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Evaluation of the genotoxicity/mutagenicity of levetiracetam, lamotrigine, and oxcarbazepine using *in silico* methods

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ABSTRACT: Epilepsy is a chronic neurological disease affecting approximately 50 million people globally, including pregnant women. The symptoms of epilepsy, obstetric complications, and the adverse effects of antiepileptic drugs on the fetus are problems for pregnant women. Medications prescribed for epilepsy treatment are typically used over an extended period. In this study, the mutagenic and genotoxic potentials of levetiracetam (LEV), lamotrigine (LTG), and oxcarbazepine (OXC), the most widely prescribed new-generation antiepileptic drugs, and their respective metabolites were evaluated using computational methods. The latest treatment protocols indicate that these drugs can be administered during pregnancy with minimal risk to the fetus compared to other antiepileptics. Metabolite analysis of LEV, LTG, and OXG was performed using MetaTox (v.2.0). The selected drugs and their respective known-predicted metabolites were analyzed for potential genotoxic/mutagenic behavior using VEGA (v.1.2.3) and EPA TEST (v.4.2.1 and 5.1.2) in both statistical-based computational methodologies. Molecular structural alerts for genotoxicity/mutagenicity were performed using the OECD QSAR Toolbox (v.4.7) with an expert rule-based approach. Metabolites of LTG and OXC, especially those formed by epoxidation, pose a risk of mutagenicity and genotoxicity. VEGA and EPA mutagenicity consensus models predicted OXC-M10, M11, M12, M13, and M14, which were formed by epoxidation, were predicted as mutagenic. Our findings indicate that LEV might be safer concerning its genotoxicity/mutagenicity potential, whereas OXC warrants cautious prescribing and further research, particularly for use during pregnancy and in long-term treatments. Further *in vivo* experimental studies are needed to analyze this risk.

KEYWORDS: Levetiracetam; lamotrigine; oxcarbazepine; toxicology *in silico*; genotoxicity; mutagenicity.

1. INTRODUCTION

Epilepsy is a chronic neurological disease that affects approximately 50 million people worldwide, including pregnant women [1]. The pregnancy period for a healthy woman is a challenging process lasting 9 months. An expectant mother with epilepsy presents with a high risk for both her and baby. The symptoms of epilepsy, obstetric complications such as pre-eclampsia, hemorrhage, and preterm labor, and the adverse effects of antiepileptic drugs on the fetus are problems for pregnant women with epilepsy [2]. The relationship between teratogenicity and conventional antiepileptic drugs such as phenytoin, carbamazepine, valproic acid, and phenobarbital has been demonstrated by various studies [3-10]. Due to the large overlap between the drug-specific syndromes of phenytoin, carbamazepine, and phenobarbital, the terms fetal hydantoin syndrome, hydantoin-barbiturate embryopathy or fetal antiepileptic syndrome are used to define these abnormalities. It is hypothesized that the malformations result from a common mechanism, such as hypoxia, and are associated with pharmacological-induced embryonic arrhythmias and episodes of hypoxia-reoxygen injury. The malformation pattern of valproic acid is markedly different, suggesting folate deficiency. Intrauterine exposure of the fetus to valproic acid may result in fetal valproate syndrome, including neural tube defects, and a prominent facial pattern including upward-sloping palpebral fissures, epicanthic folds, and posteriorly directed ears [11].

Epilepsy treatment varies based on factors including the patient's gender, age, type of epilepsy, frequency and duration of seizures, etiology, drug response and comorbidities. While medication may be discontinued after a certain period in some cases, it is often a lifelong treatment [12, 13]. Under the current

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regimens, a few new generation antiepileptics, such as levetiracetam (LEV), lamotrigine (LTG), and oxcarbazepine (OXC), can be prescribed at pregnancy with minimal risk [2, 3, 5-10, 14, 15]. LTG and OXC have similar modes of action to prevent epileptic firing by binding sodium channels, stabilizing presynaptic neuronal membranes, and inhibiting the release of presynaptic excitatory neurotransmitters, such as glutamate and aspartate [16, 17]. The antiepileptic effect of LEV is via binding to synaptic vesicle protein 2A, which is involved in the release of chemical messengers from neurons [18]. Studies have shown several reports that the use of these drugs alone or in combination with other drugs indicated a low incidence of malformations [3, 5-10, 14, 15]. However, the US Food and Drug Administration Agency (FDA) has classified LEV, LTG, and OXC as pregnancy category C [10]. This category, which falls under the earlier FDA classification, includes drugs that have demonstrated adverse effects on the fetus in animal reproduction studies. Additionally, there may be a lack of adequate and well-controlled studies in humans. However, the potential benefits of using the drug in pregnant women may justify its use despite the associated risks [19]. The ability of LEV, LTG, and OXC to cross the blood-placental barrier suggests that they or their metabolites may also reach the developing fetus [20]. Intrauterine exposure to genotoxic or mutagenic agents during pregnancy can lead to teratogenicity. Alterations in the integrity or functionality of nucleic acids during critical developmental periods can result in congenital malformations [21].

The *in-silico* prediction of genotoxicity/mutagenicity plays a vital role in the assessment of the safety and toxicity of chemicals and the drug development process [22, 23]. Although pharmaceuticals undergo stringent *in vitro* and *in vivo* toxicological testing before being marketed, computational methods are also useful for understanding drug safety profiles and adverse effects [24, 25]. *In silico* methods for predicting drug metabolism have also been developed to improve experimental evaluations [26]. Drug metabolism involves the chemical modification of drug molecules, a process that is facilitated by various enzymes responsible for metabolizing drugs. The resulting metabolites may have physicochemical, pharmacological, and even toxicological characteristics different from those of the parent drug molecule [27]. Phase I reactions often result in bioactivation. Chemicals can be activated, especially in phase I reactions, by CYP450 enzymes through oxidation to metabolites that form DNA adducts and induce mutations. The coupling of phase I and II (also termed conjugation reactions) serves to mitigate or eliminate the reactivity of these intermediate metabolites [28].

Our study aims to evaluate genotoxic/mutagenic potentials of LEV, LTG, and OXC and their respective metabolites using computational methods. The metabolite analysis of the drugs was conducted using MetaTox (v.2.0), an *in-silico* tool on the PassOnline platform (http://way2drug.com/passonline/predict.php). The known metabolites of these drugs were also gathered from the literature. LEV, LTG, and OXC and their respective known-predicted metabolites were then analyzed for their potential genotoxic/mutagenic behavior using statistical-based tools VEGA (v.1.2.3) and EPA TEST (v.4.2.1 and 5.1.2). Molecular structural alerts for genotoxicity/mutagenicity were performed using the OECD QSAR Toolbox (v.4.7), an expert rule-based tool.

2. RESULTS

2.1. Metabolites

Based on the available data in the literature, LEV has three known metabolites: (2S)-2-(4-hydroxy-2-oxopyrrolidin-1-yl)butanamide, (2S)-2-(2-oxopyrrolidin-1-yl)butanoic acid (UCB L057), and 4-{[(1S)-1-carboxypropyl]amino}-3-hydroxybutanoic acid (notated as LEV-M1, -M7, and -M8, respectively) [29]. MetaTox analysis also predicted LEV-M1. The metabolites formed by aliphatic hydroxylation (6 metabolites), hydrolysis (1 metabolite), and opening-ring (1 metabolite) are given in Scheme 1.

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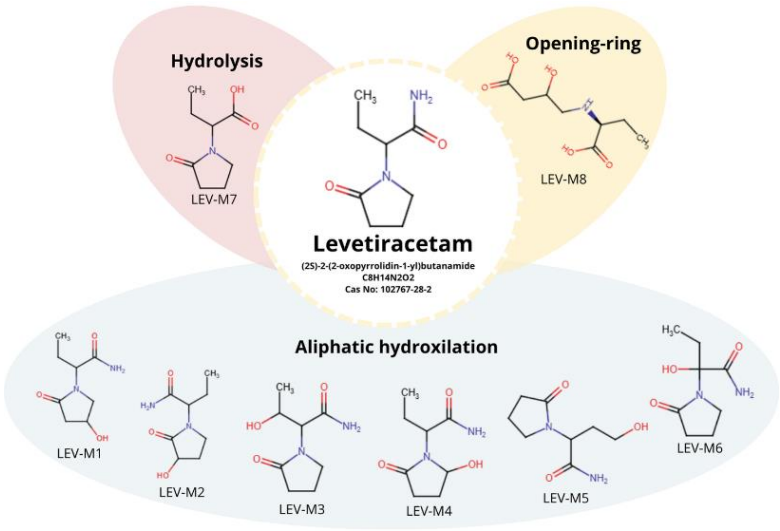
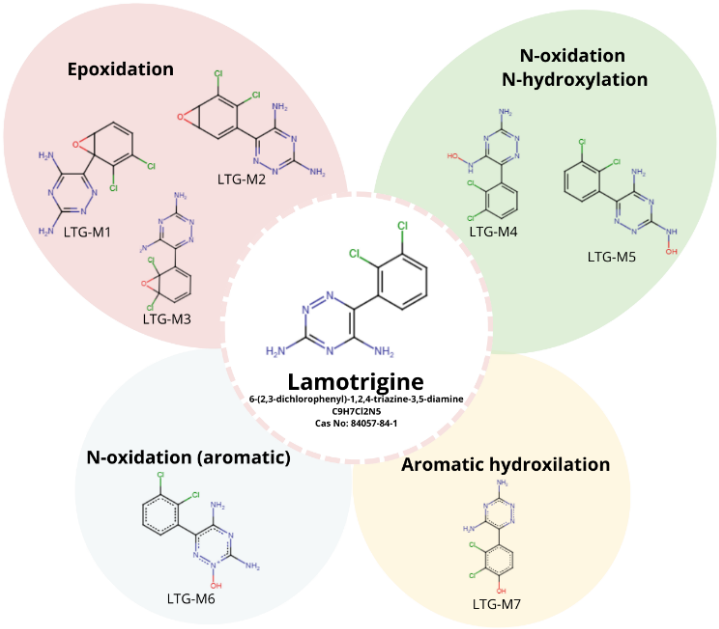


Scheme 1. Known and predicted metabolites of levetiracetam (LEV).

LTG has two known metabolites, 2N-oxide and arene oxide (notated as LTG-M6 and -M7, respectively) [30]. By the MetaTox analysis five metabolites were predicted. The predicted metabolites were formed by epoxidation and N-oxidation/N-hydroxylation. The metabolites formed through epoxidation (3 metabolites), N-oxidation/N-hydroxylation (2 metabolites), aromatic N-oxidation, and aromatic hydroxylation (1 metabolite) are given in Scheme 2.



Scheme 2. Known and predicted metabolites of lamotrigine (LTG).

OXC has two known metabolites, 10-hydroxy-10,11-dihyro carbazepine and 10,11-dihydroxy-10,11-dihyro carbazepine (notated as OXC-M8 and -M9, respectively) [31]. In addition, twelve metabolites were estimated using MetaTox. The metabolites formed by aliphatic hydroxylation (9 metabolites) and epoxidation (5 metabolites) are given in Scheme 3.

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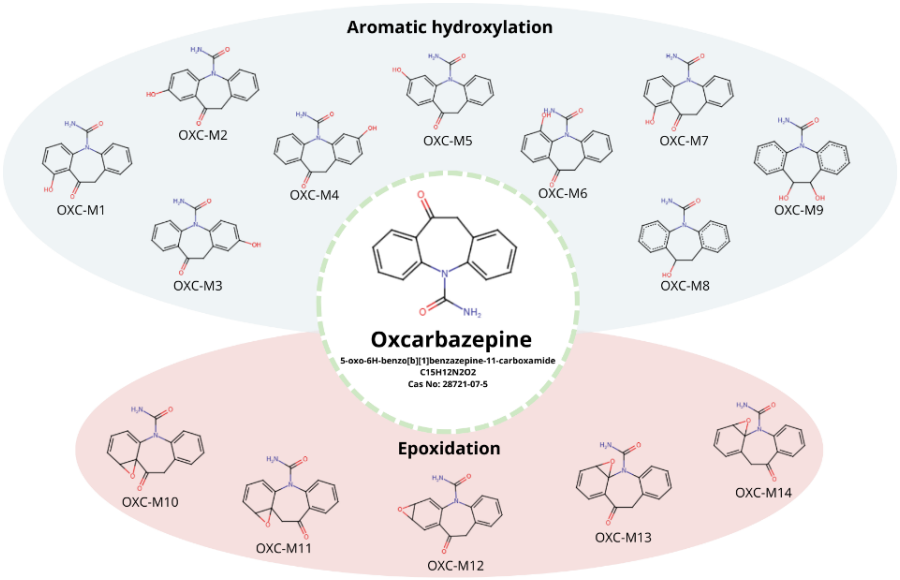

Scheme 3. Known and predicted metabolites of oxcarbazepine (OXC).

2.2. Mutagenicity and genotoxicity

Mutagenicity predictions of LEV and its metabolites were made using VEGA and EPA mutagenicity models (v.4.2.1 and 5.1.2) and the results are presented in Table 1. LEV was predicted non-mutagenic in VEGA and EPA consensus models. None of the substances were predicted as mutagenic in the CAESAR and ISS mutagenicity models. LEV-M4, -M5, -M6, and -M7 were predicted as mutagenic with moderate reliability in KNN-read across model. LEV-M4 and -M6 were estimated as mutagenic with a consensus score of 0.2 in VEGA consensus model. LEV-M5 was predicted to be mutagenicity positive with a prediction value higher than 0.5 in both EPA TEST models. Genotoxicity potential of LEV and its metabolites were assessed using VEGA models and the predictions were shown in Table 1. None of the substances were predicted active in the chromosomal aberration model. LEV was estimated inactive in *in vitro* (IRFMN-VERMEER) and *in vivo* (IRFMN) micronucleus activity models. LEV-M3, -M7, and -M8 were predicted active by *in vitro* micronucleus activity model with moderate, low, and good reliability, respectively. LEV-M1, -M2, and -M3 were predicted genotoxic with moderate reliability by *in vivo* micronucleus activity model. H-acceptor-path3-H-acceptor structural alert for *in vivo* mutagenicity was identified for LEV and all its metabolites in the OECD QSAR Toolbox evaluation (Table 2).

Mutagenicity predictions of LTG and its metabolites were made using VEGA and EPA mutagenicity models, and the results are shown in Table 1. LTG was predicted as mutagenic in the VEGA consensus model with a low consensus score (0.20), whereas mutagenicity negative in both EPA TEST models. LTG-M1, -M2, and -M3 were predicted as suspect mutagenic in the CAESAR model and mutagenic in SarPY-IRFMN models. ISS predicted LTG and all its metabolites mutagenic from low to moderate reliability. LTG-M1, -M2, and -M3 were predicted mutagenic in VEGA consensus model. TEST v.4.2.1 consensus model predicted LTG-M2, -M3, -M4, -M5, and -M7 as mutagenicity positive, while TEST v.5.1.2 predicted only LTG-M6 as positive. The genotoxicity potential of LTG and its metabolites was assessed using VEGA models, and the predictions are shown in Table 1. LTG and all its metabolites were predicted active in chromosomal aberration model from low to moderate reliability. LTG-M1, -M2, and -M3 were predicted active by *in vitro* and genotoxic by *in vivo* micronucleus models. According to the data from the OECD QSAR Toolbox presented in Table 2, LTG exhibited at least one mutagenicity alert, both *in vitro* and *in vivo*, for itself and for all its metabolites. All these alerts were associated with the categories of primary aromatic amines, hydroxylamines, and their derived esters. LTG-M1, -M2, and -M3 also identified epoxide alert as possibly related to Ames and micronucleus mutagenicity. Additionally, reactive oxygen species (ROS) formation and unimolecular nucleophilic substitution (S_N1) reaction alerts were detected for LTG-M4 and -M5.

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Table 1. Predictions from VEGA (v.1.2.3) and EPA TEST (v.4.2.1 and 5.1.2) for levetiracetam (LEV), lamotrigine (LTG), and oxcarbazepine (OXC) and their respective metabolites.

		VEGA Mutagenicity					VEGA Genotoxicity			EPA TEST Mutagenicity	
Substance		CAESAR (v.2.1.14)	ISS (v.1.0.3)	SarPy-IRFMN (v.1.0.8)	KNN-RA (v.1.0.1)	Consensus (v.1.0.4)	CA CORAL (v.1.0.1)	*In vitro* MN IRFMN-VERMEER (v.1.0.1)	*In vivo* MN IRFMN (v.1.0.2)	Consensus (v.4.2.1)	Consensus (v.5.1.2)
Levetiracetam (LEV)	LEV					Non-M (0.53)				Neg (0.23)	Neg (0.20)
	M1					Non-M (0.48)				Neg (0.26)	Neg (0.48)
	M2					Non-M (0.48)				Neg (0.19)	Neg (0.34)
	M3					Non-M (0.58)				Neg (0.24)	Neg (0.27)
	M4					Mut (0.20)			N/A	Neg (0.21)	Neg (0.18)
	M5					Non-M (0.43)			N/A	Pos (0.74)	Pos (0.51)
	M6					Mut (0.20)			N/A	Neg (0.14)	Neg (0.31)
	M7					Non-M (0.35)				Neg (0.06)	Neg (-0.03)
	M8			Possible		Non-M (0.75)				Neg (0.30)	Neg (0.31)
Lamotrigine (LTG)	LTG					Mut (0.20)		N/A		Neg (0.33)	Neg (0.08)
	M1	Suspect			N/A	Mut (0.60)				Neg (0.02)	N/A
	M2	Suspect			N/A	Mut (0.60)				Pos (0.58)	N/A
	M3	Suspect			N/A	Mut (0.20)				Pos (0.69)	N/A
	M4					Non-M (0.35)		N/A		Pos (0.68)	N/A
	M5					Non-M (0.35)		N/A		Pos (0.62)	N/A
	M6					Non-M (0.25)		N/A		Neg (0.27)	Pos (0.86)
	M7					Non-M (0.35)		N/A		Pos (0.59)	Neg (0.30)
Oxcarbazepine (OXC)	OXC					Non-M (0.65)		N/A		Neg (0.24)	Neg (0.26)
	M1					Mut (0.20)				Neg (0.17)	Neg (0.28)
	M2					Mut (0.20)				Pos (0.61)	N/A
	M3					Mut (0.20)				Neg (0.07)	N/A
	M4					Mut (0.20)				Neg (0.23)	N/A
	M5					Mut (0.20)				Pos (0.57)	Neg (0.49)
	M6					Mut (0.20)				Neg (0.47)	Pos (0.51)
	M7					Mut (0.20)				Neg (0.17)	Neg (0.28)
	M8					Non-M (0.53)		N/A		Neg (0.16)	Neg (0.26)
	M9					Mut (0.30)		N/A		Neg (0.14)	Neg (0.21)
	M10					Mut (0.35)				Pos (0.76)	Pos (0.96)
	M11					Mut (0.35)				Pos (0.91)	Pos (0.97)
	M12					Mut (0.50)				Pos (0.57)	Pos (1.01)
	M13					Mut (0.50)				Pos (0.86)	Pos (0.98)
	M14					Mut (0.35)				Pos (0.87)	Pos (0.99)

The reliability assessments of the models are given in parenthesis for each substance. Positive/mutagenic/active/genotoxic predictions are presented in red color. LR: Low Reliability; MR: Moderate Reliability; GR: Good Reliability. Non-mutagenic/inactive/non-genotoxic predictions are presented in green color. LR: Low Reliability; MR: Moderate Reliability; GR: Good Reliability. VEGA mutagenicity (Ames) consensus score (CS) near 1 indicates stronger positivity, while near 0 indicates stronger negativity. In the EPA TEST for mutagenicity (Ames), prediction score ≥0.5 is considered positive, while score <0.5 is negative. RA: Read Across; CA: Chromosomal aberration; MN: Micronucleus; CS: Consensus score; Mut: Mutagen; Non-M: Non-Mutagen; Pos: Positive for mutagenicity; Neg: Negative for mutagenicity; N/A: Not available.

Mutagenicity predictions of OXC and its metabolites were made by VEGA and EPA models and the results are shown in Table 1. OXC, itself, was not predicted to be mutagenic or genotoxic in any model tested. The CAESAR model predicted all metabolites as mutagenic from low to moderate reliability. OXC-M10, -M11, -M12, -M13, and -M14 were predicted to be mutagenic in both the ISS and SarPy-IRFMN models with low and moderate reliability, respectively. All metabolites except OXC-M8 were estimated as mutagenic in the VEGA consensus model. OXC-M12 and -M13 were predicted as mutagenic in all VEGA

and EPA mutagenicity models tested. The genotoxicity potentials of OXC and its metabolites were assessed using VEGA models and the predictions are shown in Table 1. All its metabolites, except OXC-M10 and -M13 were predicted active in the chromosomal aberration model. OXC, OXC-M8, and -M9 could not be predicted by *in vitro* micronucleus model. Except for these three substances, the other metabolites were estimated active in this model. OXC-M10, -M11, -M12, -M13, and -M14 were estimated to be genotoxic by *in vivo* micronucleus model. In the OECD QSAR Toolbox evaluation, OXC and all metabolites except OXC-M8 had a H-acceptor-path3-H-acceptor alert for *in vivo* mutagenicity. In OXC-M10, -M11, -M12, -M13, and -M14, epoxide and aziridine structural alerts for *in vitro* and *in vivo* mutagenicity were detected. OXC-M12 and -M13 had alpha, beta-unsaturated carbonyl *in vivo* micronucleus structural alerts (Table 2).

Table 2. Structural alerts from OECD QSAR Toolbox (v.4.7) for levetiracetam (LEV), lamotrigine (LTG), and oxcarbazepine (OXC) and their respective metabolites.

Substance		*In vitro* mutagenicity (Ames test) alerts by ISS	*In vivo* mutagenicity (MN) alert by ISS	DNA alerts for AMES, CA and MNT by OASIS
Levetiracetam (LEV)	LEV	No alert found	H-acceptor-path3-H-acceptor	No alert found
	M1			
	M2			
	M3			
	M4			
	M5			
	M6			
	M7			
	M8			
Lamotrigine (LTG)	LTG	Primary aromatic amine, hydroxyl amine and its derived esters	H-acceptor-path3-H-acceptor; Primary aromatic amine, hydroxyl amine and its derived esters	No alert found
	M1	Epoxides and aziridines; Primary aromatic amine, hydroxyl amine and its derived esters	Epoxides and aziridines; H-acceptor-path3-H-acceptor; Primary aromatic amine, hydroxyl amine and its derived esters	
	M2			
	M3			
	M4	Primary aromatic amine, hydroxyl amine and its derived esters	H-acceptor-path3-H-acceptor; Primary aromatic amine, hydroxyl amine and its derived esters	Radical- Radical mechanism via ROS formation (indirect); S_N1- Nucleophilic attack after metabolic nitrenium ion formation
	M5			
	M6	Primary aromatic amine, hydroxyl amine and its derived esters	H-acceptor-path3-H-acceptor; Primary aromatic amine, hydroxyl amine and its derived esters	No alert found
	M7			
Oxcarbazepine (OXC)	OXC	No alert found	H-acceptor-path3-H-acceptor	No alert found
	M1			
	M2			
	M3			
	M4			
	M5			
	M6			
	M7			
	M8	No alert found	No alert found	
	M9	No alert found	H-acceptor-path3-H-acceptor	
	M10	Epoxides and aziridines	Epoxides and aziridines; H-acceptor-path3-H-acceptor	
	M11			
	M12	Alpha, beta-unsaturated carbonyls; Epoxides and aziridines	Alpha, beta-unsaturated carbonyls; Epoxides and aziridines; H-acceptor-path3-H-acceptor	
	M13			
	M14	Epoxides and aziridines	Epoxides and aziridines; H-acceptor-path3-H-acceptor	

CA: Chromosomal aberration; MN: Micronucleus; MNT: Micronucleus test; ROS: Reactive oxygen species; S_N1: Unimolecular nucleophilic substitution.

3. DISCUSSION

Epilepsy is a chronic disease of the central nervous system, characterized by recurrent epileptic seizures. It affects about 1% of the population, including more than one million women of childbearing age. The treatment regimens that women with epilepsy may require during pregnancy face challenges, including side effects and toxicity for both mother and fetus [32]. The new generation of antiepileptic drugs, especially LEV, LTG, and OXC, are thought to be administered at lower risk during pregnancy [2, 3, 5-10, 14, 15]. Intrauterine exposure to drugs and consequent biochemical changes in pregnant women, including genotoxicity and oxidative stress, affect pregnancy outcomes. The developing embryo is more susceptible to genotoxicity due to rapid cell proliferation, cell differentiation, organogenesis, and immature DNA repair mechanisms [21, 33]. The lack of a transplacental barrier along the maternal-fetus axis to most antiepileptic drugs, also including LEV, LTG, and OXC, and consequent fetal adverse effects are of great concern [20, 34]. There may also be potential neonatal exposure to antiepileptic drugs through breastfeeding [2, 34]. The genotoxic/mutagenic properties of drugs undoubtedly pose a particular threat to the fetus. Although candidate drug molecules undergo extensive *in vitro* and *in vivo* toxicological testing, which is a requirement in preclinical studies, many drugs currently on the pharmaceutical market can be considered to lack *in silico* studies in different aspects. In the present study, *in silico* molecular structure evaluations of LEV, LTG, OXC, and their respective metabolites were performed using statistical- and expert rule-based (Q)SAR prediction tools. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline M7 (R2) Guideline [Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk] requires two complementary *in silico* methodologies for genotoxicity/mutagenicity assessments. The combined use of two complementary approaches (statistical- and expert rule-based) is predicated on the assumption that greater sensitivity in the detection of potential mutagens [22]. A substance may not raise concerns about genotoxicity or mutagenicity only if *in silico* predictions for the substance or its predicted or reported metabolites, which have been assessed as negative using a combination of valid (Q)SAR models. In such cases, it may not be necessary to conduct experimental genotoxicity/mutagenicity testing [23]. Several *in silico* models and tools have been developed based on experimental data collected over many years. Although the 3Rs principle was a primary driving force behind the development of these models, contemporary computational methodologies have extended beyond animal welfare issues. However, since *in silico* models are complex systems, expert knowledge is required to select the model to be applied. Regulatory authorities mandate assessing the applicability domain of models, as outlined in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulations and the OECD principles for (Q)SAR models [35]. The applicability domain of models is generally defined as the area where models can provide accurate and reliable predictions [36]. US EPA TEST and VEGA mutagenicity/genotoxicity models were used in our study since they provide information about the applicability domain and make reliable predictions. The OECD QSAR Toolbox, a regulatory authority-approved platform with an extensive database of endpoints and chemicals, was utilized to identify structural alerts associated with mutagenicity and genotoxicity.

LEV was not mutagenic in the Ames test or *in vitro* Chinese hamster ovary/Hypoxanthine-guanine phosphoribosyl transferase (CHO/HGPRT) gene locus assay. Neither LEV was clastogenic in the *in vitro* analysis of chromosomes of metaphase from Chinese hamster ovary cells nor in the *in vivo* mouse micronucleus test. The hydrolysis product UCB L057, which is also the main metabolite of LEV, did not show genotoxic/mutagenic effects in the Ames test or in an *in vitro* mouse lymphoma assay [29]. In human embryonic stem cells, the results of the comet assay showed that LEV induced more DNA damage than LTG, carbamazepine, and gabapentin [37]. In Sprague-Dawley rats, LEV administration at 25 or 50 mg/kg/d did not affect the sister chromosome exchange frequency or the mean number of micronucleated cells in pups in the prenatal term [38]. Rats were administered LEV at doses of 50, 300, or 1800 mg/kg/d in the diet for two years. There was no evidence of carcinogenicity reported. A study in which mice received dietary LEV at doses of 60, 240, or 960 mg/kg/d for 80 weeks found no carcinogenicity. However, it was stated that the carcinogenic response potential could not be fully evaluated because this study was not conducted at sufficient doses [29]. Our in silico findings mostly support previous *in vitro* and *in vivo* studies. Mutagenicity consensus models in VEGA and both versions of EPA assessed LEV, itself, as non-mutagenic. Chromosomal aberration and *in vivo* and *in vitro* micronucleus models also predicted LEV as inactive/non-genotoxic. LEV-M7, the active metabolite of LEV (UCB L057), was predicted as inactive in chromosomal aberration and non-

genotoxic in *in vivo* micronucleus models. *In vitro* micronucleus model predicted LEV-M7 as active, but with low reliability. No research has been identified that examines the genotoxicity/mutagenicity potential of LEV's other metabolites. Upon evaluating all predicted and known metabolites, none were simultaneously identified as positive by both genotoxicity and mutagenicity models. Therefore, our results indicate that LEV is a relatively safer antiepileptic because of its genotoxicity/mutagenicity.

LTG was not found as genotoxic/mutagenic in the Ames test and in the *in vitro* mammalian mouse lymphoma assay both in the presence or absence of metabolic activation. LTG did not increase the incidence of any structural or numerical chromosomal anomalies in human lymphocytes *in vitro* and rat bone marrow *in vivo* [30]. No evidence of carcinogenicity has been reported following oral administration of LTG for up to 2 years at maximum tolerated doses of 10-15 mg/kg/d for rats and 30 mg/kg/d for mice [30]. However, our study revealed the genotoxic/mutagenic potential of especially epoxy metabolites of LTG, although the mutagenic potential of LTG itself is low. LTG and all its metabolites were predicted active in the chromosomal aberration model from low to moderate reliability. LTG-M1, M2 and M3, all epoxy metabolites, were found genotoxic/active in *in vivo* and *in vitro* micronucleus models and structural alerts have been defined for these assays. Our findings suggest that not only LTG, but also its metabolites, may exhibit genotoxic effects, warranting caution and further research.

OXC increased mutation frequencies in the *in vitro* Ames test without metabolic activation. Both OXC and 10-hydroxy metabolite showed increased frequencies of chromosomal aberrations and polyploidy in the *in vitro* Chinese hamster ovary assay without metabolic activation. 10-Hydroxy metabolite was found non-mutagenic in the Ames test, and no mutagenicity or clastogenicity were observed with either OXC or 10-hydroxy metabolite in V79 Chinese hamster cells *in vitro*. In an *in vivo* rat bone marrow assay, OXC, and 10-hydroxy metabolite were found both negative for aneugenicity or clastogenicity [31]. In human peripheral blood lymphocyte culture, OXC significantly increased chromosomal aberrations, aberrant cells, sister chromatid exchange, and micronuclei formation in the presence and absence of S9 mix [39]. OXC was exhibited mutagenic effect in the *Drosophila melanogaster* wing spot test [40]. OXC was reported to cause DNA damage in mice following both acute and subchronic exposure using alkaline comet assay [41]. In a two-year carcinogenicity study, a dose-dependent increase in the incidence of hepatocellular adenoma was observed in mice exposed to ≥70 mg/kg/d OXC. In a two-year carcinogenicity study in rats, females treated with ≥25 mg/kg/d OXC had an increased incidence of hepatocellular carcinoma, and males and females treated with 10-hydroxy metabolite at doses of 600 mg/kg/d and ≥250 mg/kg/d, respectively, also had an increased incidence of hepatocellular adenoma and/or carcinoma. In another 2-year repeated dose study in rats, an increased incidence of benign testicular interstitial cell tumors was observed in males at doses of 250 mg OXC/kg/d and ≥250 mg 10-hydroxy metabolite/kg/d and an increased incidence of granular cell tumors of the cervix and vagina in females at doses of 600 mg 10-hydroxy metabolite/kg/d [31]. OXC was predicted as non-mutagenic in VEGA and EPA consensus models and non-genotoxic in *in vivo* and *in vitro* micronucleus, and chromosomal aberration models. OXC-M8, 10-hydroxy metabolite, was predicted active in chromosomal aberration model but inactive in *in vivo* micronucleus model, with moderate reliability. All epoxy metabolites of OXC, which are OXC-M10, -M11, -M12, -M13, and -M14, were estimated as mutagenic in VEGA and EPA consensus models. Moreover, OXC-M10, M11, M12, and M13 were predicted as genotoxic in *in vivo* and *in vitro* micronucleus models. OXC-M13 was estimated positive in all tested models. The findings from studies on the genotoxicity and mutagenicity of OXC are inconsistent with each other. Since there is no mutagenicity or genotoxicity studies on the predicted metabolites, our findings are highly significant and highlight a potential toxicity risk associated with OXC, particularly its metabolites.

4. CONCLUSION

The toxicity associated with chronic exposure to long-term antiepileptic drugs, as well as the risk of fetal toxicity during pregnancy and lactation, highlights the significance of genotoxicity and mutagenicity research. LEV, LTG, and OXC, the most frequently prescribed antiepileptics, have been observed to pose risks in this regard. Comprehensive *in silico* studies specifically targeting the metabolites and mutagenic or genotoxic potential of these drugs remain absent. Our study is the first to simultaneously investigate the genotoxic and mutagenic potential of LEV, LTG, OXC, and their metabolites. Our findings indicate that LEV is a relatively safer antiepileptic regarding its genotoxicity/mutagenicity; however, OXC is not. Especially metabolites formed by epoxidation have been determined to have mutagenic and genotoxic potential. The predictions for LTG-M1 and M2 and most metabolites of OXC (especially M10, M11, M12, M13, and M14)

are inconclusive, and there is a potential for genotoxic effects that cannot be excluded. These *in silico* data should be confirmed using *in vivo* experiments and new studies are needed, especially on the safety of OXC.

5. METHODS

5.1. Dataset

Our dataset consists of the most commonly prescribed antiepileptics LEV, LTG and OXC and their metabolites. IUPAC names, CAS numbers, structures, and canonical Simplified Molecular Input Line Entry System (SMILES) of these chemicals were searched using PubChem (https://pubchem.ncbi.nlm.nih.gov/).

5.2. Metabolism

The possible metabolites of LEV, LTG, and OXC were estimated using MetaTox (v.2.0) selecting all biotransformation reactions in the model [42]. The SMILES notation of the metabolites were elaborated using PubChem (https://pubchem.ncbi.nlm.nih.gov/) and subsequently submitted to MetaTox. Aliphatic hydroxylation, aromatic hydroxylation, C-oxidation, dehydrogenation, epoxidation, glutathionylation, hydrogenation, hydrolysis, methylation, N-acetylation, N-dealkylation, N-glucuronidation, N-hydroxylation, N-oxidation, O-dealkylation, O-glucuronidation, O-phosphorylation, O-sulfation, S-oxidation reactions were analyzed. Only Phase I predictions are selected for the further analysis, Phase 2 is excluded.

5.3. Mutagenicity

In silico mutagenicity predictions for LEV, LTG, and OXC and their respective metabolites were made using VEGA mutagenicity (Ames test) consensus (v.1.0.4), EPA TEST v.4.2.1 and 5.1.2 mutagenicity consensus [43, 44]. VEGA consensus model performs its predictions by VEGA CAESAR (v.2.1.14), ISS (v.1.0.3), SarPy-IRFMN (v.1.0.8), KNN-Read-Across (v.1.0.1) mutagenicity models [45]. Therefore, each mutagenicity model in VEGA was also analyzed. Molecular structural alerts for mutagenicity were carried out using an expert rule-based QSAR prediction tool, OECD QSAR Toolbox (v.4.7). The specific profilers used from the OECD QSAR Toolbox (v.4.7) were the '*in vitro* mutagenicity (Ames) alerts by the *Instituto Superiore di Sanità* (ISS)' in Italy, '*in vivo* mutagenicity (micronucleus) alerts by ISS', and 'DNA alerts for AMES, CA and MNT by OASIS [46].

5.4. Genotoxicity

Genotoxicity assessments for LEV, LTG, and OXC and their respective metabolites were made by models implemented inside the VEGA online platform [47]. Chromosome Aberrations (CA) were predicted by the CORAL (v.1.0.1) model in VEGA [48]. CA was determined by *in vitro* test using Chinese hamster lung (CHL) and ovary (CHO) cells, with and without S9 metabolic activation in the CORAL model. Micronucleus (MN) activity was determined using *in vitro* (IRFMN-VERMEER, v.1.0.1) and *in vivo* (IRFMN, v.1.0.2) MN models in VEGA [49]. *In vitro* MN model uses mammalian cells according to the OECD TG 487: *In Vitro* Mammalian Cell Micronucleus Test, while *in vivo* MN models follow the OECD TG 474: Mammalian Erythrocyte Micronucleus Test.

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