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Increased seizure susceptibility and other toxicity symptoms following acute sulforaphane treatment in mice

Katarzyna Socała [a,*], Dorota Nieoczym [a], Edyta Kowalczuk-Vasilev [b], Elżbieta Wyska [c], Piotr Wlaź [a]

[a] Department of Animal Physiology, Institute of Biology and Biochemistry, Maria Curie-Skłodowska University, Lublin, Poland
[b] Institute of Animal Nutrition and Bromatology, University of Life Sciences, Lublin, Poland
[c] Department of Pharmacokinetics and Physical Pharmacy, Jagiellonian University Medical College, Kraków, Poland

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ABSTRACT

Activation of Nrf2 with sulforaphane has recently gained attention as a new therapeutic approach in the treatment of many diseases, including epilepsy. As a plant-derived compound, sulforaphane is considered to be safe and well-tolerated. It is widely consumed, also by patients suffering from seizure and taking antiepileptic drugs, but no toxicity profile of sulforaphane exists. Since many natural remedies and dietary supplements may increase seizure risk and potentially interact with antiepileptic drugs, the aim of our study was to investigate the acute effects of sulforaphane on seizure thresholds and activity of some first- and second-generation antiepileptic drugs in mice. In addition, some preliminary toxicity profile of sulforaphane in mice after intraperitoneal injection was evaluated. The LD_{50} value of sulforaphane in mice was estimated at 212.67 mg/kg, while the TD_{50} value – at 191.58 mg/kg. In seizure tests, sulforaphane at the highest dose tested (200 mg/kg) significantly decreased the thresholds for the onset of the first myoclonic twitch and generalized clonic seizure in the iv PTZ test as well as the threshold for the 6 Hz-induced psychomotor seizure. At doses of 10–200 mg/kg, sulforaphane did not affect the threshold for the iv PTZ-induced forelimb tonus or the threshold for maximal electroshock-induced hindlimb tonus. Interestingly, sulforaphane (at 100 mg/kg) potentiated the anticonvulsant efficacy of carbamazepine in the maximal electroshock seizure test. This interaction could have been pharmacokinetic in nature, as sulforaphane increased concentrations of carbamazepine in both serum and brain tissue. The toxicity study showed that high doses of sulforaphane produced marked sedation (at 150–300 mg/kg), hypothermia (at 150–300 mg/kg), impairment of motor coordination (at 200–300 mg/kg), decrease in skeletal muscle strength (at 250–300 mg/kg), and deaths (at 200–300 mg/kg). Moreover, blood analysis showed leucopenia in mice injected with sulforaphane at 200 mg/kg. In conclusion, since sulforaphane was proconvulsant at a toxic dose, the safety profile and the risk-to-benefit ratio of sulforaphane usage in epileptic patients should be further evaluated.

1. Introduction

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane) is an aliphatic lipophilic organosulfur molecule obtained from cruciferous vegetables such as broccoli, Brussels sprouts or cabbages. It is produced by hydrolysis of glucoraphanin. The reaction is catalyzed by myrosinase – an enzyme released from plant vacuoles upon mechanical stress such as cutting or chewing (Houghton et al., 2016). Sulforaphane is not only consumed as a component of cruciferous vegetables but also as a dietary supplement or a drug, depending on its dosage and its intended use. It is considered to be safe and well-tolerated (Brown et al., 2015; Singh et al., 2014; Wu et al., 2016), although no toxicological data on sulforaphane exist. Neither acute nor chronic sulforaphane toxicity in animals have been established.

Sulforaphane acts as a potent activator of the nuclear factor erythroid 2-related factor 2 (Nrf2) that is a master regulator of the antioxidant response and biotransformation of xenobiotics. Nrf2 binds to antioxidant response element (ARE) and induces a variety of antioxidant and phase II detoxification genes that protect cells from electrophiles and reactive oxygen species (Thimmulappa et al., 2002; Huang et al., 2015). Considerable research has focused on the role of Nrf2 in cancer chemoprevention and sulforaphane has emerged as one of the

Abbreviations: ARE, antioxidant response element; C.L., confidence limit; CS_{50}, current strength required to induce seizure response in 50% of mice; DMSO, dimethyl sulfoxide; ED_{50}, median effective dose; ip, intraperitoneally; IS, internal standard; iv, intravenously; LD_{50}, median lethal dose; MES, maximal electroshock seizure; MEST, maximal electroshock seizure threshold; N, newton; Nrf2, nuclear factor erythroid 2-related factor 2; PTZ, pentylenetetrazole; SE, standard error of the mean; TD_{50}, median toxic dose.

* Corresponding author at: Department of Animal Physiology, Institute of Biology and Biochemistry, Faculty of Biology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19, PL 20-033 Lublin, Poland.
E-mail address: ksocala@op.pl (K. Socała).

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most promising phytochemical for cancer prevention (Houghton et al., 2016; Clarke et al., 2008). The doses of sulforaphane required to observe chemopreventive effects have not yet been determined in human clinical trials (Tortorella et al., 2015). In animal studies, the sulforaphane-induced cancer prevention was observed for example at doses of 75–150 μmol (13.3–26.6 mg) per rat (Zhang et al., 1994) and at a dose of ~1 mg per mouse (Fahey et al., 2002; Vyas et al., 2013). Alongside chemopreventive properties, activation of the Nrf2-ARE pathway with sulforaphane has been demonstrated to produce neuroprotective effects. For instance, sulforaphane treatment reduced neuronal cell loss induced by oxidative stress (Kraft et al., 2004), 6-hydroxydopamine (Siebert et al., 2009), or glutamate (Chang et al., 2010). Sulforaphane was also shown to protect immature hippocampal neurons against death caused by exposure to either oxygen and glucose deprivation or hemin (Soane et al., 2010). In animal studies, sulforaphane at a dose of 5 mg/kg (ip) decreases cerebral edema and preserved the blood brain barrier after traumatic brain injury (Zhao et al., 2005, 2007a), decreased cerebral infarct volume following focal ischemia (Zhao et al., 2006) as well as protected the brain from oxidative/cytotoxic damage and reduced behavioral deficits evoked by intracerebral hemorrhage (Zhao et al., 2007b). At a dose of 25 mg/kg (ip), it also ameliorated neurobehavioral deficits and reduced β-amyloid plaque numbers in hippocampus and cerebral cortex in mice with Alzheimer-like lesions (Zhang et al., 2015). Furthermore, sulforaphane enhanced proteasomal and autophagic activities in mice and promoted mutant huntingtin degradation (Liu et al., 2014). Given this, sulforaphane has emerged as a new therapeutic approach in the treatment of brain injury and many neurodegenerative diseases such as stroke, Parkinson's, Alzheimer's and Huntington's disease. Interestingly, a placebo-controlled clinical trial showed that sulforaphane (50–150 μmol which corresponds to 8.86–26.6 mg, for 18 weeks) improves behavior in patients with autism spectrum disorder (Singh et al., 2014).

Several recent studies demonstrated that the Nrf2-ARE signaling pathway is also implicated in the neurobiology of epilepsy (Carmona-Aparicio et al., 2015). This may be due to the fact that oxidative stress plays an important role in the initiation and progression of seizure (Shin et al., 2011). It was reported that ketogenic diet, used primarily to treat refractory epilepsy, produces mild oxidative and electrophilic stress, which in turn activates the Nrf2 pathway (Milder et al., 2010). Moreover, Nrf2 knockout mice were shown to be more vulnerable to kainate-induced seizures and death (Kraft et al., 2006). Additionally, a significant increase in Nrf2 mRNA expression in human epileptic hippocampal tissue was observed (Mazzuferi et al., 2013). Sulforaphane, as a plant-derived compound with ability to cross the blood-brain barrier after systemic administration (Innamorato et al., 2008; Jazwa et al., 2011) and no reported in vivo toxicity, seems to be a useful tool to study the involvement of the Nrf2-ARE signaling pathway in the neurobiology of seizures. There are only few studies assessing the anticonvulsant potency of sulforaphane in rodents. It was reported that activation of the Nrf2-ARE pathway by repeated (15 days) intraperitoneal (ip) sulforaphane administration suppressed the progression of amygdala kindling and ameliorated seizure-induced oxidative stress and cognitive impairment in rats (Wang et al., 2014). In another study, sulforaphane injected ip for 5 days elevated the seizure threshold in the 6 Hz seizure test, prolonged the latency to reach tonic extension in the flurothyl-induced seizure model as well as reduced the number of mice that underwent status epilepticus after pilocarpine injection. Sulforaphane was, however, not able to reduce the severity of seizures during pilocarpine-induced status epilepticus and it did not affect the thresholds for pentylenetetrazole (PTZ)-induced seizures (Carrasco-Pozo et al., 2015). In both studies, sulforaphane was injected repeatedly at a relatively small dose of 5 mg/kg. There are no studies assessing the acute effects of sulforaphane, especially administered at higher doses, on seizure activity. Likewise, interactions between sulforaphane and antiepileptic drugs have not been studied as yet.

With this background, the present study was undertaken to evaluate the acute effects of sulforaphane on the seizure thresholds in three seizure tests in mice. We also sought to investigate the acute adverse effects of high doses of sulforaphane on locomotor activity, neuromuscular strength, motor coordination, and body temperature. Some hematological and serum biochemical parameters were determined as well. Moreover, we investigated the influence of sulforaphane on the activity of selected first and second generation antiepileptic drugs. To determine the acute adverse-effect profiles for the combinations of sulforaphane with the studied antiepileptic drugs, changes in neuromuscular strength, motor coordination, and long-term memory were evaluated.

2. Materials and methods

2.1. Animals

The experiments were carried out on naïve male Albino Swiss mice weighing 23–30 g. The animals were purchased from a licensed breeder (Laboratory Animals Breeding, Ilkowice, Poland) and housed in groups of eight in standard Makrolon cages (37 cm × 21 cm × 15 cm) under a 12 h light-dark cycle with light on at 06:00 am, at a room temperature of 21–24 °C and relative humidity of 45–65%. Food pellets (Agropol S.J., Motycz, Poland) and tap water were provided ad libitum. The animals were adapted at least one week to the laboratory before being used in experiments. All experiments were performed between 8:00 a.m. and 2:00 p.m., after a minimum 30-min acclimatization to the experimental room. The animals were randomly assigned to the experimental groups. Each animal was used only once.

The study was carried out under experimental protocols approved by the Ethical Committee in Lublin. Housing and experimental procedures were conducted in accordance with the European Union Directive of 22 September 2010 (2010/63/EU) and Polish legislation concerning animal experimentation. All efforts were made to minimize the number of animals used and their suffering.

2.2. Treatment

D,L-sulforaphane (Toronto Research Chemicals Inc., Toronto, Canada) was dissolved in a small volume (250 μl) of dimethyl sulfoxide (DMSO, ICN Biomedicals, Inc., Aurora, OH, USA) and diluted to the appropriate concentration with normal saline. The final concentration of DMSO did not exceed 1%. The 60-min pretreatment time for sulforaphane was selected based on the report of Jazwa et al. (2011). Clonazepam (Clonazepamum, Polfa, Warsaw, Poland), tiagabine (Gabitril, Sanofi Winthrop, Gentilly, France), carbamazepine (kindly provided by Polpharma S.A., Starogard Gdański, Poland), and topiramate (Topamax, Janssen-Cilag International NV, Beerse, Belgium) were suspended in a 1% solution of Tween 80 (POCH, Gliwice, Poland), while valproate (as sodium salt, Sigma-Aldrich, Poznań, Poland) was dissolved in normal saline. Tiagabine and valproate were administered 15 min, clonazepam and carbamazepine 30 min, and topiramate 60 min prior to the tests. The pretreatment times for the studied antiepileptic drugs were based upon information about their anticonvulsant activity from the literature and our previous experiments (Łuszczki et al., 2010; Nieoczym et al., 2013). All drug solutions/suspensions were prepared freshly and administered ip at a volume of 0.1 ml per 10 g of body weight. Control animals received respective vehicles only.

2.3. Intravenous pentylenetetrazole (PTZ) seizure threshold test

Mice were placed in the cylindrical plastic restrainer (12-cm long, 3-cm inner diameter). The lateral tail vein was catheterized with a 2-cm long 27-gauge needle attached by polyethylene tubing PE20RW (Plastics One Inc., Roanoke, VA, USA) to a 5-ml plastic syringe containing 1% aqueous solution of PTZ (Sigma-Aldrich, St. Louis, MO,

USA). The syringe was mounted on a syringe pump (model Physio 22, Hugo Sachs Elektronik–Harvard Apparatus GmbH, March-Hugstetten, Germany). The accuracy of needle placement in the vein was confirmed by appearance of blood in the tubing. The needle was secured to the tail by an adhesive tape. After correct needle placement into the tail vein, mice were placed in a Plexiglas arena for behavioral observation. The PTZ solution was infused at a constant rate of 0.2 ml/min. The time intervals from the commencement of PTZ infusion to the onset of each of three endpoints (the first myoclonic twitch, generalized clonus with loss of the righting reflex, and tonic forelimb extension) were recorded. The seizure thresholds were calculated separately for each endpoint using the following formula: threshold dose of PTZ (mg/kg) = (infusion duration (s) × infusion rate (ml/s) × PTZ concentration (mg/ml) × 1000)/body weight (kg). Seizure thresholds were expressed as the amount of PTZ (in mg per kg) ± SE (standard error of the mean) needed to produce the first apparent sign of each endpoint in each experimental group (10–15 mice/group).

2.4. Maximal electroshock seizure test

The corneal electroshock stimulus (sine-wave pulses at 60 Hz for 0.2 s) was delivered by a constant-current stimulator (Rodent Shocker, Type 221; Hugo Sachs Elektronik, Freiburg, Germany). Before stimulation, the corneal electrodes were wet with saline to provide good electrical contact. During stimulation, mice were restrained manually and immediately following stimulation were placed in a transparent box for behavioral observation for the presence or absence of seizure activity. Tonic hindlimb extension (i.e., hindlimbs of animals outstretched at 180° to the plane of body axis) was taken as an endpoint. Two experimental approaches to induce electroconvulsions were used in the present study: (1) the maximal electroshock seizure threshold (MEST) test that employed stimulation at varied current intensities (6.6–15.1 mA) and (2) the maximal electroshock seizure (MES) test which employed stimulation at a fixed current intensity (25 mA).

In the MEST test, the current intensity was established according to an 'up-and-down' method described by Kimball et al. (1957). Current intensity was lowered or raised by 0.06-log intervals depending on whether the previously stimulated animal did or did not exert tonic hindlimb extension, respectively. The data obtained in groups of 20 animals were used to determine the threshold current causing endpoint in 50% of mice (CS_{50} with confidence limits for 95% probability).

The MES test was performed in order to determine the protective potency of carbamazepine and topiramate administered alone or in combination with sulforaphane. The animals (3–5 groups, 8 animals/group) were injected with increasing doses of antiepileptic drugs or their combinations with sulforaphane and then challenged with supramaximal MES stimulus (25 mA). The percentage of mice failing to achieve hindlimb extension was noted and a log-probit method (Litchfield and Wilcoxon, 1949) was used to determine the median effective doses (ED_{50}) of antiepileptic drugs, i.e., doses (in mg/kg) that protect 50% of animals.

2.5. Six hertz (6 Hz) psychomotor seizure test

Square-wave alternating current stimuli (0.2-ms duration pulses at 6 Hz for 3 s) were applied via saline-soaked corneal electrodes using a Grass model CCU1 constant current unit coupled to a Grass S48 stimulator (Grass Technologies, Warwick, RI, USA). Before stimulation, the corneal electrodes were wet with saline to provide good electrical contact. Mice, restrained manually during stimulation, were released immediately following the stimulation and placed in a transparent box for behavioral observation. Psychomotor seizures were characterized by immobility or stun posture, rearing, forelimb clonus, twitching of the vibrissae, and elevated tail. Lack of the features listed above or the resumption of normal exploratory behavior within 10 s after stimulation were considered as the absence of seizures. Two experimental approaches to induce psychomotor seizures were used in the present study: (1) the 6 Hz seizure threshold test that employed stimulation at varied current intensities (7.6–17.4 mA) and (2) the 6 Hz seizure test that employed supramaximal stimulation at a fixed current intensity (32 mA).

In the 6 Hz seizure threshold test, the current intensity values were chosen according to an 'up-and-down' method (Kimball et al., 1957). Each animal was stimulated only once at any given current intensity that was lowered or raised by 0.06-log intervals depending on whether the previously stimulated animal did or did not respond with convulsions. The data obtained in groups of 20 animals were used to determine the threshold current causing 6 Hz-induced seizures in 50% of mice (CS_{50} with confidence limits for 95% probability).

To evaluate the influence of sulforaphane on the anticonvulsant potency of valproate and tiagabine in the 6 Hz seizure test, 3–5 groups of mice consisting of 8 animals were treated with increasing doses of antiepileptic drugs or their combinations with sulforaphane and then stimulated with supramaximal current intensity of 32 mA. A log-probit method (Litchfield and Wilcoxon, 1949) was used to determine the ED_{50} values of antiepileptic drugs in this test.

2.6. Toxicity studies

To determine the acute toxicity of sulforaphane, mice (12 animals/group) were injected with sulforaphane at doses of 150, 200, 250, and 300 mg/kg. Control animals received 1% DMSO. After 60 min, the acute adverse effects of sulforaphane on locomotor activity, muscle strength, motor coordination, and rectal temperature were evaluated. The animals were observed continuously for the first 6 h, then every hour until 12 h after administration and then every 6 h until 48 h after sulforaphane administration, to observe intoxications syndromes such as excitation, tremors, ptosis, ataxia, and mortality. Forty eight hours after sulforaphane injection, all survived mice were decapitated and the trunk blood was immediately collected into the heparinized Eppendorf tubes for further analysis.

2.7. Blood analysis

Hematological analysis of the blood samples was performed within 2 h after sampling using an automatic hematological analyzer ABACUS Junior Vet (Automatic cell counter, Diatron, Vienna, Austria). The parameters which were evaluated included: red blood cells (RBC) count, leukocytes (WBC) count, lymphocytes (LYM) count, mid-size leukocytes (MID) count, granulocytes (GRA) count, percentage of lymphocytes (LY%), percentage of mid-size leukocytes (MI%), percentage of granulocytes (GRA%), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDWc), platelets (PLT) count, mean platelet volume (MPV), and platelet distribution width (PDWc).

Plasma for analysis of the biochemical parameters was obtained by centrifugation of whole blood at 3000 rpm for 10 min in a laboratory centrifuge (MPW-350R, MPW Medical Instruments, Warsaw, Poland) at a temperature of 4 °C. After centrifugation, biochemical plasma parameters such as creatinine, urea, and bilirubin content as well as the activity of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using reagent kits (BioMaxima, Lublin, Poland) according to the manufacturers' protocols and a random access biochemical analyzer Metrolab 2300 GL (Metrolab SA, Buenos Aires, Argentina).

2.8. Locomotor activity test

To monitor the spontaneous locomotor activity of mice, an IR Actimeter system (Panlab/Harvard Apparatus, Barcelona, Spain) was used. The

apparatus consisted of a square arena surrounded by a 25 × 25 cm frame containing a total of 16 × 16 infrared beams located on the sides. The frame was coupled to a computerized control unit. Mice were pretreated with sulforaphane or vehicle and after a given time period (60 min) they were placed individually in the actimeter, in which they were allowed to explore freely for 10 min. The arena was cleaned thoroughly with a 0.1% acetic acid solution before each mouse was placed in it. Interruptions of the photo beams were recorded automatically and analyzed with a computerized system (SedaCom32, PANLAB/Harvard Apparatus, Barcelona, Spain). Locomotor activity was defined as a horizontal activity with displacement and was expressed in terms of total number of interruptions of the photo beams. The obtained data were expressed as means of activity counts/10 min ± SE.

2.9. Grip-strength test

The acute effects of treatments on skeletal muscular strength in mice were quantified in the grip-strength test. The grip-strength apparatus (BioSeb, Chaville, France) consisted of a steel wire grid (8 × 8 cm) connected to an isometric force transducer. The mouse was lifted by its tail so that it could grasp the grid with its forepaws. The animal was then pulled back steadily by the tail until it released the grid. When the animal released the grid, the maximal grip strength value (in newtons, N) of the animal was displayed on the screen. The procedure was repeated three times and the mean force exerted by each mouse before losing grip was recorded. The mean force was then normalized to body weight and expressed in mN/g ± SE.

2.10. Chimney test

The effects of treatments on motor performance in mice were quantified with the chimney test. In this test, the inability of an animal to climb backward up through a Plexiglas tube (inner diameter 3 cm, length 30 cm) within 60 s was an indication of motor impairment.

2.11. Step-through passive avoidance test

The step-through passive avoidance test was used for testing the effect of antiepileptic drugs and their combination with sulforaphane on long-term memory impairments in mice. On the first day before training, each animal was administered with antiepileptic drug (clonazepam at 0.04 mg/kg, carbamazepine at 6.37 mg/kg, topiramate at 28.85 mg/kg, valproate at 196.67 mg/kg, and tiagabine at 1.36 mg/kg) or its combination with sulforaphane (at 100 mg/kg) and placed in the illuminated box (10 × 13 × 15 cm) connected via a guillotine door to the dark box (25 × 20 × 15 cm) with an electric grid floor. Entrance of the animal to the dark box was punished by an adequate electric shock (0.6 mA foot shock for 2 s). Twenty four hours later, animals were placed again into illuminated box and observed for up to 180 s. Mice that avoided of dark part were recognized to remember the task. The retention time (the time that the mice took to enter to the dark box) was noted and the results were presented as median latencies (retention times; in seconds) with 25th and 75th percentiles. Each group consisted of ten animals.

2.12. Rectal temperature measurement

The temperature was recorded with the usage of an electronic thermometer (ThermoWorks, Alpine, UT, USA). The rectal probe was inserted to a depth of ~2 cm into the rectum of the mouse. The temperature was recorded just before sulforaphane or vehicle injection and 60 min later. The difference between the pre- and post-injection temperature values was then calculated (Δ°C). The temperature was also recorded 24 and 48 h post sulforaphane injection to see whether it returned to the pre-treatment level.

2.13. Carbamazepine determination

In order to determine the influence of sulforaphane on serum and brain carbamazepine levels, mice were injected with carbamazepine at a dose of 6.37 mg/kg that corresponds to its ED_{50} value alone or in combination with sulforaphane at 100 mg/kg from the MES test. The animals were decapitated at time points that were identical to those in the MES test. The trunk blood was collected into Eppendorf tubes and allowed to clot at room temperature. Subsequently, it was centrifuged at 5600 rpm for 10 min and serum was collected into polyethylene tubes and frozen at −20 °C. Immediately after the decapitation, brains were dissected from the skull, washed with 0.9% NaCl and frozen at −20 °C.

Before the analysis, brains were homogenized in distilled water (1:4, w/v) with a tissue homogenizer TH220 (Omni International, Inc., Warrenton, VA, USA). Plasma (100 μl) or brain homogenate (200 μl) were spiked with 10 μl of 5 μg/ml butabarbital, which was used as an internal standard (IS) and vortexed (Reax top, Heidolph, Germany) for 10 s. The samples were extracted with 1 ml of dichloromethane for 20 min on a shaker (VXR Vibrax, IKA, Staufen, Germany). After centrifugation (EBA 12 R, Hettich, Tuttlingen, Germany) at 3000 rpm for 5 min, the organic layer was transferred to a new tube and evaporated to dryness at 37 °C under a stream of nitrogen. The residue was dissolved with 100 μl of methanol and 5 μl of this solution was injected into the HPLC system. The analysis was performed on a 250 × 4.0 mm LiChrospher®100 RP-18 column with a particle size of 5 μm (Merck, Darmstadt, Germany) protected with a guard column (4 × 4 mm) with the same packing material. The mobile phase was composed of deionized water and acetonitrile mixed at a ratio of 62:38 (v/v) and pumped at a flow rate of 1 ml/min. The column temperature was kept at 21 °C.

The HPLC system (Merck-Hitachi, Darmstadt, Germany) consisted of an L-7100 isocratic pump, an L-7200 autosampler, and a UV variable-wavelength K-2600 detector (Knauer, Berlin, Germany) set at a wavelength of 210 nm. D-7000 HSM software was used for data acquisition and processing.

The retention times of IS and carbamazepine were approximately 6.20 and 11.3 min. The calibration curve constructed by plotting the ratios of the peak height of carbamazepine to IS versus carbamazepine concentrations was linear in the tested concentration range. No interfering peaks were observed in the chromatograms. The assay was reproducible with low intra- and inter-day variation (coefficient of variation < 5%). The extraction efficiencies of carbamazepine and IS were higher than 90%. Carbamazepine concentrations were expressed in μg/ml of serum or μg/g of wet brain tissue.

2.14. Statistical analysis

The one-way analysis of variance (one-way ANOVA) followed by the Tukey post hoc test for multiple comparisons were employed to compare the changes in seizure thresholds in the iv PTZ, MEST, and 6 Hz seizure threshold tests as well as to analyze the changes in the ED_{50} values of antiepileptic drugs from the MES and the 6 Hz seizure test. For statistical analysis of the data obtained in the MEST and the 6 Hz seizure tests, the CS_{50} values with 95% confidence limits were transformed into the mean value of logarithms (of current strength) with standard deviation. Data from the locomotor activity test and the grip strength test as well as hematological and biochemical blood parameters were also compared with one-way ANOVA followed by Tukey post hoc test. Statistical analysis of data obtained in the chimney test was performed with Fisher's exact probability test. A log-probit method of Litchfield and Wilcoxon (1949) was used to calculate the median toxic dose (TD_{50} value with corresponding 95% confidence limits) of sulforaphane in the chimney test, i.e., the dose of sulforaphane that produces the impairment of motor coordination in 50% of animals. The median lethal dose (LD_{50} with corresponding 95% confidence limits) of sulforaphane and the ED_{50} values of antiepileptic drugs were also calculated with the usage of a log-probit method of Litchfield and Wilcoxon (1949). The

results obtained in the passive avoidance task were compared with Kruskal–Wallis test. Serum and brain carbamazepine concentrations were statistically analyzed using unpaired two-tailed Student's *t*-test after ascertaining normality by Shapiro–Wilk's test.

Differences were considered statistically significant when p values were equal to or <0.05. All calculations were carried out with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA.).

3. Results

3.1. Effect of sulforaphane on the seizure threshold in the iv PTZ test in mice

The effect of sulforaphane on the threshold for the first myoclonic twitch is shown in Fig. 1A (one-way ANOVA: $F(5,75) = 48.40$, $p < 0.0001$). Sulforaphane administered at doses of 10, 50 and 100 mg/kg did not affect significantly the susceptibility of mice to the PTZ-induced first myoclonic twitch. However, at the highest dose tested, 200 mg/kg, it significantly decreased the threshold for the first myoclonic twitch (Tukey post hoc test: $p < 0.001$ vs. control group).

The effect of sulforaphane on the threshold for the generalized clonus with loss of righting reflex is shown in Fig. 1B (one-way ANOVA: $F(5,74) = 30.49$, $p < 0.0001$). Sulforaphane at doses of 10, 50 and 100 mg/kg had no significant effect on the seizure threshold for the onset of clonic seizure. At the dose of 200 mg/kg, sulforaphane significantly decreased the threshold for the onset of clonic convulsions as compared to the control group (Tukey post hoc test: $p < 0.001$).

As shown in Fig. 1C, sulforaphane injected at doses of 10, 50, 100 and 200 mg/kg did not affect the susceptibility of mice to the PTZ-induced forelimb tonus (one-way ANOVA: $F(5,74) = 13.27$, $p < 0.0001$).

Valproate (a positive control) at a dose of 150 mg/kg significantly increased the threshold for the onset of all the studied endpoints (Tukey post hoc test: $p < 0.001$ vs. the control group).

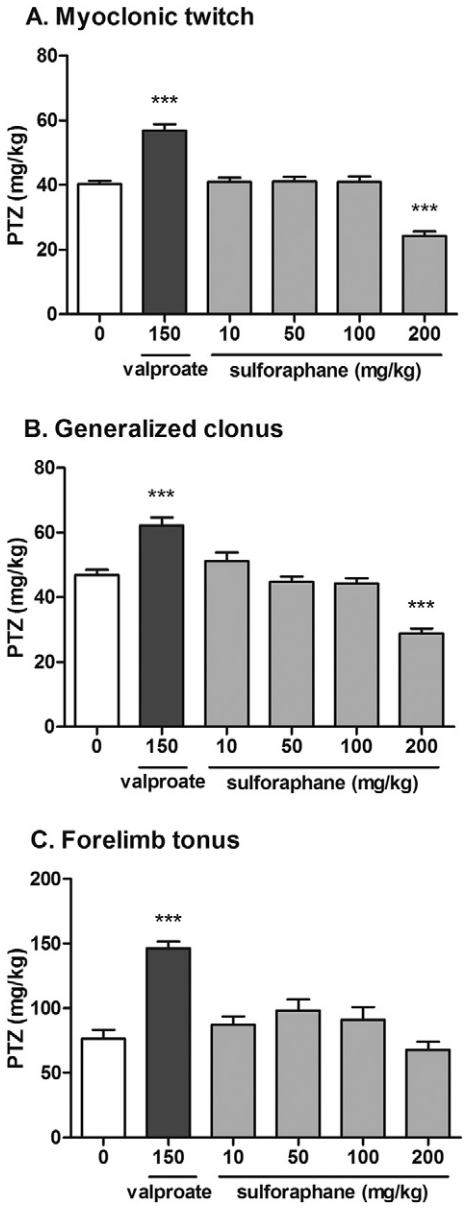

Fig. 1. Effect of sulforaphane on the threshold for the onset of first myoclonic twitch (panel A), generalized clonus (panel B), and forelimb tonus (panel C) in the iv PTZ seizure threshold test in mice. Sulforaphane and valproate (positive control) were administered ip 60 and 15 min before the test, respectively. The doses are shown on the abscissa. Control animals received 1% DMSO. Each experimental group consisted of 12–15 animals. Each bar represents the mean (mg/kg PTZ) + SE. ***$p < 0.001$ vs. the control group (one-way ANOVA followed by Tukey post hoc test).

3.2. Effect of sulforaphane on the seizure threshold in the MEST test

Fig. 2A presents the influence of sulforaphane on the threshold for the tonic hindlimb extension in the MEST test (one-way ANOVA: $F(5,49) = 44.99$, $p < 0.0001$). Sulforaphane at any doses tested (10–200 mg/kg) had no significant effect on the CS_{50} value in the MEST test. In comparison, valproate (positive control) at a dose of 150 mg/kg significantly increased the seizure threshold (Tukey post hoc test: $p < 0.001$ vs. the control group).

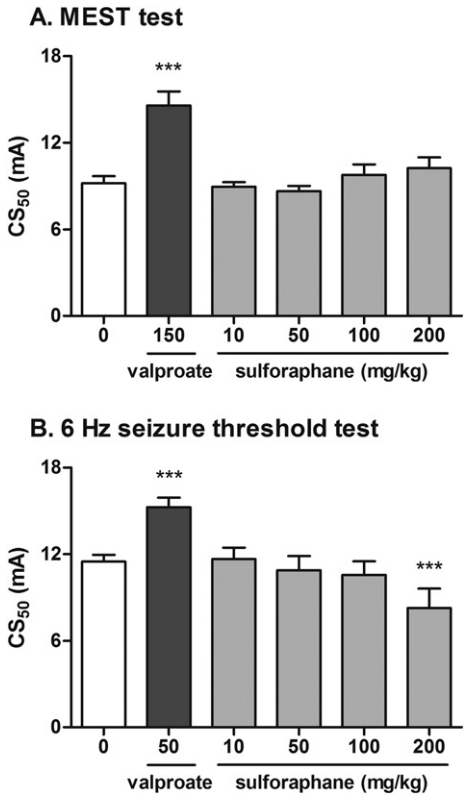

Fig. 2. Effect of sulforaphane on the seizure threshold in the MEST (panel A) and the 6 Hz seizure test (panel B). Sulforaphane and valproate (positive control) were administered ip 60 and 15 min before the test, respectively. The doses are shown on the abscissa. Control animals received 1% DMSO. Each experimental group consisted of 20 animals. Data are presented as CS_{50} (in mA) values with upper 95% confidence limits. Each CS_{50} value represents current intensity predicted to produce convulsions in 50% of mice; ***$p < 0.001$ vs. the control group (one-way ANOVA followed by Tukey post hoc test).

Table 1
Effect of sulforaphane on locomotor activity, neuromuscular strength, motor coordination, and rectal temperature in mice.

Treatment (mg/kg)	Activity counts/10 min	*n*	Neuromuscular strength (mN/g)	*n*	Impairment of motor coordination (%)	*n*	ΔT (°C)	*n*
1% DMSO	2274 ± 227.7	11	25.95 ± 1.19	12	0	12	−0.52 ± 0.18	12
Sulforaphane 150	428.9 ± 94.14***	12	27.23 ± 0.99	12	16.67	12	−4.58 ± 0.47***	12
Sulforaphane 200	394.9 ± 55.66***	11	24.33 ± 1.51	11	58.33*	12	−4.40 ± 0.28***	12
Sulforaphane 250	245.7 ± 36.18***	10	20.37 ± 1.46*	10	80**	10	−4.90 ± 0.46***	10
Sulforaphane 300	204.7 ± 48.76***	7	13.55 ± 1.50***	7	100**	9	−6.60 ± 0.40***	9

Data are presented as means ± SE of activity counts from the locomotor activity test, as grip strengths in millinewtons per gram of mouse body weight (mN/g) from the grip strength test, assessing skeletal muscular strength in mice; as a percentage of animals showing motor coordination impairment in the chimney test, and as the differences between the pre-injection and post-injection values of rectal temperature in mice (ΔT). Activity counts, grip strengths, and changes in rectal temperature were analyzed with one-way ANOVA, while statistical analysis of data from the chimney test was performed with the Fisher's exact probability test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ vs. the control group.

3.3. Effect of sulforaphane on the seizure threshold in the 6 Hz seizure threshold test

The effect of sulforaphane on the threshold for psychomotor seizure is shown in Fig. 2B (one-way ANOVA: $F(5,50) = 20.84$, $p < 0.0001$). The threshold for the 6 Hz-induced psychomotor seizure was not affected by sulforaphane administered at doses of 10, 50 and 100 mg/kg. However, sulforaphane at the highest dose tested (i.e., 200 mg/kg) significantly decreased the seizure threshold for psychomotor seizure (Tukey post hoc test: $p < 0.001$ vs. control group). Positive control (valproate at 50 mg/kg) produced a significant increase in the psychomotor seizure threshold (Tukey post hoc test: $p < 0.001$).

3.4. Acute toxicity of sulforaphane

Signs of severe toxicity including deep sedation, ataxia, ptosis, and tremors were noted several minutes after administration of sulforaphane at a dose of 300 mg/kg. All animals (twelve subjects) from this group died within 180 min from injection. Similar symptoms were seen in mice injected with sulforaphane at a dose of 250 mg/kg. Seven mice out of twelve in this group died within 240 min after injection, one mouse died over the first night and one more over the second night. In animals injected with sulforaphane at a dose of 200 mg/kg, sedation was observed in all subjects. Six mice out of twelve displayed ptosis and slight ataxia. Five mice out of twelve from this group died over the first night. When given sulforaphane at a dose of 150 mg/kg, sedation was still present. Ptosis was observed in two mice from this group (twelve subjects). Sulforaphane at a dose of 150 mg/kg produced no mortality. The LD_{50} dose was calculated to be 212.67 (95% C.L.: 191.56–236.12) mg/kg.

The acute adverse effects of high doses of sulforaphane (60 min after administration) on locomotor activity, neuromuscular strength, motor coordination, and rectal temperature changes are shown in Table 1. Sulforaphane at all doses tested (150–300 mg/kg) produced a marked sedation, as assessed in the spontaneous locomotor activity test (one-way ANOVA: $F(4,46) = 50.74$, $p < 0.0001$). The grip-strength test revealed a significant changes in neuromuscular strength (one-way ANOVA: $F(4,47) = 14.23$, $p < 0.0001$). A statistically significant decrease in neuromuscular strength was observed for sulforaphane injected at doses of 250 and 300 mg/kg (Tukey post hoc test: $p < 0.05$ and $p < 0.001$ vs. the control group, respectively). In the chimney test, no impairment of motor coordination was observed in mice administered with sulforaphane at a dose of 150 mg/kg (Fisher's test: $p > 0.05$). However, higher doses of sulforaphane produced a dose-dependent impairment of motor performance (Fisher's test: $p < 0.05$ at

Table 2
Effects of sulforaphane on the hematological and biochemical blood parameters in mice.

Parameter	Control group	*n*	Sulforaphane 150 mg/kg	*n*	Sulforaphane 200 mg/kg	*n*
RBC (10^{12}/l)	7.817 ± 0.22	11	8.030 ± 0.29	12	7.488 ± 0.44	6
WBC (10^9/l)	5.891 ± 0.60	11	5.037 ± 0.43	12	3.381 ± 0.59*	6
LYM (10^9/l)	3.598 ± 0.32	11	2.989 ± 0.29	12	1.84 ± 0.09**	5
MID (10^9/l)	0.161 ± 0.02	11	0.127 ± 0.03	12	0.124 ± 0.04	6
GRA (10^9/l)	1.838 ± 0.26	10	1.921 ± 0.31	12	1.211 ± 0.45	6
LY%	62.97 ± 3.15	11	60.26 ± 4.62	12	67.44 ± 9.22	6
MI%	2.782 ± 0.41	11	2.542 ± 0.52	12	3.356 ± 0.97	6
GRA%	34.25 ± 3.09	11	37.22 ± 4.75	12	29.19 ± 8.80	6
HCT (%)	33.31 ± 0.95	11	34.29 ± 1.24	12	35.19 ± 2.14	6
HGB (g/l)	116.0 ± 3.71	11	117.3 ± 4.32	12	110.1 ± 4.23	6
MCV (fl)	42.82 ± 0.80	11	42.92 ± 1.17	12	47.08 ± 1.60	6
MCH (pg)	14.89 ± 0.35	11	14.63 ± 0.28	12	14.89 ± 0.70	6
MCHC (g/l)	349.7 ± 8.80	11	342.8 ± 8.17	12	316.7 ± 14.91	6
RDWc (%)	22.79 ± 0.68	11	21.82 ± 0.33	11	22.47 ± 0.90	6
PLT (10^9/l)	412.2 ± 106	7	369.7 ± 56.09	9	210.4 ± 89.35	6
MPV (fl)	5.157 ± 0.16	7	5.120 ± 0.12	10	5.420 ± 0.13	6
PDWc (%)	32.26 ± 1.24	7	32.95 ± 0.75	10	33.18 ± 0.91	6
ALT (U/l)	115.4 ± 9.99	9	144.0 ± 8.75	10	95.10 ± 7.31	5
AST (U/l)	278.2 ± 25.73	10	320 ± 13.27	11	213.1 ± 21.31	6
Creatinine (μmol/l)	32.53 ± 1.23	11	33.77 ± 2.11	12	34.38 ± 3.19	7
Urea (mmol/l)	5.335 ± 0.60	11	5.055 ± 0.34	11	4.327 ± 0.15	7
Bilirubin (μmol/l)	0.947 ± 0.24	9	1.056 ± 0.34	12	2.645 ± 0.64*	7

Abbreviations: RBC, red blood cells; WBC, leukocytes; LYM, lymphocytes; MID, mid-size leukocytes; GRA granulocytes; LY%, percentage of lymphocytes; MI%, percentage of mid-size leukocytes; GRA%, percentage of granulocytes; HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDWc, red blood cell distribution width, PLT, platelets; MPV, mean platelet volume; PDWc, platelet distribution width; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Data are presented as means ± SE. The blood samples were collected 48 h after sulforaphane administration.
* $p < 0.05$.
** $p < 0.01$ vs. the control group (one-way ANOVA followed by Tukey post hoc test).

200 mg/kg, $p < 0.01$ at 250 mg/kg and 300 mg/kg). Based on the results from the chimney test, the TD_{50} dose was estimated at 191.58 (95% C.L.: 158.31–231.85) mg/kg. One hour after sulforaphane administration, a drastic decrease in rectal temperature occurred (one-way ANOVA: $F(4,50) = 37.09$, $p < 0.0001$). At 24 h after sulforaphane treatment, the body temperature normalized to the control level (as compared to the body temperature of the control mice that received 1% DMSO).

3.5. Blood parameters

Effects of high doses of sulforaphane on the hematological and biochemical blood parameters in mice are shown in Table 2. No noteworthy changes in RBC parameters were observed. Sulforaphane did not affect the total RBC count (one-way ANOVA: $F(2,26) = 0.686$, $p > 0.05$), HCT (one-way ANOVA: $F(2,26) = 0.425$, $p > 0.05$), HGB (one-way ANOVA: $F(2,26) = 0.615$, $p > 0.05$), MCV (one-way ANOVA: $F(2,26) = 3.348$, $p > 0.05$), MCH (one-way ANOVA: $F(2,26) = 0.169$, $p > 0.05$), MCHC (one-way ANOVA: $F(2,26) = 2.378$, $p > 0.05$), or RDWc (one-way ANOVA: $F(2,25) = 0.751$, $p > 0.05$). Sulforaphane at a dose of 200 mg/kg significantly reduced the WBC total count (one-way ANOVA: $F(2,26) = 4.299$, $p < 0.05$; Tukey post hoc test: $p < 0.05$ vs. control group) as well as LYM count (one-way ANOVA: $F(2,25) = 5.932$, $p < 0.01$; Tukey post hoc test: $p < 0.01$ vs. control group). However, there were no changes in MID count (one-way ANOVA: $F(2,26) = 0.501$, $p > 0.05$), GRA count (one-way ANOVA: $F(2,25) = 1.120$, $p > 0.05$), LY% (one-way ANOVA: $F(2,26) = 0.416$, $p > 0.05$), MI% (one-way ANOVA: $F(2,26) = 0.416$, $p > 0.05$), or GRA% (one-way ANOVA: $F(2,26) = 0.528$, $p > 0.05$). Although sulforaphane at a dose of 200 mg/kg caused ~50% reduction in PLT count, the statistical analysis showed that the difference was not statistically significant (one-way ANOVA: $F(2,19) = 1.470$, $p > 0.05$). Sulforaphane also did not affect MPV (one-way ANOVA: $F(2,20) = 1.319$, $p > 0.05$) and PDWc parameter (one-way ANOVA: $F(2,20) = 0.221$, $p > 0.05$).

Biochemical studies showed that serum activity of ALT and AST after administration of high doses of sulforaphane did not changed significantly compared to the control group (one-way ANOVA: $F(2,21) = 6.110$, $p < 0.01$ and $F(2,24) = 5.768$, $p < 0.01$, respectively; Tukey post hoc test: $p > 0.05$ vs. control group). There were also no significant changes in creatinine (one-way ANOVA: $F(2,27) = 0.190$, $p > 0.05$) and urea level (one-way ANOVA: $F(2,26) = 1.094$, $p > 0.05$). Sulforaphane at a dose of 200 mg/kg increased plasma bilirubin level as compared to the control group (one-way ANOVA: $F(2,25) = 4.865$, $p < 0.05$; Tukey post hoc test: $p < 0.05$ vs. control group).

3.6. Effect of sulforaphane on the activity of clonazepam in the iv PTZ seizure threshold test

As shown in Fig. 3, clonazepam (0.04 mg/kg) administered alone as well as in combination with sulforaphane (10, 50 and 100 mg/kg) significantly increased the PTZ thresholds for the onset of the first myoclonic twitch (one-way ANOVA: $F(4,58) = 22.61$, $p < 0.0001$, Fig. 3A), for the onset of generalized clonic seizures with loss of righting reflex (one-way ANOVA: $F(4,56) = 14.99$, $p < 0.0001$, Fig. 3B) and for the elicitation of tonic extension of the forelimbs (one-way ANOVA: $F(4,54) = 23.40$, $p < 0.0001$, Fig. 3C). There were no significant differences in seizure thresholds between animals injected with clonazepam alone and clonazepam in combination with sulforaphane.

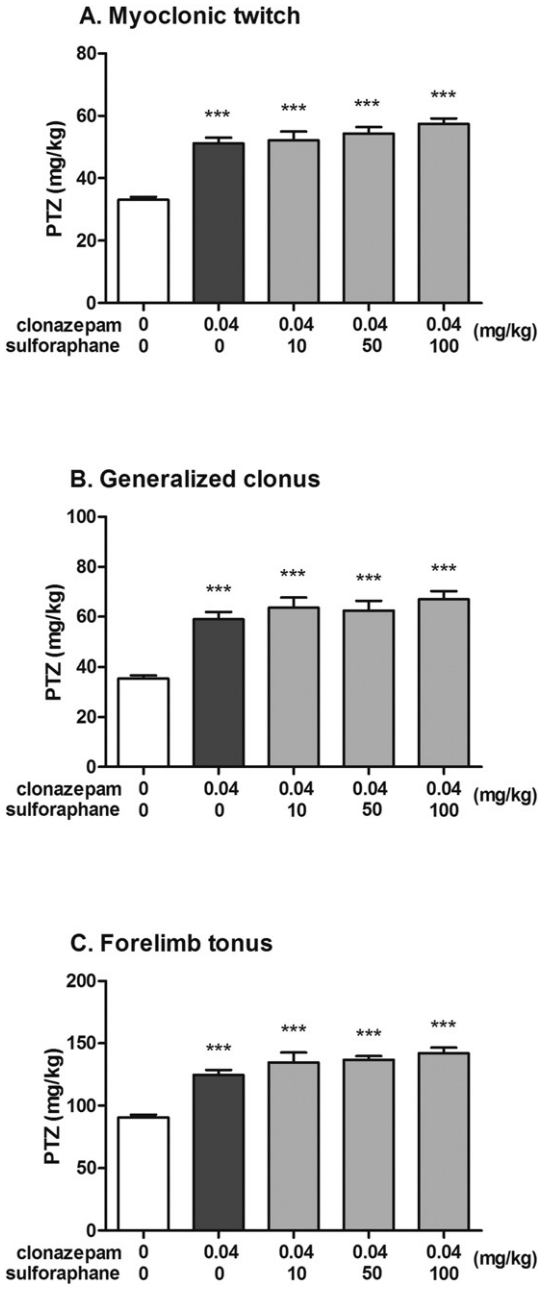

Fig. 3. Effect of clonazepam administered alone and in combination with sulforaphane on the threshold for the onset of first myoclonic twitch (panel A), generalized clonus (panel B), and forelimb tonus (panel C) in the iv PTZ seizure threshold test in mice. Sulforaphane and clonazepam were administered 60 and 30 min before the test, respectively. All the drugs were injected ip. The doses are shown on the abscissa. Control animals received vehicles only. Each experimental group consisted of 10–14 animals. Each bar represents the mean (mg/kg PTZ) + SE. ***$p < 0.001$ vs. the control group (one-way ANOVA followed by Tukey post hoc test).

3.7. Effect of sulforaphane on the activity of carbamazepine and topiramate in the MES test

The influence of sulforaphane on the anticonvulsant potency of carbamazepine and topiramate in the MES test is shown in Fig. 4 (one-way ANOVA: $F(3,86) = 3.093$, $p < 0.05$ for panel A and $F(3,76) = 0.419$, $p > 0.05$ for panel B). Both carbamazepine and topiramate administered alone exhibited anticonvulsant activity against maximal electroshock-induced seizure. Sulforaphane injected at doses of 10 and 50 mg/kg did not affect the anticonvulsant action of carbamazepine. However, sulforaphane administered at a dose of 100 mg/kg increased the ED_{50} value of carbamazepine by ~40% (Tukey post hoc test: $p < 0.05$). In contrast, sulforaphane (10, 50, and 100 mg/kg) had no significant effect on the anticonvulsant action of topiramate in the MES test in mice.

3.8. Effect of sulforaphane on the activity of valproate and tiagabine in the 6 Hz seizure test

The influence of sulforaphane on the anticonvulsant potency of valproate and tiagabine in the 6 Hz seizure test is shown in Fig. 5 (one-

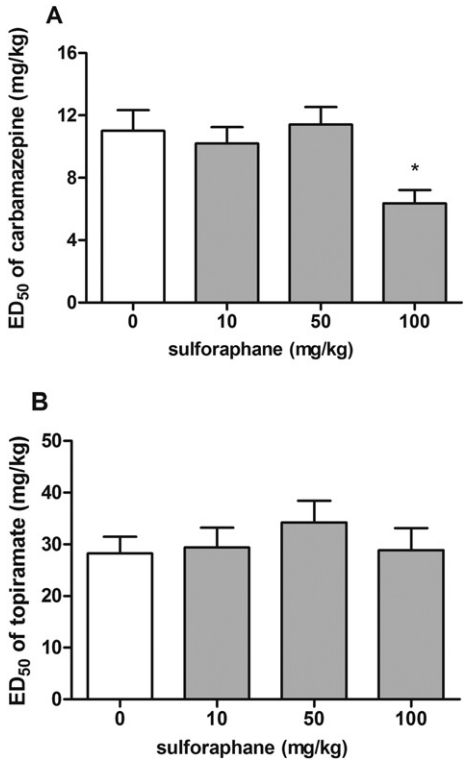

Fig. 4. Effect of sulforaphane on the anticonvulsant potency of carbamazepine (panel A) and topiramate (panel B) in the MES test in mice. Sulforaphane and topiramate were administered 60 min, while carbamazepine 30 min prior to the test. All the drugs were injected ip. Control animals received an antiepileptic drug and 1% DMSO instead of sulforaphane. Each experimental group consisted of 24–32 animals. Each ED_{50} (+SE) value represented a dose of an antiepileptic drug predicted to protect 50% of mice tested against maximal electroshock-induced convulsions in mice. *$p < 0.05$ vs. the control group (one-way ANOVA followed by Tukey post hoc test).

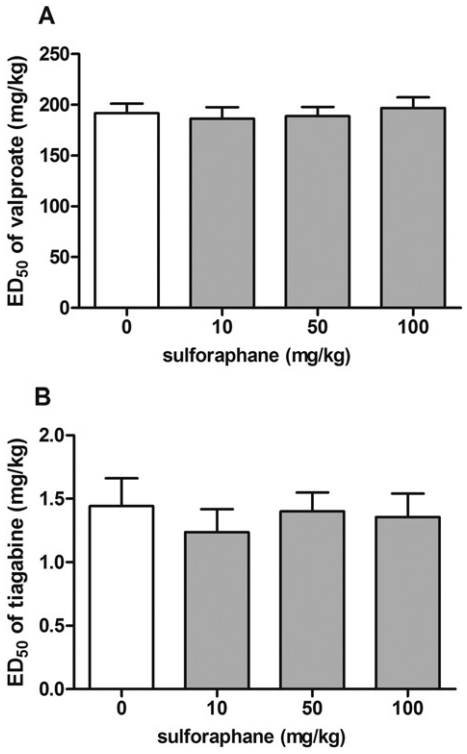

Fig. 5. Effect of sulforaphane on the anticonvulsant potency of valproate (panel A) and tiagabine (panel B) in the 6 Hz seizure test in mice. Sulforaphane was administered 60 min, while valproate and tiagabine 15 min prior to the test. All the drugs were injected ip. Control animals received an antiepileptic drug and 1% DMSO instead of sulforaphane. Each experimental group consisted of 24–32 animals. Each ED_{50} (+SE) value represented a dose of an antiepileptic drug predicted to protect 50% of mice tested against 6 Hz-induced psychomotor seizure in mice.

way ANOVA: $F(3,52) = 0.147$, $p > 0.05$ for panel A and $F(3,52) = 0.228$, $p > 0.05$ for panel B). Valproate and tiagabine administered alone produced anticonvulsant effect in the 6 Hz-induced psychomotor seizure model. Sulforaphane (at doses of 10, 50, and 100 mg/kg) did not affect the anticonvulsant potency of both of the studied antiepileptic drugs.

3.9. Effects of sulforaphane in combination with various antiepileptic drugs on muscular strength, motor coordination, and long-term memory in mice

The influence of sulforaphane in combination with various antiepileptic drugs on skeletal muscle strength is shown in Table 3, column I (one-way ANOVA: $F(2,27) = 0.638$, $p > 0.05$ for group A; $F(4,44) = 1.644$, $p > 0.05$ for group B; $F(4,45) = 2.775$, $p < 0.05$ for group C). Clonazepam, carbamazepine, topiramate, and tiagabine alone as well as in combination with sulforaphane did not affect significantly the neuromuscular strength, as assessed in the grip strength test. Valproate administered alone had no significant effect on the muscle strength, while valproate co-administered with sulforaphane reduced muscle strength in mice as compared to the control group (Tukey post hoc test: $p < 0.05$).

Neither antiepileptic drugs alone nor their combinations with sulforaphane affected significantly motor coordination, as determined in the chimney test (Fisher's test: $p > 0.05$ for all studied groups; Table 3, column II).

Likewise, no alterations in long-term memory in mice treated with antiepileptic drugs or their combinations with sulforaphane were observed, as assessed in the passive avoidance task (Kruskal-Wallis test: $KW = 0.127$, $p > 0.05$ for group A; $KW = 8.045$, $p > 0.05$ for group B; $KW = 3.721$, $p > 0.05$ for group C; Table 3, column III).

3.10. Effect of sulforaphane on carbamazepine concentration

The influence of sulforaphane on serum and total brain carbamazepine concentrations is shown in Table 4. Sulforaphane administered at a dose of 100 mg/kg significantly increased serum carbamazepine concentrations in comparison to animals that received carbamazepine alone (Student's *t*-test: $p < 0.05$). Elevated carbamazepine levels after sulforaphane co-administration were also observed in wet brain tissue (Student's *t*-test: $p < 0.01$).

4. Discussion

Plant-derived medicines are the most commonly used forms of complementary and alternative therapies and plants continue to be a promising source of novel biologically active compounds, including anticonvulsants (Atanasov et al., 2015). Sulforaphane, a naturally occurring potent activator of the Nrf2-ARE signaling pathway, has recently gained much attention as a new therapeutic option in treatment of many central nervous system related diseases.

Although the Nrf2-ARE pathway has been implicated in the pathophysiology of epilepsy (Carmona-Aparicio et al., 2015), relatively little is known about the potential anticonvulsant activity of sulforaphane. This prompted us to investigate the acute effects of sulforaphane on seizure thresholds in three seizure tests commonly used in preclinical study of new antiepileptic drugs. However, the obtained data did not confirm the previous reports demonstrating the anticonvulsant activity of sulforaphane (Carrasco-Pozo et al., 2015; Wang et al., 2014). Sulforaphane administered acutely at doses of 10–100 mg/kg did not raise

Table 3
Effects of antiepileptic drugs and their combinations with sulforaphane on muscular strength in the grip strength test (column I), motor performance in the chimney test (column II), and long-term memory in the passive avoidance task (column III) in mice.

Treatment (mg/kg)	I: Neuromuscular strength (mN/g)	II: Impairment of motor performance (%)	III: Retention time (s)
A.			
Control	28.00 ± 1.43	0	161.1 (166; 180)
Clonazepam (0.04)	28.36 ± 1.51	0	157.3 (152; 180)
Clonazepam (0.04) + sulforaphane (100)	30.20 ± 1.50	0	138.3 (75; 180)
B.			
Control	27.84 ± 0.69	0	141.2 (75; 180)
Carbamazepine (6.37)	26.28 ± 1.12	0	174 (180; 180)
Carbamazepine (6.37) + sulforaphane (100)	29.18 ± 1.81	0	180 (180; 180)
Topiramate (28.85)	27.90 ± 1.40	0	139.7 (85; 180)
Topiramate (28.85) + sulforaphane (100)	30.59 ± 0.74	0	128.5 (45.3; 180)
C.			
Control	30.69 ± 1.27	0	165.5 (180; 180)
Valproate (196.67)	26.95 ± 1.04	40	180 (180; 180)
Valproate (196.67) + sulforaphane (100)	25.64 ± 1.38*	0	161.9 (175.3; 180)
Tiagabine (1.36)	27.12 ± 0.73	0	180 (180; 180)
Tiagabine (1.36) + sulforaphane (100)	26.98 ± 1.16	0	171.5 (180; 180)

Data are presented as mean ± SE grip strengths in millinewtons per gram of mouse body weight (mN/g) from the grip strength test, assessing skeletal muscular strength in mice; as a percentage of animals showing motor coordination impairment in the chimney test, and as median retention time (in s; with 25th and 75th percentiles in parentheses) from the passive avoidance task, assessing long-term memory in mice. Each experimental group consisted of 9–10 animals. The results from the grip strength test were analyzed with one-way ANOVA. Statistical analysis of data from the chimney test was performed with the Fisher's exact probability test. Non-parametric Kruskal-Wallis ANOVA test was used to analyze the results from the passive avoidance task.

* $p < 0.05$ vs. the respective control group.

the seizure thresholds for the PTZ-induced myoclonic, clonic, and tonic seizures in mice. It also failed to increase the threshold for psychomotor seizure in the 6 Hz seizure threshold test in mice and for tonic hindlimb extension in the MEST test. On the contrary, sulforaphane injected at a dose of 200 mg/kg exerted proconvulsant activity. It caused ~40% decline in the seizure thresholds for the onset of the first myoclonic twitch and generalized clonus in the iv PTZ test as well as it lowered the seizure threshold for the 6 Hz-induced psychomotor seizure by ~30%. Sulforaphane administered at a dose of 200 mg/kg did not decrease the seizure thresholds for the onset of tonic forelimb extension and for tonic hindlimb extension in the iv PTZ-induced seizure model and the MEST test, respectively.

Wang et al. (2014) demonstrated that repeated (5 mg/kg for 15 days) injection of sulforaphane before electrical stimulation suppressed the progression of amygdala kindling in rats, while Carrasco-Pozo et al. (2015) showed anticonvulsant action of sulforaphane (5 mg/kg for 5 days) in three seizure tests in mice. In the present study, sulforaphane given acutely did not exert any protective effect against both chemically- and electrically-induced seizures in mice. This suggests that prolonged sulforaphane treatment produced some molecular and/or biochemical changes that resulted in antiepileptic effects. Wang et al. (2014) concluded that activation of Nrf2-ARE pathway by prolonged sulforaphane administration led to the up-regulation of certain cytoprotective enzymes at protein and gene level, which in turn protected the neurons from oxidative and excitotoxic insults and suppressed seizure progression. Thus, it seems that antiepileptic action of sulforaphane may be related to its neuroprotective properties and this effect may become apparent after repeated rather than chronic treatment. On the other hand, sulforaphane injected for 5 days did not protect against status epilepticus-induced hippocampal neuronal death in pilocarpine-induced seizure model in mice (Carrasco-Pozo et al., 2015). Although repeated sulforaphane administration increased the respiratory capacity of mitochondria isolated from the hippocampus and produced antioxidant effects, it was not sufficient to protect neurons from death evoked by status epilepticus (Carrasco-Pozo et al., 2015). In addition, there was a trend towards increased neuronal damage in the hippocampal CA1 area after sulforaphane treatment, which may be warning against using high amounts of sulforaphane in patients with epilepsy (Carrasco-Pozo et al., 2015). In our study, it was not only that sulforaphane injected acutely was devoid of anticonvulsant activity, but also that at high doses it reduced the seizure thresholds, which means that high doses of sulforaphane may potentially increase the risk of seizure in humans. Of note, two participants of clinical trial assessing the efficacy of sulforaphane in autism spectrum disorder had single unprovoked seizures. Despite the fact that patients with autism are predisposed to seizure, it cannot be excluded that seizures were a side effect of treating autistic patient with sulforaphane (Singh et al., 2014).

Table 4
Effect of sulforaphane on serum and brain concentrations of carbamazepine.

Treatment (mg/kg)	Serum (μg/ml)	Brain (μg/g)
carbamazepine (6.37)	1.954 ± 0.15	2.942 ± 0.31
carbamazepine (6.37) + sulforaphane (100)	2.432 ± 0.10*	3.982 ± 0.18**

Sulforaphane and carbamazepine were administered ip 60 and 30 min before decapitation, respectively. Data are presented as means ± SE. Each experimental group consisted of 9–10 animals.

* $p < 0.05$.

** $p < 0.01$ vs. carbamazepine-treated group (Student's *t*-test).

Sulforaphane is considered to be a dietary phytochemical with low toxicity (Brown et al., 2015; Singh et al., 2014; Wu et al., 2016). Depending on the study, the dosage regimen for sulforaphane varies widely, reaching up to 100 mg per kg of body weight (Poulton et al., 2013; Jakubikova et al., 2011; Wang and Wang, 2017). However, the toxicity of sulforaphane has not been thoroughly studied. Neither the TD_{50} nor the LD_{50} of sulforaphane in animals were estimated. Therefore, it was of great interest to study the toxic effects of sulforaphane. The obtained results showed that sulforaphane at high doses produced marked (over 80%) sedation, decreased muscle strength, and impaired motor performance. Moreover, sulforaphane administration resulted in severe hypothermia, which is a typical response in rodents exposed to toxic insults. This hypothermic/hypometabolic state can be protective and reduce the lethality of rodents caused by toxic insult or hypoxia (Gordon, 2001). Data from hematological studies did not reveal any significant changes in blood parameters apart from slight leucopenia. Furthermore, no impairment of renal and hepatic function was observed since there were no significant changes in creatinine and urea level as well as liver enzymes activity. Sulforaphane at 200 mg/kg did increase plasma bilirubin concentration but the observed change remained within normal ranges for mouse, which also did not indicate impaired liver function. Based on the results from toxicity studies the TD_{50} (in the chimney test) and LD_{50} values were estimated at 191.58 mg/kg and 212.67 mg/kg, respectively.

Patients with epilepsy often use dietary supplements along with antiepileptic drugs. Many natural remedies and dietary supplements may potentially interact with antiepileptic medications (Kaiboriboon et al., 2009). There is no data on the influence of sulforaphane on the efficacy of antiepileptic drugs. For this reason, we investigated the effect of sulforaphane (at 10, 50 and 100 mg/kg) on the anticonvulsant activity of several first and second generation antiepileptic drugs. The obtained results showed that sulforaphane did not alter the anticonvulsive activity of clonazepam (against the iv PTZ-induced seizures), topiramate (against the maximal electroshock-induced seizures) as well as valproate and tiagabine (against the 6 Hz-induced psychomotor seizures). Interestingly, sulforaphane at a dose of 100 mg/kg potentiated the anticonvulsant efficacy of carbamazepine in the MES test by decreasing its ED_{50} value by ~40%. The interaction between sulforaphane and carbamazepine may have either pharmacodynamic or pharmacokinetic basis. To evaluate the potential pharmacokinetic interaction between these two compounds, brain and serum concentrations of carbamazepine were determined using HPLC method. Sulforaphane co-administration with carbamazepine caused a significant increase in carbamazepine concentrations both in serum and brain tissue. This suggests that interaction between these two compounds was pharmacokinetic in nature. The mechanism underlying carbamazepine-sulforaphane interaction could have been mediated via cytochrome P450 isoenzymes. In humans, carbamazepine is primarily metabolized by CYP3A4 isoenzyme. Drugs that inhibit CYP3A4 activity thereby suppress carbamazepine metabolism and frequently lead to its accumulation and signs of toxicity. Sulforaphane was shown to markedly decrease the expression of CYP3A4, at both mRNA and activity levels, in human hepatocytes (Maheo et al., 1997; Zhou et al., 2007). Thus, it seems plausible that sulforaphane could affect the activity of murine cytochrome P450 isoenzymes involved in carbamazepine metabolism. Further studies are required to fully evaluate the safety of concomitant administration of carbamazepine and sulforaphane.

No acute adverse effects were observed in animals that received the combinations of sulforaphane with most of the studied antiepileptic drugs and subjected to the chimney test, grip-strength test, and passive avoidance task. Combination with valproate was an exception here. When injected alone, valproate did not affect the neuromuscular strength in mice, whereas combination of valproate with sulforaphane slightly weakened muscle strength in the grip strength test. This suggests that joint use of valproate and sulforaphane may produce some adverse effects, which deserves further investigation.

5. Conclusions

Among many forms of alternative and complementary therapies, natural remedies are the most popular. Their usage by patients suffering from epilepsy and taking antiepileptic drugs is widespread. Although herbs and plant-derived dietary supplements are generally consider to be safe and well-tolerated, some of them can potentially increase the risk of seizures and/or alter the response to antiepileptic drugs treatment (Haller et al., 2005; Samuels et al., 2008). In the present study, we demonstrated for the first time that a widely used phytochemical – sulforaphane was proconvulsant at a toxic dose. It also produced a pharmacokinetic interaction with carbamazepine. Moreover, the TD_{50} and LD_{50} values of sulforaphane in mice were estimated, which may be useful in selecting non-toxic dose levels for further in vivo studies.

Thus, it is possible that sulforaphane may increase seizure susceptibility and affect carbamazepine metabolism in humans. The doses of sulforaphane used in the present study were too high to achieve by a diet but when taken as a dietary supplement in excessive dose sulforaphane may have negative effects. Nevertheless, caution should be taken when extrapolating our results to humans as the response to high doses of sulforaphane and its influence on drugs metabolism may vary between mice and humans. Further detailed studies are needed to estimate the risk/benefit ratio of sulforaphane usage in epileptic patients.

Conflict of interest

None.

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