Psychoneuroendocrinology 101 (2019) 27–34

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Effects of androsterone on the protective action of various antiepileptic drugs against maximal electroshock-induced seizures in mice

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ARTICLE INFO

Keywords:
Androgens
Androsterone
Antiepileptic drugs
Epilepsy
Maximal electroshock
Seizures

ABSTRACT

This study evaluated the effect of androsterone (AND), a metabolite of testosterone, on the ability of selected classical and novel antiepileptic drugs to prevent seizures caused by maximal electroshock (MES), which may serve as an experimental model of human generalized tonic-clonic seizures in mice. Single intraperitoneal (i.p.) administration of AND (80 mg kg^{-1}) significantly raised the threshold for convulsions in the MES seizure threshold test. Lower doses of AND (5, 10, 20, and 40 mg kg^{-1}) failed to change the threshold. AND at a subthreshold dose of 40 mg kg^{-1} significantly enhanced the protective activity of carbamazepine, gabapentin, and phenobarbital against MES-induced seizures decreasing their median effective doses (ED_{50}) values ± SEM from 8.59 ± 0.76 to 6.05 ± 0.81 mg kg^{-1} ($p = 0.0308$) for carbamazepine, from 419.9 ± 120.6 to 111.5 ± 41.1 mg kg^{-1} ($p = 0.0405$) for gabapentin, and from 20.86 ± 1.64 to 10.0 ± 1.21 mg kg^{-1} ($p = 0.0007$) for phenobarbital. There were no significant changes in total brain concentrations of carbamazepine, gabapentin, and phenobarbital following AND administration. This suggests that the enhancing effects of AND on the protective activity of these antiepileptic drugs are not related to pharmacokinetic factors. A lower dose of AND (20 mg kg^{-1}) had no effect on the protective activity of carbamazepine, gabapentin, and phenobarbital. AND administered at a dose of 40 mg kg^{-1} failed to change the anticonvulsant activity of lamotrigine, oxcarbazepine, phenytoin, topiramate, and valproate in the MES test. In the chimney test, AND given at a dose enhancing the protective activity of carbamazepine, gabapentin, and phenobarbital (which alone was without effect on motor performance of mice) did not affect impairment of motor coordination produced by the antiepileptics. Our findings recommend further preclinical and clinical research on AND in respect of its use as adjuvant therapy in the management of epilepsy in men with deficiency of androgens.

1. Introduction

There are clinically important reciprocal interactions between androgens and the brain (Pack et al., 2011; Taubøll et al., 2015). Androgens can affect functions of the central nervous system (CNS) and play an important role in the pathophysiology of various neural disorders, including epilepsy (Verrotti et al., 2007; Reddy, 2013; Taubøll et al., 2015) while epilepsy and/or antiepileptic drugs can alter androgen levels to promote the development of reproductive hormonal disorders (Herzog and Fowler, 2005; Røste et al., 2005). Epilepsy in men is often associated with reduced free testosterone levels in plasma, a decreased 24-hour urinary excretion of its metabolites, androsterone (5α-androstan-3α-ol-17-one, AND) and etiocholanolone (5β-androstan-3α-ol-17-one), hypogonadism and sexual dysfunction (Reddy, 2004; Herzog and Fowler, 2005; Herzog, 2008; Yogarajah and Mula, 2017).

Testosterone is the main natural androgen and its metabolism

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https://doi.org/10.1016/j.psyneuen.2018.10.017
Received 7 August 2018; Received in revised form 20 September 2018; Accepted 20 October 2018

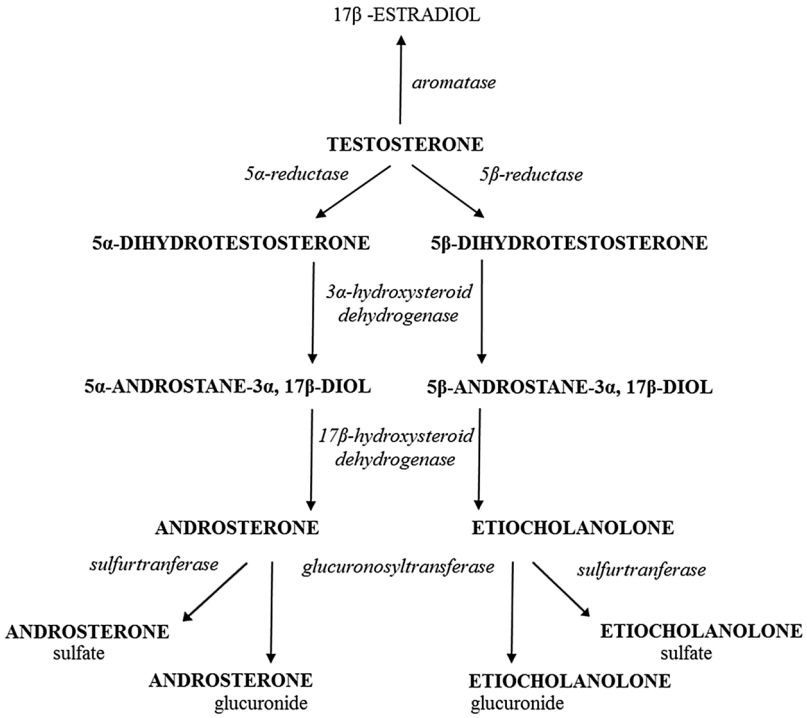

Fig. 1. Pathways of testosterone metabolism (based on Reddy, 2004; Kamiński et al., 2005, modified).

proceeds along two routes. Aromatization of ring A of the steroid skeleton by aromatase forms 17β-estradiol. Irreversible reduction of the A-ring of the steroidal backbone produces either 5α- or 5β-dihydrotestosterone which are then converted to androstanediol (5α-androstane-3α, 17β-diol and 5β-androstane-3α, 17β-diol). The final step, catalyzed by 17β-hydroxysteroid dehydrogenase, produces the two major 17-ketosteroid metabolites AND and etiocholanolone. 17-ketosteroids are then conjugated in the liver with glucuronic or sulfuric acid, forming water-soluble compounds that are readily excreted from the body. The pathways of testosterone metabolism are illustrated in Fig. 1.

Evidence in support of the involvement of androgens in the pathophysiology of epilepsy have been accumulated over the past years. The first data regarding effects of androgens on seizure susceptibility came from experiments in animal models of seizures. It was shown that male rats are less sensitive to the proconvulsive action of allyglycine and picrotoxin (Thomas and Yang, 1991; Peričić et al., 1996). Gonadectomy in male rats significantly potentiates convulsions induced by pentylenetetrazole (PTZ) and picrotoxin compared to animals with their testicles unremoved (Peričić et al., 1996; Pesce et al., 2000). PTZ-induced convulsions occur more often in 24-month-old male rats than in 9-month rats most likely due to physiological decrease in androgen secretion in older animals (Rhodes et al., 2004). Testosterone reduces incidence of PTZ-induced tonic-clonic convulsions (Frye et al., 2001) and kainic acid (KA)-induced convulsions in rats (Frye and Reed, 1998).

Nevertheless, the results of experiments in which seizures were induced in animals followed by testosterone administration were not fully conclusive, i.e. in some experiments testosterone was found to exert anticonvulsive and in others proconvulsive effects (Edwards et al., 1999; Reddy, 2004). For example, testosterone and its two metabolites, estradiol and dihydrotestosterone (DHT) enhanced the development of amygdala-kindled seizures in rats (Edwards et al., 1999).

The effects of testosterone on seizures seem to depend on its different metabolic pathways and levels of its distinct metabolites within the brain (Reddy, 2013). Aromatization of testosterone leads to formation of 17β-estradiol which is believed to have proconvulsive activity (Reddy, 2004). Inhibition of aromatase-mediated conversion to estradiol significantly attenuates the convulsant action of testosterone in tonic-clonic convulsions induced by PTZ (Reddy, 2004). Acute inhibition of testosterone aromatization by letrozole exerts anticonvulsant effect against KA-induced seizures (Iqbal et al., 2018) and development of PTZ- induced kindling in mice (Rashid et al., 2015). In turn, activation of the 5α-reductase pathway leads to formation of 5α-3α- and 5β-3α-reduced metabolites, primarily AND and etiocholanolone that both demonstrate the ability to prevent convulsions in a majority of animal studies (Reddy, 2004; Kamiński et al., 2005).

In animals, the anticonvulsant activity of AND and etiocholanolone results from their ability to modulate allosterically and positively the activity of the γ-aminobutyric acid $(GABA)_A$ receptors (Kamiński et al., 2005; Reddy, 2013). Testosterone reduced metabolites have a similar chemical structure to other GABA-mimetic neurosteroids such as allopregnanolone and tetrahydrodeoxycorticosterone (Reddy and Rogawski, 2002). The most important element of the chemical structure that determines high affinity for the $GABA_A$ receptor is the presence of the reduced A ring of the steroid backbone and the hydroxyl group at the 3α position. This chemical feature is found in androstanediol, AND and etiocholanolone, the compounds which protect against convulsions in many animal models of epilepsy (Frye and Reed, 1998; Reddy, 2004; Kamiński et al., 2005).

In humans, a deficiency of androgens may promote the development of epileptic discharges and increase seizure susceptibility (Herzog, 2008). Subnormal levels of plasma free testosterone are related to the frequency of epileptic seizures (Reddy, 2004; Herzog and Fowler, 2005). However, testosterone by itself was not reported to improve seizures occurrence clinically (Herzog et al., 1998). Therefore, in order to improve the effectiveness of treatment of epilepsy, other hormonal strategies were attempted in patients. Single reports and pilot clinical trials have indicated that any hormonal treatment of epilepsy in patients should either be aimed at blocking the estrogen pathway, or towards the induction of the 5α-reductase pathway in testosterone metabolism (Herzog et al., 1998; Harden and MacLusky, 2004; Reddy, 2004; Rhodes et al., 2004). Other therapeutic strategies that complement the deficiency of the testosterone metabolites AND and etiocholanolone, seem to be especially important and beneficial (Kamiński et al., 2005).

The effect of neuroactive 5α-3α- and 5β-3α-reduced metabolites of testosterone on the anticonvulsant activity of antiepileptic drugs has not yet been studied. An understanding of these interactions and their mechanisms is important to the comprehensive management of individuals with epilepsy (Pack et al., 2011). Therefore, the aim of this study was to determine the effects of AND on the protective activity of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, phenobarbital, phenytoin, topiramate, and valproate against convulsions caused by maximal electroshock (MES), which may serve as an experimental model of human generalized tonic-clonic seizures in mice. Additionally, the effects of AND by itself and in combination with the antiepileptic drugs on motor impairment were investigated in the chimney test. Finally, total brain concentrations of the tested antiepileptic drugs were measured to ascertain whether effects of AND were related to altered pharmacokinetics of the antiepileptic drugs.

2. Material and methods

2.1. Animals and experimental conditions

Experiments were performed on adult male Swiss mice weighing 20–25 g. After one week of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of 8–10 mice per group. The tests were performed between 8:00 and 14:00. The control groups were always tested on the same day as the corresponding experimental groups. The experimental protocol and procedures were followed according to the Guide for the Care and Use of Laboratory Animals and approved by the Medical University of Lublin Ethics Committee.

2.2. Drugs

AND (Androsterone®, Sigma-Aldrich, St. Louis, MO, USA), carbamazepine (Sigma-Aldrich, St. Louis, MO, USA), gabapentin (Gabapentin®, Teva UK Ltd, Hampden Park, England), lamotrigine (Lamitrin®, GlaxoSmithKline Export Ltd., Brentford, England), oxcarbazepine (Trileptal®, Novartis Pharma Produktions GmbH, Wehr, Germany), phenytoin (Sigma-Aldrich, St. Louis, MO, USA), and topiramate (Topamax®, Janssen Cilag, Schaffhausen, Switzerland) were suspended in a 1% solution of Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) in distilled water. Phenobarbital (Phenobarbitalum Natrium®, Polfa, Warszawa, Poland) and valproate (ICN Polfa Rzeszów S.A., Rzeszów, Poland) were dissolved in distilled water. All drugs were administered intraperitoneally (i.p.) as follows: AND (20–80 mg kg^{-1}) −15 min, carbamazepine (4–12 mg kg^{-1}), oxcarbazepine (5–10 mg kg^{-1}), and valproate (150–300 mg kg^{-1}) −30 min; phenobarbital (5–30 mg kg^{-1}), gabapentin (50–1400 mg kg^{-1}), lamotrigine (2–6 mg kg^{-1}), and topiramate (10–50 mg kg^{-1}) −60 min, and phenytoin (4–10 mg kg^{-1}) −120 min before the MES seizure test, evaluation of motor coordination, as well as, before brain sampling for the measurement of antiepileptic drug concentrations, as described in the literature and confirmed in our previous experiments (Łuszczki and Czuczwar, 2004; Tutka et al., 2013). Fresh drug solutions or suspensions were prepared *ex tempore* on each day of experimentation. Control animals were injected with adequate amounts of distilled water or 1% solution of aqueous Tween 80 via a corresponding route.

2.3. MES seizure threshold test

Electroconvulsions were induced according to Swinyard et al. (1952) with the use of alternating current impulses (50 Hz, 0.2 s) delivered from a generator (Rodent Shocker, type 221, Hugo Sachs Elektronik, Freiburg, Germany) via ear-clip electrodes.

In the first part of the experiments, the electroconvulsive threshold for administration of AND was determined. For this purpose, CS_{50} value (i.e. median current strength in mA, necessary to induce tonic hind limbs extension in 50% of animals) was calculated. Five doses of AND were screened: 5, 10, 20, 40 and 80 mg kg^{-1}. Full tonic extension of the hind limbs was taken as the end point. Mice were subjected to current stimulation with different intensities (6–12 mA) and, depending on the number of convulsing animals, an intensity-response curve was calculated according to the Litchfield and Wilcoxon method (1949). The control group of mice received the adequate amount of vehicle.

2.4. MES seizure test

In the second part of the experiments, the MES seizure model was applied to evaluate effects of AND on the anticonvulsant activity of the antiepileptic drugs. Mice were challenged with set current intensity and stimulus duration (25 mA and 0.2 s, respectively). Control groups received progressive doses of antiepileptic drug and vehicle. All animals in control groups produced seizures. The study groups received progressive doses of antiepileptic drug in combination with AND in a previously determined subthreshold dose of 40 mg kg^{-1}, i.e., the highest dose that did not significantly change electroconvulsive threshold in previous experiments. Complete protection against tonic convulsions during 1 min of observation was taken as the endpoint. Anticonvulsant activity of the antiepileptic drugs was determined in mice pretreated with these drugs before MES as ED_{50} values (i.e., doses of the drugs in mg kg^{1}, protecting 50% of animals against MES). Depending on the number of protected animals, a dose-response curve for each antiepileptic drug was calculated according to the Litchfield and Wilcoxon method (1949). The ED_{50} of the antiepileptic drug for mice pretreated with AND were compared with the respective ED_{50} of the antiepileptic drug administered separately (+ vehicle).

2.5. Chimney test

The effects of AND alone or combined with carbamazepine, gabapentin, lamotrigine, oxcarbazepine, phenobarbital, phenytoin, topiramate, and valproate on motor performance were determined by the chimney test of Boissier et al. (1960). Mice had to climb backwards up a plastic tube (3 cm inner diameter, 30 cm length). Motor impairment was indicated by the inability of animals to perform the test within 60 s. Dose-response curve calculated according to the Litchfield and Wilcoxon method (1949) and the respective median toxic doses (TD_{50} values, i.e., doses of the antiepileptics which caused impairment of motor coordination in 50% of animals) were determined. In each case, TD_{50} for the study group of mice (antiepileptic drug + AND) was compared with TD_{50} for the respective control group (antiepileptic drug + vehicle).

2.6. Measurement of total brain concentrations of the antiepileptic drugs

Pharmacokinetic estimation of total brain antiepileptic drug concentrations was performed only for combinations of AND with antiepileptic drugs whose anticonvulsant effect in the MES seizure test was significantly higher from that of control (vehicle + antiepileptic drug) mice. Animals were administered with carbamazepine, gabapentin, and phenobarbital at doses corresponding to their ED_{50} value as previously determined in the MES test and AND (40 mg kg^{-1}) or antiepileptic drug in the same ED_{50} dose and vehicle. At the time of peak concentration of the drugs, mice were decapitated and the whole brains were removed from skulls, weighed, harvested and homogenized using Abbott buffer (in the case of carbamazepine and phenobarbital) or distilled water (in the case of gabapentin) (1:2 wt/volume) in an Ultra-Turrax T8 homogenizer (IKA Werke, Staufen, Germany). The brain homogenates were centrifuged at 10,000 *g* for 10 min and the supernatant samples were analyzed by immunofluorescence with use of an Abbott TDx automatic analyzer (Abbott, Irving, TX, USA) for carbamazepine and phenobarbital or high pressure liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometer (HPLC-

APCIMS), Surveyor-LCQ Advantage MAX system (Thermo Electron Corporation, San Jose, CA, USA) for gabapentin content as described earlier (Barczyński et al., 2011). Total brain antiepileptic drug concentrations are expressed in μg ml^{-1} as the mean ± SEM of ten separate brain preparations.

2.7. Statistical analysis

The ED_{50}, TD_{50} and CS_{50} values were calculated from dose-response (intensity-response) curves using log-probit analysis (Litchfield and Wilcoxon, 1949). Statistical analysis of the CS_{50} values for increasing doses of AND was performed either with the log-probit method for a single comparison or with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test for multiple comparisons. At each time, at least four groups of mice consisting of 8–10 animals were used. Total brain antiepileptic drugs concentrations were statistically analyzed with the Student's *t*-test. The index of probability of 0.05 or less ($p < 0.05$) was considered significant in the comparative analysis.

3. Results

3.1. Effect of AND on threshold for convulsions in MES seizure threshold test

AND administered i.p. at a dose of 80 mg kg^{-1} significantly raised the threshold for electroconvulsions, increasing the CS_{50} from 7.42 ± 0.63 mA for control group to 10.33 ± 0.87 mA [$F(5;122) = 2.295$; $P = 0.049$]. Lower doses of AND failed to change the threshold (Fig. 2). The dose of AND 40 mg kg^{-1} was determined to be the subthreshold dose.

3.2. Effect of AND on the protective activity of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, phenobarbital, phenytoin, topiramate, and valproate against MES-induced convulsions

AND at a dose of 40 mg kg^{-1} administered i.p. significantly enhanced the anticonvulsant activity of carbamazepine, gabapentin, and phenobarbital. The ED_{50} value ± SEM of carbamazepine was decreased from 8.59 ± 0.76 (n = 32) to 6.05 ± 0.81 mg kg^{-1} (n = 32) [$F(2;93) = 3.613$; $p = 0.0308$] (Fig. 3A). AND at a dose of 20 mg kg^{-1} did not significantly influence the anticonvulsive activity of carbamazepine (ED_{50} = 6.25 ± 0.65 mg kg^{-1}, n = 32) (Fig. 3A).

AND at a dose of 40 mg kg^{-1} decreased the ED_{50} of gabapentin from 419.9 ± 120.6 (n = 40) to 111.5 ± 41.1 mg kg^{-1} (n = 32) [$F(2;109) = 3.302$; $p = 0.0405$] (Fig. 2B). AND at a lower dose of 20 mg kg^{-1} had no effect on the anticonvulsant activity of gabapentin (ED_{50} = 257.9 ± 49.3 mg kg^{-1}, n = 40) (Fig. 3B).

The ED_{50} value of phenobarbital decreased from 20.86 ± 1.64 (n = 32) to 10.0 ± 1.21 mg kg^{-1} (n = 32) [$F(2;93) = 7.877$; $p = 0.0007$] after i.p. administration of AND in a dose of 40 mg kg^{-1} (Fig. 3C). A lower dose of AND (20 mg kg^{-1}) did not influence the anticonvulsive activity of phenobarbital (ED_{50} = 15.48 ± 2.66 mg kg^{-1}, n = 320) (Fig. 3C).

AND administered at a dose of 40 mg kg^{-1} failed to significantly affect the anticonvulsant activity of lamotrigine, oxcarbazepine, phenytoin, topiramate, and valproate. The ED_{50} values did not show significant changes vs. respective control groups. The ED_{50} values (in mg kg^{-1}) were: for lamotrigine 2.98 ± 0.44 (n = 24) (lamotrigine + AND) vs. 4.37 ± 0.55 (n = 32) (lamotrigine alone), for oxcarbazepine 6.62 ± 0.84 (n = 32) (oxcarbazepine + AND) vs. 7.66 ± 0.87 (n = 40) (oxcarbazepine alone), for phenytoin 6.18 ± 0.78 (n = 32) (phenytoin + AND) vs. 8.30 ± 0.94 (n = 40) (phenytoin alone), for topiramate 16.40 ± 2.08 (n = 32) (topiramate + AND) vs. 24.04 ± 3.05 (n = 32) (topiramate alone), and for valproate 180.4 ± 22.9 (n = 32) (valproate + AND) vs. 179.8 ± 20.8 (n = 32) (valproate alone).

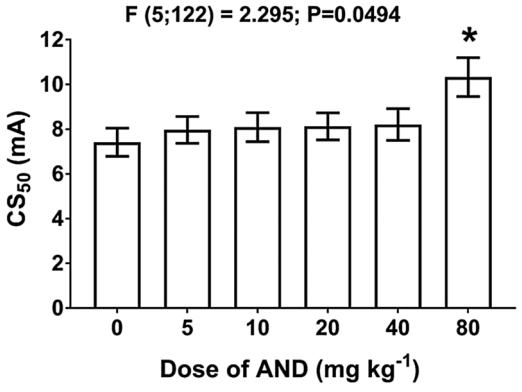

Fig. 2. Influence of androsterone (AND) upon the threshold for electroconvulsions. AND was administered i.p. at doses of 5, 10, 20, 40, and 80 mg kg^{-1} 15 min prior to the MES seizure threshold test. Each bar reflects the CS_{50} value (with SEM), i.e., median current strength in mA necessary to induce tonic convulsions in 50% of animals. For each dose of AND, five groups consisting of 8 mice per group were tested. *$p < 0.05$.vs control group. For more details see Materials and methods.

3.3. Motor performance of mice pretreated with antiepileptic drugs and AND alone or in combination

AND administered alone at a dose of 40 mg kg^{-1} did not affect motor performance in mice challenged with the chimney test. In the control group (administered with distilled water or 1% solution of Tween 80 in distilled water) no animals showed motor impairment. Carbamazepine, gabapentin, and phenobarbital in a dose-dependent manner impaired motor coordination in mice with TD_{50}s being 56.03 ± 4.07 (n = 24), 978.15 ± 86.23 (n = 24), and 89.39 ± 4.28 (n = 24) mg kg^{-1}, respectively. AND at a subthreshold dose of 40 mg kg^{-1} did not have any significant effect on impairment of motor coordination produced by carbamazepine, gabapentin, and phenobarbital. The TD_{50}s for carbamazepine, gabapentin, and phenobarbital combined with AND (40 mg kg^{-1}) were 47.15 ± 3.42 (n = 24), 871.19 ± 120.89 (n = 24), and 85.06 ± 6.30 (n = 24) mg kg^{-1}, respectively (Table 1). The combination of AND (40 mg kg^{-1}) with lamotrigine, oxcarbazepine, phenytoin, topiramate, and valproate also did not affect significantly impairment of motor coordination in mice produced by lamotrigine, oxcarbazepine, phenytoin, topiramate, and valproate (Table 1).

3.4. Influence of AND on total brain concentrations of carbamazepine, gabapentin, and phenobarbital

Total brain concentration of carbamazepine given alone in a dose of 6.05 mg kg^{-1} (i.e., at dose corresponding to its ED_{50}value from the MES test), was 1.31 ± 0.26 μg ml^{-1} and did not differ significantly from that determined when carbamazepine (6.05 mg kg^{-1}) was given in combination with AND administered at a dose of 40 mg kg^{-1} (1.43 ± 0.35 μg ml^{-1}) (Table 2). AND (40 mg kg^{-1}) co-administered with gabapentin (111.51 mg kg^{-1}) did not significantly change total brain concentration of gabapentin (27.45 ± 6.93 μg ml^{-1} for gabapentin alone vs. 31.65 ± 12.17 μg ml^{-1} for gabapentin + AND) (Table 2). Total brain concentration of phenobarbital given alone at a dose of 10.0 mg kg^{-1}, was 3.44 ± 0.37 μg ml^{-1} and did not differ significantly from that determined for phenobarbital (10.0 mg kg^{-1}) in combination with AND (40 mg kg^{-1}), which was 3.67 ± 0.22 μg ml^{-1} (Table 2).

4. Discussion

This is the first study that examined whether AND influenced the

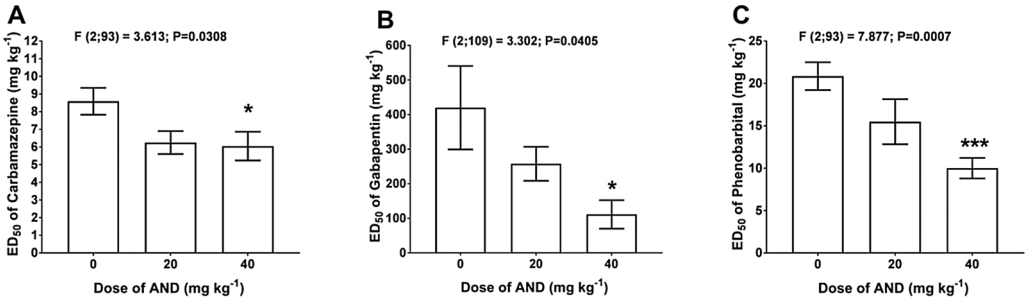

Fig. 3. A–C. Effects of androsterone (AND) on the protective activity of carbamazepine, gabapentin, and phenobarbital in the mouse MES tonic-clonic seizure test. AND was administered i.p. at a dose of 40 mg kg^{-1} 15 min before the test. The studied drugs were administered i.p.: carbamazepine −30 min, gabapentin, and phenobarbital −60 min prior to the test. Each bar reflects the ED_{50} values (median effective dose, i.e. a dose of each antiepileptic drug producing protection from tonic-clonic convulsions in 50% of mice; in mg kg^{-1}). The ED_{50}s of the antiepileptic drugs ± SEM were calculated according to the method by Litchfield and Wilcoxon. Statystical analysis of data was performed with one-way ANOVA followed by the Tukey-Kramer post-hoc test for multiple comparisons. At least four groups consisting of 8–10 of mice per group were tested. *$p < 0.05$, ***$p < 0.001$ vs respective control groups. For more details see Materials and methods.

Table 1
Effects of androsterone (AND) on motor coordination impairment caused by carbamazepine (CBZ), gabapentin (GBP), lamotrigine (LTG), oxcarbazepine (OXZ), phenobarbital (PHB), phenytoin (PHT), topiramate (TPM), and valproate (VPA) in the chimney test.

Treatment (mg kg^{-1})	TD_{50} (mg kg^{-1})
CBZ + vehicle	56.03 ± 4.07 (n = 24)
CBZ + AND (40)	47.15 ± 3.42 (n = 24)
GBP + vehicle	978.15 ± 86.23 (n = 24)
GBP + AND (40)	871.19 ± 120.89 (n = 24)
LTG + vehicle	18.96 ± 2.51 (n = 24)
LTG + AND (40)	24.79 ± 2.67 (n = 24)
OXC + vehicle	59.51 ± 4.28 (n = 24)
OXC + AND (40)	54.70 ± 4.65 (n = 16)
PHB + vehicle	89.39 ± 4.28 (n = 24)
PHB + AND (40)	85.06 ± 6.30 (n = 24)
PHT + vehicle	75.30 ± 3.75 (n = 24)
PHT + AND (40)	70.72 ± 5.57 (n = 24)
TPM + vehicle	490.15 ± 42.89 (n = 24)
TPM + AND (40)	466.87 ± 41.50 (n = 24)
VPA + vehicle	376.35 ± 22.96 (n = 16)
VPA + AND (40)	305.80 ± 16.19 (n = 16)

Data represent the TD_{50} values (median toxic dose, i.e. a dose of each antiepileptic drug producing impairment of motor coordination in 50% of mice; in mg kg^{-1}). The TD_{50}s of CBZ, GBP, LTG, OXC, PHB, PHT, TPM, and VPA ± SEM were calculated and statistically compared according to the method by Litchfield and Wilcoxon. AND was administered i.p. 15 min before the test. CBZ, OXC, and VPA were administered 30 min, GBP, LTG, PHB, and TPM −60 min, and PHT −120 min before the test. For more details see Materials and methods.

Table 2
Effect of androsterone (AND) on total brain concentrations of carbamazepine (CBZ), phenobarbital (PHB), and gabapentin (GBP).

Treatment (mg kg^{-1})	Total brain concentration (μg ml^{-1})
PHB (10.0) + vehicle	3.438 ± 0.372
PHB (10.0) + AND (40)	3.666 ± 0.221
CBZ (6.05) + vehicle	1.313 ± 0.26
CBZ (6.05) + AND (40)	1.426 ± 0.352
GBP (111.51) + vehicle	27.45 ± 6.93
GBP (111.51) + AND (40)	31.65 ± 12.17

Data are presented as mean ± SEM of 10 separate determinations. Statistical evaluation of the data was performed using the unpaired Student's *t*-test Brain tissue samples were taken at times scheduled from the MES test. For more details see Materials and methods.

protective activity of antiepileptic drugs against convulsions induced by MES in mice. First, we demonstrated that single i.p. administration of AND at a dose of 80 mg kg^{-1} caused a significant elevation of the seizure threshold for electroconvulsions. The anticonvulsant effect of AND in the MES seizure threshold test was dose-dependent.

The principal observation in this study is that AND at a subthreshold dose of 40 mg kg^{-1} significantly and dose-dependently enhanced the protective activity of three of eight selected antiepileptic drugs in the MES test. The selection of tested antiepileptic drugs was based on information that these antiepileptic drugs suppressed, in a dose dependent manner, the MES-induced convulsions in mice (Łuszczki et al., 2011; Tutka et al., 2013). In contrast, some classical and newer antiepileptic drugs, i.e., clobazam, clonazepam, lacosamide, levetiracetam, pregabalin, tiagabine and vigabatrin were not examined in this study because they are ineffective in the MES test (Łuszczki, 2009).

Our finding confirms some previous reports showing the anticonvulsant action of AND in electrically-induced seizures. It was reported that AND injected i.p. dose-dependently protected against tonic-clonic seizures in the MES test with ED_{50} being 223 mg kg^{-1} (Kamiński et al., 2005) or 227 mg kg^{-1} (Mróz et al., 2009). Furthermore, AND protected against seizures in the 6 Hz electrical stimulation test, a model of psychomotor seizures in mice thought to be a model of drug-resistant seizures (Kamiński et al., 2005). AND conferred seizure protection also in various chemically-induced convulsions in animals. AND protected against convulsions induced by 4-aminopyridine, pilocarpine, (Kamiński et al., 2005), PTZ in mice (Kamiński et al., 2005; Mróz et al., 2009), and 3-mercaptopropionic acid in hamsters (Naum et al., 2002). AND was found to exert neuroprotective activity after pilocarpine-induced status epilepticus in mice (Cho et al., 2014). On the other hand, our previous study demonstrated that AND increased the incidence of convulsions induced by i.p. KA, a model for complex partial seizures and/or status epilepticus in mice (Mróz et al., 2009). A cause of this proconvulsant action of AND in KA-induced seizures is unknown.

In this study, AND dose-dependently increased the protective activity of carbamazepine, gabapentin, and phenobarbital in the MES test. The ED_{50} of phenobarbital was reduced by more than 50% from 20.86 to 10 mg kg^{-1} ($p < 0.001$). Phenobarbital, one of the most widely used antiepileptic drugs in the developing world, exerts its anticonvulsant effect primarily through the activation of neuronal postsynaptic $GABA_A$ receptors by increasing the mean channel open duration without affecting open frequency or conductance. It results in an increase in Cl- flux, hyperpolarization of the postsynaptic neuronal cell membrane and inhibition of the transmission of epileptic activity (Kwan and Brodie, 2004). Phenobarbital also decreases the influx of Na^+ and Ca^2 and inhibits glutamatergic transmission but these mechanisms seem to be less important in the mechanism of its anticonvulsant action (Deckers et al., 2003).

As it was shown in studies with rat hippocampal slices, AND and barbiturates similarly enhanced GABA-mediated inhibitory effects in brain membranes and functional activity in Cl^- flux. However, separate sites of action were demonstrated conclusively by the observation that AND and barbiturates, when included together, gave additive or synergistic effects on binding, as well as on Cl^- flux in the absence of GABA agonist (Turner et al., 1989). In the present study, the ability of AND to enhance the anticonvulsant action of phenobarbital most likely

results from GABA-mediated inhibition of neuronal function. However, further investigations are needed concerning this effect.

Our study showed that AND enhanced the protective activity of carbamazepine in the MES test by 30% ($p < 0.05$). Carbamazepine, the mainstay of the pharmacological management of focal and generalized tonic-clonic seizures for many years and a current first-line treatment for partial onset seizures in the USA and Europe (Nevitt et al., 2017), inhibits epileptic activity by the blockade of Na^+ channels. Carbamazepine also activates K^+ channels and antagonizes the action of excitatory amino acids (Johannessen Landmark, 2008). In this study, AND did not affect the protective activity of lamotrigine, oxcarbazepine and phenytoin, drugs which exert their anticonvulsant action, at least partially, by Na^+ channel blockade (Brodie, 2017). Thus, carbamazepine was the only Na^+ channel antagonist whose protective effect was intensified by AND in the MES test.

Of the newer antiepileptic drugs used in this study, AND at a subthreshold dose enhanced the protective activity of gabapentin in the MES test. The ED_{50} of gabapentin in mice treated by AND was about 73% lower than the ED_{50} when gabapentin was given alone ($p < 0.05$). Gabapentin is approved for treatment of focal seizures (Johannessen and Ben-Menachem, 2006). The mechanism of its anticonvulsant action has not been fully described. Though similar in structure to GABA, gabapentin has not been shown to bind to GABA receptors nor transform metabolically into GABA (Honarmand et al., 2011). Gabapentin has been shown to bind to the $\alpha 2\delta$-1 subunit of voltage-gated Ca^{2+} channels, which contributes to its pain attenuation effect (Sills, 2006). It seems, however, that this action is responsible for its effectiveness in the treatment of neuralgic pain rather than its anticonvulsant effect (Landmark, 2007). Of note, we found that AND in the subthreshold dose had no effect on the protective activity of valproate and topiramate, the antiepileptics which significantly enhance GABA-mediated inhibition of epileptic activity (Perucca, 2002).

The present study is first to evaluate the nature of interaction between AND and the antiepileptic drugs in animals. Total brain antiepileptic drug concentrations were measured in our study because only total brain antiepileptic drug concentrations provide the exact classification and characterization of interactions between antiepileptic drugs and other compounds in preclinical studies (Cadart et al., 2002). Total brain concentrations of carbamazepine, gabapentin, and phenobarbital remained unchanged following AND administration. This suggests that the enhancing effects of AND on the protective activity of these drugs do not appear to have a pharmacokinetic nature, and may depend on the direct action of AND. Because AND failed to affect the anticonvulsive activity of lamotrigine, oxcarbazepine, phenytoin, topiramate, and valproate against MES-induced convulsions, we did not verify the total brain concentrations of these antiepileptic drugs.

Some studies suggested that the potential mechanism of AND effect in suppressing seizures may be attributed to its ability to positively modulate $GABA_A$ receptor activity allosterically (Turner et al., 1989; Kokate et al., 1994; Kamiński et al., 2005). Whether the action of AND on GABA-mediated neurotransmission contributes to its effect in enhancing the protective activity of carbamazepine, gabapentin or phenobarbital in the MES test remains to be determined. More advanced pharmacological studies are required to explain the mechanism(s) of such interactions.

Many clinically used antiepileptic drugs have significant adverse effects that prohibit their use in clinical settings. Therefore, the evaluation of the efficacy of antiepileptic drugs and substances with potential anticonvulsant activity should also be accompanied by an assessment of their adverse effects, including neurotoxic effects. There are many tests to determine the neurotoxic effects caused by antiepileptic drugs. Among these tests, one of the most widely used is the chimney test of Boissier et al. (1960). This test is used to quantify the motor disturbance potential of antiepileptic drugs in mice. In this study, AND administered alone at a single subthreshold dose (40 mg kg^{-1}), which enhanced the protective activity of the antiepileptic drugs in the MES test, did not affect motor coordination as determined in the chimney test. The effects of AND on motor coordination in animals were not reported in available literature. It should be noted that AND in combination with carbamazepine, gabapentin, and phenobarbital had no effect on impairment of motor coordination produced by the studied antiepileptic drugs. Thus, it seems that the interaction between AND and carbamazepine, gabapentin, and phenobarbital relies exclusively on the AND impact on the anticonvulsive activity of these antiepileptic drugs without affecting their acute side effects. The lack of a negative effect from AND on motor coordination after use of the dose increasing the protective activity effect of carbamazepine, gabapentin, and phenobarbital is beneficial from a clinical point of view.

In men with epilepsy, especially temporal lobe epilepsy, there is quite often a reduced concentration of bioavailable testosterone and, consequently, reduced production of AND (Kamiński et al., 2005; Herzog, 2008). Neuroactive sex steroids pass through the blood-brain barrier, and therefore it can be presumed that in the case of a decrease in peripheral androgen secretion, there is an AND deficiency in the CNS. A consequence of the AND deficiency may be exacerbation of the clinical course of epilepsy (Reddy, 2004; Herzog and Fowler, 2005; Herzog, 2008).

It has been suggested that the best control of epilepsy treatment is ensured by the use of drugs acting with different mechanisms (Löscher and Schmidt, 1994). Therefore, it is logical to propose the use of AND in the treatment of epilepsy, especially in men who have been shown to have a reduced level of free testosterone in plasma since the treatments used so far have not produced satisfactory therapeutic effects. AND seems to be a candidate for adjuvant therapy rather than monotherapy for epilepsy, so it is important to know about the effect of AND on the effectiveness and safety profile of antiepileptic drugs. Among the results obtained by us, the enhancing effects of AND on the protective activity of carbamazepine and phenobarbital, drugs commonly used in the treatment of epilepsy, seems particularly important. It has been known that both drugs induce the activity of hepatic cytochrome P450 enzymes (Tien et al., 2015; Sugiyama et al., 2016). The use of the liver enzyme inducing phenobarbital and carbamazepine increases serum sex hormone binding globulin (SHBG) concentrations in men with epilepsy (Isojärvi, 2008). Long-term carbamazepine treatment leads to a significantly lower testosterone/SHBG ratio (Røste et al., 2005). Over time the increase in serum SHBG levels leads to reduced activity of testosterone (Isojärvi, 2008), which may alter the epilepsy course, severity and response to anticonvulsant treatment. Thus, the induction of hepatic enzymes by carbamazepine and phenobarbital may be in a sense a self-limiting mechanism of the antiepileptic efficacy of these drugs in men with epilepsy.

As shown in this study, AND increases the antiepileptic activity of carbamazepine and phenobarbital in healthy mice. The effect of AND on the antiepileptic activity of carbamazepine and phenobarbital has not been studied in mice with decreased level of androgens. Therefore, to confirm our data, the next evaluation of the AND interaction with antiepileptic drugs should be performed in animals with a deficiency of androgens. If this is confirmed in people, this may constitute a rationale to consider modification of carbamazepine and phenobarbital dosage in epileptic patients with a deficiency of androgens. Moreover, a reduction in the dose of carbamazepine, gabapentin or phenobarbital during AND administration would reduce the risk of neurotoxic adverse effects during their clinical use.

The results of this study are less supportive for the use of AND in men treated with lamotrigine, oxcarbazepine, phenytoin, topiramate and valproate. There was no increase in the anticonvulsant effect of these drugs. On the other hand, AND did not reduce their effectiveness or intensify motor coordination impairment. Considering the fact that in addition to the anticonvulsant action, the administration of AND may be important for the treatment of fertility disorders and sexual dysfunctions in men with epilepsy, it should be noted that there are no grounds (at least experimental) against administering AND in epileptics

treated with lamotrigine, oxcarbazepine, phenytoin, topiramate and valproate.

The main limitation of this study is that the effects of AND on the anticonvulsant activity of the antiepileptic drugs were investigated in only one seizure model. To make a proper and full conclusion, a battery of acute and chronic animal models of different types of epilepsy should be used to avoid false predictions. AND testing in chronic models of epilepsy that implement the kindling model of temporal lobe epilepsy (TLE) and post-status models of TLE such as the pilocarpine or kainate models, would yield data which are more predictive of clinical efficacy. Moreover, one should consider the limitation that experimental efficacy and lack of acute side effects of a drug in animal experiments are not always correlated with its efficacy and safety in clinical practice. One reason for this discrepancy, apart from obvious differences in species, is the fact that healthy animals are used in animal studies, while in humans the drug most often administered to people with various types of CNS damage.

5. Conclusions

The presented studies have shown for the first time: 1) the anti-seizure effect of the testosterone metabolite AND in the mouse MES seizure threshold test, 2) enhancement by AND of the protective effects of carbamazepine, gabapentin, and phenobarbital against MES-induced seizures, 3) a lack of pharmacokinetic interaction between AND and carbamazepine, gabapentin, and phenobarbital in brain tissue, 4) no effect of AND on their acute side effects. Our findings recommend further preclinical and clinical research on AND in respect of its use as adjuvant therapy in the management of epilepsy in men with deficiency of androgens, including the age-related decline in androgen levels.

Author contributions

PT designed the study and contributed to preparation, planning, data analyses and interpretation, supervision of experimental part of the study and writing of the manuscript. KT and TM contributed to the execution of experiments and data collection and analyses. GB contributed to the implementation brain concentrations measurement experiments and supervision of data collection and analyses. DA and DBA contributed to the writing of manuscript drafts and provided linguistic advice. PK contributed to the writing manuscript and editorial assistance. JJŁ contributed to statistical analyses of data, consultancy and writing of the manuscript. All authors approved the final manuscript.

Funding

This study was supported by a research grant from the Medical University of Lublin (Lublin, Poland) (PW/522/08).

Declaration of interests

JJŁ has been involved in the design and development of new antiepileptic and CNS drugs. The remaining authors have no conflicts of interest to disclose.

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