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In vivo and *in silico* anti-inflammatory mechanism of action of the semisynthetic (−)-cubebin derivatives (−)-hinokinin and (−)-O-benzylcubebin

Thaís C. Lima [a], Rodrigo Lucarini [a], Angelica C. Volpe [a], Carolina Q.J. de Andrade [a], Alice M.P. Souza [a], Patricia M. Pauletti [a], Ana H. Januário [a], Guilherme V. Símaro [a], Jairo K. Bastos [b], Wilson R. Cunha [a], Alexandre Borges [c], Rosangela da Silva Laurentiz [c], Valéria A. Conforti [d], Renato L.T. Parreira [a], Carly H.G. Borges [a], Giovanni F. Caramori [e], Karla F. Andriani [e], Márcio L.A. e Silva [a,*]

[a] Núcleo de Pesquisas em Ciências Exatas e Tecnológicas, Universidade de Franca, Av. Dr. Armando Salles Oliveira 201, Franca – SP 14404-600, Brazil
[b] School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Av. do Café S/N, Ribeirão Preto – SP 14040-903, Brazil
[c] Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Av Brasil 56, Ilha Solteira – SP 15385-000, Brazil
[d] Programa de Pós-Graduação em Ciência Animal, Universidade de Franca, Av. Dr. Armando Salles Oliveira 201, Franca – SP 14404-600, Brazil
[e] Departamento de Química, Universidade Federal de Santa Catarina, Campus Universitário Trindade, CP 476, Florianópolis – SC 88040-900, Brazil

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ABSTRACT

(−)-Cubebin (CUB), isolated from seeds of *Piper cubeba*, was used as starting material to obtain the derivatives (−)-hinokinin (HK) and (−)-O-benzyl cubebin (OBZ). Using paw edema as the experimental model and different chemical mediators (prostaglandin and dextran), it was observed that both derivatives were active in comparison with both negative (5% Tween® 80 in saline) and positive (indomethacin) controls. The highest reduction in the prostaglandin-induced edema was achieved by OBZ (66.0%), while HK caused a 59.2% reduction. Nonetheless, the dextran-induced paw edema was not significantly reduced by either of the derivatives (HK or OBZ), which inhibited edema formation by 18.3% and 3.5%, respectively, in contrast with the positive control, cyproheptadine, which reduced the edema by 56.0%. The docking analysis showed that OBZ presented the most stable ligand-receptor (COX-2 – cyclooxygenase-2) interaction in comparison with CUB and HK.

Inflammation is a major component of autoimmune diseases, which are characterized by an immune response against the endogenous components identified as foreign by the immune system. This response results in an attack that produces diverse inflammation-related clinical symptoms, depending on the tissue or system affected.[1a] The drugs known as nonsteroidal anti-inflammatory drugs (NSAIDs) are able to suppress the body's defense reaction to minimize the damage and provide relief to the patient. These medications may be hormonal or non-hormonal. Steroidal anti-inflammatory drugs, also known as glucocorticoids, steroids or corticosteroids are inhibitors of prostaglandin (PG) production and leukotrienes by the inhibitory action on the enzyme phospholipase A2, through the release of lipocortin-1, a protein that acts as an anti-inflammatory mediator. The end result of anti-inflammatory action of these drugs is the partial or complete reduction in the release of leukotrienes and also PGs.[1]

The compounds produced by the cyclooxygenase pathway are well accepted as mediators of the inflammatory response, and nonsteroidal anti-inflammatory drugs are known to act on the cyclooxygenase (COX) enzyme system. The NSAIDs block the formation of PGs, thereby resulting in analgesic, antipyretic, and anti-inflammatory effects. Therefore, many cells produce PGs, and these compounds are responsible for a vast array of biological actions. PGs are also synthesized at the end of peripheral nerves and participate in the signal transmission of pain to the spinal cord and brain. It has recently been found that COX exists in two isoforms that differ in their basal expression, cellular localization, and induction during inflammation. Differences in the pharmacological profile of various NSAIDs may occur at different levels of selectivity for COX-1 and COX-2. Both potency and selectivity of NSAIDs are directly related to their toxicity to the gastric system, kidneys, and liver.[2,3]

* Corresponding author.
E-mail address: marcio.silva@unifran.edu.br (M.L.A. e Silva).

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The compounds investigated in this study belong to the chemical class of dibenzylbutyrolactone lignans. This class of compounds is widely distributed in the plant kingdom and was also found in human and other mammals' waste,[4] which might be related to hormonal activity.[5] Dibenzylbutyrolactone lignans display a wide spectrum of biological actions, such as antitumoral,[6,7] antiviral,[8] anti-inflammatory,[9,10] inhibition of platelet aggregation,[11] and trypanocidal,[12] among others. The denomination of lignan class of compounds was created in 1936 by Haworth, which consists of a phenylpropanoid dimmer.[13–15] There are eight subclasses of lignans, including furan, furofuran, dibenzylbutane, dibenzylbutyrolactone, dibenzylbutyrolactol, dibenzocyclooctadiene, aryltetraline and arylnaphthalene (Fig. S1, Supplementary material)[13,16] Several representatives of lignans, particularly the dibenzylbutyrolactone (diarylbutanolide lactone), display biological properties, including inhibition of topoisomerase, which makes them candidates for anticancer drugs, anti-platelet-activating factor (PAF), and antiviral actions.[17,18]

(−)-Cubebin (CUB) was isolated from the hexane extract of the leaves of *Zanthoxyllum naranjillo* by Bastos et al.[19] CUB is also found in the seeds of *Piper cubeba*, in amounts of 1–3%.[20,21] CUB derivatives, such as (−)-O-benzyl-cubebin (OBZ) and (−)-hinokinin (HK) (Fig. 1), are more effective as anti-inflammatory agents than CUB itself.

CUB (Figs. S2–S5) was isolated from seeds of *Piper cubeba*, bought from Floral Seeds, New Delhi, India, as previously reported,[22] and its derivatives HK (Figs. S6–S9) and OBZ (Figs. S10–S13) were obtained and chemically characterized as described and in the Supplementary material.[1,9]

Protocols for evaluating anti-inflammatory activity in experimental models typically induce inflammation via injection of chemical mediators, such as PGs, dextran, etc.[1,19,20] Prior to the beginning of the experiments, all procedures were approved by the Animal Ethics Committee of the University of Franca in accordance with the national and international accepted principles for laboratory animal use and care (Number 005/09-A). Animals used in this study were male Wistar rats (±170 g) for the paw edema test, all from the Central Animal Facility of the University of São Paulo in the city of Ribeirão Preto. The animals were caged in groups of eight at room temperature (25 ± 3 °C) with food and water *ad libitum*. Twelve hours before the beginning of the experiments, the animals were transferred to the laboratory where they continued to be provided with water *ad libitum*. The animals (n = 24 rats) used in the anti-inflammatory test were randomly assigned to one of three treatment groups (8 rats per group), as follows: (i) OBZ in saline (40 mg/kg of body weight, orally); (ii) HK in saline (40 mg/kg body weight, orally); and (iii) control (5% solution of Tween® in saline, orally). Prior to treatment, the volumes of the left and right hind paws were measured for a baseline reading using a plethysmometer (model No. 7140, Ugo Basile), as previously described by Ferreira.[5] Then, the animals received the assigned treatment (OBZ, HK, or saline). At 30 min post-treatment, a volume of 0.1 mL of either dextran (100 μg/paw) or PGE2 (10 μg/paw) was injected into the plantar area of the right hind paw of each rat to act as phlogistic agents, while the left paw was injected with an equal volume of saline (negative control). Indomethacin and cyproheptadine were used as positive controls for PGE_2 and dextran, respectively. After administration of the phlogistic agents, paw volumes were then measured hourly from the time of treatment until the fifth hour, to assess edema formation as evidenced by an increase in the volume of the paw. The control group received only saline solution with 5% Tween® 80.[5]

Injection of either prostaglandin (PGE_2) or dextran into the rats' paws induced significant edema. The peak of inflammation was observed at the 3rd hour for all tested chemical mediators. Previous treatment with CUB derivatives, administered orally, significantly inhibited edema formation by PGE_2. The group of animals injected solely with a solution of 5% Tween® 80 in saline (negative control) displayed intense edema. OBZ and HK reduced PGE_2-induced edema by 66.0% and 59.0%, respectively. Indomethacin displayed 54.0% of inhibition (Fig. 2).[1] It was included the significance level of $p < 0.05$ for both Table 1 and Fig. 2, which means confidence $\geqslant$95% in the obtained results. Previous studies of anti-inflammatory activity of semi-synthetic OBZ and HK derivatives showed that both derivatives were effective in reducing paw edema induced by carrageenan at 40 mg/kg,[1,10] which was associated with increased prostaglandin synthesis.[23] Thus, in addition to previous study showing that both HK and OBZ displayed significant anti-inflammatory effects, our results demonstrate that these compounds act by inhibiting prostaglandin synthesis.

The use of dextran, another phlogistic agent, led to intense edema formation, which was not diminished by orally treatment with 40 mg/kg CUB derivatives in comparison with negative control group. Dextran is a polymer of glucose that has a broad range of applicability. Dextransucrase is an extracellular enzyme responsible for the synthesis of dextran. It acts on the sucrose molecule, releasing and transferring fructose to a glucose molecule receptor, in the case of dextran molecules in expansion.[24] Inhibition of inflammation by OBZ and HK derivatives were 3.5% and 18.3%, respectively, while the positive control (cyproheptadine) inhibited inflammation by 56.0% (Table 1).

It is known that, unlike local anesthetics and narcotics, NSAIDs usually do not increase the pain threshold in normal tissues. OBZ

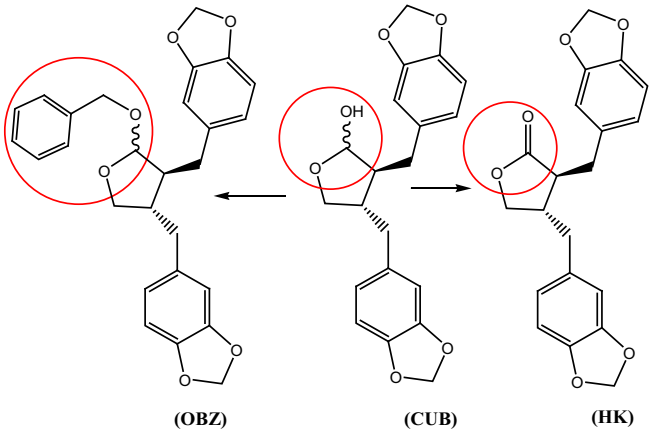

Fig. 1. Chemical structures of (−)-cubebin (CUB), (−)-hinokinin (HK) and (−)-O-benzyl-cubebin (OBZ). The red circle shows the structural differences between the compounds (lactolic group in CUB; lactonic group in HK, and lactolic with the replacement of hydrogen by a benzyl moiety turning into an acetal group in OBZ).

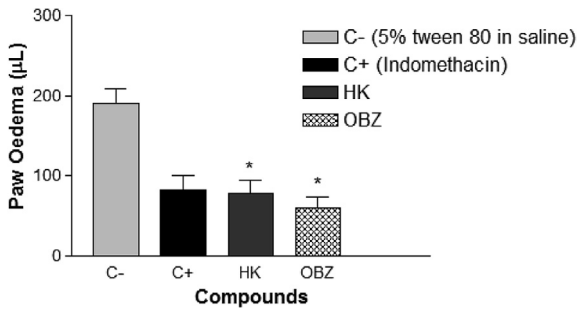

Fig. 2. Effect of (−)-cubebin derivatives (HK) and (OBZ) given orally at a dose of 40 mg/kg in rats with paw edema induced by intraplantar injection of PGE_2 (10 μg/paw) at the third hour post-injection. 5% tween in saline was given as negative control, and indomethacin, as positive control (10 mg/kg). $p < 0.05$.

Table 1
Effects of oral administration of (−)-cubebin derivatives (40 mg/kg) in the rat paw edema assay as a percentage of edema inhibition. Inflammation was induced by injection of either PGE2 or dextran into the paw.

Compounds	Percentage inhibition of paw edema	
	PGE_2	Dextran
Indomethacin	91.25 ± 20.39 (54.0%)	–
Cyproheptadine	–	156.30 ± 42.88 (56.0%)
OBZ	65 ± 13.50 (66.0%)[a]	391.30 ± 61.45 (3.5%)
HK	140 ± 14.11 (59.2%)[a]	367.50 ± 61.35 (18.3%)

[a] $p < 0.05$.

was more effective than HK in diminishing paw edema and both were more effective than indomethacin, a standard drug used as a positive control in PGE_2-induced edema protocols (Table 1). Nonetheless, OBZ and HK were much less effective in inhibiting edema caused by dextran injection. It is possible that the mechanism of action of the tested compounds might be associated with inhibition of prostaglandin synthesis, as observed for most NSAIDs.[25]

The molecular docking was performed to investigate the possible interaction sites between COX-2 (cyclooxygenase-2) and CUB, HK and OBZ ligands. The ORCA3.0.2 package[26,27] was used to obtain HK, CUB and OBZ optimized structures at BP86-D3/def2-TZVPP level of theory.[28–30] The absence of imaginary eigenvalues at Hessian matrix confirmed that all structure corresponds to local at PES. The COX-2 structure with 1.81 Å of resolution was obtained at the Protein Data Bank (PDB ID: 4ph9).[31] Due to the lack of crystallography data of COX-2 available for *Homo sapiens* organism, the **4ph9** structure was considered as the best choice (*Mus musculus* organism). For guaranteeing the chain homologous between *Mus musculus* and *Homo sapiens* organism, the BLAST was used,[32] in which 88% of concordance was obtained. To fill the lateral chains the SWISSMODEL server was used.[33–36] However all residues were found and the chain was reconstructed using PDB_HYDRO to assure that all N-terminal amino acids were taken into account.[37] Before the molecular docking at DOCKTHOR portal,[38,39] the pKa values for each residue were obtained at PDB2PQR using PROPKA[40] and the model reliability was evaluated at QMEAN server,[41] presenting a z-score of 0.812, showing good reliability. For the docking analyses at DOCKTHOR all ligands was treated as flexible allowing bond rotation. Molecular graphics were obtained with the UCSF Chimera package (Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco - supported by NIGMS *P*41-GM103311).[42]

To validate the applied methodology, the redocking analysis with B-octylglucoside (BOG) and COX-2 as receptor was performed previously. The active site of COX-2 for BOG604 remained as the same site as observed in the experimental X-ray structure (PDB ID: 4ph9) at run 13 and model 19, showing a good accuracy and reliability of the used model (Fig. S14).

The docking results of CUB, OBZ and HK, including total and interaction energies values for the first ten most stable configurations are shown in Table S1. The total energy suggests that CUB (2.74–12.79 kcal/mol) and HK (9.53 –12.45 kcal/mol) systems are energetically more stable than OBZ (25.46–30.85 kcal/mol). However, an opposite trend is observed in relation to the interaction energy between COX-2 receptor and the ligands. For instance, it was observed that OBZ presented the most stable ligand-receptor interaction (−25.13 to −27.01 kcal/mol) in comparison with CUB (−21.10 to −23.56 kcal/mol) and HK (−21.51 to −22.41 kcal/mol).

The three first configurations for CUB with the interaction energy as the limiting factor showed that both energy (−23.56 to −22.97 kcal/mol) and geometry are very close in comparison with **18.1** (Fig. 3). For the three configurations, the active site remains

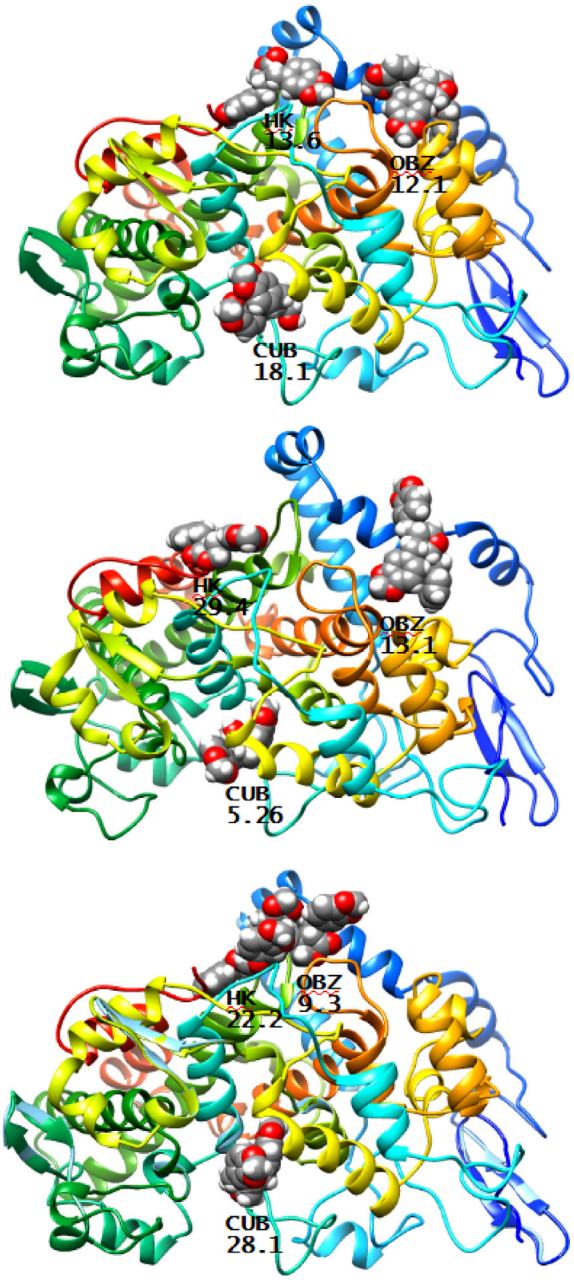

Fig. 3. Docking for COX-2 as receptor and CUB, OBZ and HK as ligands, in which the three first configurations are shown.

the same, lying between GLN172 and HIS357. Other amino acids as LEU 263, VAL 260, PHE 177 and THR 183 are also in contact with CUB ligand (Figs. 3 and S15).

Unlike COX-2 and CUB interactions, where the active sites are formed with almost the same amino acids, for COX-2 and OBZ interactions the first two configurations (**12.1** and **13.1**) are in the same active site while the third configuration (**9.3**) is almost 19.4 Å far from the others two sites (LYS442, LYS480 and TYR59), and it is also surrounded by different amino acids (ASP484, GLN162 and HYS325) (Figs. 3 and S16). For the second configuration (**13.1**), no contacts above 5 Å was observed (Fig. S16). However, the interaction energy values between these three sites are quite similar in magnitude, ranging from −26.39 to −27.01 kcal/mol.

The three first docking configurations for HK are in different amino acids domains (Figs. 3 and S17). The docking active site for HK **13.6** is very close in distance to the OBZ **9.3**, in which the HK ligand is found between (ASP316, GLN310, GLN161). The two other sites are also surrounded by different amino acids, the **29.4** lies between GLN161, SER548 and GLN319 while **22.2** in HIS320, PRO480 and GLN161. There are three active sites for COX-2 in relation to CUB, OBZ and HK ligands, in which two of them are in close distance within each other. HK and OBZ show that these studied NSAIDs should prefer to interact with COX-2 in HK and OBZ amino acids domains, due to the largest values of the interaction energy, as previously discussed. It should be pointed out that HK and OBZ might not be selective for COX-2, by acting in both COX-1 and COX-2. The molecular docking parameters for COX-1 are not well established, do not allowing an accurate analysis of docking.

In summary, the dibenzylbutyrolactone (−)-cubebin (CUB) derivatives, hinokinin (HK) and (−)-*O*-benzyl cubebin (OBZ), can be easily obtained by partial syntheses. On one hand, both compounds were active in the rat paw edema assay using prostaglandin as mediator, but on the other hand they were weakly active using dextran for the edema formation. Additionally, docking analysis showed that OBZ displayed the most stable ligand-receptor (COX-2 – cyclooxygenase-2) interaction in comparison with CUB and HK. Therefore, this class of compounds may act by inhibiting cyclooxygenases.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.11.081.

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