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Evidence for the involvement of descending pain-inhibitory mechanisms in the attenuation of cancer pain by carvacrol aided through a docking study

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ABSTRACT

Aims: The present study evaluated the carvacrol (CARV) effect on hyperalgesia and nociception induced by sarcoma 180 (S180) in mice.
Main methods: Carvacrol treatment (12.5–50 mg/kg s.c.) once daily for 15 days was started 24 h after injection of the sarcoma cells in the hind paw (s.c.). Mice were evaluated for mechanical sensitivity (von Frey), spontaneous and palpation-induced nociception, limb use and tumor growth on alternate days. CARV effects on the central nervous system were evaluated through immunofluorescence for Fos protein. Molecular docking studies also were performed to evaluate intermolecular interactions of the carvacrol and muscimol, as ligands of interleukin-10 and $GABA_A$ receptors.
Key findings: CARV was able to significantly reduce mechanical hyperalgesia and spontaneous and palpation-induced nociception, improve use paw, decrease the number of positively marked neurons in lumbar spinal cord and activate periaqueductal gray, nucleus raphe magnus and *locus coeruleus*. CARV also caused significant decreased tumor growth. Docking studies showed favorable interaction overlay of the CARV with IL-10 and $GABA_{A.}$
Significance: Together, these results demonstrated that CARV may be an interesting option for the development of new analgesic drugs for the management of cancer pain.
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Introduction

Pain is one of the most common and distressing symptoms experienced by over half of all cancer patients (Schmidt et al., 2010) and its complex pathologic process is the main challenge for an effective treatment (Sarantopoulos, 2007). This symptom can interfere with daily activities of patients with neoplasms, reducing the quality of life and promoting important psychosocial disorders, in addition to an increase in the cost of treatment (Ling et al., 2012).

Throughout history, natural products and plant food supplements have contributed unequivocally to the pain and inflammation control (Di Lorenzo et al., 2013), the example of morphine isolated from *Papaver somniferum* and more recently, ziconotide, a peptide from snails which is used for the treatment of chronic pain. Hence, herbal medicines used in pain therapy can contribute to restoring the quality of life to a patient and may enhance conventional management of different types of pain, such as rheumatologic diseases, back pain, cancer, diabetic peripheral neuropathy and migraine (Zareba, 2009).

Carvacrol (CARV), a phenolic monoterpene found in Origanum oil (Lamiaceae family), has considerable analgesic and anti-inflammatory effects; it also modulates central neurotransmitter pathways, such as dopaminergic, serotoninergic and GABAergic systems, and the release of inflammatory mediators (Guimarães et al., 2010, 2012, 2013; Melo et al., 2010, 2011; Cavalcante Melo et al., 2012; Lima et al., 2013; Zotti et al., 2013). Although several studies describe its analgesic effects, there are no reports on the central nervous system (CNS) areas activated by CARV, as well as its potential use in cancer pain.

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Due to the emerging need for new therapeutic options for the treatment of cancer pain and the vast therapeutic potential of CARV, this study aimed to evaluate its effect on the nociception induced by Sarcoma 180 in mice and neuronal regions involved. We also performed a docking study between carvacrol and $GABA_{A}$, and IL-10 receptors, and a comparative docking between the carvacrol and the muscimol, a potent, selective agonist against the $GABA_A$ receptor.

Materials and methods

Chemicals

Carvacrol (5-isopropyl-2-methylphenol, CARV, 98% purity), cremophor, sodium chloride, trypan blue, glycerol, DABCO, glycine and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Morphine and lactated Ringer's solution were purchased from Cristália (São Paulo, São Paulo, Brazil). Rabbit anti-Fos k-25 was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and the donkey anti-rabbit Alexa Fluor 488 was purchased from Life Technologies (Carlsbad, California, USA).

Animals

Male *Swiss* mice used (28–32 g; 2–3 months of age) were randomly housed in appropriate cages at 21 ± 2 °C on a 12 h light/dark cycle with free access to food (Purina®, Brazil) and water. Experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS 43/09) at the Federal University of Sergipe, and all handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines for the use of animals in pain research (Zimmermann, 1983).

Tumor cell and implantation

Sarcoma 180 (S180) tumor cells, which had been maintained in the peritoneal cavity of Swiss mice, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará. A suspension of 10^6 viable S180 cells per 25 μl of lactated Ringer's solution was implanted subcutaneously into the plantar region of mice. Animals of the sham group received only 25 μl of lactated Ringer's solution. This methodology was adapted from Kamioka et al. (1999) and Lee et al.(2009).

Treatment and behavioral studies

Twenty-four hours after administration of S180, animals (n = 10/group) were treated daily with vehicle (saline + cremophor 0.4% v/v), CARV (12.5, 25 or 50 mg/kg) or morphine (15 mg/kg) by subcutaneous route, until the fifteenth day, and submitted to behavioral evaluation on alternate days. The investigator responsible for the behavioral evaluation was blind to the experimental situation of each animal.

Mechanical hyperalgesia

The animals were screened for the sensitivity towards mechanical stimulation generated by a gradual increase in pressure of a hand-held force transducer (electronic anesthesiometer, model: EFF-301, Insight®, Brazil) adapted with a polypropylene tip. This stimulus evokes a hind paw flexion reflex that corresponds to the paw withdrawal followed by clear flinching movements.

Spontaneous and palpation-induced nociception

Mice were placed in boxes scattered and allowed to acclimate for 10 min. Flinching behaviors were counted during a 10-min observation period. Afterwards, non-noxious palpation of the tumor-bearing paw was performed during 2 min and the number of flinching behaviors was quantified for 2 min (Sabino et al., 2003).

Movement-evoked pain

In the same boxes scattered, the limb use was assessed as previously described by Luger et al. (2001), through the observation of the mouse while walking in a continuous motion. Limping and/or guarding behavior of the right (sarcoma-treated) hind limb was rated on the following scale: 0 = complete lack of use, 1 = partial non-use, 2 = limping and guarding, 3 = limping, and 4 = normal walking.

Measurement of paw volume

The effect of CARV on tumor growth caused by the plantar incubation of S180 was evaluated through right paw volume, which was measured by the dislocation of the water column of a plethysmometer (Insight®, Brazil) before (time zero) and on every other day up to 15 days.

Measurement of forelimb grip strength

In order to check for possible changes in neuromuscular function, we measured the tension force of limbs using the commercial grip strength meter (Insight®, Brazil), before the treatment (s.c.) of tumor-free animals with vehicle or CARV (12.5, 25 or 50 mg/kg) and 30, 60 and 120 min after treatment (Van Riezen and Boersma, 1969).

Immunofluorescence

Ninety minutes after the injection of CARV (50 mg/kg; s.c.), morphine (15 mg/kg; s.c.) or vehicle, the animals (n = 6, per group) were perfused and the brains and lumbar spinal cords (L4–L6) were collected and cryoprotected for immunofluorescence processing to Fos protein.

Frozen serial transverse sections (20 μm) of all brains and lumbar spinal cord lamina I were collected on gelatinized glass slides. The tissue sections were stored at −80 °C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) 5 times for 5 min. and incubated with 0.01 M glycine in PBS for 10 min. Non-specific protein binding was blocked by the incubation of the sections for 30 min. in a solution containing 2% BSA. Then, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for 2 h with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with glycerol solution (79% glycerol + 10% PBS + 1% DABCO). As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5 times for 5 min. Pictures from Fos positive brain and spinal cord areas were acquired for each animal with an Axioskop 2 plus, Carl Zeiss, Germany. The brain regions were classified according to Paxinus and Watsu Atlas, 1997. Neurons were counted by the free software ImageJ (National Institutes of Health) using a plug-in (written by authors) that uses the same level of label intensity to select and count the Fos-positive cells.

Docking studies

The docking study investigated the intermolecular interactions of the carvacrol and muscimol, as ligands (Fig. 7) and 3 macromolecules available in the Protein Data Bank: crystal structure of human interleukin-10 (PDB ID 2ILK) and $GABA_A$ receptors (PDB IDs 1KJT and 3D32). The simulations were performed on the Auto-Dock 4.2 software (Morris et al., 2009). Receptor and ligand preparation was carried out using VEGA ZZ 3.0.1 (Pedretti et al., 2004) and Molegro Molecular Viewer 2.5.

Initially, the structures were saved in pqbqt format to be used for docking calculations. PyRx 0.8 software (Wolf, 2009) was used to aid the steps of job submission and analysis of the results. The grid maps were calculated with AutoGrid. The three-dimensional grid box with 60 Å grid size (x, y, z) with a spacing of 0.300 Å, was created. Each ligand was docked into this grid with the Lamarckian algorithm as implemented in AutoDock. The genetic-based algorithm ran 12 simulations per

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