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Assessment of the genotoxicity and cytotoxicity of etazolate in cultured human lymphocytes

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ABSTRACT

Etazolate is a pyrazolopyridine compound that exhibits a range of pharmacological mechanisms, including inhibition of phosphodiesterase-4 activity and modulation of the γ -aminobutyric acid (GABA) signaling pathway. Etazolate has shown promising results in treating depression, traumatic brain injury, and Alzheimer's disease in animal models and clinical trials. However, limited information is available regarding its genotoxicity and cytotoxicity to human cells. Therefore, the current study aims at testing the effect of etazolate on the genetic material using two genotoxicity assays. In addition, the study aims to evaluate the effect of etazolate on the mitotic index (MI) of cultured human lymphocytes. Different concentrations of etazolate (0.01, 1, 10, and 50 μ M) were used. Genotoxicity was assessed using the chromosomal aberrations (CAs) assay, and oxidative DNA damage was assessed using the 8-hydroxy-2-deoxyguanosine (8-OHdG) assay, while cytotoxicity was assessed using the MI assay. Treatment of cultured human lymphocytes with etazolate did not induce CAs at all concentrations examined (p > 0.05). In addition, the normal level of 8-OHdG detected in the control group was not affected by etazolate treatment (p > 0.05). Furthermore, no effect was observed for etazolate on the MI (p > 0.05). In conclusion, current results indicate that etazolate is neither genotoxic nor cytotoxic to cultured human lymphocytes using the indicated assays at the examined concentrations.

INTRODUCTION

Etazolate is a pyrazolopyridine-derived compound that exhibits a wide range of pharmacological properties (Alzoubi *et al.*, 2018; Megat *et al.*, 2021; Siopi *et al.*, 2013). Etazolate enhances GABAergic signaling through positive allosteric modifications of GABA(A) receptors, which could explain its anxiolytic activity (Marcade *et al.*, 2008; Thompson *et al.*, 2002). Etazolate is also a selective phosphodiesterase-4 inhibitor, and thus it can be used to manage mood and cognitive disorders (Ankur *et al.*, 2013; Jindal *et al.*, 2015; Wang *et al.*, 2014). For example,

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using animal models, etazolate showed positive results in the treatment of neuropsychiatric disorders such as depression and anxiety associated with traumatic brain injury via normalization of oxidative stress and restoring the Cyclic adenosine monophosphate (cAMP) signaling pathway (Jindal et al., 2017). In addition, etazolate was shown to stimulate soluble N-terminal amyloid precursor protein fragment (sAPPalpha) secretion and subsequent elevations in the soluble amyloid precursor protein that can protect neurons from the development of beta-amyloid and Alzheimer's disease (Marcade et al., 2008). Moreover, etazolate has been shown to improve spatial learning and memory performance related to the homing task in aged rats (Drott et al., 2010). A pilot phase IIA, randomized, double-blind, multicenter clinical trial that included 159 patients with Alzheimer's disease demonstrated the safety and tolerability of etazolate and its ability to alter disease progression (Vellas et al., 2011). Similarly, data generated from phase 1 clinical trials of Alzheimer's disease showed that

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the etazolate has an acceptable safety profile with fewer side effects than other phosphodiesterase-4 inhibitors (Desire *et al.*, 2008). This made etazolate an attractive agent to be implicated in Alzheimer's disease treatment (Drott *et al.*, 2010; Vellas *et al.*, 2011). Additionally, etazolate was shown to protect against memory impairment anxiety- and depression-like behaviors induced by posttraumatic stress disorder (Alzoubi *et al.*, 2017, 2019) and Parkinson's disease (Alzoubi *et al.*, 2018).

The majority of studies with etazolate have focused on the clinical uses of this drug (Alzoubi et al., 2018; Jindal et al., 2017; Marcade et al., 2008; Megat et al., 2021). However, studies that examine the genotoxicity and cytotoxicity of etazolate to human cells are needed. Previous studies showed that some pyrazolopyridine derivatives might have DNA-binding affinity (El-Gohary and Shaaban, 2018; El-Gohary et al., 2019; Suntharalingam et al., 2010). In addition, some pyrazolopyridine derivatives showed antitumor and antiproliferative activity and could interfere with cell cycle progression (Baviskar et al., 2013; Butu et al., 2014, 2015; Gavriil et al., 2016; Rodino et al., 2014). Therefore, the current investigation aims to evaluate the genotoxicity and cytotoxicity of different concentrations of etazolate in cultured human lymphocytes using the chromosomal aberrations (CAs) assay, the oxidative DNA damage 8-hydroxy-2-deoxyguanosine (8-OHdG) assay, and the mitotic index (MI) assay.

MATERIALS AND METHODS

Blood sampling

Five healthy young men (aged 20–27 years) donated blood for laboratory experiments. Blood donors were nonsmokers and nonalcoholics and had not taken any medications or supplements for at least 3 months before donating blood (Rababa *et al.*, 2021). Blood samples were collected in lithium heparin tubes and cultured fresh within 1 hour of donation (Al-Eitan *et al.*, 2020).

Ethical approval

The study was approved by the Institutional Review Board of King Abdullah University Hospital (Approval No. 23/93/2016). Informed consent was taken from the donors before blood sampling.

Etazolate preparation

Etazolate hydrochloride (Cas No. 35838-58-5) was obtained from Bio-Apex (Houston, TX, Catalog No. B6304) as a powder. The drug was freshly prepared before each experiment by dissolving it in sterile distilled water to obtain a 10 mM stock solution. The stock solution was sterilized using a Millipore filter (0.2 μ M) before being used to treat cultured blood lymphocytes. Cultures were treated with different doses of etazolate (final concentrations: 0.01, 1, 10, and 50 μ M). These doses were within the range that was previously used with cultured cells (Barnes *et al.*, 1983; Marcade *et al.*, 2008; Mehta and Ticku, 1987).

Human lymphocyte cultures

Freshly drawn blood (1 ml) was inoculated into a tissue culture flask containing 9 ml PB-MAX Media (Thermo Fisher Scientific, MA). Cultures were incubated at 37°C, 5% CO2, and

appropriate humidity for 72 hours (Alqudah and Al-Ashwal, 2018).

CAs assay

Cultured cells were treated with different concentrations of etazolate and the positive control (cisplatin, Sigma-Aldrich, USA; final concentration 0.4 µg/ml) in the last 24 hours of the 72hour incubation period (Azab et al., 2019). After cell incubation, harvesting was initiated by arresting the cultured cells for 2 hours in the mitotic phase with 0.1 µg/ml colcemid (Sigma-Aldrich) (Mhaidat et al., 2016). Cultures were then transferred to 15 ml conical tubes and centrifuged at $500 \times g$ for 5 minutes. The supernatant was removed, and cells were then resuspended in 9 ml of prewarmed hypotonic solution (0.56% KCL) and then incubated at 37°C for 20 minutes. Swelled cells were collected by centrifugation at $500 \times g$ for 5 minutes, the supernatant was removed, and cells were then fixed in methanol: acetic acid (3:1) for 10 minutes at room temperature (Khabour et al., 2016). The cells were then washed with the above fixative $3\times$, and finally, cells were resuspended in about 1.5 ml of the fixative. The suspended cells were then dropped onto prechilled glass slides to obtain metaphase chromosomal spreads. Chromosomes were then stained by immersing the slides in slide jars containing 5% Giemsa stain (Sigma-Aldridge, USA) in a phosphate buffer for 7 minutes. Slides were then air-dried, and metaphases were visualized using a Nikon medical microscope at 1,000 × magnification. CAs were examined in 500 cells per treatment (100 cells from each donor). CAs were categorized into breaks/exchanges and gaps (Khabour et al., 2013).

MI assay

The MI is a measure of the degree of cellular proliferation and is used to measure cell division (Romansik *et al.*, 2007). The MI was assessed by random analysis >5,000 cells per drug concentration (at least 1,000 cells per concentration per donor) along with scoring of cells that were in the metaphase stage. The MI was expressed as the ratio of the number of metaphases seen over the total number of intact cells seen (Al-Sweedan *et al.*, 2012).

The 8-OHdG assay

8-Hydroxyl-2-deoxy guanosine is a naturally occurring hydroxyl radical-damaged guanosine that is generated during cellular processes (Esmadi et al., 2016). The 8-OHdG test is widely used to assess oxidative DNA damage induced by exposure to chemical agents and disease conditions (Urbaniak and Boguszewska, 2020; Valavanidis et al., 2009). Lymphocytes were cultured and incubated as described for the CAs assay (see above). After 72 hours of incubation, cultured cells were centrifuged at $500 \times g$ for 5 minutes, and the supernatant was removed. The cell pellets were then washed 5× with 9 ml of RPMI 1,640 medium each, and then the cells were resuspended in 1 ml of RPMI 1,640 medium (Alzoubi et al., 2012). Thereafter, the cell suspension was treated with etazolate or the positive control (cisplatin) for 6 hours at 37°C. Tubes were then centrifuged at 500 \times g, and 8-OHdG levels were quantified in the supernatant using an enzyme-linked immunoassay (ELISA) kit from Abcam (Cambridge, UK; Catalog No. ab201734) according to the protocol supplied with the kit. Plates were read at 450 nm using an automated ELISA reader (Bio-Tek, Winooski, VT). A standard for known concentrations of 8-OHdG was provided by the kit and was used to calculate the 8-OHdG biomarker in samples.

Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software (version 5). All parameters were compared using analysis of variance and Tukey's post hoc test. A p < 0.05 indicates a statistical difference.

RESULTS

Table 1 shows the frequency of gap aberrations after lymphocyte cultures were treated for 24 hours under different concentrations (0.1, 1, 10, and 50 μ M) of etazolate. None of the examined etazolate concentrations increased the frequency of gap aberrations observed in the control group (p > 0.05). On the other hand, the positive control cisplatin at 0.4 μ g/ml caused significant increases in the frequency of gap aberrations by several folds.

When considering CAs (breaks/exchanges), results similar to those of gap aberrations were obtained (Table 2). Thus, etazolate did not induce genotoxicity in cultured human lymphocytes at concentrations up to 50 μ M as examined using the CA assay.

Figure 1 shows the level of 8-OHdG in cultured lymphocytes treated with different concentrations of etazolate. 8-OHdG levels were not significantly different between the etazolate groups and the control group (p > 0.05). The treatment of cultures with cisplatin, the positive control, caused significant (p < 0.01) increases in 8-OHdG at a concentration of 0.4 µg/ml. Thus, etazolate did not induce oxidative DNA damage as assessed by the 8-OHdG biomarker.

The MI assay was used to evaluate the antiproliferative potential of etazolate in cultured human lymphocytes. Cultures

treated with different concentrations of etazolate showed similar mitotic indices to those of the control group (Fig. 2, p > 0.05). On the other hand, the positive control, cisplatin, at 0.4 µg/ml significantly reduced the MI (p < 0.05). Thus, etazolate did not have antiproliferative effects on cultured human lymphocytes at all examined concentrations.

DISCUSSION

In the current study, the genotoxicity and antiproliferative effects of etazolate were evaluated in cultured human lymphocytes. The results showed that etazolate at concentrations up to 50 μ M did not cause CAs or oxidative DNA damage as measured in cultured human lymphocytes by the CAs and 8-OHdG assay, respectively. In addition, etazolate did not affect the MI of cultured cells.

Etazolate is a neuroprotective agent that showed promising results in the treatment of Alzheimer's disease, depression, anxiety, and memory impairments (Alzoubi et al., 2018; Ankur et al., 2013; Drott et al., 2010; Jindal et al., 2015). Etazolate is a pyrazolopyridine derivative, and previous studies showed that some pyrazolopyridine-derived compounds could interact with DNA (El-Gohary and Shaaban, 2018; El-Gohary et al., 2019; Suntharalingam et al., 2010). In addition, some pyrazolopyridine derivatives showed antitumor and antiproliferative activity and could block the progression of the cell cycle (Baviskar et al., 2013; Gavriil et al., 2016). Furthermore, some studies have reported the ability of phosphodiesterase inhibitors to modulate DNA repair systems via alterations of the cellular cAMP-Protein Kinase A pathway (Ghorbani et al., 2015; Parkkonen et al., 2008; Sabisz and Skladanowski, 2008). These reports prompted us to evaluate the potential genotoxic effect of etazolate in cultured human cells using the CAs assay and the 8-OHdG assay. These assays are widely used, sensitive, and robust indicators of genotoxicity (Mateuca et al., 2006; Urbaniak

Treatment	Concentration	Total No. of scored cells	Frequency of gap aberrations/cell
Control	-	500	0.148 ± 0.022
Etozolate	0.1 µM	500	0.132 ± 0.042
	1 µM	500	0.138 ± 0.041
	10 µM	500	0.128 ± 0.025
	50 µM	500	0.160 ± 0.071
Cisplatin	0.4 µg/ml	500	$0.424 \pm 0.052*$

Table 1. Frequencies (mean \pm S.E.) of gap aberrations induced by treatments.

* *p*-value < 0.01 from the control group.

Table 2. Frequencies (mean \pm S.E.) of chromosomal breaks/exchanges induced by treatments.

Treatment	Concentration	Total No. of scored cells	Frequency of breaks/exchange per cell
Control	-	500	0.038 ± 0.021
Etozolate	0.1 µM	500	0.047 ± 0.005
	1 µM	500	0.036 ± 0.023
	10 µM	500	0.044 ± 0.029
	50 µM	500	0.045 ± 0.017
Cisplatin	0.4 µg/ml	500	0.241 ±0.031*

* *p*-value < 0.01 from the control group.



Figure 1. Induction of 8-OHdG in cultured human lymphocytes by different treatments. Cultures were treated with different concentrations of etazolate or cisplatin for 6 hours. Data are expressed as mean \pm SD. * indicates a significant difference from the control group ($p \le 0.01$).



Figure 2. Changes in the MI of cultured human lymphocytes induced by different treatments. Cultures were treated with different concentrations of etazolate or cisplatin for 24 hours. Data are expressed as mean \pm SD. * indicates a significant difference from the control group (p < 0.05).

and Boguszewska, 2020; Valavanidis *et al.*, 2009). According to the present results, etazolate is not genotoxic to cultured lymphocytes at the examined concentrations (range 0.01 to 50 μ M). The CAs are primarily caused by agents that directly interact with DNA or interfere with its replication. Regarding 8-OHdG, it can be generated by chemical agents that trigger oxidative stress. According to the previous literature, not all pyrazolopyridine derivatives can interact with DNA (El-Gohary and Shaaban, 2018; El-Gohary *et al.*, 2019; Suntharalingam *et al.*, 2010). In addition, etazolate may possess antioxidant and neuroprotective properties as shown using animal models (Alzoubi *et al.*, 2018; Alzoubi *et* *al.*, 2017; Jindal *et al.*, 2015, 2017). Thus, etazolate appears to be a promising and safe neuroprotective agent. Further studies using other cell types and/or other *in vivo* genotoxicity models are needed to confirm the present findings.

In the current study, etazolate at the concentrations examined did not impact the proliferative capacity of cultured human lymphocytes. In a study on *Leishmania donovani* promastigotes, etazolate was shown to have an antiproliferative effect at a dose of 24 μ M (Saha *et al.*, 2020). In addition, in an animal study on mice epidermal mitosis, etazolate has been shown to inhibit mitosis at concentrations between 10 and 100 μ M (Birnbaum *et al.*, 1976). On the other hand, etazolate has been shown to delay the apoptosis and cell death of intestinal epithelial cells induced by chemical agents (Joseph *et al.*, 2005). Thus, the potential antiproliferative effect of etazolate can vary according to the cell type and/or animal model used.

In the current study, an *in vitro* culture system was used. Previous studies have shown that metabolic activation within the body may significantly impact the genotoxicity and the cytotoxicity of chemical compounds (Ku *et al.*, 2007; Oliveira *et al.*, 2021; Zhou *et al.*, 2007). Therefore, in vivo studies are needed to confirm the present in vitro findings.

CONCLUSION

In the current study, using the CAs, 8-OHdG, and MI assays, etazolate was found to be neither genotoxic nor cytotoxic to cultured human lymphocytes. More *in vivo* studies are needed to confirm these results.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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ETHICAL APPROVALS

The study was approved by the Institutional Review Board of King Abdullah University Hospital (Approval No. 23/93/2016).

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AUTHORS' CONTRIBUTION

All authors contributed to the study design, writing of the grant proposal, conduction of experimental work, data analysis, and interpretations, participated in drafting and/or revising the manuscript, and approved the final version.

DATA AVAILABILITY

Data will be available upon request via e-mailing the corresponding author.

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