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Cytotoxicity and genotoxicity effects of pregabalin in HepG2, PC12, and L132 cells

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Abstract: Although anticonvulsants are primarily approved for epilepsy, they have also been shown to be useful in the treatment of several neurological diseases. Pregabalin is an anticonvulsant commonly prescribed for the treatment of neuropathic pain, fibromyalgia. However, the toxic effects of pregabalin have been reported in humans, involving the incidence of myoclonus. Assessing the toxicity in different cell lines provides comprehensive information on the tissue-specific activity of drugs intended to be used in human health. Few studies are using *in vitro* models, especially human cell lines, regarding the pregabalin cytotoxicity and genotoxicity. In our study, no cytotoxic effects were observed after treating HepG2, PC12, and L132 cells for 24 hours with pregabalin. Furthermore, pregabalin did not show genotoxic effects in HepG2, L132, and PC12 cell lines at all concentrations tested by comet assay. These findings are the first report regarding the pregabalin genotoxicity using *in vitro* human cell lines. Results suggested that a clinically relevant dose range of pregabalin is unlikely to induce cytotoxic and genotoxic effects when employed in the tested cell lines.**:**
Keywords: anticonvulsant, drug safety, comet assay, MTT

INTRODUCTION

Chronic neuropsychiatric diseases negatively disturb overall wellness and increase public health expenditures. Among neuropsychiatric disorders, epilepsy is the most common worldwide, affecting more than 70 million people [1]. Despite the significant growth in anticonvulsant drug development in the last three decades, finding new drugs has been hindered by the lack of knowledge regarding the physiological mechanisms associated with epileptic seizures [2]. Pregabalin is a γ-aminobutyric acid (GABA) analog anticonvulsant designed to be a gabapentin derivative (Figure 1), but presenting pharmacokinetic improvements compared to its predecessor [3]. Unlike gabapentin, the absorption of pregabalin is not dose-dependent, which provides broader pharmacokinetic linearity without transporter saturation. Also, pregabalin is promptly absorbed and reaches maximum plasma concentration in about one hour after administration [4]. Pregabalin has been reported to feature high bioavailability, which results in higher levels of pregabalin in the bloodstream when compared to other drugs [5]. Moreover, pregabalin has better cost-effectiveness than other drugs used in the treatment of fibromyalgia and neuropathic pain [6, 7].

Due to a higher analgesic potency compared with other drugs, pregabalin is commonly prescribed for the treatment of neuropathic pain, fibromyalgia, anxiety disorders, depression, and more recently used for treating postoperative pain [8]. The CDC (Centers for Diseases Control and Prevention) guidelines recommend pregabalin as the first-line agent for neuropathic pain [9]. Studies have demonstrated that pregabalin reduced postoperative pain and contributed to preventing chronic postoperative pain [10, 11].

Figure 1. Chemical structure of pregabalin, gabapentin, and GABA.

Several clinical reports have been demonstrated the positive effects of pregabalin in pain relief [12-14]. The drowsiness and dizziness associated with the treatment with pregabalin are usually reported in the first week of therapy and usually end after two weeks [15]. However, the toxic effects of pregabalin have been reported in humans, involving the incidence of myoclonus [16]. According to the United States Food and Drug Administration (U.S. FDA), pregabalin has a potential for abuse by users [17]. Cases of misuse and abuse of pregabalin have been reported in the treatment of other diseases, such as anxiety, non-neuropathic pain, insomnia, mood instability, and also for recreational use [18].

Pregabalin is prescribed for the treatment of chronic and acute diseases [19]. Therefore, it is necessary to evaluate the genotoxic and carcinogenic potentials [20]. Although experiments with human cell lines are not mandatory according to the current legislation [21], assessing toxicity in different cell lines provides comprehensive information on the tissue-specific activity of drugs intended to be used in human health. The standard *in vitro* test battery is composed of the bacterial reverse mutation assay (OECD TG 471), the *in vitro* mammalian chromosomal aberration test (OECD TG 473), the *in vitro* mammalian cell gene mutation test (OECD TG 476 [Hprt] and TG 490 [MLA/tk]), and the *in vitro* mammalian cell micronucleus test (OECD TG 487) [22]. All OECD Test Guidelines are available at the OECD website.

From the perspective of hazard identification and safety determination, the evaluation of the genotoxic potential of chemical agents is a mandatory step and should be performed using sensitive methods, following appropriate regulatory guidelines. Cellular models are powerful tools for drug screening and toxicity assessment since they can predict and reduce idiosyncratic effects by providing an early, rapid, and cost-effective feedback [23].

Among pharmacological and toxicological studies, HepG2 cells are commonly used for the evaluation of cell metabolism and toxicity [24]. These cells express critical antioxidant and metabolization enzymes that mimic *in vivo* conditions, including phase I and II drug-metabolizing enzymes [25, 26]. PC12 cells are widely used as a model in neuronal function studies [27, 28] since they can synthesize, store, and release noradrenaline and dopamine, and also expresses GABA receptors [29]. Non-cancer cell lines should be investigated to establish the specificity of the effects observed in an experimental *in vitro* system. L132 cells are derived from embryonic human lung tissue and are considered a suitable cell line in cytotoxicity and mitochondrial dysfunction experiments [30, 31].

Anticonvulsants are used worldwide for the treatment of several disorders. Pregabalin is an anticonvulsant commonly prescribed for the treatment of neuropathic pain and fibromyalgia [32]. However, the potential for pregabalin misuse or abuse and

the lack of information regarding the pregabalin cytotoxicity and genotoxicity in human cell lines, the aim of this study was to evaluate the cytotoxic and genotoxic effects of clinically relevant doses of pregabalin using *in vitro* experimental cell-based model.

MATERIAL AND METHODS

Chemicals

Pregabalin (CAS 148553-50-8) was purchased as its pharmaceutical formulation Lyrica (Pfizer, Guarulhos, Brazil). Trypan blue (CAS 72-57-1), methyl methanesulphonate (MMS, CAS 66-27-3), thiazolyl blue tetrazolium bromide (MTT, CAS 298-93-1) and neutral red (NR, CAS 553-24-2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO, CAS: 67-68-5) was obtained from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 Medium, the penicillin-streptomycin mix, fetal bovine serum, and horse serum were purchased from Gibco (Carlsbad, CA, USA). Standard and low melting point agaroses (CAS: 9012-36-6) were obtained from Invitrogen (Carlsbad, CA, USA), and GelRed™ (CAS: 7732-18-5) was purchased from Biotium (Hayward, CA, USA). All other chemicals were analytical grade products with the highest purity available.

Mammalian cell culture

The human liver cancer cell line (HepG2, HB-8065, hepatocellular carcinoma) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibiotic mix. Nontumorigenic human lung epithelial cell line L132 was obtained from Rio de Janeiro Cell Bank (BCRJ/UFRJ, Rio de Janeiro, RJ, Brazil) and cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibiotic mix. The rat pheochromocytoma cell line (PC12, CRL-1721) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI medium 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated fetal horse serum and 1% penicillin/streptomycin antibiotic mix. All cells were maintained in a humid atmosphere with 5% CO_2, 95% air in an incubator at 37 °C.

Cytotoxicity assay: mtt

The MTT assay was performed to evaluate the cytotoxicity of pregabalin in HepG2, L132, and, PC12 cells. The cells were seeded in 96-well plates (1×10^4 cells/well) for 24 hours. Cells were then treated with PBS (negative control), pregabalin (0.2, 0.4, 0.6, 0.8 or 1.0 μM), or methyl methanesulfonate (MMS, positive control, 200 μM). After 24 hours of treatment, the plates were incubated with 10 μL of MTT solution (0.5 mg/mL) for three hours. The medium was removed, and 100 uL of dimethyl sulfoxide was added to dissolve the purple formazan crystals. The absorbance of each well was measured at 570 nm by a microplate reader (Biotek ELx800, Winooski, VT, USA). Cell viability in the negative control was considered 100%, and the relative cell viability was calculated for each treatment.

Genotoxicity assay: alkaline comet assay

HepG2, L132, and PC-12 cells were seeded onto 24-well plate at 3×10^5 cells/well for 24 hours and treated with PBS, pregabalin (0.2, 0.4, 0.6, 0.8 and 1.0 μM), or MMS (200 μM) for 4 hours. Afterward, cells were harvested and the viability of each preparation performed by 4% (w/v) trypan blue exclusion method. The cell viability of all treatments was higher than 80%. Slides were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% (v/v) DMSO and 1% (v/v) Triton X-100) and incubated overnight at 4 °C. On the next day, slides were incubated in the electrophoresis solution (300 mM NaOH and 1 mM EDTA) for 20 minutes at 4 °C and transferred to a horizontal electrophoresis unit containing the same solution. The

electrophoresis settings were 25 V (0.78 V/cm) and 300 mA for 20 minutes at 4 °C. After the electrophoresis; the slides were neutralized with neutralization buffer (0.4 M Tris, pH 7.5) for 20 minutes at 4 °C and then fixed in ethanol for 5 minutes. Immediately before the analysis, the slides were stained with Gel Red® 1/10.000 (Biotium, Fremont, CA, USA) and scored with a fluorescence microscope (Axiostar, Zeiss, Germany) equipped with a 515–560 nm excitation filter, a 590 nm barrier and an integrated camera. One hundred random nucleoids were analyzed per treatment and evaluated by Comet Assay IV software (Perceptive Instruments, Suffolk, UK) at 200 x magnification. Tail Intensity (% of DNA in the tail) was scored for each nucleoid.

Statistical analysis

All data are expressed as a mean ± standard deviation. After assessing the normality of the variable distributions using the Kolmogorov–Smirnov test, data were analyzed using GraphPad Prism 6.0 (GraphPad, Carlsbad, CA, USA). Three independent experiments were performed for each assay. The results were analyzed using one-way ANOVA and *post hoc* Tukey's test and were considered significantly different if p-values were less than 0.05.

RESULTS AND DISCUSSION

A pharmacokinetic study conducted by Bockbrader, Radulovic (33) showed plasmatic concentrations up to 0.1 μM after 3 hours of administration of 300 mg of pregabalin. Our study employed a range of concentrations (0.2 – 1 μM) that are higher than the average plasmatic levels for this molecule to mimic realistic *in vivo* exposure to this compound by humans.

MTT is a well-known colorimetric assay, and it has become the gold standard for the determination of cell viability, proliferation, and cytotoxicity studies [34]. MTT has become a popular test in the academia and pharmaceutical industry because it is versatile and straightforward [35]. The MTT assay results showed no statistical difference between negative control and 0.2, 0.4, 0.6, 0.8, and 1 μM of pregabalin in HepG2, PC12, and L132 (Figure 2). All cell lines treated with MMS (positive control) showed a statistical reduction in cell viability compared with the negative control, which validates the experimental design. There are few studies using *in vitro* models to evaluate the pregabalin cytotoxicity. Our results were consistent with the limited evidence available in the literature. Salat and Librowski (36) reported no cytotoxicity in HepG2 cells treated with 1 μM of pregabalin. In another study, PC12 cells treated with 10 μM of pregabalin [37]. This is the first report of the cytotoxic evaluation of pregabalin using L132 cells.

Damage to the DNA structure can occur through two main mechanisms: spontaneous damage caused by sources inside the cell, and damage resulting from external sources such as chemical agents and radiation [38]. The DNA damage is an essential parameter to screening the machinery of genomic stability, the measurement of DNA damage would give valuable information about the health status of a cell and whole systems and organisms [39]. It is essential to evaluate the genotoxicity of pregabalin because this drug can bind directly to DNA [40]. The alkaline comet assay is a sensitive method that can detect a variety of primary DNA alterations, such as single and double-strand DNA breaks [41]. *In vitro* comet assay is recognized as an OECD test guideline (TG 489) to investigate DNA damage [42] and it has been extensively used for genotoxic screening of novel drugs, cosmetics, and nanomaterials [43].

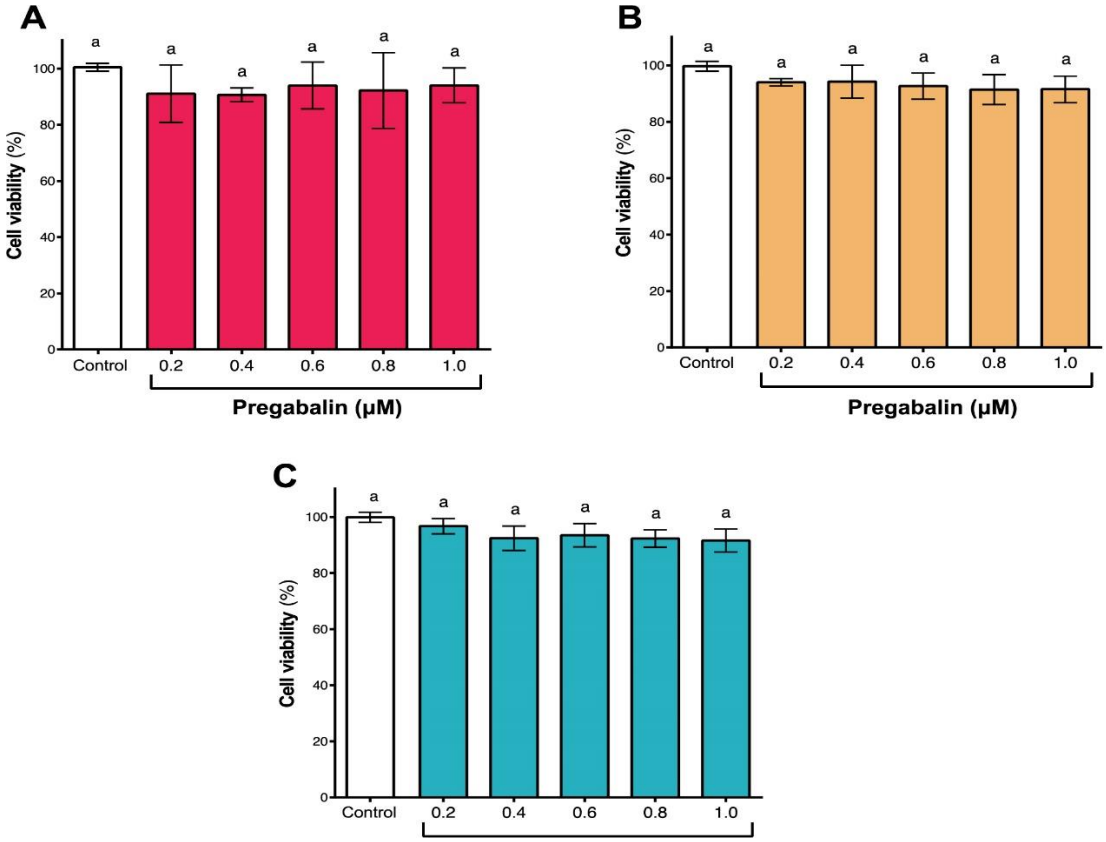

Figure 2. Viability of HepG2 (A), L132 (B), and PC12 (C) cells treated with 0.2, 0.4, 0.6 0.8 and 1.0 µM of pregabalin. Data are expressed as mean ± standard deviation of three independent experiments. Control: PBS. Different letters indicate a significant difference (One-way ANOVA-Tukey, $p < 0.05$).

Table 1. Percentage of tail DNA in HepG2, L132, and PC12 cells after treatment with 0.2, 0.4, 0.6, 0.8 and 1.0 µM of pregabalin.

Treatments	**Tail Intensity (% tail DNA)**		
	HepG2	**L132**	**PC12**
Control	8.9 ± 0.4[a]	6.0 ± 0.6[a]	6.2 ± 0.7[a]
PGB 0.2 µM	10.4 ± 1.2[a]	5.2 ± 0.3[a]	5.3 ± 1.4[a]
PGB 0.4 µM	10.3 ± 1.9[a]	5.3 ± 0.7[a]	5.3 ± 0.7[a]
PGB 0.6 µM	11.5 ± 2.2[a]	5.2 ± 0.9[a]	6.0 ± 0.8[a]
PGB 0.8 µM	11.4 ± 1.8[a]	5.9 ± 0.8[a]	6.3 ± 1.3[a]
PGB 1.0 µM	9.7 ± 0.6[a]	5.1 ± 1.3[a]	5.9 ± 3.2[a]

Data are expressed by mean ± standard deviation of three independent experiments. Control: PBS, pregabalin (PGB). Different letters indicate a significant difference (One-way ANOVA-Tukey, $p < 0.05$).

When compared to the negative control, the results obtained from comet assay showed no genotoxic effects in HepG2, PC12, and L132 cells after the treatment with 0.2, 0.4, 0.6, 0.8 and 1 μM of pregabalin (Table 1). These findings are the first report regarding the pregabalin genotoxicity in human cell lines using the comet assay method, which is the gold standard for measuring DNA strand breaks [44]. Pegg [20] reported no evidence of mutagenicity in mammalian cells after pregabalin using the micronucleus assay. A study using the wing somatic mutation and recombination test in *Drosophila Melanogaster* showed that pregabalin displayed lower genotoxicity when compared with gabapentin [40].

CONCLUSION

Pregabalin is an anticonvulsant prescribed for several types of diseases, in addition to epilepsy. Many clinical cases have shown its efficacy in the treatment of several patients. Although some cases of abuse, intoxication, and recreational use have been reported. This study aimed to investigate the cytotoxic and genotoxic potential of pregabalin using an *in vitro* cell-based model. Our findings suggested that a clinically relevant dose range of pregabalin is unlikely to induce cytotoxic and genotoxic effects when employed in the tested cell lines. The results presented in this study underscore the safety of pregabalin and contribute to filling the gap between the standard *in vitro* test battery and *in vivo* studies. More research should be done using molecular approaches and *in vivo* studies to understand the toxicological properties of pregabalin.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

CONTRIBUTIONS OF THE AUTHORS

PWSS and VPV conceived the presented idea. PWSS carried out the experiments and the analysis process. PWSS wrote the manuscript with support from VPV and LMGA. LMGA supervised the project with a critical revision of the intellectual content.

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