Acute toxicity evaluation of derivatives of 6-nitro-2*H*-Benzo[1,4]thiazin-3-one

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Abstract

The synthesis and physico-chemical properties of five derivatives of 6-nitro-2H-benzo[1,4]thiazin-3-one are described. Alkylation, amination following by benzoylation procedures were used in the molecular modification from that structure, on the positions N(4) and C(6): The derivatives 6-amino-2H-benzo[1,4]thiazin-3-one (BTZ 1R),4-methyl-6-nitro-2H-benzo[1,4]thiazin-3-one (BTZ 2), 6-amino-4-methyl-2H-benzo[1,4]thiazin-3-one (BTZ 2R), N-[4-methyl-3-oxo-3,4-dihydro-2H-benzo[1,4]thiazin-6-yl]-benzamide (BTZ 3) were synthesized and structurally characterized by spectroscopic infrared, mass, 1H-NMR and 13C-NMR. Their Lethal Doses (LD50) and toxic effects were evaluated. LD50 values were determined by probits methods. The LD50 results revealed that methylation at N-4 and the reduction of the nitro group to amine at C(6) resulted in increased toxicity.
Keywords: 6-nitro-2*H*-benzo[1,4]thiazin-3-one; LD_{50} values; *in vivo* acute toxicity; Open Field Test.

Resumo

A síntese e propriedades físico-químicas de cinco derivados de 6-nitro-2H-benzo[1,4] tiazin-3-ona são descritas. Após a alquilação, a aminação seguida de procedimentos de benzoilação foi utilizada na modificação molecular a partir dessa estrutura, nas posições N (4) e C (6): Os derivados 6-amino-2H-benzo [1,4] tiazin-3-ona (BTZ 1R), 4-metil-6-nitro-2H- benzo [1,4] tiazin-3-ona (BTZ2), 6-amino-4-metil-2H-benzo [1,4] tiazina-3- uma (BTZ 2R), N- [4-metil-3-oxo-3,4-di-hidro-2H- benzo [1,4] tiazin-6-il] -benzamida (BTZ3) foi sintetizada e estruturalmente caracterizada por espectroscopia infravermelho, massa, RMN de 1H e RMN de 13C. Suas doses letais (LD50) e efeitos tóxicos foram avaliados. Os valores de LD50 foram determinados por métodos de probits. Os resultados do LD50 revelaram que a metilação em N-4 e a redução do grupo nitro em amina em C (6) resultou em aumento da toxicidade.

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Palavras-chave: 6-nitro-2H-benzo [1,4] tiazin-3-ona; valores LD50; toxicidade aguda in vivo; Teste de campo aberto.

Resumen

Se describen la síntesis y las propiedades fisicoquímicas de cinco derivados de 6-nitro-2H-benzo [1,4] tiazin-3-ona. La alquilación, la aminación seguida por procedimientos de benzoilación se usaron en la modificación molecular de esa estructura, en las posiciones N (4) y C (6): los derivados 6-amino-2H-benzo [1,4] tiazin-3-ona (BTZ 1R), 4-metil-6-nitro-2H-benzo [1,4] tiazin-3-ona (BTZ 2), 6-amino-4-metil-2H-benzo [1,4] tiazin-3- uno (BTZ 2R), N- [4-metil-3-oxo-3,4-dihidro-2H-benzo [1,4] tiazin-6-il] -benzamida (BTZ 3) se sintetizaron y se caracterizaron estructuralmente por espectroscopia infrarrojo, masa, 1H-NMR y 13C-NMR. Sus dosis letales (LD50) y los efectos tóxicos fueron evaluados. Los valores LD50 se determinaron mediante métodos de probits. Los resultados de LD50 revelaron que la metilación en N-4 y la reducción del grupo nitro a amina en C (6) dieron como resultado un aumento de la toxicidad.
Palabras clave: 6-nitro-2H-benzo [1,4] tiazin-3-ona; valores LD50; toxicidad aguda in vivo; Prueba de campo abierto.

INTRODUCTION

The 2*H*-1,4-benzothiazine is a structural analogue of phenothiazine, where o-phenylene group is replaced by an ethylene group . The 2*H*-1,4-benzothiazines present a range of biological activities including antiproliferative, antimicrobial, immunostimulating , anti-aldose-reductase , antiinflamatory , antitumoral , anti-HIV, anti-hypertensive, anti-rheumatic, anti-allergic, vasorelaxant, anti-arrhythmic, neuroprotective/neurotoxic and 15-lipoxygenase inhibitor . Some of these activities are related in a review by Fringuelli, R., Milanese, L. Schiaffella, F., 2005.

The necessity to maximize specific pharmacological activities and reduce unwanted effects calls for the development of new therapeutic agents. The molecular modification of a prototype is the most common development methodology of new drugs. De Miranda Guarda, V. (1998) synthesized new pharmacological series unto 6-nitro-2*H*benzo[1,4]thiazin-3-one and the new derivatives showed a significant antibacterial activity.

Additionally, it is also a necessity to identify which of these compounds presents *in vivo* toxicity. The general toxicity that influences the behavior in mice can be detected by a simple method as the open-field test.

Stereotyped behavior can be induced in animals by restrictive housing conditions or by stimulant drugs. In general, artificially induced stereotypes result in increased stereotyped

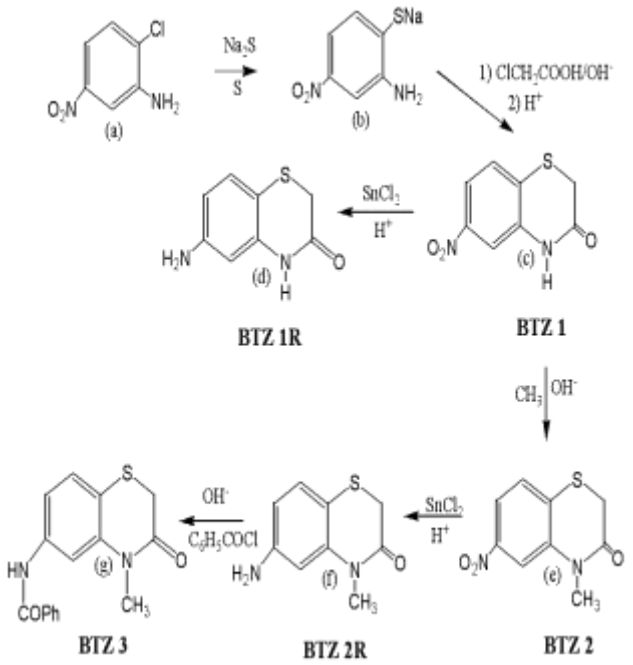
locomotors activity in open-field test. There are no scientific references about the toxicity evaluation in the central nervous system (CNS) for the 6-nitro-2*H*-benzo[1,4]thiazin-3-one derivatives. Therefore, the present study was conducted to access the general toxic effects, locomotors activities in Open-Field Test and lethal doses in mice.

METHODS

Synthesis and structural study

The benzothiazine compounds obtained from the sodium salt of the 2-amino-4-nitro-benzene-thiolate by Fries, K., Vorbrodt, M., Siebert, G., at 1927 was cyclised in the presence of monochloracetic acid in 6-nitro-2*H*benzo[1,4]thiazin-3-one, named in the present study, **BTZ 1 (**GRANDOLINI, G. et al, 1986). N-alkylation in position 4, using methyl iodide in the presence of potassium hydroxide, leads to 4-methyl-6-nitro-2*H*-benzo[1,4]thiazin-3-one, named **BTZ 2 (**NGADI, L. et al., 1990) The reduction of the nitro group at the primary function amine with tin chloride in concentrated hydrochloric acid produces 6-amino-4-methyl-2*H*-benzo[1,4]thiazin-3-one, named **BTZ 2R** or 6-amino-2*H*-benzo [1,4]thiazin-3-one, named **BTZ 1R (**CECHETTI, V., et al., 1984). The protection of the amino function by benzoylation with benzoyl chloride anhydride leads to the formation of compound named **BTZ 3**, (VOGEL, A., 1989), (scheme 1).

Scheme 1 - General scheme for the synthesis of the compounds



(c) 6-nitro-2*H*-benzo[1,4]thiazin-3-one; (d) 6-amino-2*H*-benzo[1,4]thiazin-3-one; (e) 4-methyl-6-nitro-2*H*-benzo[1,4]thiazin-3-one; (f) 6-amino-4-methyl-2*H*-benzo[1,4]thiazin-3-one; (g) N-[4-methyl-3-oxo-3,4-dihydro-2*H*benzo[1,4]thiazin-6-yl]-benzamide.

The chemical structures of the synthesized compounds were confirmed by spectroscopic infrared, mass, ^{1}H-NMR and ^{13}C-NMR . The compounds were powdered, mixed with KBr at 1% concentration and pressed into pellets before IR spectra be recorded on a (FT-IR) Nicolet spectrometer. ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker DPX 200 spectrophotometer. DMSO-d_6 was used as solvent and TMS as the reference. Chemical shifts (d) are given in parts per million (ppm), and coupling constants (J) are given in hertz (Hz). The 70 eV electronic impact mass spectra were recorded on a R-1010C Delsi-Nermag spectrometer. Fragmentations observed and the intensity of the peaks of the isotopes on electronic impact in MS are in accordance with the structures.

The synthesis and the physico-chemical characteristics of 6-nitro-2*H*-benzo[1,4]thiazin-3-one (**c**) compound are described in the literature (SOUZA, A., et al, 2006; TODOROV, D. et al, 1995).

*Amination: 6-amino-2H-benzo[1,4]thiazin-3-one (**d**) and 6-amino-4-methyl-2H-benzo[1,4]thiazin-3-one (**f**)*

6-nitro-4*H*-benzo[1,4]thiazin-3-one (**c)** or 4-methyl-6-nitro-4*H*benzo[1,4]thiazin-3-one **(e)** (3.5 mmol) was added in small quantities to a frozen solution containing 3.72 g of $SnCl_2.2H_2O$ in 4 mL of concentrated hydrochloric acid (36% m/m). The mixture was agitated for 15 min at room temperature and then refluxed for 2 h. After cooling, the precipitate in the form of hydrochlorate was washed with water. To release the amine, a solution of NaOH 20% was added until pH reached 10. The solid compounds were separated by filtration, washed with water and NaOH 10% before being purified by recrystallization in water.

*6-amino-2H-benzo[1,4]thiazin-3-one (***d***)* - $C_8H_8N_2OS$. M.p. 225-228 °C. Yield %: 91. TLC chloroform: ethanol (9:1), Rf 0.38. IR (KBr): –ν 3442, 3349, 3201, 1678, 1623, 1380, 522 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.3 - 2H, C(2), s; 5.14 - 2H, N(14), s; 6.21 - 1H, C(7), dd, *J*=9.3 and 2.7Hz; 6.23 -1H, C(5), d, *J*=1.5Hz; 6.9 - 1H, C(8), d, *J*=8.4Hz; 10.22 - 1H, N (4), s. ^{13}C NMR (DMSO-d_6,DEPT): δ 29.6 C (2), 102.5 C (7), 103.4 C(9), 109.4 C(5), 127.7 C(8), 139.1 C(10), 148.2 C(6), 165.7 C(3). MS m/z (%): 180 M^+ (100), 151 (18.1), 135 (25.5).

*6-amino-4-methyl-2H-benzo[1,4]thiazin-3-one (***f***)* - $C_9H_{10}N_2OS.HCl$. M.p. 228-230°C, $C_9H_{10}N_2OS$ 131-133°C. Yield %: 97. TLC chloroform: ethanol (9 :1), Rf: 0.61. IR (KBr): –ν 3430, 3380, 1655, 1610, 1370, 1140, 800 cm^{-1}. ^1H NMR (DMSO-d_6) δ: 3.25 3H – C(13), s; 3.31-2H, C(11), s; 5.24-2H, N(14), s; 6.28 - 1H C(7), dd, *J*=8.2 and 2.1Hz; 6.46-1H, C(5), d, *J*=2.2Hz; 6.98-1H, C(8), d, *J*=8.2Hz. ^{13}C NMR (DMSO-d_6, DEPT): δ 31.2 C(2), 31.3 C(13), 103.6 C(5), 106.7 C(9), 109.2 C(7), 128.3 C(8), 140.8 C(10), 148.5 C(6), 165.6 C(3). MS m/z(%): 194 M+ (100), 165 (17.7), 151 (29.5), 149 (28.3), 133 (11).

*Alkylation: 4-methyl-6-nitro-2H-benzo[1,4]thiazin-3-one (***e***)*

A mixture of 6-nitro-2*H*-benzo[1,4]thiazin-3-one (1.05 g, 5 mmol) in DMSO : ethanol (10:12.5 mL) and potassium hydroxide (0.56 g, 10 mmol) was agitated at room temperature. Shortly afterwards (10 min), methyl iodide (1.42 g, 10 mmol) was added dropwise and the reactional mixture was then maintained under agitation at 50 °C for 15 h. After cooling, the alkylated compound precipitated on addition of crushed ice. The methylated derivative was separated from the reactional mixture by filtration, and purified by recrystallization in ethanol 95%. $C_9H_8N_2O_3S$. M.p. 183-5 °C. Yield % 80. TLC chloroform : ethanol (9:1), Rf 0.76. IR

(KBr): ν 2940, 1690, 1510,1340, 745 cm^{-1}. ^{1}H NMR (DMSO-d6) δ: 3.43-3H, C(13), s; 3.66-2H, C(11), s; 7.7-1H, C(8) d *J*=8.4Hz); 7.9-1H, C(7), dd, *J*=8.6 and 2.2Hz), 7.98-1H, C(5), d, *J*=2.4Hz. ^{13}C NMR (DMSO-d6, DEPT) δ: 29.3 C(2), 31.5 C(13), 112.2 C(5), 117.5 C(7), 128.5 C(8), 131.9 C(9), 140.4 C(10), 146.3 C(6), 164.6 C(3). MS m/z(%): 224 M+ (100), 181(65.1), 149 (33.8), 95 (45).

*Benzoylation: N-[4-methyl-3-oxo-3,4-dihydro-2H-benzo[1,4]thiazin-6-yl]-benzamide(***g***)*

A suspension of 6-amino-4-methyl-4*H*-benzo[1,4]thiazin-3-one **(f)** (1.55 g 5 mmol) in 20 mL of NaOH 5% was added to 2 mL of benzoylchloride. The reactional mixture was maintained under strong agitation for 10 min. The benzoyl compound was precipitated as a mass. It was separated by filtration, washed with water and purified by crystallization in 95% ethanol.

$C_{16}H_{14}N_2O_2S$. M.p. 185-186. Yield % 96. TLC toluene: ethyl acetate (6:4), Rf 0.42. IR (KBr): –ν 3280, 2910, 1660, 1640, 1630, 1510, 1350, 810 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 3.32 3H- C(13), s; 3.5-2H, C(11), s; 7.36-1H C(8), d, *J*=8.4Hz; 7.53-7,57- 4H, C(7), H-C(3), H-C(4), H-C(5), m; 7.76-1H, C(5), d, *J*=2.2Hz), 7.96-2H, H-C(2), H-C(6), m; 10.33-1H, N(14), s. ^{13}C NMR (DMSO-d_6, DEPT) δ: 30.4 C(2), 31.4 C(13), 109.8 C(5), 114.9 C(7), 116.6 C(9), 127.5 H-C(2), H-C(6); 127.8 C(8), 128.3 H-C(3), H-C(5) 131.6 H-C(4); 134.6 C(16), 138.5 C(10) or C(6), 140.1 C(10) or C(6), 165.3 C(3) or C(12), 165.5 C(3) or C(12). MS m/z(%): 298 M+ (37.8), 105 (100), 77 (53.9).

In vivo Toxicity Evaluation

In this study, dimethylsulfoxide (DMSO; Synth, Brazil) and Tween 80 (Synth, Brazil) were used for compounds solubilization (DMSO: H2O: Tween - 1:8:1). All solutions to use *in vivo* experiments were prepared immediately before use.

Animals: Female Swiss albino mice weighing between 25 – 35 g (*n = 10* per group) were used. Animals were kept under standard conditions and fasted 12 h before use. All experiments were conducted in accordance with the International Standards of Animal Welfare recommended by the Society for Neuroscience and with Brazilian Federal regulations. The experimental protocols were approved by the local Animal Care and Use Committee. The smallest amount of animals and period of inspection required to obtain secure data were employed.

Determination of Lethal Doses (LD_{50}) - Toxicity was assessed by determination of the median lethal dose, LD_{50}, according to Turner (1965).

The animals received intraperitoneally (i.p.) five increasing doses of the all compounds. Control group received the corresponding vehicle. The animals were kept under inspection for a period of 24 h. The toxicological effect of mortality was expressed as LD_{50} value. In the groups with no dead animals and where all animals dead (and in the groups with only dead animals), the obtained percentages were corrected by the following formulae: For 0% dead group = 100 (0.25/*n*) and for 100% dead group = 100 [(*n* - 0.25)/*n*); where *n* symbolizes the number of animals of the group. Following the correction, all percentages were converted to probits, which were plotted *versus* log doses.

Open-Field Test - Central nervous system depressant effects were investigated by spontaneous locomotors activity and other behavioral patterns. Each animal was placed in the lower left corner of an arena constituted of 16 equal squares during a 60 s period , and the number of squares visited (two feet placed in the same square) and the frequency of rearing and grooming were counted at 0, 1, 3 and 24 h after the drug administration.

RESULTS AND DISCUSSION

Determination of LD_{50} value: The obtained data revealed that there was a similar range of LD_{50} doses for the five compounds, as shown in Table 1.

Table 1 – LD_{50} for the BTZ derivatives

Dose (mg/kg)	Log dose	Mortality (%)	Correct Mortality (%)	Probits	LD_{50} (mg/kg) graphic method
1000	3.0000	00	06.25	3.45	
1250	3.0969	20	20.00	4.16	
1500	3.1761	50	50.00	5.00	1548.82
2000	3.3010	70	70.00	5.52	
2500	3.3979	100	93.75	6.55	
100	2.0000	00	06.25	3.45	
300	2.4771	10	10.00	3.72	
500	2.6990	30	30.00	4.48	489.78
750	2.8751	60	60.00	5.25	
1000	3.0000	100	93.75	6.55	
300	2.4771	00	06.25	3.45	
1000	3.0000	20	20.00	4.16	
1500	3.1761	50	50.00	5.00	1023.29
1750	3.2430	80	80.00	5.84	
2000	3.3010	100	93.75	6.55	
100	2.0000	00	06.25	3.45	
300	2.4771	10	10.00	3.72	
500	2.6990	50	50.00	5.00	407.38
750	2.8751	80	80.00	5.84	
1000	3.0000	100	93.75	6.55	
300	2.4771	00	06.25	3.45	
1000	3.0000	10	10.00	3.72	
2000	3.3010	50	50.00	5.00	1445.44
2500	3.3979	70	70.00	5.52	
3000	3.4771	100	93.75	6.55	

Usually, behavioral changes began soon after the administration, but were reversible before 24 h for the lowest and moderate doses. A significant reduction of rearing number was observed for all compounds, although this behavior was primarily observed in the animals treated with BTZ-1, BTZ-2 and BTZ-3 compounds. Notwithstanding, the number of grooming was higher on mice treated with 300, 500 and 750 mg/kg of BTZ-2R. Statistical analysis revealed that the derivatives compounds induced a significant dose-dependent decrease in general motility and locomotors activity.

It is necessary to demonstrate that synthesized compounds don't cause motor alterations before assigning pharmacological activities to them, to avoid false-positive results. Moreover, the analysis of toxic effects of new compounds is important to generate scientific knowledge that will or will not validate future research. Based on observed results, the LD_{50} value revealed that the alkylation of nitrogen (N-4) by a methyl group in the BTZ-2 increased its toxicity. The compounds BTZ-1R and BTZ-2R also showed increasing toxicity maybe due

to the structural modifications generated by the replacement of the nitro group (-NO_2) by the amino group (-NH_2) in C(6). The compound BTZ-3 presented the lower toxicity. Motility alterations were detected primarily in the Open-Field test, which evaluated the exploratory capacity. The most common stereotypes were posture-reflex loss, convulsion, eye bleeding and shivering (Table 3). Reduction of locomotor activity was observed for all doses of compounds BTZ-1, BTZ-1R and BTZ-2, and for doses higher than 100 mg/kg of compound BTZ-2R. The compound BTZ-3 (300 and 1000 mg/kg) induced to the increase in motility. According to Choleris, E., (2001) this reduction in motility was caused by the adaptation of the animals to the environment in repetitive tests. Additionally, as aforementioned, a reduction in rearing number was observed, indicating a reduction in exploratory capacity. The Open-Field test is utilized by a myriad of authors to measure the mice's emotional behavior, exploratory and locomotors activities. The locomotion of animals in the open-field can be interpreted in several ways, but primarily as curiosity or escape trial.

Open-Field test

The general effects observed in spontaneous locomotor activity of the animals after the i.p. administration of the different doses of synthesized BTZs derivatives are summarized in Table 2.

Table 2 - Effect of BTZs derivatives on number of squares visited in one minute in Open-Field Test

Dose (mg/kg i.p.)		*0h*			*1h*			*3h*			*24h*		
BTZ 1	Control	28.5	±	1.58	22.8	±	1.82	25.3	±	2.01	29.3	±	2.84
	1000	25.3	±	2.81	1.6	±	0.27*	1.5	±	0.40*	25.9	±	2.39
	1250	22.6	±	3.04	1.3	±	0.26*	0.9	±	0.18*	11.8	±	2.47*
	1500	27.9	±	1.95	7.6	±	1.49*	3.9	±	0.80*	13.9	±	4.75*
	2000	25.6	±	1.66	0.6	±	0.16*	0.5	±	0.17*	3.6	±	2.11*
	2500	25.5	±	3.50	0.5	±	0.17*	0.3	±	0.15*	0.0	±	0.00*

Dose (mg/kg i.p.)		*0h*			*1h*			*3h*			*24h*		
BTZ 1R	Control	34.5	±	2.79	25.3	±	3.27	26.4	±	2.20	28.0	±	1.67
	100	22.7	±	2.56*	21.1	±	3.57	16.4	±	3.75*	21.3	±	3.04
	300	27.4	±	3.81	7.5	±	1.44*	9.9	±	1.83*	22.9	±	3.50
	500	27.1	±	3.97	2.6	±	0.85*	1.6	±	0.43*	13.3	±	4.15*
	750	22.8	±	2.32*	1.0	±	0.00*	0.5	±	0.17*	2.2	±	0.98*
	1000	21.5	±	2.70*	0.7	±	0.15*	0.2	±	0.13*	0.0	±	0.00*

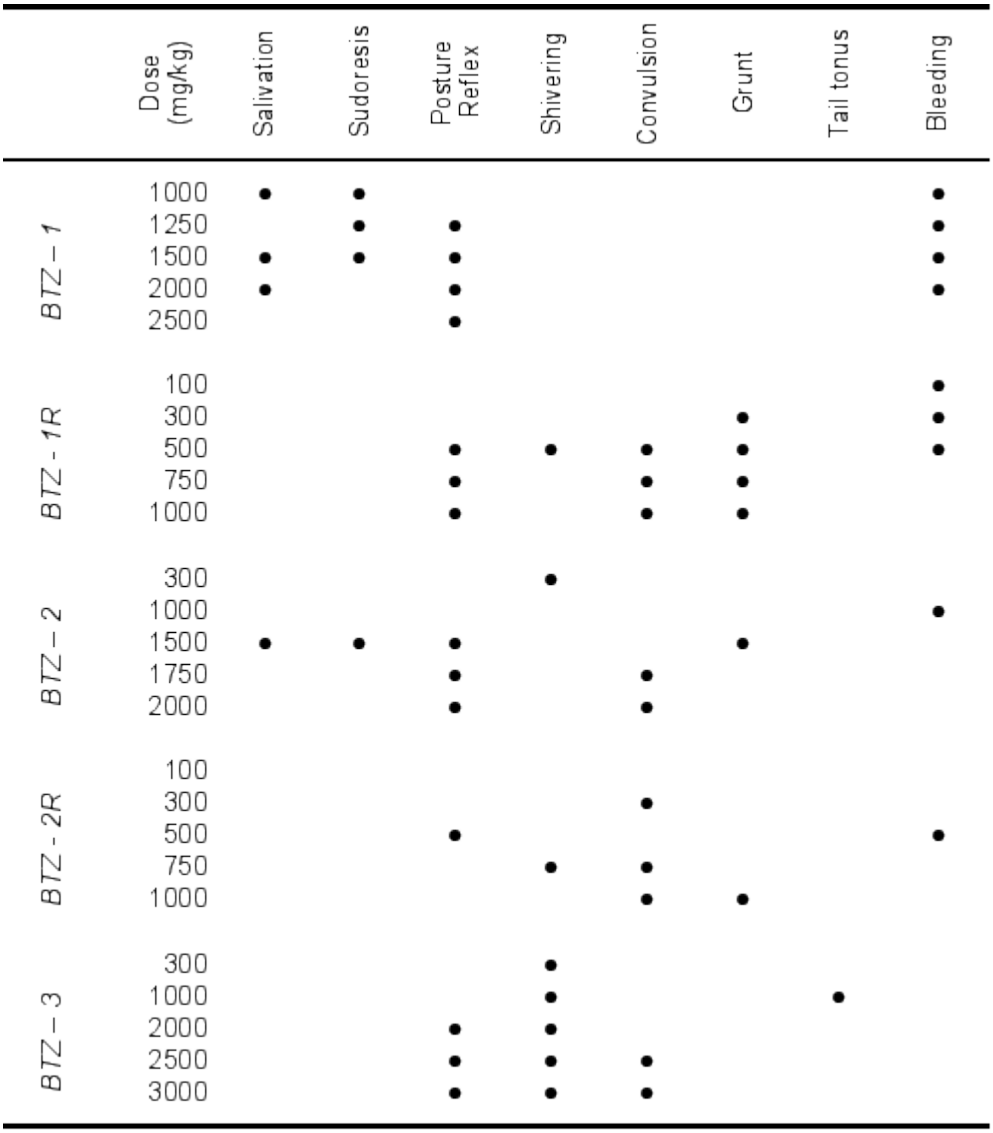
Dose (mg/kg i.p.)		*0h*			*1h*			*3h*			*24h*		
BTZ 2	Control	28.2	±	1.93	22.3	±	2.11	22.8	±	2.89	29.6	±	1.65
	300	30.6	±	3.28	5.6	±	1.79*	15.7	±	3.12	31.9	±	3.67
	1000	26.6	±	2.58	2.0	±	0.52*	0.9	±	0.10*	18.5	±	3.51*
	1500	34.0	±	3.58	2.0	±	0.47*	1.3	±	0.37*	10.6	±	3.68*
	1750	24.8	±	2.58	0.6	±	0.16*	0.4	±	0.16*	5.6	±	3.75*
	2000	25.3	±	2.58	0.3	±	0.15*	0.2	±	0.13*	0.0	±	0.00*

Dose (mg/kg i.p.)		*0h*			*1h*			*3h*			*24h*		
BTZ 2R	Control	31.2	±	3.11	21.4	±	3.04	25.5	±	3.03	30.9	±	1.79
	100	23.2	±	2.17*	12.9	±	1.49*	16.1	±	1.20*	23.4	±	1.38*
	300	23.8	±	2.18	2.7	±	0.94*	1.3	±	0.21*	20.1	±	2.57*
	500	25.6	±	2.52	1.4	±	0.22*	0.9	±	0.10*	7.3	±	2.63*
	750	27.9	±	2.73*	1.0	±	0.00*	0.5	±	0.17*	2.7	±	1.82*
	1000	27.0	±	2.50	0.7	±	0.15*	0.5	±	0.17*	0.0	±	0.00*

Dose (mg/kg i.p.)		*0h*			*1h*			*3h*			*24h*		
BTZ 3	Control	28.2	±	2.74	21.5	±	3.05	21.9	±	2.91	30.3	±	1.97
	300	28.4	±	3.54	25.8	±	3.36	27.2	±	2.75	26.9	±	2.20
	1000	26.1	±	2.71	27.1	±	3.20	31.9	±	2.62*	26.0	±	3.71
	2000	27.7	±	2.45	1.0	±	0.00*	0.7	±	0.15*	8.5	±	3.01*
	2500	27.6	±	0.93	1.0	±	0.00*	0.7	±	0.15*	4.0	±	2.12*
	3000	27.7	±	2.45	0.7	±	0.15*	0.5	±	0.17*	0.0	±	0.00*

* $p < 0,05$

Table 3 - Stereotypes observed for all doses of BTZs derivatives in the Open-Field Test



CONCLUSIONS

The data obtained by the evaluation of the *in vivo* central nervous system effects for the 6-nitro-2*H*-benzo-[1,4]thiazin-3-one derivatives suggested a sedative activity, contributing to knowledge about this compounds. Moreover, chemical and pharmaceutical studies focused on elucidating respective mechanism must be performed.

The compounds *BTZ-1R* and *BTZ-2R* showed toxic effects even in small doses, resulting in a low safety index value in the therapeutic window. This increase in toxicity may be attributed to amino group in those compounds structure.

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