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Anti-nociceptive activity and toxicity evaluation of Cu(II)-fenoprofenate complexes in mice

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ABSTRACT

The proposed curative properties of copper(II)-non-steroidal anti-inflammatory drugs (NSAIDs) have led to the development of numerous copper(II)–NSAID complexes with enhanced anti-inflammatory activity. In this work, the antinociceptive and toxic effects of two new coordination complexes: $Cu_2(fen)_4(caf)_2$ [fen: fenoprofenate anion; caf: caffeine] and $Cu_2(fen)_4(dmf)_2$ [dmf: N-N′-dimethylformamide] were evaluated in mice. The antinociceptive effect was evaluated with two models: acetic acid-induced writhing response and formalin test. For the sub-acute exposure, the complexes were added to the diet at different doses for 28 days. Behavioral and functional nervous system parameters in a functional observational battery were assessed. Also, hematological, biochemical and histopathological studies were performed. $Cu_2(fen)_4(caf)_2$ and $Cu_2(fen)_4(dmf)_2$ significantly decreased the acetic acid-induced writhing response and the licking time on the late phase in the formalin test with respect to the control and fenoprofen salt groups. The sub-acute exposure to $Cu_2(fen)_4(caf)_2$ complex increased the motor activity, the number of rearings and the arousal with respect to the control and fenoprofen salt groups. These impaired parameters in mice exposed to $Cu_2(fen)_4(caf)_2$ can be attributable to the presence of caffeine as stimulating agent. On the other hand, all exposed groups decreased the urine pools in the functional observational battery and increased the plasmatic urea. These effects could be due to the decrease in the glomerular filtration caused by NSAIDs. In conclusion, both complexes $Cu_2(fen)_4(dmf)_2$ and $Cu_2(fen)_4(caf)_2$ were more potent antinociceptive agents than fenoprofen salt. Sub-acute exposure to different doses of these complexes did not produce significant changes in the parameters that evaluate toxicity.

1. Introduction

Fenoprofen is a non-steroidal anti-inflammatory (NSAID), analgesic, with antipyretic properties used to treat mild to moderate pain. It relieves symptoms of arthritis (osteoarthritis and rheumatoid arthritis), such as inflammation, swelling, stiffness and joint pain. Evidence suggests that NSAIDs act by inhibiting cyclooxygenase (COX) and, consequently, prostaglandin synthesis (Polisson, 1996). Cyclooxigenases COX-1 and COX-2 play different roles in the inflammatory process (Hao et al., 1999; Polisson, 1996). COX-1 acts to maintain normal physiological function by its control of the renal parenchyma, gastric mucosa, platelet, and most other mammalian tissues, while COX-2 produces the prostaglandins involved in inflammation and mitogenesis (Dillon et al., 2003; Hao et al., 1999). The inhibition of COX-1 leads to gastrointestinal ulcerogenic toxicity while a drug exerting the selective inhibition of COX-2 over COX-1 is considered a safer and more effective antiinflammatory agent (Polisson, 1996).

Copper is also believed to possess anti-inflammatory and analgesic effects (Blahova et al., 1994; Okuyama et al., 1987; Sorenson, 1989). Copper ions, as centers of active site of various metalloproteins, play a vital role in a number of widely differing biological processes like electron transfer, oxidation and dioxygen transport (Mirica et al., 2004; Rosenzweig and Sazinsky, 2006).

Experimental evidences proved that the coordination of NSAIDs to copper(II) ions improves the pharmaceutical activity of the drugs themselves and reduce their undesired collateral effects in human and animals (Agotegaray et al., 2010; Blahova et al., 1994; Cini et al., 2007; Korolkiewicz et al., 1989; Lewis, 1978; Sorenson, 1992; Sorenson et al., 1995).

It is also known that many copper(II)–NSAID complexes present superoxide dismutase (SOD) (Agotegaray et al., 2010; Devereux et al., 2007; Wangila et al., 2006) and catechol oxidase activity

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(Abuhijleh et al., 1992). These enzymes protect the living cell against various pathological conditions involving cardiovascular diseases, cancer, inflammation, diabetes and aging (Valko et al., 2007). Increasing attention has been paid to the SOD activity of copper(II)-complexes with anti-inflammatory properties because superoxide is implicated in the promotion of arthritis by the degradation of hyaluronic acid, which is essential for maintaining internal joint homeostasis (Auer et al., 1990; McCord, 1974). This is supported by the complete protection against this degradation by treatment with SOD (Czapski and Goldstein, 1998; McCord, 1974). Moreover, oxygen-derived free radicals play important roles in the pathogenesis of gastric mucosal injury (Nishiyama et al., 1996) and SOD exists in the mucosal tissues of the gastrointestinal tract where it is believed to protect against such damage (Klinowsky et al., 1996).

Because the cooper(II)–NSAID complexes have a more potent pharmacologic activity and lower collateral effects than their parent drugs, and taking into account that there are no reports on the antinociceptive and toxicological properties of copper(II)-fenoprofenate complexes, we present here the study of the analgesic activity employing the acetic acid writhing and formalin tests of two new complexes [$Cu_2(fen)_4(caf)_2$ and $Cu_2(fen)_4(dmf)_2$] as well as the results of the sub-acute exposure to different doses to evaluate their toxicity.

2. Materials and methods

2.1. Materials

Fenoprofen calcium salt, carboxymethylcellulose and Tween 80 were purchased from Sigma-Aldrich. All other reagents and solvents were of analytical grade and used without further purification.

2.2. Synthesis of the complexes

The complex $[Cu_2(fen)_4(dmf)_2]\cdot 2H_2O$ was prepared according to the following procedure: a 2.0 ml dmf solution containing 0.0811 g (0.150 mmol) of racemic fenoprofen calcium salt hydrate ($Ca(fen)_2\cdot H_2O$) was added to 3.0 ml of an ethanolic solution of $CuCl_2\cdot 2H_2O$ (0.0170 g, 0.100 mmol). The resulting green solution was stirred at room temperature for about one h and then kept at the same temperature overnight. The addition of an excess of water led to the immediate precipitation of a green complex with a glassy appearance, which gradually recrystallized at room temperature after three weeks. The bright green microcrystals obtained were filtered, washed with water and air dried. Yield: 64% (0.0387 g) elemental analysis, found: C, 62.5; H, 4.9; N, 1.7%. Calc. for $C_{66}H_{70}Cu_2N_2O_{16}$: C, 62.2; H, 5.4; N, 2.1%. A slow recrystallization of the complex in dmf/water yielded green single crystals without crystallization water which were studied by X-ray diffractometry. These results were published elsewhere (Agotegaray et al., 2008). The complex $[Cu_2(fen)_4(caf)_2]$ was prepared by the addition of a solution containing 50 mg (0.040 mmol) of $Cu_2(fen)_4(dmf)_2$ in 2 ml of acetone to 31 mg (0.160 mmol) of caffeine dissolved in 2 ml of hot ethanol under stirring. This procedure led to a resulting limpid, green solution. The slow diffusion of water resulted in the formation of two layers of solvents. After a few days at 4 °C green crystals were obtained, which were washed with water and air dried. Yield: 57% (0.0340 g). The well-shaped crystals could not be solved by X-ray crystallography due to molecular disorder but it was studied by spectroscopic and thermal techniques revealing the structure proposed. Analysis calculated for $C_{76}H_{72}Cu_2N_8O_{16}$: C, 61.6; H, 4.9; N, 7.6%. Found: C, 61.3; H, 4.8; N, 7.7%.

2.3. Experimental animals

Healthy CF1 female mice 8 weeks old were used. They were obtained from the colony of the animal facility from the Biology, Biochemistry and Pharmacy Department which were maintained under constant conditions of temperature (22 ± 1 °C) and humidity (70%), in a 12 h light:12 h dark cycle (light on at 6:00 h) during all the experiment. According to the body weight (approximately 30 g) they were randomly divided into different groups of 8–10 animals which were acclimatized for a week before starting the experiment. All animals had free access to tap water and standard diet (Ganave®, Ratas y Ratones, Alimentos Pilar S.A., Argentina) throughout the experiment. The care and the handling of the animals were in accordance with the internationally accepted standard Guide for the Care and Use of Laboratory Animals (2010) as adopted and promulgated by the National Institute of Health.

2.4. In vivo analgesic activity

2.4.1. Acetic acid-induced abdominal writhing

The test was performed as described by Collier et al. (1968) and Fontenele et al. (1996). Nociception was induced by an intraperitoneal (i.p.) injection of 0.6% acetic acid solution (10 ml/kg). Mice were orally treated by gavage with 26 mg/kg $Cu_2(fen)_4(dmf)_2$ and 31 mg/kg $Cu_2(fen)_4(caf)_2$, and 1 h later the acetic acid was injected. The vehicle used for dissolution of complexes was 0.05% CMC-Na and 0.1% Tween 80. The control group received the vehicle and the positive group received 21 mg/kg fenoprofen salt. Each quantity of drug administered was equivalent to the therapeutic dose of 20 mg/kg of fenoprofen.

Immediately after the injection of acetic acid, each animal was isolated in an individual box to be observed during 20 min. The number of writhing and stretching was recorded. A writhe is indicated by abdominal constriction and stretching by full extension of hind limb.

2.4.2. Formalin test

This test was carried out as described by Hunskaar and Hole (1987). Twenty microliters of 2.5% formalin was injected into the dorsal surface of the left paw of mice 1 h after oral administration by gavage of 26 mg/kg $Cu_2(fen)_4(dmf)_2$, 31 mg/kg $Cu_2(fen)_4(caf)_2$, 21 mg/kg fenoprofen salt or vehicle (0.05% CMC (carboxymethyl cellulose) and 0.1% Tween 80). The time that animals spent on licking and biting the injected paw was recorded. On the basis of the response pattern described by Tjolsen et al. (1992), two distinct periods of intensive licking activity were identified and scored separately. The nociceptive scores normally peaked 5 min after formalin injection (early phase) and 15–30 min after the injection (late phase). The early phase is due to a direct effect on nociceptors by the formalin. The late phase seems to be an inflammatory response (Tjolsen et al., 1992).

2.5. Sub-acute toxicity

The experiment was conducted according to the protocols described by OECD Guideline No. 407 (2008). Fenoprofen salt and complexes were incorporated into the standard aliment and small pieces of food made (approximately 0.2 g) with the dose for each mouse. Before daily administration, mice were left for two h fasting. The exposure was over a period of 28 days at a dose of 21 mg/kg and 42 mg/kg body weight/day for fenoprofen salt; 31 mg/kg and 62 mg/kg body weight/day for $Cu_2(fen)_4(caf)_2$, and 26 mg/kg and 52 mg/kg body weight/day for $Cu_2(fen)_4(dmf)_2$. Control animals received standard food.

During the exposure, all the animals were observed for signs of toxicity. At the end of the exposure, behavioral and functional parameters as well as motor activity were assessed for each mouse. Subsequently, blood samples were obtained for hematological analysis by retro-orbital bleeding (Fukuta, 2004). After that, mice were euthanized and blood samples were taken by cardiac puncture for biochemical analysis. Finally, necropsy observations and histopathological examinations were realized on several tissues.

2.5.1. Functional observational battery

On the 28th day of exposure, behavioral and functional parameters of the animals were evaluated through a functional observational

battery. It included a thorough description of the animals' appearance, behavior and functional integrity (US EPA, 1998). Procedural details and scoring criteria for the functional observational battery protocol were performed according to Moser and Ross (Moser and Ross, 1996) and modified for mice (Ingman, et al., 2004; Moser and Ross, 1996; Youssed and Santi, 1997).

Briefly, measurements were first carried out in the home cage. The observer recorded each animal's posture, activity and palpebral closure. The presence or absence of clonic or tonic movements, spontaneous vocalizations and bitting were also noted. Then the animal was removed from its cage, rating the ease of removal and handling. All signs of lacrimation, salivation and piloerection were rated. Other abnormal clinical signs were also recorded. The animal was nets placed in an open field arena having a piece of clean absorbent paper on the surface, and allowed to freely explore for 3 min. During that time, the observer ranked the mouse's arousal, gait score, activity level and rears as well as any abnormal postures, unusual movements, stereotyped behaviors, pelvis elevation and tail position. At the end of 3 min, the number of fecal boluses and urine pools, and presence or absence of diarrhea was recorded. Next, sensorial responses were ranked according to a variety of stimuli (click stimulus using a metal clicker, approach and touch rump with a blunt object, pinch of the tail using forceps, and touch of the corner of the eye and the inside of the ear with a fine object).

Also, surface righting reflex and landing foot splay were evaluated. In landing foot splay, the tarsal joint pad of each hindfoot was marked with ink and the animal was then dropped from a height of 15 cm onto a recording sheet. Finally, the wire maneuver was carried out. The animal was suspended from a horizontal wire by forelimbs and released.

2.5.2. Hematological and biochemical parameters

A volume of 0.4 ml of blood was obtained from each mouse by retro-orbital bleeding under light ether anesthesia with ethylendiamine tetraacetate (EDTA) as anticoagulant (25 μl/ml of blood). These samples were used immediately for the determination of hematological parameters employing an automatic analyser (Coulter T890). The parameters measured were total erythrocytes, leukocytes and platelet counts, hematocrit and hemoglobin levels, leukocyte differential counts and erythrocyte indices. Blood samples for biochemical analysis were obtained by cardiac puncture as a terminal procedure. Heparin was used as anticoagulant (20 UI/ml of blood). The samples were centrifuged at 1500×*g* for 10 min (Rolco CM 36R centrifuge) to obtain plasma. For the hepatic function evaluation, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined, while for the renal function, urea and creatinine were evaluated. Biochemical determinations were made using specific kits (Wiener Lab, Argentina) by measurement of the optical density of reaction products at the corresponding wavelength with a spectrophotometer (Shimadzu UV-1203, UV–VIS spectrophotometer).

2.5.3. Histopathological examinations

Euthanized animals were macroscopically examined. Liver, kidneys, stomach and brain were weight and intestine total length was recorded. Representative fragments of these organs were fixed in formol 10% solution, dehydrated by serial ethanol solution and enclosed with paraffin. Sections 5 μm thick (Minot Leica model RS 2165) were stained with hematoxylin–eosin and examined under a light microscope (Olympus Bx51).

2.6. Statistical analysis

Acetic acid and Formalin test data were tested using one-way ANOVA followed by *t*-test when differences between groups were detected. Behavioral data measures in functional observational battery were continuous (providing interval data), ranked (ranks based on a defined scale), descriptive or binary (presence or absence of a sign). Continuous dates were tested using a one-way ANOVA followed by LSD test when differences between groups were detected. The ranked data were analyzed using Kruskal–Wallis nonparametric test (Zar, 1999). For descriptive or binary data, each experimental group was compared to control group and fenoprofen salt group using a chi-square test. Hematological data presented as percentages were previously transformed to $p = \arcsin\sqrt{p}$ (Zar, 1999). These data and biochemical data were tested using a one-way ANOVA followed by LSD test when differences between groups were detected.

The Kruskal–Wallis test was used for comparing the histopathological parameters between groups. Data are expressed as means ± S.E.M. Probability values less than 0.05 were considered to be significant. All statistical analysis was made using software SPSS 7.5 for Windows.

3. Results

3.1. Analgesic activity

3.1.1. Acetic acid-induced writhing

Results obtained from acetic acid-induced writhing are showing in Fig. 1. When acid-acetic test was analyzed, the one-way ANOVA reveled significant differences among the groups, $F(21,36) = 5.8$, $P<0.001$. Both complexes and fenoprofen salt showed inhibitory effects of the writhing response induced by acetic acid. Mice treated with $Cu_2(fen)_4(dmf)_2$, $Cu_2(fen)_4(caf)_2$ and fenoprofen salt decreased significantly the acetic acid-induced writhing response compared with the control group ($P<0.001$, $P<0.001$ and $P<0.05$, respectively). The percentage of inhibition was 89.7%, 92.1% and 45.2%, respectively. The difference between the groups treated with the lowest dose of both complexes and the administered with the lowest dose of fenoprofen salt was very significant ($P<0.001$). Therefore the maximal inhibition of the nociceptive response was achieved by both complexes with respect to fenoprofen salt.

3.1.2. Formalin test

In this test, the one-way ANOVA reveled significant differences among the groups, $F(3,37) = 3.8$, $P<0.02$ for the early phase, and $F(3,37) = 30.6$, $P<0.001$ for the late phase. The results obtained (Fig. 2) showed that the time spent on licking the injured paw was significantly attenuated in the early phase by both complexes and fenoprofen salt compared to the control group ($P<0.05$). In the second phase the inhibition was more significant for all drugs ($P<0.001$). Also, Cu_2

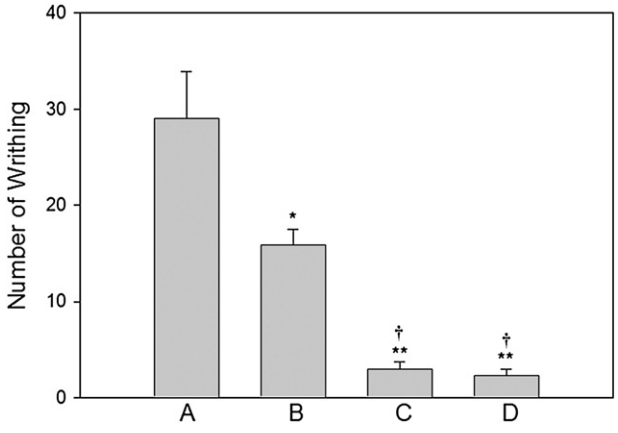

Fig. 1. Effects of fenoprofen salt and complexes in the Acetic Acid test. A, Control group; B, 21 mg/kg fenoprofen salt group; C, 26 mg/kg $Cu_2(fen)_4(dmf)_2$; and D, 31 mg/kg $Cu_2(fen)_4(caf)_2$. Mice were administered orally 1 h before of acetic acid injection (i.p.). The number of writhing and stretching per mouse was counted over a 20 min period. Fenoprofen salt group was used as positive control. Values are means ± S.E.M.; n = 9–10. $^{*}P<0.05$ and $^{**}P<0.001$ respect to the vehicle control group. $^{\dagger}P<0.001$ respect to the fenoprofen group (ANOVA followed by *t*-test).

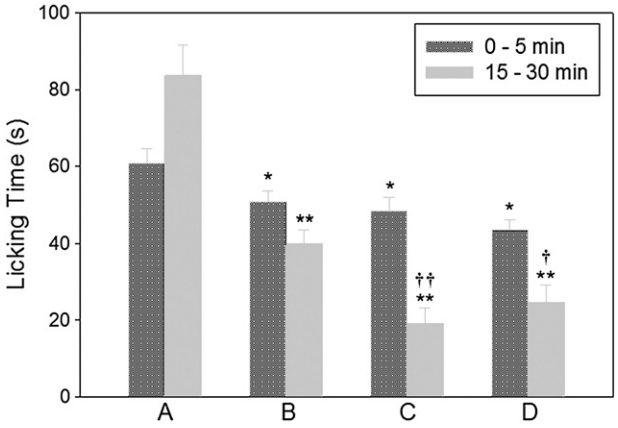

Fig. 2. Effects of fenoprofen salt and complexes in the formalin test. A, Control group; B, 21 mg/kg fenoprofen group; C, 26 mg/kg $Cu_2(fen)_4(dmf)_2$ and D, 31 mg/kg $Cu_2(fen)_4(caf)_2$. Values are expressed as the mean ± S.E.M. of time that the mouse spent on licking and biting the injected paw in the early phase (black bar) and the late phase (gray bar). n = 9–10. $^{*}P<0.05$ and $^{**}P<0.001$ respect to the vehicle control group. $^{\dagger}P<0.02$ and $^{\dagger\dagger}P<0.002$ respect to the fenoprofen group (ANOVA followed by *t*-test).

$(fen)_4(dmf)_2$ and $Cu_2(fen)_4(caf)$ showed marked inhibition of licking responses in the late phase with respect to the fenoprofen salt group ($P<0.02$ and $P<0.002$, respectively). Therefore, both complexes have a stronger effect than fenoprofen salt in inhibiting the second phase corresponding to inflammatory pain.

3.2. Sub-acute toxicity

3.2.1. Functional observational battery

Data obtained in the functional observational battery are shown in Table 1. We observed that both groups of mice exposed to both doses of $Cu_2(fen)_4(caf)_2$ presented changes in some parameters evaluated in the home cage, in the hand-held observations and during the manipulative test. Both doses of $Cu_2(fen)_4(caf)_2$ complexes increased the activity of mice in the cage ($P<0.01$) and the highest dose of caffeine complex made difficult the handling of animals ($P<0.01$) compared to the control group. On the other hand, mice administered with the highest dose of fenoprofen salt presented minor activity in the box ($P<0.01$) compared to the control group. Furthermore, the activity of mice exposed to both doses of $Cu_2(fen)_4(caf)_2$ was increased with respect to both doses of fenoprofen salt ($P<0.01$).

In the open field observations, the results showed that all groups (except the lowest dose of fenoprofen salt) increased the activity [$P<0.05$ or $P<0.01$] compared to control group. Both groups exposed to caffeine complexes were significantly more active than those exposed to both doses of fenoprofen salt ($P<0.05$). With respect to rearing and arousal, all groups increased these values compared to the control group ($P<0.05$ or $P<0.01$).

All groups exposed to different doses of fenoprofen salt, $Cu_2(fen)_4(dmf)_2$ and $Cu_2(fen)_4(caf)_2$ exhibited a significant decrease in the number of urine pools deposited compared to the control group ($P<0.01$). There were no significant changes in the number of fecal boluses compared to the control group.

Sensorial responses were ranked according to a variety of stimuli. In the touch, both doses of $Cu_2(fen)_4(caf)_2$ increased significantly the response compared to the control ($P<0.05$). In the tail pinch response, only the highest dose of $Cu_2(fen)_4(caf)_2$ increased the value with respect to the control and to the highest dose of fenoprofen salt groups ($P<0.05$). The other parameters evaluated in the open field arena were not altered in either of the experimental groups.

3.2.2. Hematological and biochemical analyses

Data from the hematology and clinical biochemistry are shown in Tables 2 and 3, respectively.

With respect to mice exposed to the lowest dose of $Cu_2(fen)_4(caf)_2$, a significant decrement in the total erythrocytes and leukocytes were detected [($P<0.05$) compared to the control group]. The decrease in the number of erythrocytes was also significant for the lowest dose of fenoprofen salt compared to the control group ($P<0.05$). However, these values are within the reference range for mice, determined approximately in 7–11 (10^6/mm^3) for erythrocytes and 2–10 (10^3/mm^3) for leukocytes (Everds, 2004). The other parameters, hemoglobin and hematrocrit, were not affected in comparison to the control mice (Table 2).

In the white blood cells, no changes were observed with respect to the control group (data not shown).

We observed a significant increase in the platelets number in mice exposed to the highest dose of fenoprofen salt, the highest dose of $Cu_2(fen)_4(caf)_2$ and both doses of $Cu_2(fen)_4(dmf)_2$, being these values within the reference range for mouse [1010–1399 (10^3/mm^3)] ($P<0.05$) (Table 2) (Everds, 2004).

When biochemical data were analyzed, a significant increase of the urea level was detected in all groups, except the one corresponding to the lowest dose of $Cu_2(fen)_4(dmf)_2$ in comparison to the control group ($P<0.05$). With respect to the creatinine level, the only group affected was the one exposed to the highest dose of $Cu_2(fen)_4(caf)_2$ ($P<0.05$). This value was higher compared to the control group (Table 3).

In the aspartate aminotransferase (AST) level, a significant increase was detected in mice exposed to the highest dose of $Cu_2(fen)_4(caf)_2$ with respect to the control ($P<0.05$). This difference was also significant in comparison to mice exposed to the highest dose of fenoprofen salt ($P<0.05$). However, no difference between groups was detected by statistical analysis in the alanine aminotransferase (ALT) level (Table 3).

3.2.3. Histopathological analysis

The histopathological examinations of liver, stomach, intestine and brain showed no changes with hematoxylin–eosine at the end of the sub-acute exposure to all the compounds here evaluated in any of the mice. The kidneys of some animals of all exposed groups exhibited a minimal change. We observed a plenty of granular interstitial tissue surrounding arteries and/or veins. Anyway, this change was statistically not significant with respect to control group.

4. Discussion

In this work, the antinociceptive and toxic effects of $Cu_2(fen)_4(caf)_2$ and $Cu_2(fen)_4(dmf)_2$ were evaluated in mice. Two different analgesic testing models were employed to identify different nociceptive pains.

The acetic acid-induced writhing is a visceral pain model used for detecting antinociceptive activity (Fukawa et al., 1980). It was reported that prostaglandin biosynthesis plays an important role in this nociceptive mechanism (Franzotti et al., 2000). Intraperitoneal administration of acetic acid releases prostaglandins and sympathomimetic mediators (Deraedt et al., 1980). In this study, both complexes reduced significantly the number of abdominal constrictions and stretching of hind limbs. The percentage inhibition was: 89.7%, 92.1% and 45.2% for $Cu_2(fen)_4(dmf)_2$, $Cu_2(fen)_4(caf)_2$ and fenoprofen salt, respectively. The visceral analgesic action of both complexes was 5–7 times more potent than fenoprofen salt at the same fenoprofen dose (20 mg/kg). Furthermore, both complexes showed longer onset and shorter duration of writhing than fenoprofen salt (data not shown). This indicates that $Cu_2(fen)_4(dmf)_2$ and $Cu_2(fen)_4(caf)_2$ present a strong analgesic activity for the visceral pain.

The formalin test is employed as model for nociceptive pain (Dubuisson and Dennis, 1977; Steinar and Kjell, 1987) being considered effective to measure nociceptive and inflammatory pain. The animals present two distinct nociceptive behavior phases. The early

Table 1
Parameters evaluated in the functional observational battery after the sub-acute exposure to different dose complexes and fenoprofen salt.

Endpoint	Control	Fenoprofen salt		$Cu_2(fen)_4(caf)_2$		$Cu_2(fen)_4(dmf)_2$	
		21 mg/kg	42 mg/kg	31 mg/kg	62 mg/kg	26 mg/kg	52 mg/kg
Home cage observations							
Normal body posture (D)	100	100	100	100	100	100	100
Activity (R)	2.60	2.30	1.90[b]	3.60[bd]	3.60[bd]	2.30	2.20
Palpebral closure (R)	0	0	0	0	0	0	0
Clonic movement (D)	0	0	0	0	0	0	0
Tonic movement (D)	0	0	0	0	0	0	0
Biting (D)	0	0	0	0	0	0	0
Vocalizations (B)	0	0	0	0	0	0	0
Hand-held observations							
Ease of removal from cage (R)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ease of handling (R)	2.00	2.00	2.00	2.30	2.90[b]	2.20	2.00
Salivation (R)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lacrimation (R)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Piloerection (B)	0	0	0	0	0	0	0
Normal fur appearance (D)	100	100	100	100	100	100	100
Normal respiration (D)	100	100	100	100	100	100	100
Normal cardiovascular signs (D)	100	100	100	100	100	100	100
Normal limb tone (D)	100	100	100	100	100	100	100
Normal abdominal tone (D)	100	100	100	100	100	100	100
Limb grasping (B)	100	100	100	100	100	100	100
Open field observations							
Activity level (R)	2.80	3.30	3.60[b]	3.90[bc]	4.10[bc]	3.40[b]	3.40[a]
Rearing (R)	1.10	3.10[b]	3.30[b]	3.10[b]	2.50[b]	2.60[b]	2.50[b]
Arousal (R)	3.40	4.10[b]	4.10[b]	4.40[b]	3.90[b]	4.00[a]	3.90[a]
Normal gait (D)	100	100	100	100	100	100	100
Stereotyped behaviors (D)	0	0	0	0	0	0	0
Pelvic elevation (R)	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Normal tail position (D)	100	100	100	100	100	100	100
Fecal boluses (C)	2.40	0.60	1.30	0.90	2.10	0.80	0.80
Urine pools (C)	1.60	0[b]	0[b]	0[b]	0[b]	0[b]	0.40[b]
Diarrhea (B)	0	0	0	0	0	0	0
Manipulative tests							
Approach response (R)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Touch response (R)	1.90	2.00	2.00	2.20[a]	2.90[a]	1.90	2.10
Click response (R)	2.00	1.90	1.90	2.00	2.20	2.00	2.00
Tail pinch response (R)	1.90	1.90	1.90	2.10	2.40[ac]	1.90	2.20
Palpebral reflex (B)	100	100	100	100	100	100	100
Pinna reflex (B)	100	100	100	100	100	100	100
Flexor reflex (B)	100	100	100	100	100	100	100
Extensor reflex (B)	100	100	100	100	100	100	100
Righting reflex (R)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Landing foot splay (C)	4.40	4.20	3.80	4.00	4.00	4.20	3.70
Wire maneuver (R)	1.10	1.10	1.00	1.00	1.00	1.10	1.00

Descriptive (D) and binary (B) date expressed as percentage of incidence (chi-square test); Ranked (R) data expressed as the mean score of the scale used (Kruskal–Wallis test); Continuous (C) data expressed as mean value (one-way ANOVA test); [a]$P<0.05$ and [b]$P<0.01$ compared to control group. [c]$P<0.05$ and [d]$P<0.01$ compared to the lowest or the highest fenoprofen dose. The lowest dose of each complex was compared with the lowest dose of fenoprofen, and the same was applied to the highest dose of complexes.

Table 2
Hematological data from mice sub-acute exposed to different doses of fenoprofen salt and complexes.

Parameter	Control	Fenoprofen		$Cu_2(fen)_4(caf)_2$		$Cu_2(fen)_4(dmf)_2$	
		21 mg/kg	42 mg/kg	31 mg/kg	62 mg/kg	26 mg/kg	52 mg/kg
Erythrocytes (10^6/mm^3)	9.4 ± 0.1	8.95 ± 0.1*	9.3 ± 0.1	9.1 ± 0.1*	9.3 ± 0.1	9.4 ± 0.1	9.3 ± 0.1
Leucocytes (10^3/mm^3)	8.6 ± 0.8	6.2 ± 0.7	9.5 ± 0.9	3.8 ± 0.2*	7.0 ± 1.4	6.0 ± 0.4	8.9 ± 1.4
Hematocrit (%)	40.1 ± 0.2	39.2 ± 0.4	40.0 ± 0.3	39.7 ± 0.3	40.3 ± 0.4	40.8 ± 0.3	40.0 ± 0.3
Hemoglobin (g/dl)	14.0 ± 0.2	13.4 ± 0.3	13.9 ± 0.3	13.6 ± 0.1	14.2 ± 0.2	14.1 ± 0.2	13.9 ± 0.2
MCV (fl)	44.4 ± 0.2	44.5 ± 0.3	44.9 ± 0.7	45.0 ± 0.3	44.9 ± 0.1	45.4 ± 0.3	44.6 ± 0.2
MCH (pg)	15.1 ± 0.2	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0	15.3 ± 0.2	15.0 ± 0.0	15.1 ± 0.2
MCHC (%)	35.6 ± 0.3	35.4 ± 0.2	35.6 ± 0.2	35.4 ± 0.1	35.5 ± 0.2	35.1 ± 0.1	35.5 ± 0.2
Platelets (10^3/mm^3)	1079.0 ± 51.7	1171.8 ± 27.8	1301.5 ± 58.9*	1163.9 ± 20.5	1229.7 ± 34.2*	1208.0 ± 47.4*	1203.6 ± 30.6*

Data expressed as mean ± S.E.M.
MCV, mean corpuscular volume.
MCH, mean corpuscular hemoglobin.
MCHC, mean corpuscular hemoglobin concentration.
* $P<0.05$ compared to control group (one-way ANOVA followed by LSD test).

Table 3
Plasma biochemical data from mice sub-acute exposed to different dose of fenoprofen salt and complexes.

Parameter	Control	Fenoprofen		$Cu_2(fen)_4(caf)_2$		$Cu_2(fen)_4(dmf)_2$	
		21 mg/kg	42 mg/kg	31 mg/kg	62 mg/kg	26 mg/kg	52 mg/kg
Creatinine (mg/dl)	7.1 ± 0.6	5.3 ± 0.5	7.5 ± 0.6	6.5 ± 0.4	9.2 ± 1.0*	5.5 ± 0.4	7.1 ± 1.1
Urea (mg/dl)	52.1 ± 1.7	60.8 ± 2.8*	69.0 ± 3.9*	72.0 ± 3.0*	65.0 ± 2.6*	59.3 ± 3.2	64.1 ± 3.3*
AST (UI/l)	64.8 ± 5.4	59.1 ± 4.9	72.8 ± 7.0	65.0 ± 6.4	99.6 ± 10.0*,**	57.8 ± 4.7	85.5 ± 11.0
ALT (UI/l)	12.3 ± 1.5	11.0 ± 1.3	7.8 ± 1.6	13.4 ± 1.3	11.9 ± 2.1	10.8 ± 1.2	8.2 ± 1.8

Data expressed as mean ± S.E.M.
AST, aspartate aminotransferase.
ALT, alanine aminotransferase.
* $P<0.05$ compared to control group (one-way ANOVA followed by LSD test).
** $P<0.05$ compared to fenoprofen salt group (one-way ANOVA followed by LSD test).

phase is initiated immediately after formalin injection and lasts about 3–5 min, resulting from chemical stimulation of nociceptors and nerve by formalin. The late phase is initiated 15–20 min after injection and lasts about 20–40 min. It has been found that NSAID agents have little effect on phase 1, reducing the nociceptive behavior of licking and biting in phase 2 (Hunskaar and Hole, 1987). It has been demonstrated that the nociception produced in phase 2 of the formalin test is a result of chemical insult resulting in tissue damage (Rosland, et al., 1990) which produces inflammation mediators (Ferreira, 1972; Otuki et al., 2001). NSAIDs block the production of prostaglandins reducing sensitization of peripheral nervous tissue, diminishing pain.

In this experiment, the analgesic effects of fenoprofen salt, $Cu_2(fen)_4(dmf)_4$ and $Cu_2(fen)_4(caf)_4$ have little effect on early phase at similar manner, but reduce the nociceptive behavior and have maximum efficacy in the late phase. Furthermore, both complexes showed marked inhibition of licking responses in the late phase versus the fenoprofen salt, indicating a potent action related to the inhibition of the pain due to the decrease of the inflammatory process.

In the acetic-acid and formalin tests we demonstrate that fenoprofen salt is less effective in treating pain from inflammatory origin due to both complexes have more potent antinociceptive activity than fenoprofen salt. Previously, we observed that these complexes have more potent antiinflammatory effect (Agotegaray et al., 2010).

Clinically, caffeine is used as an adjuvant to eliminate sleepiness induced by antihistamines and to provide better analgesic efficacy. Combinations of NSAIDs with caffeine have been analyzed in different pain models (Casteñeda-Hernandez et al., 1994; Engelhardt et al., 1997; López-Muñoz et al., 1996; Medina et al., 2006; Vinegar et al., 1996). The mechanisms that participate in the potentiation of antinociceptive effects by caffeine are not fully understood. Plasmatic level analyses of certain analgesics or caffeine have discarded that pharmacokinetic mechanisms participate in this potentiation (Casteñeda-Hernandez et al., 1994; Engelhardt et al., 1997; Gayawali et al., 1991). In the formalin model, Sawynok and Yaksh (1996) reported that caffeine only showed antinociceptive effect in the second phase at doses of 12.5–50 mg/kg. In this work, the dose of caffeine contained in the $Cu_2(fen)_4(caf)_2$ complex was 8 mg/kg. Therefore, the higher analgesic effect of $Cu_2(fen)_4(caf)_2$ with respect to the fenoprofen salt group cannot be attributed to caffeine analgesic effect, considering that $Cu_2(fen)_4(caf)_2$ did not show antinociceptive difference in the second phase with respect to $Cu_2(fen)_4(dmf)_2$.

Evidence indicates that copper possesses antiinflammatory and analgesic effects and enhances the antiinflammatory and analgesic activity of numerous NSAIDs (Beveridge et al., 1982; Blahova et al., 1994; Okuyama et al., 1987; Sorenson, 1989). By this way, the increase in the analgesic activity could be due to the presence of copper in both complexes. Evidences suggest that copper(II) salts have analgesic properties, but the antinociceptive effect is least potent than the Cu(II)–NSAID complexes (Okuyama et al., 1987). Cu–NSAIDs exhibit a marked SOD-mimetic activity. The beneficial role of copper(II) in minimizing inflammation has been attributed to its redox activity, in particular, its ability in SOD to remove the highly reactive pro-inflammatory superoxide radical anion $O_2^{-}\bullet$ (Shuff et al., 1992; Sorenson et al., 1995). Therefore, Cu(II)–NSAID complexes may act by decreasing the inflammatory process and as a result, reducing pain.

The degree of toxicity depends on many factors: the individual compound, the dose level, the duration of the period of drug administration and the pharmacokinetics and metabolism in a given species. Because of this, we evaluated the effects of sub-acute exposure of $Cu_2(fen)_4(caf)_2$ and $Cu_2(fen)_4(dmf)_2$ at two different doses: low and high therapeutic doses; the lowest dose used in the antinociceptive tests and the highest dose which could be used for patients with severe pain caused by chronic inflammatory diseases. We evaluated different parameters of toxicity after exposure of 28 days: behavioral and functional nervous system parameters in a functional observational battery and also several biochemical and histopathological studies were realized.

Both doses of caffeine complexes produced increase in the activity evaluated in the home cage compared to the control and this difference is also significant with respect to both doses of fenoprofen salt. The groups exposed to the highest dose of fenoprofen salt showed decreased activity in the home cage with respect to the control.

When the animals were removed from its cage, only those exposed to the highest dose of caffeine complexes were more difficult to handle. This difference is significant with respect to the control group and the highest dose of fenoprofen salt. We found no changes in other parameters evaluated in the home cage and handling of animals.

In the open field, the increase in the activity, number of rearings and the arousal respect to the control group, reflect a stimulant effect produced by all groups at different doses. When comparing the activity with respect to both doses of fenoprofen salt, we noted a significant increase only in the caffeine complexes exposed groups.

The principal mode of action of caffeine is blocking adenosine receptors in a nonselective manner (Fisone et al., 2004; Fredholm, 1995; Jacobson and van Rhee, 1977). Caffeine has effects on alertness (Fredholm et al., 1999) and stimulant effects have been quantified in locomotor activity studies in rodents (Snyder et al., 1981; Svenningsson et al., 1997). A study performed by El Yacoubi et al. (2000) strengthened the adenosine A_{2A} receptor participation on its stimulant effects. Current evidence suggests that the psychostimulant and reinforcing effects of caffeine may be the result of increased activity of dopaminergic neurotransmission perhaps via adenosine–dopamine interactions (Ferré, 2008; Powell et al., 2001). The fact that both groups of caffeine complexes increase the locomotor activity in the open field (in a dose dependent manner) is likely due to activation of dopaminergic transmission. Therefore, the increased activity as the difficulty in handling animals with respect to fenoprofen salt, could be due to the presence of caffeine in the complexes.

When we evaluated the sensorial response we observed that mice exposed to both doses of caffeine complexes presented extreme excitability to touch and these effects were significant with respect to

the control. Regarding to tail pinch response, the highest dose of $Cu_2(fen)_4(caf)_2$ showed higher response with respect to the control group and the highest dose of fenoprofen salt group. These higher touch and tail pinch responses can be attributed to the excitability produced by the presence of caffeine in the complex.

On the other hand, all groups exposed presented reduced number of urine pools indicating possible renal damage. NSAIDs may cause decrements in renal plasma flow and glomerular filtration. However, these effects are usually fully reversible with the discontinuation of the NSAID treatment (Murray and Craig Brater, 1993). Furthermore, in the biochemical study we observed an increase in urea levels in all groups of treated animals (except the lower dose of $Cu_2(fen)_4(dmf)_2$ group). The plasmatic urea is an indicator of renal damage taking into account that its level increases when glomerular filtration is reduced. In relation to creatinine, only the highest dose of $Cu_2(fen)_4(caf)_2$ exposed group increased the creatinine level with respect to the control. Creatinine is not a good indicator for the detection of incipient renal disease. Therefore, the increase in the plasmatic urea can be due to the decrease in the glomerular filtration caused by NSAIDs in all groups exposed to different conditions.

Even when in the hematological study we obtained a decreased number of erythrocytes in lower doses of fenoprofen salt and $Cu_2(fen)_4(caf)_2$ exposed groups and leukocytes in lower dose of $Cu_2(fen)_4(caf)_2$ group, we considered this result not relevant since this value is within reference values. The same applies to the number of platelets.

Finally, we observed an increase in AST level with the highest dose of $Cu_2(fen)_4(caf)_2$ with respect to the control and fenoprofen salt treated groups, indicating a possible liver damage.

In conclusion, in this work we identified the potent analgesic effect of $Cu_2(fen)_4(caf)_2$ and $Cu_2(fen)_4(dmf)_2$ and that this effect could be due to decrease the inflammation process. In a future, it would be interesting the elucidation the underlying mechanistic and the molecular bases of antinociceptive effect of these complexes. Furthermore, sub-acute exposure to different doses of these complexes did not produce significant changes in the parameters that evaluate toxicity in mice. Thus, these complexes may contribute to the development of novel therapeutic agents for treating inflammatory diseases due to the higher therapeutic activity and to the lower secondary effects than the parent drug.

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References

Abuhijleh, A.L., Woods, C., Bogas, E., Le Guenniou, G., 1992. Synthesis, characterization and catecholase-mimetic activity of monocuclear copper(II) aspirinate complexes. Inorg. Chem. Acta 195, 67–71.

Agotegaray, M.A., Quinzani, O.V., Faccio, R., Goyenola, C., Mombrú, A.W., 2008. Tetrakis [μ-2-(3-phenoxyphenyl)propionato-$_{κ2}$O:O]bis-[(dimethylformamide-$_{κ}$O)copper (II)]. Acta Crystallogr. 64, 1612.

Agotegaray, M.A., Boeris, M.A., Quinzani, O.V., 2010. Significant anti-inflammatory properties of a copper(II) fenoprofenate complex compared with its parent drug. Physical and chemical characterization of the complex. J. Braz. Chem. Soc. 21, S2–S3.

Auer, D.E., Ng, J.C., Seawright, A.A., 1990. Effect of palosein (superoxide dismutase) and catalase upon oxygen derived free radical induced degradation of equine synovial fluid. Equine Vet. J. 22, 13–17.

Beveridge, S.J., Whitehouse, M.W., Walker, W.R., 1982. Lipophilic copper(II) formulations: some correlations between their composition and anti-inflammatory/anti-arthritic activity when applied to the skin of rats. Agents Actions 12, 225–231.

Blahova, M., Tumova, I., Sokolik, J., Gallasova, M., Svec, P., 1994. Anti-inflammatory activity of aqua(aryloxyacetato)copper-(II) complexes. Pharmazie 49, 373–374.

Casteñeda-Hernandez, G., Castillo-Méndez, M.S., López-Muñóz, F.J., Granados-Soto, V., Flores-Murrieta, F.J., 1994. Potentiation by caffeine of the analgesic effect of aspirin in the pain-induced functional impairment model in the rat. Can. J. Physiol. Pharmacol. 72, 1127–1131.

Cini, R., Tamasi, G., defazio, S., Hursthouse, M.B., 2007. Unusual coordinating behavior by three non-steroidal anti-inflammatory drugs from the oxicam family towards copper(II). Synthesis, X-ray structure for copper(II)–isoxicam, -meloxicam and -cinnoxicam-derivative complexes, and cytotoxic activity for a copper(II)–piroxicam complex. J. Inorg. Biochem. 101, 1140–1152.

Collier, H.O.J., Dinneen, L.C., Johnson, C.A., Schneider, C., 1968. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmacol. Chemother. 32, 295–310.

Czapski, G., Goldstein, S., 1998. The uniqueness of superoxide dismutase (SOD). Why cannot most copper compounds substitute SOD in vivo? Free. Radic. Res. Commun. 4, 225–229.

Deraedt, R., Jouquey, S., Delevallée, F., Flahaut, M., 1980. Release of prostaglandins E and F in an algogenic reaction and its inhibition. Eur. J. Pharmacol. 11, 17–24.

Devereux, M., Shea, D.O., Kellett, A., McCann, M., Walsh, M., Egan, D., Deegan, C., Kedziora, K., Rosair, G., Muller-Benz, H., 2007. Synthesis, X-ray crystal structures and biomimetic and anticancer activities of novel copper(II)benzoate complexes incorporating 2-(4′-thiazolyl)benzimidazole (thiabendazole), 2-(2-pyridyl)benzimidazole and 1,10-phenanthroline as chelating nitrogen donor ligands. J. Inorg. Biochem. 101, 881–892.

Dillon, C.T., Hambley, T.W., Kennedy, B.J., Lay, P.A., Zhou, Q., Davies, N.D., Biffin, J.R., Regtop, H.L., 2003. Gastrointestinal toxicity, anti-inflammatory activity and superoxide dismutase activity of copper and zinc complexes of the anti-inflammatory drug indomethacin. Chem. Res. Toxicol. 16, 28–37.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effect of morphine, meperidine and brain stem stimulation in rats and cats. Pain 4, 161–174.

El Yacoubi, M., Ledent, C., Ménard, J.F., Parmentier, M., Costentin, J., Vaugeois, J.M., 2000. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A_{2A} receptors. Br. J. Pharmacol. 129, 1465–1473.

Engelhardt, G., Mauz, A.B., Pairet, M., 1997. Role of caffeine in combined analgesic drugs from the point of view of experimental pharmacology. Arzneimittelforschung 47, 917–927.

Everds, N., 2004. Hemathology of the mouse. In: Hedrich, H. (Ed.), The Handbook of Experimental Animals: The Laboratory Mouse. Elsevier Academic Press, USA.

Ferré, S., 2008. An update on the mechanisms of the psychostimulant effect of caffeine. J. Nerochem. 105, 1067–1079.

Ferreira, S.H., 1972. Prostaglandins, aspirin-like drugs and analgesia. Nat. New Biol. 13, 200–203.

Fisone, G., Borkvist, A., Usiello, A., 2004. Caffeine as a psychomotor stimulant: mechanism of action. Cell. Mol. Life Sci. 61, 857–872.

Fontenele, J.B., Viana, G.S., Xavier-Filho, J., de-Alencar, J.W., 1996. Anti-inflammatory and analgesic activity of a water-soluble fraction from shark cartilage. Braz. J. Med. Biol. Res. 29, 643–646.

Franzotti, E.M., Santos, C.V., Rodrigues, H.M., Mourao, R.H., Andrade, M.R., Antoniolli, A.R., 2000. Anti-inflammatory, analgesic activity and acute toxicity of Sida cordifolia L. (Malva-branca). J. Ethnopharmacol. 72, 273–277.

Fredholm, B.B., 1995. Adenosine, adenosine receptors and the actions of caffeine. Pharmacol. Toxicol. 76, 93–101.

Fredholm, B.B., Baé Ttig, K., Holmen, J., Nehlig, A., Zvartau, E.E., 1999. Action of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51, 83–133.

Fukawa, K., Kawano, O., Hibi, M., Misaki, N., Ohba, S., Hatanaka, Y., 1980. A method for evaluating analgesic agents in rats. J. Pharmacol. Meth. 4, 251–259.

Fukuta, K., 2004. Collection of body fluids. In: Hedrich, H. (Ed.), The Handbook of Experimental Animals: The Laboratory Mouse. Elsevier Academic Press, USA, pp. 543–554.

Gayawali, K., Pandhi, P., Sharma, P.L., 1991. Determination of the optimal analgesia-potentiating dose of caffeine and a study of its effect on the pharmacokinetics of aspirin in mice. Meth. Find. Exp. Clin. Pharmacol. 13, 529–533.

Guide for the Care and Use of Laboratory Animals, 2010. as adopted and promulgated by the National Institute of Health.

Hao, C.M., Komhoff, M., Guan, Y., Redha, R., Breyer, M.D., 1999. Selective targeting of cyclooxygenase-2 reveals its role in renal medullary interstitial cell survival. Am. J. Physiol. 277, 352–359.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.

Ingman, K., Sallinen, J., Honkanen, A., Korpi, E.R., 2004. Comparison of deramciclane to benzodiazepine agonists in behavioural activity of mice and in alcohol drinking of alcohol-preferring rats. Pharmacol. Biochem. Behav. 77, 847–854.

Jacobson, K.A., van Rhee, M., 1977. Development of selective purinoceptor agonists and antagonists. In: Jacobson, K.A., Jarvis, M.F. (Eds.), Purinergic Approaches in Experimental Therapeutics. Wiley-Liss, New York, pp. 101–128.

Klinowsky, E., Broide, E., Varsano, R., Eshchar, J., Scapa, E., 1996. Superoxide dismutase activity in duodenal ulcer patients. Eur. J. Gastroenterol. Hepatol. 8, 1151–1155.

Korolkiewicz, Z., Hac, E., Gagalo, I., Gorczyca, P., Lodzinska, A., 1989. The pharmacologic activity of complexes and mixtures with copper and salicylates or aminopyrine following oral dosing in rats. Agents Actions 26, 355–359.

Lewis, A.J., 1978. A comparison of the anti-inflammatory effects of copper aspirinate and other copper salts in the rat and guinea pig. Agents Actions 3, 244–250.

López-Muñoz, F.J., Díaz-Reval, M.I., Terrón, J.A., Déciga-campos, M., 1996. Analysis of the analgesic interactions between keterolac anc tramadol during arthritic nociception in rat. Eur. J. Pharmacol. 484, 157–165.

McCord, J.M., 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science 185, 529–531.

Medina, L.J.R., Domínguez-Ramírez, A.M., Jung, C.H., Bravo, G., Díaz-Reval, M.I., Déciga-Campos, M., López-Muñóz, F.J., 2006. Enhancement of entinociception by co-administration of ibuprofen and caffeine in arthritic rats. Eur. J. Pharmacol. 544, 31–38.

Mirica, L.M., Ottenwaelder, X., Stack, T.D.P., 2004. Structure and spectroscopy of copper–dioxygen complexes. Chem. Rev. 104, 1013–1046.

Moser, V., Ross, J., 1996. US EPA/AIHC Training Video and Reference Manual for a Functional Observational Battery. United States Environmental Protection Agency and Industrial Health Council.

Murray, M.D., Craig Brater, D., 1993. Renal toxicity of the nonesteroidal anti-inflammatory drugs. Annu. Rev. Pharmacol. Toxicol. 32, 435–465.

Nishiyama, J., Mizuno, M., Nasu, J., Kiso, T., Uesu, T., Maga, T., Okado, H., Tomoda, J., Yamada, G., Tsuji, T., 1996. Immunoelectron microscopic localization of copper–zinc superoxide dismutase in human gastric mucosa. Acta Histochem. Cytochem. 29, 215–220.

OECD, 2008. Test No. 407: Repeted dose 28-day oral toxicity study in rodents, OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. OECD Publishing.

Okuyama, S., Hashimoto, S., Aihara, H., Willingham, W.M., Sorenson, J.R., 1987. Copper complexes of non-steroidal anti-inflammatory agents: analgesic activity and possible opioid receptor activation. Agents Actions 21, 130–144.

Otuki, M.F., Lima, F.V., Malheiros, A., Cechinel-Filho, V., Delle Monache, F., Yunes, R.A., Calixto, J.B., 2001. Evaluation of the antinociceptive action caused by ether fraction and a triterpene isolated from resin of *Protium kleinii*. Life Sci. 28, 2225–2236.

Polisson, R., 1996. Nonsteroidal anti-inflammatory drugs: practical and theoretical considerations in their selection. Am. J. Med. 100, 31–36.

Powell, K.R., Iuvone, P.M., Holtzman, S.G., 2001. The role of dopamine in the locomotor stimulant effects and tolerance to these effects of caffeine. Pharmacol. Biochem. Behav. 69, 59–70.

Rosenzweig, A.C., Sazinsky, M.H., 2006. Structural insights into dioxygen-activating copper enzymes. Curr. Opin. Struct. Biol. 16, 729–735.

Rosland, J.H., Tjolsen, A., Maehle, B., Hole, K., 1990. The formalin test in mice: effect of formalin concentration. Pain 42, 235–242.

Sawynok, J., Yaksh, T.L., 1996. Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. Pharmacol. Rev. 45, 43–84.

Shuff, S.T., Chowdhary, P., Khan, M.F., Sorenson, J.R., 1992. Stable superoxide dismutase (SOD)–mimetic ternary human serum albumin–Cu(II) (3,5-diisopropylsalicylate) 2/Cu(II)2(3,5-diisopropylsalicylate)4 complexes in tissue distribution of binary complex. Biochem. Pharmacol. 43, 1601–1612.

Snyder, S.H., Katims, J.J., Annau, Z., Bruns, R.F., Daly, J.W., 1981. Adenosine receptors and behavioral actions of methyl-xanthines. Proc. Natl. Acad. Sci. 78, 3260–3264.

Sorenson, J.R., 1989. Copper complexes offer a physiological approach to treatment of chronic diseases. Prog. Med. Chem. 26, 437–568.

Sorenson, J.R., 1992. Use of essential metalloelement complexes or chelates in biological studies. Free Radic. Biol. Med. 13, 593–594.

Sorenson, J.R., Soderberg, L.S., Chang, L.W., 1995. Radiation protection and radiation recovery with essential metalloelement chelates. Proc. Soc. Exp. Biol. Med. 210, 191–204.

Steinar, H., Kjell, H., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.

Svenningsson, P., Nomikos, G.G., Ongini, E., Fredholm, B.B., 1997. Antagonism of adenosine A_{2A} receptors underlies the behavioural activating effect of caffeine and is associated with reduced expression of messenger RNA for NGFI-A and NGFI-B in daudate-putamen and nucleus accumbens. Neuroscience 79, 753–764.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.N., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

United States environmental Protection Agency (US EPA), 1998. Health effects test guidelines. OPPTS 870.6200. Neurotoxicity Screening Battery.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44–84.

Vinegar, R., Truax, J.F., Selph, J.L., Welch, R.M., White, H.L., 1996. Potentiation of the anti-inflammatory and analgesic activity of aspirin by caffeine in the rat. Proc. Soc. Exp. Biol. Med. 151, 556–560.

Wangila, G.W., Nagothu, K.K., Steward, R., Bratt, R., Ivere, P.A., Willingham, W.M., Sorenson, J.R., Shah, S.V., Portilla, D., 2006. Prevention of cisplatin-induced kidney epithelial cell apoptosis with a Cu superoxide dismutase mimetic [copper2II(3,5-ditertiaryutylsalicylate)4(ethanol)4]. Toxicol. In Vitro 20, 1300–1312.

Youssed, A.F., Santi, B.W., 1997. Simple neurobehavioral functional observational battery and objective gait analysis validation by the use of acrylamide and methanol with a built-in recovery period. Environ. Res. 73, 52–62.

Zar, J., 1999. Biostatistical analysis, fourth ed. Prentice Hall, New Jersey.