Genotoxicity and mutagenicity of inosine pranobex

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Abstract

   Introduction: Inosine pranobex (Methisoprinol, ISO, Isoprinosine) is an immuno-modulatory antiviral drug that has been licensed since 1971 in several countries worldwide. In humans, the drug is approved for the treatment of viral infections, and it might also have therapeutic use in animals. The aims of the presented work were to investigate the genotoxicity of inosine pranobex on BALB/3T3 clone A1 and HepG2 cell lines and to elucidate its mutagenicity using the Ames test. **Material and Methods:** The BALB/3T3 clone A1 and HepG2 cells were incubated with inosine pranobex at concentrations from 0.1 to 1,000 µg/mL. The genotoxicity was determined by comet and micronucleus assays, and the mutagenicity was determined by Ames assay. **Results:** Inosine pranobex did not induce a significant dose-related increase in the number of comets or micronuclei in BALB/3T3 clone A1 and HepG2 cells. Moreover, based on the results of the Ames test, it was concluded that inosine pranobex is not mutagenic in the *Salmonella typhimurium* reverse mutation assay. **Conclusion:** Based on the results of a comet assay, micronucleus assay, and Ames test, it was concluded that inosine pranobex is neither genotoxic nor mutagenic.

Keywords: Inosine pranobex, Methisoprinol, genotoxicity, mutagenicity.

Introduction

   Inosine pranobex (Methiosprinol, ISO, Isoprinosine) is a synthetic active substance composed of the p-acetamidobenzoate salt of N,N-dimethylamino-2-propanol and inosine at a 3:1 molar ratio (3). The drug exerts both modest antiviral (3, 17) and immuno-stimulatory and immuno-restorative effects (16, 25). It has been found effective in subacute sclerosing panencephalitis (5), herpes virus infections (17), and some other viral infections (3, 17). Treatment with inosine pranobex was also beneficial for HIV-infected patients not only due to the stimulation of the immune system but also due to its effect on folate synthesis being helpful against *Pneumocystis jiroveci* (19). Moreover, studies in animal models showed that inosine pranobex restored defective or suppressed immunity resulting, for example, from viral infections, autoimmunity, or aging (15, 16, 26). There is opportunity for the veterinary health sector to adopt this new active substance into practice.

   The safety profile of inosine pranobex in humans has been shown through clinical trials for a few indications and populations (11, 12, 18, 29). The toxicological effects of this product have not been deeply studied.

   Inosine pranobex stimulates a non-specific immune response that is independent of the specific viral antigen responsible for IL-1 production. In clinical trials, inosine pranobex has been shown to induce a type 1 T helper cell-type response in mitogen- or antigen-activated cells. This response initiates T-lymphocyte maturation and differentiation and potentiates induced lympho-proliferative responses (11, 20, 29). In like manner, this compound modulates T-lymphocyte and NK cell cytotoxicity and CD8^{+} suppressor and CD4^{+} helper cell functions and increases the number of immunoglobulin γ and complement surface markers (11, 29). The drug increases cytokine interleukin IL-1 and IL-2 production and upregulates the expression of the IL-2 receptor *in vitro* (11, 20).

Regulatory authorities for pharmaceuticals require an extensive assessment of their genotoxic potential. Comprehensive reviews have shown that many substances that are mutagenic in the bacterial reverse mutation (Ames) test are also rodent carcinogens. The use of mammalian cells in *in vitro* tests increases the sensitivity for the detection of rodent carcinogens and extends the spectrum of genetic incidences detected. A battery approach is reasonable because no single test is able to detect all genotoxic mechanisms. The general features of a standard test battery are as follows:

1. assessment of mutagenicity in a bacterial reverse gene mutation test by Ames;

2. genotoxicity evaluated in mammalian cells *in vitro* and/or *in vivo* by comet and micronuclei assays (10).

The aim of the presented work was to investigate the genotoxicity and mutagenicity of inosine pranobex on BALB/3T3 clone A1 and HepG2 cells. HepG2 cells have been adapted to the micronucleus assay and many publications suggest that a comet assay can also be performed on HepG2 cells. Accordingly, the micronucleus assay and comet assay were performed. Evaluation of the mutagenic activity of inosine pranobex was investigated in the *Salmonella typhimurium* reverse mutation assay (Ames test) (7, 8).

Material and Methods

Chemicals. Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), heat-inactivated Calf Bovine Serum (CBS), and Foetal Bovine Serum (FBS) were purchased from the American Type Culture Collection (ATCC) (USA) and Antibiotic Antimycotic solution (10,000 U/mL of penicillin, 10 mg/mL of streptomycin, and 25 μg/mL of amphotericin B), cytochalasin B, and acridine orange were sourced from Sigma-Aldrich (USA). An OxiSelect 96–well comet assay kit from Cell Biolabs (USA) was used, and a Muta-Chromo Plate bacterial strain kit from Environmental Bio-Detection Products (EBPI) (Canada) was selected. Inosine pranobex was purchased from ABC Farmaceutici S.p.A. (Italy).

Cell culture and treatment. Mouse embryo fibroblast BALB/3T3 clone A31 cells (ATCC CCL-163) and liver cancer HepG2 cells (ATCC HB-8065) were obtained from the ATCC. The cells were cultured as adherent monolayers in plastic tissue culture dishes in DMEM and EMEM, respectively, supplemented with Antibiotic Antimycotic solution (1 mL per 100 mL of cell culture medium) and 10% (v/v) heat-inactivated CBS and FBS, respectively. The cells were maintained at 37ºC in a humidified incubator, with 5% CO_2 in the atmosphere (24).

Inosine pranobex (Methisoprinol, MTP 3110261) was dissolved in deionised water at the concentration of 10 mg/mL. The final concentration was obtained by dilution in the culture medium, supplemented with serum and antibiotics (24).

In order to perform the comet and micronucleus assays, the cells were cultured on 96-well plates (2×10^5 cells/mL) in 100 μL culture medium, supplemented with serum and Antibiotic Antimycotic solution (1 mL per 100 mL of cell culture medium). After 24 h, the medium was exchanged for fresh media supplemented with inosine pranobex at final concentrations of 0.1, 0.5, 1.0, 5, 10, 50, 100, 500, and 1,000 μg/mL in a volume of 100 μL. All concentrations mentioned above were the final concentrations in the incubations. After 24 h of incubation, the comet and micronucleus assays were performed.

Comet assay. The comet assay was performed according to the manufacturer's original instructions using an OxiSelect 96- well comet assay kit.

The comet assay is a technique for measuring DNA damage in individual cells. Under an electrophoretic field, damaged cellular DNA is separated from intact DNA, yielding a classic "comet tail" shape visible under a microscope. The extent of DNA damage is visually estimated by comet tail measurement. Typical measurements are the percentage of DNA in the tail (normalised to total cell DNA) and tail moment. Tail moment is a damage measure combining the amount of DNA in the tail with the distance of migration (severity of damage).

Lysis buffer, alkaline solution, TBE electrophoresis solution and visa green dye were prepared according to the manufacturer's instruction and stored at 4ºC. Comet agarose was heated at 90–95ºC in a water bath for 20 min, following which the bottle with agarose was transferred to a water bath at 37ºC. A 96-well comet slide was heated at 37ºC for 15 min to promote agarose attachment. Cells were trypsinised, centrifuged at $700 \times g$ for 2 min, and the cell pellet washed once with ice-cold PBS (without Mg^{2+} or Ca^{2+}) and centrifuged once again. Finally, the cells were resuspended at 1×10^5 cells/mL in ice-cold PBS (without Mg^{2+} or Ca^{2+}). In a pre-warmed 96-well plate, cell samples with agarose were combined at 1:10 ratio (v/v). The mixture of cells and agarose was titrated to mix, and immediately 20 μL per well were pipetted onto the pre-warmed comet slide using a multi-channel micro-pipette. The slide was kept in the dark for 15 min at 4ºC. Next the slide was transferred to a small container containing pre-chilled lysis buffer and incubated in the dark again for 30–60 min at 4ºC. The lysis buffer was carefully aspirated from the container and replaced with pre-chilled alkaline solution and the slide was incubated in the dark in the solution for the final time for 30 min at 4ºC. Next, the alkaline aspirate solution from the container was aspirated and replaced with pre-chilled TBE electrophoresis solution. The slide was immersed in TBE electrophoresis solution for 5 min. The slide was kept perfectly horizontal and carefully transferred to a horizontal electrophoresis chamber, which was filled with cold TBE electrophoresis solution until the buffer level covered the slide. Voltage was applied to the chamber for 10–15 min at 1 volt/cm. The slide was subsequently carefully transferred from the electrophoresis chamber to a clean

small container containing pre-chilled DI H_2O where it was immersed for 2 min. Next, the water was exchanged for cold 70% ethanol. The slide was removed horizontally to allow it to air dry, and once the agarose and slide were completely dry, 50 µL/well of diluted vista green DNA dye was added. The slide was analysed to the extent of 100 cells per sample under epifluorescence microscopy using an FITC filter. The cells were analysed by Leica Application Suite version 4.4 (Leica Microsystems, Germany) (24). Experiments were performed independently six times.

Micronucleus assay. The *in vitro* Micronucleus assay was performed according to OECD guideline 487 (8).

The test detects chromosome breakage and loss by measuring the formation of micronuclei. These are small membrane bound fragments or whole chromosomes, which are unable to attach to the spindle at mitosis and appear as small bodies within the cell. Cells are treated with cytochalasin B which blocks cell division but not nuclear division, resulting in cells containing two or more nuclei. The proportion of cells that have undergone cell division and suffered chromosome breakage or loss, resulting in micronucleus formation can then be counted, giving a representation of the genotoxicity of the test item.

After 24 h of incubation with inosine pranobex, the medium was exchanged for fresh medium supplemented with cytochalasin B at a final concentration of 3 µg/mL. After 24 h of incubation, the cells were stained with acridine orange. For this purpose, cells were washed once with PBS and resuspended in acridine orange solution at a concentration of 50 µg/mL. The slide was observed under epifluorescence microscopy using an acridine orange filter. A total of 1,000 cells were analysed per sample. The results were analysed by calculating the binucleated micronucleated cells' frequency as the number of binucleated cells containing one or more micronuclei per 1,000 binucleated cells. The cells were analysed by Leica Application Suite ver. 4.4 (24). Experiments were performed independently six times.

Ames assay. The Ames test was performed according to the manufacturer's instructions provided for the Muta-ChromoPlate Bacterial Strain Kit, and according to OECD Guideline 471 (7).

The Ames assay is based on the assumption that the mutagens lead to mutations in many genes. Some of these mutations cause the reversal of ability to synthesise histidine (reverse mutations). The his-*Salmonella typhimurium* strains are mixed with the tested chemicals, and next the bacteria are incubated in the growth medium without histidine. Following this, the ratio is calculated between the surviving control bacteria not exposed to the tested substance and the surviving experimental bacteria that were exposed to it. If there are many surviving bacteria within the experimental (exposed) group, it is considered proof of mutagenicity of the tested substance.

There are several different mutant strains of *Salmonella typhimurium* that have different mutations in their DNA:
• TA 1535 has a base-pair substitution resulting in a missense mutation in the gene-encoding of the first enzyme in the histidine biosynthesis pathway. A -GGG- (proline) substitutes for a -GAG- (leucine) in the wild-type organism.
• TA 100 contains the same mutation identified in TA 1535. Its mutagenic specificity is like that of the base-pair substitutions mutagen tester strain.
• TA 98 has mutagenic specificity similar to that of the frameshift mutagen tester strain.
• TA 102 has an ochre mutation (-TAA-), which means that it has a non-sense mutation, in place of the -CAA- present in the wild-type organism. Unlike the other his-strains, this strain has an A: T base pair at the site of reversions.
• TA 97 contains an added cytosine, resulting in a run of six cytosines at the mutated site in the histidine D gene. Its mutagenic specificity is like that of the frameshift mutagen tester strain.

Between 16 and 18 h prior to the experiment, nutrient broths were transferred to the vials of lyophilised *Salmonella typhimurium* mutant strains (TA97a, TA98, TA100, TA102, and TA1535) and were incubated in the bottle overnight at 37℃.

The final concentrations of inosine pranobex (0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1,000 µg/mL) were obtained by dilution in sterile distilled water. The samples to be tested were sterilised using a 0.22 µm membrane filter. The reaction mixture was prepared according to the manufacturer's instructions as follows: 43.24 mL (A) + 9.5 mL (B) + 4.76 mL (C) + 2.38 mL (D) + 0.12 mL (E) where A is Davis-Mingioli salts (concentrate), B is D-glucose, C is bromocresol purple, D is D-biotin, and E is L-histidine.

A volume of 2.5 mL of reaction mixture was aseptically dispensed to each sterile tube. Next 17.5 mL of the sterile filtered inosine pranobex (at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 µg/mL) was added. The *S. typhimurium* mutant strains were added to each tube containing the material to be tested, in 5 µL amounts. Next, 20 mL aliquots of the mixture were dispensed into each well of a 96-well micro-titration plate using a multi-channel pipette. The plates were covered with a lid, sealed in sterile airtight plastic bag(s) to prevent evaporation, and incubated at 37°C for six days. The number of positive wells for each plate was recorded. The background plates (*i.e.*, with inosine pranobex added) showed the level of spontaneous or background mutation of the assay organisms. The results of each treatment plate were scored against the background mutation. The assay was conducted in the absence and presence of a metabolising system 5% (v/v) S9 (24) with the use of mutagen substances (Table 1).

Table 1. Positive control substances used in Ames assay

Strain	Substance
TA97a	9AA (9- aminoacridine)
TA98	2-NF (2-nitrofluorene)
TA100	NaN_3 (sodium azide)
TA102,	Cumene hydroperoxide
TA1535	NaN_3 (sodium azide)

Statistical analysis. The results were expressed as mean ±SD and the data were analysed using Student's *t*-test and Statistica software (Tibco, USA). In all cases $P < 0.05$ was considered significant.

Results

At concentrations from 0.1 to 500 µg/mL inosine pranobex did not induce a significant dose-related increase in DNA damage in either cell line (Table 2, Figs 1a and b). Table 2 and Figs 2a and b show that there was a concentration-dependent increase with statistical significance in the number of comets in cells incubated with inosine pranobex at concentrations of 1,000 µg/mL.

Table 2. The effect of inosine pranobex on comet formation

Concentration of inosine pranobex (µg/mL)	Percentage tail DNA	
	BALB/3T3 clone A31 cells	HepG2 cells
0	1	1
0.1	1	1
0.5	1	2
1	1	1
5	1	1
10	2 ± 0.1	1
50	2 ± 0.1	2 ± 0.1
100	2 ± 0.1	3 ± 0.1
500	3 ± 0.2	2 ± 0.1
1,000	4 ± 0.2*	5 ± 0.3*

* $P < 0.05$, compared with control

At concentrations from 0.1 to 500 µg/mL, inosine pranobex did not induce a significant dose-related increase in the number of micronuclei in either cell line (Table 3). Table 3 and Figs 3 and 4 show that there was a concentration-dependent increase with statistical significance in the number of micronuclei in cells incubated with inosine pranobex at concentrations of 1,000 µg/mL.
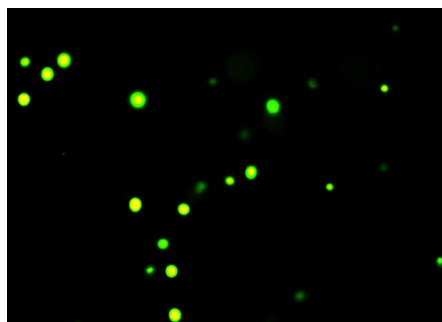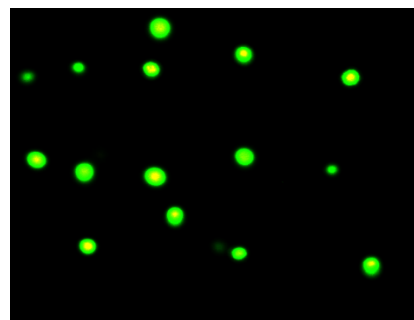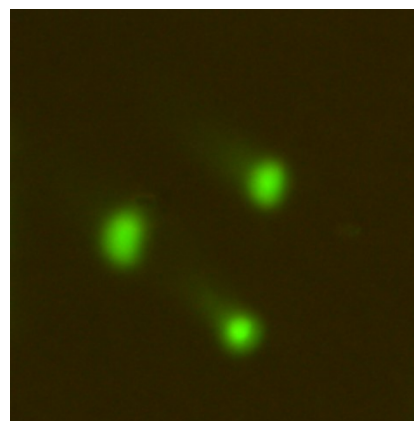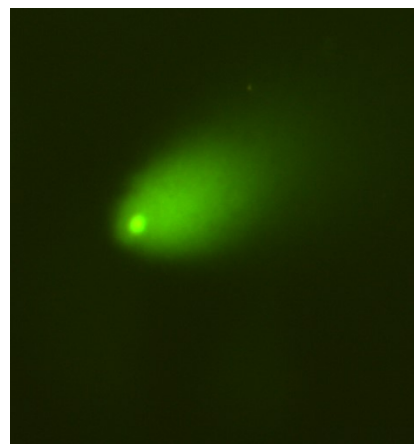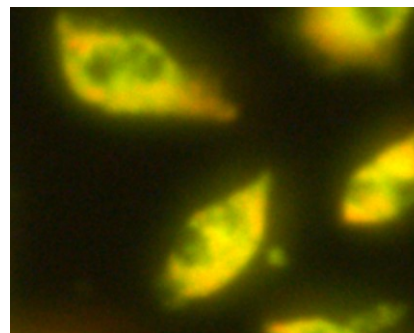

Fig. 1a. HepG2 cells: Comet assay images of untreated (control) cells, 100×

Fig. 1b. BALB/3T3 clone A31 cells: Comet assay images of untreated (control) cells, 100×

Fig. 2a. HepG2 cells: Comets in cells treated with inosine pranobex at concentration of 1,000 µg/mL, 100×

Fig. 2b. BALB/3T clone A31 cells: Comet in a cell treated with inosine pranobex at concentration of 1,000 µg/mL, 100×

Fig. 3. HepG2 cells: Micronucleus in cells treated with inosine pranobex l at concentration of 1,000 µg/mL, 100×

Table 3. The effect of Inosine pranobex on number of cells with micronuclei

Concentration of Inosine pranobex (µg/mL)	BNMN‰	
	BALB/3T3 clone A31 cells	HepG2 cells
0	0	1
0.1	1	0
0.5	2 ± 0.1	3 ± 0.1
1	2 ± 0.1	2 ± 0.1
5	2 ± 0.1	4 ± 0.2
10	3 ± 0.2	3 ± 0.2
50	4 ± 0.2	2 ± 0.1
100	4 ± 0.3	4 ± 0.2
500	5 ± 0.3	6 ± 0.3
1,000	6 ± 0.4*	10 ± 0.9*

BNMN‰ – binucleated micronucleated cells containing one or more micronuclei per 1,000 binucleated cells.
* $P < 0.05$, compared with control

Table 4. Bacterial reverse mutation results (mean number of revertant colonies per plate) in *Salmonella typhimurium* TA97a, TA98, TA100, TA102, and TA1535 strains after incubation with inosine pranobex detected with Ames assay

Concentration of inosine pranobex (µg/mL)	TA97a		TA98		TA100		TA102		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.1	4 ± 0.1	9 ± 0.7*	6 ± 0.1*	7 ± 0.3*	12 ± 1*	15 ± 1*	11 ± 1*	11 ± 1*	10 ± 0.7*	11 ± 1*
0.5	2 ± 0.1	5 ± 0.3	2 ± 0.1	3 ± 0.1	12 ± 1*	12 ± 1*	12 ± 1*	12 ± 1*	12 ± 1*	12 ± 1*
1	3 ± 0.2	4 ± 0.1	4 ± 0.2	6 ± 0.3*	2 ± 0.1	5 ± 0.1*	3 ± 0.1	3 ± 0.1	3 ± 0.1	6 ± 0.2*
5	5 ± 0.3	7 ± 0.3*	9 ± 0.5*	9 ± 0.4*	4 ± 0.1	6 ± 0.12*	4 ± 0.1	4 ± 0.1	4 ± 0.1	4 ± 0.1
10	6 ± 0.4	6 ± 0.3	6 ± 0.3*	7 ± 0.3*	8 ± 0.5*	9 ± 0.3*	8 ± 0.4*	9 ± 0.5*	8 ± 0.5*	9 ± 0.4*
50	5 ± 0.3	8 ± 0.5*	6 ± 0.4*	7 ± 0.2*	11 ± 1*	11 ± 1*	9 ± 0.6*	9 ± 0.4*	11 ± 0.9*	12 ± 1*
100	6 ± 0.4	6 ± 0.3	7 ± 0.3*	7 ± 0.3*	7 ± 0.4*	7 ± 0.4*	9 ± 0.7*	9 ± 0.5*	9 ± 0.5*	9 ± 0.6*
500	9 ± 0.6	9 ± 0.4*	11 ± 0.8*	11 ± 0.8*	11 ± 1*	11 ± 1*	10 ± 1*	10 ± 0.6*	9 ± 0.6*	10 ± 0.8*
1,000	8 ± 0.7*	8 ± 0.5*	5 ± 0.2*	7 ± 0.4*	9 ± 0.5*	9 ± 0.3*	9 ± 0.4*	9 ± 0.5*	9 ± 0.7*	9 ± 0.6*
9AA	43	43								
2-NF			69	69						
NaN₃					85	85				
Cumene hydroperoxide							84	84		
NaN₃									85	85

9AA – 9- aminoacridine, 2-NF – 2-nitrofluorene, NaN₃ – sodium azide
* $P < 0.05$ compared with control

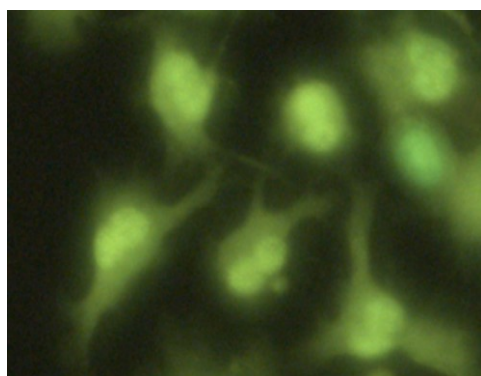**Fig. 4.** BALB/3T3 cells: Micronucleus in cells treated with inosine pranobex at concentration of 1,000 µg/mL, 100×

Inosine pranobex did not induce a dose-related increase in the number of revertant (His+) colonies in any of the five tester strains (TA1535, TA98, TA100, TA 97, and 102) in the absence or presence of S9-metabolic activation. All bacterial strains showed negative responses over the entire dose range (Table 4).

Discussion

Based on the results of the comet assay, micronucleus assay, and Ames test it has been concluded that inosine pranobex is neither genotoxic nor mutagenic.

Inosine pranobex is a synthetic active substance, consisting of inosine and p-acetamidobenzoate salt of N,N-dimethylamino-2-propanol (3). The oral dose in mucocutaneous *Herpes simplex* is 1 g four times daily for 7 to 14 days. An oral dose of 1 g three times daily is given for 14 to 28 days as an adjunct to standard topical treatment for genital warts. In subacute sclerosing panencephalitis, the oral dose is 50 to 100 mg/kg daily in divided doses given every 4 h. Inosine pranobex is

rapidly absorbed from the gastrointestinal tract with peak plasma concentrations occurring 1 h after an oral dose. Peak blood levels of N,N-dimethylamino-isopropanol (Dip) and p-acetamidobenzoic acid (PAcBA) reach approximately 4 and 7 μg/mL respectively 1 h after administration of 1 g of inosine pranobex orally. It is also rapidly metabolised, with a plasma half-life of 50 min after *per os* administration and 3 min after intravenous administration. In humans, the major excretion product of inosine is uric acid other components undergo oxidation and glucuronidation, and the metabolites are excreted in the urine. Inosine pranobex is an immuno-modulating agent which potentiates T-lymphocyte and phagocytic cell function (27, 28). It induces the gene expression of phenotypic markers of differentiation on immature precursor T cells, enhances helper or suppressor T-cell functions, and increases the production of TNF-β (2). The likely mechanism of inosine pranobex-mediated immune modulation should be further investigated *in vitro* (20). It has been proved that inosine pranobex has a comparatively low degree of both acute and chronic toxicity in both rodent and non-rodent species (23). The safety profile of inosine pranobex has been established through clinical trials for several populations (11, 12, 18). It was shown that the compound is of low toxicity, and that doses of the active substance 10 times higher than the therapeutically used dose had no cytotoxic nor immunosuppressive action (6, 29).

To establish the safety of a veterinary medicinal product, a number of toxicological studies are recommended, including investigation of any possible risk from genotoxic activity. Many substances have a genotoxic mode of action and it is wise to regard any under investigation as potential genotoxicants. In addition, substances causing reproductive and/or developmental toxicity may have a mode of action that involves genotoxic mechanisms. Sensitive tools for high-throughput toxicity screening are cultures of human cell lines, which can reduce the need for toxicological testing in animals (14). The human hepato-blastoma HepG2 cells have been well described (21) and extensively used as an *in vitro* toxicity model (4). It is proved that the sensitivity of cytotoxicity assay in HepG2 cells is 85% with a specificity of 98%. HepG2 cells were capable of detecting 75% of 60 tested substances with well-known toxicity (22). They have also been used to differentiate between genotoxic and non-genotoxic carcinogens. Literature demonstrates that HepG2 cells represent a precious *in vitro* tool for toxicity screening of active substances.

Registration of veterinary medicinal products requires assessment of their genotoxic features. Genotoxicity investigations are an integral part of regulatory toxicity evaluation in most European countries. Because there is no possibility of detecting all relevant genotoxic end-points in one single test a battery of tests for genotoxicity conducted in *in vitro* and *in vivo*

conditions is recommended by regulators. The recommended tests include *in vitro* assays for gene mutation in *Salmonella typhimurium* and in mammalian cells. An *in vitro* test for chromosomal damage in mammalian cells is recommended, either a micronucleus or metaphase chromosomal aberration assay. To the same end, an *in vivo* mammalian erythrocyte micronucleus test or *in vivo* mammalian bone marrow chromosome aberration test should also take place (7).

A sensitive and simple method for detecting DNA damage in individual cells is the comet assay or single cell gel electrophoresis (SCGE) assay. The comet assay is based upon the movement of labile nuclear DNA through an agarose gel when an electrical field is applied. The undamaged DNA retains a highly organised association with proteins in the nucleus, however, when the DNA is damaged, this organisation is disrupted. The SCGE assay's advantages over other genotoxicity tests are its requirement for small numbers of cells per sample, proven sensitivity for detecting low levels of DNA damage, flexibility, low costs, simplicity of application, and the short time needed to complete the test (25).

The micronucleus assay is an important part of genotoxicity testing. Micronucleus formation is evidence of genotoxicity. Micronuclei are chromatin-containing bodies that represent fragments or whole chromosomes that were not incorporated into a daughter cell nucleus at the end of mitosis. The aim of the assay is detection of substances which can induce chromosome damage causing micronuclei formation in interphase cells. This test is an alternative method to the *in vitro* chromosomal aberration assay. Studies described in literature show a high level of compatibility between these assays. Besides compatibility being in its favour, the *in vitro* micronucleus assay is also rapid, simpler, and has more statistical power. Moreover, this assay can also detect aneuploid-inducing substances which are very difficult to detect with the *in vitro* chromosomal aberration assay (8).

The identification of substances able to induce mutations is the most important in safety assessment, since mutagenic substances can potentially damage the germ line, lead to mutations in future generations, and also cause cancer. Gene mutations can be measured easily in bacteria. The Ames test, performed with the use of *Salmonella typhimurium*, is a widely used assay for the identification of substances that can induce gene mutations.

The Ames test typically uses TA 98, TA 100, TA 1535, TA 102, and TA 97 strains of *Salmonella typhimurium*, each carrying different mutations in various genes that render the bacteria unable to synthesise the essential amino acid histidine. If a compound induces mutations in these particular genes, it can restore gene function, allowing the cells to regain the capacity to synthesise histidine and therefore grow in its absence ("reversion assay"). The *Salmonella*

typhimurium strains have different mutations in the histidine operon and are designed to be responsive to mutagenic chemicals or drugs (1, 9, 13).

In the available literature there are no results of any research on the impact of inosine pranobex on genotoxicity and mutagenicity. In our study, inosine pranobex did not induce a significant dose-related increase in the number of comets or micronuclei in HepG2 or BALB/3T3 clone A1 cells. Moreover, based on the results of Ames tests, it has been concluded that inosine pranobex is not mutagenic in the *Salmonella typhimurium* reverse mutation assay.

Conflict of Interest Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: None required.

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