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Therapeutic properties, SOD and catecholase mimetic activities of novel ternary copper(II) complexes of the anti-inflammatory drug Fenoprofen with imidazole and caffeine

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ABSTRACT

The copper(II) ternary complexes of the non-steroidal anti-inflammatory drug Fenoprofen (Hfen) and the biologically relevant molecules imidazole (im) and caffeine (caf) as auxiliary ligands were investigated as novel anti-inflammatory agents. The new copper(II) complexes with formula $[Cu(fen)_2(im)_2]$ (**1**) and $Cu_2(fen)_4(caf)_2$ (**2**) were synthesized from the dinuclear complex $[Cu_2(fen)_4(dmf)_2]$ and characterized by IR, UV–Vis, EPR spectral and elemental analysis. The molecular structure of complex **1** was determined by X-ray crystallography. Both complexes **1** and **2** present enhanced and prolongued anti-inflammatory properties against the parent drug calcium Fenoprofenate, $Ca(fen)_2{\cdot}2H_2O$, with a better performance for complex **1**. Ternary complexes are potential models for several mono and poly-nuclear metal enzymes. The measured superoxide dismutase (SOD) mimetic activities of the complexes indicated a higher SOD mimic activity for complex **2** (IC_{50} of 0.24 μM) than complex **1** (IC_{50} of 0.70 μM), and also than the native enzyme evaluated by the same method (IC_{50} of 0.480 μM). The catecholase activity of the complexes toward the aerobic oxidation of 3,5-di-*tert*-butylcatechol (dtbc) onto 3,5-di-*tert*-butylquinone (dtbq) showed that both complexes have moderate catalytic oxidase activity.

1. Introduction

A great amount of data about the medicinal properties of small synthetic copper(II) complexes with carboxylato ligands recognized as non-steroidal anti-inflammatory drugs (NSAIDs) have been developed since the pioneering studies of Sorenson et al. [1,2]. These studies of the anti-inflammatory properties of binary and ternary copper(II) carboxylates were extended to other medicinal properties of this now wide family of compounds by several research groups. Anti-inflammatory [3], antioxidant and superoxide scavenging [3–7], catalytic and catecholase [4], anticonvulsant [8], DNA binding and nuclease [6,9–11], renal and gastrointestinal toxicity [11] and antimicrobial [5,12] properties have been reported for numerous copper(II)–NSAID complexes. A great part of those studies have been organized in an excellent review by Lay and co-workers [15].

It is known that many copper(II) complexes with non-steroidal anti-inflammatory drugs (NSAIDs) present enhanced anti-inflammatory properties when compared to the uncomplexed parent drug [15], however, this behavior has not been observed for some AINEs [16]. One mechanism of action proposed for this enhanced properties is that these complexes present antioxidant properties due to their capacity to mimic the activity of the superoxide dismutase enzyme [15]. Oxidative stress is implicated in pathophysiological processes, such as inflammation, rheumatoid arthritis and carcinogenesis [17]. The main species involved in the above mentioned processes is superoxide radical ($O_2^{\cdot-}$), an important regulator of cell death [18]. Superoxide dismutase, Cu,Zn-SOD is a metalloprotein which catalyzes the scavenging of superoxide anion O_2^-. Copper is present in the catalytic center and undergoes reduction–oxidation cycling during the dismutation of O_2^-. Although zinc ion is not involved in this cycle, it facilitates the oxidation step by maintaining the configuration of the active site [19].

The use of SOD enzyme as a diet supplement in patients presenting inflammatory and oxidative disorders is limited not only by costs, but also because of its high molecular weight and low stability. These facts make the research on SOD-mimic agents a relevant issue.

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Some copper(II) complexes also present catechol oxidase mimic activity. Catechol oxidase is a dicopper enzyme and belongs to the polyfenol oxidases [20]. It catalyzes the two-electron oxidation of o-difenols to the corresponding quinones. The role of this enzyme is related to the biological synthesis of polyfenolic pigments such as melanins, which protect tissues against pathogens [20].

Fenoprofen (Scheme 1), which systematic name is 2-(3-phenoxyphenyl)propanoic acid, is a non-steroidal anti-inflammatory drug employed in inflammatory pathologies such as rheumatoid arthritis. It exerts its anti-inflammatory activity by inhibiting ciclooxigenase enzyme, which participates in the inflammatory response [21]. In a previous work we have presented the first crystal structure of a dinuclear copper(II) complex of Fenoprofen and a full characterization of its structure and biological properties [22,23].

Motivated by the interest that represent copper(II) complexes of NSAIDs we continue our work in the synthesis of new Fenoprofen–copper(II) complexes. In this paper we present the first mononuclear copper(II) complex of Fenoprofen with imidazole as auxiliary ligand. The coordination chemistry of imidazoles has been subjected to intensive studies because the interaction of histidine residues and metal ions is relevant in many active biological systems. Herein we also report the spectroscopic and biological characterization of a new dinuclear Cu(II) complex of Fenoprofen containing caffeine in its structure. Caffeine is a xanthine alkaloid with stimulating activity. As part of the biological characterization, we compare the anti-inflammatory properties of the synthesized compounds with the uncomplexed commercial parent drug Fenoprofen calcium salt. The SOD-mimic activity is evaluated. Besides, catechol oxidase mimic activity is also presented as model for the catecholase function.

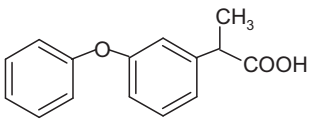
2. Experimental

2.1. General remarks

All reagents were of commercial analytical quality and used without further purification. The dinuclear complex $Cu_2(fen)_4(dmf)_2$ was synthesized following the procedure published elsewhere [8]. Chemical analyses of carbon, hydrogen and nitrogen have been performed using a Thermo Electron Flash EA instrument at UMYMFOR (FCEyN, UBA, Argentina).

2.2. Spectroscopic studies

IR spectra were recorded in KBr pellets and Nujol mulls with a Nicolet Nexus FT-IR spectrophotometer in the range 400–4000 cm^{-1}. Raman spectra of the solids, in the region between 3500 and 100 cm^{-1}, were obtained with a FRA 106 accessory mounted in a Bruker IFS 66 FTIR instrument, using a 1064 nm excitation line from an Nd-YAG laser. The UV–Vis spectra were registered on a GBC-Cintra 20 equipment in the region between 190 and 900 nm. CW EPR spectra of solid samples placed in quartz capillary tubes were obtained, at room temperature, with a Bruker ELEXSYS E500T A spectrometer, operating at X band (~9.8 GHz), with a Bruker cavity ER 4102ST and a modulation frequency of 100 KHz. Measurements of g values were made relative to TEMPO ($g = 2.0051$) [10,11].



Scheme 1. Structure of Fenoprofen (Hfen).

2.3. Preparation of complexes

2.3.1. Bis[2-(3-fenoxyfenyl)-propionato]bis(imidazole)copper(II), $Cu(fen)_2(im)_2$ (**1**)

A solution of 11.0 mg (0.160 mmol) of imidazole in 1 mL of acetone was added to another solution containing 50.0 mg (0.0400 mmol) of $Cu_2(fen)_4(dmf)_2$ in acetone. The blue solution was kept under stirring for about 30 min and then diffused with acetonitrile. After a few days at 4 °C, the obtained blue crystals were washed with acetonitrile and air dried. Yield: 28 mg (51%). Suitable single crystals were selected for X-ray diffraction analysis. *Anal.* Calc. for $C_{36}H_{34}CuN_4O_6$: C, 63.4; H, 4.9; N, 8.2%. Found: C, 62.4; H, 4.8; N, 8.3%. Several samples were used for the carbon content determination and reduced percentages were always obtained.

2.3.2. Tetrakis[μ-2-(3-fenoxyfenyl)-propionato-$\kappa^2O:O'$]bis[(1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione)copper(II)], $Cu_2(fen)_4(caf)_2$ (**2**)

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, 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: 34 mg (57%). The well-shaped crystals could not be solved by X-ray crystallography due to molecular disorder. *Anal.* Calc. 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%.

2.4. X-ray crystallography

The X-ray data collection and processing for complex **1** were performed on a Bruker APEX2/BIS/COSMO diffractometer by using graphite monochromated Mo Kα radiation ($\lambda = 0.71073$ Å) at 100(2) K. Cell refinement was done with Bruker SAINT and data reduction by Bruker SAINT/SADABS/XPREP. Programs used to solve structure were SHELX97; programs used to refine structure were SHELXL97. Images were created with Crystal Impact Diamond 3 molecular graphics [12].

2.5. Evaluation of anti-inflammatory properties

To evaluate the anti-inflammatory activity of complexes **1** and **2**, carrageenan induced paw oedema assay was carried out as described by Winter et al. [13]. Female mice of about 30 g were randomly divided into three groups. Each group contained six mice fasted for 18 h. Test animals were orally administered an aqueous suspension of the copper(II) complexes under study (31 mg/kg for complex **1** and 28 mg/kg for complex **2**) and the calcium salt of Fenoprofen (22 mg/kg). Each quantity of drug administered is equivalent to 20 mg/kg of Fenoprofen. The vehicle alone was used as excipient for the control group. The drugs were suspended in carboxymethylcellulose at 0.1% and Tween 80 at 0.05% (1:1, v/v) just before use. Drug and excipient were orally administered in 0.5 mL of the corresponding suspension to each animal one-hour before inducing oedema in the left hind paw by sub-plantar injection of 0.05 ml of a 2% suspension of carrageenan.

The length of the paw was measured with a digital electronic caliber "Caliper" (0.1 mm resolution) immediately before the injection of carrageenan and 3, 5, 7 and 9 h after the injection. The anti-inflammatory effect of the test was expressed in terms of the percent inhibition of oedema produced by each drug-treated group and was calculated as $[(\Delta C - \Delta T)/\Delta C] \times 100$ being ΔC the mean of the control group and ΔT the mean of the test group. The data were expressed as mean ± SEM.

Student's t test has been applied in order to determine significant differences between the mean responses of the treated and control groups. The difference in the mean response between the group treated with the complex and one treated with the Fenoprofen calcium salt (Aldrich) was also evaluated. Statistical significance was set as $P < 0.05$.

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 as adopted and promulgated by the National Institute of Health.

2.6. Superoxide dismutase activity assay

The SOD-mimic activity was determined by an indirect method adapted from Starha et al. [14]. This method is based on the competitive reaction of the tested compounds (**1**, **2**, Fenoprofen calcium salt and $CuCl_2$) and XTT dye [2,3-bis(2-methoxy-4-nitro-5-sulfofenyl)-2H-tetrazolium-5-carboxanilide natrium salt] (Aldrich) with a saturated DMF solution of potassium superoxide, KO_2 (Aldrich). The interaction of XTT dye with superoxide anion radical led to the formation of the XTT-formazane. Its concentration was determined by spectroscopic measurements at 480 nm. The tested complexes, which acted as the scavengers for superoxide anion radicals, decreased the absorbance at 480 nm.

The required amounts of 1.00×10^{-3} mol L^{-1} DMF solutions of the tested compounds were added to 1.0×10^{-2} M potassium phosphate buffer (pH 7.4) to provide 0.125, 0.250, 0.500, 0.750, 1.00, 2.50, 5.00, 7.50 and 10.0×10^{-6} mol L^{-1} solutions. For the cases of $CuCl_2$ and Fenoprofen calcium salt the final copper(II) concentration as well as the concentration of Fenoprofenate anions were the same than the acquired for complexes solutions in order to compare results. Subsequently, 500 μL of XTT dissolved in buffer were added. The resulting solution was mixed thoroughly. The reaction was started by the addition of 500 μL of a saturated KO_2 solution in DMF. In all cases, the final volume was 3.00 mL and five samples of each concentration level of all the copper(II) complexes were tested ($n = 5$). After 30 min of incubation at room temperature the absorbance at 480 nm was measured against a blank sample prepared without the XTT dye. The same procedure was used to prepare the control sample without the tested copper(II) complexes and the absorbance was measured against a solution containing only XTT dye. The percentages of inhibition (% INH) were determined according to the formula $(1 - X_{sample}/X_{control}) \cdot 100 \pm (S^2_{sample} + S^2_{control})^{1/2} \cdot 100\%$ where X is the media of the absorbance and S its standard deviation. The SOD-mimic activity was expressed as the IC_{50} value, calculated from the concentration-dependent curve of the inhibition of the absorbance at 480 nm at eight concentration levels. The IC_{50} values represent the concentrations of the tested copper(II) complexes reducing the XTT-formazane formation to 50%.

2.7. Catecholase mimetic activity assay

Kinetic experiments concerning the catechol oxidase activity of complexes **1** and **2** related with the oxidation of 3,5-di-*tert*-butylcatechol (3,5-dtbc) (Aldrich) were monitored spectrophotometrically by following the increase of the 3,5-di-*tert*-butyl-*o*-benzoquinone (3,5-dtbq) characteristic absorption band at 400 nm. Data of thermostated solutions (25.0 ± 0.5 °C) were collected over 10 min. The experiment was carried out by the addition of 1.50 mL of a freshly prepared (3,5-dtbc) solution in methanol (0.25, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0×10^{-3} mol L^{-1}) to a 1-cm-path-lengh cell containing 1.50 mL of a methanol solution of the copper complexes in order to obtain a final copper(II) concentration of 5.00×10^{-5} mol L^{-1}. During the kinetic run, the reaction cell was opened to the air to allow the solution to be continually equilibrated with atmospheric oxygen. The mean value of three experiments was determined using a methanolic solution of the substrate without the complex as a blank. Concentrations were evaluated from the absorbance measurements using the reported extinction molar coefficient, $\varepsilon_{400nm} = 1900$ L mol^{-1} cm^{-1} for 3,5-dtbq in methanol [17]. Time profiles were adjusted with a polynomial function and the initial rates were evaluated as the derivative of this function at $t = 0$–2 min. The same treatment was applied to a methanolic solution of $CuCl_2$ in order to obtain the same final copper(II) concentration of the complex solution, and to evaluate the catecholase activity of free cupric ions.

A kinetic treatment of the experimental data revealed a saturation Michaelis-type mechanism for the reaction and then the Michaelis–Menten approach was applied using a commercially available software.

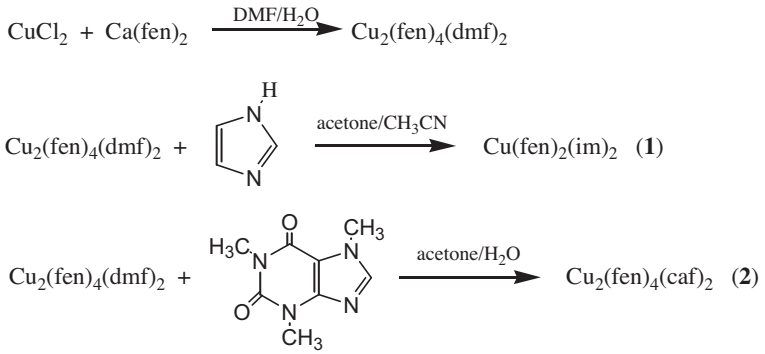
3. Results and discussion

The reaction of cupric chloride and calcium Fenoprofenate yielded the dinuclear copper(II) Fenoprofenate in quantitative amount [22]. Its reaction with a 1:4 and 1:2 molar proportion of imidazole and caffeine bases, respectively, produces the mononuclear complex $Cu(fen)_2(im)_2$ (**1**) and the dinuclear species $Cu_2(fen)_4(caf)_2$ (**2**). Both substances were soluble in acetone, DMF and DMSO, scarcely soluble in methanol and ethanol, and insoluble in acetonitrile and water. Selected crystals of complex **1** were structurally studied by X-ray diffraction techniques (Scheme 2).

3.1. Description of the $Cu(fen)_2(im)_2$ (**1**) structure based upon X-ray crystallography

The crystal data with structure refinements are given in Table 1. The molecular structure of the complex $Cu(fen)_2(im)_2$ is shown in Fig 1.

The structure consists of discrete centrosymmetric mononuclear units in which the Cu(II) ions acquire distorted octahedral environments. The almost *trans*-square plane coordination environment in the equatorial plane is built by two closest oxygen atoms (O(1)) and ($O(1^a)$) atoms of different Fenoprofenate anions and two nitrogen atoms (N(1)) of the neutral imidazole molecules. The apical coordination sites of the copper centers are occupied by other two weakly coordinating atoms (O(2)) and ($O(2^a)$) of the carboxylate anions located at a greater distance. Selected bond distances and angles of the crystal structure are shown in Table 2. The asymmetric chelate type coordination of Fenoprofenate anions (κ^2-O,O′) forces a small angle (c.a. 53°) between the square CuO_2N_2 plane and the O(2)-Cu-O(2) line. The coordination sphere of the metallic center is similar to that observed for the other few structurally resolved examples of mononuclear *bis*-imidazole adducts of copper(II) carboxylates [24–27]. Comparing with the other Cu(II)-AINE complex reported, the *trans*-$Cu(salicylate)_2(im)_2$ complex [25], the Cu–O distances are longer (2.042 Å) and the Cu–N bond lengths a little shorter (1.961 Å) than in the Fenoprofenate complex **1**. The Cu–O distances of the weakly coordinated carboxylate oxygen atoms in the axial positions for the salicylate adduct (2.87 Å) are also longer than those of the Fenoprofenate complex **1**, suggesting a closer carboxylate–Cu(II) interaction in the later. The observed distances and angles of the Fenoprofenate anions are very similar (differences in the order of 0.03 Å and 3°, respectively) to those reported for the dinuclear $Cu_2(fen)_4(dmf)_2$ complex [22]. Between the crystal packing forces there is only one intermolecular hydrogen bridge between a imidazole proton (N(2)–H(2)) and a Fenoprofenate oxygen atom ($O(2)^b$) with a total distance d(N2–H2···$O2^b$) = 2.752(2) Å.



Scheme 2. Synthetic procedure used with copper(II) Fenoprofenates.

Table 1
Crystal data and structure refinement parameters for complex **1**.

Empirical formula	$C_{36}H_{34}CuN_4O_6$
Formula weight	682.22
T (K)	100(2)
λ (Å)	0.71073
Crystal system	Monoclinic
Space group	$P2_1/c$
Unit cell dimensions	
a (Å)	10.1183(11)
b (Å)	12.881(2)
c (Å)	12.3477(17)
α (°)	90
β (°)	94.943(4)
γ (°)	90
V (Å^3)	1603.3(4)
Z	2
Density (calculated) (Mg/m^3)	1.413
μ (mm^{-1})	0.735
F(000)	710
Crystal size (mm^3)	0.23 × 0.18 × 0.18
θ (°)	2.96–30.14
Index ranges	$-14 \leqslant h \leqslant 14$, $-18 \leqslant k \leqslant 14$, $-17 \leqslant l \leqslant 16$
Reflections collected	17683
Independent reflections	4708 [R_{int} = 0.0365]
Completeness of theta = 30.14° (%)	99.3
Refinement method	Full-matrix least-squares on F^2
Data/restrains/parameters	4708/3/229
Goodness-of-fit (GOF) on F^2	1.107
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.0430, wR_2 = 0.1004
R indices (all data)	R_1 = 0.0607, wR_2 = 0.1091
Largest difference peak and hole (e Å^{-3})	0.770 and −0.635

*3.2. Description of the Cu(fen)$_2$(im)$_2$ (**1**) and Cu$_2$(fen)$_4$(caf)$_2$ (**2**) structures based upon the spectroscopic analysis*

The EPR spectra of microcrystalline samples of complexes Cu(fen)$_2$(im)$_2$ (**1**) and Cu$_2$(fen)$_4$(caf)$_2$ (**2**) confirm the stabilization of Cu(II) centers in both substances and their mononuclear and ligand-bridged dinuclear molecular structures, respectively. The room temperature EPR spectrum of complex **1** shown in Fig 2A was analyzed using the freeware WinSim 2002 program [28]. The simulation could be achieved with three nearly equal g-components $g_1 = 2.112$, $g_2 = 2.107$ and $g_3 = 2.109$ ($g_{av} = 2.11$), which is consistent with a pseudo-isotropic system (see Supplementary information S1). The X-ray structural determination indicates the presence of tetragonally elongated environments for the non-isolated Cu(II) centers (Fig 1) and an expected dx^2–y^2 ground state with g-values in the order: $g_{||} \gg g_{\perp} > 2.0$ [29,30]. The presence of two 90° misaligned chromophores (50:50%) of the single copper(II) centers in the crystalline solid (see Supplementary information S2) produces the pseudo-isotropic behavior of the substance [27].

The EPR spectra for the microcrystalline form of the dinuclear Cu(II) complex **2** (Fig 2B), recorded at room temperature, resemble those of previous reported Cu(II) Fenoprofenates [23,31] and other tetracarboxylate-bridged dinuclear Cu(II) complexes with well known paddle wheel "Cu(II)$_2$–(RCOO)$_4$" cages [32–34]. Assuming that the zero field splitting (D) is significantly greater than $h\nu$, $E \cong 0$ (axial symmetry), a singlet ground state ($S = 0$) and a thermally populated triplet state ($S = 1$), we expect to observe three absorptions of the six $\Delta M = \pm 1$ resonance fields [32]. Data obtained for complex **2** are: $Hz_1 = 370$, $H_{\perp} = 4810$, and $Hz_2 = 5990$ G, which are in good accordance to previous reports in similar Cu(II) dinuclear complexes [23,31,35]. The quality of the spectra does not allow a precise determination of the magnetic parameters. g-Values ($g_{\perp} = 2.11$, $g_z = 2.48$, and $g_{av} = 2.23$) were calculated taking into account the simplified model for moderate interacting dinuclear Cu(II) centers [32,33]. The calculated magnetic parameters are in agreement with the previous reports for dinuclear copper Fenoprofenates (Table 3) and other dicopper(II)–tetracarboxylate complexes [23,31,34,36–38].

Complexes **1** and **2** present two characteristic IR absorption bands (1578/1391 and 1602/1409 cm^{-1}, respectively for **1** and **2**) corresponding to the antisymmetric, ν_{as}(COO), and symmetric, ν_s(COO), stretching vibrations of the carboxylate groups of the Fenoprofenate anions. Those bands are shifted against Ca(fen)$_2$·H$_2$O (1560/1420 cm^{-1}) [23] indicating the coordination of the carboxylate ligands to the copper(II) centers. The separation between ν_{as}(COO) and ν_s(COO) bands (187 cm^{-1}) in complex **1** is consistent with carboxylate groups acting as unsymmetrical bidentate ligands and comparable to those reported for mononuclear copper(II)–carboxylate–imidazole complexes having essentially the CuN_2O_2 chromophore [34,39]. The great separation of the carboxylate bands for complex **2** (193 cm^{-1}) is similar to those previously reported for other Cu(II)-Fenoprofenates (Table 4) and other binuclear copper(II) complexes in which the carboxylate adducts act as bridging bidentate ligands [40,41]. The coordination of imidazole molecules to copper(II) in complex **1** is evidenced by the presence of some characteristic bands in the vibrational spectra such as that at 3138 (ν(NH)), 1330 (ν(CN)), 1073 (δ(CNC)) and 752 (γ (CH)) cm^{-1} shifted with respect to the free imidazole molecule [42]. The free caffeine molecule presents strong absorption bands at 1699 (ν(CN)), 1660 (ν_{as}(CO)) and 1542 (ν_s(CO)) cm^{-1}. These bands appear in the IR spectrum of complex **2**, shifted to higher wavenumbers, 1708, 1666 and 1582 cm^{-1}, respectively, indicating the coordination of the caffeine molecules to copper(II) [43]. A stretching vibration (ν(CC)) of the heterocyclic ring of caffeine, appearing at 1590 cm^{-1} in the free ligand, shifts to a higher Raman signal (1597 cm^{-1}) on complexation to the metal atom [44].

The electronic spectra for the mononuclear complex **1** as Nujol mull and in solution (Table 4) exhibit one weak, broad and asym-

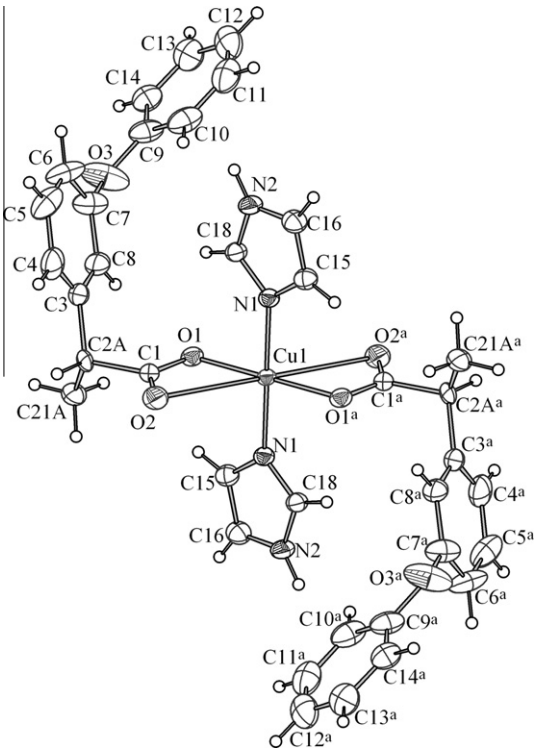


Fig. 1. ORTEP view of the copper complex **1** with 50% probability of thermal ellipsoids.

Table 2
Selected bond lengths (Å) and angles (°) for $[Cu(fen)_2(im)_2]$ (**1**).

Bond lengths (Å)		Bond angles (°)	
Cu1–O1	1.9668(13)	$O1^a$–Cu1–O1	180.00(10)
Cu1–$O1^a$	1.9668(13)	$O1^a$–Cu1–N1	90.48(6)
Cu1–O2	2.733(3)	O1–Cu1–N1	89.52(6)
Cu1–$O2^a$	2.733(3)	$O1^a$–Cu1–$N1^a$	89.52(6)
Cu1–N1	1.9815(15)	O1–Cu1–$N1^a$	90.48(6)
Cu(1)–$N(1)^a$	1.9815(15)	N1–Cu1–$N1^a$	180.00(10)
O1–C1	1.273(2)	O2–C1–O1	123.84(18)
O2–C1	1.237(3)	O2–C1–C2A	114.86(19)
C1–$C2^a$	1.542(3)	O2–Cu1–N1	90.48(6)
$O2^a$–$C1^a$	1.273(2)	O1–Cu1–O2	53.25(5)
$O1^a$–$C1^a$	1.237(3)	$O1^a$–$C1^a$–$C2A^a$	121.26(19)
$C1^a$–$C2A^a$	1.542(3)		
Hydrogen bonds			
D–H···A	d(D–H)	d(H···A)	d(D···A)
N(2)–H(2)···O(2)	0.88	1.87	2.752(2)

metric band around 660 nm and very intense ultraviolet bands. The first band is identified with d-d transitions of the Cu(II) center [38]. This band is due to the *xz*, $yz \rightarrow x^2 - y^2$ transitions with a shoulder at lower energies originated in the $z^2 \rightarrow x^2 - y^2$ transition. It appears at the expected position for mononuclear copper(II) carboxylate adducts containing two imidazole molecules [34]. The ultraviolet absorptions correspond to $\pi \rightarrow \pi^*$ transitions of the aromatic rings of imidazole and Fenoprofenate moieties.

Complex **2** electronic spectra correspond to those reported for dinuclear Cu(II)–carboxylate complexes [45,46], showing a weak asymmetric band around 690 nm and intense unresolved ultraviolet absorptions. The first, as in complex **1**, is usually assigned to the $d \rightarrow d$ transitions of tetragonally distorted Cu(II) centers. New experimental and theoretical data of the model complex $Cu_2(CH_3COO)_4{\cdot}2H_2O$ recently reported propose that not only copper(II) orbitals but also carboxylate anions orbitals are involved in the long wavelength transitions [47]. In Nujol mulls and in solutions in acetone and DMSO another very weak band is observable around 370 nm. This band corresponds to the Cu–OCO–Cu bridging linkage in dinuclear copper(II) complexes [38,45] and its charge–transfer character has been confirmed by theoretical calculations [46]. The presence of this absorption in the spectra of complex **2** clearly indicates that paddle-wheel units of the solid complex are still present in solution. Remarkably, this band is not present in the spectra of methanolic solutions, indicating the rupture of the paddle wheel "$Cu(II)_2$–$(RCOO)_4$" cages into mononuclear species, maybe to $Cu(fen)_2(caf)(MeOH)$ molecules. This behavior has been observed in methanol for other dinuclear Cu(II)–carboxylato complexes [46,48]. Following the criteria of Prenesti et al. [49] the highest energy location of the visible band of complex **1** compared to complex **2** assures the former still forms $Cu(fen)_2(im)_2$ molecular units in methanolic solutions.

3.3. Anti-inflammatory properties

The results obtained in carrageenan induced paw oedema in mice are presented in Table 5. Fenoprofen calcium salt presented very significant differences with respect to the control at the fifth hour from the beginning of the assay and significant differences at the third and at the seventh hour. The mononuclear $Cu(fen)_2(im)_2$ complex (**1**) presented a significant difference in reduction of oedema at the third hour of the experiment; meanwhile it presented very significant differences at the fifth and seventh hours when compared to the control group. These results indicate that, when comparing complex **1** and the uncomplexed parent drug Fenoprofen, even both present anti-inflammatory properties, the complex shows a more sustained activity in time. As can be seen in Fig 3, the inflammation inhibition percent approached for complex **1** was significantly higher than that for Fenoprofen calcium salt at the seventh and at the ninth hour of the assay (51.3% and 32.0% for complex **1** versus 19.9% and 10.5% for Fenoprofen calcium salt, respectively), indicating a higher capacity of the mononuclear complex to maintain the anti-inflammatory activity until the end of the assay.

When analyzing the results obtained for the dinuclear $Cu_2(fen)_4(caf)_2$ complex (**2**) the pattern for the inhibition of inflammation differs from the observed for the mononuclear complex **1**. When compared to the control, the dinuclear copper(II) complex presented very significant differences until the seventh hour of the carrageenan induced paw oedema. The percent of inhibition of inflammation was higher for this compound than Fenoprofen calcium salt and complex **1** until the seventh hour.

When comparing the complexes under study each other, it can be seen from Fig 3 that the mononuclear copper(II) complex containing Fenoprofenate anions and imidazole, presents a more sustained anti-inflammatory activity in time, but on the other hand, complex **2** containing caffeine is more effective as anti-inflammatory agent at the first time of the experiment with excellent percentages of inhibition until the fifth hour.

It is also important the comparison of the anti-inflammatory activity of the complexes here studied with the one for other Cu(II) complexes with AINEs. In the study carried out by Dillon et al. [3] it was evaluated the anti-inflammatory activity of the uncomplexed parent drug Indomethacin and the dinuclear copper(II) complex $Cu_2(Indo)_4(dmf)_2$. It was found that the most significant results were obtained at the third hour of the assay, so they only showed the results for that time, finding that the copper(II) complex presents higher anti-inflammatory properties than Indomethacin. Taking into account these results it is remarkable to mention that we have found significant results until the ninth hour from the inflammation induction for complex **1** and until the seventh hour for complex **2**, indicating a more sustained anti-inflammatory

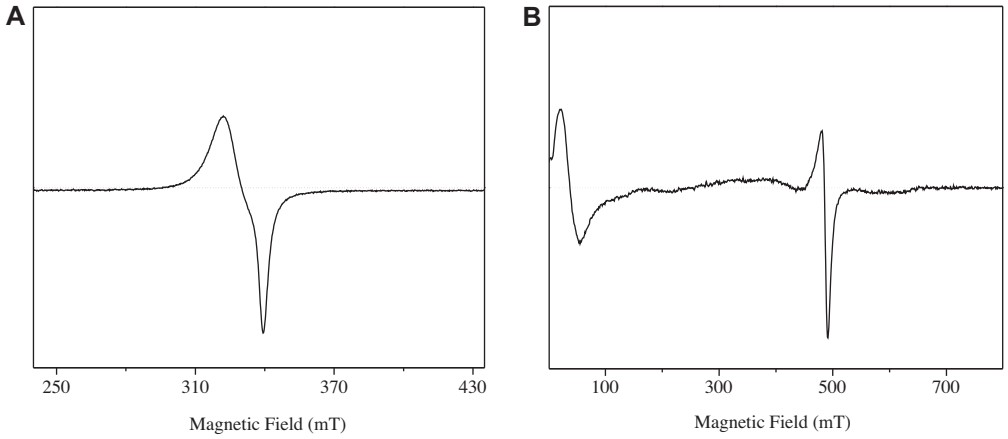


Fig. 2. 298 K X-band (9.75 MHz) spectra of microcrystalline complexes **1** (A) and **2** (B). For A: central field: 335 mT, sweep width: 80 mT; microwave power: 0.6539 mW. For B: central field: 400 mT, sweep width: 800 mT; microwave power: 43 mW.

Table 3
X-band EPR data[a] of complex **2** and other dinuclear copper(II) Fenoprofenates.

Compounds	Hz_1/G	$H_\perp$/G	Hz_2/G	$g_\perp$	g_z	Refs.
$[Cu_2(fen)_4(dmf)_2]$	502	4590	6080	2.14	2.49	[23]
$[Cu_2(fen)_4(4,4'\text{-bipy})]_n$	629	4740	6210	2.11	2.48	[31]
$[Cu_2(fen)_4(2,5\text{-}Me_2pyz)]_n$	557	4710	6080	2.20	2.52	[31]
$Cu_2(fen)_4(caf)_2$	370	4810	5990	2.14	2.49	T.w.

[a] At room temperature.

activity in time for both complexes. In another study reported by Razi et al. [50] the anti-inflammatory properties of the NSAID Diflunisal and the corresponding copper(II) complex were analyzed. Both substances were tested for 4 h employing the same technique. The increase in percentage inhibition of paw oedema for Diflunisal copper complex as compared to the drug was 6%, 9%, 11%, 12% and 11% at 30 min, 1, 2, 3 and 4 h after the carrageenan injection, respectively. For complex **1**, the increase in percentage inhibition of paw oedema with respect to Fenoprofen calcium salt was 32% and 22% at the seventh and ninth hour from the carrageenan injection and 13%, 6% and 19% at third, fifth and seventh hour of the assay for complex **2**. These differences indicate that both copper(II) Fenoprofenates here studied are very good and promising anti-inflammatory agents when compared to other copper(II) complexes with NSAIDs not only for the activity purchased but also for their capacity to sustain the effect in time.

Table 4
Selected FTIR (cm^{-1}) and UV–Vis data [λ(nm)/Molar absorptivity ε ($L\,mol^{-1}\,cm^{-1}$)] of complexes **1** and **2** and other dinuclear copper(II) Fenoprofenates.

Compound	Infrared bands		UV–Vis bands				
	ν_{as}(COO)	ν_s(COO)	Nujol	DMSO	Acetone	Methanol	Assig.[a]
$[Cu(fen)_2(im)_2]$	1578	1391	660[a]	696/124	658/156	686/85	d–d
$Cu_2(fen)_4(caf)_2$	1602	1409	680	716/404	683/423	696/136	d–d
				375/350	370/280		CTB[b]
$[Cu_2(fen)_4(dmf)_2]$ [21]	1612	1408	699			694/140	d–d
							CTB[b]
$[Cu_2(fen)_4(pyz)]_n$ [30]	1618	1409	699	704/419			d–d
				385/210			CTB[b]
$[Cu_2(fen)_4(4,4'\text{-bipy})]_n$ [30]	1626	1406	699	705/491			d–d
				380/245			CTB[b]

[a] See text for assignments.
[b] CTB: charge transfer band.

Table 5
Anti-inflammatory activity in carrageenan-induced paw oedema in mice.

Groups	Δ mm (mean ± SEM)[a]			
	3 h	5 h	7 h	9 h
Control	1.44 ± 0.09	1.70 ± 0.08	1.71 ± 0.09	1.52 ± 0.11
Fenoprofen calcium salt	1.02 ± 0.14*	0.98 ± 0.15**	1.37 ± 0.09*	1.36 ± 0.20
Indomethacin	1.28 ± 0.08	1.46 ± 0.13	1.34 ± 0.10*	1.41 ± 0.09
Complex **1**	0.97 ± 0.17*	1.02 ± 0.15**	0.83 ± 0.14**,#	1.03 ± 0.20
Complex **2**	0.83 ± 0.13**	0.88 ± 0.13**	1.05 ± 0.17**	1.42 ± 0.16

[a] Mean values of the difference of the length of the hind paw with respect to the beginning of the assay.
* $p < 0.05$ in comparison to control.
** $p < 0.01$ in comparison to control.
# $p < 0.05$ with respect to Fenoprofen calcium salt.

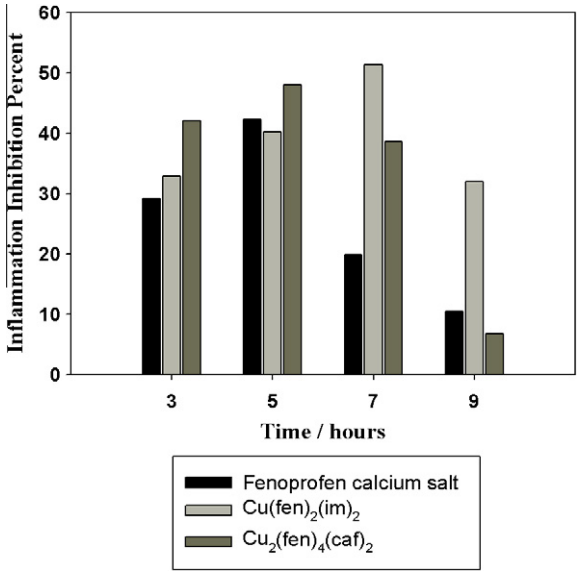


Fig. 3. Inflammation inhibition percent approached by the complexes under study and Fenoprofen calcium salt in carrageenan induced paw oedema in mice.

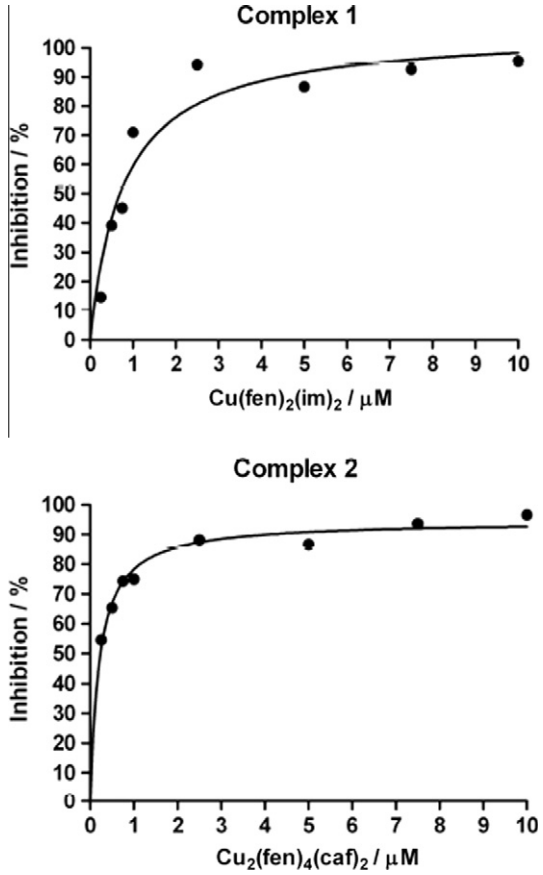


Fig. 4. Plot of percentage of inhibition of XTT-formazane against the increase in concentration of the complexes under study.

3.4. Superoxide dismutase activity assay

The superoxide dismutase mimetic activity of complexes **1** and **2** has been determined through the XTT method and is expressed as the IC_{50} value, which represents the concentration (μM) of the complex or enzyme required to reduce 50% of the superoxide radical anion ($O_2^{\cdot-}$). Fig 4 shows the percent inhibition of the formation of XTT-formazane against the increasing concentration of the complexes under study. Table 6 presents the IC_{50} values of complexes **1**, **2** and other mononuclear and dinuclear complexes evaluated by different techniques. Although sometimes the activity of the complexes is not preserved across the different assays [7] we still found that in this case the IC_{50} values obtained for the investigated complexes employing the XTT method are comparable to that obtained with other methods. Both complexes investigated in this work presented significant SOD-mimic activity.

Table 6
SOD mimic activity of the complexes under study and other Cu(II) complexes with NSAIDs.

Compound	IC_{50} (μM)	Refs.
$Cu(fen)_2(im)_2$ (**1**)[a]	0.70	T.w.
$Cu_2(fen)_4(caf)_2$ (**2**)[a]	0.24	T.w.
$Cu_2(fen)_4(dmf)_2$[a]	0.41	[31]
$Cu_2(indo)_4(dmf)_2$[b,c]	0.23	[3]
$Cu(sal)_2(1,2\text{-meim})_2$[b,d]	0.65	[51]
$Cu(sal)_2(bzdh)_2$[b,e]	0.74	[6]
Native Cu,Zn-SOD[b]	0.0042	[52]
Native Cu,Zn-SOD[a]	0.480	[14]

[a] Compound evaluated by the XTT method.
[b] Compound evaluated by the xantine–xantine oxidase/INT method.
[c] Indo: indomethacinate.
[d] sal: salicylate; meim: methylimidazole.
[e] bzdh: benzimidazole.

The SOD-mimic activity of imidazole containing complex **1** is comparable to other complexes of Cu(II) with neutral imidazole derivatives and other nitrogenated ligands. In this mononuclear compound, as well as proposed for other Cu(II)–NSAIDs complexes with the same structure, the site on Cu(II) for $O_2^{\cdot-}$ binding would be provided by the dissociation of one of the oxygen atoms from the carboxylate anions or by the imidazole nitrogen atom. This dissociation would facilitate the necessary geometrical changes to permit the reaction course. The dinuclear copper complex $Cu_2(fen)_4(caf)_2$ presents a SOD mimic activity comparable to the dinuclear copper(II) complex of Indomethacin with dmf as adduct, which is considered to be an excellent SOD mimic compound [3]. In a previous work we have evaluated the SOD mimic activity of the complex $Cu_2(fen)_4(dmf)_2$ with an IC_{50} value of 0.411 μM [31]. The replacement of the axial dmf ligands by caffeine molecules increases the activity for complex **2**. The mechanism for scavenging $O_2^{\cdot-}$ would consist, in this case, in the dissociation of one Fenoprofenate oxygen atom in the equatorial plane of a copper(II) ion. This site would be occupied by superoxide and the nitrogen atom corresponding to the caffeine molecule would provide a more favorable environment for the reaction in comparison to the oxygen atom of the dmf ligand in $Cu_2(fen)_4(dmf)_2$. Remarkably, complex **2** presents higher SOD mimic activity than the native enzyme evaluated by the same method (Table 6).

3.5. Catechol oxidase mimetic activity

The ability of the complexes under study to oxidize catechols was tested following the procedure previously reported [23]. In a kinetic run a 2.50×10^{-3} mol L^{-1} methanolic solution of 3,5-DTBC was reacted with a 5.00×10^{-5} mol L^{-1} methanolic solution of $Cu(fen)_2(im)_2$ (**1**) (a concentration of 2.50×10^{-5} mol L^{-1} was used for $Cu_2(fen)_4(caf)_2$ (**2**) in order to achieve the same conditions related to copper concentration), under aerobic conditions and the course of the reaction was followed by UV–Vis spectroscopy (Fig 5). Both complexes show moderate catecholase activity.

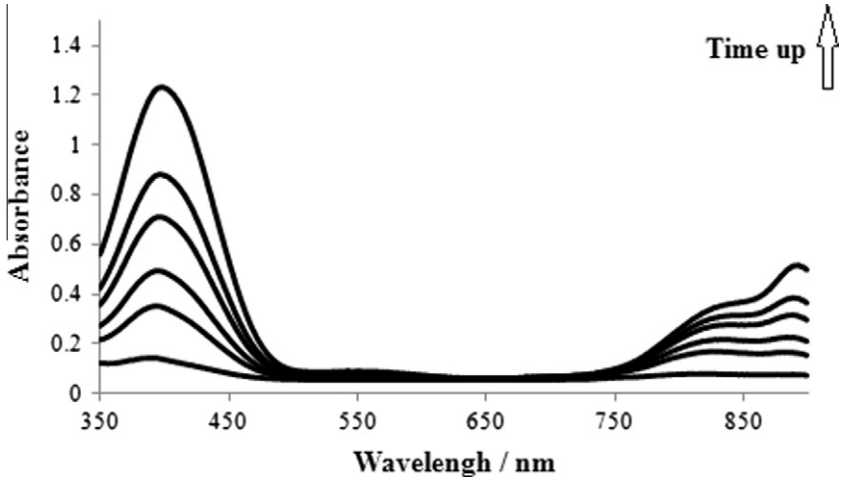


Fig. 5. Plot of the evolution of the UV–Vis spectra of the oxidation reaction of 3,5-DTBC to 3,5-DTBQ in methanol at 25.0 °C, catalyzed by the $[Cu(fen)_2(im)_2]$ complex (**2**). The spectra were recorded at the beginning of reaction and after 5, 10, 20, 30, 60 min and corrected with the blank of reaction.

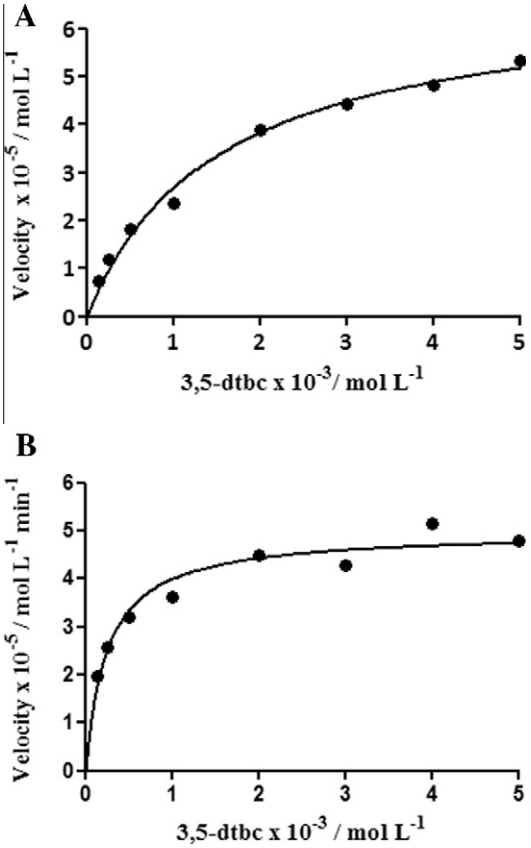


Fig. 6. Initial reaction rate (Vo) of the oxidation of dtbc to dtbq in methanol at 25.0 ± 0.5 °C: (A) $[Cu_2(fen)_4(caf)_2]$ (2.50×10^{-5} mol L^{-1}); (B) $[Cu(fen)_2(im)_2]$ (5.00×10^{-5} mol L^{-1}).

The 3,5-DTBQ concentration versus time plots were fitted with potential functions in order to recover the initial reaction rates (Vo). The dependence of Vo on the 3,5-DTBC concentrations for the oxidation reaction catalyzed by the copper(II) complexes (Fig 6) and the correlation between Vo^{-1} and $[3,5\text{-DTBC}]^{-1}$ both indicate the existence of a two step Michaelis type mechanism for the reaction [53]. A complete kinetic study was carried out on the basis of the Michaelis–Menten (M–M) model, originally developed for enzymatic reactions. The good accordance of data with this model indicates the existence of a first complexation equilibrium between the DTBC substrate and the active copper(II) species in the methanol solution. The obtained M–M parameters are shown in Table 7. As it was pointed out previously when discussing the UV–Vis spectra the active copper(II) species present in the methanolic solution could be mononuclear. The K_M value indicates that complex **1** presents lower affinity in bonding to the 3,5-dtbc molecule and then it is a better catalyst than the complexes containing dmf and caffeine as adducts. In Table 7 the catalytic behavior of the new complexes has been compared with bibliographic data of active dinuclear copper(II) complexes (as far as we know M–M parameters have not been reported yet for mononuclear species) the new compounds show greater affinity to the binding of 3,5-dtbc (lower K_M values) than dinuclear copper(II) model complexes of catechol oxidase activity and, by this way, a lower catalytic performance [54–57]. For other Cu(II)–NSAIDs complexes the catecholase mimetic activity was reported by Abuhijleh and

Table 7
Michaelis–Menten and enzymatic activity parameters for the catecholase activity of copper(II) complexes.

Compound	K_M[a]	V_{max}[b]	Enzymatic activity[c]	Refs.
$[Cu(fen)_2(im)_2]$ (**1**)	1.50 ± 0.23	6.72 ± 0.38	1.97	T.w.
$[Cu_2(fen)_4(caf)_2]$ (**2**)	0.25 ± 0.05	4.97 ± 0.19	1.34	T.w.
$[Cu_2(fen)_4(dmf)_2]$	0.13 ± 0.02	5.70 ± 0.16	1.84	[23]
$Cu_2(L1)(H_2O)(NO_3)_2$[d]	2.1	1800	n.r.	[54]
$Cu_2(Stryp)_2(H_2O)$[e]	173	53.4	n.r.	[56]
$[Cu_2(BPMP)(OAc)_2][ClO_4].H_2O$[f]	6.4	37	n.r.	[55]
$[Cu_2(L_2O)(CF_3SO_3)](CFSO_3)_2$[g]	2.92	24.6	n.r.	[57]
$[Cu(ibup)_2(im)_2]$[h]	n.r.[k]	n.r.	0.12	[3]
$[Cu(valp)_2(im)_2]$[i]	n.r.	n.r.	0.75	[58]
$[Cu_2(valp)_4]$	n.r.	n.r.	2.6	[58]
$[Cu_2(nap)_4]_n$[j]	n.r.	n.r.	1.3	[4]
$[Cu_2(ibup)_4(caf)_2]$	n.r.	n.r.	0.220	[3]

[a] In units 10^{-3} mol L^{-1}.
[b] In units of 10^{-5} mol L^{-1} min^{-1}.
[c] Amount of 3,5-dtbq (μmol) produced by 1.0 mg of the complex.
[d] L_1: 2-formil-4-metil-6R-Iminometil-fenolato.
[e] STryp: triptofane.
[f] BPMP: 2,6-bis[bis(piridin-2-yl-methylamine)methyl]-4-methylfenolate.
[g] L_2: 1,3-bis{N,N-bis(2-[2-piridyl]ethyl}-amine-2-hidroxypropane.
[h] Ibup: ibuprofenate.
[i] Valp: valproate.
[j] Nap: naproxenate.
[k] n.r.: not reported.

co-workers as the enzymatic activity [4,33,57]. The new copper Fenoprofenates showed good catecholase mimetic activity in comparison to other NSAIDs complexes.

As reported previously, when methanolic solutions of $CuCl_2$ were tested versus the 3,5-DTBC substrate, under the same conditions than the complexes under study, there was no significant catecholase mimetic activity for the solvated copper(II) ions [23]. This may be indicative that in the methanolic solutions of the complexes the active species still have the Fenoprofenates acting as ligands. In terms of the catalytic model for mononuclear copper(II) compounds proposed by Abuhijleh and co-workers [59–61] the low performance of the $CuCl_2$ solutions could be due to the absence of a proton scavenger for the production of $dtbc^{2-}$ anions.

4. Conclusions

Mixed-ligand ternary copper(II) complexes with the anti-inflammatory drug Fenoprofen as primary ligand and the biologically relevant imidazole and caffeine molecules as auxiliary ligands were synthesized and characterized by spectral methods. The crystal structure of complex **1** was determined by X-ray diffraction techniques. Complex **1** is built of mononuclear $[Cu(fen)_2(im)_2]$ units in which the copper centers have rhombically distorted octahedral $N_2O_2 + O_2$ coordination spheres. The dinuclear structure of complex **2** was elucidated by EPR spectral analysis. The molecular complex **2** is built of typical paddle-wheel binuclear "$Cu_2(fen)_4$" units with two caffeine molecules at the axial positions of the Cu(II) centers. The carrageenan induced paw oedema in mice experiments show that both complexes **1** and **2** present enhanced and lasting anti-inflammatory properties against the parent drug calcium Fenoprofenate, $Ca(fen)_2{\cdot}2H_2O$. The imidazole containing complex **1** has a similar performance than the caffeine containing complex **2** in the first phase of the experiments (3–5 h) but greater significant effects at long time exposure (7–9 h). By this way this complex could be considered as a potential therapeutic agent in the treatment of inflammatory diseases. Complex **1** has similar but lower anti-inflammatory properties than the dinuclear complex $[Cu_2(fen)_4(dmf)_2]$ [23] showing that there is not a clear correlation between structure–anti-inflammatory effects due to the unknown differences in the pharmacodinamic properties of these copper(II) complexes. Both complexes show potent superoxide dismutase (SOD) mimetic activities determined through the XTT method. The measured SOD mimetic activities of the complexes indicated a higher activity for complex **2** (IC_{50} of 0.24 μM) than complex **1** (IC_{50} of 0.70 μM), and also than the native enzyme evaluated by the same method (IC_{50} of 0.480 μM). The measured SOD mimetic activity of both complexes **1** and **2** are in the range of other copper(II)–NSAID compounds studied through the same experimental procedures [6,30,49]. The SOD activity presented by the complexes here studied permits its consideration as therapeutic agents because of their anti-oxidant activity and the low molecular weight in comparison to the native enzyme. The catecholase activities of the complexes toward the aerobic oxidation of 3,5-di-*tert*-butylcatechol (dtbc) onto 3,5-di-*tert*-butylquinone (dtbq) showed that both complexes have moderate catalytic oxidase activities.

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Appendix A. Supplementary data

CCDC 831490 contains the supplementary crystallographic data for **1**. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.poly.2011.12.005.

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