

Additive interactions between retigabine and oxcarbazepine in the chimney test and the model of generalized tonic-clonic seizures in mice

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SUMMARY

Introduction. Patients with pharmacoresistant epilepsy are usually treated with two or more antiepileptic drugs (AEDs). The search for therapeutically efficacious AED combinations is still a challenging issue for clinicians and epileptologists throughout the world.

Aim. To determine the interaction profile for the combination of retigabine (RTG) and oxcarbazepine (OXC) in both, the model of tonic-clonic seizures, the maximal electroshock (MES)-induced seizure model and chimney test (motor performance) in adult male albino Swiss mice.

Methods. Isobolographic analysis (type I) was applied to characterize interactions for the combination of RTG with OXC with respect to its anticonvulsant and acute side (neurotoxic) effects, as determined in the MES and chimney tests, respectively.

Results. The combination of RTG with OXC at the fixed-ratios of 1:3, 1:1 and 3:1 produced additive interactions in the MES test in mice. Similarly, the combination of RTG with OXC at the fixed-ratio of 1:1 produced additive interaction with a tendency towards sub-additivity in the chimney test in mice. Measurement of total brain concentrations of both AEDs revealed that RTG did not affect total brain concentrations of OXC and inversely, OXC had no impact on RTG's total brain concentrations, confirming pharmacodynamic interaction between the drugs.

Conclusions. The additive pharmacodynamic interactions in both the MES and chimney tests in mice were observed for the combination of RTG with OXC.

Key words: anticonvulsant • drug interactions • electroshock

INTRODUCTION

At present approximately 25 diverse antiepileptic drugs (AEDs) are available in the market to treat epilepsy in patients. Unfortunately, some of these patients (approximately 30%) do not successfully respond to the treatment with these AEDs used in monotherapy (Kwan et

al., 2011). Therefore, some novel therapeutic options are developed by clinicians and epileptologists in order to eliminate or drastically reduce seizure attacks along with their socio-economic consequences in epilepsy patients (Kwan and Brodie, 2006; Kwan et al.,

2011). Of these options, the application of AEDs in various combinations (either as add-on therapy or polytherapy), seems to be the most popular practice (Deckers et al., 2000).

Relatively recently, the novel AED with unique molecular mechanisms of anticonvulsant action – retigabine (RTG) has been marketed. It is prescribed as an add-on drug for adult patients with drug-resistant partial-onset seizures with or without secondary generalization, where other appropriate AED combinations have proved inadequate or have not been tolerated (European Medicines Agency, 2016).

Accumulating experimental (pre-clinical) evidence indicates that RTG combined with valproate (a classical wide-spectrum AED) exerted supra-additive (synergistic) interaction in the mouse tonic-clonic seizure model (Luszczki et al., 2009). The combination of RTG with levetiracetam (a second-generation AED) produced both, additive and supra-additive interactions in the same seizure model (Luszczki et al., 2015). Similarly, the additive interaction in the mouse MES model was observed for the combinations of RTG with carbamazepine, lamotrigine and phenytoin (three commonly used AEDs) (Luszczki et al., 2009; Żółkowska et al., 2016). Furthermore, the combinations of RTG with valproate, levetiracetam, carbamazepine and lamotrigine have been clinically confirmed as effective adjunctive therapies in patients with uncontrolled partial-onset seizures (Lerche et al., 2015).

This study was aimed at continuing preclinical experiments and characterizing the combination of RTG with oxcarbazepine (OXC – a second-generation AED) in the maximal electroshock-induced seizure (MES) model and chimney test in mice using type I isobolographic analysis of interaction.

MATERIALS AND METHODS

The choice of OXC in combination with RTG was based on both clinical presumptions concerning the use of these two drugs in patients with tonic-clonic seizures and partial onset seizures (Kwan et al., 2011), and theoretical presumptions related with their diverse molecular mechanisms of anticonvulsant action (Perucca, 1995). Of note, when two AEDs are co-prescribed, a general rule is to combine AEDs with diverse molecular mechanisms of action in order to strengthen their antiseizure effects and simultaneously to avoid the enhancement of adverse neurotoxic effects evoked by the AEDs (Deckers et al., 2000). To detect the anticonvul-

sant effects offered by RTG and OXC in combination, the mouse MES model was used because it is a model of tonic-clonic seizures and partial convulsions in humans (Loscher et al., 1991). Similarly, to detect acute adverse (neurotoxic) effects evoked by AEDs alone and in combination, the chimney test was used because it is an animal model of sedation and ataxia, manifesting as impairment of motor coordination (Loscher and Nolting, 1991).

Isobolographic analysis is based on mathematical and statistical calculations required to accurately assess types of pharmacodynamic interactions between drugs. Theoretically, one can distinguish 5 types of pharmacodynamic interactions that may occur during the combined administration of 2 or more drugs. These interactions include: supra-additivity (synergy), additivity, sub-additivity (relative antagonism), indifference and infra-additivity (absolute antagonism) (Berenbaum, 1989; Gessner, 1995; Luszczki and Czuczwar, 2004). Supra-additivity is observed when the effects produced by a drug mixture are greater than those resulting from summation of the effects of the individual drugs administered alone. Additivity occurs when the effects exerted by the drug combination are equal to the sum of effects of particular drugs comprising the investigated mixture. Sub-additivity is claimed when the effects of the drugs in mixture are lesser than those resulting from summation of effects of the individual component drugs (Berenbaum, 1989; Luszczki and Czuczwar, 2003). Indifference is observed if one of the drugs in mixture exerts placebo-like effect. The last type of pharmacodynamic interaction – infra-additivity is reported if the drugs in mixture produce the opposite effects (i.e., anti- and pro-convulsant effects) (Gessner, 1995). Additionally, the isobolographic study allowed to calculate protective indices (PI) for RTG and OXC (when administered alone) and benefit indices (BI) for the combinations of these AEDs, as recommended earlier (Luszczki and Czuczwar, 2004).

All experimental protocols described below were conducted on adult male Swiss mice in accordance with ARRIVE guidelines. The experiments were approved by the local ethics committee for animal experiments at the Medical University of Lublin (License No.: 28/2007) and conformed to the *Guide for the Care and Use of Laboratory Animals*. Each experimental group consisted of 8 mice and the total number of animals used in this study was 224.

OXC (Trileptal®, Novartis Pharma AG, Basel, Swit-

zerland) and RTG (a kind gift from GlaxoSmithKline, Brentford, UK) were suspended in a 1% aqueous solution of Tween 80 (Sigma-Aldrich) in distilled water and administered intraperitoneally (i.p.): OXC at 30 min, and RTG at 15 min., before the MES and chimney tests as well as estimation of AED concentrations.

Tonic-clonic seizures in the MES test were produced by an alternating current (sine-wave, 25 mA, 50 Hz, 500 V and 0.2 s stimulus duration) delivered via auricular electrodes by a Hugo Sachs rodent shocker stimulator (Freiburg, Germany). The anticonvulsant activity of OXC and RTG administered alone and in combination was determined as the median effective dose values (ED_{50} and $ED_{50\text{ exp}}$ in mg/kg) against MES-induced seizures, according to a log-probit method (Litchfield and Wilcoxon, 1949), as described elsewhere (Luszczki and Czuczwar, 2005; Luszczki et al., 2009; Kondrat-Wróbel and Luszczki, 2016; Luszczki, 2016). The ED_{50} and $ED_{50\text{ exp}}$ values represent doses of AEDs when injected alone or in mixtures, required to protect 50% of the mice tested against MES-induced tonic hind limb extension (seizure activity). Total number of mice used in the MES test was 96.

Deficits in motor coordination (acute side effects) in animals subjected to the chimney test manifested as the inability of the mice to climb backward up the transparent plastic tube (3 cm inner diameter, 30 cm length) within 60 seconds (Boissier et al., 1960). The acute side (neurotoxic) effects produced by RTG and OXC when administered alone and in combination were expressed as median toxic dose values (TD_{50} and $TD_{50\text{ exp}}$ in mg/kg), i.e., doses of the AEDs injected separately or in mixtures, necessary to evoke deficits in motor coordination in 50% of the animals tested. The TD_{50} and $TD_{50\text{ exp}}$ values (representing doses of AEDs injected alone or in mixtures), were determined in the chimney test by the use of a log-probit method (Litchfield and Wilcoxon, 1949), as described elsewhere (Luszczki and Czuczwar, 2004, 2005). Total number of mice used in the chimney test was 96.

Test for parallelism of dose-response lines for the studied AEDs (RTG and OXC) was performed according to the procedure described in detail earlier (Luszczki et al., 2009). It was observed that RTG had its dose-response line parallel to that of OXC in the MES test. This was the reason that the interactions between RTG and OXC were analyzed for three fixed-ratio combinations of 1:3, 1:1 and 3:1 in the MES-induced seizure test by the use of type I isobolographic analysis for parallel

dose-response lines, as published earlier (Luszczki et al., 2009). In contrast, RTG had its dose-response line non-parallel to that of OXC in the chimney test and the interactions between RTG and OXC were analyzed for the fixed-ratio combination of 1:1 in the chimney test using type I isobolographic analysis for non-parallel dose-response lines, as described in more details elsewhere (Luszczki, 2010; Luszczki et al., 2010b; Tallarida, 2011, 2012). Additionally, in this study we calculated protective indices (PI – as a quotient of the respective TD_{50} and ED_{50} values) and benefit indices (BI – as a quotient of PI_{exp} and PI_{add} values), as described earlier (Luszczki and Czuczwar, 2004).

To exclude any pharmacokinetic interaction between these AEDs, total brain concentrations of RTG and OXC were measured for the AEDs administered at doses reflecting the experimentally-derived $ED_{50\text{ exp}}$ value for the combination at the fixed-ratio of 1:1 tested in the MES test. More specifically, the animals (8 mice in each group) receiving the appropriate doses of AEDs were decapitated and their whole brains were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2 weight/volume). The homogenates were centrifuged at 10 000 g for 10 min. Subsequently, the supernatant samples (200 μ l) containing RTG and OXC were analyzed by high pressure liquid chromatography (HPLC) using an automated HPLC (Dionex, Sunnyvale, CA, USA) system. Total brain AED concentrations are expressed in μ g/ml of brain supernatants for RTG and OXC, as means \pm S.E.M. Total number of mice used in this procedure was 32.

Statistical analysis of data from the MES and chimney tests was performed with the unpaired Student's t-test as reported earlier (Tallarida, 2011, 2012). Similarly, total brain concentrations of RTG and OXC in experimental animals were statistically analyzed with the unpaired Student's t-test. Statistical significance was established at $p < 0.05$.

RESULTS

OXC and RTG administered singly produced a clear-cut anticonvulsant effect in the mouse MES test and the experimentally-derived ED_{50} values for these AEDs were 32.42 ± 2.50 and 15.92 ± 0.86 mg/kg, respectively. OXC had its dose-response line parallel to that of RTG in the MES test (figure 1A). The combination of RTG with OXC (for three fixed-ratios of 1:3, 1:1 and 3:1) produced the antiseizure activity in the MES test and the $ED_{50\text{ exp}}$ values for this combination are shown in table 1.

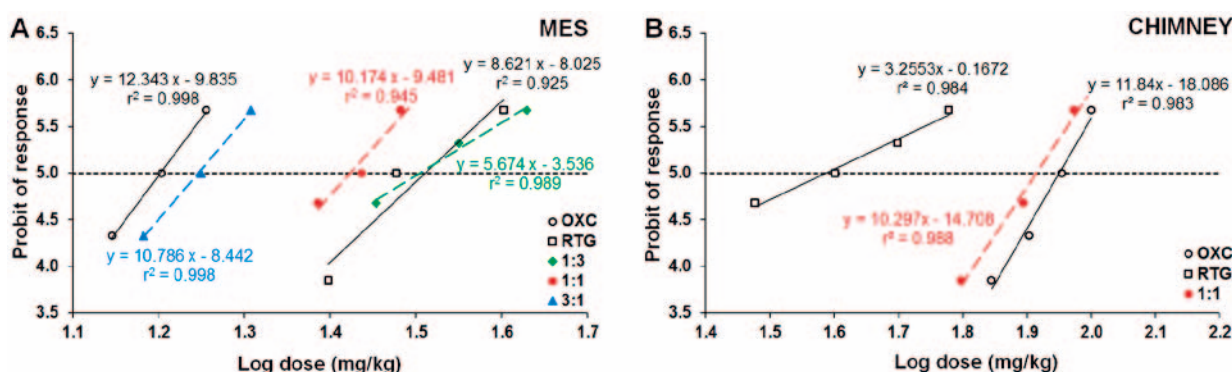


Figure 1A–D. Log-probit dose-response lines of retigabine (RTG) and oxcarbazepine (OXC) administered alone and in combinations in the maximal electroshock (MES)-induced seizure and chimney tests in mice.

Doses of RTG and OXC were transformed into logarithms, whereas the anticonvulsant and acute neurotoxic effects offered by the AEDs in the MES and chimney tests were transformed into probits. Linear regression equations of dose-response effects for RTG, OXC and their combinations are presented on the graph; where y – is the probit of response, x – is the logarithm (to the base 10) of a dose, and r^2 – is the coefficient of determination. RTG had its dose-response line parallel to that of OXC in the MES test (figure A), and simultaneously, non-parallel to that of OXC in the chimney test (figure B).

Table 1. Retigabine (RTG) in combination with oxcarbazepine (OXC) in the maximal electroshock (MES)-induced seizure model and chimney test in mice

Fixed-ratio	ED _{50 add}	ED _{50 exp}	TD _{50 add}	TD _{50 exp}	PI _{exp}	PI _{add}	BI
1:3	20.04 ± 1.27	17.63 ± 1.09	-	-	-	-	-
1:1	24.17 ± 1.68	26.50 ± 1.73	#35.94 ± 8.27	82.03 ± 6.48	3.10	1.49	2.07
1:1	24.17 ± 1.68	26.50 ± 1.73	&91.83 ± 10.40	82.03 ± 6.48	3.10	3.80	0.81
3:1	28.29 ± 2.09	31.95 ± 3.04	-	-	-	-	-

Results are median effective (ED₅₀) and median toxic (TD₅₀) doses (in mg/kg ± S.E.M.) calculated by computerized log-probit analysis. PI – protective index of the antiepileptic drugs in combination, determined experimentally (PI_{exp}) and calculated theoretically from the lines of additivity (PI_{add}). The PI values are quotients of the respective TD₅₀ and ED₅₀ values obtained experimentally or theoretically. BI – benefit index, as a ratio of PI_{exp} and PI_{add}. #TD_{50 add} and &TD_{50 add} are values calculated from the equation for the lower and upper lines of additivity, respectively. The Student's *t*-test was used to statistically analyze data.

In the chimney test, OXC and RTG produced a clear-cut acute adverse (neurotoxic) effect and the TD₅₀ values for these AEDs were 38.66 ± 6.83 and 89.10 ± 5.00 mg/kg, respectively. OXC had its dose-response line non-parallel to that of RTG in the chimney test (figure 1B). The combination of RTG with OXC (for the fixed-ratio of 1:1) exerted also acute neurotoxic effects and the TD_{50 exp} value for this combination in the chimney test is shown in table 1.

Isobolographic analysis of interaction (type I) revealed that all three fixed-ratio combinations of RTG with OXC (1:3, 1:1, and 3:1) produced additive interaction in the MES test in mice (table 1; figure 2A). In this case, the experimentally denoted ED_{50 exp} values for the combination of RTG with OXC for the fixed-ratios of 1:3, 1:1 and 3:1 did not significantly differ from the corresponding ED_{50 add} values (table 1; figure 2A). In the chimney test, the combination of RTG with OXC

for the fixed-ratio of 1:1 produced also an additive interaction (table 1; figure 2B). The experimentally-derived TD_{50 exp} value did not significantly differ from the corresponding TD_{50 add} value (table 1; figure 2B). Additionally, the isobolographically denoted BI values for the combination of RTG with OXC at the fixed-ratio of 1:1 ranged from 0.81 to 2.07 (table 1).

Total brain concentrations of OXC administered alone did not differ from those for OXC in combination with RTG at the fixed-ratio of 1:1 (figure 3A). Similarly, total brain concentrations of RTG administered singly did not statistically differ from those for RTG combined with OXC at the fixed-ratio of 1:1 (figure 3B).

DISCUSSION

Results presented in this study indicate that RTG combined with OXC produced an additive interaction with regards to their anticonvulsant and acute adverse (neu-

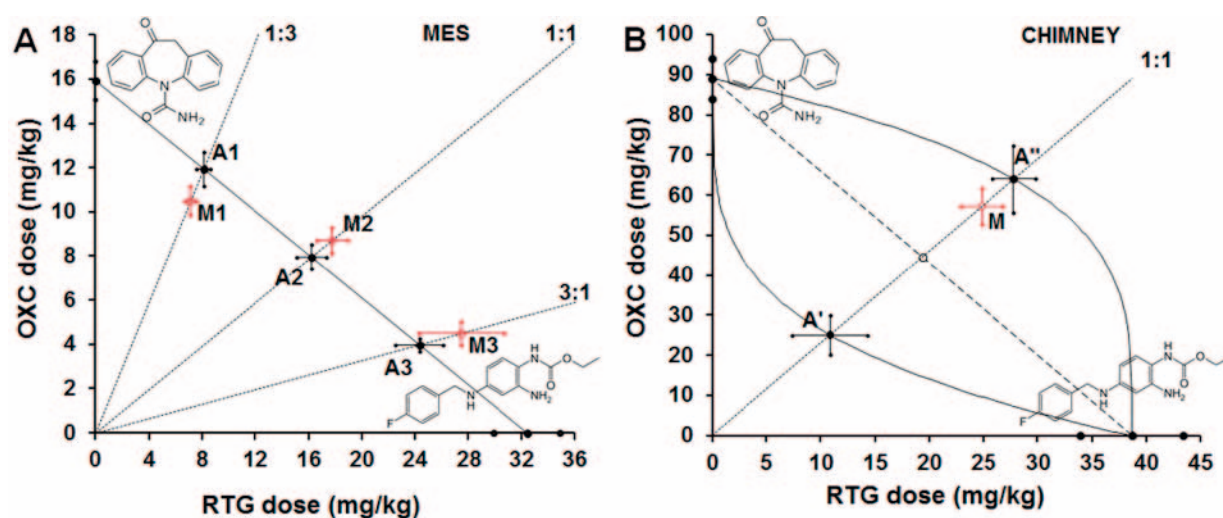


Figure 2A–B. Isobolograms with additive interactions between retigabine (RTG) and oxcarbazepine (OXC) in the maximal electroshock-induced seizure model and chimney test in mice.

The median effective doses (ED₅₀ – figure A) and median toxic doses (TD₅₀ – figure B) for RTG and OXC are plotted on the X- and Y-axes, respectively. The solid lines on the X and Y axes represent the S.E.M. for the ED₅₀ and TD₅₀ values of the AEDs administered alone. Points A1, A2 and A3 depict the theoretically additive ED₅₀ add values, whereas points M1, M2 and M3 represent the experimentally-derived ED₅₀exp values for the fixed-ratio combinations of 1:3, 1:1, and 3:1 that produced 50% anticonvulsant effects in the MES test in mice (figure A). Points A' and A'' depict the theoretically additive TD₅₀ add values, whereas point M represents the experimentally-derived ED₅₀exp value for the fixed-ratio combination of 1:1 that produced 50% acute adverse (neurotoxic) effects in the chimney test in mice (figure B).

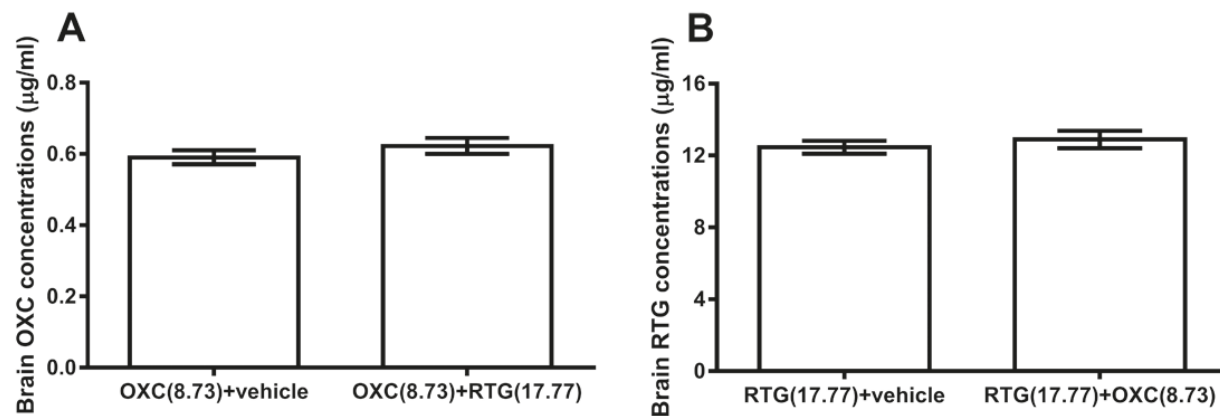


Figure 3A–B. Influence of retigabine (RTG) on total brain concentrations of oxcarbazepine (OXC), and inversely, OXC on total brain concentrations of RTG in experimental animals.

Columns are means ± S.E.M. of total brain concentrations of OXC and RTG. Each column displays results from 8 mice. The AEDs were administered i.p. at doses reflecting the ED₅₀exp value for the fixed-ratio of 1:1 from the MES test. The unpaired Student's t-test was used to statistically analyze the data.

rotoxic) effects. The additive interaction between RTG and OXC in the mouse MES model is similar to that observed earlier for the combination of RTG with carbamazepine, lamotrigine and phenytoin (Luszczki et al., 2009; Żółkowska et al., 2016). In contrast, RTG exerted synergistic interaction when combined with valproate

and levetiracetam in the mouse MES model (Luszczki et al., 2009; Luszczki et al., 2015).

On the other hand, OXC produced an additive interaction when combined with carbamazepine, phenobarbital and valproate (Luszczki et al., 2003; Luszczki and Czuczwar, 2003). Similarly, the combination of

OXC with loreclezole, pregabalin and 1-methyl-1,2,3,4-tetrahydroisoquinoline produced additivity in this seizure model (Luszczki et al., 2005b, Luszczki et al., 2010a, 2010b). In contrast, the combinations of OXC with phenytoin, felbamate and lamotrigine exerted antagonistic interactions in the mouse MES model (Luszczki et al., 2003; Luszczki and Czuczwar, 2004). It was also observed that the combinations of OXC with gabapentin, levetiracetam and topiramate exerted synergistic interaction in mice subjected to MES-induced seizures (Luszczki and Czuczwar, 2004; Luszczki et al., 2005a; Luszczki et al., 2006). In the case of the combination of OXC with clonazepam in the mouse MES model, it was documented that the two-drug mixture produced synergistic, additive and antagonistic types of interactions depending on doses of the studied drugs. For more details see (Luszczki et al., 2003; Luszczki and Czuczwar, 2003).

The above-mentioned characteristics of interactions of OXC and RTG with classical and second-generation AEDs in preclinical studies is very important because some of these AED combinations are favorable and can be recommended for clinical application. It is widely known that ineffective treatment with OXC or RTG in monotherapy may require the addition of a second AED. However, the proper selection of AEDs to the add-on treatment should be based on results from preclinical studies, involving isobolographic analysis of interaction. This was the reason to briefly describe the existing evidence on interactions of OXC and RTG with AEDs in preclinical studies based on isobolographic analysis.

Another fact is worth mentioning while interpreting the results from this study because in our experiments we determined interactions in two experimental models, the MES and chimney tests. This allowed us to calculate the BI values for the combination of RTG with OXC for the fixed-ratio of 1:1, which ranged between from 0.81 to 2.07, indicating both, neutral and advantageous combinations between these AEDs. From a theoretical point of view, the combinations of AEDs can be classified as: beneficial, neutral and unfavorable, depending on the BI values, determined for the anti-seizure and neurotoxic effects in animal models. Generally, a BI value greater than 1.3 illustrates an advantageous combination whilst a BI value lower than 0.7 indicates an unfavorable combination (Luszczki and Czuczwar, 2004). The BI values ranging from 0.7 to 1.3 reflect a neutral combination from a clinical viewpoint (Luszczki and Czuczwar, 2004). For instance, it

was reported that the combination of OXC with topiramate, producing synergy in the mouse MES model and additivity in the chimney test, has been classified as favorable with a BI values ranging from 1.35 to 1.71 (Luszczki and Czuczwar, 2004). In contrast, the combination of OXC with felbamate has been classified as unfavorable due to their antagonistic interaction in the mouse MES model and additive interaction in the chimney test with BI values ranging from 0.53 to 0.71 (Luszczki and Czuczwar, 2004). Similarly, the combination of OXC with lamotrigine has also been classified as unfavorable due to their antagonistic interaction in the mouse MES model and synergistic interaction in the chimney test with BI values ranging from 0.43 to 0.54 (Luszczki and Czuczwar, 2004).

The results from this study, reporting additivity for the combination of RTG and OXC in the mouse MES model, are generally in agreement with those reporting additive interaction for the combinations of RTG with carbamazepine, lamotrigine and phenytoin in the mouse MES model (Luszczki et al., 2009; Żółkowska et al., 2016), and can be explained in relation to their molecular mechanisms of action of OXC and RTG. There is no doubt that OXC, and also carbamazepine, lamotrigine and phenytoin, exerted additive interactions in the mouse MES model because of their blockade of voltage-gated sodium channels and calcium channels (Czapinski et al., 2005). The unique mechanisms of the anticonvulsant action of RTG are principally based on a selective M-current potassium channel opening effect and modulation of extra-synaptic GABA_A receptors containing δ -subunit (Rundfeldt and Netzer, 2000; Treven et al., 2015). Although the molecular mechanisms of the anticonvulsant actions of RTG and OXC are substantially different from each other, they do not contribute to the synergistic cooperation of both AEDs in terms of seizure suppression in the mouse MES model (Deckers et al., 2000).

Additionally, it is important to note that pharmacokinetic estimation of total brain concentrations of both AEDs in this study confirmed the pharmacodynamic nature of the interaction in the mouse MES model because RTG did not affect total brain concentrations of OXC in mice and inversely, OXC had no impact on total brain concentrations of RTG. The measurement of total brain AEDs' concentrations by use of HPLC allowed us to exclude any pharmacokinetic interactions between the AEDs in the mouse MES model, after single administration of OXC and RTG. On the other hand,

to explain the nature of interaction between RTG and OXC in the case of long-term (chronic) treatment with these AEDs, one should consider metabolic transformation of both AEDs. As regards RTG, the drug is not a substrate for the CYP450 enzymes in the liver and RTG does not induce or inhibit these isozymes (Rejdak et al., 2012; Tompson and Crean, 2014). RTG undergoes glucuronidation or acetylation to form N-glucuronide or mono-acetylated metabolites (Tompson and Crean, 2014). In the case of OXC, this prodrug is entirely converted by cytosolic aryl-ketone-reductase to the active metabolite monohydroxycarbamazepine (MHD), which subsequently undergoes glucuronide conjugation by glucuronyl transferases and renal excretion (Perucca, 2006). OXC and its active agent MHD, stimulate CYP3A4 and weakly inhibit CYP2C19 (Perucca, 2006). Thus, from a theoretical viewpoint, during a chronic administration of both AEDs, a competition between RTG and OXC for renal excretion and/or for glucuronidation may occur, resulting in an increase in AED concentrations. However, such a possible pharmacokinetic interaction between RTG and OXC has not been yet confirmed clinically.

CONCLUSIONS

The pharmacodynamic nature of interaction between RTG and OXC in both the MES and chimney tests in mice was additive. Results presented in this study confirm suggestions that some selected AED combinations, according to rational polytherapy rules, can be beneficial for patients (Brodie and Sills, 2011).

CONFLICTS OF INTEREST

Professor S.J. Czuczwar has received, within the last three years, speaker's fees from GlaxoSmithKline, Sanofi Aventis, UCB and Janssen Pharmaceuticals. Professor J.J. Luszczki has been involved in the design and development of new antiepileptics and CNS drugs. Professors S.J. Czuczwar and J.J. Luszczki have both received an unrestricted research grant from GlaxoSmithKline (Brentford, UK). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. The remaining authors have no conflict of interest to disclose.

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