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Design, synthesis and pharmacological evaluation of CVIB, a codrug of carvacrol and ibuprofen as a novel anti-inflammatory agent

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ABSTRACT

The search for new drugs with anti-inflammatory properties remains a challenge for modern medicine. Among the various strategies for drug discovery, deriving new chemical entities from known bioactive natural and/or synthetic compounds remains a promising approach. Here, we designed and synthesized CVIB, a codrug developed by association of carvacrol (a phenolic monoterpene) with ibuprofen (a non-steroidal anti-inflammatory drug). *In silico* pharmacokinetic and physicochemical properties evaluation indicated low aqueous solubility (LogP ≥5.0). Nevertheless, the hybrid presented excellent oral bioavailability, gastrointestinal tract absorption, and low toxicity. CVIB did not present cytotoxicity in peripheral blood mononuclear cells (PBMCs), and promoted a significant reduction in IL-2, IL-10, IL-17, and IFN-γ cytokine levels *in vitro*. The LD_{50} was estimated to be approximately 5000 mg/kg. CVIB was stable and detectable in human plasma after 24 h. *In vivo* anti-inflammatory evaluations revealed that CVIB at 10 and 50 mg/kg i.p. caused a significant decrease in total leukocyte count ($p < 0.01$) and provoked a significant reduction in IL-1β ($p < 0.01$). CVIB at 10 mg/kg i.p. efficiently decreased inflammatory parameters better than the physical mixture (carvacrol + ibuprofen 10 mg/kg i.p.). The results suggest that the codrug approach is a good option for drug design and development, creating the possibility of combining NSAIDs with natural products in order to obtain new hybrid drugs may be useful for treatment of inflammatory diseases.

1. Introduction

Inflammation is a multifactorial process. It reflects the response of the immune system to damage from physical, chemical and pathogenic factors and occurs in many disorders [1]. Leukotriene B4 (LTB4) and prostaglandin E2 (PGE2), synthesized from arachidonic acid *via* two pathways involving 5-lipoxygenase (5-LOX) and cyclooxygenase (COX), are principal mediators that play crucial roles in the inflammatory response. Enzymes involved in LT and PG biosynthesis are key targets in anti-inflammatory drug discovery [2,3,4].

Current treatment options are generally based on the use of non-steroidal anti-inflammatory drugs (NSAIDs) and steroids. However, we commonly observe various severe side-effects, including gastrointestinal, renal, and hepatic toxicities as well as cardiovascular

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disturbances, often limiting therapeutic adherence [5]. Limited therapeutic treatment options for chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, and gout remain great challenges [6].

Natural products are valuable sources of new biomolecules; they may be synthesized or semi-synthesized for generation of new, more effective and safer drugs for anti-inflammatory, anticancer, and analgesic use, among others [7–8].

Medicinal chemistry is a multidisciplinary area that has as one of its primary objectives to design and discover new bioactive compounds [9]. Among the strategies used in rational drug design and development (RDDD), molecular hybridization (MH) has recently gained considerable prominence due to its relative simplicity as well as a great number of successful outcomes [9–12].

Briefly, the MH strategy consists of covalently linking two or more pharmacophoric units (whole molecules, or molecular fragments) in the same compound with the hope that this new chemical entity will, at least partially, maintain the biological activities of the constituents that gave rise to it, including their mechanisms of action. Using this strategy, researchers seek to improve efficacy, affinity, pharmacodynamic and pharmacokinetic properties, to reduce drug-drug interactions, side-effects and costs, and to combat the emergence of drug resistance in microorganisms and protozoans while maintaining a multiple or at least a dual mode of action [13–15].

Ibuprofen is a commercially-available non-steroidal anti-inflammatory drug (NSAID) used to treat inflammation, pain, and fever, such as in migraines and arthritis [16]. Similar to most other commercial NSAIDs, it is a nonselective cyclooxygenase (COX) inhibitor [16].

Carvacrol is a mono-terpenoid phenol found in the essential oil of oregano, thyme and other aromatic plants [17]. Among its various associated pharmacological properties, anti-inflammatory activity is highlighted. Its mechanism of action involves immunomodulatory activity involving elevation of IL-1β, TNF-α and IL-10 levels [18], antioxidant activity [19,20] and also inhibition of both COX isoforms [21,22].

Therefore, our aim was to evaluate the anti-inflammatory potential of a new codrug, containing carvacrol and ibuprofen as pharmacophoric units (Fig. 1) in order to generate synergistic effects.

This approach has been widely successfully used in RDDD with the aim of improving physicochemical and/or biopharmaceutical properties of therapeutic compounds [23–33], resulting in marketed drugs such as sultamicillin (antibiotic), benorylate and sulfasalazine (anti-inflammatories), in which the hybrid (where subunits are linked by a labile and hydrolysable bond) presents activity profiles superior to those for the parent drugs, or even a physical mixture (parent drugs simultaneously administered) [34–39].

2. Results and discussion

2.1. Chemistry

CVIB was synthesized by direct condensation of the carboxylic acid group of (*R*,*S*)-2-[4-(2-methylpropyl)phenyl]-propanoic acid (Ibuprofen) with the phenolic hydroxyl of (5-isopropyl-2-methylphenol) (carvacrol) to generate the ester CVIB (Fig. 1). As an alternative to obtaining the target hybrid, (benzotriazol-1-yloxy)tris(dimethylamine) phosphonium hexafluorophosphonate, (BOP) was used as coupling agent.

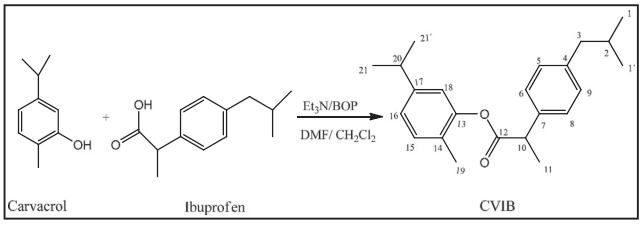

Fig. 1. Synthesis of hybrid CVIB.

BOP is a coupling agent widely used for synthesis of peptides and in other amidation reactions. It presents the great advantage of not requiring an inert atmosphere [40]. However, in this case it was used to enable an esterification.

Confirmation of the chemical structure of CVIB was determined using nuclear magnetic resonance (NMR) and mass spectrometry (MS). Interpretation of the ^{1}H, ^{13}C NMR, COSY, HMBC and HSQC spectra allowed for unequivocal attribution of the chemical shifts (σ) of all hydrogen and carbon atoms in the hybrid (see supplementary material). High resolution mass spectra (HRESI-MS) allowed identification of the molecular ion peak $[C_{23}H_{30}O_2Na]^+$ in m/z = 361.2134, and presented a fragmentation pattern compatible with that expected for the compound.

2.2. In silico pharmacokinetic and physicochemical properties

In parallel, with the free software SwissADME [http://www.swissadme.ch/index.php], several pharmacokinetic and physicochemical properties were determined, including Lipinski rule parameters, topological polar surface area (TPSA), molar refractivity (*A*) aqueous solubility (Table 1), and absorption, distribution, metabolism and elimination (ADME) parameters [41].

Most of these results are presented in the form of two parameter correlation graphs. First, the *BOILED-Egg* (Fig. 2A), which correlates TPSA with lipophilicity (LogP) and provides information related to the following: ability of the compound to cross the blood-brain barrier; passive gastrointestinal absorption; and P-glycoprotein binding potential [42]. Second, the *Bioavailability Radar* (Fig. 2B), which has a direct relationship with *drug-likeness*, where six physicochemical properties are taken into account: lipophilicity, size, polarity, solubility, flexibility and saturation, delimiting the suitable physicochemical space for the oral bioavailability zone—the reddish zone [41].

According to Table 1, CVIB presented only one violation of the Lipinski rules (LogP > 5.0) and the molar refractivity does not exceed the acceptable limits (between 40 and 130 cm^3/mol).

Nevertheless, and in accordance with the *Bioavailability Radar* graph (Fig. 2B), CVIB presented excellent conditions for oral bioavailability and absorption in the gastrointestinal tract (TGI); considering that practically all correlated physicochemical parameters were within the colored area.

These results agree with data from the work of Veber and coworkers [43], who analyzed the molecular properties that influence oral bioavailability of 1100 drug candidates and found that for good oral bioavailability, low TPSA parameters (< 140 Å), and low molecular flexibility (< 10 rotatable bonds) were more important than lipophilicity (LogP).

The *Boiled-Egg* graph confirms that CVIB presents potential TGI absorption (compound located in the white region of the Boiled-Egg), no potential to cross the blood-brain barrier (is not in the yellow part of the egg), and can be effluxed from the central nervous system (CNS) by P-glycoprotein (blue dot).

Table 1
CVIB physicochemical properties predicted using SwissADME software.

Lipinski parameters				*A*	RBN	TPSA
HBA	HBD	MW	LogP			
2	0	338	5.98	106.17	8	26.30 Å

HBA - Hydrogen Bond Acceptor, HBD - Hydrogen Bond Donor; MW- Molecular Weight; LogP – Lipophilicity; *A* – Molar Refractivity; RBN- Number of Rotatable Bonds; TPSA – Topological Polar Surface Area.

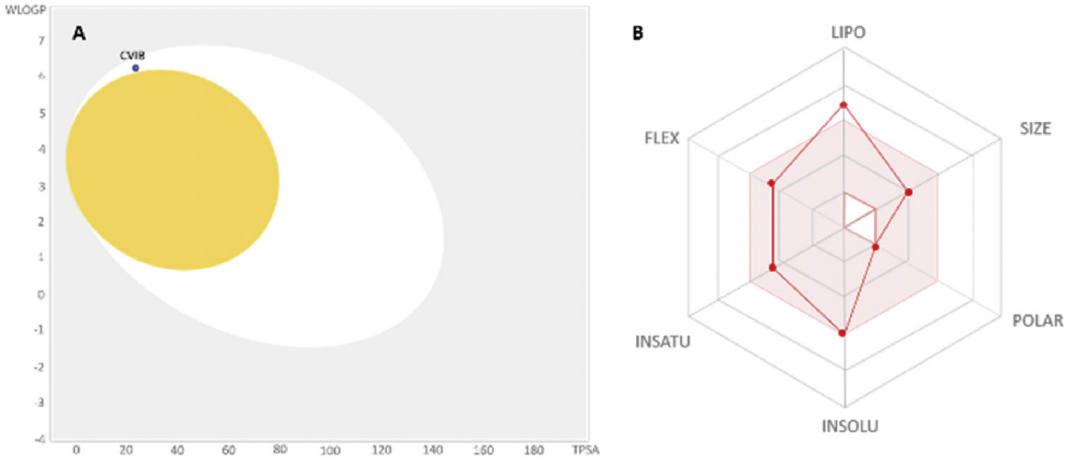

Fig. 2. A) *BOILED-Egg*: Yellow region - compound with potential to cross the blood-brain barrier; White region - compound with characteristics that can be absorbed by TGI; Gray region - compound with unfavorable pharmacokinetic parameters. B) *Bioavailability Radar*: Molecules plotted completely in the pink area present a favorable set of properties for excellent oral bioavailability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
CVIB interaction with cytochrome P-450 oxidase isoforms, as predicted by SwissADME software.

CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
No	Yes	No	Yes	No

The software also analyzed the probability of CVIB being metabolized by isoforms of the cytochrome P-450 oxidase enzyme; responsible for metabolizing xenobiotics. According to Table 2, CVIB is a potential inhibitor of only two (CYP2C19 and CYP2D6) of the five enzymes analyzed, indicating a good liver metabolism potential, thus reducing toxicity risks.

2.3. Experimental chemical and plasmatic stability studies

The chemical stability was determined in PBS for pH 7.4 and Clark-Lubs solution for pH 1.2. Results can be seen in Fig. 3.

We found that CVIB had enough stability to be absorbed because the amount in a single dose would be enough to resist most of the time the compound would be in an acidic environment. On the other hand, at pH 7.4 (the pH circulating blood), CVIB had a rapid decrease in the first hour, and another rapid decrease after 8 h, becoming undetectable by the 24 h time point; this could be an issue. Nevertheless, the compound was very stable in human plasma, probably because of some level of plasma protein binding. Because the compound must be free to be chemically degraded, this binding helped to improve the compound's stability. Furthermore, the plasma stability of CVIB suggests that if future studies show a short half-life of this compound *in vivo*, it would be related to organ metabolism, instead of plasma hydrolysis.

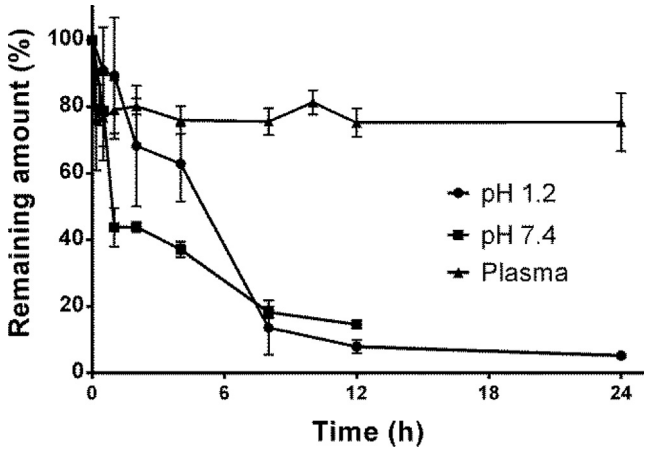

Fig. 3. Stability of CVIB in buffer solutions (pH 1.2 and 7.4) and in human plasma. Data are expresses as mean ± SEM of three independent experiments.

2.4. CVIB experimental log P

The experimental log P assay was performed using the correlation of log P of standard compounds to their capacity factor. The linearity equation was $y = 0.304x - 0.2636$ and the correlation coefficient was higher than 0.98. To obtain the CVIB log P, its capacity factor would be interpolated in the constructed curve, however CVIB have shown a capacity factor higher than the highest standard compound, DDT. Therefore, the CVIB log P is reported as higher than 6.2, which is in agreement to the aforementioned *in silico* physicochemical property.

Even though log P is essential for the compound BBB crossing ability of the compound, other properties, such as TPSA, the number of hydrogen bond donors and acceptors, and dipole moment, also play important role in this characteristic [44]. Taking the partition coefficient into account, the experimental log P higher than 6.2 brings more evidence for the Boiled-Egg graph results. Still, it is out of the suggested log P range for increasing the potential for BBB penetration, which is between 2 and 5 [45,46].

2.5. In vitro cytotoxicity

The *in vitro* cytotoxicity of CVIB, carvacrol ibuprofen and physical mixture carvacrol + ibuprofen was evaluated in peripheral blood mononuclear cells (PBMCs) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. All molecules tested presented mean cell viability of the three independent experiments above 94% at concentrations of 25 and 50 μM, thereby showing no cytotoxicity. Considering these results, we chose concentrations of 25 and 50 μM for evaluation of *in vitro* immunomodulatory activity.

2.6. In vitro cytokine evaluation

In the evaluation of the immunomodulatory effects of CVIB in PBMC from healthy controls stimulated with anti-CD3 and anti-CD28, we observed that the CVIB promoted a statistically significant reduction in IL-2, IL-10, IL-17, and IFN-γ cytokine levels (Fig. 4).

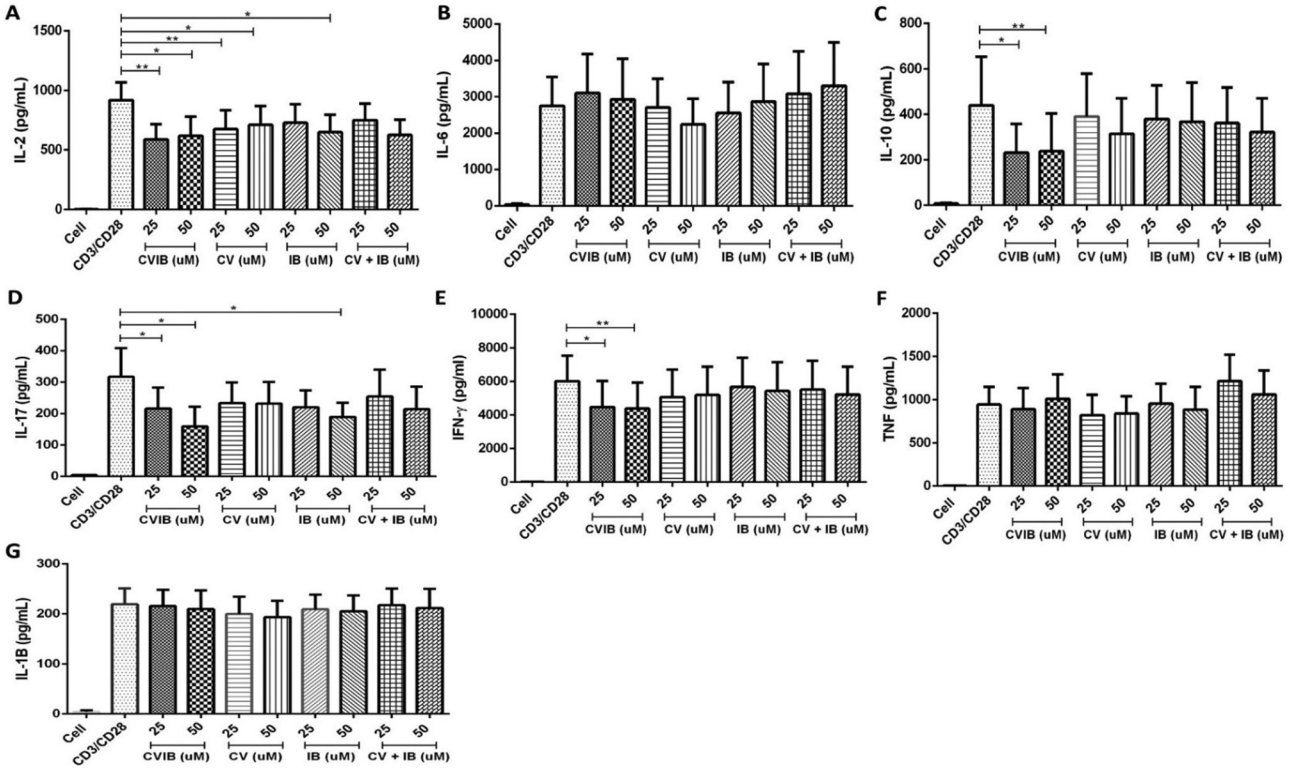

Fig. 4. Immunomodulatory activity of CVIB, carvacrol (CV), ibuprofen (IB) and physical mixture carvacrol + ibuprofen (CV + IB) in PBMC cells. The bars represent cytokine levels IL-2 (A), IL-6 (B), IL-10 (C), IL-17 (D), IFN-γ (E), TNF-α (F), and IL-1β (G) at two different concentrations (25 and 50 μM), non-stimulated (Cell), and stimulated cell (anti-CD3 and anti-CD28 antibodies) conditions. *$p < 0.05$; **$p < 0.01$ (Mean ± SEM, followed by Wilcoxon's signed rank test).

IL-2 (Fig. 4A), IL-10 (Fig. 4C), IL-17 (Fig. 4D) and IFN-γ (Fig. 4E) levels were significantly reduced following treatments with CVIB at 25 and 50 μM ($p = 0.007$ and $p = 0.023$ for IL-2, respectively; $p = 0.031$ and $p = 0.031$ for IL-10, respectively; $p = 0.046$ and $p = 0.031$ for IL-17, respectively; and $p = 0.023$ and $p = 0.039$ for IFN-γ, respectively). All comparisons were performed under stimulation with anti-CD3 and anti-CD28 antibodies. We did not observe significant differences in TNF-α, IL-6 and IL-1β levels after CVIB treatment in PBMCs from healthy controls (Fig. 4B, F and G).

When evaluated separately, carvacrol (CV) significantly reduced IL-2 levels at 25 μM and 50 μM ($p = 0.007$ and $p = 0.023$, respectively) (Fig. 4A). No significant differences were observed in the levels of the other evaluated cytokines. On the other hand, ibuprofen (IB) promoted a significant reduction in the levels of IL-2 (Fig. 4A) and IL-17 (Fig. 4D) at 50 μM ($p = 0.015$ for both). The physical mixture CV + IB at various concentrations did not significantly change the levels of any of the evaluated cytokines (Fig. 4A–G).

Cytokines are produced by a variety of cells and act as important mediators of the immune response. Exacerbated production of these mediators may result in pathological processes such as inflammatory and autoimmune diseases [47–49]. Drugs that reduce cytokine production may represent good therapeutic strategies for inflammatory conditions.

2.7. Evaluation of acute nonclinical toxicity

Hybrid CVIB at 300 mg/kg induced no animal deaths. Following guideline n° 423 from the OECD, the experiment was repeated with the same dose and the results were the same. Then, we tested CVIB at 2000 mg/kg and this too did not induce deaths (Table 3).

In the first 30 min, CNS depressant effects such as ocular reflex loss were observed at both the 300 mg/kg dose and the 2000 mg/kg dose; however this effect disappeared after a 1-h period at both doses. LD_{50} was estimated to be approximately 5000 mg/kg.

Table 3
Evaluation of acute nonclinical toxicity. Effects of single doses (v.o.) of CVIB in mice (n = 3).

Groups	Dose	M/T[a]	Behavioral effects
Control	–	0/3	Loss of ocular reflex
CVIB	300 mg/kg	0/3	Loss of ocular reflex
CVIB	300 mg/kg	0/3	Loss of ocular reflex
CVIB	2000 mg/kg	0/3	Loss of ocular reflex
CVIB	2000 mg/kg	0/3	Loss of ocular reflex

[a] M/T = Number of dead mice/number of treated mice.

2.8. In vivo anti-inflammatory evaluation by carrageenan-induced pleurisy assay

In order to evaluate the *in vivo* anti-inflammatory profile of the CVIB hybrid (10, 50 mg/kg, i.p.), we performed the carrageenan-induced pleurisy test. This acute inflammatory model evaluates the participation of various mediators as well as the effects induced by putative drugs in an inflammatory response. The administration of carrageenan increases local vascular permeability, leading to fluid extravasation and leukocyte infiltration [50–52]; it activates immune cells, causing greater production and release of the pro-inflammatory cytokines TNF-α and IL-1β [50,52,53].

The CVIB anti-inflammatory profile data (10, 50 mg/kg, i.p.) are summarized in Fig. 5. We found that the administration of carrageenan into the pleural space in mice induced an inflammatory process with significant increases in total leukocyte counts ($p < 0.001$) and increases in TNF-α ($p < 0.001$), and IL-1β ($p < 0.001$) levels. Treatment with CVIB (10, 50 mg/kg, i.p.) caused a significant decrease in total leukocyte counts ($p < 0.01$), and provoked a significant reduction in IL-1β ($p < 0.01$) production in the pleural fluid when compared

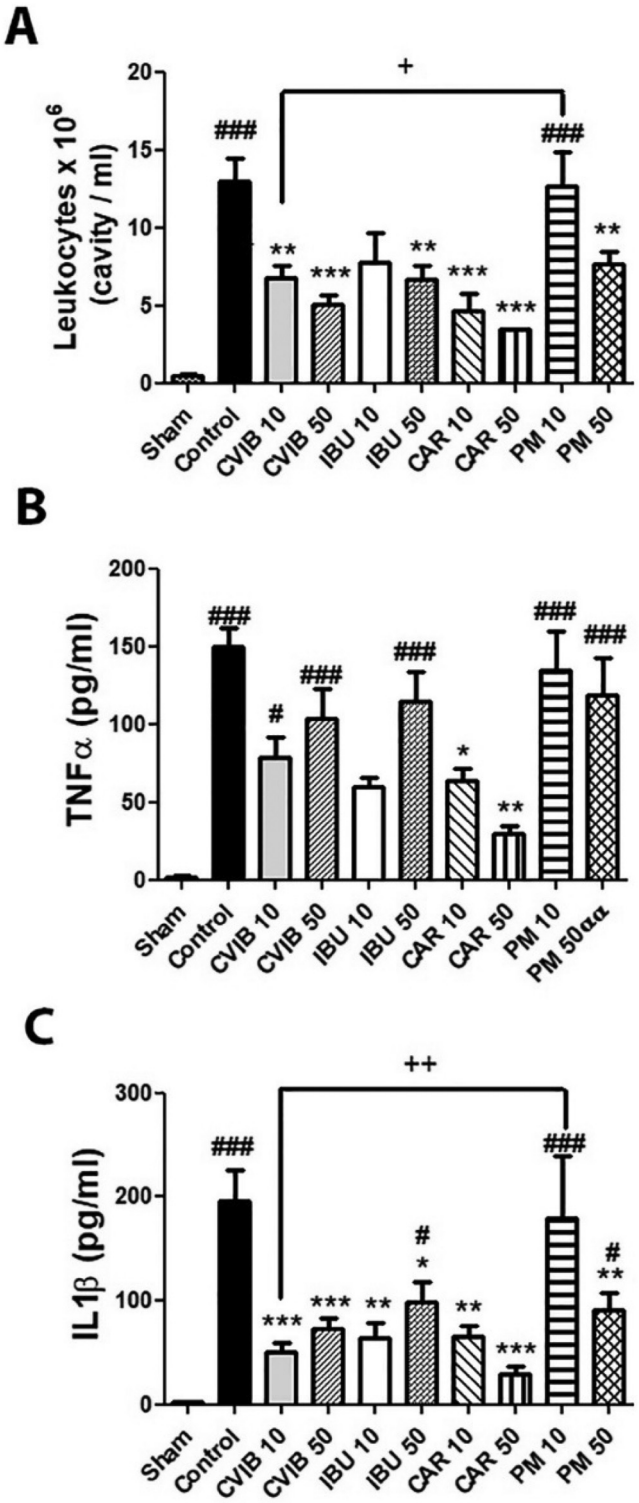

Fig. 5. Effect of CVIB (10, 50 mg/kg, i.p.), carvacrol (10, 50 mg/kg, i.p.), ibuprofen (10, 50 mg/kg, i.p.) and physical mixture (PM) of carvacrol and ibuprofen (10, 50 mg/kg, i.p.) on carrageenan induced pleurisy in mice. The analyses were performed at 4 h after carrageenan injection to evaluate the recruitment of total leukocytes (A), levels of TNF-α (B), and IL-1β (C). Data were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs* control groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs* sham groups; $^{+}p < 0.05$ and $^{++}p < 0.05$ *vs* the physical mixture (10 mg/kg) groups (one-way ANOVA, followed by Tukey's test).

to the control group. However, no significant difference was observed for TNF-α production in the CVIB-treated mice ($p > 0.05$) (Fig. 5A, B and C).

2.9. Pleural fluid cytokine determination

To perform a comparative analysis, cytokines IL-1β and TNF-α were quantified from the pleural fluid of mice in all groups (ibuprofen, carvacrol, physical mixture and CVIB), in the same procedures to those preformed with carvacrol alone [54]. Treatment with ibuprofen (50 mg/kg, i.p.), carvacrol (10 and 50 mg/kg, i.p.), and the physical mixture (50 mg/kg, i.p.) inhibited cell influx induced by carrageenan ($p < 0.01$; $p < 0.001$; $p < 0.01$ respectively), and significantly reduced IL-1β content ($p < 0.05$; $p < 0.01$; $p < 0.01$, respectively).

In addition, carvacrol treatment (10, 50 mg/kg) decreased TNF-α production ($p < 0.01$). Interestingly, for the physical mixture (10 mg/kg i.p.) in the treated-group when compared to the control group ($p > 0.05$), at 4 h after carrageenan injection, no significant changes were observed in total leukocyte counts or in IL-1β or TNF-α levels. However, CVIB (at all doses) produced significant reductions ($p < 0.01$ or $p < 0.001$) in pro-inflammatory cell migration and IL-1β when compared to the control group. However, in this case, CVIB was ineffective in modifying TNF-α levels (Fig. 5: A, B and C). There was also a significant difference between CVIB (10 mg/kg, i.p.) and physical mixture (10 mg/kg, i.p.) in terms of total leukocyte counts ($p < 0.05$) and in IL-1β production ($p < 0.01$) (Fig. 4: A and C). No significant difference was observed for CVIB (50 mg/kg, i.p.) when compared with the physical mixture (50 mg/kg, i.p.), carvacrol (50 mg/kg, i.p.), or ibuprofen (50 mg/kg, i.p.) groups ($p < 0.05$) for leukocyte counts, showing that all were effective anti-inflammatory in this animal model (Fig. 5: A, B and C).

Our findings showed that all doses of CVIB blocked carrageenan-induced cell migration while the physical mixture was ineffective at the lowest dose. This effect was associated with proinflammatory cytokine IL-1β inhibition in the CVIB-treated group; an effect sought in new drugs to mitigate the effects of neuroinflammation, Alzheimer's disease, signaling mediated by caspase-1, and for certain types of chronic dysfunctional pain [55,56]. In inflammatory reactions, IL-1β exerts critical modulatory activity, leading to increases in prostanoid levels and activation of inflammatory pathways [50]. It is possible the CVIB possesses anti-inflammatory activity associated with inhibiting inflammatory mediator production.

We note that CVIB at 10 mg/kg decreased inflammatory parameters; however, the physical mixture (with the same amount of carvacrol and ibuprofen as the hybrid at 10 mg/kg) presented no alterations in inflammatory mediators. Interestingly, and as already described in the literature, carvacrol strongly reduced both cell migration and *in vivo* model tested cytokine levels [54].

Therefore, the approach employed in the present study of codrug assembly provided an improvement in anti-inflammatory properties when compared to simultaneous administration of the parent drugs (the physical mixture). The profiles corroborate formation of a new compound (CVIB) that presents properties different from those of isolated carvacrol and ibuprofen.

Similar results were reported by Theoduloz et al. [39] in which the combined hybrid compound of a terpene, oleanolic acid, and ibuprofen presented better inhibition, being more active than the parent compounds in two inflammation models. The hybrid tested (ARN2508) was a prototype of a class of hybrid compounds that simultaneously inhibit FAAH, COX-1, and COX-2 with high potency, selectivity and oral bioavailability [57]. Other hybrid compounds with great potential anti-inflammatory activity are described in the literature, as in the Pedrosa et al. study [13].

Carvacrol-ibuprofen hybridization reduces the active dose commonly used in current pharmacological posology and may diminish the adverse effects of both carvacrol and ibuprofen. CVIB appears to be a

promising compound for chronic inflammation or chronic inflammatory pain (including neuropathic pain) because of its apparent involvement with the IL-1β pathway [58].

3. Conclusions

This work again demonstrates the success of molecular hybridization and codrug strategy in drug design and development. The chemical link between the carvacrol and ibuprofen pharmacophores generated a new hybrid chemical entity, a codrug, with satisfactory enzymatic stability, promoting improvements in *in vitro* and *in vivo* anti-inflammatory properties, reducing inflammation and leukocyte migration, and inflammatory mediator production, yet maintaining low toxicity.

The fact that the hybrid compound presented a superior anti-inflammatory profile to that of the physical mixture (carvacrol + ibuprofen simultaneously administered) leads us to believe that certain possibilities need to be better investigated. Will the hybrid have a distinct pharmacokinetic profile that allows the parent drugs to remain in the body for longer periods, amplifying the observed effects, and acting as a "prolonged-release pseudo-system"? Could the hybrid bind to different receptors, and or act by different mechanisms of action from its drug components?

Despite requiring these answers, the data suggest that the codrug approach is a viable option in drug design and development, and encourage continued realization of hybridizations between NSAIDs and natural products to obtain useful novel hybrid drugs for the treatment of inflammatory diseases.

4. Experimental section

4.1. Chemistry

4.1.1. General

All chemicals (reagent grade) were purchased from Sigma-Aldrich or Fisher Scientific and were used without further purification. Reactions were monitored by TLC, run on silica gel coated aluminum sheets (silica gel 60 GF_{254}, E. Merck, Germany) and visualized in UV light (254 nm). Purification was performed using column chromatography with silica gel 60 (200–300 mesh ASTM, E. Merck, Germany). The synthesized compound (CVIB), was characterized using ESIMS (high-resolution), using microTOF II (Bruker) and by ^{1}H, ^{13}C and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer in $CDCl_3$ solution referenced to the solvent residual peak.

The chemical shift (σ) was reported in parts per million (ppm) downfield to tetramethylsilane (σ = 0). The coupling constant, *J*, was expressed in Hertz (Hz). The data were reported in the following order: chemical shift, number of protons, multiplicity, coupling constant, and proton/carbon assignment. The multiplicity of proton signals were reported as s: singlet, d: doublet, t: triplet, q: quartet, quint: quintet; sept: septet and dd: doublet of doublets;

4.1.2. General procedure for the preparation of CVIB

Ibuprofen (1.44 mmol) was dissolved in 6 mL of DMF, 6 mL of dichloromethane and 6 mL of triethylamine. The solution was cooled in an ice-water bath and 1.1 eq. mmol of the BOP (1.58 mmol) was added. The solution was kept under stirring for 30 min. In parallel, the mixture of carvacrol (1 eq., 1.44 mmol), dichloromethane (3 mL) and triethylamine (1.8 mL) was kept under stirring for 30 min. The carvacrol solution was slowly added to the previous solution of ibuprofen + BOP and the reaction was followed by 4 h at 40 °C under stirring. Solvents were removed under reduced pressure and the solution was diluted with 10 mL of water. The product was extracted three times with ethyl acetate (20 mL). The organic phase was washed successively with 1 M HCl, water, 1 M $NaHCO_3$, and water, and the organic phase was collected and evaporated. The residue was purified on a silica gel column (eluent: hexane:ethyl acetate, 9.5:0.5). The calculated yield for the reaction was 28.37%.

4.1.3. 5-isopropyl-2-methylphenyl-2-(4-isobutylphenyl) propanoate (CVIB)

Yellowish oil, yield 28.37%, R*f* 0.66 (hexan: ethyl acetate, 9.5:0.5), ^{1}H NMR: δ 0.90 (6H, d, *J* = 6.6 Hz, H-1/H-1′), 1.202 (6H, d, *J* = 7.2 Hz, H-21/21′), 1.63 (3H, d, *J* = 6.9 Hz, H-11), 1.84 (3H, s, H-19), 1.87 (1H, appears sept, 1H, *J* = 6.3 Hz, H-2), 2.48 (2H, d, *J* = 7.2 Hz, H-3), 2.83 (1H, quint, *J* = 6.9 Hz, H-20), 3.96 (1H, q, *J* = 7,8 Hz, H-10), 6.78 (1H, d, *J* = 1.8 Hz, H-18), 6.96 (1H, dd, *J* = 7.5 Hz, *J* = 1.8 Hz, H-16), 7.06 (1H, d, *J* = 7.5 Hz, H-15), 7.14 (2H, d, *J* = 8.1 Hz, H-5/H-9), 7.32 (2H, d, *J* = 7.5, H-6/H-8). ^{13}C NMR: δ 22.284 (C-1/C-1′), 30.208 (C-2), 45.243 (C-3), 140.822 (C-4), 129.388 (C-5/C-9), 127.411 (C-6/C-8), 137.296 (C-7), 45.013 (C-10), 18.145 (C-11), 172.870 (C-12), 149.190 (C-13), 127.227 (C-14), 130.798 (C-15), 123.885 (C-16), 147.872 (C-17), 119.670 (C-18), 15.371 (C-19), 33.518 (C-20), 23.847 (C-21/C-21′). HRESI MS calculated for $C_{23}H_{30}O_2Na$ $[M]^+$ = 361.4722, found 361.2134;

4.2. In silico studies

4.2.1. Pharmacokinetics and physicochemical properties

The "*in silico*" study of pharmacokinetic and physicochemical properties, including: the parameters of the Lipinski rule, topological polar surface area (TPSA), aqueous solubility and absorption, distribution, metabolism and elimination (ADME) parameters were performed using the free software SwissADME available in http://www.swissadme.ch/index.php.

4.3. Stability studies

CVIB quantitation for chemical stability studies were performed using high performance liquid chromatography with a Waters Acquity UPLC system equipped with an UV/Vis detector. The separation was achieved using an Acquity UPLC CSH C18 (2.1 × 100 mm, 1.7 μm) column at 35 °C and the analyte was detected at 200 nm. The mobile phase was acetonitrile:water (80:20) in isocratic mode at a flow rate of 0.3 mL/min. The injection volume was 5 μL and running time was 10 min [59].

4.3.1. Chemical stability

The chemical stability studies were performed in Clark-Lubs buffer for pH 1.2 and PBS for pH 7.4. For these experiments CVIB was prepared at 1.5 μg/mL as triplicate in each of the buffers. The solutions were then placed under agitation (150 rpm) and controlled temperature of 37 °C. At time points 0.5, 1, 2, 4, 8, 12, and 24 h, 200 μL of sample were taken for analyte quantitation.

4.3.2. Enzymatic stability

The enzymatic susceptibility of CVIB to degradation was evaluated using human plasma. CVIB was prepared in triplicate at 50 μM in plasma by dilution of a stock solution. This mixture was placed under agitation (150 rpm) and controlled temperature of 37 °C. Then, samples (100 μL) were collected at time points 10, 20, and 30 min, and 1, 2, 4, 8, 10, 12 and 24 h. The extraction procedure was performed using acetonitrile precipitation with 200 μL of cold acetonitrile, containing triphenylamine 50 μg/mL as internal standard, to 100 μL of a plasma sample. This mixture was vortexed for 30 s and centrifuged (12,000 rpm, 4 °C). Then, 200 μL of supernatant was separated, filtered using 0.22 μm membranes, and transferred to a new clean vial for chromatographic analysis.

4.4. Experimental log P determination

In order to obtain the log P of CVIB, the method by High

Performance Liquid Chromatography based on OECD guideline 117 was used. Eight healthy controls (HCs) were included in the study. A UHPLC system equipped with a UV–Vis detector was used. The separation was performed using an Acquity BEH C18 (2.1 × 50 mm; 1.7 μm) column protected by a BEH VanGuard® (2.1 × 5 mm; 1.7 μm) guard column at 30 °C. The mobile phase was a 0.1% aqueous formic acid:methanol (30:70, v/v) mixture in isocratic mode. The flow rate was 0.4 mL/min and the injection volume was 2 μL.

The partition coefficient was deduced from the capacity factor k, which is obtained from the retention time. The compounds 2-butanone, benznidazole, acetanilide, nifedipine, benzene, probenecid, diazepam, chlorobenzene, thymol, phenanthrene, tryphenylamine, and DDT were used to build the calibration curve of log k versus log P.

4.5. Study population

Eight healthy controls (HCs) were included in the study. Controls were excluded if they regularly used any medication, had a rheumatic disease, or consumed alcohol or smoked in the previous 15 days. The study protocol was approved by the ethics committee of Universidade Federal de Pernambuco (CEP/CCS/UFPE/CAAE N° 63517517.1.0000.5208).

4.5.1. PBMC isolation and culture

PBMCs were isolated from the peripheral blood of HCs (n = 8) after collection in heparinized tubes. The blood was subjected to Ficoll Paque Plus (GE Healthcare Biosciences, Pittsburgh, PA, USA) centrifugation for PBMC separation. After separation, the cells were counted and placed in 24-well plates (1×10^6 cells/well) in RPMI-1640 medium (Gibco), supplemented with L-Glutamine, 10% FBS (Gibco), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid) (Gibco), and 200 U/mL penicillin/streptomycin (Gibco). After plating the cells, they were stimulated with anti-CD3 and anti-CD28 (eBioscience, San Diego, CA, US) in the presence or absence of CVIB at concentrations of 10, 25 and 50 μM.

4.5.2. Cytokine quantification

Cytokines IL-2, IL-6, IL-10, IL-17, IFN-γ, TNF-α and IL-1β were evaluated in the culture supernatants. Cytokines were quantified using human ELISA kits: BD Biosciences (San Jose, CA, US) for IFN-γ, TNF-α, IL-6 and IL-10; eBioscience for IL-2 and IL-17, and Invitrogen (Carlsbad, CA, US) for IL-1β. All tests were performed according to the manufacturer's recommendations.

4.6. In vitro cytotoxicity

The MTT assay was used to evaluate the cytotoxic activity of CVIB, carvacrol, ibuprofen and physical mixture carvacrol + ibuprofen in PBMCs. Cells were plated in 96-well plates (1×10^4 cells/well). Subsequently, the cells were exposed to the drugs and physical mixture at 25 and 50 μM for 48 h. Cell viability was measured using the yellow dye MTT [3-(4,5-dimethylthiazol-2-)-2,5-diphenyltetrazolium bromide] reduction to blue formazan product. After the incubation period with the molecule, 20 μL of 0.5 mg/mL MTT were added to each well of the plate; 130 μL of 20% SDS was added 3 h later. Absorbance was measured after 24 h at 570 nm using a multi-plater reader (Elx808, Biotek, EUA). Three independent experiments were performed.

4.7. Animals

Male albino Swiss mice (3-month-old, 28–32 g) were randomly housed in appropriate cages (22 ± 2 °C) with a 12-h light:dark cycle (light from 06:00 to 18:00), with free access to food (Purina®, Brazil) and water. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS: 23/2017). The ethical principles established by the Brazilian Society for Laboratory Animal Science (SBCAL) and by the National Institutes of Health (NIH) were respected.

4.8. Evaluation of acute nonclinical toxicity

An acute preclinical toxicity study protocol was performed according to the Guidelines for Testing of Chemicals n° 423 from Organization for Economic Co-operation and Development (OECD). For this study, Swiss mice (n = 3/group) were treated with single doses of 300 or 2000 mg/kg of CVIB intraperitoneally (i.p.) and the control group received vehicle alone (12% Tween-80 in saline). Careful observation was performed at the intervals of 0, 15, 30, 60 min, and 4 h, and daily for 14 days, recording death and signs referable to the CNS or autonomic nervous system (ANS), and the lethal dose for 50% of the animals tested (LD_{50}) was estimated [60].

4.9. In vivo anti-inflammatory evaluation using the carrageenan-induced pleurisy model

Adult male Swiss mice (28–32 g) were treated with CVIB (10 and 50 mg/kg, i.p.), carvacrol (10 and 50 mg/kg, i.p.), ibuprofen (10 and 50 mg/kg, i.p.), physical mixture (PM) of carvacrol and ibuprofen (10 and 50 mg/kg, i.p.) - consisting of the same amount of carvacrol and ibuprofen in carvacrol-ibuprofen hybrid solution, carvacrol (10 and 50 mg/kg, i.p.), ibuprofen (10 and 50 mg/kg, i.p.) or vehicle (distilled water 0,1% Tween-80, 0.1 ml/10 g, i.p.) 30 min before carrageenan pleural injection. Pleurisy was induced by intrapleural administration of 100 μL of 1% (w/v) carrageenan suspension in sterile saline solution [61]. An adapted 13 × 5 needle was inserted into the right side of the thoracic cavity for injection of the carrageenan solution. Four hours after carrageenan administration, the animals were euthanized and the pleural inflammatory exudates were collected using pleural lavage with 1 mL of PBS containing ethylene diamine tetraacetic acid (EDTA; 10 mM). The exudates were centrifuged (1500 rpm, 10 min), and the supernatants were collected for determination of cytokines levels in the pleural fluid. The cells were resuspended in 500 μL PBS and an aliquot of 10 μL was diluted with Turk's solution (1:20). The total leukocytes were counted in a Neubauer chamber using a light microscope, examining four external quadrants [62].

4.10. Determination of TNF-α, IL-1β and IL-10 levels in the pleural fluid

Tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) levels in the pleural cavity were assessed 4 h after the injection of carrageenan. The pleural lavage recovered was centrifuged at 1500 rpm for 10 min. TNF-α and IL-1β levels were quantified in supernatants free of cells by ELISA following the manufacturer's protocol (BD-Bioscience Pharmingen, San Diego, CA).

4.11. Statistical analysis

For the cytokine quantification in vitro, GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) was used for data analysis. The results of the continuous variables were expressed as mean/standard error of mean (Mean ± SEM). Significant differences between the treatments were calculated using Wilcoxon's signed rank test. Differences were considered significant when $p < 0.05$.

For the in vivo anti-inflammatory evaluation and for the determination of cytokines in the pleural fluid, the data were expressed as mean ± SEM. Differences of groups were analyzed using one-way analyses of variance (ANOVA) followed by Tukey's test. Differences were considered significant if $p < 0.05$. The statistical analyses were assessed using GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA, USA).

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Declaration of competing interest

All the authors declare they have no conflict of interests for this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105856.

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