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$Cu(Nor)_2 \cdot 5H_2O$, a complex of Cu(II) with Norfloxacin: theoretic approach and biological studies. Cytotoxicity and genotoxicity in cell cultures

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Abstract Norfloxacin is a fluoroquinolone antibiotic used in the treatment of bacterial infections. In this article, we studied the potential antitumoral action of a complex of Norfloxacin with Cu(II), $Cu(Nor)_2 \cdot 5H_2O$ on osteosarcoma cells (UMR106) and calvaria-derived cells (MC3T3-E1), evaluating its cytotoxicity and genitoxicity. We have also elucidated the more stable conformation of this complex under physiologic conditions by Molecular Dynamic simulations based on the model of the canonical ensemble and PM6 force field. When solvent effect was taken into account, the complex conformation with both carbonyl groups in opposite sides displayed lower energy. $Cu(Nor)_2 \cdot 5H_2O$ caused an inhibitory effect on the proliferation on both cell lines from 300 μM ($P < 0.01$). Nevertheless, the decline on cell proliferation of UMR106 cells was more pronounced (45 % vs basal) than in MC3T3-E1 cells (20 % vs basal) at 300 μM ($P < 0.01$). $Cu(Nor)_2 \cdot 5H_2O$ altered lysosomal metabolism (Neutral Red assay) in a dose-dependent manner from 300 μM ($P < 0.001$). Morphological studies showed important transformations that correlated with a decrease in the number of cells in a dose-dependent manner. Moreover, $Cu(Nor)_2 \cdot 5H_2O$ caused statistically significant genotoxic effects on both osteoblast cell lines in a lower range of concentrations (Micronucleus assay) ($P < 0.05$ at 10 μM, $P < 0.001$ from 25 to 50 μM). UMR106 cells displayed a dose-related genotoxic effect between 5 and 25 μM while the MC3T3-E1 cells showed a narrower concentration dependent range. Altogether, these results suggest that $Cu(Nor)_2 \cdot 5H_2O$ is a good candidate to be further evaluated for alternative therapeutics in cancer treatment.

Keywords Copper · Norfloxacin · Cytotoxicity · Genotoxicity · Osteoblast cells

Introduction

Norfloxacin (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) is a member of the fluoroquinolone group of antibiotics. It is a wide-ranging drug used in the treatment of bacterial infections of the skin, the urinary, and respiratory tracts, among others [1]. Many drugs possess modified toxicological and pharmacological properties when they are in the form of metal complexes [2].

Metals are essential cellular components that function in several indispensable biochemical processes for living organisms. Relevant metal properties include redox activity, variable coordination modes, and reactivity toward organic substrates. Owing to the importance of their functions in living organisms, metals are tightly regulated under normal conditions and aberrant metal ion concentrations are associated with various pathological disorders, including cancer.

The most widely studied metal in this context is copper(II) which, due to its anti-inflammatory properties, has proved to be beneficial in diseases with an inflammation base such as tuberculosis, gastric ulcers, rheumatoid arthritis, and cancers [3–6]. Moreover, copper is a biometal found in all living

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organisms and is a crucial trace element in redox chemistry, cell growth, and development. On the other hand, copper is also a necessary component to support angiogenesis, that is, the formation of new blood vessels in tumors, increasing their metastatic potential, the major cause of morbidity in cancer patients. So, to some extent, lowering copper concentration reduces the risk to develop tumors [7].

One of the main goals of the Medicinal Chemistry is to obtain metal-based drugs with different mechanisms of action to improve or promote the healthy status of the living organisms. Based on the assumption that endogenous metals may be less toxic, several researchers have investigated copper compounds of fluoroquinolones [8, 9].

Norfloxacin was considered the best of the second generation Quinolone family for its antibacterial properties [10]; as a *N*-heterocyclic compound, Norfloxacin easily interacts with metal ions and forms complexes. Quinolone antibiotics can participate in the formation of complexes in a number of ways [11–15]. In the case of isolated drugs, these complexes in acidic media usually contain singly and/or doubly protonated Quinolones that are incapable of bonding to the metal ions and in these cases only electrostatic interaction was observed between the drug and the metal ions [11–13]. On the other hand, it was found that neutral Quinolones in the zwitterionic state are capable of forming simple complexes (bidentate chelating). The Quinolones can also act as bridging ligands and thus are capable of forming polynuclear complexes [15, 16]. There are several reports about Norfloxacin complexes with metals [17–19]. In the form of metallic complexes, Norfloxacin and related compounds possess modified pharmacological properties [9]. Various studies showed that antibacterial activity of many Norfloxacin–metal complexes is different from Norfloxacin [9, 20, 21]. In addition, the studies on the interaction between Quinolone–metal complexes and DNA exemplify the importance of the metal ions for the biological activity of these drugs [22, 23]. This interaction with DNA contributes to the main pharmacological antibacterial activity of the antibiotics [24], and it can also be responsible, at least in part, for other pharmacological effects on mammalian cells such as antiproliferative actions. Therefore, the investigation of the biological properties of these complexes is important for a better understanding of their therapeutic efficacy.

The synthesis and physicochemical characterization of a complex of Norfloxacin with Cu(II), $Cu(Nor)_2{\cdot}5H_2O$ have been previously reported by Ruiz et al. [17]. In order to obtain a deeper insight into the putative conformation of this complex, which its X-ray structure could not be determined, we report herein a theoretic approach by the semiempirical quantum method PM6 for optimize all geometries obtained and calculate heats of formation for compounds of interest in biochemistry. This method is somewhat better than Hartree–Fock (HF) or B3LYP DFT methods using the 6-31G(d) basis set. For a representative set of compounds, PM6 gave an average unsigned error (AUE) of 4.4 kcal mol^{-1}; for the same set, HF and B3LYP had AUE of 7.4 and 5.2 kcal mol^{-1}, respectively [25]. Moreover, as a part of a project devoted to the development of transition metal complexes with potential pharmacological properties, the cytotoxic and genotoxic effects of this complex have been tested in two osteoblast cell lines of murine origin (MC3T3-E1 preosteoblast cells of normal phenotype and UMR106 osteosarcoma cells).

Materials and methods

Materials

Norfloxacin was purchased from Provefarma S.A. (Colombia). Other chemicals such as Metanol and $Cu(NO_3)_2{\cdot}3H_2O$ were obtained from Merck. Dulbecco's Modified Eagles Medium (DMEM) was purchased from GBO Argentina and fetal bovine serum (FBS) from Internegocios SA (Buenos Aires, Argentina); trypsin–EDTA was provided by Gibco (Gaithersburg, MD, USA); Neutral red (NR) and Cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Theoretic approach

The theoretic study was performed using the semiempirical quantum chemical PM6 method [25].

Experimental

Synthesis of $Cu(Nor)_2{\cdot}5H_2O$ $Cu(Nor)_2{\cdot}5H_2O$ (Cu–Norfloxacin) was synthesized according to literature [17]. Briefly, 0.15 mmol of $Cu(NO_3)_2{\cdot}3H_2O$ in 15 mL of distilled water was poured with continuous stirring into an aqueous NaOH solution (0.3 M) of Norfloxacin (0.3 mmol). Final pH 9.4. The green solid obtained was filtered and washed with methanol: water 50:50 (2 × 25 mL) and air dried. Infrared spectroscopy (IR) and Differential Scanning Calorimetry (DSC) were used to identify Norfloxacin raw material as Norfloxacin dihydrate [26]. Besides, the same methods were employed to identify the powder obtained in the chemical synthesis of the complex. The IR spectrum of the complex exhibited a broad band at 3400 cm^{-1} which can be assigned to the O–H stretching vibrations of water molecules and also includes the N–H stretching vibration of the ligand. Besides, the IR spectrum presented a very strong band around 1624 cm^{-1} which can be assigned to ν(CO) of the ketone

group and to νa(OCO) of the carboxylate group, indicating the deprotonation of this moiety and its interaction with the metal ion. Besides, the intense band at ca 1385 cm^{-1} can be assigned to νs(OCO). These results agreed to those previously reported by Ruiz et al. [17].

Preparation and stability of $Cu(Nor)_2{\cdot}5H_2O$ solutions Fresh stock solutions of the complex and the free ligand were prepared in DMSO at 20 mM and diluted according to the concentrations indicated in the legends of the figures. The stability of the complex solution in DMSO was followed by UV–vis spectroscopy between 200 and 800 nm. The absorption band at 285 nm remains unchanged after 48 h. This band has been assigned to the coordination of copper ions to the carboxylic group and ketone groups of Norfloxacin [27].

Cell culture and incubations Two osteoblastic cell lines were used for the culture experiments: MC3T3-E1 cells derived from mouse calvaria and UMR106 osteoblast-like cells derived from a rat osteosarcoma [28]. Both UMR106 and MC3T3-E1 cell lines were grown in DMEM containing 10 % FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5 % CO_2 atmosphere. Cells in a 75-cm^2 flask were grown until reach 70–80 % of confluence. Then, the cells were subcultured using 0.1 % trypsin–1 mM EDTA in a Ca^{2+}–Mg^{2+}-free phosphate buffered saline (PBS). For experiments, cells were grown in multiwell plates. When cells reached the desired confluence, the monolayers were washed with DMEM and were incubated under different conditions according to the experiments.

Cell proliferation: crystal violet assay A mitogenic bioassay was carried out as described by Okajima et al. [29] with some modifications. Briefly, the cells were grown in 48-well plates (30.000 cells/well) for 24 h and incubated with medium alone (basal) and different concentrations (50–500 μM) of the complex for 24 h at 37 °C. After this treatment, the monolayers were washed with PBS and fixed with 5 % glutaraldehyde/PBS at room temperature for 10 min. After that, they were stained with 0.5 % crystal violet/25 % methanol for 10 min. Then, the dye solution was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 mL/well 0.1 M glycine/HCl buffer, pH 3.0/30 % methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution.

Cell morphology Cells were grown on glass coverslips and incubated under control conditions (without complex addition) or with different concentrations of $Cu(Nor)_2{\cdot}5H_2O$ in serum-free DMEM for 24 h. Then, the cells were fixed with methanol at −20 °C and stained with Giemsa 5 % [30, 31]. Samples were observed under light microscopy.

Cytotoxicity: neutral red (NR) assay The NR accumulation assay was performed according to Borenfreund and Puerner [32]. Cells were plated in 96-well culture plates (2.5×10^4 cells/well). Cells were treated with different $Cu(Nor)_2{\cdot}5H_2O$ concentrations for 24 h at 37 °C in 5 % CO_2 in air. After treatment, the medium was replaced by one containing 100 μg/mL NR dye and cells were incubated for other 3 h. Then, NR medium was discarded, the cells were rinsed twice with warm (37 °C) PBS (pH 7.4) to remove the non-incorporated dye, and 100 μL of 50 %ethanol and 1 % acetic acid solution was added to each well to fix the cells releasing the NR into solution. The plates were shaken for 10 min, and the absorbance of the solution in each well was measured in a Microplate Reader (7530, Cambridge technology, Inc, USA) at 540 nm, and compared with wells with untreated cells. Optical density was plotted as percentage of control.

Genotoxicity: cytokinesis-block micronucleus (MN) assay Cytokinesis-block micronucleus technique was set up with cultures in the log phase of growth. Cells were seeded onto pre-cleaned coverslips in 35-mm Petri dishes at a density of 3×10^4 cells/dish and incubated at 37 °C for 24 h. Then, the cells were treated with different concentrations of the complex with cytochalasin B (4.5 μg/mL). After 24 h, the cells were rinsed and subjected to hypotonic conditions with 0.075 % KCl at 37 °C for 5 min, fixed with pure methanol at −20 °C for 10 min and stained with 5 % Giemsa solution. After staining, the coverslips were air dried and placed down onto pre-cleaned slides using Depex mounting medium.

For MN assay, 500 binucleated (BN) cells were scored at 400× magnification per experimental point from each experiment. The examination criteria employed were reported before [33]. Briefly, the criteria employed in identifying micronuclei (MNi) were diameter smaller than 1/3 of that of the main nuclei, non-refractibility, same staining intensity as or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, and MNi boundary distinguishable from the main nuclei boundary.

Results

$Cu(Nor)_2{\cdot}5H_2O$ was synthesized according to literature [17]. Infrared spectroscopy (IR) and Differential Scanning Calorimetry (DSC) were used to identify Norfloxacin raw material as Norfloxacin dihydrate [26]. Besides, the same

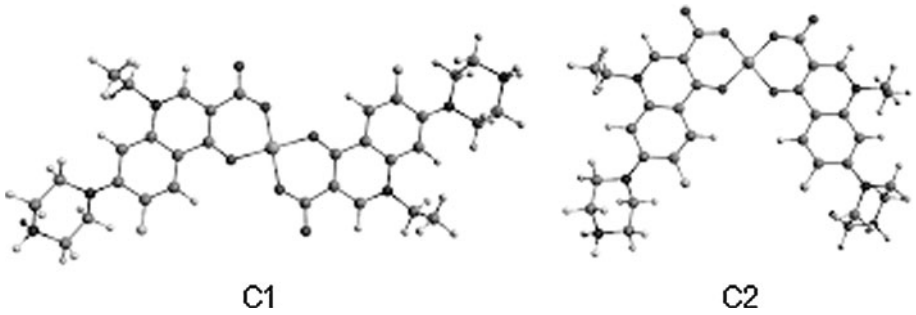

Fig. 1 Conformations of the complex $Cu(Nor)_2 \cdot 5H_2O$. *Circle* represents the Cu^{2+} ions. Conformation C1 is the one that has both carbonyl groups in the same side, and conformation C2 presents those groups in opposite side of the complex molecule

methods were employed to identify the powder obtained in the chemical synthesis of the complex.

Theoretic approach

To elucidate the more stable conformation of this complex under physiologic conditions (aqueous solution and pH 7.4), Molecular Dynamic simulations of Norfloxacin were carried out based on the model of the canonical ensemble and PM6 force field, using a Berendsen thermostat at 900 K. This method was successfully used to reproduce atomic distances in many copper species of importance in biochemistry [25]. Run total time was 40 ps with equilibration time of 10 ps and time step of 1 ps. Conformations were saved every 2 ps obtaining a total of twenty which were then optimized at the level of the semiempirical method PM6 [25]. Besides, the 20 conformations were also optimized with DFT BLYP functional and cc-pVTZ basis set for C and H atoms and cc-aug-pVTZ basis set for N, O, and F atoms for validating the semiempirical method. The lowest energy conformation of Norfloxacin was used for binding to the copper atom to build the complex.

Two possible conformations for the complex $Cu(Nor)_2 \cdot 5H_2O$ were tested and both optimized at PM6 level. Molecular modeling was done considering Norfloxacin with its carboxylate group deprotonated because it corresponds to synthesized complex in solid state. Crystallization water molecules were not considered for modeling. We called conformation 1 (C1) to that which has both carbonyl groups in the same side, and conformation 2 (C2) the one which presents those groups in opposite side of the complex molecule (Fig. 1). Optimization was performed in vacuum and, under this condition, the calculated heat of formation of C1 for $Cu(Nor)_2$ was 3.98 kcal more stable than C2. This geometry is in agreement with x-ray crystallographic structure reported for the binary complex $[Cu(HNor)_2]Cl_2 \cdot 2H_2O$ [17]. When solvent effect was taken into account setting the dielectric constant of water, C2 resulted 1.51 kcal lower than C1.

Experimental

Effect of $Cu(Nor)_2 \cdot 5H_2O$ on cell proliferation

Figures 2 a and 2b show the effect of the free ion, Cu (II), the free ligand (Norfloxacin), and the complex on osteoblast-like cell proliferation. As can be seen from Fig. 2a, in UMR106 osteosarcoma cells, Norfloxacin slightly decreased cell proliferation (ca 20–30 %) at the higher concentrations (300 and 500 μM), while the complex produced a stronger decrease (ca 45–50 %) at the same concentrations. Moreover, Cu(II) did not exert any significant action in the whole range of concentrations.

On the other hand, as can be seen from Fig. 2b, in MC3T3-E1 cell line, Norfloxacin displayed a different behavior than in the tumoral cells since it stimulated cell proliferation in the whole range of tested concentrations ($P < 0.01$). Besides, copper(II) and the complex acted in a similar way, causing a slight decrease of cell proliferation at 300 μM (ca 15 %), while at 500 μM, the inhibitory action was similar to that observed for the tumoral cell line (ca 55 %, $P < 0.01$). Comparing the effect of the complex, both cell lines were affected from 300 μM. Nevertheless, the decline on cell proliferation of UMR106 osteosarcoma cells (Fig 2a) was more pronounced than in the MC3T3-E1 calvaria-derived cells (Fig 2b) ($P < 0.01$), which makes $Cu(Nor)_2 \cdot 5H_2O$ a good candidate to be further evaluated as a potential antitumoral drug.

Effect of $Cu(Nor)_2 \cdot 5H_2O$ on cell morphology

From Fig. 3, it can be seen that as the complex concentration increases, a reduction in the cell number per field can be observed for both cell lines. Besides, at 500 μM, UMR106 osteosarcoma cells only show well stained although pyknotic nuclei, while MC3T3-E1 osteoblasts display fairly well-maintained morphological features. These results are in agreement with the experiments on cell proliferation since from a morphological point of view, the complex was also more deleterious in the tumoral osteoblasts.

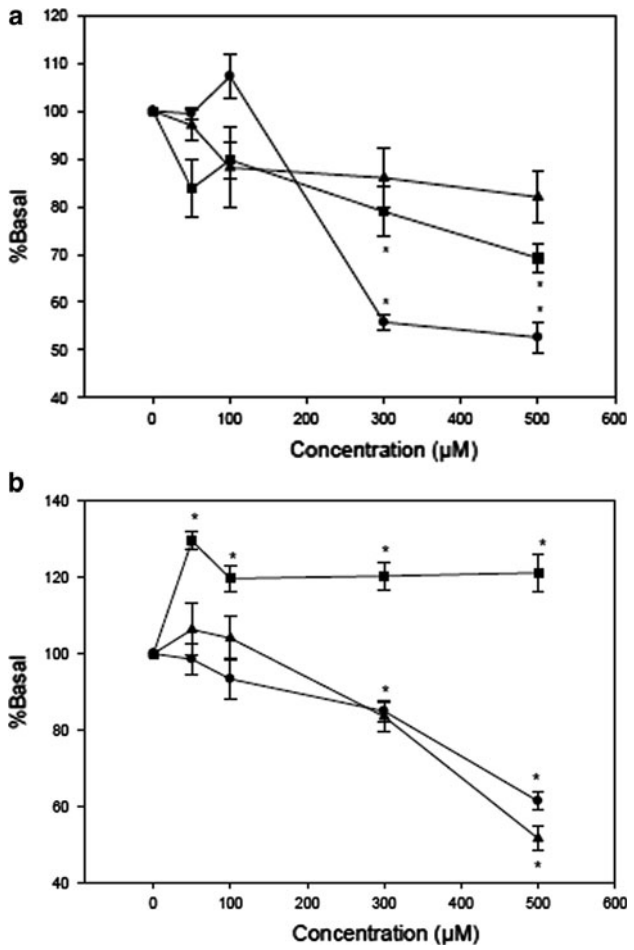

Fig. 2 Crystal violet assay. UMR106 cells (**a**), MC3T3-E1 cells (**b**). Effect of $Cu(Nor)_2{\cdot}5H_2O$, Norfloxacin, and Cu^{2+} on osteoblast-like cell proliferation. Cells were incubated in serum-free DMEM without (0 represents basal condition) or with different concentrations of $Cu(Nor)_2{\cdot}5H_2O$ at 37 °C for 24 h. Results are expressed as % basal and represent the mean ± SEM ($n = 9$) * Significant differences versus control, $P < 0.01$. *Triangle* correspond to Cu^{2+}, *square* represent Norfloxacin, *circle* correspond to $Cu(Nor)_2{\cdot}5H_2O$

Effect of $Cu(Nor)_2{\cdot}5H_2O$ on cell cytotoxicity

The Neutral Red uptake assay is suitable for the assessment of lysosome metabolic activity. A complex concentration-dependent decrease in both cell lines can be observed from Fig. 4a, b. UMR106 cells showed a significant reduction from 300 μM ($P < 0.001$), while MC3T3-E1 displayed this decrease only at 500 μM. These results agree with the effect of the complex on cell proliferation (Fig 2a, b) since it was more deleterious for the tumoral cell line on the basis of both determinations. Moreover, the effects of Norfloxacin and $CuCl_2$ were also evaluated. None of them showed any effect in MC3T3-E1 cells in the studied concentration range, and both compounds produced a slight, but significant, decrease in UMR106 cells at 500 μM. The cytotoxic effects of the complex on osteoblast-like cells may be due to the functional disruption of organelles like the lysosomes.

Induction of micronuclei by $Cu(Nor)_2{\cdot}5H_2O$

The genotoxic effect was studied by the assessment of micronuclei induction. The micronucleus assay (MN) is a test used in toxicological screening for potential genotoxic compounds. A micronucleus is the erratic (third) nucleus that is formed during the anaphase of mitosis or meiosis. Micronuclei are cytoplasmic bodies having a portion of acentric chromosome or the whole chromosome which were not carried during the anaphase to the opposite poles, finally resulting in missing of part or whole chromosomes for the daughter cell. These chromosome fragments or whole chromosomes normally develop nuclear membrane and form the micronuclei as a third nucleus [34]. Since the genotoxicity is a process that can begin before the alterations at the cytoplasm level could be detected, we investigated the effect of $Cu(Nor)_2{\cdot}5H_2O$ at the cell nuclear level by the MN assay in the range of 5–50 μM. The effect of $Cu(Nor)_2{\cdot}5H_2O$ on the induction of micronucleus in binucleated cells can be observed from Fig. 5. The complex induced micronuclei formation from 10 μM concentration ($P < 0.05$) with a great increment in the damage from 25 μM ($P < 0.001$) in both cell lines compared with control conditions. Besides considering the effects of different doses in each cell line, the results indicate that the increase in MN frequency on MC3T3-E1 cells treated with $Cu(Nor)_2{\cdot}5H_2O$ showed a concentration-related manner from 5 to 10 μM. At the latter concentration, the effect reached a *plateau*. On the other hand, the effect on UMR106 cells showed a concentration–response curve from 5 to 25 μM without any further variation. Bleomycin was used as positive control. It demonstrated a similar effect compared to the complex at the highest concentration (50 μM) in both cell lines.

Discussion

A very high significant number of compounds derived from Quinolone family have been developed up to now [35, 36]. The coordination chemistry of these drugs of biological and pharmaceutical importance with metal ions such as copper is of considerable interest. Antibiotics of the Quinolone family can participate in the formation of metal complexes in different ways [19]. The zwitterionic state of neutral Quinolones is the chemical form predominant under physiologic conditions (pH 7.4). This Quinolone zwitterion form is able to chelate metal ions forming simple metal complexes by the coordination of the deprotonated carboxylate group at

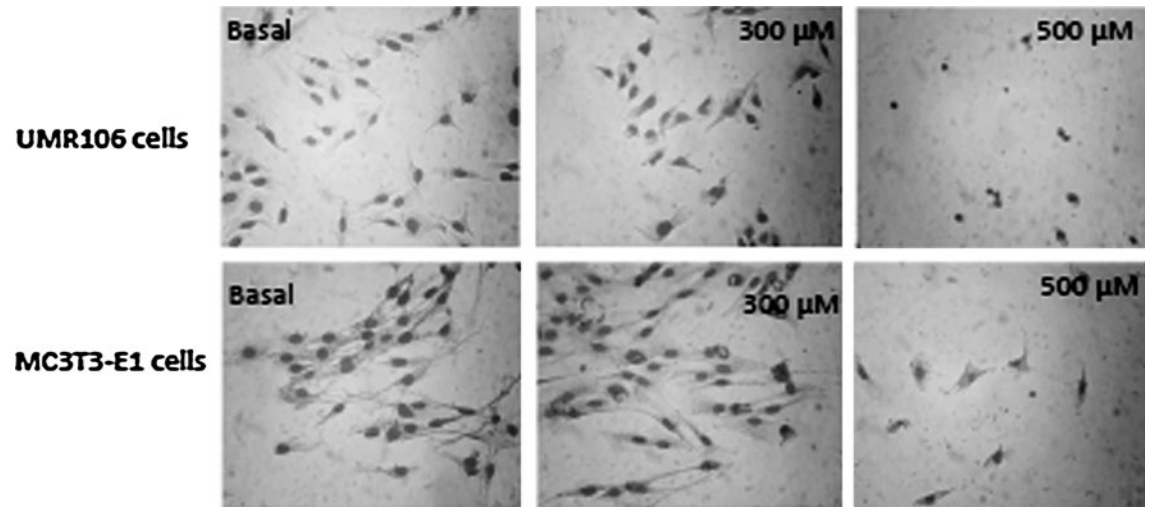

Fig. 3 Morphological study. Effect of $Cu(Nor)_2{\cdot}5H_2O$ on osteoblast-like cells (UMR106 cells: *upper panel* and MC3T3-E1 cells: *lower panel*). Cells were incubated with $Cu(Nor)_2{\cdot}5H_2O$ for 24 h. Then, the cells were stained with Giemsa and observed by light microscopy (magnification ×100)

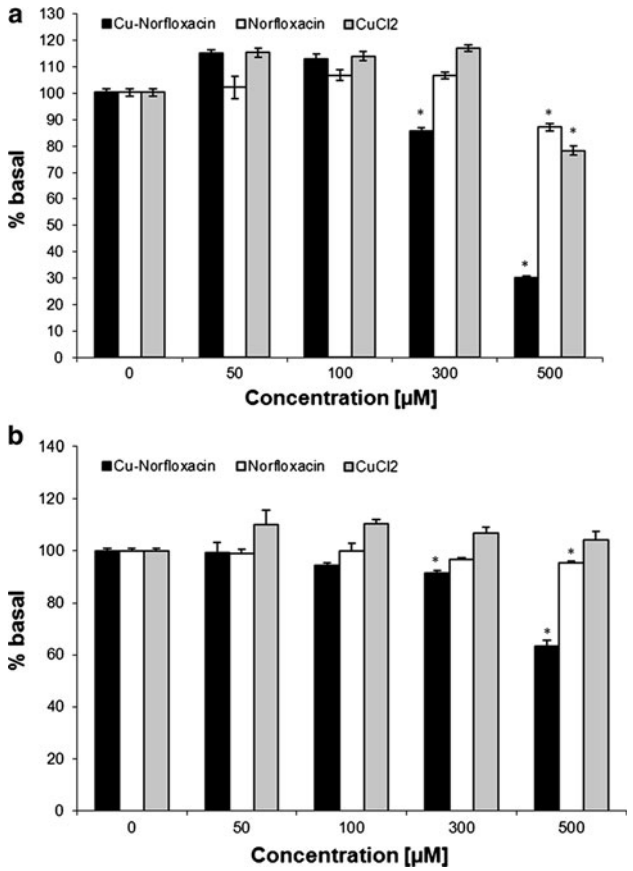

Fig. 4 Neutral Red uptake assay. UMR106 cells (**a**), MC3T3-E1 cells (**b**). Cells were incubated with different doses of $Cu(Nor)_2{\cdot}5H_2O$, Norfloxacin and Cu^{2+} for 24 h at 37 °C. After incubation, cell viability was determined by the uptake of NR. The dye taken up by the cells was extracted and the absorbance read at 540 nm. Results are expressed as % basal and represent the mean ± SEM ($n = 16$), $^*P < 0.001$

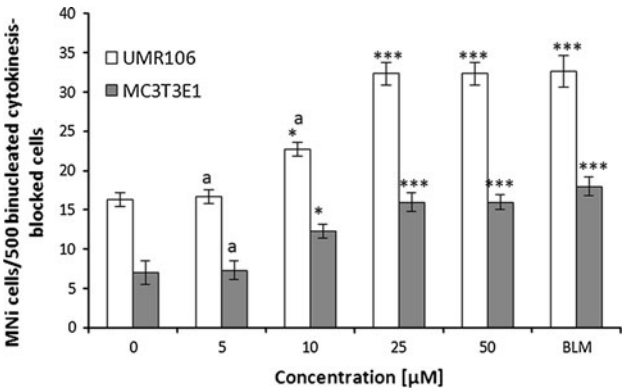

Fig. 5 Micronuleus assay. Micronuclei (MNi) induction in osteoblast-like cells (UMR106 and MC3T3-E1) after 24 h exposure to $Cu(Nor)_2{\cdot}5H_2O$. Significant difference at $^*P < 0.05$, $^{***}P < 0.001$, (*a*) is the significant difference. BLM stands for bleomycin, it is used as positive control

position 1 and the oxygen of the ring carbonyl group at position 3 [19, 21, 37].

The accuracy of PM6 method used to predict the heats of formation for compounds of interest in biochemical systems is somewhat better than HF or B3LYP DFT methods using the 6-31G(d) basis set [38, 39]. Because of its construction and parametrization for more than 80 elements, PM6 is superior to other similar methods [25]. PM6 has been successfully used to reproduce atomic distances in many copper species of importance in biochemistry [25]. The more stable conformation under vacuum (C1) presents a coordination sphere around the copper atom that matches with the x-ray crystallographic structure previously reported for another binary copper complex of Norfloxacin $[Cu(HNor)_2]Cl_2{\cdot}2H_2O$ [17]. The fact that C2 displays

lower energy than C1 when taking into account the solvent effect for the two conformations could mean that in aqueous solution the presence of water molecules would allow the approach of fluorine atoms, which stabilizes the structure by hydrogen bonding. We can suggest that $Cu(Nor)_2{\cdot}5H_2O$ complex could have a structure as C1 in the solid state because the energy barrier of 3.8 kcal is high to convert to C2. On the other hand, it is probably that the complex adopts a structure similar to C2 when it is in aqueous solution even though a significant contribution of C1 is also possible considering the little difference in the heat formation of both conformations.

Metal-based antitumor drugs play a relevant role in cancer treatment. One of the most effective drugs in this group is Cisplatin even though severe toxicity and drug resistance has been reported [40]. Therefore, in recent years, there has been a rapid expansion in research and development of new metal-based anticancer drugs to reduce the toxicity and to improve clinical effectiveness [41]. Among non-Pt compounds, copper complexes are potentially attractive as anticancer agents in Medicinal Chemistry [8, 42, 43]. Copper compounds are very attractive based on the assumption that endogenous metals may be less toxic. It has been established that the properties of copper-coordinated compounds are largely determined by the nature of ligands and donor atoms bound to the metal ion $Cu(Nor)_2{\cdot}5H_2O$ [8]. Norfloxacin is a synthetic antibacterial agent of the Quinolone group used to treat different infections, mainly complicated urinary tract infections [1]. Recently, it has been an increased interest in antibacterial and antitumoral actions of Quinolones. These effects are based on their inhibitory action on DNA gyrase and topoisomerase II in bacteria and mammals, respectively [44]. It is well known that the interaction of metal ions with antibiotics may change the pharmacological and toxicological properties of both components [6]. Besides, many drugs exhibit or modified their pharmacological and toxicological properties when they are in the form of metallic complexes.

A deleterious effect of the complex on the proliferation of UMR106 and MC3T3-E1 osteoblast-like cells in culture could be seen with a stronger effect on the tumoral cells. This finding is in agreement with previous papers reporting the antitumoral action of copper–Norfloxacin complexes in other tumoral cell lines [9, 45]. $Cu(Nor)_2{\cdot}5H_2O$ exhibited a better inhibitory effect on the osteosarcoma cells comparing to the organic moiety of the molecule, Norfloxacin, and to copper (II) ion, demonstrating the usefulness of the complexation.

Very few data concerning the cellular mechanisms underlying the antitumoral activity of copper compounds are available. The results obtained in the proliferation and the morphological studies point to the importance to search the toxicity of the complex in osteoblast-like cells in culture. For this reason, we focused our investigation on the cytotoxicity and genotoxicity of the $Cu(Nor)_2{\cdot}5H_2O$.

The uptake of the supravital dye NR is a good method to measure lysosome functionality. It is one of the mostly used cytotoxicity tests with many biomedical and environmental applications. This test is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes [46]. Viable cells will take up the dye so any decrease in the uptake will serve as an indicator of cytotoxicity. Changes of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such alterations brought about by the action of xenobiotics result in a decreased uptake and binding of NR. [47]. After 24-h incubation with different concentrations of the complex, a reduction in this important metabolic function could be determined in both cell lines. The results of the cytotoxicity test are in accordance with that of the Crystal Violet bioassay since the dose related cytotoxic effect is higher for the tumoral cell line than for the normal osteoblasts. Besides, this effect is also stronger in UMR106 osteosarcoma derived cell line than the effects of the free ligand and free ion. Antitumoral actions of another Cu(II) with a Quinolone derivative in different tumoral cell lines were previously reported by [48]. An in vitro cytotoxicity study of copper(II) mixed–ligand complexes with 2-phenyl-3-hydroxy-4(1H)-quinolinone, has shown significant activity against human osteosarcoma (HOS) and human breast adenocarcinoma (MCF7) cell lines with IC_{50} near 2 μM.

Copper compounds cause genotoxicity since they may exert antiproliferative effects via interactions with DNA. The strong interactions of copper(II) mixed–ligand complexes of Quinolinone derivatives with calf thymus DNA and their high ability to cleave pUC19 DNA plasmid have been previously reported [48]. These authors found a good correlation between the in vitro cytotoxicity and genotoxicity with DNA cleavage studies of the complexes. MN is a test used in toxicological screening for potential genotoxic compounds. The assay is one of the most successful and reliable tests for genotoxic carcinogens, i.e., carcinogens which act by causing genetic damage according to OECD [49] (Organization for Economic Co-operation and Development). In our study, alterations in the nuclei of the cells began at low concentration in both cell lines. Nevertheless, UMR106 tumoral cells displayed a dose-related effect between 5 and 25 μM, while the normal osteoblasts showed a narrower concentration-dependent range. The increment in the MN frequency may be due to an effect displayed by the Norfloxacin. It is well known that Norfloxacin disrupts topoisomerase II activity in bacteria and in mammalian cells [50, 51]. Moreover, the increase in MN frequency can also be related to the effect of copper(II) complexes on DNA cleavage as it has been previously reported for other organic copper compounds [37, 52, 53]. In addition, a third possibility for the genotoxicity of

$Cu(Nor)_2 \cdot 5H_2O$ could be attributed to the action of both moieties on DNA.

Conclusions

In conclusion, from a physicochemical point of view, the IR spectrum of $Cu(Nor)_2 \cdot 5H_2O$ confirms that the carboxylate group COO^- and the ring carbonyl of Norfloxacin are involved in the complexation of copper(II). Moreover, the observed biological responses may be mainly attributed to the species with C2 conformation, the predominant lower energy form under physiologic conditions.

The results of the biological cell culture study demonstrate the stronger antiproliferative effect of the complex $Cu(Nor)_2 \cdot 5H_2O$ in the osteosarcoma UMR106 cells than in the normal cell line MC3T3-E1. These results are in agreement with the NR uptake cytotoxicity assay and with the MN assay, the test chosen to determine the genotoxicity.

Altogether, taking into consideration that all the performed tests show the more deleterious action of the complex on tumoral osteoblasts than on the normal cells, further studies are of great interest in the field of Medicinal Chemistry for the potentiality of this complex for clinical treatment of cancer.

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References

1. Padeiskaia EN (2003) Norfloxacin: more than 20 years of clinical use, the results and place among fluoroquinolones in modern chemotherapy for infections. Antibiot Khimioter 48:28–36
2. Anacona JR, Da Silva GJ (2005) Synthesis and antibacterial activity of cefotaxime metal complexes. J Chil Chem Soc 50:447–450
3. Williams DR (1971) The metals of life: the solution chemistry of metal ions in biological systems. Van Nostrand-Reinhold, London
4. Sorenson JRJ (1976) Copper chelates as possible active forms of the antiarthritic agents. J Med Chem 19:135–148
5. Brown DH, Lewis AE, Smith WE, Teape JW (1980) Antiinflammatory effects of some copper complexes. J Med Chem 23:729–734
6. Arayne S, Sultana N, Haroon U, Mesaik MA (2009) Synthesis, characterization, antibacterial and anti-inflammatory activities of enoxacin metal complexes. Bioinorg Chem Appl 2009:914105
7. Pan Q, Kleer CG, van Golen KL, Irani J, Bottema KM, Bias C, De Carvalho M, Mesri EA, Robins DM, Dick RD, Brewer GJ, Merajver SD (2002) Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res 62:4854–4859
8. Marzano C, Pellei M, Tisato F, Santini C (2009) Copper complexes as anticancer agents. Anticancer Agents Med Chem 9:185–211
9. Katsarou ME, Efthimiadou EK, Psomas G, Karaliota A, Vourloumis D (2008) Novel copper(II) complex of *N*-propyl-Norfloxacin and 1,10-phenanthroline with enhanced antileukemic and DNA nuclease activities. J Med Chem 51:470–478
10. King DE, Malone R, Lilley SH (2000) New classification and update on the Quinolone antibiotics. Am Fam Physician 61:2741–2748
11. Turel I, Gruber K, Leban I, Bukovec N (1996) Synthesis, crystal structure, and characterization of three novel compounds of the Quinolone family member (Norfloxacin). J Inorg Biochem 61:197–212
12. Turel I, Leban I, Zupancic M, Bukovec P, Gruber K (1996) An adduct of magnesium sulfate with a member of the Quinolone family (Ciprofloxacin). Acta Crystallogr C 52:2443–2445
13. Turel I, Leban I, Klintschar G, Bukovec N, Zalar S (1997) Synthesis, crystal structure, and characterization of two metal-Quinolone compounds. J Inorg Biochem 66:77–82
14. Chen ZF, Xiong RJ, Zuo JL, Guo Z, You XZ, Fun KH (2000) X-Ray crystal structures of Mg^{2+} and Ca^{2+} dimers of the antibacterial drug Norfloxacin. J Chem Soc, Dalton Trans 22:4013–4014
15. Al-Mustafa J (2002) Magnesium, calcium and barium percholate complexes of ciprofloxacin and Norfloxacin. Acta Chim Slov 49:457–466
16. Ruiz M, Perello L, Cario JS, Ortiz R, Granda SG, Diaz MR, Canton E (1998) Cinoxacin complexes with divalent metal ions. Spectroscopic characterization. Crystal structure of a new dinuclear Cd(II) complex having two chelate-bridging carboxylate groups.Antibacterial studies. J Inorg Biochem 69:231–239
17. Ruiz P, Ortiz R, Perello L, Alzuet G, Gonzalez-Alvarez M, Liu-Gonzalez M, Sanz-Ruiz F (2007) Synthesis, structure, and nuclease properties of several binary and ternary complexes of copper(II) with Norfloxacin and 1,10 phenantroline. J Inorg Biochem 101:831–840
18. Gao F, Yang P, Xie J, Wang H (1995) Synthesis, characterization and antibacterial activity of novel Fe(III), Co(II), and Zn(II) complexes with Norfloxacin. J Inorg Biochem 60:61–67
19. Sadeek SA, El-Shwiniy WH, Zordok WA, El-Didamony AM (2009) Synthesis, spectroscopic, thermal and biological activity investigation of new Y(III) and Pd(II) Norfloxacin complexes. J Argent Chem Soc 97:128–148
20. Ibrahim IT, Motaleb MA, Attalah K (2010) Synthesis and biological distribution of Tc-99m–Norfloxacin complex, a novel agent for detecting sites of infection. J Radioanal Nucl Chem 285:431–436
21. Refat MS (2007) Synthesis and characterization of Norfloxacin-transition metal complexes (group 11, IB): spectroscopic, thermal, kinetic measurements and biological activity. Spectrochim Acta A 68:1393–1405
22. Jimenez-Garrido N, Perello L, Ortiz R, Alzuet G, Gonzalez-Alvarez M, Canton E, Liu- Gonzalez M, Garcia-Granda S, Perez-Priede M (2005) Antibacterial studies, DNA oxidative cleavage, and crystal structures of Cu(II) and Co(II) complexes with two Quinolone family members, ciprofloxacin, and enoxacin. J Inorg Biochem 99:677–689
23. Drevensek P, Turel I, Ulrih NP (20039 Influence of copper(II) and magnesium(II) ions on the ciprofloxacin binding to DNA. J Inorg Biochem 96:407–415
24. Song GW, He Y, Cai ZX (2004) The interaction between levofloxacine hydrochloride and DNA mediated by Cu^{2+}. J Fluoresc 14:705–710
25. Stewart JJP (2007) Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements. J Mol Model 13:1173–1213
26. Chongcharoen W, Byrn SR, Sutanthavibul N (2008) Solid state interconversion between anhydrous Norfloxacin and its hydrates. J Pharm Sci 97:473–489
27. Refat MS (2007) Synthesis and characterization of Norfloxacin-transition metal complexes (group 11, IB): spectroscopic, thermal, kinetic measurements and biological activity. Spectrochimica Acta A 68:1393–1405

28. Barrio DA, Etcheverry SB (2006) Vanadium and bone development: putative signaling pathways. Can J Physiol Pharmacol 84:677–686
29. Okajima T, Nakamura K, Zhang H, Ling N, Tanabe T, Yasuda T, Rosenfeld RG (1992) Sensitive colorimetric bioassays for insulin-like growth factor (IGF) stimulation of cell proliferation and glucose consumption: use in studies of IGF analogs. Endocrinology 130:2201–2212
30. Cortizo AM, Bruzzone L, Molinuevo MS, Etcheverry SB (2000) A possible role of oxidative stress in the vanadiuminduced cytotoxicity in the MC3T3-E1 osteoblast and UMR106 osteosarcoma cell lines. Toxicology 147:89–99
31. Etcheverry SB, Ferrer EG, Naso L, Barrio DA, Lezama L, Rojo T, Williams PAM (2007) Losartan and its interaction with copper(II): biological effects. Bioorg Med Chem 15:6418–6424
32. Borenfreund E, Puerner JA (1984) A simple quantitative procedure using monolayer culture for toxicity assays. J Tissue Cult Methods 9:7–9
33. Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455:81–95
34. Stopper H, Muller SO (1997) Micronuclei as a biological endpoint for genotoxicity: a minireview. Toxicol In Vitro 11:661–667
35. Castillo-Blum SE, Barba-Behrens N (2000) Coordination chemistry of some biologically active ligands. Coord Chem Rev 196:3–30
36. Turel I (2002) The interaction of metal ions with Quinolone antibacterial agents. Coord Chem Rev 232:27–47
37. Macías B, Villa MV, Rubio I, Castiñeiras A, Borrás J (2001) Complexes of Ni(II) and Cu(II) with Nofloxacin. Crystal structure of a new Cu(II) Nofloxacin complex. J Inorg Biochem 84:163–170
38. Lee C, Yang W, Parr RG (1988) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. Phys Rev B 37:785–789
39. Becke AD (1993) Density-functional thermochemistry. III. The role of exact exchange. J Chem Phys 98:5648–5652
40. Wang X (2010) Fresh platinum complexes with promising antitumor activity. Anticancer Agents Med Chem 10:396–411
41. Frezza M, Hindo S, Chen D, Davenport A, Schmitt S, Tomco D, Dou QP (2010) Novel metals and metal complexes as platforms for cancer therapy. Curr Pharm Des 16:1813–1825
42. Tardito S, Marchio L (2009) Copper compounds in anticancer strategies. Curr Med Chem 16:1325–1348
43. Tisato F, Marzano C, Porchia M, Pellei M, Santini C (2010) Copper in diseases and treatments, and copper-based anticancer strategies. Med Res Rev 30:708–749
44. Harris AL, Hochhauser D (1992) Mechanisms of multidrug resistance in cancer treatment. Acta Oncol 31:205–213
45. Shingnapurkar D, Butcher R, Afrasiabi Z, Sinn E, Ahmed F, Sarkar F, Padhy S (2007) Neutral dimeric copper–sparfloxacin conjugate having butterfly motif with antiproliferative effects against hormone independent BT20 breast cancer cell line. Inorg Chem Commun 10:459–462
46. Repetto G, del Peso A, Zurita JL (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3:1125–1131
47. Ivanova L, Uhlig S (2008) A bioassay for the simultaneous measurement of metabolic activity, membrane integrity, and lysosomal activity in cell cultures. Anal Biochem 379:16–19
48. Buchtik R, Travnicek Z, Vanco J, Herchel R, Dvorak Z (2011) Synthesis, characterization, DNA interaction and cleavage, and in vitro cytotoxicity of copper(II) mixed-ligand complexes with 2-phenyl-3-hydroxy-4(1H)-quinolinone. Dalton Trans 40:9404–9412
49. OECD Guideline http://www.oecd.org/document/35/0,3746,en_2649_34377_45773411_1_1_1_1,00.html
50. Coughlin SA, Danz DW, Robinson RG, Klingbeil KM, Wentland MP, Corbett TH, Waud WR, Zwelling LA, Altschuler E, Bales E, Rake JB (1995) Mechanism of action and antitumor activity of (S)-10-(2,6-dimethyl-4-pyridinyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7Hpyridol[1,2,3-de] [1,4] benzothiazine -6-carboxylic acid. Biochem Pharm 50:111–122
51. Clement JJ, Burres N, Jarvis K, Chu DT, Swiniarski J, Alder J (1995) Biological characterization of a novel antitumor Quinolone. Cancer Res 55:830–835
52. da Silveira VC, Benezra H, Luz JS, Georg RC, Oliveira CC, Ferreira AM (2011) Binding of oxindole-Schiff base copper(II) complexes to DNA and its modulation by the ligand. J Inorg Biochem 105:1692–1703
53. Lee WY, Yan YK, Lee PP, Tan SJ, Lim KH (2012) DNA binding and nucleolytic properties of Cu(II) complexes of salicylaldehyde semicarbazones. Metallomics 4:188–196