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Evaluation of Genotoxic Potential of 2-(Bromoacetamido) Phenylboronic Acid on Balb/C Mice Peripheral Blood Cells Using In Vivo Micronucleus Assay

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ABSTRACT

Boronic acids and their ester derivatives are important compounds in medicinal chemistry. Most boronic acids present no particular toxicity compared to other organic compounds. Despite of this promising sign and the recognition of the therapeutic potential of some boronic acid derivatives by the US Food and Drug Administration, medical applications of these compounds are still restricted due to lack of complete toxicity profile. A novel phenylboronic acid derivative; 2-(bromoacetamido) phenylboronic acid (2-BAPBA) has been recently prepared. In this study, we examined capacity of 2-BAPBA to cause DNA damage in erythrocytes of mice expressed as micronuclei (MN). The test chemical (2-BAPBA) was dissolved to homogeneity in 50% in dimethyl sulfoxide and solution was used at varying doses. The 24h i. p. LD50 of 2-BAPBA was estimated to be 20mg/kg. At the highest 2 doses; 80mg/kg and 100mg/kg of the test chemical, no animal survived. In contrast, no death or toxic symptoms were observed among animals received 5mg/kg and 10mg/kg. Reduced activity was observed in all mice treated with a dose ≥20mg 2-BAPBA /kg b. w. No significant changes in body weight were noted in all mice groups. The increases in percent of micronucleated polychromatic erythrocytes and decreases in percent polychromatic erythrocytes, at doses ranging between 5mg/kg and 35mg/kg for 48 and 72h, were statistically significant ($P<0.01$) compared with parallel control group. The results suggest that the compound, under present experimental conditions, is cytotoxic and genotoxic. To reach more definitive conclusions about this subject, further research with different test systems is needed

Keywords: Balb/c Micronucleus assay, 2-(Bromoacetamido) phenylboronic acid, Cytotoxicity, Genotoxicity, Normochromatic erythrocytes, Polychromatic erythrocytes.

1. INTRODUCTION

The continuous discovery and development of new chemical, biological and physical agents necessitates utilization of rapid and reliable test methods and biomarkers for screening of genotoxicity [1]. Boronic acids and their derivatives are used as biochemical tools for various purposes, including interference in signaling pathways, enzyme inhibition and cell delivery systems [2]**.** The phenyl boronate ligands are useful tool for the specific capture and isolation of cis-diol molecules, such carbohydrates, glycoproteins, enzymes, RNA, nucleotides, etc. Many sensors for detection of sugars and glycoproteins have been prepared based on the interactions between BA and sugars in hydrochloric acid aqueous solution with sodium fluoride as hybridization agent [3-6].

The wide spread protein-based glucose-responsive systems using glucose oxidase and lectin have not achieved success in clinical trials because of their low biostability and potential cytotoxicity [7]. 3-aminophenylboronic acid (3-APBA) was reported to be toxic in Swiss albino mice [8]. This raised concerns about the potential cytotoxic or genotoxic hazards resulting from the exposure to such compounds. Recent epidemiological, in vitro, and animal

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studies revealed a possible role for boric acid, the most abundant physiological form of boron in the plasma, as a chemotherapeutic agent [9-11]. In a nude mouse model of prostate cancer, boric acid decreased tumor size, insulin-like growth factor 1 (**IGF-1**) serum levels and **prostate-specific antigen (**PSA) levels and proteolytic activity[10]. Boric acid at 1mM significantly decreased migration and proliferation of the prostate cancer cell line DU-145 in vitro [11]. These antimetastatic and antiproliferative effects were correlated with dietary intake of boric acid[12]. Other results[13-14] suggested that phenylboronic acid (PBA) is more potent than boric acid in targeting actomyosin-based contractility of metastatic and proliferative properties of prostate and breast cancer cells.

Phenyl BA was also conjugated onto low molecular weight polyethylenimine to generate nanovector which facilitates cancer-targeted RNA delivery [15]. These compounds and other BA have initiated significant interest in boron based small molecules in drug discovery. A novel PBA derivative; 2-(bromoacetamido)phenylboronic acid (2-BAPBA, Figure 1), is an innocuous prospective candidate of 3-APBA that may be useful in treatment of the human or animal diseases. It has been synthesized and patented by the Jordan Company for antibody Production (MONOJO) in collaboration with HIKMA chemicals[16].

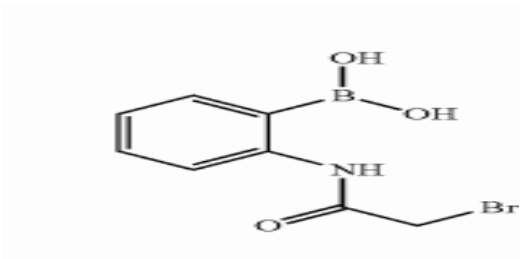

Figure 1: Structure of 2-(bromoacetamido) phenylboronic acid

An extensive review of the literature has shown that little information on the potential risk of this compound on human health has been published. 2-BAPBA was reported to have cytotoxic effects on Chinese hamster ovary (CHO) cell line as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[16]. In this study, the in vivo conventional mouse peripheral blood microscopy micronucleus (MN) test, which does not require metabolic enzymatic transformation activation of a toxic metabolite, was used as a tool to supplement our previous knowledge of the genotoxic properties of 2-BAPBA as a drug candidate[17].

2. EXPERIMENTAL

Chemical

2-BAPBA was a generous gift from (MONOJ, Al-Jubaiha, Amman, Jordan). Methyl methane sulfonate (MMS; CAS number 67–27-3), Giemsa stain, modified solution (CAS number 51811-82-6) and dimethyl sulfoxide (DMSO; CAS number 67–68-5) were purchased from Sigma Aldrich (St. Louis, MO, USA). Colcemid (CAS number 477–30-5) was obtained from Life Technologies/Gibco (Carlsbad, CA, USA). All other chemicals were of analytical grade.

Animals

Six to eight week-old male Balb/C mice (average weight 25g) were obtained from the breeding colonies in the Animal House Facility at Yarmouk University, Irbid/Jordan. The animals were maintained under standard conditions for 5 days for acclimatize before experiment. They were kept in plastic cages in an experimental room under controlled conditions of temperature (22 ± 2 °C), humidity (55 ± 10%), 12-h light/dark cycles and *ad libitum* access. Animals were handled and treated humanely according to Institutional Animal Ethics Committee Guidelines. The study design was approved by the Animal Ethical Committee of Yarmouk University.

LD50 determination

The 2-BAPBA powder was dissolved to homogeneity in 50% DMSO. Further working concentrations were prepared by dilution in normal saline. The LD50% was determined by intraperitoneal (i. p.) administration of a single injection of 0.2ml of 2-BAPBA homogenous solution at doses of 5, 10, 20, 40, 60, 80 and 100mg/kg body weight (6 mice/dose). The negative control group received 0.2ml of 50% DMSO. All animal groups were

monitored frequently for 24 h for signs of toxicity and mortality. The experiment was repeated twice. At the end of the experiments, the dose which killed 3 mice out of 6 (LD50) was recorded.

Micronucleus assay

Mice were randomly distributed in eight groups (4 animals per group). Mice in group 1 (positive control) received 0.2ml MMS solution (40 mg/kg b. w) intraperitoneally. Individuals in group 2 (negative control) received 0.2ml of the highest concentration of the solvent (50% DMSO). The other 6 groups served as experimental groups. Each animal was given the test chemical at a dose of 5, 10, 20, 25, 30 or 35 mg/kg b .w. These doses were chosen on the basis of LD50 preliminary experiments (20mg/kg).

Blood collection and smear preparation

After 48h or 72h of experimental treatment as recommended by the Collaborative Study Group for the Micronucleus Test (CSGMT) [18], peripheral blood samples were collected in heparinized capillaries from retro- orbital vein through a small puncture in the eye socket of the mouse. About 5µl of blood were immediately mixed with equivalent volume of 3% ethylene diamine tetraacetic acid (EDTA) solution (1.5 mg per ml of blood) and smeared onto a clean prewashed glass slide. At least 2 slides were prepared from each animal and smears were fixed in 100% methanol for 1 min and allowed to dry at 40°C in an incubator for an overnight. The slides were double stained as described previously [19] with Harris hematoxylin (10 min) and 0.1% Giemsa (15 min). Slides were rinsed thoroughly in tap water, then differentiated for 10 min in Sorensen's buffer (pH 6.8) and allowed to air-dry. Under blind code, cytotoxicity was followed by determining percentage of polychromatic erythrocytes (%PCE) in a sample of 2000 red blood cells. As an indicator of in vivo mutagenicity, 2000 PCE per mouse were screened for presence of MN and percent of micronucleated PCE (%MNPCE) was recorded.

Statistical analysis

Separate triplicate experiments were carried out. The means, standard deviations and standard errors of the MN frequencies and PCE percentages were calculated. Data were analyzed using Minitab v.14 (Minitab inc., State College, PA, USA). For the determination of the significance among the means, an independent t-test was conducted ($p < 0.01$).

RESULTS

The 24 h LD50 of 2-BAPBA in Balb/C mice was found to be 20mg/kg b. w. (Table 1). No animal survived the highest 2 doses; 80mg/kg and 100mg/kg of the test chemical. In contrast, no death or toxic symptoms were observed among animals received 5mg/kg and 10mg/kg.

Table 1. The death rate of Balb/C mice after 24 h of treatment intraperitoneally with 2-BAPBA dissolved in 50%DMSO

2-BAPBA Dose (mg/kg)	Mice Number	No. of dead mice	% Death
Control (50% DMSO)	6	0	0
5	6	0	0
10	6	0	0
20	6	3	50
40	6	4	67
60	6	5	83
80	6	6	100
100	6	6	100

Reduced activity was observed in all mice treated with a dose ≥20mg 2-BAPBA /kg b. w. This effect started 1h

post treatment, however, the normal activity was recovered after 4h of treatment. Dying experimental animals exhibited sever pathological signs including corner setting, loss of appetite, occasional pawing, burrowing, fast pulse, convulsion and death. Control animals looked healthy without clear signs of death or behavioral changes. No significant changes in the body weight were noted in treated and untreated mice.

Photomicrographs of NCE and PCE with micronuclei are shown in figure (2). Although most cells exhibited a single micronucleus, two micronuclei were encountered in some cells.

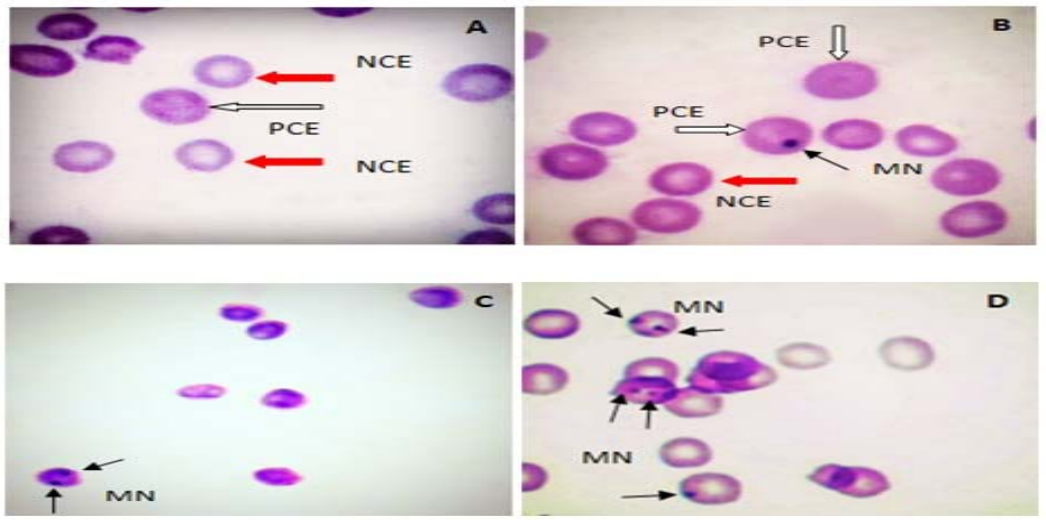

Figure 2: Photomicrographs of peripheral blood erythrocytes of Balb/C mice stained by hematoxylin and 0.1% Giemsa treated with different doses of 2-BAPBA. A: polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE); B: micronucleated erythrocyte (MN); C: bimicronucleated erythrocyte; D: MN observed at high doses of 2-BAPBA and positive control. Magnification 1000X

The % PCE among 2000 erythrocytes is shown in figure (3). The incidence of micronucleated mature erythrocytes (MNNCE) was very low at the 48h and 72h sampling times, therefore, only %MNPCE is presented in figure (4). Test chemical induced statistically significant ($P<0.01$) increases in % MNPCE when compared with the parallel control group. The incidence of MN formation was time-independent.

The effect, at both sampling times, was weak at low doses of 2-BAPBA (less than 3-fold), which became more clear (5-fold) at doses higher than 10 mg/kg. At levels of 35mg/kg (48h treatment) as well as 25mg/kg, 30mg/kg and 35mg/kg (72h treatment) data on % MNPCE were not recorded because mice could not withstand these doses. The positive control (MMS) exerted significant effects upon %PCE and %MNPCE. In contrast, at both treatment times, 10mg/kg dose of 2-BAPBA resulted in significant elevations of %PCE compared to negative control. However, following this initial increase, proportion of PCE started to decline significantly at doses higher than 20mg/kg.

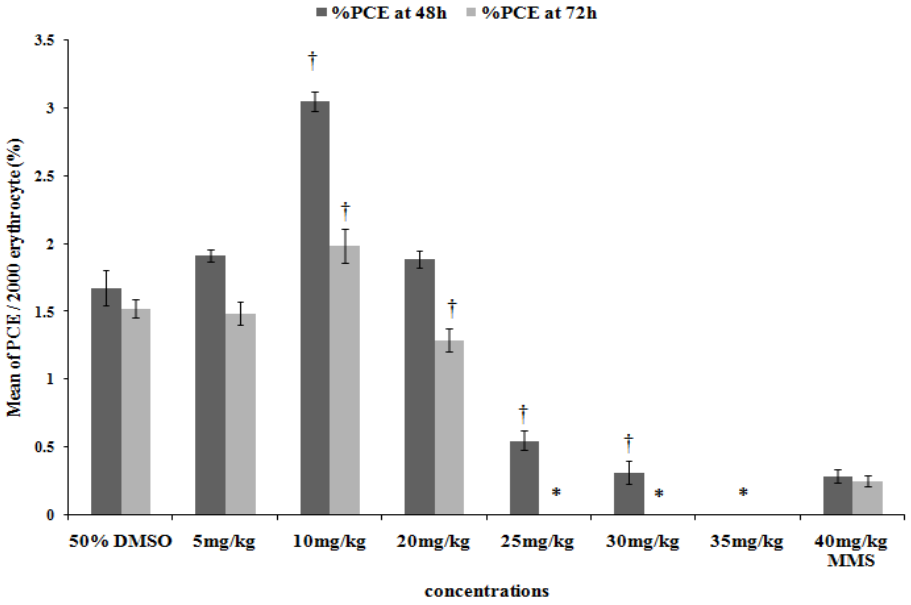

Figure 3: Effect of different 2-BAPBA doses on percent polychromatic erythrocytes (%PCE) in 2000 peripheral blood cells of Balb/C compared with negative control (50%DMSO) and positive control (40mg/kg MMS). Data represent means ± SD for groups of 4 animals. † Significant at $p < 0.01$; independent t-test.* No animals survived at these dose levels. At 20 mg/kg, only half of the animals survived

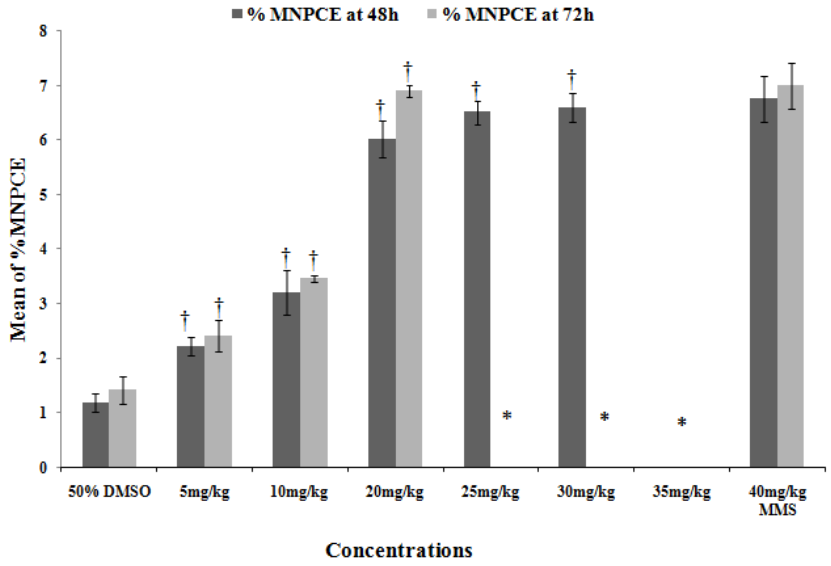

Figure 4: Effect of different 2-BAPBA doses on percentage of micronucleated polychromatic erythrocytes (%MNPCE) among 2000 peripheral blood polychromatic cells compared with negative control (50%DMSO) and positive control (40mg/kg MMS). Data represent means ± SD for groups of 4 animals. † Significant at $p < 0.01$; independent t-test.*No animals survived at these dose levels. At 20 mg/kg, only half of the animals survived

4. DISCUSSION

One of the most important end points in the evaluation of safety is the potential of a compound to become genotoxic. The procedures and experimental design employed in the present study complied with the recommendations of the CSGMT [18] and guidelines of the OECD[20]. The 24h mouse i p. LD50 of 2-BAPBA was estimated to be 20mg/kg. The oral LD50 of the compound in rats was reported by others[16] in the vicinity of 130 mg/kg. The absolute LD50 value for a compound varies among different laboratories, and these variations have been attributed to differences in e.g., protocol details such as solvent and route of administration, animal strains, caging, and test-chemical source [20]. Although it is difficult to extrapolate the finding in one species to those in another species, our results using mice and the i. p. route could be compared to a published Scale for toxicity [22], which is based the oral dose given to rats. Thus, 2-BAPBA may be classified as moderately toxic compound (50-500 mg/kg).

Erythrocyte is particularly well suited to analysis for MN because during maturation of erythroblast to PCE (a period of about 6 h following final mitosis), nucleus is extruded, making detection of MN easier because any MN that has been formed may remain behind in the otherwise anucleated cytoplasm [23]. Furthermore, the PCE still contains rRNA, and so it stains blue-grey with Giemsa. This allows differentiation from the smaller, nonspherical, mature, hemoglobin-containing erythrocytes (NCE), which stain less bluer with Giemsa. This facilitates identification of cells where MN induced by a test substance may be present.

The main finding of the present research is that 2-BAPBA is cytotoxic and genotoxic as evidenced by its ability to induce changes in %PCE and % MNPCE in Balb/C mice peripheral blood. This supports and extends our previous results using CA in *Allium cepa* as an indicator for the test chemical[17]. The test chemical was administered once with doses in the range of 5mg/kg to 35mg/kg. For each dose, one group was sacrificed 48 h and another at 72 h after treatment.

This corresponds to the time necessary for absorption and metabolism of the chemical, the completion of the erythroblast cell cycle, including any test chemical-induced cell-cycle delay, and for extrusion of the erythroblast nucleus[24]. The increase in the frequency of micronucleated cells is an indication that 2-BAPBA is a potential clastogenic or aneugenic chemical[20]. In toxicity studies, the MN assays have been used to assess the carcinogenic potential of compounds and elevated levels of MN have been associated with tumor predisposition in genetically modified mice[25]. The observed general trend of non-linear increment and decline of MN frequencies after a longer duration of exposure to the test chemical could be the result of overdose and toxic effect in this test system. These conditions may have led to cellular death resulting from deletion of primary genes[24, 26-28].

The possibility that the death of the animals might be due to different toxic reasons, for example bromine, rather than the effect on erythrocyte and bone marrow cannot be excluded. It is supposed that the increase in %PCE value probably resulted from an increase in PCE in bone marrow due to either direct or indirect stress of 2-BAPBA on erythropoiesis. At the present time, we do not have a clear evidence or a satisfactory explanation for the cause of this stress. However, it can be suggested that the decrease of %PCE at high doses was attributable to an increase in the numbers of NCE, resulted from rapid differentiation and multiplication or denucleation of erythroblasts. Alternatively, the toxicity of high doses of 2-BAPBA caused significant reduction in erythropoiesis so that PCE could not be produced but NCE in the circulation remained there because there life span reached its maximum i.e. effect was on generated cells but bulk was mature and cycled in blood. The drastic change in erythropoiesis in bone marrow induced by 2-BAPBA treatment would affect fluctuations of %PCE or % MNPCE per non-micronucleated erythrocytes in the MN test.

Unfortunately, no studies similar to ours have been conducted on 2-PABA to compare them with the obtained data. However, in bacteria, a number of boronic acids have been tested using *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli*

strain WP2uvrA(pKM101)[29]. The results with some compounds proved mutagenicity in both TA100 and WP2uvrA (pKM101).

On the contrary, it has been reported that there was no significant genotoxic effects evaluated with all of the boron concentrations by the somatic mutation and recombination test (SMART) on *Drosophila* [30]. In mammalian systems, some boron containing acid like boric acid did not increase the rate of sister-chromatid exchanges (SCE) in CHO cells [31] or in human lymphocytes [32]. Most short-term mutagenicity studies that have been conducted [33] indicated that boron is not genotoxic. In this regard, it was found that none of the boron containing compounds they used; BA, borax pentahydrate (BP) and disodium pentaborate decahydrate (DPD) have significant effects on the level of existing cytogenetic end-points in CCL 62 (HeLa contaminant) human amniotic epithelial cell line [34]. Similarly, treatment of male CD1 mice at different doses of boric and BA showed that most of the tested boron containing acids has very low toxicity [35]. Further contradictory results have been recently reported [36], since boron was able to abolish the genotoxic effects induced by ethyl methane sulfonate (EMS). They found that BA pre-treatment significantly reduced the DNA damaging capacity of H_2O_2 at each tested BA concentration in V79 cells.

5. CONCLUSIONS

The outcomes of data extracted from the present research suggest that MNPCE induced by application of high doses of 2-BAPBA may cause chromosomal damage or damage to the mitotic apparatus. This is of practical interest and should be considered as a warning or an indicator that the tested chemical may cause a risk to human health. However, the results do not preclude the therapeutic consumption of 2-BAPBA in clinical trials, but before this, further research should be performed with different test systems to reach more definitive conclusions about this subject. Some of these activities are being done in our laboratory. The effect of presence of bromine in the test compound is not known and therefore the observed toxicity and lethality may be related to bromine. Modification of the structure of boronic acid-based therapeutics by changing type and/ or position of groups on benzene ring may prove useful in having less toxic derivatives.

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تقييم قدرة حمض برومو أسيتا أميدو فينل بورونك على إحداث سمية جينية باستخدام اختبار تكوين النوى الصغيرة في خلايا دم الفئران البيض

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كلية العلوم، قسم العلوم الحياتية، جامعة اليرموك، إربد، الأردن.

ملخص

حموض البورونك ومشتقاتها من الاسترات مركبات مهمة في الكيمياء الطبية. ومقارنة بغيرها من المركبات العضوية، فإنها غير سامة. وعلى الرغم من هذه الإشارات المشجعة واعتراف إدارة الأغذية والعقاقير بالإمكانات العلاجية لبعض مشتقات حموض البورونك، إلا أن التطبيقات الطبية لهذه المركبات لا يزال مقيداً بسبب عدم وجود التصور الوافي لسميتها. وفي الآونة الأخيرة، تم تصنيع واحدا من مشتقات حمض فينيلبورونيك الجديدة؛ حمض برومو أسيتا أميدو فينل. في هذه الدراسة، قمنا باستقصاء قدرة هذا المركب على إحداث أضرار في الحمض النووي في خلايا الدم الحمر المأخوذة من سلالة الفئران المخبرية البيض. وتظهر هذه الأضرار على هيئة نوى صغيرة يمكن الكشف عنها باستخدام المجهر. وتشير النتائج إلى أن هذا المركب، في ظل الظروف الحالية التجريبية، قادر على استحداث تلك الأضرار الجينية، بل وزيادة نسبة الخلايا الحاملة لتلك الأضرار، بمستويات ذات دلالات إحصائية. للتوصل إلى استنتاجات نهائية أكثر شمولية حول هذا الموضوع، ينبغي إجراء مزيد من البحوث باستخدام نظم تجريبية مختلفة.

الكلمات الدالة: حمض برومو أسيتا أميدو فينل، تكون النوى الصغيرة، التسمم الوراثي، خلايا الدم الحمر، الفئران المخبرية.

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