IN VIVO ANALYSIS OF THE GENOTOXIC POTENTIAL OF 14-HYDROXYLUNULARIN, A MOLECULE WITH LEISHMANICIDAL EFFECT

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ABSTRACT

In this study, we evaluated whether 14-hydroxilunularin, a hydroxyibibenzyl derivative that showed *in vivo* and *in vitro* leishmanicidal activity, has genotoxic effects in bone marrows of treated mice by analyzing the percentages of chromosomal aberrations (CA) in metaphasic cells and micronucleated polychromatic erythrocytes (MNPCE). The frequencies of CA in bone marrow cells of the mice treated with three different concentrations of 14-hydroxylunularin were not statistically significant when compared with the negative control group. The frequencies of MNPCE in bone marrow cells of mice treated with three different concentrations of 14-hydroxylunularin were similar to the frequency found in the negative control group. The results indicate that the 14-hydroxylunularin does not induce an increase of cytogenetic damage in bone marrow cells of mice exposed to the same, treated for 48 hours. *www.relaquim.com*

Keywords: 14-hydroxilunularin, leishmanicidal, genotoxic, chromosomal aberrations, micronucleated polychromatic erythrocytes, urethane.

RESUMEN

En este trabajo analizamos si la 14-hidroxilunularina, un derivado hidroxibibencílico que mostró actividad leishmanicida *in vivo* e *in vitro,* tiene efectos genotóxicos en la médula ósea de ratones tratados analizando los porcentajes de aberraciones cromosómicas (AC) en células metafásicas y de eritrocitos policromáticos micronucleados (EPCMN). Las frecuencias de AC en células de médula ósea de ratones tratados con tres concentraciones diferentes de 14-hidroxilunularina no fueron significativas estadísticamente cuando se compararon con el grupo control negativo. Las frecuencias

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de EPCMN en células de médula ósea de ratones tratados con tres concentraciones diferentes de 14-hidroxilunularina fueron similares a las frecuencias encontradas en el grupo control negativo. Los resultados indican que la 14-hidroxilunularina no induce un aumento del daño citogenético en células de médula ósea de ratones expuestos al mismo, tratados durante 48 horas. *www.relaquim.com*

Palabras clave: 14-hidroxilunularina, leishmanicida, genotóxico, aberraciones cromosómicas, eritrocitos policromáticos micronucleados, uretano.

INTRODUCTION

Leishmaniasis is a disease found in five continents, and endemic in tropical and sub-tropical regions of 88 countries. Twelve million cases are estimated worldwide while one to two million estimated new cases occur each year and 350 million people are at risk (WHO, 2011).

This disease is treated with antimonials, which are expensive and administered intravenously for several weeks under strict medical supervision. Other drugs such as amphotericin B and pentamidine are used in cases unresponsive to antimonials but the former is considerably toxic and the latter seems to be less effective in controlling *L. braziliensis* infections (Andersen *et al.,* 2005). All these drugs should be handled with caution due to side effects which in the case of antimonials are arthralgia, myalgia, headache, loss of appetite, vomiting and diarrhea, cardio, nephro and hepatotoxicities, which constitute a major constraint to their safety. Some side effects of amphotericin B are nausea, vomiting, fever, hypokalemia, renal insufficiency, anemia and cardiac disorders (Gontijo *et al.,* 2003). Additionally, there have been strains with specific resistance to antimonials because of its use in the treatment of dogs infected with visceral leishmaniasis (Dupouy-Camet, 2004). Some treatment strategies based on liposomal amphotericin B (AmBisome) were found to be very effective but the current drug cost hinders the massive use of this drug (Meheus *et al.,* 2010). A new alkyl-phospholipid, miltefosine, has been very active against visceral leishmaniasis resistant to antimonial therapy and is administered orally but its use is not recommended during pregnancy and a contraceptive method is necessary even after completion of treatment in women of reproductive age (Sindermann *et al.*, 2006). Due to the side effects, cost and emergence of resistant strains is necessary to continue the search for new drugs for the treatment of leishmaniasis.

Hydroxybibenzyl compounds are attributed a great variety of biological effects including antifungal, antimicrobial and cytotoxic among others. In particular, liverworts are a rich natural source of these hydroxybibenzyl compounds as lunularin and 14-hydroxilunularin (Zinsmeister *et al.,* 1991; Hernández-Romero *et al.,* 2005; Niu *et al.,* 2006; Salem *et al.,* 2006; Zhang *et al.,* 2007). The 14-hydroxilunularin, is a hydroxyibibenzyl derivative, isolated from a bryophyte, which showed leishmanicidal activity when its effects were tested both *in vitro* and *in vivo* (Roldos *et al.,* 2008). The analysis of the genotoxic potential of new drugs is a crucial step in their research and development (Andrighetti-Fröhner *et al.,* 2006).

The possible genetic damage caused secondarily by physical, chemical or biological agents is analyzed by cytogenetics or molecular biology techniques. The cytogenetic assay for analysis of chromosomal aberrations provides important information on the genotoxic effects of potential new drugs (Preston *et al.,* 1987) and is widely used for the verification of clastogenic (break in

the chromosome) and/or anuegenic effects (loss of whole chromosomes). For its part, the cytogenetic assay for analysis of micronucleus formation provides key data on the genotoxic effects of the agents tested. The micronucleus assay is also used for verification of clastogenic (structural damage) or aneugenic (chromosomal loss) effects. This technique is highly sensitive, easy to perform and relatively inexpensive (Patlolla & Tchounwou, 2005).

In this paper, we evaluated whether 14-hydroxylunularin, which has shown potent anti-parasitic effect at a concentration of 25 mg/kg in a *in vivo* treatment (Roldos *et al.,* 2008), has genotoxic effects in bone marrow of mice by analyzing the percentage of chromosomal aberrations (CAs) in metaphasic cells and analysis of the percentage of micronucleated polychromatic erythrocytes (MNPCE) in treated animals.

MATERIALS AND METHODS

Experimental drug: 14-hydroxylunularin was obtained by synthesis in the Laboratory of Organic Synthesis of the Department of Chemistry from the Universidad de la Republica (Montevideo, Uruguay) and gently provided for this work. Colchicine, KCl, Giemsa dye, ethyl carbamate (urethane) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA) while dimethyl sulfoxide (DMSO) was purchased from Merck (Germany).

Animals and treatment: This was an experimental work that used Swiss albino mice selected by simple random sampling. Animals were male, 6-8 weeks old and maintained in the animal facility of the Instituto de Investigaciones en Ciencias de la Salud (IICS, UNA) on a 12h day/night cycle with food and water *ad libitum*. When possible, suffering was avoided to animals and the minimum number required for this test was used. There were five treatment groups: Group I: negative control, treated with 20 µL of DMSO diluted in distilled water (used to dissolve the 14-hydroxylunularin), Group II: treated with 12.5 mg of 14-hydroxylunularin, Group III: treated with 25 mg of 14-hydroxylunularin; Group IV: treated with 50 mg of 14-hydroxylunularin; Group V: positive control, treated with 750 mg of urethane, which was dissolved in distilled water. The treatment of each group was repeated five times, totalling five animals per concentration. Animals treated with 14-hydroxylunularin received two administrations by intraperitoneal (i.p.) via at 48 hours and 24 hours before sacrifice, while the positive and negative controls received an administration of urethane or DMSO diluted in water, respectively, 24 hours before sacrifice. Each treated animal was used for both assays; a femur was used for analysis of CA and the other for the analysis of MN. In order to have a larger number of metaphasic cells, all animals were treated with 4 mg/kg of colchicine by i.p. via 1h before sacrifice. In general, we followed the International Principles and Guidelines for Biomedical Research prepared by the Council for International Organizations of Medical Sciences (CIOMS, 2007) for the handling of animals and the study was previously approved by the Ethics Committee of the IICS.

Chromosomal aberrations assay (CA): We used the technique of Preston *et al.* (Preston *et al.,* 1987). The animals were sacrificed by cervical rupture 48 hours after first treatment**,** femurs were then removed and the bone marrow was extracted with hypotonic solution maintained at 37°C. The homogenized material was transferred to a conical tube and centrifuged at 1,000 rpm for 5 minutes. Then the supernatant was discarded and the samples were washed by adding methanol:acetic acid fixative solution (3:1) and centrifuged again. The supernatant was discarded and samples were prepared with the remaining cells. Samples were allowed to air dry and stained with

2% Giemsa stain diluted with distilled water. Then, samples were analyzed under an optical microscope, counting 100 metaphasic cells per treated animal, including those with chromosomal aberrations.

Micronucleus assay (MN): We used the method of Schmid (Schmid, 1975) for the micronucleus assay. The animals were sacrificed by cervical rupture 48 hours after first treatment. Femurs were removed, bone marrow extracted with fetal calf serum and maintained at 37°C. The material was homogenized, transferred to a conical tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and samples were prepared with the remaining cells. Samples were allowed to air dry and 24 hours after they were fixed in absolute methanol during 5 minutes. Samples were stained with 2% Giemsa stain diluted with distilled water. Samples were analyzed using an immersion microscope and 1,000 polychromatic erythrocytes (PCE) per animal were counted, including those with micronuclei (MNPCE).

Statistical analysis: The proportions of Chromosomal Aberrations (CA) (Bousimi *et al.*; 2008; Ayed *et al.*; 2011) and polychromatic erythrocytes (PCE) (Mazzei *et al.*; 2007, Kawanishi *et al.*; 2009) were added up for each group treated with 14-hydroxylunularin and urethane and they were then compared, independently, with the results of the negative control using the Chi^2 test, which is recommended for the analysis of two independent events within the same study with only one sample.

RESULTS

Analysis of the frequency of chromosomal aberrations and micronuclei

To reduce the number of dead animals, the same animal from each treatment was used for both analyses. Colchicine treatment an hour before sacrifice does not interfere with results, as it has been shown that it does not induce MN in bone marrow up to 10 h after treatment (Vallarino-Kelly & Morales-Ramírez, 2001).

Analysis of chromosomal aberrations: Table 1 shows the percentages of chromosomal aberrations (CA) of the animals treated with three different concentrations of 14-hydroxylunularin and with urethane as well as the results obtained in negative control animals. The percentage of CA in the negative control group was 0.4%. In the group treated with 12.5 mg of 14-hy-

Table 1. *Frequency of Chromosomal Aberrations and Micronuclei.* Frequency of CA in 500 cells of mice analyzed and Micronuclei induced in bone marrow cells by the effect of three concentrations of 14-hydroxylunularin. % and mean ± SD of micronucleated cell frequency based on 5,000-cell observation

Treatment groups	Analyzed cells	Total of cells with Chromosomal Aberrations (%)	PCE	MNPCE	% MNPCE (± S.D.)	Hours post-treatment
CTL-	500	02 (0.4)	5,000	6	0.12 (1,30)	24 hs
Uret 750 mg	500	49* (9.8)	5,000	6	0.12** (0,84)	24 hs
VP7 12.5 mg	500	05** (1.0)	5,000	5	0.10** (1,41)	48 hs
VP7 25 mg	500	03** (0.6)	5,000	8	0.16** (1,14)	48 hs
VP7 50 mg	500	02** (0.4)	5,000	59	1.18 *** (2,0)	48 hs

CTL-: negative control; VP7: 14-hydroxylunularina; Uret urethane;
* Statistically significant $p < 0.05$ compared with negative control group
** Statistically not significant $p > 0.5$ compared with negative control group
*** Statistically significant $p < 0.001$ compared with negative control group

droxylunularin was 1.0%, in the 25 mg group was 0.6% and in the 50 mg group was 0.4%. The percentage of CA in the positive control group treated with a single dose of urethane was 9.8%, an increase of almost 20 times compared to the negative control group and the groups of animals treated with 14-hydroxylunularin.

The frequency of metaphasic cells with CA in bone marrow cells of the mice treated with urethane was significant when compared with the negative control group. CA frequencies in bone marrow cells of the mice treated with three different concentrations of 14-hydroxylunularin product were not statistically significant when compared with the negative control group.

Micronucleus analysis. Table 1 also shows the results of the analysis of micronucleus induction in bone marrow of mice. Percentage of MNPCE in the negative control group was 0.12%, 0.12% in the group of 12.5 mg of 14-hydroxylunularin, 0.10% in the 25 mg group and 0.16% in the 50 mg group. The percentage of MNPCE in the positive control group treated with a single dose of urethane was 1.18%. This value was almost ten times higher compared with the negative control group, and almost seven times higher than in the groups treated with 14-hydroxylunularin. The frequency of micronuclei in bone marrow cells of mice treated with urethane was high compared with the negative control group and the difference was significant. The frequencies of MN in bone marrow cells of mice treated with three different concentrations of 14-hydroxylunularin were similar to the frequency found in the negative control group.

DISCUSSION

In the present study, we evaluated the mutagenic effects of 14 -hydroxylunularin in mice. We analyzed bone marrow cells of mice treated with 14-hydroxylunularin for 48 hours, using MN and AC tests. The micronuclei in young erythrocytes arise primarily from chromosomal fragments that are not incorporated into daughter cells upon cell division of erythropoietic cells and it is considered that the variations in the incidence of MNPCE reflect chromosomal damage (Vallarino-Kelly & Morales-Ramírez, 2001). This assay is valid to study either genotoxic (clastogenic or aneugenic) effects of some substances or the possible protective effects of others (Vallarino-Kelly & Morales-Ramírez, 2001; Premkumar *et al.,* 2003; Celik *et al.*¸ 2005). The type of chromosomal aberration detected by the MN test is specifically related to chromosomal breaks or spindle disruption. The analysis of AC frequency is used to identify different types of structure damages such as chromatid and chromosomal breaks, gaps or exchanges (Azevedo *et al.,* 2010). In this study, it was used as complementary analysis.

In the *in vitro* and *in vivo* analysis of 14-hydroxylunularin conducted to assess its leishmanicidal activity, the concentration of 25 mg/kg had a significant antiparasitic activity (Roldos *et al.,* 2008). The concentrations used in this study represent 50, 100 and 200% of that concentration and were chosen to determine the limits of use of the compound. The results of this study indicate that the 14-hydroxylunularin does not induce an increase of cytogenetic damage in bone marrow cells of mice exposed to the same treatment for 48 hours. The result of MNPCE percentage in the negative control group was 0.12%, a value within the normal range (Topaktas *et al.,* 1996), and the data obtained in the positive control group were validated, as they coincide with the data reported in the literature (Choy *et al.,* 1995; Hoffler *et al.,* 2005; Kirkland *et al.,* 2007). We found that the percentage of induction of metaphasics cells with chromosomal aberrations was not significant when compared to the percentage in the negative control group. In the present study, we evaluated the mu-

tagenic effects of 14-hydroxylunularin in mice and the results of the MN test and AC analysis did not show a significant increase in MNPCE or AC frequencies. These results suggest that the 14-hydroxylunularin does not produce mutagenic effects as these results could be attributed to a true innocuity of the compound or to the exposure time we used. Therefore, future studies using longer exposure time are necessary to confirm the innocuity of 14-hydroxylunularin in mammals.

CONCLUSIONS

The results of this study indicate that the 14-hydroxylunularin does not present significant genotoxic (clastogenic and/or aneugenic) effects on bone marrow cells of mice exposed to it in a treatment regimen of 48 hours and under these experimental conditions. This information is very important in the validation process of this compound with very promising features in the finding of an alternative treatment for leishmaniasis.

REFERENCES

Andersen, E.M., Cruz-Saldarriaga, M., Llanos-Cuentas, A., Luz-Cjuno, M., Echevarria, J., Miranda-Verastegui, C., Colina, O., Berman, J.D. (2005) Comparison of meglumine antimoniate and pentamidine for Peruvian cutaneous leishmaniasis. *American Journal of Tropical Medicine and Hygiene* **72** : 133-137.

Andrighetti-Fröhner, C.R., Kratz, J.M., Antonio, R.V., Creczynski-Pasa, T.B., Barardi, C.R., Simões, C.M. (2006) *In vitro* testing for genotoxicity of violacein assessed by Comet and Micronucleus assays. *Mutation Research* **603** : 97-103.

Ayed, Y., Ayed-Boussema, I., Ouanes, Z., Bacha, H. (2011) *In vitro* and *in vivo* induction of chromosome aberrations by alpha- and beta-zearalenols: Comparison with zearalenone. *Mutation Research* **726**(1):42-6. [Epub ahead of print].

Azevedo L, Dragano NR, Sabino AP, Resck MC, Alves de Lima PL, Gouvêa CM. (2010) *In vivo* antimutagenic properties of transgenic and conventional **soybeans 13**(6):1402-8 *Journal of Medicinal Food. Dec.*

Bousimi, A., Bouaziz, C., Ayed-Boussema, I., Hassen, W., Bacha, H. (2008) Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells. *Toxicology* **251**:1-7.

Celik A, Ogenler O, Cömelekoglu U. (2005) The evaluation of micronucleus frequency by acridine orange fluorescent staining in peripheral blood of rats treated with lead acetate. Mutagenesis vol. **20** no. 6 pp. 411–415.

Choy, W.N., Black, W., Mandakas, G., Mirro, E.J., Black, H.E. (1995) A pharmacokinetic study of ethanol inhibition of micronuclei induction by urethane in mouse bone marrow erythrocytes. *Mutation Research* **341** : 255-263.

Council for International Organizations of Medical Sciencies. May of 2007. (1985) International Guiding Principles for Biomedical Research Involving Animals. URL available in: http://www.cioms.ch/1985_texts_of_guidelines.htm. Last accessed on October 2011.

Dupouy-Camet, J. (2004) New drugs for the treatment of human parasitic protozoa. *Parassitologia* **46** : 81-84.

Gontijo, B.Y., Carvalho, M.L.R. (2003) Leishmaniose tegumentar americana. Artigo de Atualizacao. *Revista da Sociedade Brasileira de Medicina Tropical* **36** : 71-80.

Hernández-Romero, Y., Acevedo, L., Sánchez, M. de L., Shier, W.T., Abbas, H.K., Mata, R. (2005) Phytotoxic activity of bibenzyl derivatives from the orchid *Epidendrum rigidum. Journal of*

Agricultural and Food Chemistry **53** : 6276-6280.

Hoffler, U., Dixon, D., Peddada, S., Ghanayem, B.I. (2005) Inhibition of urethane-induced genotoxicity and cell proliferation in CYP2E1-null mice. *Mutation Research* **572** : 58-72.

Kawanishi, M., Watanabe, T., Hagio, S., Ogo, S., Shimohara, C., Jouchi, R., Takayama, S., Hasei, T., Hirayama, T., Oda, Y., Yagi, T. (2009). Genotoxicity of 3,6-dinitrobenzo[e]pyrene, a novel mutagen in ambient air and surface soil, in mammalian cells *in vitro* and *in vivo*. *Mutagenesis* **24** (3) : 279-84.

Kirkland, D., Whitwell, J., Deyo, J., Serex, T. (2007) Failure of antimony trioxide to induce micronuclei or chromosomal aberrations in rat bone-marrow after sub-chronic oral dosing. *Mutation Research* **627** : 119-128.

Mazzei, J.L., da Silva, D.N., Oliveira, V., Hosomi, R.Z., do Val, R.R., Pestana, C.B., Felzenszwalb, I. Absence of mutagenicity of acid pyrogallol-containing hair gels. *Food Chemical Toxicology* **45** : 643-648.

Meheus, F., Balasegaram, M.,Olliaro, P., Sundar, S., Rijal, S., Abul Faiz, M.D., Boelaert, M. (2010) Cost-Effectiveness Analysis of Combination Therapies for Visceral Leishmaniasis in the Indian Subcontinent. *PLoS Neglected Tropical Diseases* **4** : 8-18.

Niu, C., Qu, J.B., Lou, H.X. (2006) Antifungal. Bis[bibenzyls] from the Chinese Liverwort *Marchantia polymorpha. L. Chemistry & Biodiversity* **3** : 34-40.

Patlolla, A.K., Tchounwou, P.B. (2005) Cytogenetic evaluation of arsenic trioxide toxicity in Sprague-Dawley rats. *Mutation Research* **587** : 126-33.

Premkumar, K., Bowlus, C.L. (2003) Ascorbic acid reduces the frequency of iron induced micronuclei in bone marrow cells of mice. *Mutation Research* **542** : 99-103.

Preston, R.J., Dean, B.J., Galloway, S., Holden, H., McFee, A.F., Shelby, M. (1987) Mammalian *in vivo* cytogenetic assays. Analysis of chromosome aberrations in bone marrow cells. *Mutation Research* **189** : 157-165.

Roldos, V., Nakayama, H., Rolón, M., Montero-Torres, A., Trucco, F., Torres, S., Vega, C., Marrero-Ponce, Y., Heguaburu, V., Yaluff, G., Gómez-Barrio, A., Sanabria, L., Ferreira, M.E., Rojas de Arias, A., Pandolfi, E. (2008) Activity of a hydroxybibenzyl bryophyte constituent against *Leishmania spp*. and *Trypanosoma cruzi*: *In silico*, *in vitro* and *in vivo* activity studies. *European Journal of Medicinal Chemistry* **43** : 1797-1807.

Salem, M., Werbovetz, K. (2006) Natural products from plants as drug candidates and lead compounds against leishmaniasis and trypanosomiasis. *Current Medicinal Chemistry* **13** : 2571-2598.

Sindermann, H., Engel, J. (2006) Development of miltefosine as an oral treatment for leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **100**: S17-20.

Schmid, W. (1975) The micronuclei test. *Mutation Research* **31** : 9-15

Topaktas, M., Rencuzogullari, E., Ila, H.B. (1996) *In vivo* chromosomal aberrations in bone marrow cells of rats treated with Marshal. *Mutation Research* **371** : 259-264.

Vallarino-Kelly, T., Morales-Ramírez, P. (2001) Kinetics of micronucleus induction and cytotoxic activity of colchicine in murine erythroblast *in vivo*. *Mutation Research* **495** : 51–59.

World Health Organization (2001). Parasitic Diseases: Leishmaniasis. Available in: http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index3.html. Last accessed on October 2011.

Zhang, X., Xu, J.K., Wang, J., Wang, N.L., Kurihara, H., Kitanaka, S., Yao, X.S. (2007) Bioactive bibenzyl derivatives and fluorenones from *Dendrobium nobile*. *Journal of Natural Products* **70** : 24-28.

Zinsmeister, H., Becker, H., Eicher, T. (1991) Bryophytes, a Source of Biologically Active, Naturally Occurring Material. *Angewandte Chemie International Edition* **30** : 130-147.