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ANTICONVULSANT ACTIVITY OF NEWLY SYNTHESIZED 2*H*-CHROMENE BASED HYDRAZONES IN ICR MICE

Violina T. Angelova, Pavlina A. Andreeva-Gateva*, Nikolay G. Vassilev, Radka Tafradjiiska-Hadjiolova***, Slavina Surcheva*, Jana Tchekalarova*****

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Abstract

Two 2*H*-chromene based hydrazones, structurally related to the previously described potent hydrazone analogues, were synthesized and evaluated for their anticonvulsant activity and neurotoxicity. Initial anticonvulsant screening was performed using maximal electroshock induced seizure tests (MES) and subcutaneous pentylenetetrazol (scPTZ) in ICR mice. Although not so efficient like the standard drug phenytoin, the tested compounds, at a dose of 300 mg/kg and 30 mg/kg, respectively, showed 50% protection and tendency to alleviate the mortality in MES test. Unlike diazepam (2.5 mg/kg), at tested doses 2H-chromene based hydrazones were unable to exhibit 100% suppression of clonic seizures in scPTZ test. However, the two compounds increased dose-dependently the latency to onset of clonic seizures and showed a tendency to alleviate their incidence. Incidence of tonic hind limb extension and mortality were significantly suppressed with the highest doses of hydrazones. Motor impairment, evaluated with the rotarod test, was minimal at highest doses tested. Further studies need to be carried out on other seizure tests and models of epilepsy to ascertain the precise mechanism of action of these molecules.

Key words: anticonvulsant activity, 2*H*-Chromene, hydrazide/hydrazone, PTZ test, MES test, rotarod, mice

Introduction. Epilepsy is a brain disorder characterized by a persistent predisposition for generation of epileptic seizures. It is related with neurobiological, cognitive, psychological and social consequences [1]. Epileptic seizures are episodes of sudden qualitative and quantitative disturbances of consciousness, and of sensor, motor and autonomic function. They reflect hypersynchronized discharges of cortical neurons and clinical presentations are in dependence of

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their cortical localization and spread. Epilepsy is the second most prevalent neurological disorder (after stroke) in the industrial world [2].

By tradition, pharmacological strategies for treatment of epilepsy target suppression of generation and propagation of pathological neuronal activity, rather than causative epileptogenic process [3]. Targets for antiepileptic drugs in the synapses are mechanisms enhancing the inhibitory GABA mediation or suppressing the excitatory glutamate mediation, voltage-gated sodium or calcium channels and interference with intracellular transduction pathways [4].

The "rational" development of new antiepileptic drugs started in the 1980s targeting GABA-mediation and until 2015 more than 30 antiepileptic drugs were registered [5]. Despite the high number of antiepileptic medications, however, still 20–40% of patients are with drug-resistant epilepsy [6].

The contemporary screening of new antiepileptic drugs is focused on research of promising compounds coping with refractory epilepsy, epileptogenesis, disease progression and comorbidity. At least three strategies are used to this purpose [7]: (1) screening of newly synthetized substances with different structure and unknown mechanism of action; (2) structure variations of well-known antiepileptic drugs; (3) rational drug design with development of drugs that are selectively synthesized for acting on target epileptogenic mechanism.

Growing data were published about the biological activity of substances containing N-N bounding, i.e. hydrazones (-CH=N-NH-), hydrazides (-CO-NH-N=), semicarbazones (-CH=N-NH-CO-N=), semicarbazides (=N-NH-CO-NH_2), etc. [8]. Pharmacological screening revealed their potential antimicrobial, analgesic, anti-inflammatory, antitumor and anticonvulsant activities. 2*H*-chromene is a heterocycle which appears as an important structural component in natural and synthetic compounds and possesses biological activities, including potential as anticonvulsant [9–11]. Inspired by this fact it was planned to synthesize 2*H*-chromene derivatives bearing hydrazide/hydrazone motif as potential anticonvulsants.

Our aim was to test for a potential anticonvulsant activity newly synthetized 2*H*-chromene based hydrazones.

Methods. ***Chemistry.*** The melting points were determined using a Buchi 535 apparatus. The FTIR spectra were recorded on a Nicolet IS10 FT-IR Spectrometer from Thermo Scientific (USA) using ATR technique. All NMR experiments were carried out on a Bruker Avance spectrometer II+600 MHz at 20 °C in DMSO-d_6 as a solvent, using tetramethylsilane (TMS) as an internal standard. The precise assignment of the ^{1}H and ^{13}C NMR spectra was accomplished by measurement of 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C–H) correlations (HMQC and HMBC). Mass spectra were measured on a LTQ Orbitrap Discovery® spectrometer (ThermoFisher, Germany) equipped with electrospray ionization module Ion Max® (Thermo Scientific Co, USA) operating in positive mode. All chemicals and solvents were either purchased puriss p.a. from commercial suppliers or purified by standard

techniques. Preparation of 2*H*-chromene-3-carbaldehyde **1** was described elsewhere [12]. Yellow solid, yield: 62%, m.p.: 42–43.5 °C, lit. m.p. [13] 42–44 °C, lit. m.p. [14] 69–71 °C. HRMS (ESI); Calcd for $C_{10}H_8O_2[M + H]^+$161.0603. Found: 161.05990.

General synthetic procedure for compounds **3 a, b**. To a stirred solution of 2*H*-chromene-3-carbaldehyde (2.0 mmol) **1** in 10–15 ml anhydr. ethanol appropriate hydrazides 4-chlorobenzohydrazide **2 a** and furan-2-carbohydrazide **2 b** (2.0 mmol) was added. The reaction mixture was vigorously stirred for 15 min at room temperature. The resulting pale yellow precipitate was filtered and recrystallized from EtOH. (Table 1).

Pharmacology. Animals. The experiments were carried out on male ICR mice (20–25 g) obtained from the breeding house of the Institute of Neurobiology, Bulgarian Academy of Sciences. The mice were housed in standardized conditions (12 h/12 h light/dark cycle, lights on at 07:00 h, temperature 20–23 °C, 50% relative humidity) with free access to food (standard laboratory chow) and water. They were habituated in the animal facilities for at least one week. The experiments were performed between 10.00 a.m. and 01.00 p.m. The procedures used in this study were in agreement with the European Communities Council Directive 2010/63/EU. The experimental procedures were approved by the Local Ethics Committee of Institute of Neurobiology, BAS.

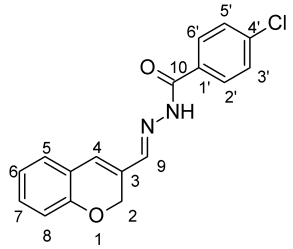
We followed guidelines of the National Institute of Neurological Disorders and Stroke (NINDS) in USA, incorporated in the Anticonvulsant screening program (ASP), available at: `https://panache.ninds.nih.gov`. This program aims to encourage and facilitate the discovery of new therapeutic agents for epilepsy. Since its establishment in 1975 till May 2015, ASP was used for screening of approximately 30 000 compounds, bringing 10 antiseizure drugs to market since 1990 [15]. According to this program, standard anti-ictal screening is performed at the beginning with qualitative screens following doses of 30, 100 and 300 mg/kg of test compound, using maximal electroshock seizure test (MES), pentylenetetrazol test (PTZ) and acute toxicity-assessment of motor impairment.

Anticonvulsant testing. Maximal electroshock seizure test (MES). Animals ($n = 8$) were injected with vehicle (control) or compounds **3 a, b** (30, 60, 100 and 300 mg/kg), dissolved in 10% DMSO and applied at a volume of 10 ml/kg. Phenytoin (12 mg/kg) was used as a standard drug. Corneal electrodes were applied followed by an electric stimulus of 50 mA, 60 Hz delivered for 0.2 s (Constant Current Shock Generator) 30 min later. Results are expressed as the percentage of mice protected from tonic hind limb extension (THLE) and incidence of mortality.

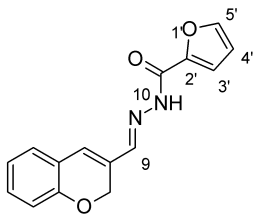
Pentylenetetrazol test (PTZ). PTZ (75 mg/kg, s.c.) was dissolved in 0.9% NaCl. Tested compounds **3 a, b** (30, 60, 100 and 300 mg/kg) and diazepam (DZP) as a standard drug were administered 30 min before PTZ. Each mouse was placed into a separate transparent cage and seizure stage was evaluated for 30

Table 1

Physical properties, FTIR (ATR), HRMS, ^{1}H-NMR and ^{13}C-NMR spectral data of **3 a** and **3 b**



(3 a)



(3 b)

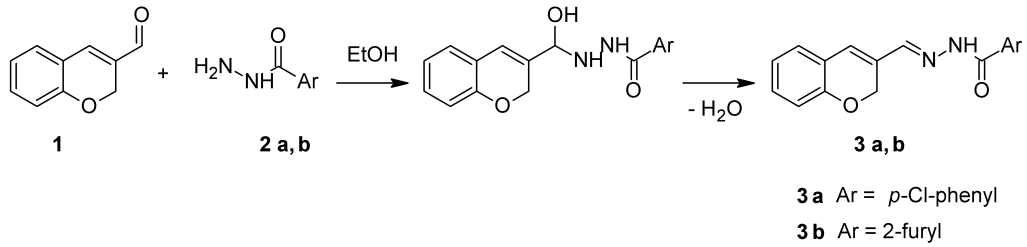
Compd	Name	Yield (%)	M.p (°C)	FTIR ν_{max} (cm^{-1})	HRMS (ESI) m/z	^{1}H-NMR (DMSO-d_6) (δ, ppm)	^{13}C-NMR (DMSO-d_6) (δ, ppm)
3 a	*4-Chloro-N'-[2H-chromen-3-ylmethylidene] benzohydrazide or 2H-chromen-3-carbaldehyde 4-chlorobenzoyl-hydrazone*	0.424 g (68%), yellow crystals	190–192	3378, 3195, 3031, 1653, 1623.	Calcd: $[M^+ + H]$ 313.07383 Found: $[M^+ + H]$ 313.07384	5.055 (s, 2H, H-2), 6.835 (d, J=8.3 Hz, 1H, H-8), 6.929 (dt, J=0.9, 7.4 Hz, 1H, H-6), 6.955 (s, 1H,H-4), 7.18 – 7.21 (m, 2H, H-7 and H-5), 7.569 (d, J=8.5 Hz, 2H, H-3' and H-5'), 7.857 (d, J=8.5 Hz, 2H, H-2' and H-6'), 8.110 (s, 1H, H-9), 11.970 (s, 1H, NH)	64.35 (C-2), 116.23 (C-8), 122.29 (C-4a), 122.50 (C-6), 128.50 (C-5), 129.30 (C-2' and C-6'), 129.50 (C-3), 129.89 (C-4), 130.10 (C-3' and C-5'), 131.31 (C-7), 132.23 (C-1'),137.47 (C-4'), 147.70 (C-9), 154.63 (C-8a), 163.26 (C-10)
3 b	*N'-[2H-Chromen-3-ylmethylidene] furan-2-carbohydrazide or 2H-chromen-3-carbaldehyde 2-furylhydrazone*	0.386 g (72%), yellow crystals	204–205	3246, 3030, 2850, 1652, 1632, 1602, 1576, 1538.	Calcd: $[M^+ + H]$ 269.09206 Found: $[M^+ + H]$ 269.09198	δ = 5.034(s, 2H, H-2), 6.670 (dd, J = 1.7, 3.5 Hz, 1H, H-4'); 6.826 (d, J = 8.3 Hz, 1H, H-8), 6.922 (dt, J = 1.0, 7.4 Hz, 1H, H-6), 6.929 (s, 1H, H-4), 7.184 (dt, J = 1.6, 8.3 Hz, 1H, H-7), 7.192 (d, J = 7.4 Hz, 1H, H-5), 7.257 (d, J = 3.0 Hz, 1H, H-3'), 7.856 (bs, 1H, H-5'), 8.110 (s, 1H, H-9), 11.959 (s, 1H, NH)	64.33 (C-2), 112.88 (C-4'), 116.11 (C-3'), 116.21 (C-8), 122.29 (C-4a), 122.44 (C-6), 128.47 (C-7), 129.50 (C-3), 129.74 (C-4), 131.26 (C-5), 146.62 (C-2'), 146.66 (C-9), 146.62 (C-5'), 154.61 (C-8a), 155.11 (C-10)

min. The seizure scoring scale was according to MEDINA et al.[16]. The following parameters were evaluated: the latency to onset of clonic seizures and THLE, the incidence of clonic seizures, THLE and mortality.

Rotorod test. In mice, the rotorod procedure is used to identify minimal muscular or neurological impairment [17]. The motor coordination was tested in mice using rotorod procedure. Inability of a treated mouse to maintain equilibrium for at least 1 min in three consecutive trials on a 25 mm diameter slowly rotating rod (6 rpm) was used as the endpoint indicating motor impairment.

Statistical analysis. Results were expressed as means±S.E.M. and analyzed with one-way ANOVA and the t-Student-Neuman-Keuls test as post hoc test. All analyses were performed using SigmaStat®. The incidence of seizures and mortality was evaluated by Fisher's exact test. The level of statistical significance was set at 5%.

Results and discussion. ***Chemistry.*** Our aim was to obtain the new 2*H*-chromen-3-carbaldehyde 4-chlorobenzoylhydrazone **3 a** and 2*H*-chromen-3-carbaldehyde 2-furylhydrazone **3 b** by simple and efficient classic reaction. The synthetic procedure (Scheme 1) involved a condensation reaction of equimolecular amount of 2*H*-chromene-3-carbaldehyde **1** with 4-chlorobenzoylhydrazide **2 a** and 2-furylhydrazone **2 b** at room temperature under vigorous stirring. The progress of the reaction was monitored by TLC. The products were obtained in good yields and excellent purity.



Scheme 1. Synthetic route for 2*H*-chromene containing hydrazone derivatives **3 a, b**

The synthesized 2*H*-chromene-3-carbaldehyde **1** and products **3 a, b** were characterized by FTIR, ^{1}H NMR and ^{13}C NMR, 2D (^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and HMBC) and mass spectral techniques. The spectral data and the physical properties for structure determination of test compounds are given in Table 1.

Pharmacology. The anticonvulsant activity and neurotoxicity of the newly synthetized 2*H*-chromene based hydrazones **3 a, b** were evaluated after intraperitoneal administration in MES and scPTZ test. The results were summarized along with data for standard drugs phenytoin and diazepam, respectively, in Table 2 and Table 3. Both compounds **3 a, 3 b** dose-dependently alleviated the onset of THLE and the incidence of mortality in MES test but they did not demon-

strate strong anticonvulsant activity in the tested dose range compared with the standard drug phenytoin (Table 2). The compound **3 b** showed more promising results in MES test with 50% suppression of THLE produced by the lowest dose of 30 mg/kg while similar activity was achieved by compound **3 a** with the highest dose of 300 mg/kg.

T a b l e 2

Effects of compound **3 a, b** on MES-induced seizures and mortality in ICR mice. Compounds **3 a, b** and the standard drug phenytoin were administered 30 min before the MES test. The data (mean±SEM, $n = 8 - 10$) were analyzed by Fisher's exact probability test. $^{*}p < 0.05$ vs control group

Treatment (mg/kg)	Incidence of THLE (%)	Mortality (%)
Vehicle	100	73
Compound 3 a		
30	67	33
60	60	10
100	90	50
300	50	11
Compound 3 b	50	12
30	67	33
60	89	67
100	67	13
300		
Phenytoin	13^{*}	0^{*}
25		

Although not as effective as diazepam (2.5 mg/kg), the compound **3 a** exhibited higher activity compared to compound **3 b** in the scPTZ test. However, both compounds were unable to exhibit 100% suppression of clonic seizures at tested doses. One-way ANOVA demonstrated a significant dose-dependent effect for the latency for onset of PTZ-induced clonic seizures in mice treated with the compound **3 a** [$H = 8.744$, $p = 0.033$]. Post hoc test showed that a dose of 60 mg/kg significantly decreased the latency for clonic seizures while the highest dose of 300 mg/kg showed a tendency to 38% in PTZ test (Table 3). Furthermore, doses of 100 and 300 mg/kg significantly decreased the incidence of THLE and mortality of mice tested with the compound **3 a** (Fisher exact test: $p < 0.05$).

Analyses of variance showed a main dose effect for the latency for onset of clonic seizures in mice treated with compound **3 b** [$H = 13.689$, $p = 0.008$]. Post hoc test confirmed that highest dose of 300 mg/kg decreased the latency for clonic seizures and showed a tendency to attenuate their incidence to 50% (Table 3). For the incidence of THLE, a significant alleviation was shown for a dose of 300 mg/kg while for the incidence of mortality the significant effect was detected for doses of 100 and 300 mg/kg, respectively, (Fisher exact test: $p < 0.05$).

Table 3

Effects of compound **3 a, b** on scPTZ-induced seizures and mortality in ICR mice. Compounds **3 a, b** and the standard drug diazepam were administered 30 min before PTZ. The data (mean±SEM, $n = 8 - 10$) were analyzed by one-way ANOVA. $^{*}p < 0.05$ vs control group

Treatment (mg/kg)	Latency to clonus (sec)	Latency to HLTE (sec)	Incidence of clonic seizure (%)	Incidence of HLTE (%)	Mortality (%)
Vehicle	122.2 ± 28.5	439.3 ±148.8	100	80	80
Compound 3 a					
30	138.8±26.5	388.8±139.8	100	75	75
60	388.8±139.8*	×	85	29	0*
100	69.9± 14.1	×	75	0*	0*
300	×	×	38	13*	13*
Compound 3 b					
30	132.8± 10.6	371.6+139.3	100	89	89
60	287.7±141.3	×	75	25	25
100	323.3±104.7	×	75	38	13*
300	399.0± 64.0*	×	50	6*	0*
DZP					
2.5	×	×	0*	0*	0*

Like the two standard drugs, the compounds **3 a, b** did not demonstrate obvious neurotoxic effects at the tested doses while the highest dose of **3 a** showed a minor impairment of 13% at the highest dose of 300 mg/kg in the rotarod test.

Two 2*H*-chromene based hydrazone structurally related to the previously described potent hydrazone analogues, were designed, synthesized, and their anticonvulsant activity and neurotoxicity were evaluated after intraperitoneal administration in two routine seizure tests – the MES and scPTZ. Our results showed that that these compounds represent a promising candidate for further investigation. The compound **3 b** showed higher protection against MES test, indicative of its ability to inhibit the seizure spread. The compound **3 a** exhibited higher activity in scPTZ test, used to identify compounds that elevate seizure threshold. Both compounds showed minor neurotoxicity in the highest administered dose of 300 mg/kg. Further studies need to be carried out on other seizure tests and models of epilepsy to ascertain the precise mechanism of action of these molecules.

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Department of Chemistry, Faculty of Pharmacy, MU-Sofia
15 Acad. Ivan Geshov Blvd, 1431 Sofia, Sofia, Bulgaria
[*]*Department of Pharmacology, Medical Faculty, MU-Sofia*
15 Acad. Ivan Geshov Blvd, 1431 Sofia, Bulgaria
[**]*Laboratory of Nuclear Magnetic Resonance, Institute of Organic Chemistry with Centre of Phytochemistry, BAS, Acad. G. Bonchev St, Bl. 9, 1113 Sofia, Bulgaria*
[***]*Department of Pathophysiology, Medical Faculty, MU-Sofia*
15 Acad. Ivan Geshov Blvd, 1431 Sofia, Bulgaria
[****]*Institute of Neurobiology, BAS, Acad. G. Bonchev St, Bl. 23, 1113 Sofia, Bulgaria,*
e-mail: `janetchekalarova@gmail.com`