*	17–23	32	77
	500	toxic	N/A	5	61
MMC (positive control)	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.

Table 4. DNA–damaging activity of PTP and PTB in the comet assay

Chemical agent	Dose (μg/ml)	Tail moment (arbitrary units)
PTP	50	5.47 ± 2.00*
	100	6.27 ± 2.39*
PTB	100	2.91 ± 0.15*
	250	4.11 ± 1.27*
EMS (positive control)	300	5.84 ± 1.54*
DMSO (negative control)	1	0.99 ± 0.17

*$p < 0.001$ compared to negative control.

Table 5. Micronucleus inducing activity of PTP and PTB in bone marrow polychromatic erythrocytes of Swiss albino mice

Chemical agent	Dose in mg/kg (number of administration)	Number of MN (‰, mean ±SE)	Range per 1000 PCE	Percent of PCE
PTP	185 x 2	8.5 ± 1.1*	6.5–11.5	49.8 ± 1.5
	72 x 2	2.6 ± 0.9	1–5	53.4 ± 1.1
PTB	100 x 2	16.7 ± 1.4*	13.5–20	44.8 ± 1.9*
	40 x 2	9.1 ± 0.9*	7.5–11.5	51.8 ± 1.3
	20 x 2	4.4 ± 0.6*	3–6	54.6 ± 0.9
Cyclophosphamide (positive control)	25 x 2	22.1 ± 1.7*	18–26	52.4 ± 1.4
DMSO (negative control)	0.2 ml x 2	2.0 ± 0.6	0–3	51.8 ± 1.7

*$p < 0.001$ compared to negative control; every group consisted of 5 mice; 2,000 PCEs were studied from each mouse.

doses used — $^1/_2$, $^1/_5$ and $^1/_{10}$ of LD_{50}. At equitoxic doses PTB induced more than 2–fold and 3.5–fold increased level of MN compared with PTP ($^1/_2$ and $^1/_5$ of LD_{50}, respectively). Cyclophosphamide, the drug permanently used in our laboratory as a positive control, induced 21.7 ‰ PCEs with MN, very close to our recent data [10, 11]. It should be noted that the acute toxicity of PTB for mice is close to that of cyclophosphamide — about 200 mg/kg [12, 13]. But, at the same time, cyclophosphamide is much more active MN inductor and induces significantly higher level of PCEs with MN after administration at doses equal to $^1/_8$ of LD_{50} than PTB at doses equal to $^1/_2$ of LD_{50} ($p < 0.02$).

Hence, we can conclude that PTB is more potent MN inductor than PTP both *in vivo* and *in vitro*.

It is noteworthy that the replacement of =NH group to =O group in chemical structure leads to substantial (1.85–fold) increase in acute toxicity and MN induction activity both *in vivo* and *in vitro*. It is well known that any changes in chemical structure may lead to substantial changes in biological properties (toxicity, mutagenicity, antitumor activity). The study of mutagenic activity of three quinoline–2–amine derivatives have shown that an addition of one methyl group to the chemical structure decreased mutagenic potency of the substance, and elimination of dihydro– group increased significantly the mutagenic activity of chemical in the Ames assay [14]. Substantial changes in cytotoxicity and genotoxicity depended on chemical structure was observed among 14 substituted 4–anilinoquinazolines [15]. Another good example are *cis*– and *trans*–platin. The first one is a potent antitumor agent with high toxic and mutagenic potency, while the second one possesses substantially less antitumor activity, toxicity and mutagenicity [16]. But the only difference between two compounds is the position of Pt in the compounds (*cis*– or *trans*–).

PTB possessing antitumor activity *in vitro*, comparatively low acute toxicity and MN induction activity is a good candidate for *in vivo* studies on antitumor action.

In conclusion, we obtained the data on relationship between chemical structure and genotoxicity of two newly synthesized pyrrolinone derivatives. It has been shown that L5178Y mouse lymphoma cells model is a good predictor of mutagenic action and toxicity of studied substances *in vivo*.

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