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A Combined Study Using Ligand-Based Design, Synthesis, and Pharmacological Evaluation of Analogues of the Acetaminophen *Ortho*-Regioisomer with Potent Analgesic Activity

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A ligand-based drug design study was performed to acetaminophen regioisomers as analgesic candidates employing quantum chemical calculations at the DFT/B3LYP level of theory and the 6-31G* basis set. To do so, many molecular descriptors were used such as highest occupied molecular orbital, ionization potential, H–O bond dissociation energies, and spin densities, which might be related to quench reactivity of the tyrosyl radical to give *N*-acetyl-*p*-benzosemiquinone-imine through an initial electron withdrawing or hydrogen atom abstraction. Based on this *in silico* work, the most promising molecule, orthobenzamol, was synthesized and tested. The results expected from the theoretical prediction were confirmed *in vivo* using mouse models of nociception such as writhing, paw licking, and hot plate tests. All biological results suggested an antinociceptive activity mediated by opioid receptors. Furthermore, at 90 and 120 min, this new compound had an effect that was comparable to morphine, the standard drug for this test. Finally, the pharmacophore model is discussed according to the electronic properties derived from quantum chemistry calculations.

Key words: acetaminophen, analgesic effect, ligand-based drug design, molecular modeling, opioid derivatives

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Acetaminophen (ACP) or paracetamol is a widely used over-the-counter analgesic and antipyretic drug, and it appears to be safe when administered according to the therapeutic dosage. However, when an overdose is taken, it produces hepatic and/or renal injury in humans and in experimental animals (1,2). The pharmacological effects are generally considered to be based on the inhibition of prostaglandin synthesis (3–5). The cyclooxygenase (COX)-inhibiting activity was suggested to be related to its capacity to quench the tyrosyl radical present in prostaglandin endoperoxide synthase (PGES) (6), as shown in Figure 1.

This drug is metabolized primarily by glucuronidation and sulfation. Nevertheless, a small amount is probably metabolized *via* a third metabolic pathway, through an oxidation by the microsomal cytochrome P450-containing mixed-function oxidase system to form *N*-acetyl-*p*-benzoquinone-imine (NAPQI). 1,4-Michael adduct of NAPQI-glutathione and the corresponding cysteine conjugate and mercapturic acid breakdown products were found in human urine after the ingestion of ACP. However, hepatotoxic activity appears to be limited to compounds that are capable of forming quinoid structures, which are susceptible to both irreversible and reversible attack by soluble and non-soluble thiols (7–9).

Many reports describe investigations that aim at performing structural modifications of the ACP scaffold to improve its analgesic and safety properties. Besides efforts to modulate its toxicity or to understand the toxic mechanism, important progress has been made as well by modifying the substituents to generate homologous and congeners obtained by alkylations, halogenations, and addition of heteroatom, just to cite a few (10–15). Furthermore, based on calculations at the DFT/B3LYP level of theory, we have found that, in general, molecules with bond dissociation energies (BDE_{OH}) lower than 341.42 kJ/mol were more potent (16,17).

The field of structure-based drug design is a rapidly growing area in which many success cases were reported lately. The explosion of genomic, proteomic, and structural information has been providing hundreds of new targets and opportunities for future drug lead discovery. Usually, drug design is based on ligand (a small molecule) and/or on target (a macromolecule) structure and the receptor–drug interactions. Moreover, calculations involving macromolecules commonly applied molecular mechanics or semiempirical methods to save computational time, but semi-empirical and HF methods could provide the best geometry and electronic distribution of a molecule. By contrast, DFT proved to be reliable in the study of the energetic and geometrical properties of electrons, hydrogens, proton transfers, or other ion–molecule reactions in structure–activity relationship studies (16,18).

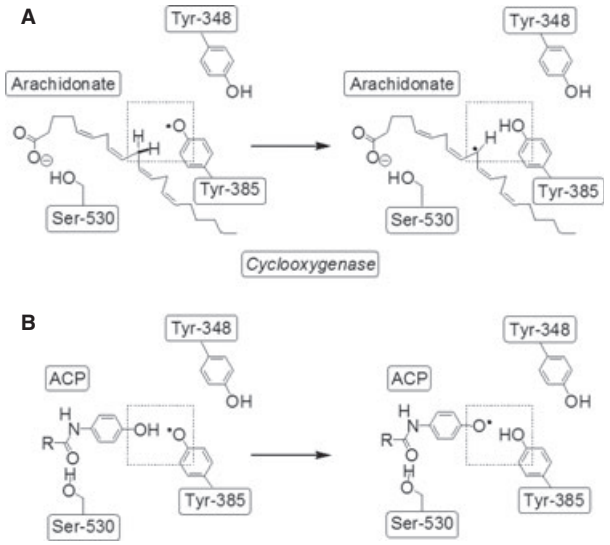

Figure 1: Hypothetical radical pathway for the cyclooxygenase inhibition by tyrosyl quenching.

In a continuous effort to develop new analgesic drug candidates, we report in this study the design, synthesis, and pharmacological evaluation of *N*-(2-hydroxy-phenyl)-acylamide derivatives. The design concept considered the need to carry out structural modifications of the ACP toxicophoric unit, avoiding its biotransformation to the reactive metabolite NAPQI. The structural design of this new series of analgesics was accomplished by applying a rational design method, represented by the replacement of the phenolic group in the *para* position to *ortho* position (Figure 2). Furthermore, to investigate the importance of solubility and stereoelectronic parameters to the analgesic activity, the chemical nature of the methyl moiety was modified by the introduction of a phenyl unit. Therefore, in this work, we discuss the design, synthesis, and the analgesic effect of an ACP regioisomer by means of quantum chemical calculations and *in vivo* preclinical assays.

Material and Methods

Calculation

All structures were submitted initially to a conformational search with the semi-empirical PM3 method (19). In addition, the geometries were optimized using density functional theory (DFT) (20). Calculations were performed with the Gaussian 03 molecular package (21). One hybrid functional of the DFT method, which consists of the Becke's three parameters exact exchange functional (B3) combined with the non-local gradient corrected correlation functional of Lee–Yang–Parr (LYP), known as B3LYP (22,23), was used with the 6-31G* basis set (24) for the final geometry optimizations.

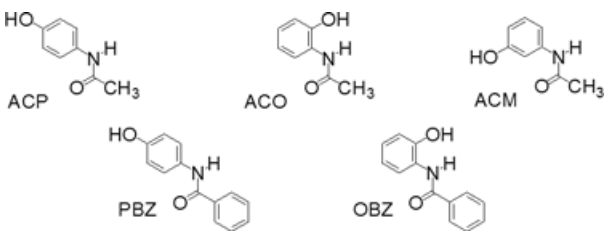

Figure 2: Structures of acetaminophen regioisomers.

The ionization potential (IP) was calculated as energy differences between the neutral molecule and its respective cation free radical as shown in eqn 1.

$$IP = E\text{RCONHC}_6\text{H}_4\text{OH}^{\cdot+} - E\text{RCONHC}_6\text{H}_4\text{OH} \quad (1)$$

Bond dissociation energies of the phenol group or the *N*-acetyl-*p*-benzosemiquinone imine (NAPSQI) formation were calculated as energy differences between the neutral molecule and its respective semiquinone plus hydrogen radical as defined in eqn 2.

$$\text{BDE}_{\text{OH}} = (E\text{NAPSQI}^{\cdot} + E\text{H}^{\cdot}) - E\text{RCONHC}_6\text{H}_4\text{OH} \quad (2)$$

To achieve our aim, we calculated: (i) highest occupied molecular orbital (HOMO) energies, (ii) IP, and (iii) BDE of ACP regioisomers and (iv) spin density distributions.

Chemistry

Reagents for synthesis such as 2-aminophenol and benzoyl chloride were obtained from the Merck Inc., (Germany) while solvents with satisfactory degree of purity were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). The compound *N*-(2-hydroxy-phenyl)-benzamide, named orthobenzamol (OBZ), was obtained according to the methodology described in the literature (25,26).

Biological assays

Animals

Male Swiss albino mice (20–30 g) were used. All animals (obtained from colonies maintained at the Instituto Evandro Chagas (IEC), Belém, Brazil) were housed in a group of cages and maintained on a 12 h light/12 h dark cycle, with free access to food and water at all time (27). Experiments were carried out according to a protocol approved by the Animal Welfare Committee of the Federal University of Para (UFPA) in agreement with the ethical guidelines for investigation of experimental pain in conscious animals.

Reagents

Acetic acid (Merck), morphine sulfate (Dimorf-Cristalia-BR), and ACP (Sigma-Aldrich) were obtained from commercial sources. A solution of formalin 1% was prepared with formaldehyde (Merck) in saline solution (NaCl 0.9%). Orthobenzamol was used in suspension with gum arabic in all experiments *via* oral administration.

Acetic acid-induced writhing

This test was performed as described by Koster *et al.* (25). Groups of 10 mice were fasted overnight prior to the beginning of the experiment, while free access to water was given. Acetic acid (0.6% v/v) was administered i.p. in a volume of 0.1 mL per 10 g of

animal. Mice were then placed in boxes, and the number of writhes, a response consisting of contraction of the abdominal wall and pelvic rotation followed by hinding limb extension, was counted during continuous observation for 20 min, starting after 10 min of the acetic acid injection. Orthobenzamol (5, 10, 25, and 50 mg/kg), indomethacin (5 mg/kg), and equivalent volumes of vehicle (0.1 mL per 10 g) were administered 60 min before the acetic acid injection. Indomethacin, a well-known peripheral analgesic drug, was used as positive control. Antinociceptive activity was expressed as percentage of inhibition of the usual number of writhings observed in control animals. ID_{50} values for orthobenzamol were determined by linear regression from individual experiments using BioStat® software.

Hot plate test

Mice were preselected using the hot plate at 50 °C (±1 °C). Animals showing a reaction time (latency for jumping or licking the feet) >20 seconds were discarded. Groups of 10 mice were then treated with OBZ (22.5 mg/kg, p.o.), morphine (10 mg/kg, s.c.), and equivalent volumes of the vehicle (NaCl 0.9% solution, 10 mL/kg). The reaction time (spanning 40 seconds) for each mouse was determined on the hot plate before and after drug administration at intervals of 30 min (28–30).

Formalin-induced nociception

The procedure reported here was already described (31,32). Animals received 20 μL of formalin solution (1% formaldehyde in saline) administered to mice *via* intraplantar route, and immediately the licking time was registered for 5 min to determine the first phase (i.e., neurogenic). 15 min after the beginning of the experiment, the licking time was registered for another 15 min, corresponding to the second phase (inflammatory). To assess the possible participation of the opioid system in the antinociceptive effect, the animals were pretreated with naloxone (1 mg/kg, i.p.) 15 min before the administration of OBZ (22.5 mg/kg, p.o.), morphine (4 mg/kg, s.c.), or vehicle (NaCl 0.9% solution, 10 mL/kg). The responses caused by first and second phases of the formalin test were recorded for 60 min after drug and vehicle administration. The other group of mice received morphine 30 min before the formalin injection.

Statistical analysis

Data obtained from *in vivo* experiments were expressed as mean and standard error (mean ± SEM). Statistical differences between the treated and the control groups were evaluated by ANOVA followed by Student–Newman–Keuls or Dunn's tests. $p < 0.05$ was considered to be significant.

Results and Discussion

Theoretical study of acetaminophen regioisomers

The theoretical study was determined to four different ACP analogues. The selection of these compounds was based on the chemical structure characteristics and properties computed for ACP. Aiming at establishing the potential of theoretical descriptors to qualify *para*-, *ortho*- and *meta*-derivatives with acetyl or benzoyl moiety as drug candidates, we calculated the overall score values using the HOMO, IP, and BDE, as shown in Table 1.

It can be seen that the regioisomerization strategy to produce orthoacetamol (ACO) and metacetamol (ACM) resulted in a decrease in HOMO and an increase in IP values. Therefore, the hydroxyl group at the *para* position has more inductive effect than the *ortho* or *meta* positions.

Similar results were obtained in another strategy that involved the modification of acylamide to benzamide. The major exception was parabenzamol (PBZ) because of its electronic properties of phenyl when compared with methyl moiety of ACP.

It is interesting to note that all molecules with benzamide increased HOMO and decreased IP values. This result indicates that the resonance effect of the benzene moiety is more important to the nucleophilicity than the inductive effect of the methyl moiety.

The analgesic potency of ACP has been suggested to be related to its oxidizability. This link suggests that one- or two-electron oxidation is a prerequisite for PGES inhibition (33). Furthermore, other studies claim that there is an interaction of carbonyl moiety with the positive charge of the ferric complex, while a reduction of the tyrosyl (Tyr385) radical of COX is promoted by ACP phenolic group (17). Therefore, the COX-inhibiting activity of ACP regioisomers was suggested to be related to its capacity to quench the tyrosyl radical present in PGES inhibition (6), as shown in eqn 3.

$$TyrO^{\cdot} + CH_3CONHC_6H_4OH \rightarrow TyrOH + RCONHC_6H_4O^{\cdot} \quad (3)$$

In this case, the BDE_{OH} for ACP regioisomers were calculated. This value represents the easiness of hydrogen donation of these regioisomers to yield semiquinone derivatives by tyrosyl reaction, because the hydrogen abstraction is the main mechanism proposed for the PGES inhibition by phenol derivatives (6,16,17).

The BDE_{OH} value of paracetamol was 341.42 kJ/mol. Nonetheless, a difference was observed for the hydroxy isomerization to *ortho* position for which BDE_{OH} decreased to 320.19 kJ/mol. The same tendency was not observed by the hydroxy isomerization in *meta*, as BDE_{OH} value increased to 358.75 kJ/mol.

Table 1: Theoretical properties of acetaminophen regioisomers

Compound	HOMO (eV)	IP (kJ/mol)	BDE_{OH} (kJ/mol)
ACP	−5.45	714.49	341.42
ACO	−5.63	731.94	320.19
ACM	−5.79	747.39	358.75
PBZ	−5.45	699.76	341.47
OBZ	−5.64	715.64	318.99

BDE, bond dissociation energies; HOMO, highest occupied molecular orbital; IP, ionization potential.

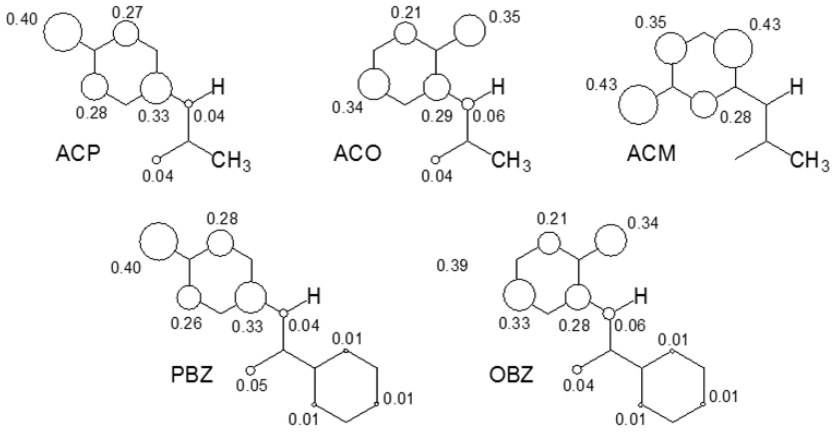

Figure 3: Spin density distribution of phenoxyl radical of the acetaminophen regioisomers.

Similar electronic effects were obtained for the benzamide analogues. In fact, the BDE_{OH} value of benzamide group in *para* position was 341.47 kJ/mol (PBZ), and the BDE_{OH} value of benzamide group in *ortho* position was 318.99 kJ/mol (OBZ). These results are opposite to that of HOMO and IP values, showing that semiquinone at the *ortho* position with benzene ring was more stable than the cation free radical. In other words, the hydrogen transfer was favored over electron transfer for these ACP regioisomers.

These results could be confronted mainly by the stability of the semiquinone radical generated after hydrogen abstraction, such as between tyrosyl and phenol reaction. This reaction can be also related to the stability of semiquinone form, as reported in previous works (18,34,35). Therefore, the resonance structures of semiquinone free radicals generated by hydrogen abstraction of the semiquinone regioisomers of NAPSQI can be observed by the spin density distribution (Figure 3).

The calculated spin density to initial hydrogen abstraction of ACP regioisomers showed that the contribution of the phenoxy moiety in *ortho* position was decreased from 0.40 to 0.34–0.35, as well as the global contribution of the benzene ring from 0.88 to 0.82–0.84. At the same time, it was observed an increment of the nitrogen atom contribution from 0.04 to 0.06. However, the phenoxy group in *ortho* position had an increased contribution from 0.40 to 0.43. The global contribution of the benzene ring was also raised from 0.88 to 1.06, while no contribution is showed for nitrogen atom and carbonyl moiety. Additional contribution was observed by the replacement of methyl by benzene ring. Therefore, the more stable compound had shown to harbor the *ortho*-phenoxy group.

Furthermore, the amide and hydroxy moieties at the 1,2-positions can favor the complex formation of these molecules with certain metal ions, inactivating the ions so that they cannot normally react with other elements or ions to produce enzyme inactivation such as PGES.

Chemistry

The synthesis of orthobenzamol was undertaken using a classical methodology, based on amine functional group interconversions, employing the benzyl chloride as the key reagent. The benzoylation of 2-aminophenol with benzoyl chloride produced the OBZ at 90% yield.

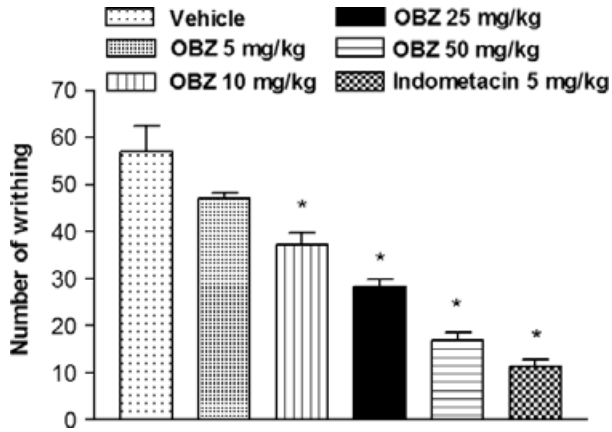

Figure 4: Orthobenzamol effects on abdominal constrictions induced by i.p. injection of acetic acid. Each group represents the mean of six animals, with the standard error. *Significantly different from control ($p < 0.05$, ANOVA, Tukey's test) at a given time.

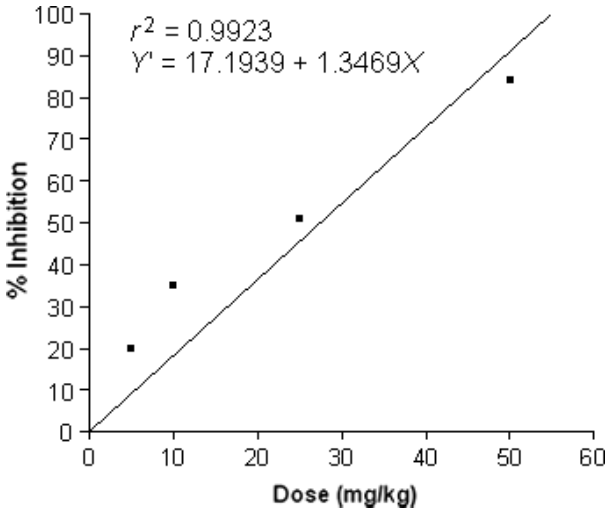

Figure 5: Determination of ED_{50} in mice by linear regression of the orthobenzamol administration (5, 10, 25, 50 mg/kg, p.o.) on the writhing induced by acetic acid 0.6% i.p., r^2 = correlation coefficient.

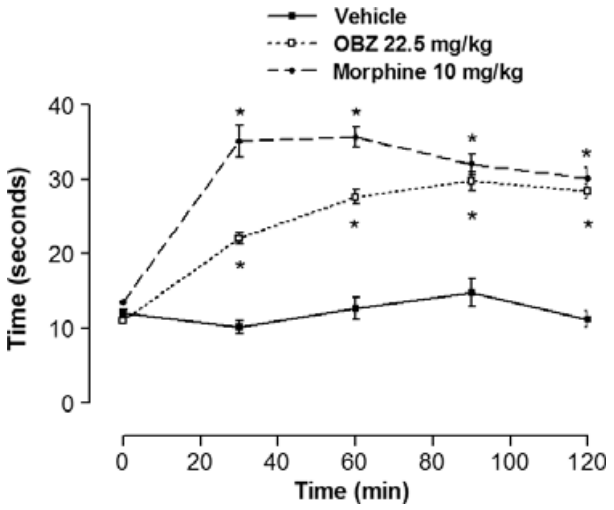

Figure 6: Time course of orthobenzamol (OBZ) effects on thermal nociception. Time (min) after OBZ (oral) or morphine (s.c.) administration. Ordinate latency time (s) for the response to thermal stimulation (50 ± 1 °C, mean ± SEM, $n = 10$) for OBZ 22.5 mg/kg dose. *Significantly different from control ($p < 0.05$, ANOVA, Dunn's test) at a given time.

This molecule was then crystallized in acetone. The structural determination was compared and confirmed by spectral properties and a melting point of 163.7–164.2 °C as described in the literature (26,36).

Pharmacological evaluation

The analgesic activity of OBZ was initially checked by employing the acetic acid-induced abdominal writhing model in mice (25). This model of pain detects both central and peripheral analgesia (37), being a screening tool for the evaluation of analgesic and anti-inflammatory activity of new agents (38,39).

In this model, OBZ was evaluated at doses of 5, 10, 25, and 50 mg/kg by oral administration using indomethacin (5 mg/kg) as the standard treatment. The results (Figure 4) showed that all doses of OBZ produced a remarkable inhibition of the acetic acid-induced writhing response by 20%, 35%, 51%, and 84%, respectively. At 50 mg/kg, OBZ was almost as potent as indomethacin (60%). OBZ produced a dose-dependent inhibition of acetic acid-induced abdominal constrictions in mice, with ID_{50} values of 22.5 mg/kg, as depicted in Figure 5. Thus, this dose was selected to study its central antinociceptive activity in the hot plate test (28,29,40) using morphine (10 mg/kg, i.p.) as the standard therapy.

The hot plate test evaluate the analgesic activity mediated by central mechanisms, because the heat is often used as a painful stimulus in models of acute pain, mediated by activation of nociceptors (Aδ and C fibers) (30). This test is commonly used to test compounds for the narcotic analgesic effect. Nevertheless, indomethacin and other non-steroidal anti-inflammatory drugs (NSAIDs) had no effect in this assay (29,41,42).

As shown in Figure 6, morphine significantly increased the latency period at 30, 60, 90, and 120 min after its subcutaneous administration when compared with the control group. The oral administration of orthobenzamol also significantly increased the latency at the same time of the nociceptive response in the hot plate test. It is important to note that at 30 and 60 min, morphine showed a more significant effect than OBZ to increase the latency. However, at 90 and 120 min, no significant difference was observed between morphine and OBZ when compared with

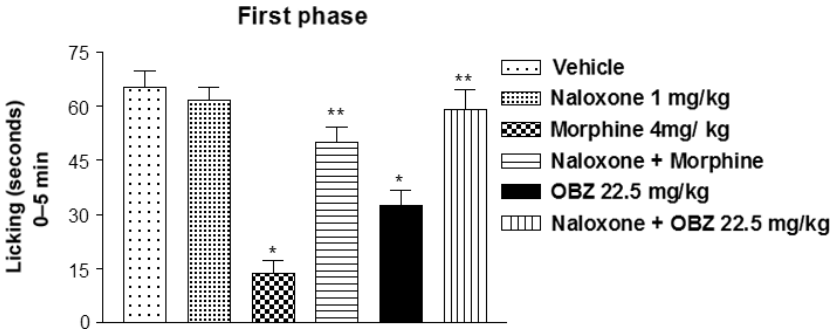

Figure 7: Orthobenzamol effects on first phase of the formalin test. Each group represents mean ± SE for 10 animals. *$p < 0.05$ when compared to control value; **$p < 0.05$ when compared to agonist plus antagonists versus agonist alone (ANOVA, Newman–Keuls test).

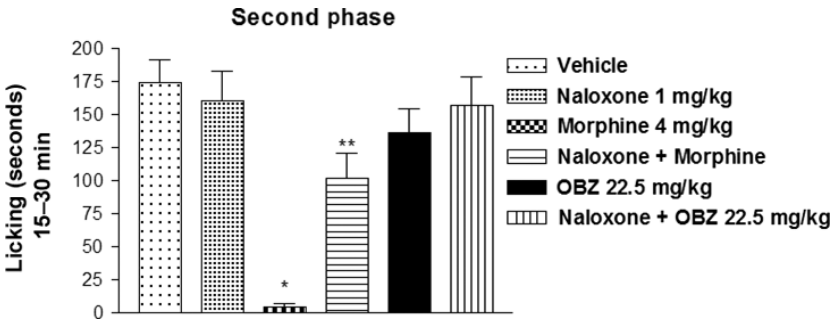

Figure 8: Orthobenzamol effects on second phase of the formalin test. Each group represents mean ± SEM of 10 animals. *$p < 0.05$ when compared to control value; **$p < 0.05$ when compared to agonist plus antagonists versus agonist alone (ANOVA, Newman–Keuls test).

the control group, where both acted effectively increasing the latency time. The observations that OBZ increased the baseline of animals in the hot plate model suggest that this compound presents supraspinal analgesic activity.

The antihyperalgesic activity of OBZ was determined using the formalin test (32,43). This test assesses two distinct phases of pain that are consequences of the release of different mediators (31,32). Currently, this is the model that most closely matches the chronic pain (44). The first phase begins immediately after the injection of formalin, lasting 5 min. It is believed that this effect happens as a result of the direct stimulation of afferent nociceptors C and Aδ by chemical mediators such as substance P, glutamate, and bradykinin, which are responsible for the neurogenic nociception (32,43). This phase can be suppressed by opioid analgesic drugs such as morphine (43,45,46).

The second phase of this model takes place between 15 and 30 min after formalin injection, and it is mainly related to the release of various pro-inflammatory mediators such as bradykinin, histamine, prostaglandins, and serotonin, among others. These mediators induce functional changes in neurons of dorsal horn that, over time, promotes the facilitation of transmission at the spinal level. This evidence suggests that the process of peripheral inflammation is involved in the second phase (43,47,48). The results in Figure 7 showed a significant antinociceptive response observed in the first phase (i.e., central phase) of the formalin-induced pain after oral administration (22.5 mg/kg) of OBZ, using morphine (4 mg/kg, i.p.) as positive control and naloxone (1 mg/kg, i.p.) as negative control. As these effects were reversed by naloxone association, we can infer that compound OBZ had analgesic activity in the hot plate assay.

On the other hand, the analysis of the second phase of the formalin test (i.e., inflammatory phase) did not indicate an antihyperalgesic activity of OBZ, as shown in Figure 8. These data suggested that the opioid system may be involved in the antinociception exerted by orthobenzamol (49) in the formalin test.

Conclusions

In the present work, the theoretical properties computed for ACP regioisomers had been studied following the ligand-based drug design approach. The lowest values of bond dissociation energy of the hydroxy moiety were related to an increment of the analgesic activity, while the chemical stability was linked to the spin density for the initial hydrogen abstraction of ACP analogues. These relationships led us to the synthesis of the most promising compound, which was then validated in *in vivo* tests. The results of the biological assays described in this work showed that *ortho*-benzamides, that is, OBZ, had significant antinociceptive activity. The mechanism involved is not completely understood, but the results suggested that the opioid receptors could be involved in the antinociceptive action observed for the tested compound. Based on these results, we propose that the OBZ can be used as a novel candidate for future development as an analgesic drug.

Acknowledgments

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Note

[a]BioStat Version 5.0. AnalystSoft Inc (Belém, Brazil).