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Synthesis, properties and biological activity of a novel phosphines ligand derived from ciprofloxacin

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ABSTRACT

Novel potential antibacterial agents derived from ciprofloxacin (**HCp**) were synthesized in the reaction of this antibiotic with hydroxymethyldiphenylphosphine. Detailed analysis by the NMR, IR and MS techniques attested to the identity and structure of the obtained compounds. In addition, the structure of phosphine PPh_2CH_2-Cp was unambiguously confirmed by X-ray crystal structural analysis. PPh_2CH_2-Cp exhibited an antibacterial activity comparable to that of the original drug. Cytotoxicity studies revealed that this compound was characterized by lower toxicity against mammalian cells than **HCp**. Besides, it did not induce a morphological change in cells after its action and was unable to degrade plasmid DNA, as well as parent antibiotic. Our results open up new possibilities in designing novel, less toxic and comparably effective antibiotics drugs.

1. Introduction

Increasing microbial resistance against antibiotics has been recognized by the World Health Organization as one of the significant health dangers in the early twenty-first century [1,2]. Large genetic variations of pathogenic organisms and excessive frequency of antibiotics usage result in the development of increasingly effective defense mechanisms by microorganisms. Another problem is the speed with which they inactivate drugs used in therapies [3–6].

For this reason, search for new antimicrobial agents is extremely important and desirable. It can be performed in two ways. One involves structural modifications of substances already used in medicine, and the other – synthesis of completely new antibiotics. The first approach is safer but it carries the risk of rapid resistance acquisition. The other approach involves a much lower risk but is more expensive and time consuming [7]. We have undertaken a modification of ciprofloxacin (**HCp**), a second generation fluoroquinolone, by enriching its molecule with a new functional group.

HCp is a broad-spectrum antibiotic, commonly used in the urinary tract, respiratory and digestive infections. It is active against Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) and some Gram-positive (*Staphylococcus aureus*, *Mycobacterium tuberculosis*) bacteria. The mechanism of its action is based on blocking the DNA replication process by binding gyrase or topoisomerase IV [8–11].

In this paper we present the synthesis and the biological properties of **HCp** modified by substitution with diphenylphosphinomethyl group. The presence of the $-CH_2PPh_2$ residue could potentially generate additional interactions with bacteria. Therefore it may alter the biological activity of the parent drug and impact its bioavailability or toxicity level. Due to the presence of a secondary nitrogen atom in the piperazine ring (Fig. 1), **HCp** can be easily converted into phosphine ligand [12–14]. Possibility of such modification seems especially attractive, because a number of papers show that phosphines, as well as their derivatives and the metal ion complexes (i.e. copper, platinum, gold) exhibit promising anticancer, antibacterial or antiarthritic activity [14–21]. On the other hand, phosphines are very often characterized by a high cytotoxicity [15].

HCp is an antibiotic that has already been subjected to structural modifications. They are associated with the introduction of additional groups, including hydroxybisphosphonate [22], benzenesulphoamide [23–29] or acyl [30]. Complexes in which **HCp** is coordinated to the d-electron (Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}) [31–34] and f-electron (La^{3+}, Eu^{3+}, Tb^{3+}) [35–37] metal ions have also been

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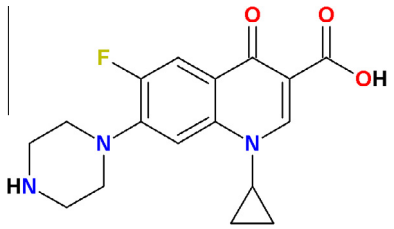

Fig. 1. Molecular scheme of a ciprofloxacin molecule (**HCp**).

reported. Besides, there are known ternary systems containing this drug, metal ion and diimine ligands [31,34,38]. It should be noted that the modified antibiotic and **HCp** complexes with transition metal ions often exhibit a higher activity against pathogens than the parent drug [24,27,30–34].

2. Experimental

2.1. General methods

The reagents (ciprofloxacin, triethylamine, hydrogen peroxide, diphenylphosphine, formaldehyde, hydrochloric acid) and solvents were purchased from Sigma–Aldrich and used without further purification. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer with traces of solvent as an internal reference for ^{1}H and ^{13}C spectra and 85% H_3PO_4 in H_2O as an external standard for ^{31}P. Chemical shifts are reported in ppm (parts per million) and coupling constants are reported in Hz. Mass spectra were registered on a Bruker Daltonics micrOTOF–Q Mass spectrometer equipped with electrospray ionization (ESI) source and operated in positive ion mode. IR spectra were recorded from 4000 to 400 cm^{-1} on Bruker 113v FTIR spectrophotometer as a KBr pellets. Elemental analysis was performed with a Vario EL3 CHN analyzer.

Reactions were carried out under a nitrogen atmosphere using standard Schlenk techniques. All solvents were deaerated prior to use. $[PPh_2(CH_2OH)_2]^+Cl^-$ was synthesized according to the literature method [39].

2.1.1. Synthesis of PPh_2CH_2-Cp (**PCp**)

Triethylamine (10 ml) was added drop wise to a solution of 1.2789 g of $[PPh_2(CH_2OH)_2]^+Cl^-$ (4.53 mmol) in 30 ml of methanol on the ice bath. After 30 min of stirring water suspension (20 ml) of ciprofloxacin **HCp** (1.5 g; 4.53 mmol) was added. The mixture was stirred for 1 h at room temperature. The obtained white solid was centrifuged, washed twice with water and dried under reduced pressure. Yield 88%. *Anal.* Calc. for $C_{30}H_{29}FN_3O_3P$ (529.5): C, 68.04; H, 5.52; N, 7.93. Found: C, 67.81; H, 5.75; N, 7.89%.

NMR ($CDCl_3$, 298 K): $^{31}P\{^1H\}$: −27.42 s; ^{1}H: H^{3Pb}: 15.01 s, H^{2P}: 8.71 s, H^{5P}: 7.95 d *J = 13.80*, $H^{Ph(o),(m),(p)}$: 7.47–7.34, H^{8P}: 7.47–7.34, H^{1Pa}: 3.53 m, $H^{3P'}$: 3.37, $H^{1P'}$: 3.29 d *J* = 2.87, $H^{4P'}$: 2.90, H^{1Pb}: 1.39 m, H^{1Pb}: 1.18 m; $^{13}C\{^1H\}$: C^{4P}:177.15 s, C^{3Pa}: 167.09 s, C^{6P}: 154.77 and 152.77, C^{2P}: 147.44 s, C^{7P}: 145.98 d *J* = 10.39, C^{9P}: 139.18 s, $C^{Ph(i)}$: 138.12 d *J* = 12.40, $C^{Ph(o)}$: 132.94 d *J* = 18.50, $C^{Ph(p)}$: 128.85 s, $C^{Ph(m)}$: 128.61 d *J* = 6.63, C^{10P}: 119.76 d *J = 7.93*, C^{5P}: 112.39 d *J* = 23.50, C^{3P}: 108.14 s, C^{8P}: 104.91 d *J* = 3.35, $C^{1P'}$: 61.31 d *J* = 4.06, $C^{4P'}$: 54.20 d *J* = 9.29, $C^{3P'}$: 49.95 d *J* = 5.04, C^{1Pa}: 35.41 s, C^{1Pb}: 8.32 s.

MS ($CHCl_3$): 344.1 $[CH_2C_{17}H_{17}FN_3O_3]^+$; 247.1 $[C_{13}H_{10}FNO_3]^+$; 199.1 $[PPh_2CH_2]^+$; 530.2 $[MH]^+$; 546.2 $[MOH]^+$; 285.1 $[PPh_2CH_2C_4H_{10}N_2]^+$; 504.1 (503.1?) $[PPh_2CH_2C_{15}H_{16}FN_3O_3]^+$;

IR (KBr): ν = 1727.2 cm^{-1} (vs) –C═O.

2.1.2. Synthesis of $O = PPh_2CH_2$-Cp (**OPCp**)

OPcp was obtained in the reaction in dichloromethane-acetonitrile mixture (1:1) (10 ml) of **PCp** (0.1081 g; 0.204 mmol) placed in ice bath with equimolar amount of H_2O_2 (30% solution in water) (20.8 μl; 0.204 mmol). Solvent was evaporated under vacuum to dryness and obtained yellowish solid was washed twice with water. Yield 91%. *Anal.* Calc. for $C_{30}H_{29}FN_3O_4P$ (545.5): C, 66.05; H, 5.36; N, 7.70. Found: C, 65.88; H, 5.56; N, 7.67%.

NMR ($CDCl_3$): $^{31}P\{^1H\}$: 27.70 s; ^{1}H: H^{3Pb}: 14.49 s, H^{2P}: 8.72 s, H^{5P}: 7.94 d *J* = 13.03, $H^{Ph(o),(m),(p)}$: 7.80–7.49, H^{8P}: 7.30 d *J* = 7.17, H^{1Pa}: 3.50 m, $H^{3P'}$: 3.29, $H^{1P'}$: 3.34 d *J* = 6.31, $H^{4P'}$: 2.92, H^{1Pb}: 1.37 m, H^{1Pb}: 1.17 m; $^{13}C\{^1H\}$: C^{4P}:177.18 s, C^{3Pa}: 167.09 s, C^{6P}: 154.74 and 152.73, C^{2P}: 147.49 s, C^{7P}: 145.87 d *J* = 10.35, C^{9P}: 139.16 s, $C^{Ph(i)}$: 132.35 d *J* = 98.11, $C^{Ph(o)}$: 132.13 d *J* = 2.69, $C^{Ph(p)}$: 131.26 d *J* = 8.92, $C^{Ph(m)}$: 128.75 d *J* = 11.54, C^{10P}: 119.84 d *J* = 7.80, C^{5P}: 112.43 d *J* = 23.57, C^{3P}: 108.19 s, C^{8P}: 104.97 d *J* = 3.26, $C^{1P'}$: 58.05 d *J* = 87.34, $C^{4P'}$: 55.06 d *J* = 8.01, $C^{3P'}$: 49.90 d *J* = 4.93, C^{1Pa}: 35.40 s, C^{1Pb}: 8.33 s.

MS ($CDCl_3$): 546.2 $[MH]^+$; 344.1$[CH_2C_{17}H_{17}FN_3O_3]^+$;

IR (KBr): ν = 1725.2 cm^{-1} (vs) –C═O; ν = 1186.2 cm^{-1} (s) –P═O.

2.1.3. X-ray structure of PPh_2CH_2-Cp (**PCp**)

The colourless plate shaped crystal of dimensions 0.21 × 0.14 × 0.10 mm was used for X-ray data collection. Data were collected at low temperature (100 K) using an Oxford Cryosystem device on a Kuma KM4CCD κ-axis diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The crystal was positioned at 65 mm from the CCD camera. 612 frames were measured at 0.75° intervals with a counting time of 10 s at low theta angles and 764 frames were measured at 0.6° intervals with a counting time of 30 s at high theta angles. 'Multi-scan' absorption correction was applied. Data reduction and analysis were carried out with the Oxford Diffraction programs [40,41]. The structure was solved by direct methods (program SHELXS97 [42] and refined by the full-matrix least-squares method on all F^2 data using the SHELXL97 [42] programs.

Crystal/refinement data: PCp ≡ $C_{30.02}H_{29.02}FN_3O_3P$, M_r = 529.82, Crystal size: 0.21 × 0.14 × 0.10 mm, Crystal system: Monoclinic, Space group: *P*21/*c*, Unit cell: *a* = 7.595(3) Å, *b* = 28.100(12) Å, *c* = 11.892(7) Å, β = 97.05(3)°, *V* = 2519(2) $Å^3$, D_{calc}(*Z* = 4) = 1.396 g/cm^3, θ range for data collection: 3.07–36.99° deg, Mo Kα radiation (λ = 0.71073 Å), μ_{Mo} = 0.156 mm^{-1}, T_{min} = 0.949, T_{max} = 1.000, Reflections collected/unique 45398/12212 [R_{int} = 0.0379], Final *R* indices [$I > 2\sigma(I)$] R_1 = 0.0439, wR_2 = 0.1060, *R* indices (all data) R_1 = 0.0687, wR_2 = 0.1125, Goodness-of-fit = 1.020, Largest difference in peak and hole: 0.580 and −0.314 e$Å^{-3}$, Data/restraints/parameters: 12212/0/401; *T* = 100(2) K.

2.2. Antibacterial activity

The antimicrobial activity was evaluated by the serial dilutions method using the Antibiotic Assay Medium (Assay Broth; pH 7.0 ± 0.2; dextrose 1.0; K_2HPO_4 3.68; beef extract 1.5; peptone 5.0; KH_2PO_4 1.32; NaCl 3.5; yeast extract 1.5 g/L) according to Grove and Randall [43]. The following strains were employed: *S. aureus* PCM 2054 (=ATCC 25923), *E. coli* PCM 2057 (=ATCC 25922) from the Polish Collection of Microorganisms of the Institute of Immunology and Experimental Therapy in Wroclaw, as well as *P. aeruginosa* isolated from clinical samples. The latter strain was identified using conventional methods and miniaturized identification systems (ID 32 C and API 20 NE [BioMérieux], respectively). An overnight culture of strain tested was diluted 1:1000 in the AB. To a series of tubes containing appropriate amounts of compounds (as the films on the tube walls), 0.9 mL of AB and 0.1 mL of microbial dilution were added. Following concentrations of each compound were obtained [μg/mL]: 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1.

Drug-free purity and growth controls were included. The tubes were incubated at 37 °C for 24 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of compound that inhibited microbial growth. For each compound, tests were performed in triplicate and results shown are the average of independent experiments.

2.3. Cytotoxic assay on RK-13 according to EN 14675

2.3.1. Cells

The cell line (rabbit kidney), passages 119–156, incubated in 96-well polystyrene plate for 24 h were used in this project.

2.3.2. Cytotoxic assay

Substances were tested using EN 14675 [44] with own modification. Product test solutions were prepared in Minimum Essential Medium (MEM) supplemented with additional 2% Fetal Bovine Serum (FBS) and L-glutamine. Solutions of the compounds in concentrations from 2 mM to 2×10^{-8} mM were prepared and transferred (100 μl) into cell culture units (wells of microtitre plates) containing suspended cells in 100 μl. Final concentrations of the complexes were: 1, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} and 1×10^{-8} mM. Eight units were inoculated with each concentrations. Plates were incubated in 37 °C/5%CO_2 and observed daily for up to 96 h for the development of cytopathic effect (CPE), using an inverted microscope [44] (Olympus Corp., Hamburg Germany; Axio Observer, Carl Zeiss MicroImaging GmbH).

2.4. Cytotoxic properties on mesenchymal stem cells (HMSC-ad)

Mesenchymal stem cells (HMSC-ad) isolated from horse adipose tissue (passage 3) were seeded into 96-well plate (20×10^3 per well) and incubated in 37 °C/5%CO_2 for 24 h for adhesion in DMEM supplemented by 10% FBS and 1% penicillin/streptomycin/amphotericin b [45–47]. HMSC-ad were treated with **HCp** and **PCp** of various concentrations. Their influence on the cell metabolism was determined by spectrophotometrical measurement of resazurin bioreduction ratio at 600 nm, with distraction of absorbance at 690 nm wavelength as a background, according to manufacturer's protocol. Additionally, control wells were included, with addition of pure compounds solvent at concentrations reflecting the research group. Viable cells reduce the amount of dye oxidized, blue form and increase the amount of red, reduced. Non-toxic concentrations of the compounds were established in comparison to normal, not treated control cells [46,47]. The research was supported by microscopic observations and fluorescent stainings. Actin chains, as active element of cell cytoskeleton were visualized for evaluation of cell general morphology and condition, with fluorophore-labeled phalloidine. Detection of apoptosis was done by immunocytochemical assay, using antibodies against active caspase 3 – protein directly committed in apoptosis. Additional DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) staining showed the form and condition of nuclei. Connection of these two approaches resulted in receiving detailed picture of pharmaceutics' toxicity and their neutral concentrations for cells *in vitro* [48,49–51].

2.4.1. Metabolic tests (alamar blue cytotoxic assay)

Examined substances were added to culture medium (end volume 200 μl) at different concentrations (2×10^{-1}, 1×10^{-1}, 2×10^{-2}, 1×10^{-2}, 2×10^{-3}, 1×10^{-3}, 2×10^{-4} and 1×10^{-4} mM) for 30 min. After that time, medium was replaced by fresh with an addition of 10% resazurin (Sigma) and cultures were incubated for 4 h. After incubation, the changes in absorbance were measured with plate reader (SpectroStar) in a wavelength of 600 nm, with respect to not treated control wells. The results from all repeats ($n = 3$) were averaged and compared with control cells [45–47].

2.4.2. Immunofluorescence

Cells were washed with PBS/1% FBS, fixed with 4% paraformaldehyde for 10 min at RT and permeabilized with 0,1% Triton X-100/3%BSA for 10 min in room temperature. After that, cells were washed again and incubated with primary antibody against active caspase 3 c-terminal fragment (polyclonal IgG produced in rabbit, Sigma) for 60 min in 37 °C/5%CO_2. Cells were than washed again three times and incubated with secondary antibody with fluorescent conjugate (AlexaFluor-594 nm, anti-rabbit IgG produced in goat, Sigma) and with fluorescent-labeled phalloidine (Atto488, Sigma) and were incubated for one hour in 37 °C/5%CO_2. Finally, cells were washed three times, counterstained with DAPI, treated with mounting medium (Fluoromount, Sigma), observed and photographed with inverted, fluorescent microscope with digital camera attached (Cannon PC1200). Picture of cells were taken also with microscope contrast-phase light option enable [48,49–51]. Pictures were merged and partially pseudocoloured for best visualization.

2.5. DNA strands break analysis

The ability to induce strand breaks by the studied compounds was tested with the application of pUC18 plasmid. The solution of DNA in 5 mM sodium phosphate buffer (pH 7.4) was mixed with compounds. After 1 h incubations at 37 °C, reaction mixtures (20 μL) were mixed with 3 μL of loading buffer (bromophenol blue in 30% glycerol) and loaded on 1% agarose gels, containing ethidium bromide, in TBE buffer (90 mM Tris–borate, 20 mM EDTA, pH 8.0). Gel electrophoresis was done at constant voltage of 100 V (4 V cm^{-1}) for 60 min. The gel was photographed and visualized with a Digital Imaging System (Syngen Biotech).

3. Results and discussion

3.1. Chemistry

Phosphine derivative (**PCp**) of ciprofloxacin was obtained in the reaction of **HCp** with hydroxymethyldiphenylphosphine. Then, this new ligand was transformed into an oxide derivative (**OPCp**, Fig. 2).

The IR spectra confirm the presence of synthesized compounds. On the **HCp** spectrum the C=O stretching vibrations of the group from quinolone ring are observed at 1723.3 cm^{-1}. Since our modifications do not include the ring, the band of C=O groups is also present on the spectra of **PCp** and **OPCp** at 1727.2 cm^{-1} and 1725.2 cm^{-1} respectively. For **OPCp** derivative the band associated with P=O bond stretching is observed at 1186.2 cm^{-1}. Further confirmation of the molecular structures of these derivatives was obtained from the Mass spectra.

The reaction products were described in detail using NMR spectroscopy (see the Section 2 and Table S1 in Supplementary data). We assigned all signals relying on the NMR data of **HCp** obtained in other solvents [52,53]. Apparently, the formation of a C–N bond in the course of the reaction of hydroxymethyldiphenylphosphine with **HCp** leads to significant changes in the $^{31}P\{^1H\}$ NMR spectra in $CDCl_3$. A singlet at −11.46 ppm, characteristic for Ph_2PCH_2OH, is moved to higher fields (−27.42 ppm) for **PCp**, which is associated with the change of the substituents at P atom. On the other hand, oxidation of the phosphorus atom and – in consequence – formation of the phosphine oxide (**OPCp**) causes a significant downfield shift of the phosphorus atom signal. A similar trend was observed for other diphenylphosphines [54,55].

In contrast to the phosphorus spectra, ^1H NMR spectra do not undergo considerable changes. Signals of the Ph_2P– group and of

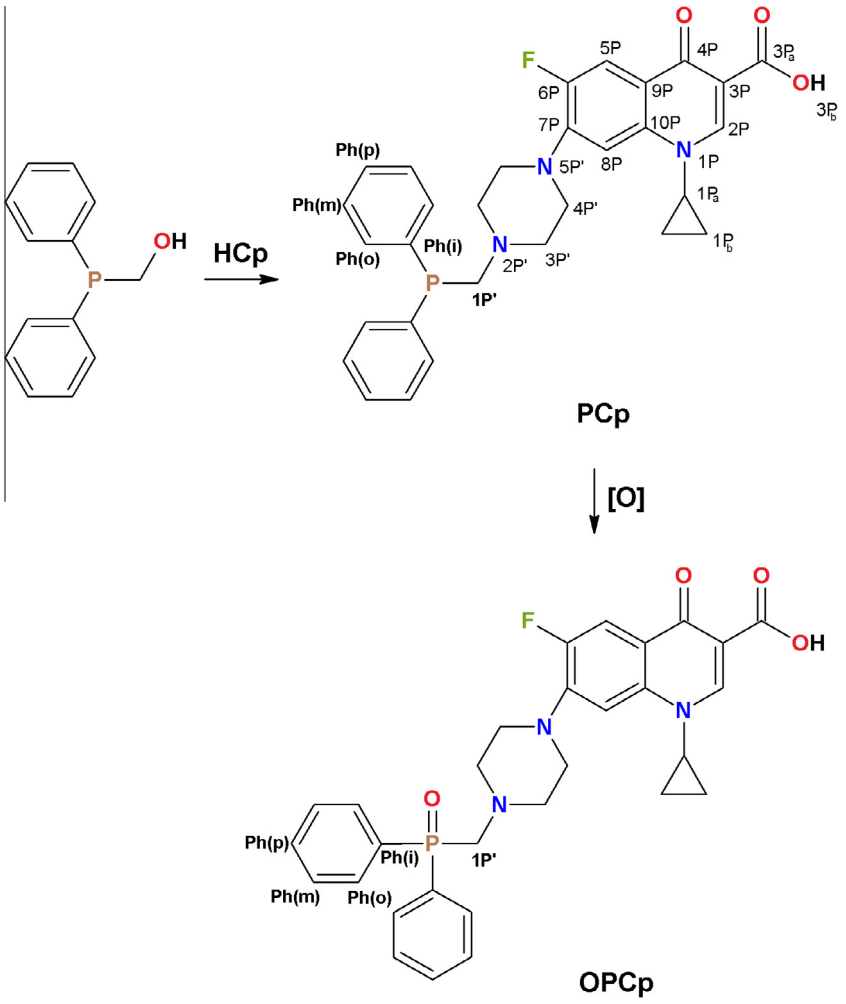

Fig. 2. Scheme of formation of **PCp** and **OPCp**.

–Cp fragment have similar values of chemical shifts for the substrates and obtained derivatives. As expected, the largest changes caused by the formation of the phosphine and its oxide are observed for a doublet originating from $H^{1P'}$ protons. Forming of a C–N bond results in a significant upfield shift of the signal with a simultaneous decrease of the $^{2}J(H^{1P'}–P)$ spin–spin coupling constant. After oxidation, this signal is shifted to lower fields with a parallel increase of the above coupling constant.

Due to the low solubility of **HCp**, $^{13}C\{^{1}H\}$ NMR spectra were measured only for Ph_2PCH_2OH, **PCp** and **OPCp**. The available data indicate that the formation of **PCp** ligand does not involve significant changes of the phenyl rings carbon atoms signals. However, for the $–CH_2–$ group directly associated with the P atom, a significant decrease of the $^{1}J(C^{1P'}–P)$ coupling constant is observed. The formation of the oxide triggers alterations in the chemical shifts and coupling constants for carbon atoms of the phenyl rings and the $=PCH_2–$ fragment. The signal of the latter atom shifts to higher fields and $^{1}J(C^{1P'}–P)$ dramatically increases from 4.06 to 87.34 Hz.

The structure of **PCp** was unambiguously confirmed by the crystallographic analysis. This compound crystallized as a strongly disordered molecule in which the $–N(CH_2CH_2)NCH_2PPh_2$ fragment occupies two completely different positions. The X-ray structure of **PCp** in the dominating conformation is shown in Fig. 3. The geometrical parameters of the shared parts (i.e. quinolone and

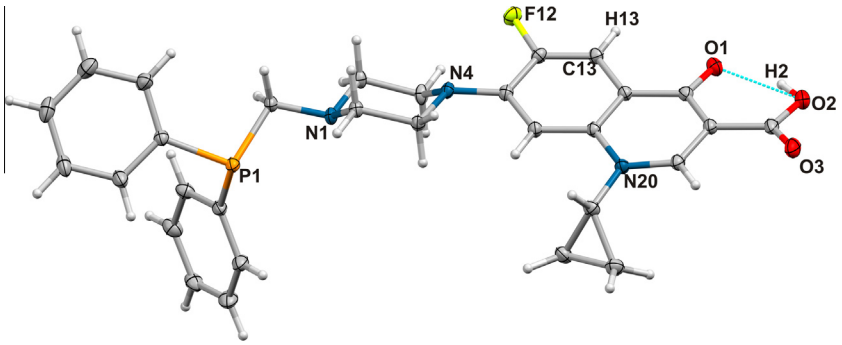

Fig. 3. X-ray structure of a disordered **PCp** molecule.

Table 1
Antibacterial activity (MIC [μg/ml]) of **HCp** and its derivatives.

	E. coli	*S. aureus*	*P. aeruginosa*
HCp	<0.1	0.5	0.5
PCp	0.2	0.5	1
OPCp	2	10	50

piperazine rings) of **PCp** and **HCp** hexahydrate [56] are similar (see Tables S2 and S3 in the Supplementary data) however there are some differences resulting from the variant character of these molecules. The molecule of **HCp** is zwitterionic while the molecule of **PCp** is not. This is confirmed by the values of the angle between the plane of the fused rings and the plane of the carboxylic group, which is equal to 28.33° in **HCp** and to only 15.30° in **PCp**. The lower value for **PCp** is most likely a result of a strong intramolecular hydrogen bond: O2–H2···O3 (d_{D-H} = 0.82 Å; d_{H-A} = 1.79 Å; d_{D-A} = 2.551(2) Å; α = 153.5°) in its molecule (Fig. 3).

3.2. Antibacterial activity

The antibacterial activity of **HCp**, **PCp** and **OPCp** was preliminarily determined *in vitro* against Gram-negative (*E. coli*, *P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria. MIC values are presented in Table 1. The collected data demonstrate that **PCp**, a phosphine derivative of ciprofloxacin, inhibits the growth of bacterial strains at relatively low concentrations. Moreover, it exhibits an activity against the tested strains comparable to the parent drug. An analogous trend was observed for another derivative of **HCp**: Cp–CH_2–C_2N_3H–$(CH_2)_3$–$C(PO(OH)_3)_2OH$ [22]. However, the activity of **OPCp** is much lower and the inhibition of microbial growth for all tested strains was observed at concentrations at least one order of magnitude higher than for other compounds.

3.3. Cell viability studies

Due to the low antimicrobial activity of **OPCp**, cytotoxicity was determined only for **HCp** and **PCp**. Both compounds were tested in the rabbit kidney cell (RK-13) and mesenchymal stem cells isolated from adipose tissue horse (HMSC-ad).

3.3.1. Cytotoxic assay according to EN 14675

A newly synthesized compound **PCp** and an unmodified antibiotic **HCp** were evaluated for their cytotoxic activities on the RK-13 cell line using the European standard EN 14675 [44]. A cytopathic effect was observed after 24 h of incubation with different concentrations of the tested compounds and it remained unchanged throughout the incubation time. **HCp** was cytotoxic in the concentrations range from 1 to $1{\cdot}10^{-6}$ mM, whereas **PCp** only in the range from 1 to $1{\cdot}10^{-1}$ mM (Fig. 4). This indicates an unexpected low-toxicity of the **PCp** derivative in comparison with the parent antibiotic.

3.3.2. Metabolic test – alamar blue cytotoxic assay

Cell viability assays were also performed on the HMSC-ad cell line. In contrast to the results obtained for the cell line RK-13, the activities of **HCp** and **PCp** against HMSC-ad were not so divergent (Fig. 5). However, we observed different concentration profiles of the cell response for both compounds. As can be seen from the smooth course of the plot, the cell metabolic activities at various concentrations of **HCp** do not differ so much at the full range of concentrations. In the case of **PCp**, we observed a distinct shape of the curve. This derivative does not affect the metabolic activity up to 2×10^{-3} mM, while at 1×10^{-1} and 2×10^{-1} mM it causes a significant decrease of the cell viability.

Cell imaging using three different techniques was performed to visualize changes in the cells caused by the tested compounds (Fig. 6). The phase contrast pictures (photos A 1–3) show that, after treatment with **HCp** (A 2), the monolayer of the cell culture thinned down with the necrosis effect and cell remnants are visible. In the case of **PCp** synthesized by us (A 3), normal cells morphology with limited cluster formation was observed. There were virtually no changes in comparison with the control cultures (A 1). These data are fully consistent with the images obtained using immunofluorescent staining (B1–3). HMSC-ad cells treated with **HCp** (B 2) are characterized by altered morphology, with cells detached or undergoing necrosis. After treatment with **PCp** (B 3) the cells did not undergo morphological changes and the only alternation was the formation of clusters. What is very important, their image was more similar to that of the control group (B 1), than to the image of the cells treated with **HCp**. Photos C 1–3 show the nuclei of HMSC-ad cells stained with DAPI. The nuclei were not damaged upon treatment with **HCp** (C 2) and **PCp** (C 3). For both compounds their pictures look like the control (C 1). For **PCp**, however, some clusters are formed with incidental occurrence of pyknotic nuclei [48].

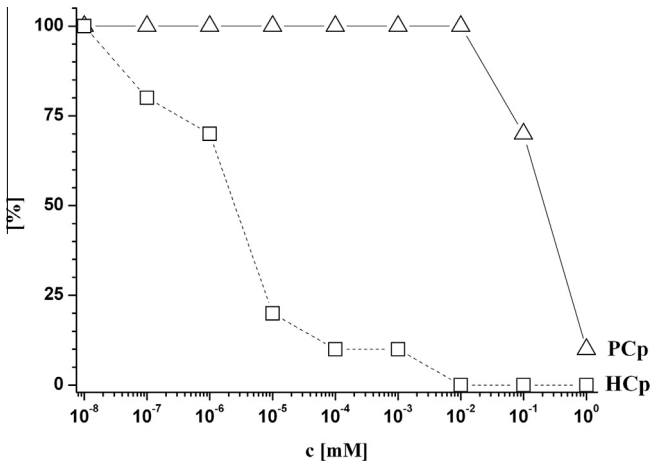

Fig. 4. Cytotoxic assay performed on RK-13 cell line for **HCp** (squares) and **PCp** (triangles). *X*-axis: the concentration of the compound [mM] in the logarithmic scale; *Y*-axis: the percent of the viable cells.

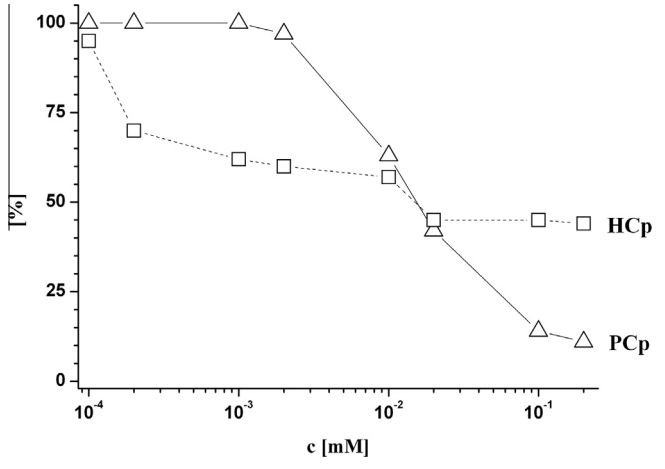

Fig. 5. Metabolic tests performed on the mesenchymal stem cells (HMCS-ad) isolated from horse adipose tissue for **HCp** (squares) and **PCp** (triangles). *X*-axis: the concentration of the compounds [mM] in the logarithmic scale; *Y*-axis: the percent of the metabolically active cells.

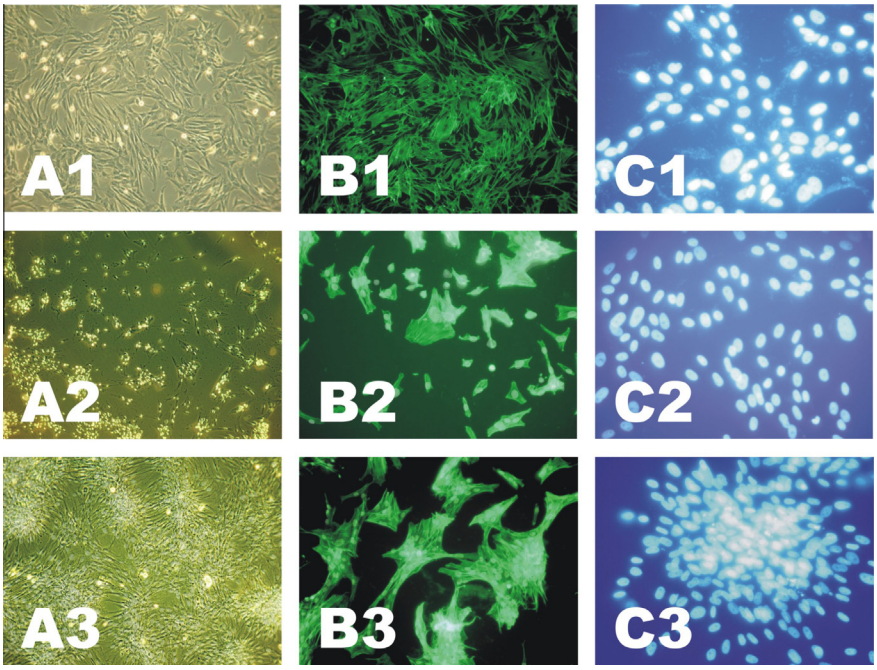

Fig. 6. Imaging of activity of HMSC-ad cell line treated with **PCp** (**3**) ($c = 2 \times 10^{-2}$ mM) and **HCp** (**2**) ($c = 2 \times 10^{-2}$ mM) together with the untreated cells (**1**). (A) Phase-contrast picture of monolayer HMSC-ad (Mag. 100×); **(B)** Immunofluorescent staining with phalloidine (Mag. 200×); (C) DAPI stained cells (Mag. 400×) (nuclei presented in white).

The results presented above suggest that the influence of a novel phosphine derivative of ciprofloxacin on the morphology of the investigated cells is considerably smaller than the impact of the unmodified antibiotic, despite their comparable cytotoxicities at 2×10^{-2} mM.

3.4. Interactions with pUC18 plasmid

The influence of **HCp**, **PCp** and **OPCp** on the DNA plasmid was determined for their different concentrations at the physiological pH. None of the compounds were able to induce single and/or double strand cleavage in a DNA molecule. The analysis of the electropherogram shows that regardless of the concentration, the tested compounds are unable to degrade the plasmid. This result clearly indicates that **PCp** and **OPCp** are not mutagenic, which can be important in the context of their potential medical applications.

4. Conclusions

In this paper we presented the spectroscopic profile and biological activity of the novel phosphine (**PCp**) and phosphine oxide (**OPCp**) derived from ciprofloxacin (**HCp**). A detailed analysis using the NMR, IR, MS and X-ray techniques attest to the identity and structure of the obtained compounds.

Biological activity experiments showed that **PCp** was able to inhibit the growth of microorganisms at the same level as **HCp**. Additional studies revealed that phosphine in higher concentrations was characterized by a lower *in vitro* toxicity against mammalian cells than the parent drug. It did not induce any morphological changes in cells after its action, which was definitely a positive result. In addition, **PCp** does not show any mutagenic activity similar to **HCp** what was confirmed by testing its interactions with DNA. This is relevant in terms of their potential medicinal usage.

The presented results confirm that the antimicrobial activity and *in vitro* cytotoxicity of **PCp** are similar to those of the parent drug. Therefore it makes this phosphine a smart choice for designing new biological agents based on metal ions. We are currently focusing on interactions of **PCp** with biomolecules and verifying its mechanism of action. We also work on syntheses and characteristics of potentially active [17] copper(I) complexes with this interesting molecule.

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Appendix A. Supplementary data

CCDC 922239 contains the supplementary crystallographic data for **PCp**. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.poly.2013.04.059.

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