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Anticonvulsant effects of acute treatment with cyane-carvone at repeated oral doses in epilepsy models

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ABSTRACT

Epilepsy affects about 40 million people worldwide. Many drugs block seizures, but have little effect in preventing or curing this disease. So the search for new drugs for epilepsy treatment using animal models prior to testing in humans is important. Increasingly pharmaceutical industries invest in the Research & Drug Development area to seek safe and effective new therapeutic alternatives to the currently available epilepsy treatment. In this perspective, natural compounds have been investigated in epilepsy models, particularly the monoterpenes obtained from medicinal plants. In our study we investigated the effects of cyane-carvone (CC), a synthetic substance prepared from natural a monoterpene, carvone, against pilocarpine- (PILO), pentylenetetrazole- (PTZ) and picrotoxine (PTX)-induced seizures in mice after acute treatment with repeated oral doses (CC 25, 50 and 75 mg/kg) for 14 days. CC in all doses tested showed increase in latency to first seizure, decrease in percentages of seizuring animals as well as reduction percentages of dead animals ($p < 0.05$) in PILO, PTZ and PTX groups when compared with vehicle. However, these effects were not reversed by flumazenil, benzodiazepine (BZD) antagonist used to investigate the CC action mechanism. Our results suggest that acute treatment with CC at the doses tested can exert anticonvulsant effects in PILO, PTZ and PTX epilepsy models. In addition, our data suggest that CC could act in an allosteric site of $GABA_A$, which would be different from the site in which BDZ acts, since flumazenil was not able to reverse any of CC effects on the modulation of seizure parameters related with epilepsy models investigated. New studies should be conducted to investigate CC effects in other neurotransmitter systems. Nevertheless, our study reinforces the hypothesis that CC could be used, after further research, as a new pharmaceutical formulation and a promising alternative for epilepsy treatment, since it showed anticonvulsant effects.

1. Introduction

Epilepsy is a disease that affects about 40 million people worldwide (Njamnshi et al., 2010). Many drugs block seizures, but have little effect in preventing or curing this disease, and the search for new drugs for epilepsy treatment using animal models prior to testing in humans is important (Loeb, 2011). Among the drugs that act on the central nervous system, altering cognition and psychomotor activity, benzodiazepines can be highlighted for their therapeutic effects like sedation, hypnosis and muscle relaxation, but have high rates of tolerance and dependence (Telles Filho et al., 2011). Given this information, it is plausible to investigate new compounds with anticonvulsant properties devoid of these side effects. In this regard, various studies have investigated the anticonvulsant effects of natural compounds, as well as synthetic compounds derived from essential oils isolated from monoterpenes.

Carvone (p-mentha-6,8-dien-2-one) is a monoterpene ketone found as the main active component of various essential oils. It is obtained by distillation and occurs naturally as the enantiomers (+)- and (−)-carvone (Gonçalves et al., 2010). Lippia alba (Mill.) N.E. Brown (Verbenaceae) is widely used in different regions of Central and South America as a tranquilizer and (R)-(-)-carvone is the main constituent of this plant (Hatano et al., 2012). (-)-Carvone is also found in spearmint (Mentha spicata var. crispa) essential oil, which is widely used as an odor and flavor additive (Souza et al., 2013). In studies conducted by De Sousa et al. (2007), (+)-carvone significantly

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increased the latency of pentylenetetrazol- and picrotoxin-induced seizures in mice. This information reinforces the need to investigate the effects of cyane-carvone, a carvone derivative, obtained from essential oils in epilepsy models as a new therapeutic approach to epilepsy treatment.

In addition, cyane-carvone showed a potential antioxidant activity in vitro, by its capacity to inhibit hydroxyl radical formation inhibition and remove nitric oxide, and also by preventing thiobarbituric acid reactive substances (TBARS) production (Costa et al., 2012c). Costa et al. (2012a) have shown that cyane-carvone possesses anticonvulsant activity probably due to the modulation of cholinergic system and reduction of neuronal oxidative stress in vivo mainly through free radical scavenging capacity. Their study showed that cyane-carvone may be helpful to produce neuronal protection and may be considered as a potential natural anticonvulsant. However, additional studies should be conducted to determine its action mechanism, which further reinforces the need for realize this study.

Cyane-carvone was used at doses up to 2000 mg/kg without causing any death of mice (Costa et al., 2012b) and presented a larger therapeutic window than diazepam, whose lethal dose 50% (LD_{50}) is of 700 mg/kg (Clark, 1978), suggesting that cyane-carvone could be used safely and effectively in epilepsy models. Thus, in this research, the potential pharmacological activity of cyane-carvone was studied in experimental models to elucidate its anticonvulsant effects in mice. This work was performed to investigate the cyane-carvone effects on pilocarpine- (PILO), pentylenetetrazole- (PTZ) and picrotoxine (PTX)-induced seizure models as well as its anticonvulsant action mechanism in mice after acute treatment with repeated oral doses for 14 days.

2. Material and methods

2.1. Cyane-carvone preparation

The compound cyane-carvone (Fig. 1) was prepared in our laboratory as previously described (De Sousa et al., 2010). The purity of cyane-carvone was 90%. The substance is insoluble in water and soluble in chloroform.

2.2. Drugs and reagents

Pilocarpine (PILO), pentylenetetrazole (PTZ), picrotoxin (PTX), polyoxyethylene-sorbitan monolated (Tween 80) and flumazenil were purchased from Sigma (USA) and diazepam (DZP) from Cristália (Brazil). Agents were orally (*p.o.*, by gavage) or intraperitoneally (*i.p.*) administered at a dose volume of 0.1 mL/10 g.

2.3. Animal and experimental protocol

Swiss male mice *Mus musculus* (25–30 g; 2-month-old) were used. Animals were housed in cages with free access to food and water and were kept under standard light–dark cycle (lights on at 07:00 a.m.) and controlled temperature (25 ± 2 °C). Animals were tested during the light period and observed in a closed room with controlled temperature (25 ± 2 °C). Protocol and procedures were approved by the Ethics Committee in Animal Experimentation of UFPI (CEEA/UFPI Number 016/2011). The experiments were performed according to the Guide for Care and Use of Laboratory of US Department of Health and Human Services, Washington, DC (1985).

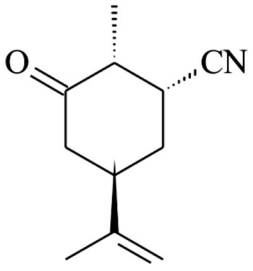

Fig. 1. Chemical structure of cyane-carvone [(1S,2S,5R)-5-isopropenyl-2-methyl-3-oxocyclohexanocarbonitrile] (Costa et al., 2012c).

To investigate the cyane-carvone effects, the animals received acute treatment at repeated oral doses for 14 days. Vehicle group was treated with 0.05% Tween 80 dissolved in 0.9% saline (0.1 mL/kg of weight/day, p.o., $n = 12$; negative control). CC 25, CC 50 and CC 75 groups were treated with cyane-carvone in emulsified in 0.05% Tween 80 dissolved in 0.9% saline at doses of 25, 50 and 75 mg/kg (o.r., $n = 12$), respectively. DZP group was treated with diazepam (5 mg/kg, p.o., $n = 12$; positive control) emulsified vehicle.

In turn, to clarify the cyane-carvone action mechanism, other groups were treated with flumazenil, DZP and CC at a dose of 75 mg/kg and associations. The FLU group was treated with flumazenil (5 mg/kg, p.o., $n = 12$) emulsified vehicle. The DZP + FLU group ($n = 12$) was pretreated with flumazenil (5 mg/kg, o.r.) and, after 15 min, treated with diazepam (5 mg/kg, o.r.). The CC 75 + FLU group ($n = 12$) was pretreated with flumazenil (5 mg/kg, o.r.) and, after 15 min, treated with cyane-carvone (75 mg/kg, o.r.).

2.4. Pilocarpine-induced seizure test

This model was developed by Turski et al. (1983). After 30 min from the last dose of CC, all groups received pilocarpine (400 mg/kg, i.p.; PILO). Direct observation by the researcher was performed for 4 h to monitor latency to the first seizure (tonic–clonic seizures with or without rearing), the number of animals that seized and death (Freitas, 2009).

2.5. Pentylenetetrazole-induced seizure test

Pentylenetetrazole (PTZ) was used to induce clonic convulsions (Smith et al., 2007). After 30 min from the last dose of CC, all groups received PTZ (60 mg/kg, i.p.) and animals were observed for 4 h to monitor the same parameters of the previous test.

2.6. Picrotoxin-induced seizure test

After 30 min from the last dose of CC, all groups were treated with picrotoxin (PTX) at dose 8 mg/kg (i.p.). Immediately after the administration of the convulsant agent, the animals were observed for 24 h to monitor the same previous parameters (Lehmann et al., 1988; Bum et al., 2001).

2.7. Statistical analysis

The data were shown as mean ± standard error mean. For statistical analysis, latency to the first convulsion was examined through analysis of variance (ANOVA) for multiple comparisons and Student–Newman–Keuls as *post hoc* test by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). Chi square test was used to analyse percentage of animals with seizures and mortality rate. Differences were considered statistically significant when $p < 0.05$.

3. Results

Cyane-carvone effects in PILO-induced seizures in mice are presented in Table 1. CC 25, CC 50 and CC 75 groups showed a significant increase in latency to first seizure [$F_{(19.9)} = 174.8\%$, $F_{(23.6)} = 225.9\%$ and $F_{(34.6)} = 381.0\%$, respectively; $p < 0.05$] when compared with the vehicle group, and this effect was not reversed by flumazenil. Percentages of number of animals with seizure decreased significantly in CC 25, CC 50 and CC 75 groups [$F_{(75)} = 25\%$, $F_{(50)} = 50\%$ and $F_{(25)} = 75\%$,

Table 1
Effects of acute treatment with cyane-carvone (25, 50 and 75 mg/kg) at repeated oral doses on PILO-induced seizure test in mice.

Treatments	Dose (mg/kg)	Latency (s)	Seizure (%)	Death animals (%)
Vehicle	–	7.2 ± 1.1	100	100
DZP	5	27.7 ± 1.4[a]	16.7[b]	16.7[b]
FLU	5	7.4 ± 1.5	100	100
CC	25	19.9 ± 1.0[a]	75[b]	75[b]
	50	23.6 ± 0.7[a]	50[b]	50[b]
	75	34.6 ± 0.6[a,g,h]	25[b]	25[b]
DZP + FLU	5 + 5	7.2 ± 0.8[c]	100[e]	100[e]
CC + FLU	75 + 5	35.7 ± 1.3[d]	16.7[f]	16.7[f]

Values were shown as mean ± S.E.M. for 8 mice (per group). [a]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from vehicle. [b]$p < 0.01$ (χ^2 test), significantly different from vehicle. [c]$p < 0.001$, (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the DZP group. [d]$p < 0.001$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 75 group. [e]$p < 0.01$ (χ^2 test), significantly different from the DZP group. [f]$p < 0.01$ (χ^2 test), significantly different from the CC 75 group. [g]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 25 group. [h]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 50 group.

respectively; $p < 0.05$] when compared with the vehicle group. Percentages of number of dead animals decreased significantly in CC 25, CC 50 and CC 75 groups [$F_{(75)} = 25\%$, $F_{(50)} = 50\%$ and $F_{(25)} = 75\%$, respectively; $p < 0.05$] when compared with the vehicle group. Flumazenil significantly reversed the effects of diazepam 5 mg/kg; however, it did not reverse the effects of cyane-carvone 75 mg/kg on the parameters tested in PILO-induced seizures.

The evaluation of cyane-carvone effects in PTZ-induced seizures in mice is shown in Table 2. CC 25, CC 50 and CC 75 groups showed a significant increase in latency to first seizure [$F_{(99.6)} = 33.3\%$, $F_{(123.8)} = 65.8\%$ and $F_{(237.9)} = 218.7\%$, respectively; $p < 0.05$] when compared with the vehicle group, and this effect was not reversed by flumazenil. Percentages of number of animals with seizure decreased significantly in CC 25, CC 50 and CC 75 groups [$F_{(91.7)} = 8.3\%$, $F_{(75)} = 25\%$ and $F_{(58.4)} = 41.7\%$, respectively; $p < 0.05$] when compared with the vehicle group. Percentages of number of dead animals decreased significantly in CC 25, CC 50 and CC 75 groups [$F_{(91.7)} = 8.3\%$, $F_{(75)} = 25\%$ and $F_{(58.4)} = 41.7\%$, respectively; $p < 0.05$] when compared with the vehicle group. Similarly to the previous test, flumazenil also significantly reversed the anticonvulsant effects of diazepam 5 mg/kg; however, it did not reverse the effects of cyane-carvone 75 mg/kg on the parameters analyzed in PTZ-induced seizures.

The analyses of cyane-carvone effects in PTX-induced seizures in mice are shown in Table 3. CC 50 and CC 75 groups showed a significant increase in latency to first seizure [$F_{(531.9)} = 9.6\%$ and $F_{(1003.2)} = 106.7\%$, respectively, $p < 0.05$] when compared with the vehicle group. Percentages of number of animals with seizure decreased significantly in CC 50 and CC 75 groups [$F_{(83.3)} = 16.7\%$ and $F_{(66.7)} = 33.3\%$, respectively; $p < 0.05$] when compared with the vehicle group. Percentages of number of dead animals decreased significantly in CC 50 and CC 75 groups [$F_{(83.3)} = 16.7\%$ and $F_{(66.7)} = 33.3\%$, respectively; $p < 0.05$] when compared with the vehicle group.

Table 2
Effects of acute treatment with cyane-carvone (25, 50 and 75 mg/kg) at repeated oral doses on PTZ-induced seizure test in mice.

Treatments	Dose (mg/kg)	Latency (s)	Seizures (%)	Death animals (%)
Vehicle	–	74.7 ± 1.5	100	100
DZP	5	879.4 ± 0.9[a]	30[b]	30[b]
FLU	5	73.0 ± 2.0	100	100
CC	25	99.6 ± 1.3[a]	91.7[b]	91.7[b]
	50	123.8 ± 2.8[a]	75[b]	75[b]
	75	237.9 ± 2.2[a,g,h]	58.4[b]	58.4[b]
DZP + FLU	5 + 5	75.0 ± 0.6[c]	100[e]	100[e]
CC + FLU	75 + 5	234.7 ± 2.8[d]	66.7[f]	66.7[f]

Values were shown as the mean ± S.E.M. for 12 mice (per group). [a]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from vehicle. [b]$p < 0.01$ (χ^2 test), significantly different from vehicle. [c]$p < 0.001$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the DZP group. [d]$p < 0.001$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 75 group. [e]$p < 0.01$ (χ^2 test), significantly different from the DZP group. [f]$p < 0.01$ (χ^2 test), significantly different from the CC 75 group. [g]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 25 group. [h]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 50 group.

Table 3
Effects of acute treatment with cyane-carvone (25, 50 and 75 mg/kg) at repeated oral doses on PTX-induced seizure test in mice.

Treatments	Dose (mg/kg)	Latency (s)	Seizures (%)	Death animals (%)
Vehicle	–	485.3 ± 4.8	100	100
DZP	5	1267.9 ± 4.1[a]	20[b]	20[b]
CC	25	378.5 ± 0.6	91.7	91.7
	50	531.9 ± 0.4[a,c]	83.3[b]	83.3[b]
	75	1003.2 ± 1.8[a,c,d]	66.7[b]	66.7[b]

Values were shown as the mean ± S.E.M. for 8 mice (per group). [a]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from vehicle. [b]$p < 0.01$ (χ^2 test), significantly different from vehicle. [c]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 25 group. [d]$p < 0.05$ (ANOVA followed by *t*-Student–Neuman–Keuls as *post hoc* test), significantly different from the CC 50 group.

4. Discussion

New treatments to epilepsy should fight a large number of brain insults capable of producing seizures. Literature shows that there is a long period of time between latency and the insult for the first clinical seizure, but our knowledge of the epileptogenic process itself is still limited (Loeb, 2011). Epilepsy is a common disease and has a negative impact on the patient's life, whose treatment cannot cure. Investigation of epilepsy development or epileptogenesis has yielded new insights into potential therapies that may prevent epilepsy (Blumenfeld, 2011).

Among models used to study epilepsy, PILO model in rodents reproduces the main features of mesial temporal lobe epilepsy related to hippocampus sclerosis (MTLE-HS) in humans (Lopes et al., 2013). Costa et al. (2012a) observed the anticonvulsant and antioxidant effects of acute treatment with cyane-carvone on pilocarpine model, but their results were not generalizable to other seizure models. Pilocarpine is a cholinergic drug, and so, even the cyane-carvone can be affecting the cholinergic system in pilocarpine model. The possible effectiveness of cyane-carvone in protecting the brain from neurotoxicity that follows pilocarpine-induced seizures by stimulation of the AChE activity is described.

In this work, following the administration of PILO in the vehicle group, mice showed some behavioral changes: piloerection, akinesia, ataxia, tremor, automatisms and wet-dog shakes. These behavioral changes were continuous until the installation of seizures, including clonic movements of the upper extremities, which occurred in 100% of animals at 7.2 ± 1.1 s after administration of PILO. In the same group, the seizures progressed to the development of status epilepticus in 100% of the animals and the survival rate was 00%.

Cyane-carvone administration increased installation time of first seizure and reduced both the number of animals showing seizures and the number of dead animals when compared with vehicle. The results suggest that cyane-carvone can present a significant anticonvulsant action in seizure model induced by PILO and are in agreement with a previous study, probably due to the modulation of the cholinergic system (Costa et al., 2012).

The induction of epileptic seizures by pentylenetetrazol is other validated model to study epileptic seizures. According to Okoye et al. (2013), herbal preparations of *Annona senegalensis* Pers. (Annonaceae) root bark are used in Nigerian ethnomedicine for epilepsy treatment and febrile seizures. The researchers found that kaurenoic acid is the active constituent that exhibited activity against PTZ-induced seizures in

mice. In addition, Ramos et al. (2012) have suggested that the GABAergic, glutamatergic and adenosinergic systems are involved in PTZ-induced seizures, as well as alterations in NMDA, kainite, A1 receptors and benzodiazepine binding site densities.

Cyane-carvone protected mice against PTZ and significantly delayed the onset of myoclonus jerks and tonic seizures. We assessed the CC effects (25, 50, and 75 mg/kg i.p.) on PTZ-induced generalized seizures in adult male mice. The results suggest that cyane-carvone can also present a significant anticonvulsant action in seizure model induced by PTZ.

Flumazenil is a benzodiazepine antagonist (Saxona et al., 2010). The pretreatment with flumazenil prior to administration of diazepam blocked the anticonvulsant effect of the benzodiazepine in the PILO and PTZ-induced seizures tests. However, the benzodiazepine antagonist did not block cyane-carvone anticonvulsant effect. Thereat, cyane-carvone could act either in an allosteric site of $GABA_A$, which would be different from the site in which BZD acts, or in other neurotransmitter systems.

Epilepsy is a chronic neurological disorder characterized by recurrent seizures. However, approximately one-third of epilepsy patients still suffer from uncontrolled seizures. Effective treatments for epilepsy are yet to be developed. N(6)-(3-methoxyl-4-hydroxybenzyl) adenine riboside is a N(6)-substitued adenosine analog that had a dose-related anticonvulsant effect in PTX-induced seizure model (Li et al., 2013). Epileptic seizures have been related to both increase in glutamatergic activation and decrease in GABAergic inhibition (Kesim et al., 2012). It has been shown that GABAergic and glutamatergic synaptic activity are very important on epileptic seizures in hippocampal pyramidal CA3 cells, and epileptic seizures are caused by the decrease in the gabaergic activity (Lasztoczi et al., 2009).

PTX is a specific $GABA_A$ chloride channel blocker (Kesim et al., 2012). In the PTX-test, cyane-carvone increased installation time of first seizure and reduced the number of animals showing seizures and the number of dead animals when compared to the vehicle group. These results suggest that cyane-carvone can also present a significant anticonvulsant action in seizure model induced by PTX.

Fig. 2 shows the possible mechanism of action of cyane-carvone, involving binding site (diferent of BDZ binding site) in $GABA_A$ receptor.

5. Conclusion

Acute treatment with cyane-carvone at repeated oral doses for 14 days showed anticonvulsant effect in the PILO, PTZ and PTX models of induced seizures in mice, noting that cyane-carvone can act either in an allosteric site of $GABA_A$, which would be different from the site in which BZD acts, or in other neurotransmitter systems. Thus, the results support the knowledge that cyane-carvone can be used, after further research, as a new pharmaceutical formulation and a promising alternative for epilepsy treatment.

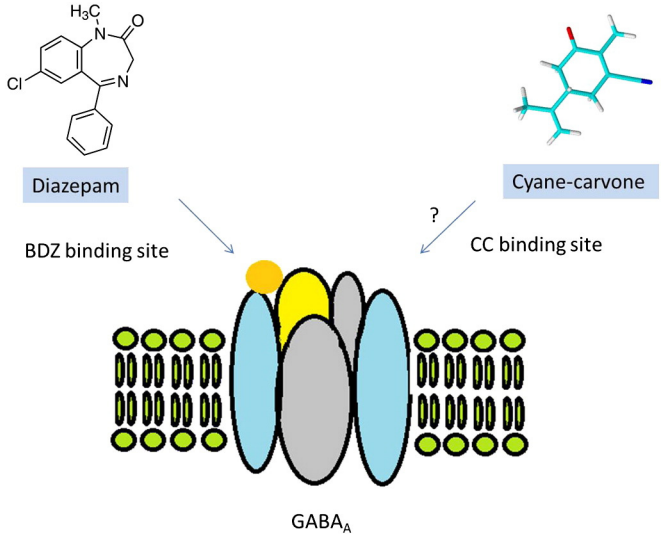

Fig. 2. Possible action mechanism of cyane-carvone in the $GABA_A$ receptor.

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