Article

Antioxidant Activity of a Novel Acetylcholinesterase Inhibitor: *In Vivo* and *Ex vivo* Studies

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Abstract: The acetylcholinesterase (AChE) inhibitors are the main drugs for symptomatic treatment of neurodegenerative disorders like Alzheimer's disease. A recently designed, synthesized and tested hybrid compound between the AChE inhibitor galantamine (GAL) and the antioxidant polyphenol curcumin (CU) showed high AChE inhibition *in vitro*. Here, we describe tests for acute and short-term toxicity in mice as well as antioxidant tests on brain homogenates measured the levels of malondialdehyde (MDA) and glutathione (GSH). Haematological and serum biochemical analyses were also performed. In the acute toxicity tests, the novel AChE inhibitor given orally in mice showed LD50 of 49 mg/kg. The short-term administration of 2.5 and 5 mg/kg did not show toxicity. In the *ex vivo* tests, the GAL-CU hybrid performed better than GAL and CU themselves. In a dose of 5 mg/kg, it demonstrates 25% reduction in AChE activity, 28% and 73% increase in the levels of MDA and GSH, respectively. No significant changes in blood biochemical data were observed. The GAL-CU hybrid is a novel non-toxic AChE inhibitor with high antioxidant activity which makes it a perspective multitarget drug candidate for treatment of Alzheimer's disease.

Keywords: galantamine; curcumin; in vivo AChE inhibition; acute toxicity in mice; antioxidant activity; malondialdehyde levels; glutathione levels; brain homogenate; complete blood count; biochemical serum parameters

1. Introduction

The neurodegenerative disorders like Alzheimer's and Parkinson diseases rise exponentially every ten years and during the next few decades will overtake cancer and become the second leading cause of death after cardiovascular diseases [1]. The successful treatment of these disorders requires reliable tests and biomarkers for early diagnosis, identification of translational drug targets and fast discovery and development of multitarget drugs and therapeutic strategies to tackle the complex pathogenesis of the neurodegeneration.

In the last 20 years, the main drugs used to cope with some of the symptoms of Alzheimer's disease (AD) are inhibitors of the enzyme acetylcholinesterase (AChE). The reversible AChE blockage increases the levels of acetylcholine (ACh) – the main neurotransmitter associated with the cognitive and motor brain functions. Galantamine (GAL) (Figure 1) is among the few AChE inhibitors approved for treatment of AD [2]. Additionally, GAL is an allosteric modulator of nicotinic and muscarinic ACh receptors [3,4], increases the phagocytosis of amyloid β (Aβ) peptide in rat microglia [5], exhibits a moderate scavenging effect in antioxidant studies [6].

The oxidative stress hypothesis in AD dates back to the late 1990s [7,8]. According to this hypothesis, the mammals' brain is very sensitive to oxidative stress because of the abundant presence of polyunsaturated fatty acids and transition metals like iron, copper

and zinc [9] and the relative shortage of antioxidant ability to detoxify the free radicals [10]. Most of the clinical trials report associations between antioxidant use and better cognitive functions [11-15]. Curcumin (CU) (Figure 1) is a natural polyphenol with a powerful antioxidant activity [16] and ability to reduce oxidative stress and amyloid pathology in transgenic mice [17]. Additionally, it was found that CU binds to Aβ oligomers and fibrils and inhibits the β-sheet formation [18]. We simulated the primary nucleation of Aβ peptide by molecular dynamics and showed that CU molecules inhibit the process by intercalating among the Aβ chains [19]. Even more, CU is able to disintegrate preformed Aβ fibrils [18], reduce insoluble Aβ deposits [20] and amyloid plaques [21].

Recently, we designed a combinatorial library of GAL-CU hybrids, screened for optimal ADME properties and blood-brain permeability and docked on AChE [19]. The 14 best performing hybrids were synthesized and tested for neurotoxicity and AChE inhibition *in vitro*. Five of them showed less toxicity than GAL and CU and AChE inhibition between 41 and 186 times higher than GAL. Here, we describe the *in vivo* evaluation of acute and short-term toxicity and the *ex vivo* antioxidant properties of the best performing inhibitor – compound **4b**. As the present study is a continuation of our previous research [19] and will be followed by other studies on this particular compound, we prefer to keep the same compound ID. As positive controls in the study are used GAL and CU.
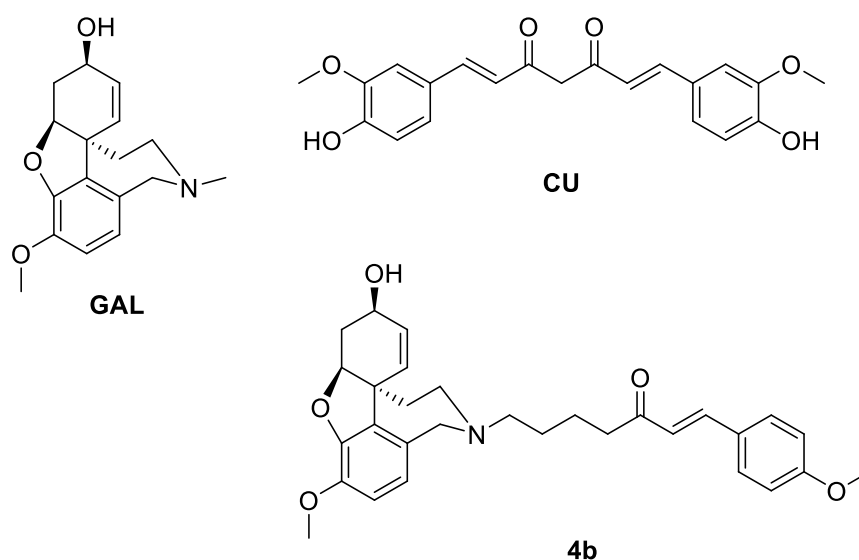

Figure 1. Structures of Galantamine (GAL), Curcumin (CU) and their hybrid **4b**.

2. Materials and Methods

2.1. Materials

In the present study were used the following materials: galantamine HBr (Galen-N Ltd., Sofia, Bulgaria, Mw = 368.3 g/mol, purity > 98%), curcumin (BioXtract, Belgium, Mw = 368.4 g/mol, purity > 98%), bovine serum albumin (Sigma Aldrich, Germany, Mw ≈ 66 kD, purity > 96%), thiobarbituric acid (Sigma Aldrich, Germany, Mw = 144.15 g/mol, purity > 98%), trichloroacetic acid (Sigma Aldrich, Germany, Mw = 163.39 g/mol, purity > 99%), acetylthiocholineiodide (Sigma Aldrich, Germany, Mw = 289.18, purity > 98%), 2,2-Dinitro-5,5 dithiodibenzoic acid (DTNB) (Sigma Aldrich, Germany, Mw = 396.35, purity > 98%), Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Germany, Mw = 292.24, purity > 99%).

The synthesis of **4b** and detailed analytical data is already reported in the context of the synthesis of a series of GAL-CU hybrids [19]. A contribution of the herein reported synthetic procedures is upscale and optimization of the protocols.
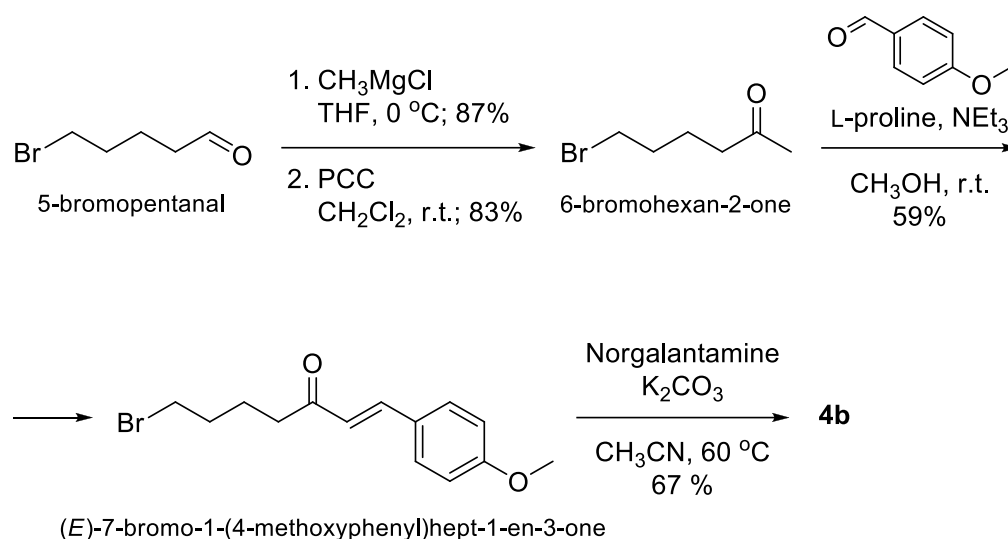
Scheme 1. Synthesis of **4b**.

A stirred solution of 5-bromopentanal (1.038 g, 6.29 mmol) in dry THF (25 ml) under argon atmosphere was treated dropwise at 0 oC with CH3MgCl (3M in THF, 4.2 ml, 12.58 mmol). The reaction was stirred for 30 min at r.t., cooled down to 0 oC and quenched with 10 ml sat.aq.NH4Cl. The resulting mixture was extracted (ether), dried (MgSO4), filtered, concentrated and purified by flash column chromatography to give 6-bromohexan-2-ol in 87% yield. A suspension of 6-bromohexan-2-ol (0.782 g, 4.32 mmol), PCC (1.862 g, 8.64 mmol) and Celite (0.500 g) in CH2Cl2 (25 ml) was stirred for 18 h at r.t. The mixture was diluted with petroleum ether (75 ml), filtered through a pad of Celite, concentrated and subjected to flash column chromatography to give 6-bromohexan-2-one in 83% yield.

A solution of 6-bromohexan-2-one (0.615 g, 3.435 mmol), p-methoxybenzaldehyde (0.562 g, 4.122 mmol), L-proline (0.06 g, 0.515 mmol) and NEt3 (0.104 g, 1.031 mmol) in methanol (7 ml) was stirred at r.t. for 5 days. The mixture was concentrated and directly subjected to flash column chromatography to give (E)-7-bromo-1-(4-methoxyphenyl)hept-1-en-3-one in 59% yield. The latter bromide (0.248 g, 0.834 mmol) and anhydrous K2CO3 (0.314 g, 2.276 mmol) were added to a solution of Norgalantamine (0.207 g, 0.759 mmol) in anhydrous acetonitrile (20 mL) under argon atmosphere. After stirring for 24 h at 60 oC, the mixture was filtered through a pad of Celite, concentrated and purified by flash column chromatography to give 0.249 g (67%) the desired product 4b.

2.2. Animals

Male and female ICR mice (6 weeks old, 25–35 g) obtained from the National Breeding Center, Sofia, Bulgaria were used in the experiments. As a more sensitive sex [22], 18 females were used in the acute toxicity test and 30 males in the short-term toxicity test. Mice were housed in Plexiglas cages (6 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72% ± 4%) with free access to water and standard pelleted food № 53-3, produced in accordance with ISO9001:2008. Prior to the start of the experiment, the mice were acclimatized under vivarium conditions for seven days and their health was monitored daily by a veterinarian. The vivarium (registration certificate № 15320139/01.08.2007) was inspected by the Executive Agency for Medicines in order to verify the conditions for keeping laboratory animals (№ A-16-0532/14.10.2016). All experiments strictly followed the principles

set out in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123) (Council of Europe, 1991). Efforts have been made to minimize animal suffering. The Animal Care Ethic Committee approved the study protocol and Ethic clearance (№ 273 from 02/06/2020) was issued for the study by the Bulgarian Agency for Food Safety.

2.3. Acute Toxicity Protocol

Acute toxicity test was performed on female mice after oral (po) administration of **4b** using a simplified Lorke's method [23]. Three animals per dose were used, starting with a dose of 10 mg/kg and gradually increasing it every 3 h until the first lethal case. Compound **4b** was solubilized with Tween 80 (0.1%) in distilled water and administered orally in a volume of 0.1 ml/ 10 g.

The LD_{50} is calculated using the following equation:

$$LD_{50} = \sqrt{(D_0 \times D_{100})},$$

where D_0 is the highest dose that does not cause mortality and D_{100} is the lowest dose that results in death.

Surviving animals were observed for 24 hours and then for up to 14 days. On day 14, they were euthanized after anesthesia with ketamine/xylazine (80/10 mg/ kg, i.p.) and an examination of the internal organs for possible macroscopic abnormalities (organ color, consistency, neoplasms, etc.) was made.

2.4. Short-Term Toxicity Protocol

The short-term toxicity test was performed on male ICR mice at the same age of 6 weeks and approximately 30-35 g. Three drugs (GAL, CU and **4b**) were administered daily for 14 days orally with a dosing needle at approximately the same time of day, 11.00 h. Based on the LD_{50} value ≈ 50 mg/ kg, derived in the acute toxicity test, two doses of 2.5 mg/kg and 5 mg/kg (1/20 and 1/10 of the LD50) were selected for multiple administration of **4b**. The animals were divided into 5 experimental groups of 6 mice (n = 6). Group 1 was the control group treated with physiological saline via oral gavage (0.1 ml/10 g bw); group 2 was treated orally with the positive control GAL in dose of 3 mg/kg dissolved in distilled water [24]; group 3 was treated orally with the second positive control CU at a dose of 25 mg/kg [25]; group 4 was treated orally with **4b** at a dose of 2.5 mg/kg (1/20 LD_{50}); group 5 was treated orally with **4b** at a dose of 5 mg/kg (1/10 LD_{50}).

Animals were observed daily for behavioral changes and signs of toxicity.

On day 15, after 14-day treatment, the animals were anesthetized with ketamine/xylazine (80 mg/10 kg ip) and decapitated. Blood was taken in vacutainers for complete blood count and biochemistry measurment. The brains were dissected, measured and prepared for the assessment of AChE inhibition, MDA and GSH levels. Protein content of brain homogenates was measured by the method of Lowry [26] using bovine serum albumin as a standard.

2.5. Measurement of Acetylcholinesterase (AChE) Inhibition in Brain Homogenate

Brains were homogenized with 0.1 M, phosphate buffer, pH 7.4. Aliquots of brain homogenates from different groups were used to measure AChE activity for 10 min by the method of Ellman [27]. AChE activity was calculated and expressed as nmol/min/mg protein using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$.

2.6. Measurement of Malondialdehyde (MDA) Levels in Brain Homogenate

Brains were homogenized with 0.1 M phosphate buffer and EDTA, pH 7.4. Aliquots of the homogenates were heated for 20 minutes on a water bath (100°C) with thiobarbituric acid. The amount of thiobarbituric acid-formed reactive species (TBARS) (expressed as MDA equivalents) was measured spectrophotometrically by the method of Deby and Goutier [28] at a wavelength of 535 nm. The concentration of MDA was calculated using a molar absorption coefficient of $1,56 \times 10^5$ $M^{-1}cm^{-1}$ and expressed in nmol/g tissue.

2.7. Measurement of Glutathione (GSH) Levels in Brain Homogenate

GSH was evaluated by measuring non-protein sulfhydryls after trichloroacetic acid (TCA) protein precipitation by the method described by Bump et al. [29]. Brains were homogenized in 5% TCA (1:10) and centrifuged for 20 min at 4 000 × g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05M phosphate buffer (pH = 8), and 0.02mL DTNB reagent. Absorption was determined at a wavelength of 412 nm and the results were expressed as nmol/g tissue.

2.8. Measurement of Haematological and Serum Biochemical Data

Whole blood was analyzed by a semi-automated hematological analyzer BC-2800 Vet, (Mindray, China) according to the manufacturer's instructions. The count of leukocytes (WBC), erythrocytes (RBC, Er), platelets (PLT), amount of hemoglobin (Hb), hematocrit (Ht), as well as the differential count of leukocytes were measured. The biochemical serum data as blood sugar (GLU), cholesterol (CHOL), triglycerides (TRIG), urea (UREA), creatinine (CREAT), total protein (TP), albumin (ALB), total bilirubin -Bil), direct bilirubin (D-Bil), calcium (Ca), uric acid (Uric acid), as well as the activity of the enzymes aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and amylase (AMYL) were measured using automated biochemistry analyzer kits (BS-120, Mindray, China), following the manufacturer's instructions.

2.9. Statistical Analysis

The MEDCALC statistical program was used to analyze the *in vivo* data. Results are expressed as mean ± SD for six animals in each group. The significance of the data was assessed by a nonparametric Mann-Whitney U test. Values of $p \leq 0.05$ are considered as statistically significant.

3. Results

3.1. Acute Toxicity in Mice

The median lethal dose (LD_{50}) of **4b** in mice was 49 mg/kg. No serious toxic effects or mortality were observed in mice at doses of 10 and 20 mg/kg. At dose of 40 mg/kg, tachypnea and mild tremor for up to 2 hours were observed with no other visible signs of toxicity. At dose of 60 mg/kg, one fatal outcome was observed 3 h post-dosing with accelerated breathing, piloerction, mild tremor and seizures. Forthteen days later, the animals were euthanized and a macroscopic examination of the internal organs was performed. No changes in the size, color and consistency of the lungs, liver, heart, kidneys, stomach, spleen, and intestines were observed. No abnormalities in the morphology of the gonads and brain were detected.

3.2. Short-term Toxicity in Mice

The short-term toxicity of **4b** was assessed by daily administration of 2.5 mg/kg (1/20 of LD_{50}) and 5 mg/kg (1/10 of LD_{50}) for 14 days, according to the protocol described in Materials and Methods. As positive contols were used GAL in dose of 3 mg/kg (1/10 of LD_{50}) and CU in dose of 25 mg/kg (1/10 of LD_{50}). A control (placebo) group also was included in the test. Animals were examined daily and no behavioral changes and signs of toxicity were observed. On day 15, the animals were anesthetized and decapitated. Blood from each group was collected for haematological and serum biochemical analuses. Brains from each group were collected as described in Material and Methods and prepared for measurements of AChE inhibition, MDA and GSH levels.

3.3. AChE Inhibition in Mice Brain Homogenate

After 14-day treatment of mice by GAL, CU, **4b** or placedo, the AChE activity was measured for 10 min by the Ellman's method as described in Materials and Methods. The

results are given in Figure 2. CU caused a mild decrease in AChE activity, followed by GAL and **4b**. The AChE decrease of **4b** is dose-dependent. The dose of 5 mg/kg causes 25% inhibition of enzyme activity compared to the control group.
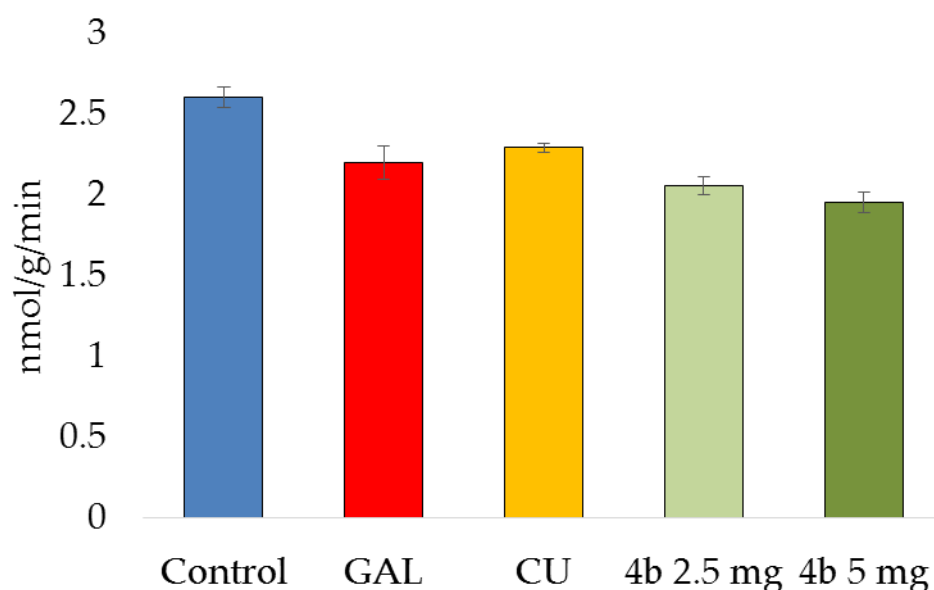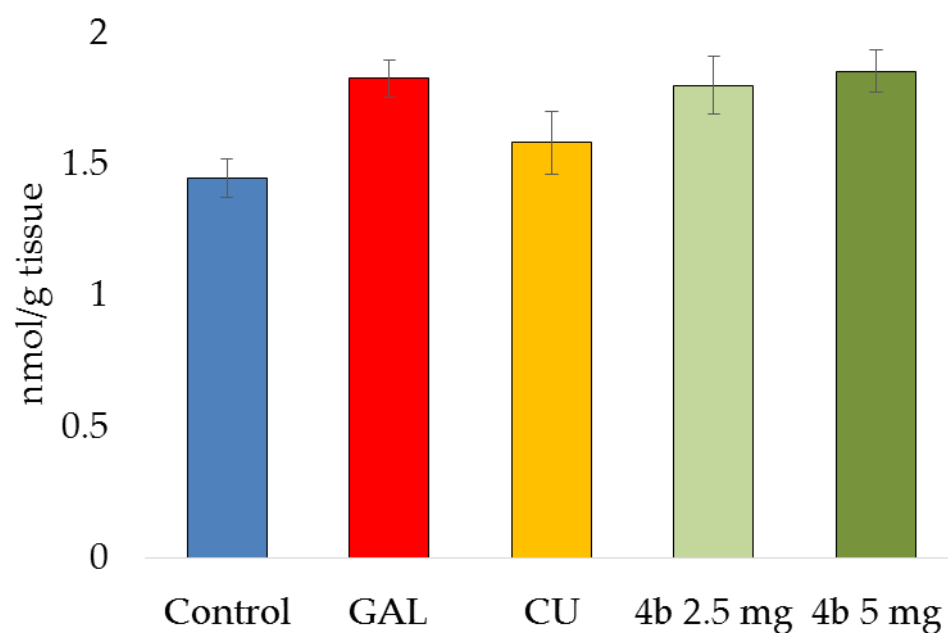

Figure 2. AChE activity measured for 10 min in brain homogenates derived from mice treated 14 days by GAL, CU, **4b** in two doses (2.5 mg/kg and 5 mg/kg) or placebo (control group). $p < 0.05$ *vs.* control.
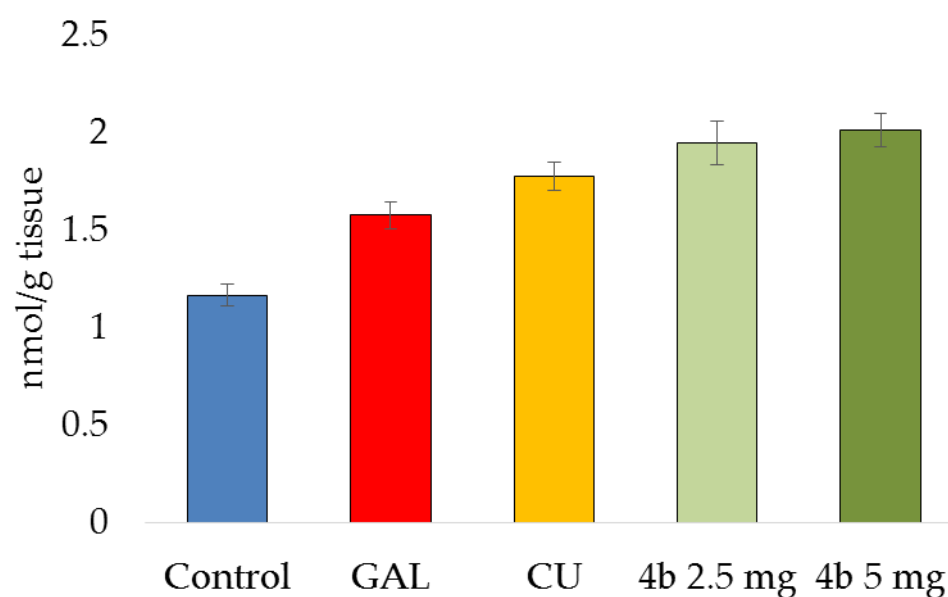
3.4. Antioxidant Activity in Mice Brain Homogenate

The antioxidant activity of the tested compounds after 14-day treatment, was assessed by measuring the levels of malondialdehyde (MDA) and gluthathione (GSH) in mice brain homogenates as described in Materials and Methods. MDA is a specific marker for lipid peroxidation. The thiobarbituric acid (TBA) test is widely used to measure the formation of red pigment, which is an adduct of TBA with aldehydes, MDA, ketones and others. The effects of the tested compounds on MDA levels are given in Figure 3a. All compounds caused increase of MDA levels: CU – by 9%, GAL – by 26%, **4b** in dose 2.5 mg/kg – by 24% and **4b** in dose 5 mg/kg – by 28% compared to control. The effect of **4b** on MDA levels is dose-dependent.

The oxidative stress causes decrease in GSH levels [9]. All tested compounds in the present study elevated the GSH levels (Figure 3b) showing antioxidant activity. The most prominent effect has **4b** in dose of 5 mg/kg (73%), followed by **4b** in dose of 2.5 mg/kg (67%), CU (52%) and GAL (35%). Here again, the effects of **4b** on GSH levels is dose-dependent.

(a)

(b)

Figure 3. Antioxidant activity measured by MDA (**a**) and GSH levels (**b**) in brain homogenates derived from mice treated 14 days by GAL, CU, **4b** in two doses (2.5 mg/kg and 5 mg/kg) or placebo (control group). $p < 0.05$ vs. control.

3.5. Haematological and Serum Biochemical Data

The haematological data after 14-day treatment by GAL, CU, two doses of **4b** or placebo are summarized in Table 1. There are no statistically significant deviations from the reference values for mice (embedded in the analyzer's software), indicating no adverse effect of the test substances on hematopoiesis.

Table 1. Haematological data after 14-day treatment by GAL, CU, two doses of **4b** or placebo. Values are means ± standard deviation (n = 6). Mice reference ranges are given according to analyzer's software.

Complete Blood Count	Control	GAL	CU	4b 2.5 mg/kg	4b 5 mg/kg	Mice Reference Range
WBC x 10^9/L	4,8 ± 0,62	3,9 ± 0,43	2,9 ± 0,22	5,4 ± 0,37	4,1 ± 0,2	2,9 - 15,3
LYM x 10^9/L	3,4 ± 0,56	2,6 ± 0,72	2,6 ± 0,66	3,9 ± 0,5	3,0 ± 0,7	2,6 - 13,5
MO x 10^9/L	0,7 ± 0,04	0,7 ± 0,05	0,5 ± 0,08	0,8 ± 0,04	0,6 ± 0,03	up to 0,8
GRA x 10^9/L	0,6 ± 0,03	0,5 ± 0,04	0,4 ± 0,02	0,5 ± 0,03	0,5 ± 0,03	0,4 - 3,2
LYM %	71,6 ± 2,3	67,7 ± 2,8	68,9 ± 3,2	73,1 ± 3,6	70,1 ± 2,6	63,7 - 90,1
MID % (Mo+Eo+Ba)	15,5 ± 1,2	16,9 ± 1,4	14,2 ± 1,6	16,2 ± 1,3	17,4 ± 1,6	10 - 20
GRA %	12,9 ± 1,6	15,4 ± 1,8	16,9 ± 1,2	10,7 ± 1,4	12,7 ± 1,8	7,3 - 30,1
RBC x 10^{12}/L	7,79 ± 0,3	7,45 ± 0,3	7,14 ± 0,2	7,77± 0,26	7,42 ±0,13	5,6 - 7,89
Hgb g/L	144 ± 7,8	139 ± 8,2	147 ± 5,4	150 ± 4,8	138 ± 5,8	120 - 150
HCT %	40,4 ± 1,8	37,7 ± 2,1	42,2 ± 1,6	40,2 ± 1,2	42,2 ± 2,2	36 - 46
MCV fl	53,2 ± 1,2	54,6 ± 0,9	53,8 ± 1,1	53,8 ± 1,4	63,8 ± 3,4	53,0 - 68,8
MCH pg	19,5 ± 1,3	18,6 ± 1,2	19,2 ± 1,2	18,9 ± 0,9	17,6 ± 1	16,0 - 23,1
MCHC g/L	341 ± 5,4	338 ± 5,8	332 ± 4,2	323 ± 3,8	340 ± 2,2	300 – 341
RDW %	14,7 ± 1,1	13,6 ± 1,2	12,6 ± 1,4	12,4 ± 1,1	11,4 ± 2,1	11,0 - 15,5
PLT 10^9/L	736 ± 159	589 ± 143	430 ± 136	538 ± 151	632 ± 161	100 – 1610
PCT %	0,256 ± 0,06	0,272 ± 0,07	0,258 ± 0,1	0,267 ± 0,04	0,233 ± 0,06	0,1 - 0,48
MPV fl	6,2 ± 1,2	6,2 ± 1,6	6,0 ± 1,0	6,1 ± 1,4	6,0 ± 0,4	3,8 - 6,2
PDW fl	7,7 ± 1,5	9,2 ± 1,6	7,8 ± 1,2	8,3 ± 1,1	7,3 ± 1,6	7 – 23

The serum biochemical data collected after 14-day treatment by GAL, CU, **4b** in two doses or placebo are given in Table 2. No abnormalities on the hepatic or renal functions were observed apart from transaminases (ALAT and ASAT) and uric acid. An increase in both transaminases by **4b** was detected within the reference range. ALAT levels after treatment by the higher dose of **4b** are higher than the upper reference limit. The levels of uric acids were decresed by CU and **4b** relatively to the control group but within the mice reference range.

Table 2. Serum biochemical data after 14-day treatment by GAL, CU, two doses of **4b** or placebo. Values are means ± standard deviation (n = 6). Mice reference ranges are given according to Ref. 30 and 31. In bold are given the increased or decreased values.

Serum Biochemical Data	Control	GAL	CU	4b 2.5 mg/kg	4b 5 mg/kg	Mice Reference Range
GLU mmol/L	6,2 ± 0,82	6,1 ± 0,77	5,3 ± 0,76	6,1 ± 0,84	6,0 ± 0,36	4,2 - 7,5
UREA mmol/L	5,1 ± 0,32	4,0 ± 0,36	3,8 ± 0,28	4,5 ± 0,22	4,2 ± 0,32	3,27 - 12,1
CREAT μmol/L	68 ± 2,3	62 ± 28,8	65 ± 6,6	46 ± 5,6	56 ± 4,6	35 – 120
TP g/L	58 ± 2,2	53 ± 3,1	54 ± 2,6	53 ± 3,6	54 ± 2,6	53 – 63
ALB g/L	27 ± 1,8	26 ± 1,7	26 ± 2,2	26 ± 3,1	27 ± 2,3	26 - 29
ASAT U/L	83 ± 4,5	96 ± 5,2	102 ± 3,6*	**123± 4,1****	**123 ± 5,3****	65 - 122
ALAT U/L	58 ± 2,2	56,6 ± 3,1	62,2 ± 3,3	**81,4 ± 3,4****	**96 ±4,7****	55 - 80

T-Bil μmol/L	5,6 ± 0,42	6,0 ± 0,38	5,6 ± 0,44	6,3 ± 0,28	7,2 ± 0,18	3.9 - 9.6
D-Bil μmol/L	2,4 ± 0,32	2,5 ± 0,44	2,4 ± 0,46	2,9 ± 0,34	3,6 ± 0,24	0 - 6.8
Ca mmol/L	2,04 ± 0,11	2,14 ± 0,15	2,04 ± 0,21	2,1 ± 0,2	2,2 ± 0,1	2.0 - 2.8
Uric acid μmol/L	195 ± 18,4	196 ± 20,3	**133 ± 23,7***	**94 ± 16,7***	**104 ± 26,7***	0 - 300
CHOL mmol/L	2,3 ± 0,35	2,61 ± 0,28	2,6 ± 0,44	2,16 ± 0,36	2,11 ± 0,23	1.77 - 2.9
TRIG mmol/L	1,04 ± 0,12	1,29 ± 0,18	1,02 ± 0,2	1,14 ± 0,22	1,04 ± 0,32	0.85 - 2.23
ALP U/L	148 ± 12,9	131 ± 13,6	122 ± 14,9	113 ± 13,8*	123 ± 11,6	80 - 168
GGT U/L	13 ± 2,1	10 ± 2,6	10 ± 1,8	6 ± 3,2*	8 ± 4,4	6 - 14
AMYL U/L	1021 ± 86,2	1179 ± 74,6	1036 ± 64,3	1263 ± 66,7*	1068 ± 53,4	512 - 1312

*$p < 0.05$ *vs* control; **$p < 0.05$ *vs* reference values

4. Discussion

In the present study, we performed *in vivo* and *ex vivo* studies of a newly synthesized hybrid between GAL and CU aiming to combine the AChE inhibitory properties of GAL with the powerful antioxidant properties of CU. Both properties have beneficial effect on the delay of neurodegeneration. As a result, an AChE inhibitor was generated, 186 times more potent than GAL *in vitro* (IC$_{50}$ of compound **4b** = 20 nM *vs*. IC$_{50}$ of GAL = 3.52 μM) [19].

The LD$_{50}$ of **4b** registered in the acute toxicity test on mice is 49 mg/kg orally. The LD$_{50}$ of GAL in mice is 10 mg/kg i.p. [32] and between 15 and 45 mg/kg (median 30 mg/kg) orally [33]. The oral LD$_{50}$ of CU is more than 2000 mg/kg (at highest given dose 2000 mg/kg) [34,35]. Obviously, the presence of CU fragment in the molecule of **4b** reduces its overall toxicity. The symptoms of toxic damage that were observed, expressed in breathing difficulty, initially rapid and then severely delayed, ataxia, lack of coordinated movements, tremor and tonic-clonic seizures, are characteristic of acetylcholine (ACh) neurotoxicity due to inhibition of AChE. In mammals, respiratory failure caused by inhibition of cerebral AChE is recognized as a cause of death [36]. In fish, the relationship between AChE inhibition and mortality is unclear, as some species are able to survive a high rate (90-95%) of brain enzyme inhibition [37-39]. In this regard, some authors have found that 50% of AChE inhibition may indicate intoxication or poisoning [40]. No changes in the morphology of the internal organs (lungs, liver, heart, kidneys, stomach, spleen, intestines, gonads and brain) were observed.

The daily administration of **4b** in doses of 2.5 mg/kg (1/20 of LD$_{50}$) and 5 mg/kg (1/10 of LD$_{50}$) for 14 days did not show any sign of toxicity in animals' behavior. The *ex vivo* AChE activity measured after this period showed that **4b** inhibits the enzyme in a dose-dependent manner: 21% inhibition by the dose of 2.5 mg/kg and 25% - by the dose of 5 mg/kg compared to the control group (Figure 2). Such extent of AChE inhibition is not enough to cause toxic effects or mortality, but it leads to moderate increase in the levels of brain ACh. Any elevation of ACh levels in the brain are associated with improved cognitive effects [41], which was the purpose of the design of GAL-CU hybrids. The protective effect of GAL on mild to moderate Alzheimer's disease is due to its inhibitory effect on AChE, which was confirmed in the present study by the 16% reduction in AChE activity in mice brain homogenates. The neuroprotective and AChE inhibitory effects of curcumin and other curcuminoids is well known [42-44]. Our study also confirmed these effects: CU showed 12% reduction in the AChE activity in the tested brain

homogenates. Being a congener of GAL and CU, **4b** accumulates and fortifies these effects and exerts more powerful AChE inhibition than both leads.

Tested *in vitro* **4b** showed 186 times higher inhibitory effect on AChE than GAL [19]. In the present *ex vivo* studies, the difference in the reductions of AChE activity by **4b** and GAL is only 5-9% in healthy animals. Some authors report reactivation of AChE, or even overproduction of an enzyme in response to inhibition [45]. The mild difference in the *ex vivo* AChE inhibition between **4b** and GAL might be indicative for such recovery of AChE levels due to high spontaneous reactivation of the enzyme associated with rapid synthesis and release of a new enzyme from the liver. This possible mechanism requires further investigation.

The AChE inhibition *in vivo* is associated with increased oxidative stress [46,47]. In response, the increased oxidative stress mediates an increase in the AChE activity. The central nervous system is very sensitive to oxidative stress. The brain has many areas of high iron content, an activator of oxidative reactions, and neuronal mitochondria generate large amounts of hydrogen peroxide [9]. Neuronal membranes are rich in polyunsaturated fatty acids, which are also particularly susceptible to oxidative stress [9]. Due to the oxidative stress, the cholinergic neurons undergo a degeneration leading to impairments in cognition and memory. One of the strategies for prevention and treatment of Alzheimer's disease is focused on the suppression of oxidative damage [7,8].

The association between AChE inhibition and increased oxidative stress also was observed in our MDA test. MDA is a biomarker of the oxidative stress relating to the lipid peroxidation. In the present study, we found that all tested AChE inhibitors elevated the MDA levels (Figure 3a). As stronger is the AChE inhibition, as bigger is the increase of MDA levels. In the same time, it was found in our GSH test that all tested compounds increased the GSH levels (Figure 3b). GSH is an important endogenous cellular protector and antioxidant. The increased GSH levels might be interpreted as a compensatory response of the brain to overcome the lipid peroxidation induced by the decreased AChE activity. Kaur и Sandhir [48] conducted acute and chronic toxicity studies in Wistar rats with two different oral doses of insecticide (AChE inhibitor). In this study, rats showed a decrease in GSH levels after acute pesticide exposure and an increase in GSH levels during chronic repeated administration. The authors suggest that GSH elevation is an adaptive response to increased oxidative stress.

GAL has an antioxidant activity related with the enolic OH group [6]. One of the mechanisms by which GAL improves the mental function in Alzheimer's dementia is thought to be its antioxidant effect [49-51]. CU also is a proven antioxidant and brain protector [52-54]. These effects were also confirmed in the present study, where CU and GAL significantly increased GSH levels by 52% and 35%, respectively, compared to the control group. As a hybrid of GAL and CU, **4b** accrues and augments the antioxidant effects of its leads.

The analyses of the haematological and serum biochemical data point to some concern about the elevated values of ASAT and ALAT by **4b**. ASAT levels are slightly higher than the upper limit but ALAT levels rise by 20% above the upper limit of the mice reference range. CU and **4b** decrease the serum levels of uric acid. CU inhibits xanthine oxidase and increases uricosuric activity [55,56]. As a CU congener, **4b** might be also involved in the same mechanism.

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