Effect of levofloxacin and 3-carboethoxy-4-quinolone on the β-amyloid fibrils formation

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ABSTRACT

Alzheimer´s disease (AD) is a progressive and irreversible neurological disorder associated with age-related dementia. Extracellular deposition of fibrils derived from β-amyloid peptide (Aβ) is one of the most important pathological hallmark of AD. Here we present results concerning the investigation of the in vitro inhibitory effect of the quinolones levofloxacin (3) and 3-carboethoxy-4-quinolone (4) on the Aβ fibrillation. $A\beta_{42}$ peptide solutions were incubated in the presence of either compound 3 or 4 and the fibrillation was assessed by thioflavin-T fluorescence assay and atomic force microscopy (AFM). The results indicate that compounds 3 and 4 are inhibitors of Aβ fibrillization, being the latter more potent. Quinolone 4 also promotes the disaggregation of pre-formed Aβ fibrils. We concluded that both compounds interfere with the fibrillization process.

Keywords: Alzheimer´s disease, β-amyloid, levofloxacin, quinolones, fibrillation
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INTRODUCTION

Alzheimer´s disease (AD) is a progressive and irreversible neuropathology that affects cognitive functions including learning and memory. More than 10% of people over 65 years old have AD. Moreover, as the median age of population is increasing, a proportional increase in the number of AD victims is expected.[1] AD is characterized by intracellular neurofibrillary tangles formed by fragments of hyperphosphorylatedτ-protein, and amyloid plaques derived from extracellular deposition of amyloid peptides.[2] The most affected brain region is the hippocampus. Amyloid peptides containing 40-43 amino acid residues derived from the abnormal metabolism of the amyloid precursor protein (APP) are the main constituent of the senile plaques. These plaques also contain glycosaminoglycans, apolipoprotein E and metal ions, among other constituents.[3,4] Despite recent advances in understanding the pathophysiology of AD, the pharmacological therapy is still limited to the use of acetylcholinesterase inhibitors,[5] such as donepezil and galantamine, and/or memantine, a glutamate antagonist.[6] Recent studies have implicated copper and zinc accumulation in the central nervous system, in the process of β-amyloid aggregation.[7] The discovery that clioquinol**1** (Figure 1), inhibits Aβ deposit formation [8] has raised several investigational studies on the anti-amiloydogenic effect promoted by metal chelators, like hydroxypyridinones**2**.[9] Recently, we have prepared and characterized zinc and copper complexes having carboxyquinolones as ligands.[10] The role of these metals in the neurodegenerative process associated to the AD,

attracted our attention to a possible effect of carboxyquinolones on the Aβ fibrils formation. Here, we tested the effects of levofloxacin (**3**) and 3-carboethoxy-4-quinolone (**4**) in the process of β-amyloid fibrillization. These effects were evaluated by thioflavin-T fluorescence assay and atomic force microscopy.
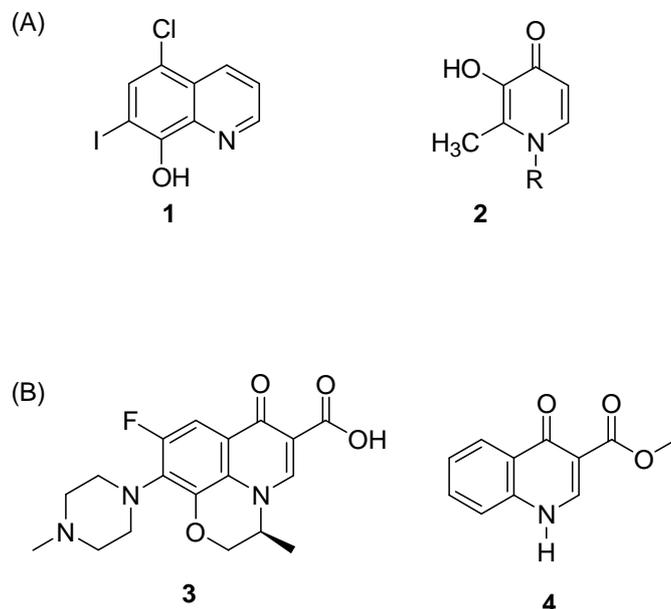

Figure 1. (A) Inhibitors of the aggregation of Aβ peptide: clioquinol (1) and dihydropyridones (2); (B) Compounds evaluated in this study: levofloxacin (3) and 3-carboethoxy-4-quinolone (4)

EXPERIMENTAL SECTION

1.1. Chemistry

^{1}H and ^{13}C spectra were recorded on a Bruker 250 or Avance 500, operating at 250/500 and 62.5/125 MHz, respectively. IR spectra were obtained in a BOMEN- MBSERIES instrument. Melting points were measured in open capillary tubes using an Electrothermal model 9100, and are uncorrected. Purification and separations by column chromatography were performed on silica gel, using flash chromatography and TLC visualization employed UV light. All chemical reagents were purchased from Aldrich and were used without previous purification. Levofloxacin (>98%) was kindly provided by EMS S.A. (Hortolândia, Brazil).
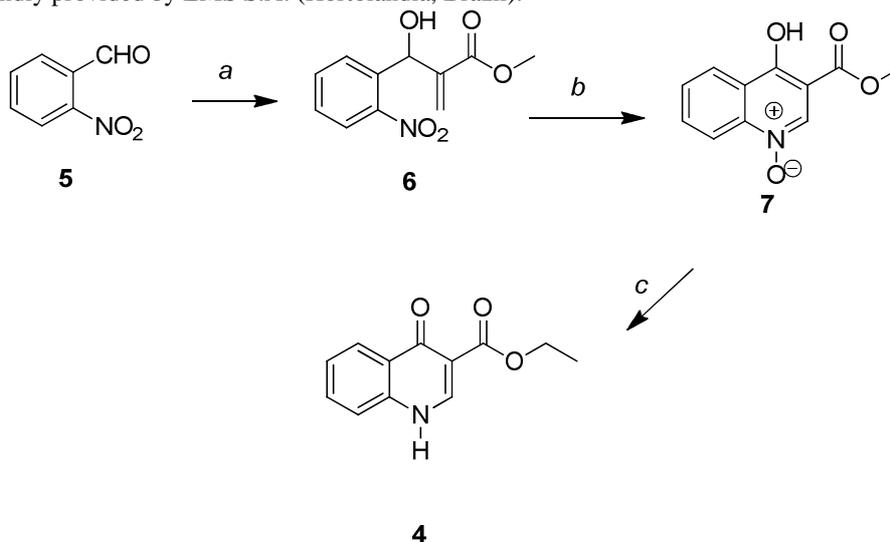

Figure 2. Scheme of the preparation of the quinolone 4; reagents and conditions: [a] methyl acrylate, DABCO, overnight, 98% yield; [b] TFA, 70-75 °C, 20 h, 75% yield; [c] Mo(CO)$_6$, anhydrous ethanol, 50 min, 55% yield

Quinolone **4** was synthesized in three steps-sequence from *2*-nitrobenzaldehyde **5** in a 41% overall yield as depicted in the scheme represented in Figure 2, All compounds were characterized by spectroscopic methods and all spectral data are in agreement with the precedent literature. [10-12]

1.2.Biological assays
1.2.1.Preparation of $A\beta_{42}$ films
$A\beta_{42}$ peptide was purchased from American Peptide Co. Lyophilized peptide (1 mg) was stored in ultra-freezer at -80 °C. Prior to resuspension, the vial was allowed to equilibrate to room temperature for 30 min and dissolved in 275 μL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to reach 1 mmol.L^{-1} concentration. Then, the resulting clean solution was aliquoted in six micro-centrifuge tubes. The HFIP was allowed to evaporate in a fume hood, and the resulting films were dried under vacuum in a SpeedVac. Films were stored at -80°C until use.

1.2.2.Preparation of $A\beta_{42}$ fibrils
A positive control ($A\beta_{42}$fibrilar) was prepared by resuspending the peptide film in 6 μL of sterile dimethylsulfoxide to 6 mmol.L^{-1} final concentration and sonicated for 10 min. The resulting solution was diluted with 29 μL of a10 mmol.L^{-1} solution ofHCl to reach 1mmol.L^{-1} final concentration and immediately mixed for 30 seconds. The resulting solution was incubated at 37 ^{o}C for 24 hours under stirring. The formation of fibrils was confirmed by analyzing ThT fluorescence emission and atomic force microscopy.

1.2.3. Preparation of samples for evaluation of the anti-fibrillogenic activity
To study the effect of levofloxacin and quinolone **4** on the $A\beta_{42}$fibrillization process, the peptide film was resuspended in 6 μL of a 0.2 mmol.L^{-1} solution of the test-compounds in sterile dimethylsulfoxide and then sonicated. As a way of inducing the formation of fibrils, 30 μL of a 10 mmol.L^{-1}HCl solution were added. Aliquots (5 μL) of the peptide solution were added to 1 mL of solution of ThT in phosphate buffer pH 7.4 (0.05 mmol.L^{-1}) before analyzing. Samples were immediately analyzed (t_0) and after incubation for 24 h at 37 °C (t_{24}). A solution of ThT in buffer pH 7.4 (0.05 mmol.L^{-1}) was used as control. Experiments were performed in duplicate.

1.2.4. Preparation of samples for the disaggregation studies
$A\beta$ fibrils prepared as above described were resuspended with 6 μL of a solution of test-compound in buffer (0.2 mmol.L^{-1}). The resulting mixture was incubated for 24h at 37 °C and after this period, the ThT fluorescence emission was measured.

1.2.5.Thioflavin T fluorimetry assay
A stock solution of thioflavin-T (ThT) was preparing by adding 8 mg of ThT to 10 mL phosphate buffer pH 7.4 and filter through a 0.2 μm syringe filter. This stock solution was stored in the dark and is stable for about one week. On the day of analysis, the stock solution was diluted into phosphate buffer (1mL of ThT stock to 50 mL buffer), to generate the working solution. The fluorescence intensity was measured by adding 5μL of protein solution to the cuvette containing 1mL working solution, stirred for 1 min, and measured the intensity of fluorescence by excitation at 450 nm (slitwidth 10 nm) and emission at 485 nm (slitwidth 10 nm). The results are the mean of ten fluorescence spectra measures. Fluorescence measurements were carried in a Varian Cary Eclipse spectrofluorimetric apparatus at 450 and 485 nm excitation and emission wavelengths respectively.

1.2.6.Atomic Force Microscopy (AFM)
Peptide preparations, such as $A\beta_{42}$ in DMSO (A), $A\beta_{42}$ in HCl (B), $A\beta_{42}$ in HCl in the presence of levofloxacin **3** (C) and in the presence of quinolone **4** (D) were characterized using and atomic force microscope Topometrix TMX 2010 using blinking mode (non-contact). Samples were analyzed immediately preparation (t_0) and 24h after incubation (t_{24}). A silicon cantilever was used with resonance frequency in the range of 180-191 KHz. Samples were prepared by spotting 10-50 μL of peptide solutions at 10 μmol/L concentration on freshly cleaved mica and then, dried at room temperature under vacuum, for 1 h.

RESULTS AND DISCUSSION

The development of new pharmaceuticals is a long time and costly process. The strategy named drug repositioning or repurposing can be of great benefit and consists in identifying a new use for existing approved drugs. [13] Since the development of a new drug for treating AD is a high-risk process for the pharmaceutical industry, this approach

can greatly contribute.[14] A number of approved drugs have been tested for control symptoms, including anti-inflammatory, antidepressants and antimicrobial agents.[15]

Here, we report the results of studying the effect of the antimicrobial agent levofloxacin (**3**) and 3-carboethoxy-4-quinolone (**4**) on the Aβ fibrils formation. The former compound was selected for this study due to the ability to chelate divalent cations and the well-known pharmacokinetic profile. Moreover, an almost planar geometry seems to be one of the common structural characteristic of all non-peptide inhibitors of Aβ aggregation like clioquinol and hydroxypyridinones. As well as levofloxacin (**3**), quinolone **4** has chelating properties. [10] The fluorine atom at the C-6 of the aromatic ring of levofloxacin was not considered when choosing the quinolone **4**, since its presence has been reported as a structural feature related to the increase of the antimicrobial activity.[16]

Effect of levofloxacin and quinolone 4 on the fibrillization of Aβ peptide

The evaluation of the Aβ peptide fibrils formation can be carried out by several methods, including thioflavin-T fluorescence emission, microscopy, gel electrophoresis and/or circular dicroism. [17] Here, the effect of levofloxacin and quinolone on Aβ fibrils formation was evaluated by Tht fluorescence emission assay and atomic force microscopy. Samples were prepared as described in the Experimental Section.

2.1.ThT-fluorescence emission assay

In order to induce the fibrils formation, we choose the method described by Dahlgren and co-workers [18] using acidic conditions (HCl solution) to produce a positive control for the fibrils formation. Zhou and co-workers reported the Aβ fibril formation using phosphate buffer, but this process takes about 7 days. [19]
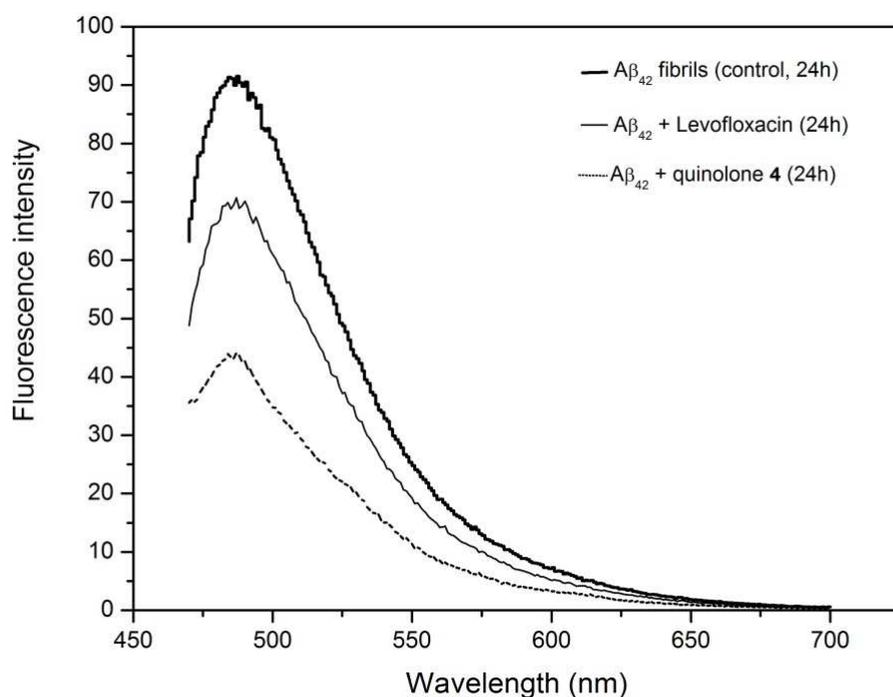

Figure 3. ThT fluorescence emission of Aβ$_{42}$ positive control (bold line), levofloxacin (levo) + Aβ$_{42}$ (normal line) and quinolone + Aβ$_{42}$ (dashed line)

Thioflavin-T has a typical fluorescence spectra change when bound to amyloid fibrils.[20] As more fibrils are attached, greater is the fluorescence intensity. Prior to analyzing the effect of levofloxacin and quinolone **4** on the

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$A\beta_{42}$fibrillization, fluorimetric spectra of these compounds were recorded and we found that they did not interfere with the ThT emission.

As shown in Figure 3, co-incubation of $A\beta_{42}$ with levofloxacin for 24 h (normal line) reduces the ThT fluorescence emission, compared to the control (bold line). A similar result was observed when levofloxacin was replaced by quinolone **4** (dashed line), which exhibited a pronounced effect on the ThT fluorescence emission.

The more effectiveness of quinolone **4** in inhibit the $A\beta_{42}$fibrillizationmay be related to a more planar geometry compared to levofloxacin (Figure 4). Short peptides containing phenylalanine residues were found to bind to amyloid peptides, leading to well-ordered fibrils.[21-23] These observations raised the hypothesis that π-stacking interactions have a possible role in the self-assembly of Aβ fibrils.[24] In fact, a planar geometry seems to be a molecular recognition pattern for all reported inhibitors of fibrillogenesis.

Figure 4. Optimized geometries of levofloxacin 3 (left) and quinolone 4 (right)

The decrease in the intensity of ThT fluorescence emission associated to the Aβ fibrils formation, could be attributed to the inhibition of fibrils formation or/and the solubilization of pre-formed fibrils. In order to clarify this point, a study on the disaggregation of pre-formed $A\beta_{42}$ fibrils was conducted. Thus, Aβ peptide was treated with HCl, as above mentioned, and the fibrils formation was verified by measuring the ThT fluorescence emission after 24 h. The sample containing $A\beta_{42}$ fibrils was then incubated in the presence of levofloxacin (0.02 mmol. L^{-1}) for additional 24 h and the ThT fluorescence was measured. The same procedure was adopted to evaluate the effect of quinolone **4** (0.02 mmol. L^{-1}) on Aβ fibrils. In the presence of levofloxacin, there was an increase in the ThT fluorescence emission, suggesting that levofloxacin (dashed line, Figure 5A) is not able to disaggregate the pre-formed fibrils, while quinolone **4** (dashed line, Figure 5B) promoted a decreasing in the ThT fluorescence emission, when incubated with pre-formed fibrils. We concluded that in addition to reduce the fibrils formation, quinolone 4 disaggregates pre-formed ones.
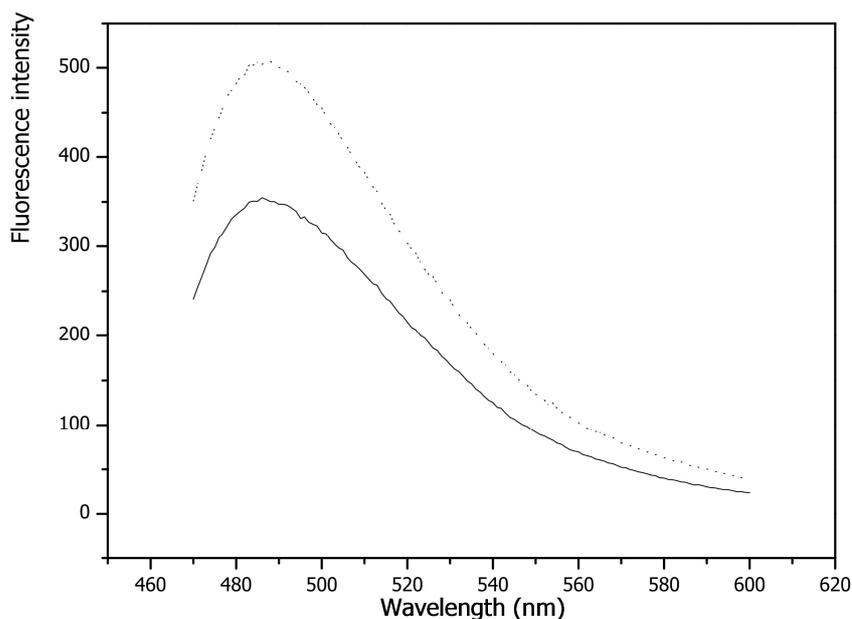
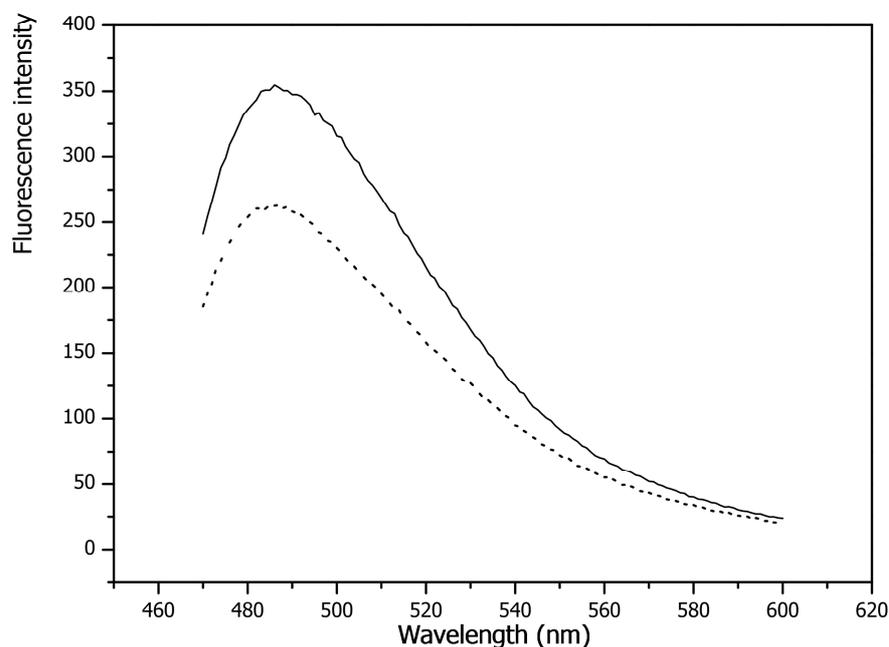**Figure 5. Top: ThT fluorescence emission of Aβ$_{42}$ fibrils (bold line), Aβ$_{42}$ fibrils + levofloxacin (dashed line). Bottom: Aβ$_{42}$ fibrils (bold line) + quinolone 4 (dashed line)**

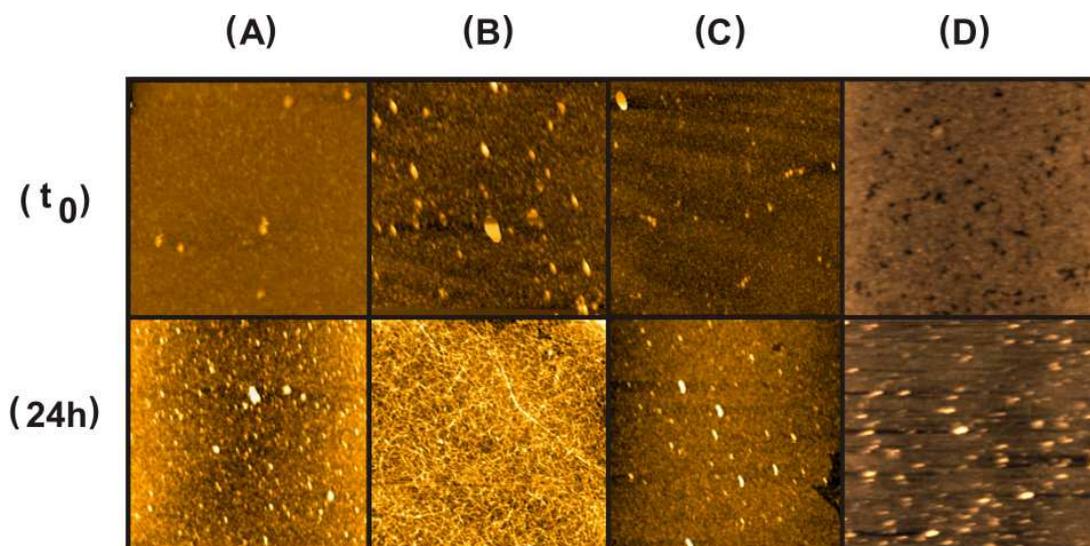**Figure 6. Morphological studies of Aβ$_{42}$ immediately after mixing (top) and after 24h of incubation. (A) in DMSO, (B) in HCl, and in the presence of levofloxacin (C) and quinolone 4 (D)**

2.2. Atomic Force Microscopy (AFM)

Morphological studies of samples were performed in a Topometrix TMX 2010 atomic force microscope using blinking mode (non-contact). A silicon cantilever in a 180-191 kHz resonance frequency range was used. AFM is a technique that allows the detection of Aβ-peptide aggregates measuring ~1 nm and micrometer fibrils. Thus, Aβ$_{42}$ peptide solutions (10-50 µL) at 10 µmol.L^{-1} concentration, in the presence and without test compounds, were spotted on freshly cleaved mica. The final preparations were dried at room temperature under vacuum in a SpeedVac for 1 h. Samples were analyzed at t$_0$ (Figure 6, top) and after 24 h of incubation (Figure 6, bottom). AFM images showed

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that $A\beta_{42}$ in DMSO (without HCl addition) was not converted into fibrils, but some aggregates were observed (Figure 6A). On the other hand, when $A\beta_{42}$ was incubated with HCl, fibrils measuring 6 x 70 x 3000 nm were detected (Figure 6B). According to the literature, $A\beta$ fibrils are larger than 1 μm. [25] In contrast, fibrils were not detected when $A\beta$ was incubated with HCl in the presence of levofloxacin or quinolone for 24 h (Figures 6C and 6D). Only a little amount of oligomers measuring 8 nm and 4 nm were formed in the presence of levofloxacin and quinolone **4**, respectively.

CONCLUSION

Alzheimer´s disease has a multipathogenic nature and numerous research groups are involved in finding potential drugs to combat this disease, based on the design of β-secretase and γ-secretase inhibitors and substances able to reduce the aggregation and/or the formation of amyloid fibrils. Since the accumulation of copper and zinc in the CNS was implicated in the physiopathology of AD, metal chelators have been explored as prototypes for anti-amyloidogenic drugs. The ability of levofloxacin and other quinolones to form metal complexes attracted our attention and commercial sample of levofloxacin and that quinolone were evaluated as potential inhibitors against $A\beta_{42}$ aggregation. Quinolone **4** was synthesized in a four step sequence from the 2-nitrobenzaldehyde in 41% overall yield. In our studies on the effect of levofloxacin and 4-quinolone on the aggregation of $A\beta_{42}$ peptide, we have found that both compounds decrease the ThT fluorescence emission. Quinolone **4** showed to be more effective than levofloxacin in decreasing ThT fluorescence emission. Quinolone **4** is equivalent to the planar moiety of levofloxacin and, as previously mentioned, a planar geometry seems to be one common structural characteristic of all non-peptide inhibitors of the $A\beta$ fibrillization. In view of the most pronounced effect of this quinolone, compared to the levofloxacin, a more planar geometry could be involved in the effectiveness of the candidates to inhibit the $A\beta$ fibrils formation.

Moreover, preparations of the $A\beta_{42}$ peptide incubated with levofloxacin and quinolone **4** in the aggregation conditions were analyzed by atomic force microscopy and showed no fibrils. Additional ThT fluorescence emission studies indicated that quinolone **4** dissolves pre-formed fibrils, while levofloxacin has no effect.

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