Bioorganic & Medicinal Chemistry Letters 24 (2014) 5446–5449

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Seizure prevention by the naturally occurring phenols, carvacrol and thymol in a partial seizure-psychomotor model

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ARTICLE INFO

Article history:
Received 2 July 2014
Revised 3 October 2014
Accepted 8 October 2014
Available online 14 October 2014

Keywords:
Isopropyl phenol
Anticonvulsant
Antiseizure
Antiseizure model

ABSTRACT

The natural compounds carvacrol and thymol completely prevented seizures in the 6 Hz, 32 mA partial seizure model. Carvacrol and thymol, both exhibited an ED_{50} = 35.8 mg/kg, ip and yielded protective indices of 5.3 and 3.4, respectively. At 44 mA current intensity, carvacrol and thymol exhibited ED_{50}s of 88.82 mg/kg (PI = 2.15) and 73.0 mg/kg (PI = 1.65), respectively. Thymol, but not carvacrol showed partial inhibitory activity in the maximal electroshock (MES), sc Metrazol (scMET) and Corneal-kindled models. These results suggest that carvacrol and thymol are more efficacious anticonvulsants than suggested by their lower efficacies in the conventional MES and scMET tests.

The alkyl phenolic compound, carvacrol (5-isopropyl-2-methyl phenol) and its structural isomer thymol (2-isopropyl-5-methyl phenol) occur as natural products in oregano, thyme and other herbs.[1] Studies have shown that they exhibit a number of beneficial effects in mammals and other biological systems. Both compounds have antioxidant,[2] antimicrobial[3] and anti-inflammatory effects.[4–6] Carvacrol has been shown to have anticancer,[7] antinociception and antiviral effects.[8]

The effects of these compounds on the central nervous system (CNS) are less well known. As low molecular weight isopropyl phenols, they are closely related to the potent anesthetic and sedative propofol (2,6-diisopropylphenol). In addition to causing anesthesia, propofol exerts anticonvulsant properties in sub-anesthetic doses. Propofol is emerging as a preferred therapy in aborting seizures in human status epilepticus.[9] It is believed to primarily depress CNS activity by acting as a positive allosteric modulator of $GABA_A$ receptors.[10] Other mechanisms may include inhibition of sodium channels[11,12] and NMDA receptors.[13]

Rodent studies show that thymol and carvacrol are likewise active in the CNS. Carvacrol can increase dopamine and serotonin in the brains of rats.[14] Both can enhance cognition in animals that are challenged with β-amyloid or scopolamine.[15] Carvacrol exhibits anxiolytic properties in mice.[16] Mechanistically, thymol and carvacrol have been shown to potentiate $GABA_A$ receptors.[17,18] Even though closely related in structure, evidence has been presented that thymol binds to $GABA_A$ receptors at sites that are distinct from those that bind propofol.[17]

The anticonvulsant activities of carvacrol and thymol have not been fully characterized. Quintans-Júnior et al.[19] showed that carvacrol could increase seizure latency in the maximal shock (MES) and pentylenetetrazole (scPTZ or scMET) mice models. At high doses (>100 mg/kg) it provided incomplete protection suggesting that it is not an highly efficacious anticonvulsant. Recently, it was reported that thymol increases latencies to seizures in the acute MES (maximal electroshock) and scMET animal models. However, complete protection was not achieved.[20]

Certain molecules may be useful anticonvulsants even though they are partially or ineffective in preventing seizures in the conventional scMET and MES models. Levetiracetam lacks efficacy in the scMET and MES models. However, it was found to be effective in the 6 Hz minimal clonic seizure model (also known as the psychomotor model or partial seizure model).[21] It has become a successful anticonvulsant drug in humans with much less sedative potential than most marketed anticonvulsants. This and other findings support the notion that the 6 Hz model is useful for screening for anticonvulsant compounds that have unique mechanisms of action. The lesser ability of other classes of anticonvulsants to prevent seizures in the 6 Hz model screening also contributed to the thinking that the 6 Hz model is a model of therapy resistant seizures.[22] This study sought to clarify the anticonvulsant properties of carvacrol and thymol with a focus on their anticonvulsant effectiveness in the 6 Hz model.

Carvacrol (5-isopropyl-2-methyl-phenol), Thymol (2-isopropyl-5-methyl-phenol), 2-isopropyl phenol, 3-isopropyl phenol, and

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http://dx.doi.org/10.1016/j.bmcl.2014.10.028
0960-894X/

4-isopropyl phenol were purchased from Sigma–Aldrich (St. Louis, MO). Each compound was further purified by column chromatography before submitting to NINDS Anticonvulsant Screening Program (ASP).

Compounds were screened by the NINDS Anticonvulsant Screening Program in whole mouse models: 6 Hz psychomotor seizure model of partial epilepsy (32 and 44 mA),[23–25] the maximal electroshock model (MES), the scMET test (pentylenetetrazole, Metrazol).[26] Adult male CF No 1 albino mice (26–30 g) were used for the 6 Hz test and 18–25 g mice were used in the MES and scMET tests. The animal are housed, fed and handled in a manner consistent with the recommendations in the National Council Publication, 'Guide for Care and Use of Laboratory Animals'. All compounds were administered ip and polyethylene glycol in saline was used as the solvent.

The 6 Hz test (minimal clonic seizure, or psychomotor test) involved delivering a corneal stimulus of 32 or 44 mA for 3 s.[21] This current elicits psychomotor seizures in 97% of untreated animals. Seizures consist of a minimal clonic phase followed by stereotyped, automatistic behaviors. Animals not showing such behaviors were considered protected. Behavioral toxicity (psychomotor or neurotoxicity) was determined by the rotorod test. Each animal is placed on a rotorod at 6 rpm following dosing. The animal is considered neurotoxic if it falls off the rotorod three times in a one minute period. Dose response curves were determined from 4 to 5 doses with 8 animals receiving each dose. The ED_{50}, TD_{50} (neurotoxic dose), and confidence intervals were determined by probit analysis. Protective index (PI) is defined as TD_{50}/ED_{50}.

In brief, the MES model, which generates generalized tonic-clonic seizures, involved pre-treating mice with experimental compound, and administering 60 Hz of alternating current (50 mA) for 2 s via corneal electrodes.[23] Prior to current administration, the animal's corneas were treated topically with tetracaine. Animals are considered protected if the hind limb tonic extensor component is absent. The scMET test was done by pre-treating mice with experimental compound and administering 85 mg/kg Metrazol into the loose folds of the skin in the middle of the neck. Eighty-five mg/kg Metrazol is the dose that causes seizures in 97% (CD97) of control animals. The animals were observed for 30 min for progression or absence of seizure which consists of clonic spasms of 3–5 s of the forelimbs, hindlimbs, jaws or vibrasse.[24]

In the corneal kindling model,[27] mice are kindled electrically with 3 s stimulation, 8 mA, 60 Hz, with corneal electrodes to a criterion of 10 consecutive Stage 5 seizures (facial clonus and head nodding progressing to forelimb clonus, and finally rearing and falling accompanied by a generalized clonic seizure as described by Racine[28]). Stage 5 is generally reached after twice daily stimulation for 8 days. With continued stimulation once a day, animals usually progress to a reproducible Stage 5 after 10–14 additional days. At least 72 h after the mice have been kindled, the test substance is administered ip at the previously determined time to peak effect and each animal is given the electrical stimulus indicated above. Following stimulus, the animals are observed for the presence or absence of the rearing and falling criteria of a Stage 5 seizure. Treated animals not displaying a Stage 3, 4, or 5 seizures are considered protected. In the Pilocarpine seizure model, seizures were induced in rats by systemic administration of pilocarpine hydrochloride (50 mg/kg, ip), a muscarinic cholinergic agonist.[29] Administration of lithium chloride (20 mg/kg; ip) 24 h prior to pilocarpine reduces the dose of pilocarpine needed to induce status epilepticus (SE). Pilocarpine induces seizures within a few minutes and those animals showing no seizures after 45 min of pilocarpine were removed from the study. At the time of the first stage 3 (Racine scale)[28] seizure or higher, rats were randomized into treatment groups and given test compound 30 min after the first stage 3 seizure. Animals were observed and scored for seizure severity for 1.5 h before being returned to their home cages.

All compounds were initially screened in the 6 Hz test using a current intensity of 32 mA and a dose of 100 mg/kg, ip. All compounds presented in Figure 1 provided complete protection (4 out of 4 animals) at one or more time points of 0.25, 0.5, 1, 2 or 4 h after dosing. Each showed low neurotoxicity except for phenol **5** (4-isopropylphenol) which caused neurotoxicity in 4 of 4 animals at 4 h after dosing with 100 mg/kg.

The ED_{50} and TD_{50} for each phenol were determined following assessment of time-to-peak effect and time-to-peak neurotoxicity at doses that did not cause a complete protection (Table 1). Phenol **1** (Carvacrol) and **2** (Thymol) yielded ED_{50} values lower than those of phenols **3**, **4**, and **5**. Furthermore, their ED_{50}s were nearly identical at 36 mg/kg. TD_{50}s determined for phenol **1** and **2** were 190 and 120 mg/kg, respectively, which yielded protective indices of 5.3 and 3.3, respectively. In addition to yielding higher ED_{50}s, phenols **3** and **4** had PIs lower than those of phenol **1** and **2**, reflecting their lower TD_{50}s. Phenol **5** was found to be highly neurotoxic as assessed by the rotorod test. Also, multiple animal deaths occurred in subsequent 2–24 h periods at doses from 100 to 200 mg/kg, the latter being the highest dose tested. For that reason a TD_{50} was not determined. No deaths occurred from phenol **5** at doses of 75 mg/kg and below.

Compound screening in the 6 Hz test using a 44 mA current intensity showed that phenol **1** and phenol **2** retain effectiveness in this more stringent model (Table 2). Thymol (**2**) caused complete protection at 0.25 h and carvacrol (**1**) caused near complete protection (3/4) at 4 h following dosing with 100 mg/kg. Phenol **4** caused complete protection at 1 h. Carvacrol (**1**) and Thymol (**2**) were quantitatively assessed in the 6 Hz 44 mA model and yielded ED_{50}s of 88.82 (95% CI = 76.12–101.35) at 4 h and 73 mg/kg (95% CI = 61.74–87.83) at 0.5 h, respectively. Thus, carvacrol and thymol have PIs of 2.15 and 1.65, respectively, in the 44 mA model.

As a group phenols **1**, **2**, **3** and **4** were less effective in inhibiting seizures in the MES and scMET models (Table 3). Among the phenols tested, thymol (**2**) caused the greatest inhibition of seizures in the MES model where protection of 3 of 4 animals occurred at 0.25 and 4 h at 100 mg/kg. Phenol **3** showed low protection where only one of four animals was protected at several time points. Carvacrol (**1**) in contrast, showed no activity the MES test at any time point. In the scMET test, phenol **2** (Thymol) and **3** caused partial protection, whereas phenol **1** (Carvacrol) caused none.

Carvacrol (**1**) and thymol (**2**) were compared in the corneal-kindled model at their ED_{50} doses determined in the 6 Hz 32 mA test, 36 mg/kg. Carvacrol (**1**) provided no protection at this dose whereas thymol (**2**) provided slight protection (Table 4). Testing of carvacrol and thymol in the Li-pilocarpine at 65 mg/kg ip showed that neither inhibited seizures resulting from this cholinergic overstimulation model (Table 5). Phenol **3** inhibited seizures in only 1 of 7 rats. Neither compound prevented weight loss caused by Li-pilocarpine treatment.

Animal screening provides useful information regarding potential antiseizure effectiveness of experimental compounds in humans. All marketed anticonvulsants are active in suppressing seizures in one or more of the MES, scMET, or 6 Hz acute seizure models.[27] Prior screening of propofol and several propofol

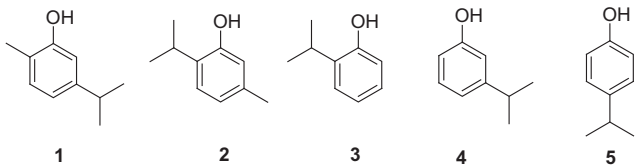

Figure 1. Isopropyl phenols