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Article

Multifunctionality of βCD/Ofloxacin and HPβCD/Ofloxacin Complexes: Improvement of the Antimicrobial Activity and Apoptosis Induction on Lung Adenocarcinoma A549 Cells

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The ofloxacin (OFLOX) is a second-generation synthetic antibiotic that can be classified as a multifunctional drug, but is a poorly soluble drug, which influences its efficiency. The inclusion complexes of OFLOX with β-cyclodextrin (βCD) or hydroxypropyl-β-cyclodextrin (HPβCD) can improve the chemical characteristics of the drug; however, studies showing the biological activity of these inclusion complexes are still scarce. The present work aimed to investigate the multifunctionality of the OFLOX and their inclusion complexes. Thus, the 1:1 βCD/OFLOX or HPβCD/OFLOX were prepared and analyzed by Fourier transform infrared spectroscopy (FTIR), thermogravimetric and differential thermal analysis (TGA and DTA), ^{1}H nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC). The antitumor and antibacterial effects were assessed. The results confirm the formation of the inclusion complexes, which had lower minimum inhibitory concentration (MIC) values, higher cytotoxicity and promoted the apoptosis. The present study showed, for the first time, the promising effects of the inclusion complexes as antitumor, improving the biological activities of the uncomplexed ofloxacin.

Keywords: complexation, MTT, flow cytometry, toxicity, MIC

Introduction

Almost all drugs have more than one pharmacological property. The ability of drugs to have more than one pharmacological activity suggests the possibility of use of known substances, which safety and efficacy are already well established by surveillance agencies, in the control of diseases other than those already described in the state of the art. In this context, the search for multifunctional drugs through the investigation of new action mechanisms for common drugs can be an alternative strategy for the development of new medicines.[1,2]

The 7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid (ofloxacin) is a second-generation synthetic antibiotic of the class of fluoroquinolones, that was synthesized from the nalidixic acid by the addition of a fluorine at the 6-position, an *N*-methylpiperazine ring at the 7-position and the formation of an oxazine ring between the 1-nitrogen and 8-carbon of the quinolone nucleus.[3,4] Although the antibacterial effect of fluoroquinolones is already well established, different studies show that this is a multifunctional class of compounds, being also promising for the treatment of other diseases, including cancer.[1,5-7]

The ofloxacin (OFLOX) is a poorly soluble drug, existing as a cationic form in acidic pH and zwitterionic form in neutral pH. Concerning this, the supramolecular inclusion complexes of OFLOX with β-cyclodextrin (βCD),[8] methyl-β-cyclodextrin (MeβCD)[9] and hydroxypropyl-β-cyclodextrin (HPβCD)[10] have been studied and all

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of them showed an expressive solubility improvement. However, studies evaluating the biological effects of these inclusion complexes are still scarce.

Cyclodextrins (CD) are cyclic oligosaccharides, formed by units of the D-glucopyranose that are naturally produced, with the main forms found being αCD, βCD and γCD, composed of 6, 7 and 8 units of the D-glucopyranose, respectively.[11-13] CD are widely used because of their capacity to accommodate a large number of molecules in their cavity and to be already approved as excipient for oral formulations.[13]

Although there is cited in the literature[8-10] a row of studies showing physical-chemistry characterizations of OFLOX inclusion compounds, highlighting the improvement of the solubility of the drug in the presence of cyclodextrins, until the present moment, it was found few studies[14-18] reporting the biological activities of fluoroquinolones/CD complexes, the most of them, reporting only the antimicrobial activity while the antitumor potential has not been yet explored.

Thus, the main objective of the present work was to provide new insights about the β-cyclodextrin/ofloxacin (βCD/OFLOX) and hydroxypropyl-β-cyclodextrin/ofloxacin (HPβCD/OFLOX) complexes, focusing especially in their multifunctional potential as an antimicrobial agent against *Escherichia coli* and *Staphylococcus aureus* and simultaneously as an antitumor agent on adenocarcinomic human alveolar basal epithelial cells (A549).

Experimental

Reagents

OFLOX was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and βCD and HPβCD (substitution degree 5-8 and molecular weight (Mw) ca. 1400 g mol^{-1}) were obtained from Cerestar Company (Hammond, Indiana, USA). All the other materials and solvents were of analytical reagent grade and used as received.

Inclusion complex preparation

The 1:1 βCD/OFLOX and HPβCD/OFLOX inclusion complexes were prepared by the freeze-drying method and they were used for the physicochemical characterization in solid state and in the assessment of biological activities. Briefly, ofloxacin and βCD, or ofloxacin and HPβCD, were dissolved in Milli-Q® water (Merck, Darmstadt, Germany) at a 1:1 molar ratio. These aqueous solutions were stirred for 24 h and then submitted to the freeze-drying process to achieve the solid inclusion complex.[19,20] The 1:1 molar ratio was used based on the relative size of molecules as well as the work from Li and Zhang,[21] who proposed the 1:1 stoichiometries for both βCD/OFLOX and HPβCD/OFLOX systems.

Infrared analysis

The infrared spectra (4000 to 400 cm^{-1}) of OFLOX, βCD, HPβCD, βCD/OFLOX, HPβCD/OFLOX and the respective mechanical mixtures were recorded on a PerkinElmer Fourier transform (FTIR) model Spectrum Two™, from pellets containing potassium bromide (KBr) (Sigma-Aldrich, St. Louis, Missouri, USA). The spectra were obtained as the mean of 4 consecutive scans, with a resolution of 2 cm^{-1} and a wave range of 4000 to 400 cm^{-1}.

Thermogravimetric and differential thermal analysis (TGA and DTA)

Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were recorded for OFLOX, βCD, HPβCD, βCD/OFLOX, HPβCD/OFLOX and mechanical mixtures in a TGA/DTA modulus STA7200RV from Hitachi (Tokyo, Japan). TGA/DTA experiments were performed using platinum crucibles with ca. 5 mg of sample under heating rate of 10.0 °C min^{-1}. The range of temperature was scanned from 30 to 800 °C.

Nuclear magnetic resonance (NMR)

^1H nuclear magnetic resonance of OFLOX, βCD, HPβCD, βCD/OFLOX and HPβCD/OFLOX were acquired in a Bruker Avance III HD spectrophotometer (Billerica, Massachusetts, USA) at 500 MHz. The samples were dissolved in deuterium oxide (D_2O) (Sigma-Aldrich, St. Louis, Missouri, USA) at 1.0 mM. The spectra were edited in MestreNova® 12.0 software.[22]

Isothermal titration calorimetry (ITC)

Calorimetric titrations were carried out in duplicate using a Microcal VP-ITC microcalorimeter (Malvern Panalytical, Almelo, Netherlands) at 298.15 K, after electrical and chemical calibration. In this study, each titration experiment consisted of 41 successive injections of 20.0 mM βCD or HPβCD in mixture dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA)/water (40/60) (Milli-Q® water) into the reaction cell charged with 1.5 mL of 1.0 mM OFLOX in the same solvent. The first injection of 1.0 μL was discarded to eliminate diffusion effects from syringe material in the calorimetric cell. The subsequent injections were applied at a constant

volume of 6.3 μL of βCD or HPβCD. The raw data were analyzed by the Microcal Origin 9.0 software[23] for ITC, after subtracting the blank experiment (dilution of βCD or HPβCD in solvent). During the fitting procedure by using the Wiseman isotherm[19,24,25] as the curves did not show the typical sigmoidal profile, the stoichiometries were fixed as 1:1 based on the relative size of the molecules.

Dynamic light scattering (DLS)

The size of hydrophobic nanoprecipitates[24,26,27] formed by OFLOX or inclusion complexes (βCD/OFLOX or HPβCD/OFLOX) in DMSO/water solvent was measured by the average hydrodynamic diameter (D_h) of the particles through DLS, which was performed in a Malvern Zetasizer Nano ZS90 particle analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK), at 25 °C with thermostatization via Peltier system. The samples were submitted to a monochromatic light (4 mW He-Ne laser at 633 nm) and the scattered light intensity was measured at 90° angle. The D_h was determined by the average of five independent measurements, each of them obtained as the mean of five counts. 40 mM solutions of OFLOX, βCD/OFLOX or HPβCD/OFLOX, solubilized in DMSO, were used for the execution of this experiment. Subsequently, 30 injections of 10 μL of these solutions in 1.5 mL of ultrapure water were performed in order to obtain the hydrophobic nanoprecipitates.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method.[28] Different concentrations of OFLOX, βCD/OFLOX or HPβCD/OFLOX, ranging from 11.05 to 0.003 μM, were incubated with 5×10^5 colony forming unit (CFU) mL^{-1} from *Escherichia coli* and *Staphylococcus aureus* strains. Sterilized controls were included for each trial, and the tests were performed on three occasions in triplicate. Cells no treated were used as positive controls. The microdilution plates were incubated under aerobic conditions at 35 °C for 24 h. MICs were defined as the lowest concentration that completely inhibited the growth of *E. coli* and *S. aureus* and they were confirmed by colorimetric analysis using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, Missouri, USA) as indicator.

Antimicrobial time-kill curves

Tests were performed using two strains of *E. coli* (American Type Culture Collection (ATCC) 25922 and C10) and two strains of *S. aureus* (ATCC 27853 and C4). The time-kill curve assays were performed as described previously, with adaptations.[29] Bacterial inoculums, prepared to achieve a final concentration of 5×10^5 CFU mL^{-1} were treated with MIC or 2MIC of OFLOX, βCD/OFLOX or HPβCD/OFLOX in Mueller-Hinton broth (BD Difco, Franklin Lakes, New Jersey, USA). Aliquot of bacterial inoculums were removed from each well at 0, 3, 6, 12 and 24 h and serially diluted in sterile saline (NaCl, 0.9%), before plating on Mueller-Hinton agar (BD Difco, Franklin Lakes, New Jersey, USA) for colony count determinations. The plates were incubated at 35 °C for 24 h prior to colony counting. The data were plotted as total number of viable cells *per* mL (mean of CFU mL^{-1} ± standard deviation (SD)) on a logarithmic scale against time.

MTT assay

A549 cell line was placed at 2×10^5 cells mL^{-1} in 96-well plates containing Roswell Park Memorial Institute 1640 (RPMI-1640) (Gibco, Grand Island, New York, USA) supplemented with 2.0 mM L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA), 100.0 μg mL^{-1} of streptomycin and penicillin (Sigma-Aldrich, St. Louis, Missouri, USA) and 5% fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA). The cells were incubated at 37 °C in 5% CO_2 atmosphere in the presence of OFLOX, βCD/OFLOX or HPβCD/OFLOX (at 50, 100 or 300 μM). OFLOX, βCD/OFLOX and HPβCD/OFLOX were diluted in RPMI-1640 before use. Cellular viability was measured using MTT assay. After 48 and 72 h of culture the supernatant was removed, and the cells were incubated with 100 μL of supplemented RPMI-1640 medium and 10 μL of MTT (5.0 mg mL^{-1}) during 4 h at 37.0 °C in 5% CO_2. After purple formazan crystal formation, the supernatant was gently removed, and crystal products were solubilized and incubated with DMSO. The complete solubilization was obtained by shaking the plates for 10 min. The optical density (OD) values were determined in the microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Waltham, MA, USA) at 560 nm wavelength.[30] The cellular viability were calculated using the formula $(\overline{x}_1 / \overline{x}_2)100$, considering $\overline{x}_1$ the mean OD of treated cells and $\overline{x}_2$ the mean OD of untreated cells. The compounds were considered cytotoxic when the viability was lower than 70%.[30]

Analysis of apoptosis by flow cytometry

For apoptosis detection the Annexin V Apoptosis Detection Kit (Invitrogen by Thermo Fisher Scientific,

Waltham, MA, USA) was used. To perform the assay 1×10^6 cells mL^{-1} A549 cells were cultured in 96-well microplates and incubated in the presence or absence of OFLOX, βCD, HPβCD, βCD/OFLOX or HPβCD/OFLOX at 300 μM. After 36 h of culture, the cells were trypsinized and washed with phosphate buffer. After washing, the cells were labeled with Annexin V-FITC and propidium iodide (PI), according to the manufacturer's instructions. The cells were incubated at room temperature for 15 min in the dark, then acquired in FACSVerse (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), and the apoptosis (Annexin V^+/PI^-) analyzed in the FCS Express software.[31]

Statistical analysis

The results represent at least three independent experiments and are presented as the mean ± SEM (standard error of the mean). All data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni posttests (GraphPad Prism 5.00),[32] and the differences were considered significant at $p < 0.05$.

Results and Discussion

Physical-chemistry characterizations

The FTIR spectra of OFLOX, βCD, HPβCD, βCD/OFLOX, HPβCD/OFLOX, and their respective mechanical mixtures (MM), were recorded to confirm the formation of intermolecular interactions between CD and OFLOX in the solid state (Figures S1a and S1b, Supplementary Information (SI) section).

In the OFLOX, βCD and HPβCD spectrum, it was possible to observe the major absorption bands in accordance with those described in the literature.[8-10,33] In the spectra of the MM, it was observed mainly the overlap of the bands from OFLOX and CD.

In the spectra of βCD/OFLOX and HPβCD/OFLOX it was observed the disappearance and/or strong attenuation of bands in the region from 3100 to 2750 cm^{-1} associated to the CH stretching aromatic, alkenes and alkanes groups from OFLOX. It was also observed a pronounced attenuation of the band associated to the stretching vibration of OFLOX acid carbonyl C=O (1715 cm^{-1}). Moreover, other OFLOX bands at 1629 (NH bending vibration of quinolones), 1289 (OH bending vibration of carboxylic acid), 1240 and 1201 cm^{-1} (stretching vibration of ketone groups) were attenuated while the bands at 879 and 805 cm^{-1} (ascribed as out of plane bending vibration of double bonded alkenes = CH groups) disappeared (Figures S1a and S1b, SI section). The attenuation of the vibrational modes from guest molecule is commonly observed in spectra of the inclusion complexes and attributed to vibrational restrictions due to inclusion.[34]

The TGA and DTA thermal analysis were performed in order to corroborate the occurrence of interactions in the solid state, as well as to determine the effect of CD in the thermal stability of the compounds. Thus, the experiments were recorded for OFLOX, βCD, HPβCD, βCD/OFLOX, HPβCD/OFLOX and MM (Figures S2a-S2d, SI section), in order to compare the thermal profile of the inclusion complexes with their precursors and MM, aiming to find differences in the thermal profile of the inclusion compounds.

The TGA/DTA data obtained for OFLOX, βCD and HPβCD were all in accordance with those described in the literature.[35,36] For the two inclusion complexes, it was possible to observe changes in the thermal profile, corroborating the formation of new interactions in solid state. For βCD/OFLOX, its decomposition was observed at 236 °C, below than the decomposition of pure OFLOX at 255 °C. On the other hand, for HPβCD/OFLOX it was observed an improvement of thermal stability, so that its decomposition begun at 269 °C (Figures S2a-S2d, SI section). Moreover, TGA and DTA curves indicated significant differences in the intermolecular interactions established in the inclusion compounds compared with the MM, as result of the contact in the liquid phase as described in "Inclusion complex preparation" sub-section. In addition, DTA curves for the inclusion compounds showed only very simple decomposition profiles, without well-defined transitions, suggesting amorphization of the samples after complex formation.

In order to evaluate the local topology of βCD/OFLOX and HPβCD/OFLOX assemblies in solution, ^{1}H NMR experiments (D_2O, 300 K and 1.0 mM solutions) were performed and compared with the spectrum of the respective precursors at the same concentration. As shown in Figures S3 and S4 (SI section), clear changes in the shape and position of the peaks can be observed for all hydrogens of OFLOX and the cyclodextrins as result of disturbance of electronic density of the hydrogens, confirming thereby the occurrence of interactions in solution.

^{1}H chemical shifts of free and complexed OFLOX and CD are summarized in the Tables S1 and S2 (SI section). As can be seen, for the two inclusion complexes, the higher chemical shift changes occurred in the H26 of methylene group from piperazine ring of OFLOX (δ_{H26} = 2.9511), suggesting the inclusion through that edge. For the βCD/OFLOX system, it is also possible to observe a greater change in the H3 hydrogen from βCD cavity (δ = 3.9121), confirming the inclusion. In other hand, for the HPβCD/

OFLOX system, the chemical shift changes in the cyclodextrin hydrogens were less expressive than that observed for the βCD/OFLOX system, probably due to the spatial hindering caused by the hydroxypropyl groups from HPβCD.

In order to assess the thermodynamic parameters of binding, Gibbs free energy ($\Delta_b G°$), enthalpy ($\Delta_b H°$), entropy ($T\Delta_b S°$) and binding constant (K_b), ITC experiments were performed with titration of 20.0 mM βCD or HPβCD in an OFLOX solution at 0.5 mM. The curves were subtracted from the blank experiments (titration of cyclodextrin in the solvent), in order to mathematically reduce the effects of solvent in the interactions.

In the Figures S5a and S5b the titration curves for the two systems are shown. As observed, they did not show the sigmoidal profile, suggesting the occurrence of very weak interactions. However, the βCD/OFLOX system showed an unconventional profile, with an increase of injection heat ($dQ/d[CD] = \Delta_{inj}H°$) until the molar ratio close to [βCD]/[OFLOX] ca. 0.5. After this point, the $\Delta_{inj}H°$ decreased until the end of the titration. This uncommon behavior suggests the occurrence of more than one stoichiometry over molar ratio. At the start of titration, when there is excess of OFLOX, some kind of high stoichiometry βCD/OFLOX supramolecular complex might be formed. When the βCD/OFLOX concentrations are equalized, this supramolecular structure is spontaneously converted in a more likely 1:1 stoichiometry.

The data obtained for the first interaction are unreliable due to the low number of points considered in the fit (only six points). Therefore, the increase of $\Delta_{inj}H°$ suggests that the formation of high order supramolecular complex is exothermic. On the other hand, the 1:1 complex formed at equalized concentrations of βCD and OFLOX, with a binding constant of $K_b = 880$, was endothermic ($\Delta_b H° = +1241.0$ cal mol^{-1}) and entropic driven ($T\Delta_b S° = +5247.4$ cal mol^{-1}), being ascribed to the desolvation of species upon reorganization.

In the titration with HPβCD, the $\Delta_{inj}H°$ was endothermic in overall range, and decreasing with the molar ratio. For this system the "one set of site", provided by Wiseman isotherm, was used fixing the 1:1 stoichiometry. The process was endothermic ($\Delta_b H° = +3871$ cal mol^{-1}) and entropy driven ($T\Delta_b S° = +6350.3$ cal mol^{-1}), also attributed to desolvation upon inclusion. However, the binding constant was very low ($K_b = 65.2$) being ascribed to the steric hindering caused by hydroxypropyl groups present in the outer surface of the HPβCD. The greater polarity of this cyclodextrin might also contribute to reducing the affinity between the molecules.

The changes in the shape and position of the peaks are observed for all hydrogens of βCD/OFLOX and HPβCD/OFLOX systems. These major changes are in agreement with the interactions observed in the solid state by FTIR data, corroborating the occurrence of new interactions.

In literature, there are several thermodynamic data for OFLOX/cyclodextrin complexes. However, all of them were obtained by spectroscopic data, where the enthalpy and equilibrium constant were determined by Van't Hoff approach.[9,10] In the present study, thermodynamic parameters of interaction for βCD/OFLOX and HPβCD/OFLOX inclusion complexes were obtained by ITC and, different from the spectroscopic data described in literature, here it was used a mixture of solvent (DMSO:H_2O 40:60 v/v), where it is expected low or no ionization of OFLOX molecules. The magnitude of the binding constants obtained in the present study was different from those obtained by fluorescence in aqueous environment, which can be attributed to the differences in the solvent used in the two experimental approaches.[21] Moreover, this author also found greater values of K_b for the βCD/OFLOX system if compared with the HPβCD/OFLOX system, corroborating the hypothesis about the occurrence of steric hindering in the presence of HPβCD.

Hydrophobic nanoprecipitates (HNPs) are solid particles of very low solubility, usually produced in solvents where the water is the component of greater proportion (> 90%). The greater advantage of these materials is the possibility to produce simple formulations without the need to use costly additives as surfactants or polymers, besides acting as controlled release system driven by dissolution of the solid phase.

Considering the potentiality of producing formulations through the hydrophobic nanoprecipitation strategy, DLS experiments were performed in order to evaluate the effect of CD on the average size of particles in a liquid medium.[24,26,27]

In the Figure S6 (SI section), it is shown the D_h values of HNPs in relation to the increment in the nominal concentration of OFLOX, βCD/OFLOX or HPβCD/OFLOX. As observed, the inclusion complexes showed lower values of size if compared with free OFLOX. The reduction in the size of the inclusion complexes could be explained by the presence of hydroxyls in the CD, which result in higher water interaction in relation to pure OFLOX, and by the increase of solubility of the inclusion compounds. This better affinity to water, probably due to the formation of hydrogen bonding with the solvent, contributes to the reduction of aggregation and the coalescence of the particles.[27]

These data are relevant because the reduction in the size of the HNPs ensures that a larger superficial area of the particle is exposed to the biological environment, enabling new patterns of interactions and consequently increasing its bioavailability.[37]

Furthermore, the HPβCD/OFLOX (364.55 ± 64.51 nm) showed lower average size when compared to βCD/OFLOX (860.17 ± 88.64 nm), as can be seen in Figure S6 (SI section). This result could be explained by the high water solubility of HPβCD (> 600 mg mL^{-1}) when compared to βCD (18.5 mg mL^{-1}).[11] Moreover, according with the data from the literature,[10,38] the HPβCD/OFLOX complex is at least three times more soluble than pure OFLOX, while the βCD is 2.1 times more soluble than pure OFLOX, corroborating thereby the hypothesis that the greater solubility of HPβCD facilitates the interactions of these nanoparticles with water.

Biological evaluation

The lung cancer is the major cause of death between the neoplastic diseases, which can aggravate with bone metastasis, turning more expensive the treatment and reducing the life quality of the patient.[39] It affects both men and women and there is a clear association with smoking in the development of the disease, although it is not the only risk factor.[40,41] The non-small cell lung cancer (NSCLC) type, that affects 85% of the patients, can present subtypes as adenocarcinoma or squamous cell carcinoma, due the different origin site.[42] The other patients are affected by the small cell lung cancer type, although rare cases such as sarcomatoid carcinoma can be found.[40,42]

The effect of fluoroquinolones in cancer cells has already been evaluated, showing that enoxacin, norfloxacin, ciprofloxacin and levofloxacin can reduce the proliferation of human NSCLC (NCI-H460), with levofloxacin inducing the apoptosis in lung cancer cells.[1,43,44] However, the effect of βCD/OFLOX and HPβCD/OFLOX inclusion complexes, or even ofloxacin, on A549, an NSCLC,[45,46] had not yet been investigated. In the present study the inclusion complexes (HPβCD/OFLOX and βCD/OFLOX) reduced the viability of the lung cancer cell and increased the apoptosis in this cell line, more than pure OFLOX.

In this study βCD/OFLOX and HPβCD/OFLOX were capable of inhibiting the bacterial proliferation, with MICs values lower than OFLOX for *E. coli* and *S. aureus* (Table 1). It is believed that the CD improve the time of contact of the drug with cell membrane surface, forming hydrogen bonding with components of the membrane, guaranteeing a greater time of residence of the drug around bacterial cells.

For the time-kill curves, there were no statistical differences between the kinetic profiles of OFLOX, βCD/OFLOX and HPβCD/OFLOX, even with the decrease in MICs. For the *E. coli*, the compounds generated a bactericidal curve, with a reduction of 99.9% in viable bacterial density after 24 h compared to initial inoculum (Figure 1). For the *S. aureus*, all compounds provided a bacteriostatic curve, since there is a slight reduction (1-2 log 10) of the amount of CFU mL^{-1} compared to the initial inoculum showed in Figure 2.

Table 1. Minimal inhibitory concentrations of the OFLOX, βCD/OFLOX and HPβCD/OFLOX

Strain	MIC $value_{100\%}$ / μM		
	OFLOX	βCD/OFLOX	HPβCD/OFLOX
Escherichia coli ATCC25922	1.38	0.16	0.07
E. coli EVM	1.38	0.16	0.14
E. coli C2	1.38	0.16	0.14
E. coli C4	1.38	0.08	0.03
E. coli C7	1.38	0.08	0.07
E. coli C8	0.34	0.08	0.07
E. coli C9	0.34	0.08	0.03
E. coli C10	1.38	0.08	0.03
E. coli C13	0.34	0.04	0.03
Staphylococcus aureus ATCC 27853	2.76	0.33	0.14
S. aureus C923	1.38	0.33	0.14
S. aureus C602	2.76	0.33	0.14
S. aureus C6	1.38	0.33	0.14
S. aureus C5	1.38	0.33	0.14
S. aureus C4	1.38	0.16	0.14
S. aureus C601	1.38	0.33	0.14
S. aureus C003	1.38	0.33	0.14
S. aureus C707	1.38	0.33	0.14

MIC: minimal inhibitory concentration; OFLOX: ofloxacin; βCD/OFLOX: β-cyclodextrin/ofloxacin; HPβCD/OFLOX: hydroxypropyl-β-cyclodextrin/ofloxacin; ATCC: American Type Culture Collection; EVM: environmental; C: clinical.

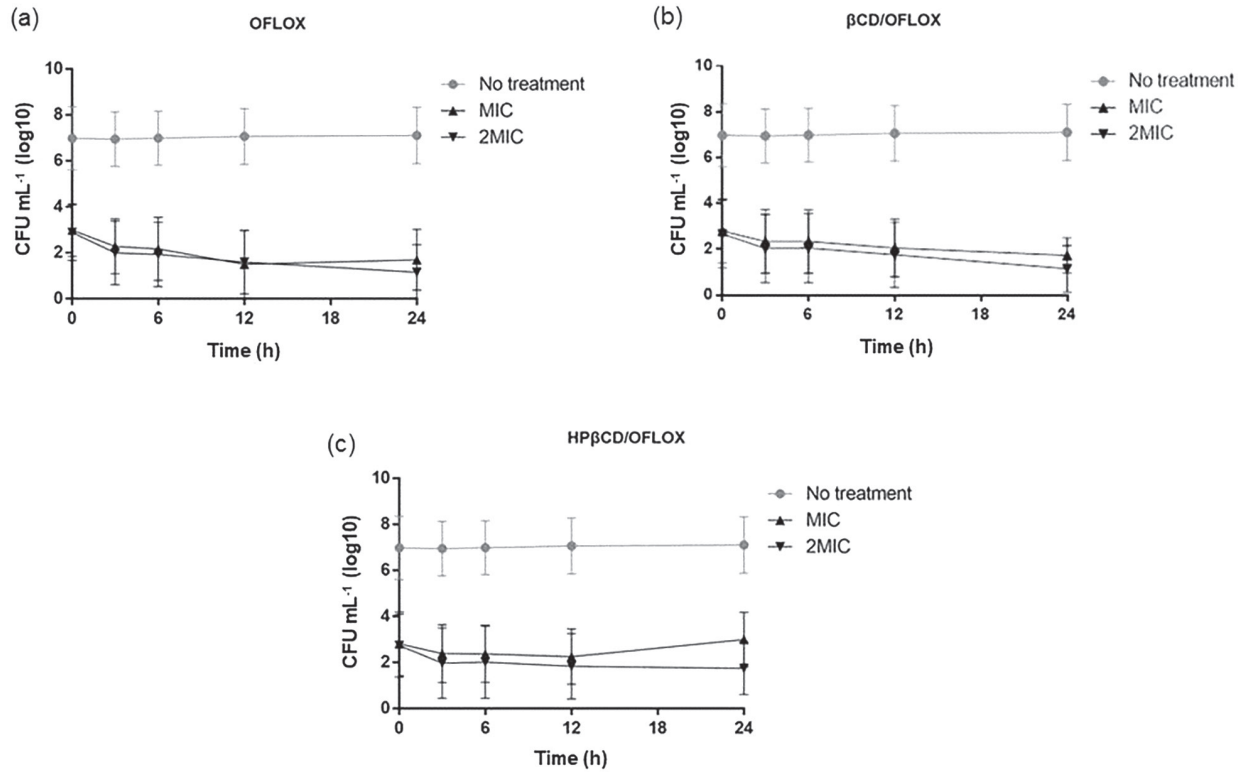

Figure 1. Time-kill curves of *Escherichia coli* (C10). The curve was generated at different times. The *E. coli* was treated (a) with ofloxacin (OFLOX); (b) β-cyclodextrin/ofloxacin (βCD/OFLOX); (c) hydroxypropyl-β-cyclodextrin/ofloxacin (HPβCD/OFLOX) or not treated. MIC: minimal inhibitory concentration; 2MIC: twice the minimal inhibitory concentration.

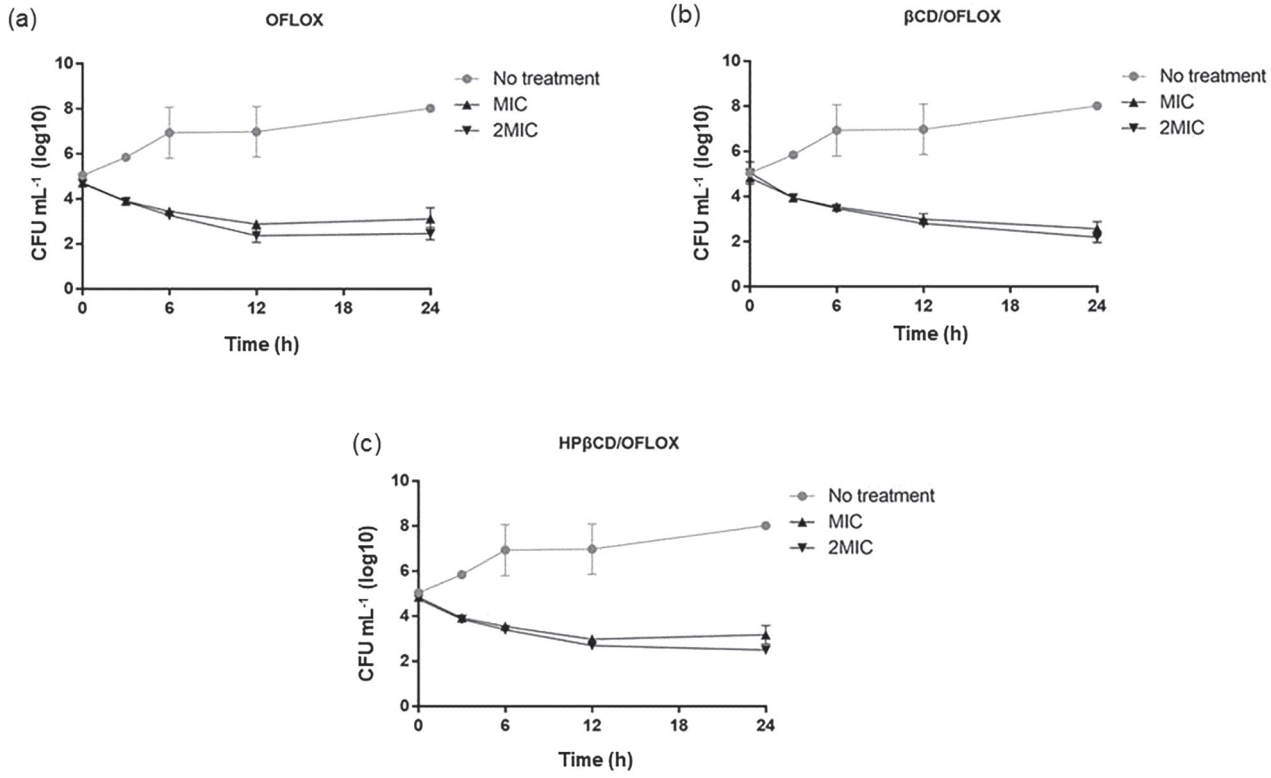

Figure 2. Time-kill curves of *Staphylococcus aureus* (C4). The curve was generated at different times. The *S. aureus* was treated with (a) ofloxacin (OFLOX); (b) β-cyclodextrin/ofloxacin (βCD/OFLOX); (c) hydroxypropyl-β-cyclodextrin/ofloxacin (HPβCD/OFLOX) or not treated. MIC: minimal inhibitory concentration; 2MIC: twice the minimal inhibitory concentration.

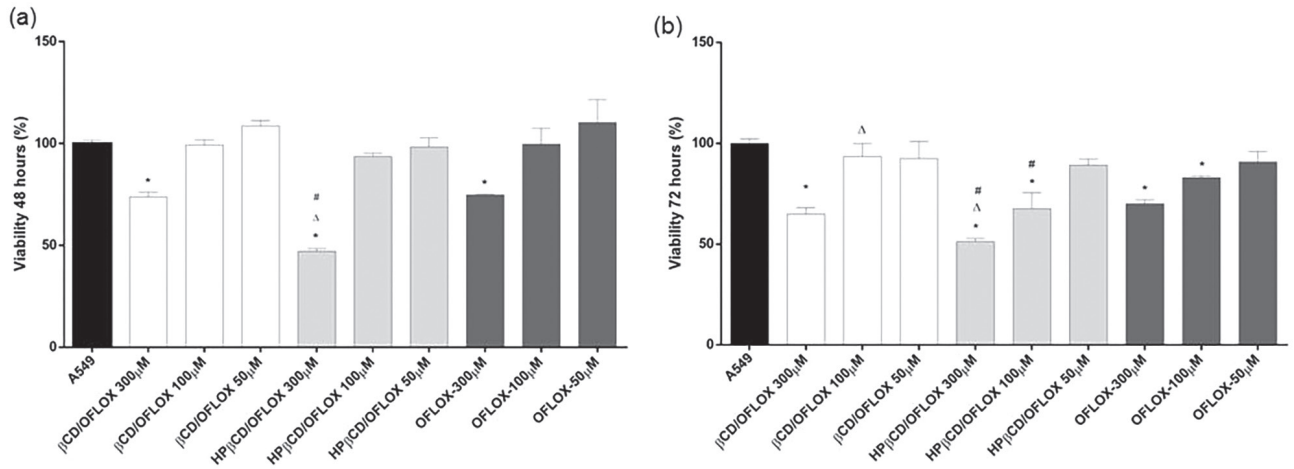

Figure 3. MTT assay. A549 cells were maintained for (a) 48 and (b) 72 h in the presence or absence of ofloxacin (OFLOX), β-cyclodextrin/ofloxacin (βCD/OFLOX) or hydroxypropyl-β-cyclodextrin/ofloxacin (HPβCD/OFLOX) at 50, 100 and 300 μM. The cellular viability was measured through MTT assay. The results were calculated as percentage of cell viability relative to not treated control (A549). *: $p < 0.05$ *versus* A549 not treated, #: $p < 0.05$ *versus* βCD/OFLOX; Δ: $p < 0.05$ *versus* OFLOX.

In the present study, since OFLOX is a known antimicrobial drug, the antimicrobial activity of the inclusion complexes was evaluated with the objective to verify the enhancing in the activity of OFLOX. In relation to the antimicrobial activity, the inclusion complexes showed lower MIC values if compared with pure OFLOX, suggesting a greater antimicrobial activity, probably due to the increase of the residence time around the cells. Moreover, the MIC values obtained for HPβCD/OFLOX were lower than those observed for βCD/OFLOX complex, for almost all strains. This difference was ascribed to the lower binding constant for the HPβCD/OFLOX inclusion complex, which could facilitate the dissociation on the bacterial surface.

The cytotoxic activity of the βCD/OFLOX and HPβCD/OFLOX was evaluated by the determination of cell viability (Figure 3). Both complexes reduced the viability of the A549 cell at 300 μM, more than OFLOX. There were no statistical differences on the viability of A549 for pure βCD or HPβCD treatment. Moreover, βCD/OFLOX and HPβCD/OFLOX were able to induce apoptosis in A549 cell lines at 300 μM, which was more effective than OFLOX for HPβCD/OFLOX (Figure 4).

The better results obtained for HPβCD/OFLOX corroborate with the hypothesis raised in the microbiological studies about the facility of this complex to dissociate on the surface of the cells, improving the targeting of the compound and its bioavailability. Thus, it can be observed that the inclusion complexes showed better antitumor and antimicrobial activity with elevated cytotoxicity of A549 cells and lower MIC, when compared to pure OFLOX, being the first time that these activities were demonstrated. Furthermore, the activity of OFLOX as an antitumor compound was shown.

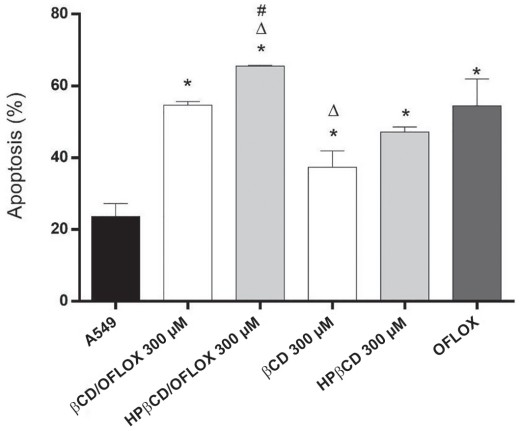

Figure 4. Apoptosis by flow cytometry. A549 cells were maintained for 36 h in the presence or absence of ofloxacin (OFLOX), β-cyclodextrin (βCD), βCD/OFLOX, hydroxypropyl-β-cyclodextrin (HPβCD) or HPβCD/OFLOX at 300 μM. The apoptosis (annexin V^+/PI^-) was measured by flow cytometry. *: $p < 0.05$ *versus* A549 not treated; #: $p < 0.05$ *versus* βCD/OFLOX; Δ: $p < 0.05$ *versus* OFLOX.

Conclusions

The OFLOX and their inclusion complexes (βCD/OFLOX and HPβCD/OFLOX) showed to be a multifunctional drug, improving its biological activities, with promising effects as anti-proliferative cancer cells and antimicrobial. The better microbiological and cytotoxic results observed for HPβCD/OFLOX was correlated with its lower binding constant, which could facilitate its dissociation on the surface of the cells, improving the targeting of the compound and its bioavailability. The beneficial effect of these compounds, *in vivo*, needs to be investigated.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Bolivar R. Amaro contributed in running the laboratory work and supporting the analysis of data and data curation; Paloma E. Carvalho contributed in running the laboratory work and supporting the analysis of data and data curation; Cleonice A. Souza contributed in supporting the laboratory work and data analysis; Oswaldo Cardoso Jr. contributed in supporting the laboratory work and data analysis; Caio C. S. Alves contributed in the conceptualization, biological analysis, funding acquisition and writing review and editing the manuscript; Gabriella F. Ferreira contributed in microbiological analysis, funding acquisition and writing original draft; Jeferson G. da Silva contributed in chemical analysis, funding acquisition and writing original draft; Alessandra P. Carli contributed in biological analysis and critical review of the manuscript; Fabiana S. Machado contributed in the resources and critical review of the manuscript; Ângelo M. L. Denadai contributed in conceptualization, funding acquisition, resources and writing original draft; Sandra B. R. Castro lead the conceptualization, project administration, funding acquisition, visualization and writing the original draft.

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