Current Topics in Toxicology
Vol. 10, 2014

Original Communication

Evaluation of toxicity, cytotoxicity and mutagenicity of an imidazole derivative on *Allium cepa* L.

Manoel Pinheiro Lucio Neto[1], Alisson Ferreira Dantas[1], Débora Cássia Vieira Gomes[1], Márcia Fernanda Correia Jardim Paz[2], Ana Maria Oliveira Ferreira da Mata[1], Marcus Vinícius Oliveira Barros de Alencar[1,*], Md. Torequl Islam[3] and Ana Amélia de Carvalho Melo-Cavalcante[1]

[1]Postgraduate Program in Pharmaceutical Sciences, Federal University of Piauí, Teresina, Brazil; [2]Laboratory of Genetic Toxicology, Postgraduate Program in Genetic Toxicology, Lutheran University of Brazil, Canoas, Brazil. [3]Department of Pharmacy, Southern University Bangladesh, Chittagong, Bangladesh.

ABSTRACT

The compound PT-31, 3-(2-cloro-6-fluorobenzil)-imidazolidine-2,4-dione, is a promising derivative of imidazolidine with proven agonist activity at the α2A-adrenergic receptors, that displays analgesic dose-dependent and synergistic action profile with morphine. This study evaluates the toxicity, cytotoxicity and mutagenicity of PT-31 on *Allium cepa* L. Three graded dosages with dilution ranging from 0.5 to 5.0 mg/mL were used in the present study, with dechlorinated water and copper sulphate (0.0006 mg/mL) as negative and positive controls, respectively. After 72 hours of exposure, the roots were measured and removed. Analyses of root growth and mitotic index were used to assess the toxicity and cytotoxicity. Mutagenicity was identified by the frequencies of chromosomal aberrations and of micronuclei. The PT-31 inhibited the mitotic index and root growth at a dose of 5.0 mg/mL ($P < 0.001$) and increased frequencies of chromosomal aberrations and micronuclei at a dose of 1.0 mg/mL ($P < 0.05$). These data indicate toxicity, cytotoxicity and mutagenicity of PT-31. Given the clinical potential of PT-31, we suggest additional studies to elucidate the mechanisms that generate the effects observed. The *A. cepa* test, as well as the Ames test, may be used for pre-clinical tests related to toxicity, cytotoxicity and mutagenicity, and may predict an initial profile of safety and efficacy of the new molecule.

KEYWORDS: *Allium cepa*, cytotoxicity, imidazolidine-2,4-dione, mutagenicity, toxicity

*Corresponding author
Marcus Vinícius Oliveira-Barros de Alencar,
Laboratório de Pesquisa em Genética Toxicológica,
Federal University of Piauí, Campus Universitário
Ministro Petrônio Portella, Bairro Ininga,
CEP 64049-550 Teresina, Piauí, Brazil.
marcus-alencar@msn.com

INTRODUCTION

The compound PT-31, 3-(2-chloro-6-fluorobenzil)-imidazolidine-2,4-dione is an imidazolidine derivative, a structural analogue of clonidine. Studies by [1] revealed that the PT-31 has high affinity for the active site of adrenergic receptors α2A, which can generate a dose-dependent antinociception, acting in synergy with the opioid morphine that makes it an interesting drug with clinical potential in pain management and anesthesiology. The clinical advantages of α2-adrenergic agonists for pain management are clear, but only a few drugs exhibit such pharmacological activity.

Identification of new compounds with this profile is clinically relevant. The first α2-agonist drugs were synthesized in the early 1960s and used in clinical practice, initially as nasal decongestants

and antihypertensive agents [2]. Subsequent studies have attributed to this group, analgesic, sedative, anxiolytic and sympathomimetic activities also [3, 4]. In prescribing a drug, knowledge of the risk/benefit ratio is of fundamental importance. Among the various adverse reactions that a drug can cause, the occurrence of genotoxic and mutagenic effects is clinically relevant, in view of its direct relationship with the increased incidence of cancer, chronic degenerative diseases, premature aging, infertility problems, endocrine, neurological and behavioral disorders [5].

The DNA may be the target of drug metabolites that react directly or indirectly, with the incorporation of nucleotide analogs, or blocking metabolic functions such as DNA polymerases and DNA topoisomerases. Two strategies are involved in responses to DNA damage: the damage is repaired or tolerated, or cells are removed by apoptosis. Not repairing leads to consequences such as chromosomal aberrations, gene mutations and malignant transformation [6].

Superior plants are recognized as excellent genetic models to detect mutagens and are often used in many biomonitoring studies [7]. The results of bioassays of plants are highly relevant, considering that a chemical capable of inducing damage to chromosomes of plants, can also pose risks to other eukaryotes [8]. Among the plant species, *Allium cepa* has been reported to assess damage to DNA, such as chromosomal aberrations, micronuclei and the mitotic cycle disturbances induced by a large number of chemical agents [9]. The *A. cepa* test is considered favorable because of certain characteristics such as the presence of small number of chromosomes (2n = 16) that are large in size [10].

Technical modifications in *A. cepa* test were made to allow a more comprehensive assessment of chemicals, such as complex mixtures, which comprise the majority of environmental samples and pure substances [11]. The *A. cepa* test also allows evaluation of different parameters: the frequencies of chromosomal aberrations and micronuclei have been the most commonly used to detect mutagenicity, while the mitotic index and some nuclear abnormalities are used to evaluate cytotoxicity [12]. In addition, *A. cepa* test system provides important information for the identification of mechanisms of action of an agent on the genetic material (clastogenic and/or aneugenic). An advantage of this test is the presence of an oxidase enzyme system, which enables a pre-mutagenic evaluation, and the lack of a pretreatment of the sample, which differentiates it from other tests, such as Ames test, that requires processes of extraction and concentration of samples [11].

Tests for genotoxicity and mutagenicity *in vivo* are sensitive tools for the detection of action affecting the genetic material and the carcinogenic potential of several molecules [13]. In the case of new pharmaceuticals, health authorities in most countries advocate such tests prior to clinical trials [14]. This study aims to investigate the possible genotoxic effects of imidazolidine derived PT-31 in *A. cepa* meristematic cells.

MATERIALS AND METHODS

Chemical synthesis of PT-31

The imidazolidine derivative PT-31 was synthesized by [1] in the Center for Therapeutic Research in Innovation at the Federal University of Pernambuco, Recife, Brazil. The compound was kindly provided to the Center for Pharmaceutical Technology, Federal University of Piauí - UFPI, Teresina, Piauí, Brazil for pre-clinical evaluations.

Allium cepa test

Conditions for root growth, concentrations of the test and treatments

The *Allium cepa* test was performed according to the description of [10, 15], with some adjustments, as described by [16]. Each experimental group consisted of dilutions of the compound PT-31 at doses of 0.5, 1.0 and 5.0 mg/mL in 0.5% dimethyl sulfoxide (DMSO) solution. As a negative control, we used dechlorinated water, and as positive control copper sulphate 0.0006 mg/mL.

Each experiment consisted of 5 (five) bulbs. Each test solution or substrate was distributed in 5 mL glass containers previously sterilized, and a bulb was placed in each container with the root surface area in contact with the solution, and left to germinate at 18-22 °C. The volume of solution absorbed was replenished daily, aiming at maintaining the roots soaked. After 72 hours of exposure, the roots were measured with the aid of ruler, roots that were very short or very long and those that

can induce toxicity were ignored. After the measurement, they were placed in Carnoy fixative solution (99% ethanol and glacial acetic acid, 3:1 v/v) for 24 hours and thereafter, in 70% ethanol, and kept under refrigeration until the histological slides were prepared. The test was conducted at a controlled temperature of 20 °C on a bench without vibration and direct lighting.

Slide preparation

For the preparation of the slides, two to three roots were removed from the 70% ethanol, washed in distilled water (three baths of 5 min) and hydrolyzed in HCl (1 N) at 60 °C for 8 min. Then the roots were transferred to dark bottles (amber), containing Schiff's solution (basic fuchsin and sodium metabisulfite, 3:1 w/w) for approximately 2 hours. Using tweezers with a scalpel blade the hood (the apical portion of the root) of approximately 1 mm to 2 mm in length was collected, discarding rest of the portion. Two drops of 2% acetic carmine was added and left to stain for 10 minutes. Then the tips of the roots were transferred to a slide. The coverslip was placed on the slide softly by the thumb. The prepared material was then observed under a compound light microscope at 1000 X and subsequently photographed for a more efficient reading.

Analysis of toxicity, mutagenicity and cytotoxicity

A total of 5000 cells per test group were examined. The following parameters were observed: (a) mitotic index (MI) (1000 cells per bulb), (b) chromosome aberrations (CA's) in the mitotic cycle, and (c) presence of micronuclei (MN) (1000 cells per bulb). MI corresponds to the ratio between the number of cells in mitosis (prophase, metaphase, anaphase and telophase) and the total number of cells. To determine chromosome aberrations, various types of parameters such as anaphase bridge, chromosomal fragments, delayed chromosomes and micronucleated cells were taken into consideration.

Statistical analysis

Statistical analysis was conducted using the program GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA), using descriptive statistics and nonparametric ANOVA with Tukey's test for multiple comparisons between groups of the test system, with significance level $*P < 0.05$.

RESULTS

The analysis results for the toxic and cytotoxic effects of PT-31 in root meristems of *Allium cepa* are shown in Table 1. The effects of PT-31 on the growth of root meristems of *A. cepa* indicate that doses of 0.5 and 1.0 mg/mL showed no significant reduction but the dose of 5.0 mg/mL produced significant ($P < 0.001$) reduction in comparison to the negative control (dechlorinated water). The same dose (5.0 mg/mL) significantly ($P < 0.001$) inhibited the mitotic index of meristems of *A. cepa* when compared to negative control group, indicating it's cytotoxic effects.

Table 1. Mitotic index and size of the roots of species of *Allium cepa* exposed to the test drug and the controls.

Group	Mitotic index (in process of cell division/1000)	Size (cm)
Negative Control[a]	80.90 ± 10.45	0.66 ± 0.25
Positive Control[b]	37.00 ± 8.07***	0.32 ± 0.06***
DMSO (0.5%)	78.40 ± 4.41	0.71 ± 0.24
0.5 mg/mL	73.20 ± 5.59	0.65 ± 0.12
1.0 mg/mL	72.06 ± 6.30	0.45 ± 0.11
5.0 mg/mL	46.16 ± 8.42***	0.30 ± 0.04***

$***P < 0.001$ compared to the negative control and DMSO (ANOVA-Tukey's test).
Values are expressed as mean ± standard deviation (SD); [a]Dechlorinated water; [b]Copper sulfate 0.0006 mg/mL.

The results for the mutagenic parameters - frequency of chromosomal aberrations and micronuclei are shown in Table 2. In this microscopic analysis lots of loose, delayed, bridged and fragmented chromosomes were observed in the anaphase cell division phase (Figure 1). There was significant ($P < 0.05$) increase in the overall frequency of aberrations only for the dose of 1.0 mg/mL. Similarly, a significant ($P < 0.05$) increase in the overall frequency of micronuclei was observed only for the dose of 1.0 mg/mL. This suggests possible mutagenic effect of the compound (PT-31) at the dose of 1.0 mg/mL.

DISCUSSION

The *Allium cepa* test has shown high sensitivity and good correlation when compared with other test systems, for example, in mammals, despite that the oxidase enzyme system of higher plants presents a low concentration and limited specificity to different substrates, in relation to mammalian cytochrome P_{450}. Chemicals were evaluated by *A. cepa* test, and through these studies, the test was considered as a standard for the determination of chromosomal damage induced by chemicals. Its sensitivity was reported as similar to tests with human lymphocytes, presenting it more sensitive than Ames test and MicroScreen [12].

In the experiments, we observed significant inhibition of root growth and mitotic index in meristems of *Allium cepa* only for doses of 5.0 mg/mL ($P < 0.001$), suggesting that the compound PT-31, in this concentration, has toxic and cytotoxic effects (Table 1). According to [12], analysis of the mitotic index (MI) was used in different studies and most of them showed satisfactory results for the analysis proposed. The MI is an important parameter to evaluate the cellular toxicity of various substances, where the cytotoxicity of certain chemical agents can be determined by the increase or decrease in MI. Mitotic index greater than the negative control is the result of an increase in cell division, which may be harmful to cells, leading to uncontrolled cell proliferation and even the formation of tumorous tissues. The reduction of the MI may indicate changes caused by chemical agent, which influence growth and development of the exposed organism. According to Turkoglu [16], the reduction of MI may be due to an inhibition of DNA synthesis or a blockage of G2 phase of the cell cycle, preventing the cell from mitosis.

In the experiment, the analysis of the size of the roots exposed to the test drug, PT-31 induced a reduction in root growth. Thus, these parameters suggest evidence of toxicity and cytotoxicity to the dose of 5.0 mg/mL concentration and indicate that this process interferes with DNA synthesis. Cytotoxicity is not well known in response to genotoxic agents; this is because the responses to genotoxic damage are complex and can take place anywhere from repairs, fixing damage, mutations, deletions, damage and cell death [17, 18]. It is worth mentioning that clonidine, an imidazolidine-2,4-dione, analogous to the structure of the compound PT-31, was not carcinogenic, but presented negative results in some genotoxicity and mutagenicity assays

Table 2. Chromosomal aberrations and micronuclei in *Allium cepa* exposed to the test drug and the controls.

Group	Anaphase bridges	Chromosomal fragments	Delayed anaphase	Microscopic effect	Micronucleated cells (MN/2000)
Negative Control[a]	0.02 ± 0.04	0.08 ± 0.08	0.04 ± 0.05	0.02 ± 0.04	0.02 ± 0.04
Positive Control[b]	0.02 ± 0.04	0.06 ± 0.05	0.02 ± 0.04	0.48 ± 0.32*	0.48 ± 0.16*
DMSO (0.5%)	0.01 ± 0.03	0.24 ± 0.36	0.06 ± 0.09	0.10 ± 0.12	0.04 ±0.05
0.5 mg/mL	0.06 ± 0.13	0.00 ± 0.00	0.22 ± 0.13	0.12 ± 0.13	0.12 ± 0.18
1.0 mg/mL	0.18 ± 0.20	0.12 ± 0.22	0.26 ± 0.27	0.16 ± 0.36	0.34 ± 0.18*
5.0 mg/mL	NO	NO	NO	NO	0.14 ± 0.09

*$P < 0.05$ compared to the negative control and DMSO (ANOVA-Tukey's test).
Values are expressed as mean ± standard deviation (SD); NO: not observed; [a]Dechlorinated water; [b]Copper sulfate 0.0006 mg/mL.

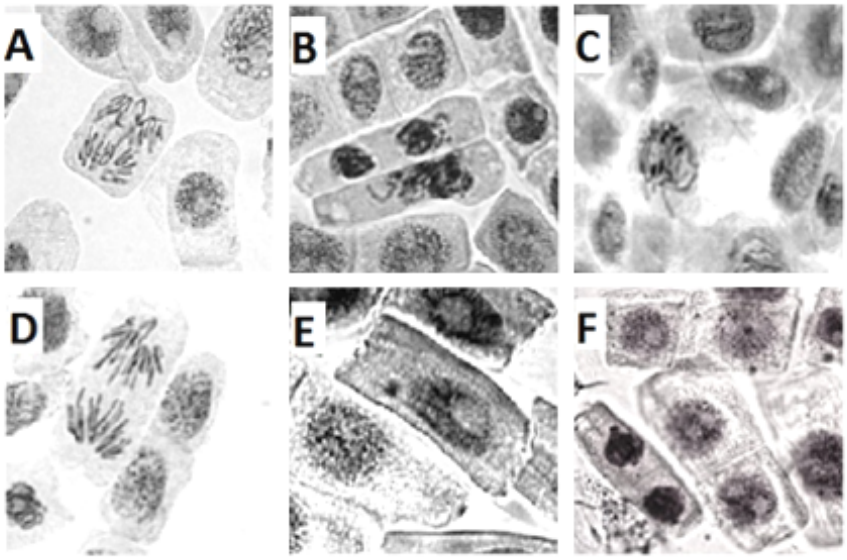

Figure 1. Photomicrographs (1000x magnification) of chromosomal aberrations and micronuclei in specimens of *Allium cepa* exposed to concentrations of 0.5, 1.0 and 5.0 mg/mL of the compound PT-31. A: anaphase bridge, B&C: chromosomal fragments, D: delayed chromosomes, E&F: micronuclei.

(reverse mutations in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA as well as unscheduled DNA synthesis in rat hepatocytes.) [14]. However, there are reports that phenytoin, belonging to the same group of imidazolidine-2,4-dione, during studies of its bioactivation *in vitro* produces reactive intermediate compounds that induce free radicals and may have teratogenic effects in mice by the action of one of its metabolites especially epoxides [19].

Microscopic analysis showed the chromosomal aberrations like - delayed, bridged and fragmented chromosomes in anaphase (Figure 1). The results presented in Table 2 show that the PT-31 has mutagenic activity in meristems of *A. cepa* only at a concentration of 1.0 mg/mL, as indicated by the significant increase ($P < 0.05$) in the overall frequency of chromosomal aberrations. Chromosomal aberrations are characterized as changes in structure or number of chromosomes, which may occur spontaneously or as a result of exposure to physical and chemical agents. Structural changes in chromosomes can be induced by broken DNA tapes, inhibition of the DNA synthesis, overall change in replication process and so on. Numerical changes, for example aneuploidy and polyploidy, may occur spontaneously or by the action of aneugenic agents [20, 21]. The analysis of the frequency of mitotic aberrations has been highlighted as a marker of damage to genetic material, mostly used in *A. cepa*. The presence of these cellular changes does not mean, necessarily, the occurrence of permanent damage to the cell, since they can be the target of enzyme systems that ensure the integrity of the genome of the cell [22].

Chromosome bridges or inter-chromatide connectors are composed of chromatin fibers that bind sister chromatids in metaphase and keep them together until the anaphase or telophase [23]. Lagging chromosomes are the result of a delay due to a failure to shift the poles of the cell. Loose chromosomes may be the result of whole chromosomes, with aneugenic origin, that continue as laggards. Fragments can be derived from the action of chemical agents that induce chromosome breaks (clastogenic) and its interference in the chromosomes is associated with breaks in the DNA molecule [16]. There were no significant changes to the chromosomal aberration parameters by the test doses. But the dose, 1.0 mg/mL showed an increase in number of micronucleated cells (Table 2). This suggests possible aneugenic/ clastogenic effects in this PT-31 concentration in meristems of *A. cepa*.

According to Paz *et al.* and Flores & Yamaguchi [12, 24] the micronuclei (MN) are defined as small spherical masses, extra-nuclear, consisting of chromatin not incorporated into the core of the cell during the final stages of mitosis, viewable only in dividing cells. Their formation is extensively used in molecular epidemiology as a biomarker of chromosome damage, genetic instability and possible cancer risk.

The occurrence of micronuclei represents an integrated response to instability of the chromosomes, phenotypes and cellular changes caused by genetic defects and/or exposure to exogenous genotoxic agents, reflecting a number of chromosomal alterations important in carcinogenesis [25]. Thus, the concentration of 1.0 mg/mL PT-31 is capable of inducing an increased frequency of micronuclei, in a way similar to its analog, phenytoin, which can induce oxidative damage in rodents. According to the Center for Drug Evaluation and Research phenytoin belongs to the group 2B, hence considered a possible carcinogen to humans by way of evaluating *in vitro* cytogenetic tests, as well as tests with bacteria, cytogenetic tests *in vivo* in rodents and also carcinogenicity tests. Recent studies corroborate the preceding information confirming that phenytoin can induce cancer of the esophagus, liver, lung and lymphocytic leukemia [26].

CONCLUSION

In conclusion, the new PT-31 derived imidazolidine, an agonist of α2A-adrenergic receptor showed signs of toxicity and cytotoxicity at the dose of 5.0 mg/mL and mutagenicity at the dose of 1.0 mg/mL. The lowest concentration tested (0.5 mg/mL) demonstrated negligible results in comparison to the negative control group. These results suggest that the compound PT-31 at a concentration of 5.0 mg/mL can probably inhibit DNA synthesis or block the G2 phase of the cell cycle, preventing the cell from mitosis, and the dose, 1.0 mg/mL can cause clastogenic or aneugenic damage in root cells of *Allium cepa*. With regard to the *A. cepa* test system, it is observed that, as with the Ames test, it can be used as a method for preclinical screening and analysis of parameters related to toxicity, cytotoxicity and mutagenicity, and predictive profile of an initial safety and efficacy of new molecules.

ACKNOWLEDGEMENT

We thank the Center for Therapeutic Research in Innovation at the Federal University of Pernambuco - UFPE, Recife, Brazil, who synthesized the molecule and provided us with the PT-31, thus allowing this work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

1. Sudo, R. T., Calasans-Maia, J. A., Galdino, S. L., Lima, M. C., Zapata-Sudo, G., Hernandes, M. Z. and Pitta, I. R. 2010, J. Pain, 11, 71-78.
2. Stähle, H. 2000, Best. Pract. Res. Clin. Anaesthesiol., 14, 237-246.
3. Park, J. W., Chung, H. W., Lee, E. J., Jung, K., Paik, J. and Lee, K. 2013, Neurosci. Lett., 541, 184-189.
4. Shujaa, N., Za´Dori, M. Z. S., Rossi, M., Ma´Tyus, P., Ne´Meth, J., Hein, L. and Gyires, K. 2011, Neurochem. Int., 58, 708-713.
5. Słoczynska, K., Waszkielewicz, A. M. and Marona, H. 2014, Mutat. Res., 768, 8-13.
6. Kultz, D. 2005, Annu. Rev. Physiol., 67, 225-257.
7. Goujon, E., Sta, C., Trivella, A., Goupil, P., Richard, C. and Ledoigt, G. 2014, Pestic. Biochem. Phys., 113, 47-54.
8. Ozakca, D. U. and Silah, H. 2013, Pestic. Biochem. Phys., 107, 38-43.
9. Hoshina, M. M. and Marin-Morales, M. A. 2009, Ecotox. Environ. Safe., 72, 2090-2095.
10. Fiskejõ, G. 1985, Hereditas, 102, 99-112.
11. Leme, D. M. and Marin-Morales, M. A. 2009, Mutat. Res., 682, 71-81.
12. Paz, M. F. C. J., Alencar, M. V. O. B., Soares, R. L. L., Costa, D. A. F., Nunes, A. T. and Melo-Cavalcante, A. A. C. 2013, Rev. Interdisciplinar, 6, 52-61.
13. Maluf, S. W. and Erdtmann, B. 2003, Genética toxicológica, Alcance, Porto Alegre.
14. Brambilla, G. and Martelli, A. 2009, Pharmacol. Res., 60, 1-17.

15. Mitteregger, H. J., Silva, J., Arenzon, A., Portela, C., Ferreira, I. and Henriques, J. A. P. 2007, Chemosphere, 67, 1211-1217.
16. Turkoglu, S. 2008, Food Chem. Toxicol., 46, 2035-41.
17. Fritz, G. and Kaina, B. 2006, Curr. Cancer Drug Targets, 6, 1-142.
18. Kaina, B. 2003, Biochem. Pharmacol., 66, 1547-1554.
19. Wells, P. G., Mccallum, G. P., Chen, C. S., Henderson, J. T., Lee, C. J., Perstin, J., Preston, T. J., Wiley, M. J. and Wong, A. W. 2009, Toxicol. Sci., 108, 4-18.
20. Gamal-Eldeen, A. M., Abo-Zeid, M. A. M. and Ahmed, E. F. 2013, Exp. Toxicol. Pathol., 65, 27-34.
21. Rossner, J. R., Rossnerova, A. and Sram, R. J. 2011, Mutat. Res., 707, 34-41.
22. Ma, T. H., Xu, Z., Xu, C., Mcconnell, H., Rabago, E. V., Arreola, G. A. and Zhang, H. 1995, Mutat. Res., 334, 185-95.
23. Murnane, J. P. 2012, Rev. Saúde e Pesquisa, 1, 337-340.
24. Flores, M. and Yamaguchi, U. M. 2008, Health Res., 1, 337-40.
25. Fernandes, T. C. C., Mazzeo, D. E. C. and Marin-Morales, M. A. 2007, Pestic. Biochem. Phys., 88, 252-259.
26. Friedman, G. D., Jiang, S. F., Udaltsova, N., Quesenberry, J. R., Chan, J. and Habel, L. A. 2009, Int. J. Cancer, 125, 2173-2178.