



Mutagenicity and genotoxicity of isatin in mammalian cells *in vivo*

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ABSTRACT

Isatin (1H-indole-2,3-dione) is a synthetically versatile substrate used for the synthesis of heterocyclic compounds and as a raw material for drug synthesis. Isatin and its derivatives demonstrate anticonvulsant, antibacterial, antifungal, antiviral, and anticancer properties. We evaluated the genotoxic and mutagenic effects of acute (24 h) and repeated (14 d) exposure to isatin *in vivo*, using the comet assay and the micronucleus test. Three doses (50, 100, and 150 mg/kg b.w.) were administered to mice via gavage. Doses were selected according to the LD₅₀ of isatin, estimated in a preliminary test to be 1 g/kg b.w. To evaluate the results, parametric (ANOVA/Tukey) and non-parametric (Kruskal–Wallis/Dunn's *post hoc* test) tests were used, according to the nature of the data distribution. At all doses (50, 100 and 150 mg/kg b.w.), after acute treatment with isatin, alterations in DNA migration (comet assay) were not observed and mutagenic effects were not seen (micronucleus test on peripheral blood cells). After repeated doses, only the highest dose of isatin (150 mg/kg b.w.) induced alterations in the DNA that gave rise to micronuclei in the bone marrow and peripheral blood cells of the mice. Our results show that the mutagenic and genotoxic effects of isatin depend on dose and on period of exposure.

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1. Introduction

Natural products, including medicinal plants, have an important role in the discovery of novel compounds for drug development [1], especially in developing countries [2]. However, medicinal plants may be consumed with little or no evidence of pharmacological properties. Many medicinal plants have substances that are potentially antagonistic to secondary metabolites; for this reason, they should be used with caution and with an appreciation of their toxicological risks [3].

Alkaloids are of great interest in medicine and are among the best-characterized secondary metabolites in pharmaceutical chemistry [4,5]. Isatin (1H-indole-2,3-dione) was discovered by Erdmann and Laurent in 1840 as a product of the oxidation of indigo with nitric and chromic acids [6]; it is a synthetically versatile substrate used for the synthesis of a variety of heterocyclic compounds, such as indoles and quinolines, and as a raw material for drug synthesis. Silva et al. [6] and Vine et al. [7] reported that isatin is found in plants of the *Isatis* genus, *Couroupita guianensis* Aubl. and *Calanthe discolor* Lindl., and is secreted from the parotid gland of *Bufo* frogs. Various derivatives of isatin have also been identified in

plants, for example, the melosatin alkaloids (methoxy phenylpentyl isatins) obtained from the Caribbean tumorigenic plant *Melochia tomentosa*, fungi, and marine mollusks.

Isatin and its derivatives demonstrate diverse biological and pharmacological activities, including anticonvulsant, antineoplastic, antimicrobial and antiviral properties [6]. A recent review of isatin focused on its cytotoxic and anticancer properties [7]. However, *in vivo* studies of the genotoxic and mutagenic potential of isatin are lacking. The aim of our work was to evaluate, using the comet assay and the micronucleus test, the genotoxic and mutagenic potentials of isatin, administered to mice in acute or repeated doses.

2. Materials and methods

2.1. Test compound

Isatin P.A. (≥99.0%) was purchased from Fluka (CAS: 91-56-5) and diluted in distilled water. The LD₅₀ of isatin (1 g/kg b.w.) was estimated using the acute oral toxicity test, according to OECD protocols [8]. The doses selected for the genotoxicity and mutagenicity tests (50, 100, and 150 mg/kg b.w.) correspond to 5, 10, and 15% of the estimated LD₅₀. For each treatment, isatin was administered by gavage to groups of eight animals. These doses are similar to doses of isatin or its derivatives that have been used in animal models to investigate biological activities, such as anticonvulsant effects (30–200 mg/kg b.w.) [9–11], anxiogenic and sedative effects (10–300 mg/kg b.w.) [12,13], activity against Ehrlich's ascites carcinoma (50 mg/kg b.w.) [14], and amelioration of Parkinson's disease (100 mg/kg b.w.) [15].

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2.2. Positive control chemical

Cyclophosphamide (CP; CAS:50-18-0) an alkylating agent that induces chromosomal damage in bone marrow and peripheral blood cells [16], was purchased from Sigma Chemical Co. (St. Louis, MO), diluted in distilled water, and used as a positive control.

2.3. Animals

Male and female albino Swiss mice (*Mus musculus*), 7–8 wk old and ~30 g at the beginning of the experiments, were obtained from the breeding colonies of the State University of Londrina (Paraná, Brazil) and housed individually in polypropylene cages, with *ad lib.* access to food and water throughout the treatment period. All experiments were performed according to the standards of the *Canadian Council on Animal Care* [17]. For the acute and repeated dose studies, two groups of 40 animals were used for each test.

2.4. Experimental design

To investigate the genotoxic and mutagenic effects in mice after acute or repeated exposure to isatin, mice were divided into groups of eight mice (four males and four females) for each treatment. Mice were treated with each solution of isatin, 0.1 ml for every 10 g of body weight (b.w.), at three doses: 50, 100, or 150 mg/kg b.w. After the treatments, the animals were anesthetized and euthanized with a 1:1 mixture of 2% xylazine HCl and 10% ketamine HCl. All animals were weighed at the beginning and end of each treatment.

2.4.1. Acute treatment

To assess the genotoxic and mutagenic effects of isatin, the animals were administered a single dose of one of the three concentrations, by gavage, for 24 h. The negative control group received only water, by gavage, and the positive control group was treated with CP (50 mg/kg b.w.) intraperitoneally (i.p.) for 24 h.

For the peripheral blood cell micronucleus test, blood samples were obtained from the tail veins of mice, before gavage (T0) and 36 (T1), 48 (T2), and 72 (T3) h post-gavage. For the comet assay, tail vein blood leukocyte samples were obtained 4 (T1) and 24 h (T2) post-gavage, according to the protocols of the OECD [18] and Tice et al. [19], respectively.

2.4.2. Repeated-dose treatment (14 d)

For the evaluation of the genotoxic, cytotoxic, and mutagenic effects of isatin, the animals received, via gavage, the same doses of the drug used in the acute arm of the study, but for 14 consecutive days. All doses were administered daily, at 24 h intervals. The negative control group received drinking water daily for two weeks, via gavage; the positive control group was treated with CP (50 mg/kg b.w., i.p.) on day 14. CP was administered in a single dose. According to Anderson et al. [16], OECD [18], and Hayashi et al. [20], mouse bone marrow cells and peripheral lymphocytes have similar sensitivity in acute and chronic exposure (28 d) to CP, and it is acceptable that the positive control be administered by a route different from that of the test substance, and sampled at an appropriate time after a single treatment.

For the micronucleus test of bone marrow cells, the animals were euthanized 24 h after the last treatment. For the comet and micronucleus analyses of peripheral blood cells, samples of blood were obtained 4 and 36 h, respectively, after the last treatment, according to the protocols of Tice et al. [19] and the OECD [18], respectively.

Appropriate statistical tests were used for each assay. The linear-trend test was carried out by analysis of the mean values obtained from three assays: comet assay (score and damaged cells), MNPCes, and MNRETS.

2.5. Bone marrow micronucleus test

The micronucleus test was performed using the method of Schmid [21], with some modifications. Animals were euthanized 24 h after treatment; both femurs were immediately removed and the bones were freed from the muscles. The epiphyses were cut and the bone marrow was flushed with fetal calf serum (Gibco). The cell suspension was centrifuged at 900 rpm for 10 min and the supernatant was discarded. A small drop of the resuspended cell pellet was spread onto a clean glass slide, which was air-dried and fixed in absolute methanol for 10 min. The smears were stained with Leishman's eosin blue for detection of micronucleated polychromatic erythrocytes (MNPCE). For each animal, three slides were prepared, and 2000 polychromatic erythrocytes (PCE) were counted to determine the frequency of MNPCE. To evaluate bone marrow toxicity, the ratio of PCE/PCE + NCE (normochromatic erythrocytes) was calculated by counting a total of 200 erythrocytes on these slides.

According to the Bartlett test, the data were not homogeneous with regard to variance, so the Kruskal–Wallis test, followed by Dunn's *post hoc* test, was used to analyze the results.

2.6. Micronucleus test of peripheral blood cells

The micronucleus test on peripheral blood cells was performed as described by Hayashi et al. [22], who used slides pre-stained with acridine orange (CAS: 494-38-2). Glass slides were heated to 70 °C on a hot-plate, and an aqueous solution of the dye (10 µL, 1 mg/mL) was placed on each slide and spread evenly over the surface with the end of a second well-cleaned slide. After drying, the slides were kept in the dark at room temperature for at least 24 h.

An internal control was established for each animal by preparing a test slide with a drop of blood taken from its tail before the first treatment (T₀). After treatment, blood (5 µL) was sampled by perforating the tail vein of the mouse with a needle. The blood sample was placed at the center of a pre-stained slide and covered with a coverslip (24 mm × 60 mm). These slides were then kept in the dark at –20 °C for a minimum of 24 h before cytological examination of the blood cells.

The cell preparations were examined under a fluorescence microscope (Nikon) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter and using an immersion objective. Reticulocytes (1000 per treated animal) were analyzed and the proportion of micronucleated cells was determined.

To compare the results, parametric (ANOVA/Tukey) or non-parametric tests (Kruskal–Wallis/Dunn's *post hoc*) were used, according to the nature of the data distribution.

2.7. Comet assay

The alkaline version of the comet assay was performed as described by Singh et al. [23], with slight modifications [24]. Four and 24 h after the treatments, a sample (20 µL) of heparinized peripheral blood leukocytes was mixed with 0.5% low-melting-temperature agarose (120 µL) in phosphate-buffered saline (PBS) and applied to microscope slides pre-coated with 1.5% agarose in PBS. The slides were covered with a coverslip and refrigerated for 5 min. After the gel had solidified, the slides were immersed in ice-cold alkaline lysing solution (2.5 M NaCl, 10 mM tris, 100 mM EDTA, 10% dimethyl sulfoxide and 1% triton X-100, pH 10.0) for at least 1 h. The slides were then incubated for 20 min in ice-cold electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), followed by electrophoresis at 25 V:300 mA (1.25 V/cm) for 20 min [25]. All of the above steps (preparation of slides, lysis and electrophoresis) were conducted without direct light exposure, to prevent additional DNA damage. After electrophoresis, the slides were neutralized (0.4 M tris, pH 7.5) and stained with ethidium bromide (20 µg/mL). One hundred cells per animal (two slides, 50 cells each) were analyzed under a fluorescence microscope (Nikon-Brasil) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter.

DNA damage was quantified by visual scoring, and the cells were placed into four classes according to tail size, between class 0 (undamaged cells) and maximum comet length (class 3) [26]. The frequency of damaged cells (DCs) was calculated as the sum of the number of undamaged cells (class 0) and DCs (classes 1, 2 and 3) divided by the total number of cells analyzed. A total score for 100 nucleoids was obtained by multiplying the number of cells in each class by the damage class, according to the formula modified from Manoharan and Banerjee [27]: total score = $(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3)$, where n = the number of cells analyzed in each class. Thus, the total score ranged from 0 (100 undamaged cells) to 300 (all cells presenting damage class 3).

To compare the results, parametric (ANOVA/Tukey) and non-parametric (Kruskal–Wallis/Dunn's *post hoc*) tests were used, according to the nature of the data distribution.

3. Results

No significant differences were observed between results for males and females (Students' *t*-test); consequently, data for both sexes were combined (Tables 1–5).

3.1. Acute tests

In the comet assay, the DNA lesions observed 4 and 24 h after acute treatment with isatin were not statistically significant from the negative control group (Table 1). For all doses (50, 100, and 150 mg/kg b.w.) and times of evaluation (36, 48, and 72 h), no significant ($P < 0.05$) increases in peripheral blood cell micronucleus frequency were seen, compared to the negative control group (Table 2).

3.2. Repeated doses tests (14 days)

Variance analysis showed no significant ($P < 0.05$) differences among the median body weights of the groups of mice treated with

Table 1

Detection of DNA damage in 100 cells per mouse ($n=8$ mice per group) after acute treatment with three different concentrations of isatin. The blood leukocytes samples were obtained 4 and 24 h after exposure to water, cyclophosphamide (CPA) and isatin.

| Exposure time (h) | Treatments (mg/kg b.w.) | Levels of damage | | | | Score (mean \pm SD) | Frequency of DC (mean \pm SD) | |
|-------------------|-------------------------|------------------|-----------------|-----------------|-----------------|-----------------------|---------------------------------|------------------------------|
| | | 0 | 1 | 2 | 3 | | | |
| 4 | Water | | 93.6 \pm 2.06 | 6.37 \pm 2.06 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 6.37 \pm 2.20 ^a | 0.06 \pm 0.02 ^a |
| | CPA | 50 | 45.2 \pm 2.62 | 34.4 \pm 3.42 | 16.6 \pm 2.39 | 3.75 \pm 0.66 | 78.9 \pm 5.05 ^b | 0.53 \pm 0.06 ^b |
| | | 50 | 91.4 \pm 2.73 | 8.62 \pm 2.73 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 8.62 \pm 2.92 ^a | 0.09 \pm 0.03 ^a |
| | Isatin | 100 | 89.5 \pm 2.60 | 10.0 \pm 2.34 | 0.50 \pm 0.50 | 0.00 \pm 0.00 | 11.0 \pm 3.12 ^a | 0.10 \pm 0.03 ^a |
| | | 150 | 89.5 \pm 3.53 | 9.62 \pm 2.87 | 0.87 \pm 0.78 | 0.00 \pm 0.00 | 11.4 \pm 4.53 ^a | 0.10 \pm 0.04 ^a |
| 24 | Water | | 93.4 \pm 1.93 | 6.62 \pm 1.93 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 6.62 \pm 2.06 ^a | 0.07 \pm 0.02 ^a |
| | CPA | 50 | 32.4 \pm 3.15 | 42.0 \pm 4.61 | 20.5 \pm 3.43 | 5.12 \pm 1.16 | 97.6 \pm 5.18 ^b | 0.67 \pm 0.03 ^b |
| | | 50 | 93.4 \pm 1.65 | 6.62 \pm 1.65 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 6.62 \pm 1.77 ^a | 0.07 \pm 0.02 ^a |
| | Isatin | 100 | 91.4 \pm 2.50 | 7.87 \pm 2.03 | 0.75 \pm 0.66 | 0.00 \pm 0.00 | 9.37 \pm 3.25 ^a | 0.09 \pm 0.03 ^a |
| | | 150 | 90.5 \pm 2.69 | 9.00 \pm 2.40 | 0.50 \pm 0.50 | 0.00 \pm 0.00 | 9.87 \pm 3.14 ^a | 0.09 \pm 0.03 ^a |

Mean \pm SD: mean \pm standard deviation; water: negative control; CPA: positive control. DC: damaged cells.

Means with the same letter do not differ statistically ($P < 0.05$).

Table 2

The frequency of micronucleated reticulocytes (MNRETs) in a total of 1000 analyzed cells per mouse ($n=8$ mice per group) to evaluate the mutagenicity of acute treatments of three different doses of isatin and the positive and negative control groups.

| Treatments (mg/kg b.w.) | T0 (0 h) | | T1 (36 h) | | T2 (48 h) | | T3 (72 h) | |
|-------------------------|----------|----------------------|-----------|------------------------------|-----------|------------------------------|-----------|-----------------------------|
| | MNRETs | Mean \pm SD/animal | MNRETs | Mean \pm SD/animal | MNRETs | Mean \pm SD/animal | MNRETs | Mean \pm SD/animal |
| Water | 21 | 2.6 \pm 0.52 | 15 | 1.9 \pm 0.64 ^a | 17 | 2.1 \pm 0.64 ^a | 19 | 2.1 \pm 0.83 ^a |
| CPA | | | | | | | | |
| 50 | 15 | 1.9 \pm 0.64 | 180 | 22.5 \pm 2.07 ^b | 107 | 13.4 \pm 1.68 ^b | 41 | 5.1 \pm 1.64 ^b |
| Isatin | | | | | | | | |
| 50 | 17 | 2.1 \pm 0.83 | 13 | 1.6 \pm 0.92 ^a | 14 | 1.7 \pm 0.46 ^a | 14 | 1.7 \pm 0.70 ^a |
| 100 | 13 | 1.6 \pm 0.74 | 15 | 1.9 \pm 0.64 ^a | 16 | 2.0 \pm 0.75 ^a | 14 | 1.7 \pm 0.89 ^a |
| 150 | 13 | 1.6 \pm 0.74 | 27 | 3.4 \pm 1.19 ^a | 13 | 1.6 \pm 0.74 ^a | 11 | 1.4 \pm 0.74 ^a |

Mean \pm SD: mean \pm standard deviation; water: negative control; cyclophosphamide (CPA): positive control.

Means with the same letter do not differ statistically ($P < 0.05$).

Table 3

Detection of DNA damage in 100 cells per mouse ($n=8$ mice per group) after repeated doses treatment (14 days) of three different concentrations of isatin. The blood leukocytes samples were obtained 4 h after the last treatment.

| Treatments (mg/kg b.w.) | Levels of damage | | | | Score (mean \pm SD) | Frequency of DC (mean \pm SD) |
|-------------------------|------------------|------------------|-----------------|-----------------|---------------------------------|---------------------------------|
| | 0 | 1 | 2 | 3 | | |
| Water | 91.1 \pm 2.20 | 8.87 \pm 2.20 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 8.87 \pm 2.36 ^a | 0.09 \pm 0.02 ^a |
| CPA | | | | | | |
| 50 | 24.5 \pm 5.15 | 55.7 \pm 3.53 | 15.1 \pm 2.26 | 4.62 \pm 1.41 | 99.9 \pm 7.85 ^b | 0.75 \pm 0.05 ^b |
| Isatin | | | | | | |
| 50 | 89.6 \pm 2.17 | 8.62 \pm 1.93 | 1.75 \pm 0.97 | 0.00 \pm 0.00 | 12.12 \pm 2.95 ^a | 0.10 \pm 0.02 ^a |
| 100 | 84.5 \pm 2.18 | 13.12 \pm 1.45 | 2.25 \pm 1.09 | 0.00 \pm 0.00 | 17.62 \pm 3.42 ^{a,c} | 0.15 \pm 0.02 ^{a,c} |
| 150 | 71.9 \pm 3.82 | 26.6 \pm 2.82 | 2.50 \pm 1.00 | 0.00 \pm 0.00 | 31.62 \pm 2.33 ^{b,c} | 0.29 \pm 0.02 ^{b,c} |

Mean \pm SD: mean \pm standard deviation; water: negative control; CPA: positive control. DC: damaged cells.

Means with the same letter do not differ statistically ($P < 0.05$).

isatin vs the negative control group, during the repeated doses tests (data not shown). Table 3 shows the results of the comet assay for isatin. Only the highest dose tested (150 mg/kg b.w.) induced DNA damage at a level statistically significantly different from the negative control (genotoxic activity). Most of the cells were class 1, with a few class 2 and no class 3 cells.

The micronucleus tests in bone marrow and peripheral blood showed that only the 150 mg/kg b.w. dose of isatin resulted in significantly higher frequencies of micronucleated cells compared to the negative control (Tables 4 and 5).

To evaluate the cytotoxicity of this substance in the repeated-dose treatment, the PCE/NCE ratio was calculated. Cytotoxic effects in mice bone marrow cells were not seen, even at higher doses of isatin or in the positive controls (Table 4). The two lower doses did not induce statistically-significant increases in the frequencies

Table 4

The frequency of micronucleated polychromatic erythrocytes (MNPCEs) in a total of 2000 analyzed cells per mouse ($n=8$ mice per group) to evaluate the mutagenicity of repeated doses (14 days) of three different concentrations of isatin. The blood samples were obtained 24 h after the last treatment.

| Treatments (mg/kg b.w.) | MNPCEs | Mean \pm SD | PCEs/PCEs + NCEs (mean \pm SD) |
|-------------------------|--------|--------------------------------|----------------------------------|
| Water | 34 | 4.2 \pm 2.12 ^a | 0.761 \pm 0.04 |
| CPA | | | |
| 50 | 341 | 42.6 \pm 6.02 ^b | 0.771 \pm 0.02 |
| Isatin | | | |
| 50 | 80 | 10.0 \pm 2.14 ^{a,c} | 0.747 \pm 0.03 |
| 100 | 81 | 10.1 \pm 1.25 ^{a,c} | 0.742 \pm 0.02 |
| 150 | 128 | 16.0 \pm 1.93 ^{b,c} | 0.755 \pm 0.02 |

Mean \pm SD: mean \pm standard deviation; water: negative control; cyclophosphamide (CPA): positive control.

Means with the same letter do not differ statistically ($P < 0.05$).

Table 5

The frequency of micronucleated reticulocytes (MNRETs) in a total of 1000 analyzed cells per mouse ($n=8$ mice per group) to evaluate the mutagenicity of repeated doses (14 days) of three different concentrations of isatin. The blood samples were obtained before treatment (0 h) and 36 h after the last treatment.

| Treatments (mg/kg b.w.) | T0 (0h) | | T1 (36h) | |
|----------------------------|---------|----------------|----------|------------------------------|
| | MNRETs | Mean \pm SD | MNRETs | Mean \pm SD |
| Water | 13 | 1.6 \pm 0.74 | 9 | 1.1 \pm 0.99 ^a |
| CPA | 11 | 1.4 \pm 0.92 | 193 | 24.1 \pm 1.13 ^b |
| Isatin | | | | |
| 50 | 8 | 1.0 \pm 0.53 | 10 | 1.2 \pm 0.46 ^a |
| 100 | 14 | 1.7 \pm 0.89 | 18 | 2.2 \pm 0.89 ^a |
| 150 | 12 | 1.5 \pm 0.75 | 26 | 3.2 \pm 0.71 ^c |

Mean \pm SD: mean \pm standard deviation; water: negative control; cyclophosphamide (CPA): positive control.

Means with the same letter do not differ statistically ($P < 0.05$).

of scores, damaged cells, MNPCEs, or MNRETs. Nevertheless, we observed a dose-dependent increasing trend in the mean values of these parameters. Indeed, the linear-trend test confirmed the presence of dose–response trends in the comet assay (score: $F=277.2$; $P < 0.0001$); damaged cells ($F=422.5$; $P < 0.0001$); bone marrow cell micronucleus ($F=140.4$; $P < 0.0001$); and peripheral blood reticulocyte ($F=34.3$; $P < 0.0001$) data sets.

4. Discussion

The therapeutic use of natural products, including medicinal plants, has become increasingly prevalent. Many pharmacognosical and pharmacological investigations have been performed to identify lead compounds for drug development [1]. Evaluation of the genotoxic/mutagenic potential of such agents is essential [28–30], and the comet assay and micronucleus test are effective tests, in this context [19,31–34]. Metabolism, including detoxication, is an important characteristic of any substance being tested, and metabolic activation may be very different between *in vivo* and *in vitro* experiments [35].

The mouse model has often been used for the micronucleus test and the comet assay [36,37]. Mice and humans have many similar metabolic, biochemical, and physiological pathways [38,39]. We also chose to administer isatin by the same route of exposure that humans would experience with the drug.

In the present study, no primary DNA damage was observed with the comet assay after acute (4 or 24 h) isatin, in agreement with the protocol suggested by Tice et al. [19]. The absence of mutagenic effects of this compound 36, 48, or 72 h after treatment was also demonstrated via the micronucleus assay with peripheral blood cells.

It has been suggested that alkaloids are potentially mutagenic, because their therapeutic actions involve interactions with DNA [4,40–42]. This possibility was corroborated in the present study only when mice were submitted to the highest dose of isatin (150 mg/kg b.w.) for 14 consecutive days. The results obtained in the micronucleus (RET and PCE) and comet assays clearly showed that isatin induced primary DNA damage, and breaks and/or loss of entire chromosomes, which gave rise to micronuclei in the treated animals.

Fouremant et al. [43] demonstrated that isatin was mutagenic in the sex-linked recessive lethal (SLRL) assay in male *Drosophila melanogaster* germ cells. Nevertheless, no mutagenic effects of isatin were seen in the Ames test [44] at concentrations up to 8 μ mol per plate or, with derivatives of isatin, by Bacchi et al. [45]. In our study, a mutagenic effect was observed only at the highest isatin dose tested (150 mg/kg b.w.).

A mutagenic agent can weaken an animal by promoting cachexia and weight loss. However, under our experimental conditions, isatin did not affect mouse body weight. The cytotoxic effect of isatin after repeated doses was determined by the PCE/NCE relationship in the micronucleus test with mouse bone marrow cells. When a toxic agent affects the normal proliferation of bone marrow cells, the number of immature erythrocytes (PCE) decreases relative to mature erythrocytes (NCE) [46]. In our study, isatin did not affect the proliferation of bone marrow cells. Nevertheless, isatin inhibits the proliferation of several tumor cell lines *in vitro* [47–51]. Sathisha et al. [14] also showed that *bis*-isatin thiocarbohydrazone metal complexes showed good activity in an Ehrlich's ascites carcinoma model in Swiss albino mice.

The genotoxic and mutagenic effects observed after repeated doses, but not after acute treatment, showed that induction of DNA damage by isatin is dependent on both the dose and the duration of exposure. Because therapeutic treatments usually involve repeated doses and extended time periods, use of elevated doses of this compound may be hazardous. We hope that data presented here will contribute to understanding of the potential application of this alkaloid as a chemotherapeutic agent.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

- [1] D.J. Newman, G.M. Cragg, K.M. Snader, Natural products as sources of new drugs over the period 1981–2002, *J. Nat. Prod.* 66 (2003) 1022–1037.
- [2] R.A. Mothana, U. Lindequist, R. Gruenert, P.J. Bednarski, Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqatra, *BMC Complement. Altern. Med.* 9 (2009), doi:10.1186/1472-6882-9-7.
- [3] V.F. Veiga Junior, A.C. Pinto, M.A.M. Maciel, Plantas medicinais: cura segura? *Quím. Nova* 28 (2005) 519–528.
- [4] M. Beljanski, M.S. Beljanski, Selective inhibition of *in vitro* synthesis of cancer DNA by alkaloids of beta-carboline class, *Exp. Cell Biol.* 50 (1982) 79–87.
- [5] A.M. Rodríguez, J.R. Camargo, F.J.B. García, Actividad *in vitro* de la mezcla de alcaloides de *Ervatamia coronaria* (Jacq) Staff. Apocynaceae sobre amastigotes de *Leishmania braziliensis*, *Rev. Bras. Farmacogn.* 18 (2008) 350–355.
- [6] J.F.M. Silva, S.J. Garden, A.C. Pinto, The chemistry of isatins: a review from 1975 to 1999, *J. Braz. Chem. Soc.* 12 (2001) 273–324.
- [7] K.L. Vine, L. Matesic, J.M. Locke, M. Ranson, D. Skropeta, Cytotoxic and anticancer activities of isatin and its derivatives: a comprehensive review from 2000–2008, *Anticancer Agents Med. Chem.* 9 (2009) 397–414.
- [8] Organization for Economic Co-operation and Development (OECD), OECD guideline for testing of chemicals, n.º. 423, Acute Oral Toxicity – Acute Toxic Class Method, 2001.
- [9] A.E. Medvedev, A. Clow, M. Sandler, V. Glover, Isatin: a link between natriuretic peptides and monoamines? *Biochem. Pharmacol.* 52 (1996) 385–391.
- [10] S.K. Sridhar, S.N. Pandeya, J.P. Stables, A. Ramesh, Anticonvulsant activity of hydrazones, Schiff and Mannich bases of isatin derivatives, *Eur. J. Pharm. Sci.* 16 (2002) 129–132.
- [11] S.N. Pandeya, S. Smitha, M. Jyoti, S.K. Sridhar, Biological activities of isatin and its derivatives, *Acta Pharm.* 55 (2005) 27–46.
- [12] S.K. Bhattacharya, S.B. Acharya, Further investigations on the angiogenic effects of isatin, *Biogenic Amines* 9 (1993) 453–463.
- [13] N. Hamaue, M. Minami, Y. Kanamaru, M. Ishikura, N. Yamazaki, H. Saito, S.H. Parvez, Identification of isatin, an endogenous MAO inhibitor, in the brain of stroke-prone SHR, *Biogenic Amines* 10 (1994) 99–110.
- [14] M.P. Sathisha, V.K. Revankar, K.S.R. Pai, Synthesis, structure, electrochemistry, and spectral characterization of *bis*-isatin thiocarbohydrazone metal complexes and their antitumor activity against Ehrlich ascites carcinoma in Swiss albino mice, *Met Based Drugs* (2008), doi:10.1155/2008/362105.
- [15] N. Hamaue, M. Minami, M. Terado, M. Hirafuji, T. Endo, M. Machida, T. Hiroshige, A. Ogata, K. Tashiro, H. Saito, S.H. Parvez, Comparative study of the effects

- of isatin, an endogenous MAO-inhibitor, and selegiline on bradykinesia and dopamine levels in a rat model of Parkinson's disease induced by the Japanese encephalitis virus, *Neurotoxicology* 25 (2004) 205–213.
- [16] D. Anderson, J.B. Bishop, R.C. Garner, P. Ostrosky-Wegman, P.B. Selby, Cyclophosphamide: review of its mutagenicity for an assessment of potential germ cell risks, *Mutat. Res.* 330 (1995) 115–181.
- [17] E.D. Olfert, B.M. Cross, A.A. McWilliam, Guide to the Care and Use of Experimental Animals, 2nd ed., Canadian Council on Animal Care, Ottawa, 1993.
- [18] Organization for Economic Co-operation and Development (OECD), OECD guideline for the testing of chemicals, n°. 474, Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test, 1997.
- [19] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.-C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, *Environ. Mol. Mutagen.* 35 (2000) 206–221.
- [20] M. Hayashi, J.T. MacGregor, D.G. Gatehouse, I.-D. Adler, D.H. Blakey, S.D. Dertinger, G. Krishna, T. Morita, A. Russo, S. Sutou, *In vivo* rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring, *Environ. Mol. Mutagen.* 35 (2000) 234–252.
- [21] W. Schmid, The micronucleus test, *Mutat. Res.* 31 (1975) 9–15.
- [22] M. Hayashi, T. Morita, Y. Kodama, T. Sofuni, M. Ishida Jr., The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides, *Mutat. Res.* 245 (1990) 245–249.
- [23] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* 175 (1988) 184–191.
- [24] J. da Silva, T.R.O. de Freitas, J.R. Marinho, G. Speit, B. Erdtmann, An alkaline single-cell gel electrophoresis (comet) assay for environmental biomonitoring with native rodents, *Genet. Mol. Biol.* 23 (2000) 241–245.
- [25] F. Nessler, N. Zennouche, S. Simar-Meintières, I. Talahari, E.N. Nkili-Mboui, D. Marzin, *In vivo* comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds, *Mutat. Res.* 630 (2007) 28–41.
- [26] H. Kobayashi, C. Sugiyama, Y. Morikawa, M. Hayashi, T. Sofuni, A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay, *MMS Commun.* 3 (1995) 103–115.
- [27] K. Manoharan, M.R. Banerjee, β -Carotene reduces sister chromatid exchanges induced by chemical carcinogens in mouse mammary cells in organ culture, *Cell Biol. Int. Rep.* 9 (1985) 783–789.
- [28] A. Bast, R.F. Chandler, P.C. Choy, L.M. Delmule, J. Gruenwald, S.B.A. Halkes, K. Keller, J.H. Koeman, P. Peters, H. Przyrembel, E.M. de Ree, A.G. Renwick, I.T.M. Vermeer, Botanical health products, positioning and requirements for effective and safe use, *Environ. Toxicol. Pharmacol.* 12 (2002) 195–211.
- [29] J.L. Santos, E.A. Varanda, L.M. Lima, C.M. Chin, Avaliação da atividade mutagênica da talidomida pelo teste de Ames, *Rev. Eletr. Farm.* 4 (2007) 154–158.
- [30] I. Rodeiro, L. Cancino, J.E. González, J. Morffi, G. Garrido, R.M. González, A. Nuñez, R. Delgado, Evaluation of the genotoxic potential of *Mangifera indica* L. extract (Vimang), a new natural product with antioxidant activity, *Food Chem. Toxicol.* 44 (2006) 1707–1713.
- [31] M. Fenech, N. Holland, W.P. Chang, E. Zeiger, S. Bonassi, The human micronucleus project – an international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans, *Mutat. Res.* 428 (1999) 271–283.
- [32] G. Krishna, M. Hayashi, *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation, *Mutat. Res.* 455 (2000) 155–166.
- [33] I. Witte, U. Plappert, H. de Wall, A. Hartmann, Genetic toxicity assessment: employing the best science for human safety evaluation part III: the comet assay as an alternative to *in vitro* clastogenicity tests for early drug candidate selection, *Toxicol. Sci.* 97 (2007) 21–26.
- [34] B. Burlinson, R.R. Tice, G. Speit, E. Agurell, S.Y. Brendler-Schwaab, A.R. Collins, P. Escobar, M. Honma, T.S. Kumaravel, M. Nakajima, Y.F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, Fourth international workgroup on genotoxicity testing: results of the *in vivo* comet assay workgroup, *Mutat. Res.* 627 (2007) 31–35.
- [35] D.J. Tweats, D. Blakey, R.H. Heflich, A. Jacobs, S.D. Jacobsen, T. Morita, T. Nohmi, M.R. O'Donovan, Y.F. Sasaki, T. Sofuni, R. Tice, Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards, *Mutat. Res.* 627 (2007) 78–91.
- [36] M. Hayashi, R.R. Tice, J.T. MacGregor, D. Anderson, D.H. Blakey, M. Kirsch-Volders, F.B. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou, B. Vannier, *In vivo* rodent erythrocyte micronucleus assay, *Mutat. Res.* 312 (1994) 293–304.
- [37] A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud, R.R. Tice, Recommendations for conducting the *in vivo* alkaline comet assay, *Mutagenesis* 18 (2003) 45–51.
- [38] C.W. Boone, G.J. Kelloff, W.E. Malone, Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: a review, *Cancer Res.* 50 (1990) 2–9.
- [39] C.A. Argmann, P. Chambon, J. Auwerx, Mouse phenogenomics: the fast track to "systems metabolism", *Cell Metab.* 2 (2005) 349–360.
- [40] J.A.P. Henriques, P.R.H. Moreno, G.L. Von Poser, C.C. Querol, A.T. Henriques, Genotoxic effect of alkaloids, *Mem. Inst. Oswaldo Cruz* 86 (1991) 71–74.
- [41] Z. Taira, S. Kanzawas, C. Dohara, S. Ishida, M. Matsumoto, Y. Sakiya, Intercalation of six beta-carboline derivatives into DNA, *Jpn. J. Toxicol. Environ. Health* 43 (1997) 83–91.
- [42] C. Ansah, A. Khan, N.J. Gooderham, *In vitro* genotoxicity of the West African anti-malarial herbal *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine, *Toxicology* 208 (2005) 141–147.
- [43] P. Fourreman, J.M. Mason, R. Valencia, S. Zimmering, Chemical mutagenesis testing in *Drosophila*. IX. Results of 50 coded compounds tested for the national toxicology program, *Environ. Mol. Mutagen.* 23 (1994) 51–63.
- [44] U. Rannung, H. Bramstedt, U. Nilsson, The presence of genotoxic and bioactive components in indigo dyed fabrics—a possible health risk? *Mutat. Res.* 282 (1992) 219–225.
- [45] A. Bacchi, M. Carcelli, P. Pelagatti, G. Pelizzi, M.C. Rodriguez-Arguelles, D. Rogolino, C. Solinas, F. Zani, Antimicrobial and mutagenic properties of organotin (IV) complexes with isatin and *N*-alkylisatin bithiocarbonohydrazones, *J. Inorg. Biochem.* 99 (2005) 397–408.
- [46] J.B. Vilar, F.L. Ferreira, P.H. Ferri, L.A. Guillo, L. Chen Chen, Assessment of the mutagenic, antimutagenic and cytotoxic activities of ethanolic extract of araticum (*Annona crassiflora* Mart. 1841) by micronucleus test in mice, *Braz. J. Biol.* 68 (2008) 141–147.
- [47] A. Cane, M.-C. Tournaire, D. Barriault, M. Crumeyrolle-Arias, The endogenous oxindoles 5-hydroxyoxindole and isatin are antiproliferative and proapoptotic, *Biochem. Biophys. Res. Commun.* 276 (2000) 379–384.
- [48] N. Igosheva, C. Lorz, E. O'Conner, V. Glover, H. Mehmet, Isatin, an endogenous monoamine oxidase inhibitor, triggers a dose- and time-dependent switch from apoptosis to necrosis in human neuroblastoma cells, *Neurochem. Int.* 47 (2005) 216–224.
- [49] M.E. Matheus, F.A. Violante, S.J. Garden, A.C. Pinto, P.D. Fernandes, Isatins inhibit cyclooxygenase-2 and inducible nitric oxide synthase in a mouse macrophage cell line, *Eur. J. Pharmacol.* 556 (2007) 200–206.
- [50] K.L. Vine, J.M. Locke, M. Ranson, K. Benkendorff, S.G. Pyne, J.B. Bremner, *In vitro* cytotoxicity evaluation of some substituted isatin derivatives, *Bioorg. Med. Chem.* 15 (2007) 931–938.
- [51] L. Hou, C. Ju, J. Zhang, J. Song, Y. Ge, W. Yue, Antitumor effects of isatin on human neuroblastoma cell line (SH-SY5Y) and the related mechanism, *Eur. J. Pharmacol.* 589 (2008) 27–31.