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Article

Anticonvulsant profile of 2-ethylthio-7-methyl-4-(4-methylphenyl) pyrazolo[1,5-*a*][1,3,5]triazine

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This work evaluates the central nervous effects in ICR strain mice of 2-ethylthio-7-methyl-4-(4-methylphenyl)pyrazolo[1,5-*a*][1,3,5]triazine (MH4b1), a compound obtained by an efficient one-step reaction of *S,S*-diethyl 4-methylbenzoylimidodithiocarbonate with 5-amino-3-methyl-1*H*-pyrazole, in order to assess its neuro-pharmacological profile. The tests applied were: maximal electroshock seizure (MES), pentylenetetrazole (PTZ) seizures, forced swimming, plus maze, marble burying, sleeping time, rota-rod and catalepsy. In addition, MH4b1 binding to the benzodiazepine site of the GABA-A receptor and MH4b1 inhibition of monoamine oxidase (MAO) subtypes A and B were evaluated. MH4b1 showed anticonvulsant effects in a dose dependent manner (30-300 mg/kg, p.o.) against MES and inhibition of MAO-B (IC_{50}: 24.5 μM) without activity at the benzodiazepine site. These data suggest that MH4b1 has anticonvulsant properties related to MAO-B inhibition.

Uniterms: Pyrazolo-triazine/anticonvulsant profile. Anticonvulsants/experimental study. Monoamine oxidase/inhibitors.

Este trabalho avalia o efeito do 2-etiltio-7-metil-4-(4-metilfenil)pirazol[1,5-*a*][1,3,5]triazina (MH4b1) no sistema nervoso central de camundongos ICR. O MH4b1 foi obtido por a reação de 4-metilbenzoilimidoditiocarbonato de *S,S*-dietil e 5-amino-3-metil-1*H*-pirazol em uma única etapa. O perfil neurofarmacológico foi realizado por testes de convulsão induzida por eletrochoque (MES) e pentilenotetrazol (PTZ) e por testes de nado forçado, labirinto em cruz, esconder as esferas, sono barbitúrico, *rota-rod* e catalepsia. Também foi avaliada a união do MH4b1 ao o local de ligação de benzodiazepínicos do receptor GABA-A e a capacidade inibitória do MH4b1 sobre a monoaminoxidase (MAO) A e B. O MH4b1 mostrou efeito anticonvulsivante dependente da dose (30-300 mg) no teste do MES e apresentou atividade inibitória da MAO-B (CI_{50}: 24.5 μM) sem interagir com o local de ligação de benzodiazepínicos do receptor. Os resultados sugerem que o MH4b1 tem atividade anticonvulsivante relacionada com a inibição da MAO-B.

Unitermos: Pirazol-triazina/perfil anticonvulsivante. Anticonvulsivantes/estudo experimental. Monoaminoxidase/inibidores.

INTRODUCTION

The pyrazolo[1,5-*a*][1,3,5]triazine system has been a pharmacological structure for the development of drugs that are potentially useful for a wide range of disorders, including asthma (Junien *et al*., 1998), cancer (Nie *et al*., 2008; Popowycz *et al.,* 2009), inflammatory (el-Hawash *et al*., 1998), thrombogenic (Raboisson *et al.,* 2002) and affective disorders such as major depression and pathological anxiety (Gilligan *et al.,* 2009). An efficient method for the preparation of pyrazolo[1,5-*a*][1,3,5]triazines by the interaction of *S,S*-diethyl aroyliminodithiocarbonates with 5-amino-3-methylpyrazole to obtain novel 4-aryl-2-ethylthio-7-methylpyrazolo[1,5-*a*][1,3,5]triazines

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was developed (Insuasty *et al.,* 2006a,b). However, the pharmacological profiles of these compounds have not been assessed until now.

The GABA-A receptor and monoamine oxidases A and B are targets in the central nervous system for compounds with potential anxiolytic, anticonvulsant, antidepressant and/or anti-Parkinsonian properties (Trincavelli *et al.,* 2012; Kulkarni *et al*., 2009; Youdim *et al*., 2006) In view of the global and increasing impact of major affective, neurologic and neurodegenerative disorders, there is a need to look for new pharmacological alternatives for their treatment.

Pyrazolo-triazines are sources of potential drugs for the treatment and/or prevention of disorders of the central nervous system, including anxiety, convulsions and cognition enhancement. These effects seem to be related to their affinity for GABA-A receptors (Atack, 2011a,b; Guerrini *et al*., 2010). Furthermore, adenosine A(1) receptor antagonism of some of these compounds could add to their potential value in the treatment of cognitive dysfunction (Harvey *et al*., 2012). Also, corticotropin-releasing factor antagonism could explain anxiolytic properties some of them possess (Li *et al*., 2003). In addition, some pyrazolotriazine derivatives have shown affinity for central monoamine oxidases (Carotti *et al*., 2007), target molecules in the search for agents to treat neuroprotective and/or affective disorders (Moussa *et al*., 2006).

This work presents the effects of 2-ethylthio-7-methyl-4-(4-methylphenyl)pyrazolo[1,5-*a*][1,3,5]triazine (MH4b1) in screening models in mice and shows its profile as a potential anticonvulsant agent with MAO-B properties.

MATERIAL AND METHODS

MH4b1 synthesis

MH4b1 was synthetized by heating a solution of equimolar amounts of *S,S*-diethyl 4-methyl-benzoylimidodithiocarbonate 1 (0.003 mol) and 5-amino-3-methylpyrazole 2 (0.003 mol) in dimethylformamide (DMF, 2 mL) under reflux for 0.5–1.5 h, according to the method described by Insuasty *et al.* (2006). The solid product was precipitated by the addition of cold water to the reaction mixture. The precipitate was collected by filtration and purified by column chromatography on silica gel using a mixture of hexanes/ethyl acetate (4:1) as eluent.

Animals

The Animalarium in the Pharmacy Department of the National University of Colombia provided male 7–9 week-old ICR strain albino mice, between 20 and 30 g, which were maintained under conditions of controlled temperature and humidity with a photoperiod of 12 hours light/darkness and free access to food and water. The animals were subjected to a fasting period of no more than six hours during the test day to prevent effects on the convulsive threshold. Six to seven animals per treatment and per dose were used, except in the preliminary observational Irwin test, where three animals were used (Roux *et al*., 2003). A digital video camera was used to examine the behaviour of each animal in the absence of the experimenter. All procedures were conducted following the care principles for the management of laboratory animals. The Ethics Committee of the Faculty of Science at the National University of Colombia approved this study (Act 4, 27/04/2009).

Behavioural tests

Irwin test

This test was used to assess the preliminary effects of the drug on the behavioural and physiological states of the mice and to select the screening dose to be used in the following *in vivo* models. Animals were treated with 30, 60 and 100 mg/kg, p.o. of MH4b1 or vehicle (control), in a mixture of glycerine (10%), propylene glycol (10%), polysorbate (5%) and distilled water (75%) in a volume of 0.1 mL/10 g, p.o. The animals were observed at 0, 15, 30, 60, 120 and 180 min, and 24 h after administration. The presence or absence of the following effects was analysed: mortality, seizure, erection of the tail (Straub sign), sedation, excitation, abnormal gait, jumps, motor incoordination, abdominal torsion, piloerection, stereotypy, ticks, and increase or decrease in respiration (Roux *et al*., 2003).

Maximum electroshock seizure (MES)

This test was used to identify potentially effective agents in preventing tonic clonic seizures. Groups of seven animals were treated with MH4b1 (100 mg/kg, p.o.), sodium phenytoin (positive control, 20 mg/kg, p.o.) or vehicle (negative control) one hour prior to exposure to an electric shock of 50 mA, 60 Hz and 130 ms through corneal electrodes (A13-65 *Coulbourn Instruments*® stimulator). A protective effect was assumed when the drug prevented the tonic extension of the hind legs at an angle greater than 45 degrees (Swinyard *et al.,* 1989).

Seizures induced by pentylenetetrazole (PTZ)

This test was used to detect potentially effective agents to prevent absence seizures. Groups of

seven animals treated one-hour prior with MH4b1 (100 mg/kg, p.o.), clonazepam (positive control, 0.5 mg/kg, p.o.) or vehicle were administered PTZ (GABA antagonist, 85 mg/kg, s.c.). Animals that did not show clonic seizures in their heads, backs or limbs for more than five seconds over the 30-minute observation period after PTZ administration were considered to be protected (Swinyard *et al.,* 1989).

Plus maze

This test was used to detect potentially effective anxiolytic agents. Animals (six per group) were treated first with MH4b1 (100 mg/kg, p.o.), diazepam (positive control, 0.5 mg/kg, i.p.) or vehicle. After one hour, animals were individually placed into the central area (5x5 cm) of the plus maze platform formed by two opposing open arms (30x5x25 cm) and two opposing closed arms (30x5x25). The percentage of time that a mouse remained in the open arms during five minutes was recorded (Lister, 1987).

Marble burying

This test was used to detect potentially effective anxiolytic agents (Njung'e, Handley, 1991). Animals (six per group) were treated first with MH4b1 (100 mg/kg, p.o.), clonazepam (positive control, 0.5 mg/kg, i.p.) or vehicle. After one hour, animals were individually placed into an acrylic clear box measuring 30×20×15 cm containing sawdust bedding that was 4 cm in depth, with twenty-five marbles 2 cm in diameter. Testing was conducted for a 15-min period. The number of marbles buried was recorded. Marbles were considered buried if at least one half of their surface was covered with bedding.

Rota-rod

This test was used to detect potentially neurotoxic agents (Swinyard *et al.,* 1989; Löscher, Nolting, 1991). Three groups of six animals were trained to maintain their balance on an axle three cm in diameter powered by a small engine at 12 rpm. Mice were previously trained on the rota-rod for 3 min at a speed of 12 rpm. Mice that were able to remain on the rod for this time period were selected. For testing, the animals were placed on the rota-rod one hour after administration of MH4b1 (100 mg/kg, p.o.), clonazepam (0.5 mg/kg, p.o.) or control (vehicle, 0.01 mL/g). The time that each animal was able to maintain its balance upon the rota-rod was recorded.

Barbiturate-induced sleep period

This test was used to detect agents with sedative effects. Animals (three groups of six) were treated first with MH4b1 (100 mg/kg, p.o.), diazepam (0.5 mg/kg, i.p.) or vehicle and were then treated with pentobarbitone (40 mg/kg, i.p.). Sleep periods were recorded from the moment the animals completely lost their gait until the moment gait was recovered (Lapa *et al*., 2002).

Forced swimming (FST)

This test was used to detect agents with antidepressant potential. Animals (three groups of six) were treated with MH4b1 (100 mg/kg, p.o.), imipramine (positive control, 20 mg/kg, i.p.), or vehicle. After one hour, the animals were individually placed into plastic cylinders (35x24 cm) containing water (25 °C) to a height of 13.5 cm. The total time immobile, defined as movements only necessary for the animal to stay afloat, was recorded over five minutes (Porsolt *et al.,* 1977).

Catalepsy test

This test is used to detect anti-extrapyramidal drugs, such as anticholinergic and anti-Parkinsonian agents (Abraham, 2003). Three groups of six animals were treated with either MH4b1 (100 mg/kg, p.o.), biperiden (reference drug, 3 mg/kg, i.p. [Makoto *et al*., 1979]) or vehicle (control) and then, after one hour, the animals were administered haloperidol (2 mg/kg, i.p.). The time required for each animal to return to a natural position with its four legs on the floor after placing his forepaws on a metal bar situated four inches above floor level was determined (Costall, Naylor, 1974).

In vivo dose-response effect of MH4b1

According to the results obtained in the *in vivo* screening test, MES was the test selected to perform a dose response experiment. Groups of mice treated with 30, 100 and 300 mg/kg p.o. of MH4b1 were subjected to the same protocol described previously.

Determination of monoamine oxidase (MAO) isoform activity

The effects of MH4b1 on MAO-A and MAO-B activities were studied using a crude rat brain mitochondrial suspension as a source of enzymes. Serotonin (100 µM) and 4-dimethylaminophenethylamine (5 µM) were used as selective substrates for MAO-A and -B, respectively. The methodological details are described in Hurtado *et al*., (2004). Substrates and their metabolites were detected by HPLC-ED after 10 and 8 minutes of reaction to the MAO-A and MAO-B isoforms, respectively. The IC_{50} value was calculated from at least two independent experiments, each performed in triplicate.

Binding to the benzodiazepine site on the GABA-A receptor

An assay was performed to estimate the level of inhibition produced by MH4b1 on the binding of tritiated flunitrazepam (^{3}H-FNZ) to the GABA-A receptor in synaptosomal membranes of rat cerebral cortex containing 0.2–0.4 mg of protein (Fernández *et al.,* 2004). The membranes were suspended in the presence of MH4b1 (300 µM) and radiolabeled ligand (0.6 nM) in a final volume of 1 mL of buffer solution (25 mM Tris-HCl, pH 7.3). The incubation was carried out at 4 °C for 1 hour. This assay was performed in triplicate. The displacement of ^{3}H FNZ by MH4b1 was determined using liquid scintillation.

Experimental design and data analysis

The experiments followed a randomized design with 6–7 replicates per treatment. The results are expressed as the arithmetic mean values ± standard error of the mean (SEM) in all tests except the MES and PTZ tests, which are expressed as the number of animals with or without convulsion. The Fisher test was applied to the MES and PTZ tests due to their dichotomous responses. One-way analyses of variance followed by Tukey's comparison test was applied to the plus maze, marble burying, sleeping time, forced swimming, rota-rod and catalepsy tests. A semi-logarithmic regression analysis was applied to the MAO-inhibition experiments in order to determine the inhibitory concentration 50 (IC_{50}) values. The data were analysed with the statistical package Graph Pad®. A p value of 0.05 was assumed to be statistically significant.

RESULTS

Identification of MH4b1

Its melting point value (112 ºC) as well as infrared, ^{1}H and ^{13}C magnetic resonance spectra were consistent with the proposed structure of 2-ethylthio-7-methyl-4-(4-methylphenyl)pyrazolo[1,5-*a*][1,3,5]triazine (Figure 1, molecular weight: 284). The structure of this compound was additionally corroborated by X-ray diffraction analysis (Insuasty *et al.,* 2006a,b).

Data: Green solid, mp 112 °C (63%). ^{1}H NMR (400 MHz, DMSO): δ 1.40 (t, 3H, CH_3), 2.45 (s, 3H, 7-CH_3), 3.19 (c, 2H, CH_2), 6.33 (s, 1H, 8-H), 7.42 (t, 2H, H*m*), 8.58 (d, 2H, H*o*). ^{13}C NMR (DMSO): δ 13.6 (CH_3), 13.8 (7-CH_3), 20.5 (CH_2), 93.3 (C-8), 126.5 (C_i), 128.3 (C*m*), 130.4 (C_o), 143.1 (C_p), 150.5 (C-4), 151.3 (C-8a), 156.8 (C-7), 164.6 (C-2). EIMS: m/z: 284 (M^+, 100), 269 (22), 251 (57), 139 (27%), 118 (24), 70 (15), 39 (15) (Figures 1, 2).

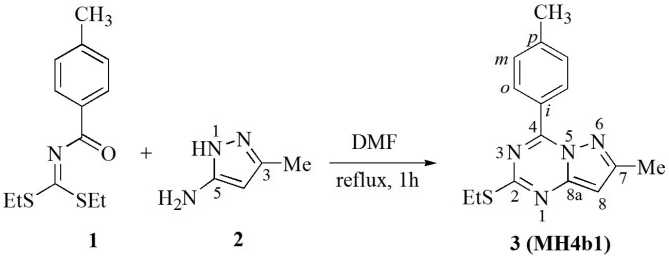

FIGURE 1 - Schematic synthesis of 2-ethylthio-7-methyl-4-(4-methylphenyl)pyrazolo [1,5-*a*][1,3,5]triazine (MH4b1).

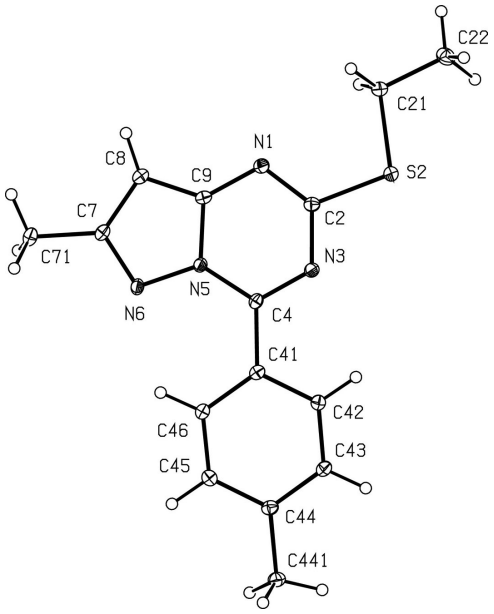

FIGURE 2 - 2-Ethylthio-7-methyl-4-(4-methylphenyl) pyrazolo[1,5-*a*][1,3,5]triazine (MH4b1), a compound obtained by an efficient one-step reaction from *S,S*-diethyl 4-methyl-benzoylimidodithiocarbonate and 5-amino-3-methylpyrazole, displayed anticonvulsant properties in mice.

In vivo tests

No obvious neurological or autonomic changes were observed in the Irwing test in treated animals, even at 100 mg/kg p.o. of MH4b1. Therefore, the 100 mg/kg dose of this compound was selected for use in screening tests. According to the guidelines to screening compounds administered p.o., a 1 h interval was chosen between administration of the compound and each behavioural test (Voguel, 2002).

MH4b1 showed significant effects in the MES and sleep period tests, but was not active in the PTZ, plus maze, marble burying, forced swimming, rota-rod and

TABLE I – Effects of MH4b1 in screening behavioural models in ICR mice. *: p≤0.05. NS: Not significant. ǂDose (mg/kg). MES: maximal electroshock seizures. PTZ: pentylenetetrazole

Test	Effect	Treatment	Dose[ǂ]	n	Result	*p* value
MES	Hind limb extension	Control	(-)	7	7/7	(-)
		Phenytoin	20	7	0/7	*
		MH4b1	100	7	2/7	*
PTZ	Myoclonus	Control	(-)	7	7/7	(-)
		Clonazepam	0.5	7	0/7	*
		MH4b1	100	7	7/7	NS
Plus maze	Percent time/frequency in open arms	Control	(-)	6	21±5/37±5	(-)
		Diazepam	0.5	6	46±5/51±3	*
		MH4b1	100	6	39±6/36±6	NS
Marble burying	No. of buried marbles	Control	(-)	6	21±5	(-)
		Clonazepam	0.5	6	46±5	*
		MH4b1	100	6	39±6	NS
Rota rod	Time on rota rod (s)	Control	(-)	6	18±3	(-)
		Clonazepam	0.5	6	8±2	*
		MH4b1	100	6	18±2	NS
Sleeping period	Time asleep (min)	Control	(-)	6	17±2	(-)
		Diazepam	0.5	6	34±2	*
		MH4b1	100	6	32±5	*
Forced swimming	Time immobile (s)	Control	(-)	6	172±19	(-)
		Imipramine	0.5	6	65±12	*
		MH4b1	100	6	207±12	NS
Catalepsy	Time cataleptic (min)	Control	(-)	6	683±33	(-)
		Biperiden	3	6	417±82	*
		MH4b1	100	6	669±35	NS

catalepsy tests. The effect of MH4b1 was dose dependent (30 – 300 mg/kg, p.o.) in the MES test (Table I, Figure 3). The reference drugs gave positive results in all models.

In vitro tests

MH4b1 showed a selective inhibitory activity toward MAO-B (Figure 4). The IC_{50} obtained against MAO-B was 24.5 [17.1 – 34.2] μM. The percent inhibition of MAO-A was only 16% at the highest concentration tested (100 μM). MH4b1 did not show activity at the benzodiazepine binding site of the GABA-A receptor, not even at 300 mM (data not shown).

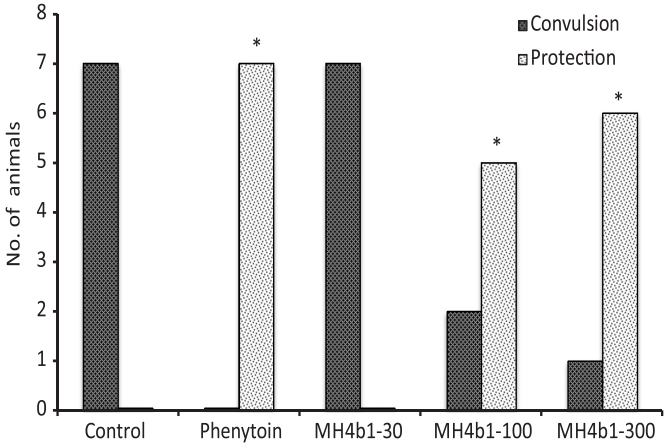

FIGURE 3 - Effect of MH4b1 (30, 100 and 300 mg/kg, p.o) in the MES test in ICR mice (50 mA, 60 Hz, 130 ms), *:p≤0.05 against control (vehicle). Phenytoin (20 mg/kg p.o) was used as a reference.

DISCUSSION

The results of this study show that MH4b1 exerted significant effects in the MES and sleeping period

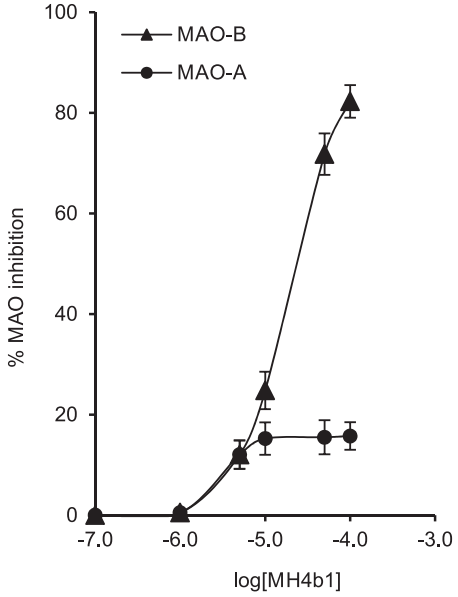

FIGURE 4 - Concentration-response curves of MH4b1 (10^{-7} – 10^{-4} M) against percent inhibition of MAO-A and MAO-B. Data are the mean ± S.E.M. *p<0.05.

screening tests and selective activity against MAO-B, whereas it lacked effect in pentylenetetrazole, plus maze, marble burying, rota-rod and catalepsy tests, and is devoid of binding to the benzodiazepine site of the GABA-A receptor.

The duration of the tonic hindlimb extensor phase in the MES test is a parameter used by some researchers in order to evaluate seizure severity and the protection conferred by a potential anticonvulsant drug (Manocha *et al.*, 2003). The latency period until seizure induced by pentilenetetrazol is considered in a similar fashion (Roeloffs *et al*., 2008). However, the predictive value of a potential anticonvulsant only considering the capacity to prevent the seizure could be a better parameter, because the anticonvulsant should not only be able to decrease the length of time of an experimental seizure but also prevent it. In the search for anti-absence drugs for instance, drugs should prevent the PTZ induced seizures (Löscher *et al.*, 1991).

In this work, MH4b1 was able to prevent the MES but not PTZ seizures, suggesting that GABA-A and T calcium channel binding properties were not implicated in the mechanisms of action of MH4b1. Agents with this mechanism of action, like benzodiazepines and valproic acid, protect against PTZ seizures (White, 1999; Macdonald, Kelly, 1995).

MH4b1 could have an anticonvulsant activity against tonic clonic seizures, considering that MES is a useful screening model to identify drugs for this kind of seizure (Swinyard *et al.,* 1989). Some anticonvulsants like lamotrigine and zonizamide are effective against MES, preventing tonic clonic seizures in man and displaying inhibitory effects against MAO-B (Muck-Seler *et al.,* 2008; Sonsalla *et al.,* 2010). This property suggests a neuroprotective effect due to a reduction in reactive oxygen species generated in the central nervous system by this enzyme, a characteristic that could be of special interest in searching for modern anticonvulsant drugs (Marzo *et al*., 2004).

MAO inhibition has been described as a possible anticonvulsant mechanism of action of some agents. In addition, MAO inhibition is also implied in sedative properties (Kohli *et al*., 1967). This fact is in accordance with the increase in the sleeping period obtained with MH4b1 in pentobarbitone tests in mice. Anticonvulsant GABA-A agonists, like benzodiazepines drugs, protect against MES and PTZ seizures and increase time latency and duration in pentobarbitone tests (Lapa *et al*., 2002). The lack of an effect of MH4b1 against pentylenetetrazole and in the plus maze tests supports the idea that MH4b1 does not act at the benzodiazepine site of the GABA-A receptor (Fradley *et al.,* 2007; Duarte *et al.,* 2008). This presumption was confirmed in the *in vitro* test with tritiated flunitrazepam (^{3}H-FNZ).

Interactions at voltage dependent sodium channels are frequently observed with drugs that are effective in the MES model (Holmes and Zhao, 2008). Such is the case with anticonvulsants like lamotrigine and zonizamide (Vohora *et al.,* 2010), which, in addition, possess MAO-B inhibitory properties. Therefore, this mechanism of action should be considered in future experiments with MH4b1.

It is also interesting that at the dose tested, MH4b1 did not affect motor coordination according to the rotarod test, whereas clonazepam, the reference drug test, did. This could suggest a good safety profile for MH4b1 as an anticonvulsant (Kamiński *et al.,* 2008). Moreover, the lack of activity of MH4b1 in the haloperidol induced catalepsy test could imply that this moiety does not have pivotal central anticholinergic properties. In addition, the negative results against forced swimming are in accordance with a MAO-B inhibitory profile, like that of deprenyl, which displays good activity against MAO-B at inactive doses in FST (Shimazu *et al*., 2005).

According to these results MH4b1, with a positive effect in the MES test but a negative effect in the PTZ test, along with a sedative but not anxiolytic or anti-depressant-like profile, could be consistent with MAO-B inhibition properties, although another mechanism, like sodium channels antagonism, should be considered.

The structure of MH4b1 seems to show that the 4-methylphenyl moiety could play a key role in

the anticonvulsant properties of this compound. The electron-releasing, steric and lipophilic characteristics of the substituent at position 4 of the aryl ring would be important for the activity of this pyrazolotriazine, but further structure activity relationship experiments are needed to verify this proposition.

CONCLUSION

In conclusion, 2-ethylthio-7-methyl-4-(4-methylphenyl)pyrazolo[1,5-*a*][1,3,5]triazine (MH4b1) displays anticonvulsant effects in mice and selective inhibition of MAO-B. More experiments are needed in order to pinpoint its precise mechanism of action.

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CONFLICTS OF INTEREST

There have not been any conflicts of interest in carrying out this work.

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