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Article

Moderate Toxicity of Potential Boron-containing Therapeutic, Dipotassium-trioxohydroxytetrafl uorotriborate - $K_2(B_3O_3F_4OH)$ in Rats and Mice

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Biological activity of boron-containing compounds (BCCs) has been well-known. Growing interest and numerous applications for BCCs have been reported. Boron and boron-containing acids show low acute toxicity in mammals but data on halogenated boroxine (HB) - dipotassium-trioxohydroxytetrafluorotriborate, $K_2(B_3O_3F_4OH)$ acute toxicity have not been reported before. This compound, characterized as a potential therapeutic for skin changes, exhibits no observable genotoxicity in doses lower that 0.1 mg/ml *in vitro* and 55 mg/kg *in vivo.* It has also been confirmed as an antitumour agent both *in vitro* and *in vivo* as well as an inhibitor of enzymes involved in antioxidant mechanisms. The aim of this study was to assess the acute toxicity of HB and to determine the maximum tolerated dose as well as a dose free of any signs of toxicity in different test organisms. Acute toxicity of HB was tested in Sprague-Dawley and Wistar rats and BALB/c mice after single parenteral application of different doses. We determined doses free of any sign of toxicity and LD_{50} after single dose administration. LD_{50} of HB ranges from 63 to 75 mg/kg in different test models, meaning that HB shows moderate toxicity.

Keywords: Halogenated boroxine. BALB/c mice. Wistar rats. Sprague-Dawley rats. drug development.

INTRODUCTION

Boron-containing compounds (BCCs) are widely used in antiseptics, antibiotics and cosmetics, therefore human exposure to BCCs is quite common (Soriano-Ursúa, Das, Trujillo-Ferrara, 2014a). BCCs are toxic at high doses but do not show mutagenic or carcinogenic effects (Soriano-Ursúa *et al.*, 2014b). Biological activity of BCCs has been reported and resulted in their increased use in anti-cancer or anti-inflammatory therapies (Paramore, Frantz, 2003; Ciani, Ristori, 2012; Das *et al.*, 2013). Boric and boronic acid are of the highest medical interest due to their reactivity and diversity (Guo, Shin, Yoon, 2012). The acute toxicity (LD_{50}) of boric acid is 3-6 g for infants and 15-20 g for adults. Clinical symptoms of boron toxicity, in the range 0.1 to 55.5 g per dose, vary according to individuals' age and body weight (Bakirdere, Örenay, Korkmaz, 2010). Boronic acid and certain structurally related compounds do not exhibit toxic effects in humans (Soriano-Ursúa, Das, Trujillo-Ferrara, 2014a; Fu *et al.*, 2014) and show low mutagenic and cytotoxic effects when pan-mechanistic eukaryotic GADD45a genotoxicity assays, BlueScreen HC and GreenScreen HC were applied (Scott, Walmsley, 2015). Generally, inorganic borates show low acute toxicity when applied orally, dermally and by inhalation (Hubbard, 1998). BCCs are considered safe to be used as potential therapeutic agents (Soriano-Ursúa *et al.*, 2014b). Novel structure-

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activity association studies of BCCs give promising results in development of new preventive, diagnostic and therapeutic drugs with a low toxicity risk (Farfán-García *et al*., 2016). Inorganic compound dipotassium-trioxohyd roxytetrafluorotriborate, $K_2(B_3O_3F_4OH)$, is a member of halogenated boroxines, derivatives of cyclic anhydride of boronic acid (Hall, 2005). Cyclic anhydride form of modified dipeptidyl boronic acid is also present in the antineoplastic agent bortezomib (*Velcade®, Millennium Pharmaceuticals*) (Paramore, Frantz, 2003). The primary structure of $K_2(B_3O_3F_4OH)$ halogenated boroxine (HB) contains 4 fluorine atoms substituted in 6-membered ring. It structurally resembles the BRAF (B-Raf Serine/ Threonine Kinase enzyme) and MEK (mitogen-activated protein kinase kinase enzyme) inhibitors often used for treatment of metastatic melanoma caused by mutations in *BRAF* or *MEK* genes. Since the patenting of HB as a prevention and therapeutic agent for various skin changes (Galic, 2012; 2013), numerous *in vitro* analyses were conducted to investigate its biological activities (Haveric *et al*., 2011; Hadzic *et al*., 2015; Haveric *et al*., 2016; Hadzic *et al*., 2019). So far, anti-tumour activity of HB, particularly towards mammary carcinoma (4T1), melanoma (B16F10) and squamous cell carcinoma (SCCVII) *in vitro* has been proven (Ivankovic *et al*., 2015). It also exhibits significant inhibitory effect on tumour growth *in vivo* regardless of the application route (oral, intramuscular or topical application on skin) with the characteristics similar to the antitumour drug 5-fluorouracil (Ivankovic *et al*., 2015). In GR-M cell line, HB changes expression of more than 30 genes (common anti-tumour drug targets), with significant downregulation of *IGF-1* and *hTERT* genes (Pojskic *et al*., 2016), which indicates antitumour activity of HB. Within physiological concentration of Ca^{2+} ions, HB *in vitro* inhibits the growth of tumour cells while inhibitory effect in normal cells is insignificant (Ivankovic *et al*., 2016).

Because BCCs are prone to interacting with proteins that expose hydroxyl groups, HB reduces catalase activity that is possible apoptosis-inducing mechanism in tumour cells (Islamovic, Galic, Milos, 2014). HB also reduces activity of superoxide dismutase (Herenda *et al*., 2018) and horseradish peroxidise by conformational changes in its structure (Ostojic *et al*., 2017). HB is a strong inhibitor of human carbonic anhydrase, especially hCA IX (transmembrane) isoform (Vullo *et al*., 2015), a marker of hypoxia, that is highly expressed in solid tumours and thus a potential target for their treatment (McDonald *et al*., 2012).

Current data on HB indicate its bioactivity at low concentrations, low toxicity and significant antitumour potential. Although boronic acid is not toxic, due to the proposed and confirmed therapeutic properties of its derivative, this study aimed to assess the acute toxicity of HB in order to establish the maximum tolerated dose and a dose free of any signs of toxicity in different organisms.

MATERIAL AND METHODS

Chemicals

Dipotassium trioxohydroxytetrafluorotriborate, $K_2(B_3O_3F_4OH)$ (HB) (synonym: potassium tetrafluorotriborate), is white powder, soluble in water (7.1% at 20ºC), ethanol and DMSO. The substance was synthesized at the Department of Chemistry, Faculty of Science, University of Sarajevo, according to the modified method described by Ryss and Slutskaya (1951). The process of HB synthesis yields 99.99% purity of the substance with less than 0.01% of total impurities. Molecular weight (salt form) is 251.6 g/mol. Input compounds, the highest purity reagents, used in this study were commercially obtained from Sigma-Aldrich (St. Louis, MO).

Test system

The research consisted of two independent experimental lines. The first experiment was conducted at the Centre de Recherches Biologiques (CERB), France on the SPF (Specific Pathogen Free) Sprague-Dawley - Crl: OFA (SD) rats while the other was conducted at the Faculty of Medicine, University of Sarajevo, Bosnia and Herzegovina, on the Wistar rats and BALB/c mice.

Animals were housed in the laboratory for a minimum of five days. Only animals with no visible signs of illness were used for the study. The rats were kept on a standard diet with free access to water. The

animals were housed in groups of 5 (Wistar rats) or 2 or 3 (Sprague-Dawley rats) in cages of standard dimensions with sawdust bedding under the appropriate standard conditions of temperature, humidity and day/night cycle according to the Animal Welfare Law (Official gazette of BiH No. 25/09) and OECD Guidelines (2001) adapting to technical progress for the 29th time Council Directive No. 67/548/EEC and subsequent amendments.

The research on the Sprague-Dawley rats was conducted on the total of 8 females, 7-8 weeks old, nulliparous and non-gravid. The experiment also included 42 BALB/c mice, 6-7 weeks old (21 males and 21 females), and 42 Wistar rats, 8-10 weeks old (21 males and 21 females) at the time of HB administration. All animals were marked individually by a marker pen. For Wistar rats and BALB/c mice, six animals (3 females and 3 males) were treated for each applied dose.

The Ethics Committee of the Institute for Genetic Engineering and Biotechnology approved the study on Wistar rats and BALB/c mice (Approval No. 13-1/14). The study plan related to the study on Sprague-Dawley rats (Study No. 20130224TRP) has been approved by the CERB Internal Ethics Committee. Animal use and care were in accordance with the Directive 86/609/EEC European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Administration of the HB

The test substance was administered dissolved in 0.9% NaCl. Formulations were prepared and kept at room temperature and used within 2 hours. Each preparation was mixed by vortexing followed by magnetic stirring until dissolution was achieved.

Each dose of HB was expressed in mg/kg/body weight (bw) and the administered volume was adjusted to the individual bw determined just before administration.

In the Sprague-Dawley rats test substance was administered as a single dose by the intravenous (i.v.) route in the caudal vein using a stepwise procedure. The starting dose in one Sprague-Dawley female was 50 mg/kg/bw. As no symptoms occurred in this female, another female was treated a higher dose of 100 mg/kg. As mortality occurred in this second female, three females were treated with 75 mg/kg/bw. After mortality occurred in one of these females, three females were finally treated with 60 mg/kg/bw of HB. The intraperitoneal (i.p.) administration of test substance was used for the Wistar rats. At the start, six animals, three males and three females, were treated with 30 mg/kg/bw; as no mortality occurred, six animals were treated with 40 mg/kg/bw also with no lethal outcome. Subsequently, equivalent groups were treated with doses ranging from 50 to 90 mg/kg/bw of HB, with 10 mg/kg bw increments Mortality, behaviour and clinical signs were observed. BALB/c mice were treated by the intraperitoneal (i.p.) route in the single administration. Groups of three males and three females were treated with doses ranging from 10 to 70 mg/kg/bw with 10 mg/kg/bw increments. We observed behaviour and clinical symptoms or mortality. Observations of all the surviving animals were continued over subsequent fifteen days.

Median lethal dose (LD_{50}) calculation

Acute toxicity (LD_{50}) testing has been performed using combination of stepwise and up-and-down procedure (OECD, 2008). LD_{50} was calculated in R using the *ecotoxicology* (1.0.1) package according to Miller-Tainter methodology. Statistics and plotting were completed in R (3.6.1) using *tidyverse* (1.3.0). The data underlying our calculations are available in supplemented materials.

Observation of animals

The body weight of the animals was measured at the beginning of the study in order to determine administered volume of HB solution. The general disposition, behaviour and activity of all surviving animals were observed for 15 days after the experiment conclusion. The animals were examined once before dosing, twice on the day of treatment (90 minutes post-dosing and between 3rd and 4th hour post-dosing) and daily from the 2nd to the 14th day of experiment.

Cadavers found after the dosage administration were subjected to immediate necropsy. Surviving

animals were euthanized and subjected to necropsy on the 15th day of extended observation. The organs (brain, liver, spleen, kidneys, stomach, intestines, gonads/reproductive tract, lungs and heart and injection site) were examined macroscopically.

RESULTS

Acute toxicity of HB was investigated in three animal strains (Sprague-Dawley and Wistar rats and BALB/c mice). Clinical and necropsy findings were evidenced but not quantified. Summarized data are given in Table I.

TABLE I - Dose-dependent clinical and necropsy findings among used animals and strains

Animal strain	Dose mg/kg	Clinical findings	Necropsy
Sprague-Dawley rats	60; 75	body sag or decrease in grip strength, cyanosis, hind limb reflex or loss of pinna reflex, increased drinking or change in body posture, lacrimation, noisy breathing, piloerection, tremors,	
	75	polypnea, reduced spontaneous locomotion with abnormal gait,	
	75; 100	polypnea, reduced spontaneous locomotion with abnormal gait, vocalization	enlarged spleen enlarged adrenals
Wistar rats	40	hypothermia, leg stretching, passivity,	
	40; 60	lying on the side	
	60	body stretching, dyspnea, vocalization	
	80; 90	absence of spontaneous locomotor activity, cyanosis, tremors, absence of reactivity	bleeding in abdomen and heart, lung clots
	40; 80; 90	bradypnea	
BALB/c mice	30	lying on the side, scab around the site of administration, hypothermia,	
	30; 60	body stretching	
	60	bradypnea, convulsions, tail pitching, vocalization	
	70		blood in lungs and pleura, severe abdominal bleeding

Acute toxicity in Sprague-Dawley rats

Body weight of Wistar rats ranged from 152 to 183 g with the mean of 162.75 ± 12.91. Stepwise procedure was used for testing toxic effects of HB (Table II). At first, one animal was treated with the starting dose of 50 mg/kg/bw. As no mortality occurred, another animal was treated with 100 mg/kg/bw of tested substance. After mortality occurred in the 100 mg/kg/bw dose, three females were treated with 75 mg/kg/bw of HB. Mortality occurred in one of these females thus three females were treated at 60 mg/kg/bw with no lethal consequence.

TABLE II - Acute toxicity screening of i.v. HB administration in Sprague-Dawley rats

Test system	Dose per day (mg/kg)	Treated animals			Mortality (x/N)		
		Male (♂)	Female (♀)	Total	Male (♂)	Female (♀)	Total (%)
Sprague-Dawley rats	50	0	1	1	0/0	0/1	0/1 (0%)
	60	0	3	3	0/0	0/3	0/3 (0%)
	75	0	3	3	0/0	1/3	1/3 (33.33%)
	100	0	1	1	0/0	1/1	1/1 (100%)

In both cases the death occurred approximately 45-50 minutes post-dosing. In the female treated with 75 mg/kg/bw, clinical findings before the death included reduced spontaneous locomotion with abnormal gait 10 minutes post-dosing. The absence of spontaneous locomotor activity and polypnea occurred 30-45 minutes post-dosing. Cyanosis appeared 45 minutes after the treatment. Necropsy performed approximately 2 hours after the death revealed no unusual findings. In the female treated with 100 mg/kg/bw, except vocalization and restlessness during the intravenous administration, no clinical sign was recorded before the death. At immediate necropsy, there were no major findings.

The observations at 50 mg/kg/bw revealed no clinical signs after dosing, only a scab in the site of injection from the 1st up to the 3rd day. At 60 and 75 mg/kg/bw (3/3 and 2/3 females, respectively) doses a scab in the site of injection from the 5th up to the 14th day with a scab along the tail from 7th day in 1/2 surviving female treated at 75 mg/kg appeared. A number of clinical findings were observed on the day of treatment (mainly 1 hour post-dosing). Those included: noisy breathing, increased drinking or change in body posture (recumbent position or lying on side), and central excitation (tremors, absence of startle response or insensitivity to pinching of tail), changes in mood or awareness (vocalization, passive response to finger approach, passivity when touched or absence of reactivity), changes in motor activity (absence of spontaneous locomotor activity, reduced or increased spontaneous locomotor activity), autonomic profile or miscellaneous (lacrimation, piloerection, polypnea or cyanosis), and in muscle tone (body sag or decrease in grip strength, body tone, abdominal tone or limb tone), changes in reflexes (hind limb reflex or loss of pinna reflex) or in motor coordination (staggering gait). The observed clinical findings occurred in one of the three animals treated with 60 mg/kg and were more common in animals treated with 75 mg/kg/bw (Table I). No clinical findings were recorded from the 2nd up to the 14th day in all surviving animals treated with these doses.

On the 15th day, at necropsy, there were no macroscopic findings on organs of animals treated at 50 or 60 mg/kg/bw doses. In the two surviving animals treated with 75 mg/kg/bw, macroscopic examination revealed enlarged spleen and enlarged adrenals.

Overall, clinical signs were registered at 60 mg/kg/bw and LD_{50} in Sprague-Dawley rats is estimated to be 74.99 ± 2.06 mg/kg/bw (Figure 1).

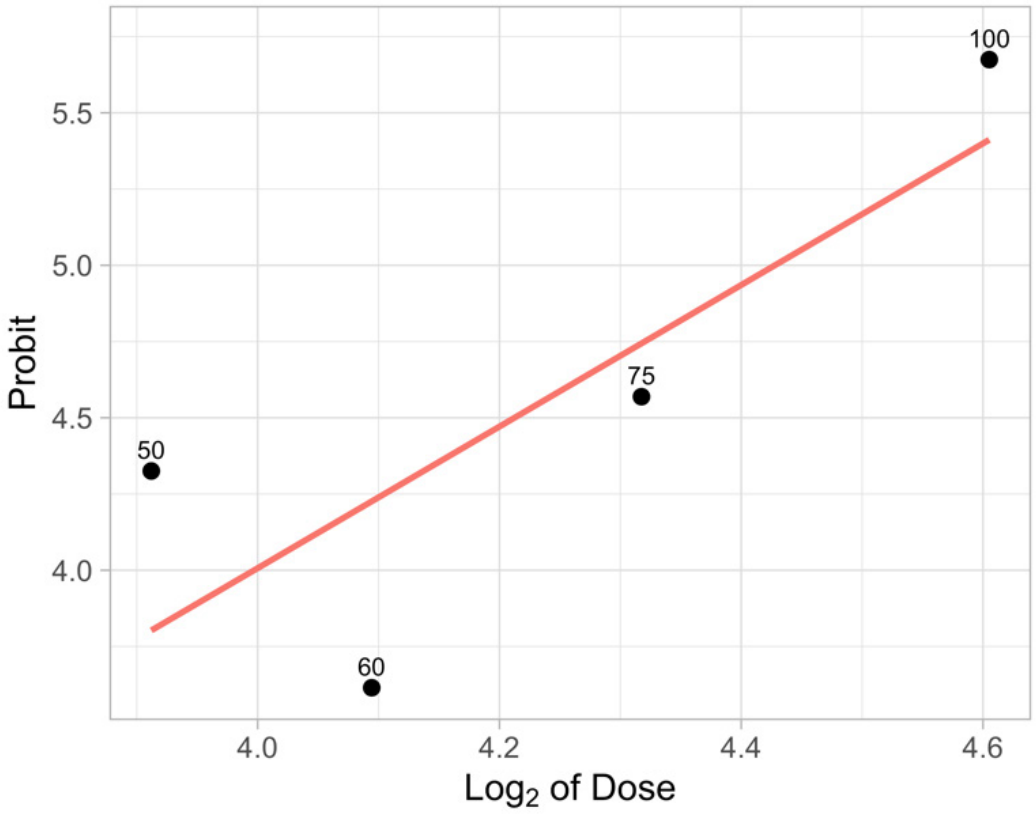

FIGURE 1 - Plot of log-doses versus probits for i.v. LD_{50} calculation of HB in Sprague-Dawley rats.

Acute toxicity in Wistar rats

In this experimental line, body weight of Wistar rats ranged from 200 to 278 g with the mean of 232.76 ± 23.67 g. Animals were not further subjected to weighting in the treatment follow up. In rats treated with 30 and 40 mg/kg/bw, no mortality occurred. Mild clinical signs at dose of 40 mg/kg/bw included lying on the side, leg stretching, bradypnea, and hypothermia (expressed as shivering), passive response to finger approach, passivity when touched while reacting to sounds (Table I). In 4-8 hours, post-dosing, all the treated animals recovered.

Three males and three females were treated with 50 mg/kg/bw of HB with the fatal outcome in two males and all females 2-4h post-dosing (Table III). When equivalent groups were treated with 60 mg/kg of HB, clinical signs in 60 mg/kg dose included dyspnea, lying on the side, body stretching, vocalization, spontaneous tail pitching. Mortality occurred in two males and one female within 24 hours. Mortality rate in females and males treated with 70 mg/kg of HB was 2/3 registered 1-3 hours post-dosing. In 80 and 90 mg/kg treatment, overall mortality rate was 83.33% occuring within 60 minutes post dosing. Clinical signs in those treatments included tremors, absence of spontaneous locomotor activity, lying on the side or on the back, bradypnea, cyanosis, vocalization, absence of reactivity (Table I). Necropsy in dead animals treated with 80 and 90 mg/kg revealed bleeding in abdomen and heart as well as lung clots. Intraperitoneal LD_{50} in Wistar rats is estimated at 62.84 ± 2.13 mg/kg/bw for both sexes (Figure 2); 52.31 ± 2.41 mg/kg/bw for females and 66.44 ± 4.8 mg/kg/bw for males.

TABLE III - Toxicological study of different doses of HB (i.p. administration) in Wistar rats

Test system	Dose per day (mg/kg)	Mortality (x/N)		
		Male (♂)	Female (♀)	Total (%)
Wistar rats	30	0/3	0/3	0/6 (0%)
	40	0/3	0/3	0/6 (0%)
	50	2/3	3/3	5/6 (83.33%)
	60	2/3	1/3	3/6 (50%)
	70	2/3	2/3	4/6 (66.67%)
	80	2/3	3/3	5/6 (83.33%)
	90	2/3	3/3	5/6 (83.33%)

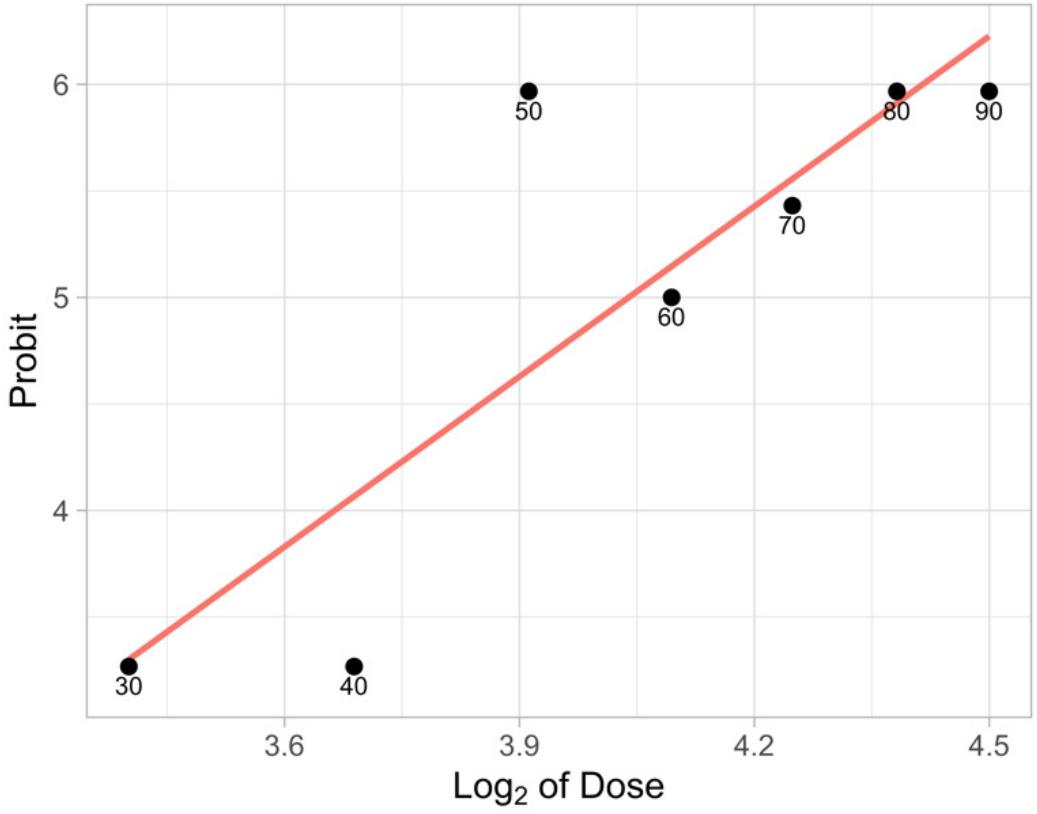

FIGURE 2 - Plot of log-doses versus probits for i.p. LD_{50} calculation of HB in Wistar rats.

Acute toxicity in BALB/c mice

Body weight of BALB/c mice ranged from 22 – 30 g (25.2 ± 1.99 g). Treatments of BALB/c mice with HB in doses of 10, 20, 30 and 40 mg/kg/bw, regardless the sex used, were not lethal (Table IV). Observation of animals treated with doses higher than 30 mg/kg revealed mild symptoms such as lying on the side, scab around the site of administration, body stretching in the first hour post dosing. Two to five hours post dosing, the mice were hypothermic, slightly sensitive to touch and sound. After five hours of application mice were sleepy and still but with the response to touch. After 24 hours, animals were fully recovered. The treatment with 60 mg/kg resulted in more pronounced symptoms including body stretching and convulsions, bradypnea with vocalization, tail pitching, and absence of the response to touch and locomotor activity with the rejection of food (Table I). These symptoms subsided 8 hours following the treatment, with full recovery of surviving animals within 24 hours. In this treatment, 50% of animals were found dead, two within 24 hours post-dosing, one on the 15th day post-dosing indicating possible prolonged toxicity and the need to assess chronic toxicity of HB. Dose of 70 mg/kg immediately induced strong mortality and death on the 1st, 2nd and 3rd day post-dosing (4/6 treated animals). At necropsy, severe abdominal bleeding was revealed with the blood present in lungs and pleura. Differences in macroscopic findings between males and females were not registered. Summarized results of acute toxicity estimations in BALB/c mice are presented in Table III and log-doses presented in Figure 3. LD_{50} in BALB/c mice is estimated to be at 61.27 ± 1.67 mg/kg/bw for all treated animals; 70.17 ± 4.63 mg/kg/bw for females and 51.32 ± 1.89 mg/kg/bw for males.

TABLE IV - Toxicological study of different doses of HB (intraperitoneal administration) in BALB/c mice

Test system	Dose per day (mg/kg)	Mortality (x/N)		
		Male (♂)	Female (♀)	Total (%)
BALB/c mice	30	0/3	0/3	0/6 (0%)
	40	0/3	0/3	0/6 (0%)
	50	1/3	1/3	2/6 (33.33%)
	60	2/3	1/3	3/6 (50%)
	70	3/3	1/3	4/6 (66.67 %)

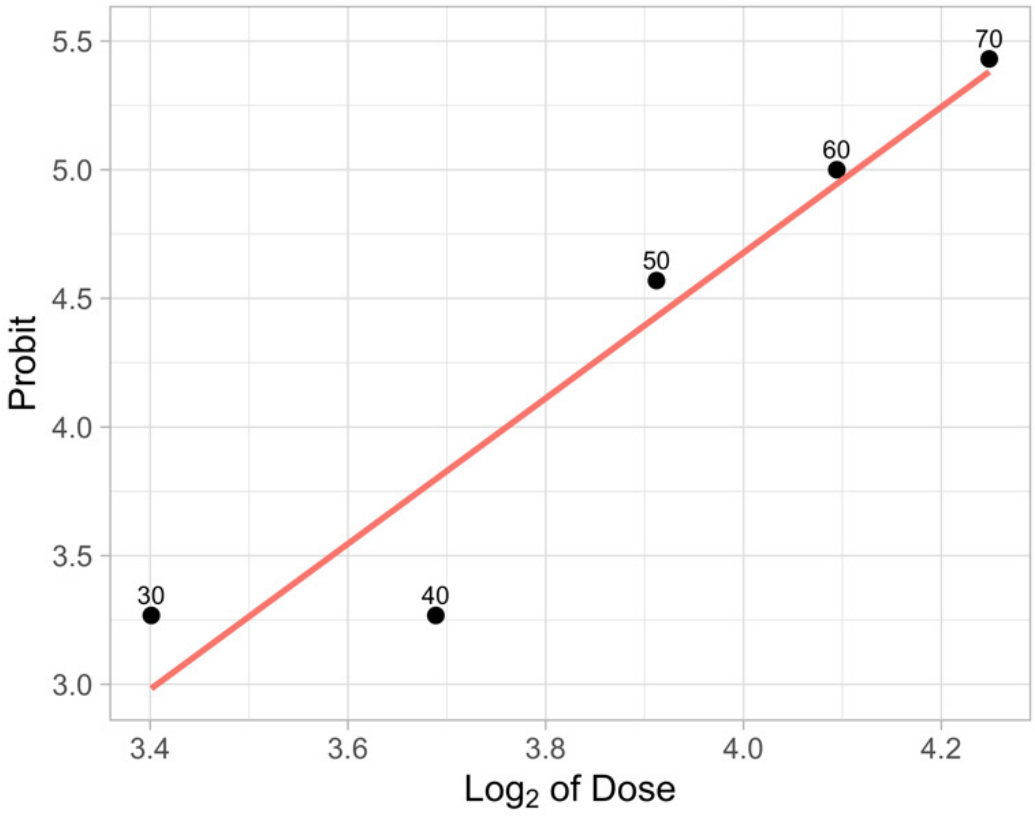

FIGURE 3 - Plot of log-doses versus probits for i.p. LD_{50} calculation of HB in BALB/c mice.

DISCUSSION

Acute toxicity of HB has been investigated in various organisms and two research institutions (local and international one as a blind control), in order to gain the first and broader observation and the most relevant data that would be further used in additional studies. Because of the intended future clinical use of HB, three strains of animals were used to avoid or reduce possible strain specific HB response (Weber *et al.*, 2011). In the experiment, we used i.v. (Sprague-Dawley rats) and i.p. (Wistar rats and BALB/c mice) administration routes that are considered as the most common routes. When the aim is to evaluate the effect(s) of target engagement rather than properties of a drug formulation and/or its pharmacokinetics i.p. is considered as the efficient and more preferable way of administration (Al Shoyaib, Archie, Karamyan, 2019). It is also preferred over the oral route, when avoiding the gastrointestinal tract is needed because of potential degradation/modification of administered agent (Al Shoyaib, Archie, Karamyan, 2019). However, various molecules in i.p. and i.v. administration exhibit different effects (Liu *et al.*, 2016) and in assessment of the overall effect of an agent *in vivo* it is necessary to eliminate possible differences between exposure routes that can affect LD_{50} estimation (Wang *et al.*, 2015). Additionally, as potential clinical application routes for HB are still under investigation, we decided to test two the most invasive and the most common administration procedures.

According to the OECD (2000) toxicity scale, HB belongs in the group of substances with moderate toxicity, with the LD_{50} between 50 and 500 mg/kg/bw (74.99 ± 2.06 mg/kg/bw in Sprague-Dawley rats, 62.84 ± 2.13 mg/kg/bw in Wistar rats and 61.27 ± 1.67 mg/kg in BALB/c mice). Sex and strain dependent HB toxicity was observed as LD_{50} in Wistar rat females (52.31 mg/kg/bw) was lower than LD_{50} in BALB/c and Sprague-Dawley

females (70.17 and 74.99 mg/kg/bw respectively). On the contrary, in Wistar males higher LD_{50} was detected (66.44 mg/kg/bw) than in BALB/c mice (51.32 mg/kg). These differences may be related to differences in cytochrome P450s (CYPs) genes expression in different species and between sexes in rodents (Renaud *et al*., 2011). Female-male differences in response to xenobiotics are observable in behaviour, exposure, anatomy, physiology, biochemistry, and genetic influence toxicokinetics (TK) and toxicodynamics from molecular to organism level in humans and animals (Gochfeld, 2017). As expected, we observed higher toxicity with i.v. administration compared to the i.p. administration that requires higher applied dose to produce similar effect.

Determined LD_{50} values for HB, present the first data of acute toxicity assessment conducted on three different animal strains and provide the basis for future toxicological and pharmacological studies and potential therapeutic use as well. The requirement for HB acute toxicity screening arose from previous studies (Haveric *et al*., 2011; Islamovic, Galic, Milos, 2014; Ivankovic *et al*., 2015; Vullo *et al*., 2015; Pojskic *et al*., 2016; Haveric *et al*., 2020) that proved a pronounced bioactivity and antitumour potential of this BCC. Toxicological profiles of some BCCs, along with boron-containing therapeutics, in humans, based on the environmental, experimental and clinical studies, include following clinical findings: vomiting, diarrhoea, hypotension, metabolic acidosis, gastrointestinal and central nervous system damage etc (Farfán-García *et al*., 2016). Antineoplastic therapeutic agent bortezomib, chemically related to HB, can induce dose-dependent peripheral neuropathy as the most severe side effect of chemotherapy against multiple melanomas, mainly after pre-treatment with neurotoxic agents or in condition of pre-existing neuropathy (Argyriou *et al*., 2014). According to other studies of BCCs chronic toxicity, symptoms of respiratory irritation such as dry cough, nose bleeds, and sore throat were reported in exposed workers (Garabrant *et al*., 1985). In our study, respiratory difficulties were also registered and along with necropsy findings of extensive internal bleeding and clot formation in the lungs and abdomen, they require additional chronic toxicity evaluation and confirmation, especially with the mortality case on the 15th day post-dosing in one BALB/c mouse.

In our recent study, Wistar rats received single intraperitoneal doses of 10, 25, 35 and 45 mg/kg/bw and biochemical parameters were observed 24 and 96 h following the treatment. Effects of HB on biochemical blood parameters were also observed 24 h after continuous nine-days' application of 10 mg/kg i.p and 50 mg/kg/bw per os. Kidneys, liver, spleen, lungs and heart were subjected to histomorphological observation therefore it was not repeated in this study. Mild to moderate HB dose-dependent lesions in the kidneys and livers have been registered (Haveric *et al*., 2020) and toxic effects in kidneys were already reported for boronophenylalanine and some other BCCs (Farfán-García *et al*., 2016). Increased serum levels of creatinine and urea correlate with the observed lesions in kidneys. Urea and creatinine, although significantly increased when compared with control and 10 mg/kg/bw treatment, are prone to reversal towards the expected values 96 h following the HB administration (Haveric *et al*., 2020). However, HB has no adverse effects in single dose administration lower than 35 mg/kg or in repeated dosages at 10 mg/kg/bw (Haveric *et al*., 2020).

When antitumour activity of HB was studied in BALB/c mice syngeneic with 4T1 mammary adenocarcinoma cells, C57BL/6 syngeneic with B16F10 melanoma cells and C3H/H syngeneic with SCCVII squamous cell carcinoma cells, HB slowed the growth of all three tested tumours compared to controls regardless the administration route (intraperitoneal, intratumour, *per os* or topically). This confirms stability and targeted selective activity of HB (Ivankovic *et al*., 2015) mitigating the investigation of acute toxicity of HB in various application routes of administration. In the same study, effects of HB were compared to those of 5-fluorouracil (5-FU). In the mammary adenocarcinoma 4T1 transplanted mice, HB and 5-FU were injected in a dose of 10 mg/kg once a day for five consecutive days starting from 10th day of tumour transplantation. I.p. application significantly reduced the growth of adenocarcinoma 4T1 and reduction was also significant compared to the 5-FU treatment while 5-FU alone had no significant effect on tumour growth compared to the control. When injected into

tumour as a single dose of 50 mg/kg at 10th day after tumour transplantation, 5-FU efficiently inhibited tumour growth but also induced mortality in more than 50% of animals in the group. In contrast, no lethal cases were registered for the same dose of HB (Ivankovic *et al*., 2015). Although we report the first acute toxicity data for HB, the main limitations of present study include the lack of the skin toxicity studies with the eye and skin irritation tests and multiple dosing because HB is intended for the treatment of skin changes.

Under the presented experimental conditions, intraperitoneal administration of HB to Wistar rats in 60 mg/kg dose led to 50% of mortality (LD_{50}= 62.84 ± 2.13 mg/kg/bw). Mild clinical signs were registered at 40 mg/kg/bw dose. HB administered once by the intravenous route at 50 mg/kg/bw induced no sign of toxicity in the female Sprague-Dawley rat. Since there were clinical signs at 60 mg/kg/bw, the NOAEL for Sprague-Dawley rats could be set between 50 and 60 mg/kg/bw and LD_{50} 74.99 mg/kg/bw. The median lethal dose (LD_{50}) of intraperitoneally administered HB in BALB/c mice is estimated at 61.27 mg/kg/bw. The obtained results indicate that HB shows moderate toxicity. These data are consistent with the proposed therapeutic doses and present important basis for further studies regarding chronic and developmental toxicity that should provide additional information.

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Conflicts of Interest/Competing Interests

The authors declare no conflict of interest.

Ethics Approval

The Ethics Committee of the Institute for Genetic Engineering and Biotechnology approved the study on Wistar rats and BALB/c mice (13-1/14). The study plan related to the study on Sprague-Dawley rats (20130224TRP) has been approved by the CERB Internal Ethics Committee.

Authors' Contributions

GB, HA and HS designed the research. HM and HA conducted the experiment. EJ and CT performed data analysis and interpretation. HA and HS wrote the manuscript. All the authors read and approved the manuscript.

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