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ORIGINAL ARTICLE

Genotoxic activity of four newly synthesized pyrrolin-2-one derivatives

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Summary

Purpose: *To study the genotoxic activity of 4 newly synthesized derivatives of pyrrolin-2-one by means of the micronucleus (MN) assay both in vivo and in vitro, and the DNA-damaging activity of these substances by means of the comet assay on murine cells in vitro.*

Materials and methods: *The following compounds were studied: [3-(imidazolyl-1)-propylamide-4,5,5–trimethyl-pyrrolin-2-one] (IPA-TP); [3-cyclohexylamide-4,5,5 –trimethyl- pyrrolin-2-one](CH-TP); [3-piperonylamide-4,5,5–trimethyl-pyrrolin-2-one] (PA-TP); and [3-phenethylamide-4,5,5–trimethyl-pyrrolin-2-one] (PHA-TP). L5178Y mouse lymphoma cells were used to study the activity of the compounds by the MN and the comet assays. The acute toxicity and MN-inducing activity of the 4 compounds was assessed on Swiss albino mice.*

Results: *IPA-TP, PA-TP, and PHA-TP were very weak MN inducers in mouse lymphoma cells, which induced MN only at toxic for lymphoma cells concentrations. No dose-effect relationship was registered. CH-TP was tested at low concentration because of bad solubility and was not MN-inducer. IPA-TP and CH-TP were not active in the comet assay, while both PA-TP and PHA-TP were active. The study of acute toxicity showed the following results: LD_{50} of IPA-TP, CH-TP, PA-TP and PHA-TP were 460 mg/kg, 650 mg/kg, 370 mg/kg, and 350 mg/kg, respectively. The substances were studied using the MN assay on mouse bone marrow polychromatic erythrocytes (PCEs), and all of them were active only at doses equal to 1/2 of LD_{50}. The increase of bone marrow cells with MN was 2.5-5.8-fold compared with the background MN level. Lower doses (1/5 of LD_{50}) of all substances were not effective.*

Conclusion: *A good agreement between in vivo and in vitro genotoxicity was obtained. IPA-TP, PA-TP, PHA-TP with potential antitumor activity, comparatively low acute toxicity and genotoxicity are good candidates for in vivo studies of antitumor activity.*

Key words: comet assay, genotoxicity, L5178Y mouse lymphoma cells, micronucleus assay, pyrrolin-2-one derivatives, Swiss albino mice

Introduction

The creation of new antitumor drugs with low toxicity and high specific activity is an extremely important issue. It has been shown that some derivatives of pyrrolin-2-one possess antitumor activity *in vitro* (on 3 murine tumor cells lines: MG-22A – hepatoma, B-16 – melanoma, Neuro2A – neuroblastoma) [1]. Four derivatives of pyrrolin-2-one were synthesised recently [2]. A computer-based study of chemical structure - biological activity relationship has shown that the mentioned compounds can have antitumor activity [2]. 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.

L5178Y mouse lymphoma cells are a cell system suitable for assessing the mutagenic, clastogenic, aneugenic, and genotoxic (DNA-damaging activity) properties of various agents [3-5]. All of the compounds tested for mutagenicity should be studied using the mentioned cell system according to the US

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Environmental Protection Agency (EPA) and the United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (UKCOM) recommendations [6].

MN form from chromosomal fragments and/or whole chromosome that are not included in the main daughter nuclei during nuclear division [3]. Hence, scoring of MN provides a measure of both structural (clastogenic) and numerical (aneugenic) chromosomal aberrations [3]. The MN assay *in vivo* is widely used for the assessment of clastogenic and aneugenic effects of new compounds, and the mentioned test is used by the pharmaceutical industry in Europe, Japan and the USA for the evaluation of the mutagenic potential of new drugs [7]. This assay is recommended by EPA and UKCOM for evaluation of the mutagenic potential of various agents as well [6].

Single-cell gel electrophoresis (the comet assay) is a rapid and well-established test system for detecting DNA primary lesions [8].

The aim of the present work was to study the MN-inducing activity of 4 substances in bone marrow PCEs of mice and in L5178Y mouse lymphoma cells, and to evaluate the DNA-damaging activity of these substances by means of the comet assay in L5178Y mouse lymphoma cells.

Materials and methods

Chemicals. In 1993 Melikyan et al. proposed a new method of synthesis of substituted pyrrolinones [8]. New derivatives of pyrrolin-2-one were synthesized according to the proposed method. The following compounds (lactames) were studied: IPA-TP; CH-TP; PA-TP and PHA-TP.

Cell culture. L5178Y $tk^{+/-}$ mouse lymphoma cells were routinely cultured in suspension in RPMI-1640 medium supplemented with 98 u/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). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37° C.

In vitro micronucleus assay. In the experiments, exponentially growing L5178Y cells (1×10^6 cells in 5 ml medium) were treated with the substances at doses of 500 and 1,000 ng/ml overnight, and at doses of 1 and 10 μg/ml. Since we did not register either the toxic or the MN-inducing effect, we used another treatment schedule – incubation of the lymphoma cells with the 4 substances for 4 h at concentrations of 50-1000 μg/ml [5]. After removing of the chemicals by centrifugation and medium replacement, the cells were incubated for 18 h (expression time). As positive control mitomycin C (MMC) (50 μg/ml) and as negative (solvent) control DMSO were 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. The slides were washed twice with buffer and mounted for microscopy. The numbers of nuclei and MN were scored at a magnification of 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 the size of the main nucleus, and showed staining similar to the main nucleus.

Comet assay. This assay was performed according to Singh et al. method [9] with slight modifications. Exponentially growing L5178Y cells (1×10^6 cells in 5 ml medium) were treated with the 4 substances or solvent control (DMSO) for 4 h. The doses of the substances were chosen according to the active doses in the MN assay. CH-TP was tested at maximum soluble dose. As positive control ethyl methansulphonate (EMS) was used (300 μg/ml), and as negative control a solvent of the substances. After removing of the chemicals by centrifugation and medium replacement, the cells were incubated for 18 h (expression time). Then, cells (3×10^3/slide) were embedded in low melting agarose (0.5%) which was layered onto fully frozen 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 for 1h. The slides were then 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 3-5 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 analysed under 1250× magnification and using computer-aided image analysis. Images of 50 cells (25 from each slide) were evaluated using the software program NIH Image 1.54 (NIH, USA). The tail moment was evaluated and presented in arbitrary units.

In vivo micronucleus assay. To study the MN-inducing activity of the substances *in vivo* we first studied their acute toxicity using the Lorke's approach [10].

Swiss albino male mice (22-25 g) obtained from the Animal House of the Institute of Fine Organic Chemistry (Yerevan, Armenia) were used in the experiments on toxicity and mutagenicity. IPA-TP, PA-TP and PHA-TP were dissolved in DMSO (Sigma, USA), and were injected intraperitoneally into the mice. Because of limited solubility in DMSO, CH-TP was dispersed in 1% solution of starch, and administered by means of a tube into the stomach of the mice according to the recommendations for prescreening of new drugs [11]. To study the MN-inducing activity of the substances we used the protocol described by Kirkhart [12]. Each experimental group was consisted of 5 mice. All substances were administered to mice twice at 0 and 24h at doses equal to 1/2 and 1/5 of LD_{50}, and mice were sacrificed at 48 h. IPA-TP, PA-TP, PHA-TP were dissolved in DMSO and administered intraperitoneally. As positive control cyclophosphamide (Mosmedpreparati, Russia) was used at a dose of 25 mg/kg (dissolved in 0.2 ml saline), according to the schedule and route of administration of the substances. As negative control the vehicle of the substances was used (0.2 ml DMSO and 0.2 ml of starch). 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-Grunwald-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 PCEs was calculated among erythrocytes.

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

Results

Incubation of lymphoma cells with all substances at doses of 500 and 1,000 ng/ml overnight did not induce a significant increment of MN frequency (data not shown). No change of the number of cells was observed in comparison with controls. The increase of the doses of the substances in the same experimental protocol to 1.0 and 10 μg/ml again did not induce any significant increase in MN frequency in lymphoma cells (data not shown). In this case also no significant change of the number of cells was registered, and, hence, the studied substances were not toxic for cells. The results of two experiments showed that in the mentioned experimental conditions and doses all substances were not toxic or mutagenic.

As all 4 substances were not active in the mentioned experimental protocol, we applied another one. In this case the substances were incubated with lymphoma cells for 4 h, washed from the substances using centrifugation and replacement of cell medium,

Table 1. MN-inducing activity of IPA-TP, CH-TP and PA-TP 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*
IPA-TP	50	6.3±1.7	5-8	88	58
	100	8.7±1.2	8-10	86	78
	500	12.0±2.3*	11-15	79	57
	1000	16.7±2.3*	15-19	81	39
CH-TP	50	4.0±1.2	3-5	89	100
	100	5.7±2.3[1]	5-9	69	76
	500	5.0±2.3[1]	4-8	92	55
PA-TP	50	4.3±2.3	2-6	78	81
	100	6.7±1.2	6-8	76	89
	500	9.0±4.0	5-12	81	53
	1000	10.3±1.7*	9-12	91	45
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

[1]lot of crystals in cell medium; *p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

Table 2. MN-inducing activity of PHA-TP 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*
PHA-TP	100	9.0±2.9	7-12	87	84
	1000	10.7±1.7*	9-12	46	49
MMC (positive control)	50	69.7±15.6*	58-85	88	
DMSO (negative control)	1.0	4.7±1.7	3-6	100	

*p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

and then incubated for 18 h (expression time). The results are presented in Tables 1 and 2. The substances were studied for MN-inducing activity in a concentration range of 50-1000 μg/ml.50 μg/ml is the maximum dose CH-TP can be used in experiments because of bad solubility in DMSO. At higher CH-TP concentrations there were a lot of crystals in the cell medium. IPA-TP induced MN only at high doses (3- and 4-fold higher than in negative control at doses of 500 μg/ml and 1000 μg/ml, respectively).

It is interesting that, after 18 h incubation with lymphoma cells, IPA-TP significantly decreased the number of cells (57% and 39% at doses 500 μg/ml and 1000 μg/ml, respectively, compared with negative control –100%). Hence, we can conclude that IPA-TP is MN inducer only at doses close to toxic ones. The increase of IPA-TP concentration 10 and 20 times led to the increase of MN number 1.9 and 2.7 times, respectively. So, IPA-TP is a very weak MN inducer, functioning only at toxic concentrations for lymphoma cells. CH-TP did not induce any increase of the level of MN compared with negative control at all concentrations used. But as was mentioned above, 50 μg/ml was the highest concentration being used because of bad solubility. PA-TP induced significant increase of MN level only at highest toxic concentration for cells. The increase of concentration of the compound by 20 times in the cell medium led to the increment of MN only 2.4 times. It is noteworthy, that there was no difference in MN numbers induced by the compound at concentration of 500 μg/ml and 1000 μg/ml. Like IPA-TP and PA-TP, PHA-TP induced significant increase of MN number only at the toxic highest concentration used (1000 μg/ml). There was no difference in MN numbers induced by the compound at concentrations of 500 μg/ml and 1000 μg/ml, like in the case of PA-TP.

Table 3. DNA-damaging activity of IPA-TP and CH-TP in the comet assay (experiment 1)

Chemical agent	*Dose (μg/ml)*	*Tail moment (arbitrary units, mean ± SE)*
IPA-TP	500	2.28±0.21
CH-TP	50	1.25±0.28
EMS	300	14.16±0.61
DMSO	1	6.32±0.49

For abbreviations see text

Table 4. DNA-damaging activity of IPA-TP and CH-TP in the comet assay (experiment 2)

Chemical agent	*Dose (μg/ml)*	*Tail moment (arbitrary units, mean ± SE)*
IPA-TP	1000	0.88±0.08
CH-TP	50	3.07±0.29
EMS	300	14.16±0.61
DMSO	1	6.32±0.49

For abbreviations see text

Table 5. DNA-damaging activity of PHA-TP in the comet assay

Chemical agent	*Dose (μg/ml)*	*Tail moment (arbitrary units, mean ± SE)*
PHA-TP	1000	5.28±0.64*
EMS (positive control)	300	11.89±0.68*
DMSO (negative control)	1	0.72±0.18

*p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

Table 6. DNA-damaging activity of PA-TP and PHA-TP in the comet assay

Chemical agent	*Dose (μg/ml)*	*Tail moment (arbitrary units, mean ± SE)*
PA-TP	500	5.47±0.59*
	1000	6.27±2.39*
PHA-TP	500	4.49±1.83*
	1000	3.25±0.62*
EMS (positive control)	300	5.84±1.54*
DMSO (negative control)	1	0.99±0.17

*p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

All substances were studied using the comet assay (Tables 3-6). IPA-TP and CH-TP were not active. IPA-TP was tested at concentration, which induces significant increase of MN. CH-TP was tested at maximum available concentration due to bad solubility. Both PA-TP and PHA-TP were active at concentrations which induced significant increase of MN level in lymphoma cells. So we obtained good agreement between the results in MN assay and the comet assay for PA-TP and PHA-TP, but not for IPA-TP, which was MN inducer at concentrations of 500 μg/ml and 1000 μg/ml. It is noteworthy that PA-TP and PHA-TP induced significant increase of the number of MN only at concentration of 1000 μg/ml, but were active in the comet assay at 2-fold low concentrations. It was impossible to study the real potency of CH-TP because of its limited solubility.

All substances were studied for acute toxicity. For IPA-TP the following results were obtained: LD_{50}: 460 mg/kg (here and for all the 4 substances, 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 – 0/3; 370 mg/kg – 1/3; 600 mg/kg – 2/3). The following results were obtained for CH-TP: LD_{50} 650 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 – 2/3; 600 mg/kg – 1/3). For PA-TP the following results were obtained: LD_{50} is 370 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 – 1/3; 600 mg/kg – 3/3). For PHA-TP the following results were obtained: LD_{50} 350 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 – 2/3; 600 mg/kg – 3/3).

In MN test *in vivo*, all studied substances were active only at doses equal to 1/2 of LD_{50} (Tables 7 and 8). They increased the number of MN 5.3–, 2.5–, 4.3–, and 5.8-fold compared with appropriate negative controls, respectively. Lower doses (1/5 of LD_{50}) of all substances were not effective**.** There was no statistically significant difference between the numbers of MN induced by IPA-TP, PA-TP, PHA-TP at high doses. IPA-TP and PHA-TP at highest doses used decreased significantly the number of PCEs, meaning that at the mentioned doses both substances were tox-

Table 7. MN-inducing activity of IPA-TP, PA-TP and PHA-TP in bone marrow PCEs of Swiss albino mice

Chemical agent (LD_{50}, mg/kg)	*Dose in mg/kg (number of administrations)*	*Number of MN (‰, mean ±SE)*	*Range per 1000 PCEs*	*Percent of PCEs (mean ± SE)*
IPA-TP(460)	230×2	10.6±1.6*	7-14.5	45.0±1.5*
	92×2	3.1±0.8	1.5-5	51.8±1.3
PA-TP (370)	185×2	8.5±1.1*	6.5-11.5	49.8±1.5
	72×2	2.6±0.9	1-5	53.4±1.1
PHA-TP (350)	175×2	11.6±1.9*	5.5-14.5	46.2±1.3*
	70×2	2.9±0.9	1.0-5.0	54.0±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	51.8±1.7

*p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

Table 8. MN-inducing activity of CH-TP in bone marrow PCEs of Swiss albino mice

Chemical agent (LD_{50}, mg/kg)	*Dose in mg/kg (number of administrations)*	*Number of MN (‰, mean ±SE)*	*Range per 1000 PCEs*	*Percent of PCEs (mean ± SE)*
CH-TP(650)	325×2	5.6±1.6*	2-9.5	48.2±1.4
	130×2	3.6±0.8	1.0-5.5	52.8±1.3
Cyclophosphamide (positive control)	25×2	18.4±1.7*	13-21	54.2±1.0
Starch (negative control)	0.2 ml×2	2.2±0.6	1-3	54.0±1.5

*p <0.001 compared to negative control (Student's t-test)
For abbreviations see text

ic for hemopoietic cells. The other 2 compounds were not toxic for hemopoietic cells, even at high doses.

Discussion

IPA-TP, PA-TP, PHA-TP were very weak MN inducers, which induced MN only at toxic concentrations for lymphoma cells. No dose-effect relationship was registered. As for CH-TP, it was tested only at low concentration (because of weak solubility in DMSO) and was not MN inducer. PA-TP and PHA-TP, but not IPA-TP and CH-TP, induced DNA damage in lymphoma cells. Maybe at higher concentrations CH-TP might induce both MN and DNA damage in lymphoma cells. This case is a limitation of L5178Y mouse lymphoma cells and other *in vitro* cell systems. Based on the obtained data we can conclude that IPA-TP, PA-TP and PHA-TP are weak mutagens and genotoxins, which are active only at high, close to toxic doses. As for CH-TP, it is not genotoxic in *in vitro* assays, but is slightly mutagenic *in vivo* on mice.

It is well known that any changes in the chemical structure of a substance may lead to substantial changes in its biological properties (toxicity, mutagenicity, antitumor activity). The best examples 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 [13]. In addition, the first one is soluble in water and normal saline, while the second one is soluble only in DMSO. The only difference between the 2 compounds is the position of Pt (cis- or trans-). Another good example is quinoline-2-amine derivatives. It has been shown that the addition of one methyl group to the chemical structure decreased the mutagenic activity of the substance, and elimination of the dihydro- group increased significantly the mutagenicity of the compound in the Ames assay [14]. In our case changes in chemical structure of compounds did not lead to substantial changes in biological activity.

Change of -(imidazolyl-1)-propylamide group in the chemical structure of IPA-TP to piperonylamide, or phenethylamide in PA-TP and PHA-TP, respectively, didn't change substantially the acute toxicity (all mentioned compounds are low toxic – LD_{50} values are 350-460 mg/kg; values are between 101 and 1000 mg/kg), MN-inducing and DNA-damaging activities. Only replacement of -(imidazolyl-1)-propylamide group (IPA-TP) by -phenethylamide- one (CH-TP) led to the decrease of toxicity (although it is a substance with low toxicity as well, and in addition it was tested after gastric administration unlike the other 3 compounds which were tested after intraperitoneal injection), solubility, and MN-inducing activity as well.

A good agreement between *in vivo* and *in vitro* genotoxicity results was obtained.

IPA-TP, PA-TP, PHA-TP with high probability to possess antitumor activity, comparatively low acute toxicity and genotoxicity are good candidates for *in vivo* studies of antitumor activity.

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