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Short Communication

Genotoxicity test study of bioactive protein fraction from Lumbricus rubellus, DLBS1033

Agung Heru Karsono[1], Lidwina Tri Kristanti Setiawan[1], Raymond Rubianto Tjandrawinata[1,2*]

1. Dexa Laboratories of Biomolecular Sciences, PT Dexa Medica, Industri Selatan V Block PP No. 7, Jababeka Industrial Estate II, Cikarang, West Java, 17550, Indonesia
2. Center for Pharmaceutical and Nutraceutical Research and Policy, Atma Jaya Catholic University of Indonesia, Jakarta Selatan 12930, DKI Jakarta, Indonesia

ABSTRACT

Introduction: Genotoxicity is one of the safety concerns that can lead to damage at the genetic level. The observation of DLBS1033 using AMES and micronucleus tests was conducted to ensure the safety precautions.

Methods: Ames test was done using 3 strains of Salmonella typhimurium (TA98, TA100, and TA1535) with or without metabolic activation (S9 enzyme). In vitro micronucleus test was performed with prolonged exposure without S9. The variation of DLBS1033 concentrations were 100, 600, and 1200 µg/ml.

Results: The results of the Ames test (with/without S9) and in vitro micronucleus (prolonged exposure without S9) showed that DLBS1033 did not have a mutagenic effect.

Conclusion: DLBS1033 was safe and did not show any genotoxic activity.

Keywords:
Ames test
Genotoxicity
Lumbricus rubellus
Micronucleus test

Introduction

DLBS1033 is a bioactive protein fraction (BAPF) derived from the earthworm *Lumbriculus rubellus*. Earthworms have been extensively researched and are widely known to possess various benefits such as antimicrobial, anti-inflammatory, and anticancer properties. The protein content of earthworms varies depending on the species and their environment. *Lumbricus rubellus* earthworms are reported to contain lumbrokinase, a fibrinolytic enzyme that can be used as a thrombolytic agent (Rong et al., 2010). DLBS1033, as a BAPF from *Lumbricus rubellus*, also exhibits antithrombotic and thrombolytic activities (Trisina et al., 2011; Tjandrawinata et al., 2014; Christy et al., 2015). Ongoing research and development are being conducted. A recent study by Stephani et al. (2023) discovered the presence of potential fibrinolytic enzymes in DLBS1033.

A study regarding the toxicity of DLBS1033 has been conducted. DLBS1033 was tested on mice, and the results showed its safety (Sukandar et al., 2014). Furthermore, safety studies on humans have also been carried out. Some of these include safety tests on healthy volunteers (Tjandrawinata et al., 2016; Gayatri et al., 2018) and patients with acute ischemic stroke (Pinzon et al., 2021). The outcomes of both studies demonstrated a safe profile.

* Corresponding author: Raymond Rubianto, raymond@dexa-medica.com
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There are several chemical, physical, and biological factors that can lead to cell death. Additionally, there are other factors that are non-lethal but damage genetic material, known as genotoxins. The study proceeds to examine the genotoxicity profile. The Ames and micronucleus tests are conducted following the guidelines of the Organization for Economic Cooperation and Development (OECD). The AMES test is conducted using three strains of *Salmonella typhimurium*, namely TA98, TA100, and TA1535, with or without metabolic activation by S9. The present study is expected to provide additional safety-related information about DLBS1033.

Materials and Methods

Materials

DLBS1033 was prepared by PT. Dexa Medica. *Salmonella typhimurium* (strains TA98, TA100, and TA1535), the media, post mitochondrial supernatant (S9), and other reagents required for conducting the Ames test were obtained from the Salmonella Mutagenicity Test Kit (Moltox, Boone, NC, USA). Genotype confirmation and S9 preparation were performed according to the manufacturer's instructions. The sources of positive control articles for the Ames test were as follows: Daunomycin (Moltox, NC, USA), Sodium azide (MP Biomedicals, CA, USA), and 2-Aminoanthracene (MP Biomedicals, CA, USA). The source of positive control articles for the *in vitro* micronucleus test was Mitomycin-C (Roche, TX, USA). RPMI-1640 culture medium, Fetal Bovine Serum (FBS), and Penicillin/Streptomycin were purchased from Gibco (MA, USA).

Ames test

The assay was carried out in accordance with OECD guideline 471 and the manufacturer's instructions. Briefly, three concentrations of DLBS1033 were used: 100, 600, and 1200 µg/ml. The dilutions were prepared immediately prior to use. Two milliliters of molten top agar were added to a tube containing 100 µl of the vehicle or test article and 100 µl of the tester strain. For the S9 additional treatment, 500 µl of the S9 mix solution was added to a test article-contained tube before the strain tester was added. After all components were added, the mixture was vortexed to homogenize and then overlaid onto the surface of 25 ml of minimal glucose agar in a 15× 00 mm petri dish. After the overlay solidified, the plates were inverted and incubated at 37°C for 48 hours. Revertant colonies were then counted manually. Positive controls included daunomycin (6 µg/plate) and sodium azide (1.5 µg/plate) for conditions without S9, and 2-aminoanthracene (10 µg/plate) for conditions with S9. These were plated using the same volumes as the test article.

In vitro micronucleus test

The assay was carried out in accordance with OECD guideline 487. Briefly, blood samples were collected from consenting donors using heparinized anticoagulant tubes. Several 15 ml centrifuge tubes were prepared according to the number of treatments. Each tube contained 0.5 ml of whole blood, 4.5 ml of culture medium (RPMI-1640) supplemented with 10% heat-inactivated FBS, and 1% penicillin-streptomycin. Phytohaemagglutinin (PHA) (Thermo, MA, USA) was added to each culture at a final concentration of 20 µg/ml to stimulate cell division. The blood was then cultured at 37°C, 5% CO_2. Twenty hours after PHA induction, mitomycin-C was added to the culture. At 44 hours post-PHA induction, DLBS1033 (100, 600, and 1200 µg/ml) or negative control (phosphate buffer) was added to the respective cultures. Afterward, 20 µl of 1.5 mg/ml cytochalasin B was added to each culture to inhibit cell division. The cultures were terminated at 72 hours post-PHA stimulation.

Statistical analysis

Statistical differences between the test and negative control groups were determined by student's t-tests (two-sided, $P<0.05$).

Results

Ames test assessment

The strain confirmation and the number of strain colonies were consistent with the theoretical results provided by the Moltox kit. The mutagenicity result for each group was considered positive if the number of revertant colonies was at least twofold higher than that of the negative control. However, this threshold could increase to threefold if the number of colonies in the negative control was too low (< 20 colonies), provided there was a dose-response relationship in at least one strain (Hamel et al., 2016; OECD, 2017; Levy et al., 2019). The results of the Ames test are presented in Table 1. None of the positive criteria was found in the study.

TABLE 1: Ames test result

Test Substance	Concentration	Without metabolic activation (-S9)			With metabolic activation (+S9)		
		Frameshift mutation	Base-pair substitution	Frameshift mutation	Frameshift mutation	Base-pair substitution	Frameshift mutation
		TA98	TA100	TA1535	TA98	TA100	TA1535
DLBS1033 (µg/ml)	0	16 ± 6	101 ± 3	6 ± 3	15 ± 1	123 ± 3	5 ± 0
	100	15 ± 1	112 ± 4	8 ± 2	21 ± 0*	124 ± 11	7 ± 4
	600	16 ± 1	122 ± 13	13 ± 0*	21 ± 2	158 ± 22	9 ± 3
	1200	26 ± 4	139 ± 10*	11 ± 1	24 ± 13	153 ± 37	11 ± 1
Sodium azide (µg/plate)	1.5	-	600 ± 3*	607 ± 15*	-	-	-
Daunomycin (µg/plate)	6	446 ± 10*	-	-	-	-	-
2-aminoanthracenea (µg/plate)	10	-	-	-	367 ± 13*	TMTC[b]	121 ± 0

Data are presented as the mean ± SD of two replicates
* $P<0.05$ compared to negative control (0 µg/ml)
[a] Positive control
[b] TMTC = too many to count

TABLE 2: Micronucleus test result

Test Substance	Concentration (µg/ml)	Long exposure without S9
		Percentage of micronuclei per 1000 binucleate cells
DLBS1033	0	0.6 ± 0.2
	100	0.8 ± 0.2
	600	0.7 ± 0.1
	1200	0.7 ± 0.1
Mitomycin-C[a]	0.1	2.5 ± 0.5*

Data are presented as the mean ± SD of two replicates
* $P<0.05$ compared to negative control (0 µg/ml)
[a] positive control

In vitro micronucleus test assessment

According to OECD guideline 2016, the test substance is considered positive if it meets the following criteria: a) the increase of micronucleus number is dose-related in at least one experimental condition, b) at least one of the test concentrations exhibits a statistically significant increase compared to a concurrent negative control, c) any of the results are outside the distribution of the historical negative control data (95% control limits of the distribution). The results of the *in vitro* micronucleus test are presented in Table 2. The micronucleus percentage per 1000 binucleate of negative control at prolonged exposure without S9 should be around 0.1%–0.9% (95% control limits of the distribution) by the historical data (data not shown). None of the test results were found in the positive criteria.

Discussion

DLBS1033 is a bioactive protein fraction that continues to be developed. It has been used as a drug substance with anti-thrombotic and thrombolytic properties. Genotoxicity testing is necessary to enhance our understanding of the presence or absence of genotoxic activity and to provide greater assurance regarding the safety of the

DLBS1033 formulation. The effects of genotoxins can have adverse consequences, such as triggering cancer (Philips and Arlt, 2017). Moreover, genotoxicity testing has long been performed using the Ames test and Micronucleus test (Sommer et al., 2020) and is continuously evolving, such as the use of bacterial bioreporters (Biran et al., 2010).

Safety studies on the mutagenicity of DLBS1033 have been conducted using two tests: the bacterial reverse mutation assay (Ames test) and the *in vitro* micronucleus test (long exposure without S9). These tests were performed in accordance with the guidelines outlined in ICH S2(R1) (2011). In the Ames test, some concentrations of DLBS1033 showed a significant increase in colony numbers for certain strains. However, this increase did not reach double or more than double, nor was a dose relationship observed. The Ames test indicated that DLBS1033 does not possess mutagenic effects. On the other hand, the negative results from the *in vitro* micronucleus long exposure without S9 were also observed. There was no significant increase observed in micronucleus compared to the control. Based on the results of both genotoxicity tests, DLBS1033 does not exhibit mutagenic effects. The in vitro study data obtained can complement the previously completed in vivo toxicity data (Sukandar et al., 2014).

Lumbrokinase, a component of DLBS1033, contributes to its anti-thrombotic and thrombolytic activities. Although numerous studies have investigated the activity and mechanisms of lumbrokinase, studies on its mutagenicity remain limited. To date, we have found no published studies specifically addressing the mutagenicity of lumbrokinase. Safety studies related to its activity are also limited, such as a recent study by Agoncillo (2021). However, we have conducted safety studies on DLBS1033 itself, as mentioned earlier, and the results support its safety (Tjandrawinata et al., 2016; Gayatri et al., 2018; Pinzon et al., 2021).

DLBS1033 is a bioactive protein fraction that continues to be studied and developed. It has been used as a drug substance with demonstrated efficacy as an anti-thrombotic and thrombolytic agent. The results of the Ames test using *Salmonella typhimurium* (strains TA98, TA100, and TA1535) showed no significant increase in colony counts in any of the strains, supporting the conclusion that DLBS1033 is non-mutagenic. The conclusion was also in line with the *in vitro* micronucleus test (prolonged exposure without S9), which was also negative. Further genotoxicity studies will be conducted as part of ongoing research.

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