"POST PRINT"

Adsorption of the cis-[Pt(NH$_3$)$_2$(P$_2$O$_7$)]$^{2-}$ (*phosphaplatin*) on hydroxyapatite nanocrystals as a smart way to selectively release activated cis-[Pt(NH$_3$)$_2$Cl$_2$] (*cisplatin*) in tumor tissues

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Abstract

The relevant adsorption of *cis*-$[Pt(NH_3)_2(P_2O_7)]^{2-}$ (*phosphaplatin*) on hydroxyapatite nanocrystals (nHAP) was observed and studied in water suspension. *Phosphaplatin* cytotoxicity, which is very low for HeLa, MCF-7 and HS-5 cell lines could be enhanced, reaching that of *cisplatin*, by interaction with solid nHAP. This effect stems from nHAP ability to catalyze the *phosphaplatin* hydrolysis, producing the same hydrolytic species responsible for *cisplatin* antitumor activity.

Keywords

Cisplatin; *phosphaplatin*; hydroxyapatite; nanocrystals; antitumor drug.

Introduction

After discovery of its antitumor activity, the *cis*-$[PtCl_2(NH_3)_2]$ (*cisplatin*) became one of the lead drugs for cancer therapy, notwithstanding the relevant disadvantages, as side effects and the possible intrinsic or acquired resistance of some tumors, Figure 1.[1-4] This is the reason why, after introduction of *cisplatin*, many other platinum compounds were studied and tested,[3,5-16] with consequent development of new generations of platinum drugs, characterized by substitution of only the two chlorido or both amino and chlorido ligands, with other ligands. *Carboplatin*, *cis*-diamine-1,1'-ciclobutandicarboxilate-*O,O'*-platinum(II), and *oxaliplatin*, *trans-L*-diamminocyclohexane-oxalato-platinum(II), are today the best seller 2nd and 3rd generation platinum drugs, Figure 1. In particular, *carboplatin* is extensively used for its milder side effects as a substitute of *cisplatin*, instead *oxaliplatin* is used for its ability to circumvent the resistance to *cisplatin* of some tumors, as in the case of colon rectal cancer.[2] Notwithstanding the improvements of antitumor therapy, today the research of innovative methods remains a very active research field, for the multiplicity of unsolved therapeutic problems.[2,4,17]

Bose et al. discovered a new class of platinum based antitumor active molecules with two *cis N*-donors and a chelate pyrophosphate ligand, generally named as *phosphaplatins*, Scheme 1.[18-21] The *in vivo* tolerability of *phosphaplatins* is generally similar to that of *carboplatin*.[18] The higher solubility and stability in water of *phosphaplatins*, with respect to *cisplatin*, contributed to improve the pharmacological interest toward these complexes.[18] In fact, they generally react slowly with thiols involved in the deactivation of platinum drugs.[22] Notwithstanding the presence of a coordinated chelate pyrophosphate (well known as a bad living group), these molecules generally show a good antitumor activity, sometimes even on human tumors resistant to *cisplatin*.[18-21] This may be the consequence of a different mechanism of action, with respect to *cisplatin*, producing apoptosis without involvement of genomic targets.[18-21]

To date, a lot of research activity is directed to the development of new strategies for the controlled delivery of drugs. This aspect is particularly critical in the case of antitumor drugs, where therapeutic and toxic doses are generally similar, causing severe side effects. In this context, the use of nanoparticles has recently been proposed for their peculiar drug releasing properties.[23-27] In particular, nanocrystalline hydroxyapatite (nHAP) has largely been studied as a nanocarrier for the localized delivery of drugs.[28-33] This stems from its high biocompatibility and biodegradability, being hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ one of the main inorganic constituents of bones and teeth.[34] An advantage in the use of nHAP as a drug vector is the possible gradual and controlled release of active species, which in this way could be maintained for a prolonged time. In this respect, by studying the adsorption of *cisplatin*, its hydrolytic species, and other antitumor drugs on nHAP, it has been recently demonstrated that phosphate and/or Cl^-, both important components of biological buffers, can induce an enhanced release of these drugs.[35-37]

Results and Discussion

Adsorption of phosphaplatin on nHAP. In the family of *phosphaplatins,* the species $Na_2\{cis$-$[Pt(NH_3)_2(P_2O_7)]\}$ (indicated in short as *phosphaplatin*), is characterized by a low cytotoxicity, thus preventing its use as antitumor drug. Interestingly, this complex bears both a chelating pyrophosphate ligand and a net negative charge (at neutral pH), useful for adsorption on hydroxyapatite, as previously demonstrated.[38]

The effective adsorption of *phosphaplatin* on nHAP, in water at neutral pH, was quantified by ICP-AES analysis of the residual amount of the complex in solutions, after treatment with nHAP. Indeed, an adsorption of about 72% was found when the starting *phosphaplatin* concentration and nHAP dispersion were of about 10 μM and 250 mg/L, respectively.

Cytotoxic activity of nHAP. MTT cytotoxicity tests on HeLa, MCF-7 and HS-5 cells, in the 24-72 h time interval, demonstrated that the sole plate shaped nanocrystalline hydroxyapatite (in short nHAP, see Figure 2) exhibits, depending concentration and type of treated cells, no or low cytotoxicity, Figure 3. This is in line with previously reported literature data.[39] Interestingly, HeLa cells, differently from the other considered MCF-7 and HS-5 cells, seem to be able to react, in some way, to a prolonged presence of the nHAP, by developing a sort of acquired resistance after about 48 h. On the other hand, a possible use of the nHAP in cell functions and a consequent very low cytotoxicity were already observed on HeLa cells.[40,41] This could explain why the HeLa cells survival, after an initial decrease to about 63% of the control, 24h from the initial treatment with 250 mg/L of nHAP, start to increase to about 81% and 137% of the control, after 48 and 72h, respectively.

Cytotoxic activity of phosphaplatins adsorbed on nHAP. MTT cytotoxicity tests were used to evaluate the effects of administration to cultured cells (of different origin and known for their differential sensitivity to platinum complexes) of preparations containing both *phosphaplatin* and nHAP dispersed in water.

The effect of a constant amount of *phosphaplatin* (10 μM) in the presence of a variable nHAP concentration (0-500 mg/L), was preliminarily tested on HeLa cells cultures. The cytotoxicity evaluated, for this fixed 10 μM *phosphaplatin* concentration, gradually increases with the nHAP increase in the 0-100 mg/L range interval, reaching a maximum for a nHAP concentration between 100 and 250 mg/L, Figure 4. For higher nHAP concentrations (between 250 and 500 mg/L) the observed *phosphaplatin* cytotoxicity gradually decreases. This effect appears essentially due to the observed compensative stimulation of cell growth, although a possible concomitant competitive adsorption of cytotoxic species in the excess nHAP could not be excluded.

It should be noted that the optimum *phosphaplatin*/nHAP ratio is equal to 10 μM/250 mg/L. In fact, in this case the cytotoxicity increases faster with time, with respect to other *phosphaplatin*/nHAP ratios, being pronounced even after 24h and 48h from treatment. This confirms the rational of adopting the 10 μM/250 mg/L ratio in the here reported cytotoxicity evaluations of water dispersed *phosphaplatin*/nHAP mixtures. Indeed, we could interpret these findings considering that nHAP, characterized by low cytotoxicity on HeLa cells,[41] may be of some use in cell functions [40] resulting in an overall HeLa cells growth stimulation, see Figure 3. On the other hand, the *phosphaplatin*, which exhibit a very low cytotoxicity on its own, can be activated by interaction with nHAP. The combined use of variable *phosphaplatin*/nHAP mixtures produces therefore a sort of hormetic effect, as clearly observed in Figure 4.

The cytotoxicity level of *phosphaplatin*, alone or adsorbed on nHAP, was compared with that of *cisplatin*. The analysis was carried out on three different cell lines, HeLa (*cisplatin*-sensitive human cervical cancer cells) MCF-7 (*cisplatin*-resistant human breast cancer cells) and HS-5 (immortalized human bone marrowstromal cells derived from a normal donor). The results of MTT assays at the different concentrations of the platinum drugs (0-100 μM) and at different time points (24-72 h time interval) are comprehensively reported in Figure 5.

The results of the MTT tests performed on the *cisplatin* sensitive HeLa cell line in the 24-72 h time interval show that *cisplatin* is very cytotoxic ($IC_{50} \approx 1$ μM at 72 h), if compared with *phosphaplatin* ($IC_{50} \approx 10\text{-}100$ μM at 72 h). Moreover the cytotoxicity of *cisplatin* is not affected by the presence of nHAP whereas that of *phosphaplatin* strongly increases in the presence of nHAP, approximately reaching that of *cisplatin* ($IC_{50} \approx 1$ μM at 72 h), Figure 5A.

The time-course analysis at 10 μM (Figure 6A) on HeLa cells clearly shows that *cisplatin*, as reported, [10] exhibits a strong cytotoxicity ($IT_{50} \approx 24$ h), even at short incubation times whereas *phosphaplatin* has a mild cytotoxicity even at longer incubation times (72 h), never reaching the IT_{50} level. Interestingly, when nHAP is added, the cytotoxicity of *cisplatin* is only slightly reduced while that of *phosphaplatin* is strongly increased ($IT_{50} \approx 24$ h). In other words, when nHAP is added, both *cisplatin* and *phosphaplatin* show nearly the same cytotoxicity.

The results of the MTT tests performed on the MCF-7 *cisplatin* resistant cell line (Figure 5B) show that *cisplatin* and *phosphaplatin* both exhibit low cytotoxicity ($IC_{50} \approx 10$ μM and $IC_{50} > 100$ μM at 72 h, for *cisplatin* and *phosphaplatin*, respectively). The cytotoxicity of *cisplatin* is not affected by hydroxyapatite, whereas that of *phosphaplatin* is increased by the presence of nHAP, reaching eventually nearly that of *cisplatin* ($IC_{50} \approx 10$ μM at 72 h, for both *cisplatin* and *phosphaplatin*). This confirms that also for MCF-7, as already observed for HeLa cells, both *cisplatin* and *phosphaplatin* show the same cytotoxicity in the presence of nHAP, Figure 5B.

The time-course analysis at 10 μM (Figure 6B) clearly shows that in the absence of nHAP *cisplatin* is slightly more cytotoxic than *phosphaplatin* on MCF-7 cells although it reaches the IC_{50} cytotoxicity level only at long incubation time ($IT_{50} \approx 72$ h). In the presence of nHAP the *phosphaplatin* cytotoxicity increases reaching in the 24-72 h time interval that of *cisplatin*, which remains unaffected by nHAP addition.

The results of the MTT test performed on the HS-5 cell line (Figure 5C) show that both *cisplatin* and *phosphaplatin* are cytotoxic ($IC_{50} \approx 1$ and $10\ \mu M$, respectively). Again the cytotoxicity of *cisplatin* is not affected by the presence of nHAP, instead that of *phosphaplatin* increases reaching that of *cisplatin* in the 24-72 h time interval ($IC_{50} < 1\ \mu M$).

The time-course analysis at $10\ \mu M$ (Figure 6C) clearly shows that *cisplatin* is more cytotoxic than *phosphaplatin* on HS-5 cells and that both *cisplatin* and *phosphaplatin* reach the IC_{50} cytotoxicity level, for long incubation time ($IT_{50} \approx 48$ and 72 h, respectively). When nHAP is added the cytotoxicity of *cisplatin* remains unchanged while *phosphaplatin* reaches the *cisplatin* IC_{50} in the 24-72 h time interval.

Altogether, our results confirm that *phosphaplatin* is generally less cytotoxic than *cisplatin* for all the tested cell lines, in the 24-72 h time interval and show that addition of nHAP to the cell cultures has negligible effects on the *cisplatin* cytotoxicity [35] while induces a 10-100 times increase of *phosphaplatin* cytotoxicity, reaching that of *cisplatin*. In all MTT tests the modulation of *cisplatin* cytotoxicity, due to the presence of nHAP, resulted negligible, consistently with literature data.[35] Differently, the cytotoxicity of *phosphaplatin*, for all tested cell lines, can be strongly increased by the presence of nHAP, reaching that of *cisplatin*, in the 24-72 h time interval. This means that a joint administration of *phosphaplatin* and hydroxyapatite is able to induce a 10-100 times increase of cytotoxicity which may therefore reach that of *cisplatin* (Figures 5A-C and 6A-C).

Although the sole nHAP seems able to enhance cell growth, the increased cytotoxicity of *phosphaplatin,* reaching that of *cisplatin* in the presence of nHAP, suggests an enhanced hydrolysis (and therefore enhanced toxicity) of *phosphaplatin* in the presence of nHAP. The combination of the two contrasting effects occuring at the same time results in the observed hormetic trend.

nHAP as activating agent for phosphaplatins. In an effort to rationalize the previous findings, we comparatively followed the evolution of the ^{195}Pt NMR signals of *cisplatin* and *phosphaplatin* in neutral water solutions, both alone and in the presence of nHAP.

In neutral water the *cisplatin* solution slowly hydrolyzes giving both the *mono-* and *diaquo* species *cis*-[Pt(NH$_3$)$_2$Cl(H$_2$O)]$^+$ and *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$, respectively, while the *phosphaplatin* does not undergo hydrolysis of the chelate pyrophosphate ligand, within a few days, as previously reported by Bose et al..[18-21] The addition of a nHAP suspension to both the *cisplatin* and *phosphaplatin* solutions does not change appreciably the *cisplatin* hydrolytic rate, while strongly speeds up the *phosphaplatin* hydrolysis, see Figure 7. The displacement of the platinum metal core from the pyrophosphate ligand is probably caused by the entrance of chlorido ions into the platinum coordination sphere, as previously shown for similar complexes.[42,43] This substitution is a common trend observed in this kind of inorganic conjugates and is probably due to a synergistic effect between calcium ions present on the nHAP surface, which anchor the pyrophosphate ligand, and the chloride ions present in the buffer used for the MTT cytotoxicity tests.[43] It is noteworthy that, in the presence of nHAP, both platinum complexes hydrolyze showing the ^{195}Pt NMR signals of the mono- and di-aquo hydrolytic species *cis*-[Pt(NH$_3$)$_2$Cl(H$_2$O)]$^+$ and *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$, as shown by ^{1}H{^{195}Pt} HSQC NMR spectrum reported in Figure 7. Since *phosphaplatin*, in the presence of nHAP, can produce the same *cisplatin* hydrolytic species, corresponding to its most active metabolites, nHAP can therefore render the low cytotoxic *phosphaplatin* as cytotoxic as *cisplatin*, see Scheme 1. The hydrolysis reaction of *phosphaplatin* in the presence of nHAP was also followed by ^{195}Pt NMR spectroscopy determining a half-life value for the *phosphaplatin* hydrolysis of about 8h in the adopted experimental conditions, Figure 8.

Previous results suggest the possible use of a novel therapeutic approach based on nHAP acting as a trigger to activate *phosphaplatin*. Several *phosphaplatins* have been tested in various *in vivo* preclinical studies [21] moreover recent studies suggested the possible use of nHAP water suspensions for a direct injection in blood vessels.[44] This opens the possibility to administer various kinds of drugs and/or diagnostic agents adsorbed on nHAP. It is well known that blood vessels are more porous in tumor tissues, producing an easy local uptake of macromolecules, polymers and

nanoparticles.[25-27] Accordingly, the nHAP (administered as a suspension) could be able to accumulate preferentially in tumor tissues. Further general administration of the low toxic *phosphaplatin*, could thus result in a specific activation of the latter only where nHAP particles are localized. The selective release in tumor tissues of the same hydrolytic species normally produced by *cisplatin* but originated from the much less toxic *phosphaplatin* used as prodrug could be the very interesting novelty of this approach. On the other hand, the selective cytotoxicity toward those tissues able to selectively absorb hydroxyapatite nanoparticles, could produce a lowering of the typical *cisplatin* related systemic side effects.

Conclusion

The here reported adsorption tests of *phosphaplatin* on nHAP confirm the importance of a negative molecular charge and the relevance of the presence of phosphate groups, as key factors in the adsorption phenomena on hydroxyapatite matrices.[38] Although *phosphaplatin* is much less cytotoxic with respect to *cisplatin*, our studies demonstrate that its cytotoxicity can be strongly enhanced by simple absorption on solid nHAP matrices. This occurs due to the release from *phosphaplatin* of the same antitumor active hydrolytic species produced by *cisplatin*. The high superficial area of the nHAP indeed enhances the hydrolytic effects. The cytotoxicity of *phosphaplatin* can therefore reach that of *cisplatin*, in the 24-72 h time interval, if nHAP suspensions are administered together with *phosphaplatin*, as observed in HeLa, MCF-7 and HS-5 treated cell cultures. Overall, our results suggest the possible use of nHAP as a trigger to activate *phosphaplatin* generally characterized by a much lower cytotoxicity with respect to *cisplatin*. In this respect, administration of nHAP before or together with *phosphaplatin* could represent a smart system to activate, by selective adsorption in tumor tissues, this and similar platinum complexes, to be used as antitumor prodrugs.

Experimental Section

Synthesis of complexes. All solvents and reagents, except otherwise stated, were purchased from Aldrich Chemical Company and used as received. *Cisplatin*, *cis*-[PtCl$_2$(NH$_3$)$_2$],[45] and *phosphaplatin*, Na$_2${*cis*-[Pt(NH$_3$)$_2$(P$_2$O$_7$)]},[18-21] were prepared from K$_2$[PtCl$_4$], as previously described.

Synthesis of hydroxyapatite nanocrystals. Plate shaped hydroxyapatite nanocrystals, were synthesized according to a previously published method,[46] with some modifications, according to a recent procedure, as reported by some of us.[47] The TEM image of the obtained synthetic hydroxyapatite nanocrystals (nHAP), with plate-shaped morphology is reported in Figure 2.

NMR spectroscopy. The NMR spectra were recorded on a Bruker Avance DPX 600 or 400, at 300 K, using H$_2$O/D$_2$O = 90/10 mixtures. The spectra were referenced to the residual H$_2$O signal (^{1}H) and H$_2$PtCl$_6$ (^{195}Pt) used as internal and external standard, respectively. Minimum amounts of HCl or NaOH were added to the solutions to adjust the pH to the desired values. LC-MS analysis for *phosphaplatin* dissolved in H$_2$O (pH = 7) was performed on Agilent Technologies LC-MS ion Trap-VL_01002. Negative ion mode (electrospray) was used at a drying gas temperature of 350 °C. The sample was injected by infusion.

Analysis by ICP-AES. For the determination of platinum concentration, each sample was previously heat treated with 0.5 mL of 67% ultrapure nitric acid and 0.2 mL of hydrogen peroxide, to obtain a clear solution. The samples were then diluted to a final volume of 5 mL, in order to obtain a suitable concentration for the acid used in the mineralization process and avoid damage to the system. Each sample was filtered before analysis (0.45 μm) to prevent the entry of any remaining suspension in the measuring instrument. The platinum concentration in the analyzed samples was determined by Thermo iCAP 6000 spectrometer. The spectrophotometer was calibrated with a calibration line consisting of four points each corresponding to a concentration of the element: 1 μg/L, 10 μg/L, 100 μg/L, and 1000 μg/L.

Cell cultures. HeLa (human epithelial carcinoma) and MCF-7 (human breast adenocarcinoma) cells were maintained in DMEM (Gibco - Life Technologies) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin). HS-5 (human bone marrow stromal) cells were maintained in RPMI 1640 (Gibco - Life Technologies) containing 10% FBS and antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin). All cell lines were cultured at 37 °C in a humidified incubator, 5% CO_2, and the culture medium was changed every 2 days.

MTT assay. The cytotoxicity of nHAP, platinum complexes and nHAP/platinum complexes mixtures were measured by MTT assays [Sigma Aldrich: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Briefly, cells were seeded in 96-well plates (1×10^4 cells/well) and treated with the different molecules, alone or in combination, for the indicated concentrations and times. After treatment, MTT solution (5 mg/mL in PBS) was added to reach a final concentration of 0.5 mg/mL and plates were incubated at 37 °C for 3 h. Then 100 µL of 2-propanol/HCl 4N was added to the wells. The solubilized formazan was measured at 595 nm using EnVision® Multilabel Plate Reader (PerkinElmer). Each treatment was assayed in triplicate and data, reported as % of control, represent the mean ($\pm$ SEM) of three independent experiments.

Statistical analysis. Significant differences ($p < 0.05$) were assessed by *t*-test for unpaired samples or two-way analysis of variance (ANOVA), as appropriate.

Identification of the phosphaplatin hydrolitic species, formed in the presence of nHAP. We were able to identify, by $^1H\{^{195}Pt\}$ HSQC NMR spectroscopy, the hydrolytic species formed as a consequence of *phosphaplatin* ($\approx$ 1.5 mM) hydrolysis, in the presence of KCl ($\approx$ 3 mM) and nHAP (250 mg/L), dispersed in H_2O/D_2O = 90/10, at pH $\approx$ 5.5. After stirring for three days, a 600 µL aliquot of this mother solution, were taken and inserted into a NMR tube. The minimum amount of HNO_3 was then added to dissolve the residual nHAP solid phase, so inducing release of adsorbed species (final pH $\approx$ 3).

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Determination of half-life for the hydrolysis of phosphaplatin in the presence of nHAP. A mother solution of $Na_2[Pt(NH_3)_2(P_2O_7)]$ (*phosphaplatin*) ($\approx$ 10 mM), KCl ($\approx$ 100 mM), and nHAP ($\approx$ 10 g/L) in H_2O/D_2O = 90/10, at pH $\approx$ 6.5 was putted under stirring at 300 K. Aliquots of this mother solution were taken at regular time intervals (hours), to collect the ^{195}Pt NMR spectra. In this way we could follow the reduction of the *phosphaplatin* ^{195}Pt NMR signal intensity, due to hydrolysis, see Figure 8. To generate a reference signal we inserted in the NMR tube a capillary, filled with a solution containing K_2PtCl_4 ($\approx$ 40 mM), KCl ($\approx$ 400 mM) and HCl (the amount necessary to reach a pH $\approx$ 2), in H_2O/D_2O = 90/10. Immediately before acquisition of the ^{195}Pt NMR, the minimum amount of HCl was added to the NMR tube, in order to dissolve the solid nHAP suspension and induce release of adsorbed species (final pH $\approx$ 3) without decomposition of the residual phosphaplatin. These data permitted calculation of a half-life of about 8 h for *phosphaplatin* hydrolysis in the presence of nHAP at a $[Cl^-] \approx$ 100 mM (similar to that occurring in blood plasma, where the *phosphaplatins* are generally known to be stable).[48]

Keywords: *cisplatin*, *phosphaplatin*, platinum drug, antitumor drug, hydroxyapatite, nanoparticles.

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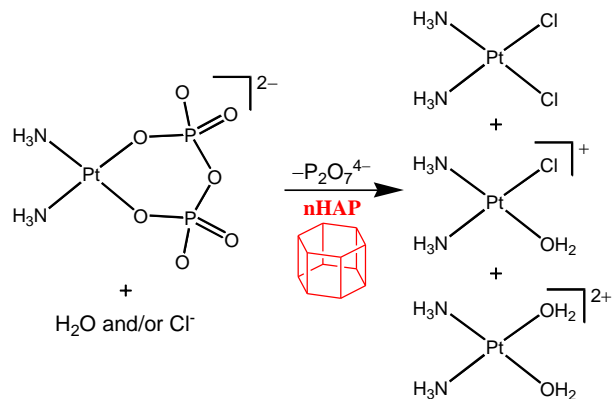
Scheme 1. Hydrolysis of *cis*-[Pt(NH$_3$)$_2$(P$_2$O$_7$)]$^{2-}$ (*phosphaplatin*) catalyzed by nHAP in water solution, producing *cis*-[PtCl$_2$(NH$_3$)$_2$] (*cisplatin*) and related hydrolitic *mono* and *diaquo*-species, *cis*-[Pt(NH$_3$)$_2$Cl(H$_2$O)]$^+$ and *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$, respectively.
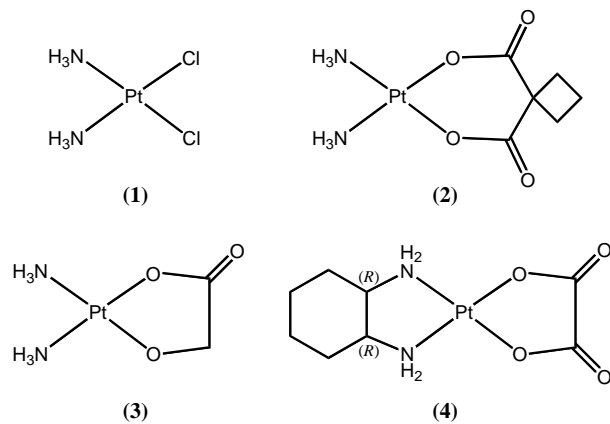
Figure 1. Comparison between the structures of the clinically used *cisplatin* (**1**), *carboplatin* (**2**), *nedaplatin* (**3**) and *oxaliplatin* (**4**) antitumor drugs.
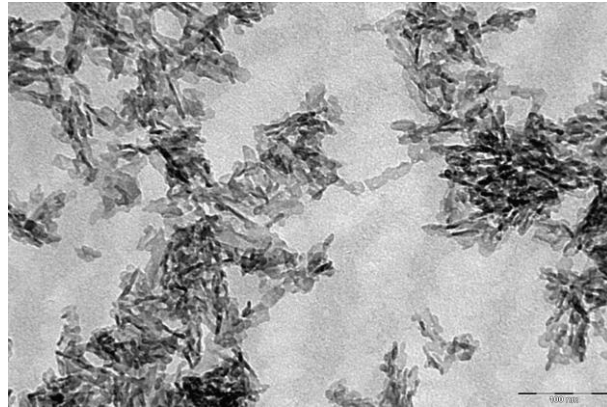
Figure 2. TEM image of synthetic hydroxyapatite nanocrystals (nHAP) with plate-shaped morphology (scale bar 100 nm).
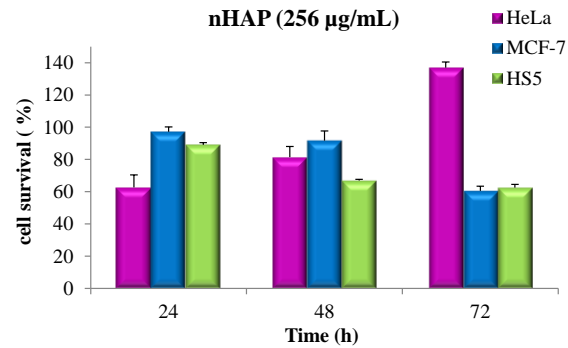
Figure 3. Cytotoxicity of here used plate shaped nanocrystalline hydroxyapatite (nHAP) at the 250 mg/L concentration, toward (A) cancerous *cisplatin*-sensitive HeLa (human epithelial carcinoma), (B) *cisplatin*-resistant MCF-7 (human breast adenocarcinoma) and (C) non cancerous bone tissue HS-5 (human bone marrow stromal) cells, after incubation times of 24, 48 and 72 h. Viable cell number was determined by MTT assay; data represent the mean ± SD for three different experiments, each with eight replicates, and are presented as a percentage of the control.
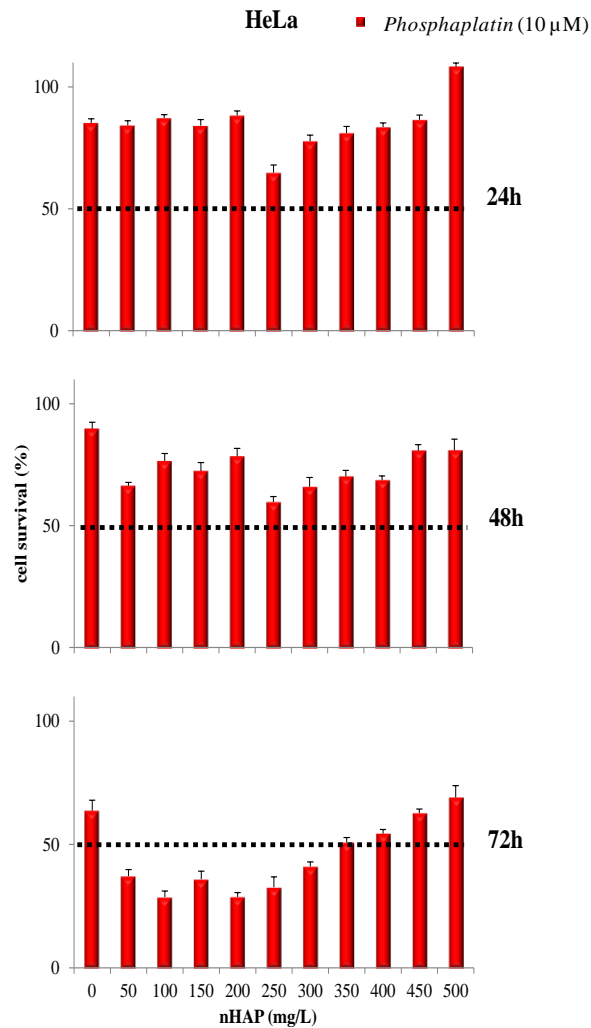
Figure 4. Cytotoxicity of *phosphaplatin*, $Na_2\{cis\text{-}[Pt(NH_3)_2(P_2O_7)]\}$, at 10 μM concentration, in the presence of plate shaped nanocrystalline hydroxyapatite (nHAP) in the 0-500 mg/L concentration range, toward cancerous *cisplatin*-sensitive HeLa (human epithelial carcinoma) cells, after incubation times of 72 h. Viable cell number was determined by MTT assay; data represent the mean ± SD for three different experiments, each with eight replicates, and are presented as a percentage of the control.
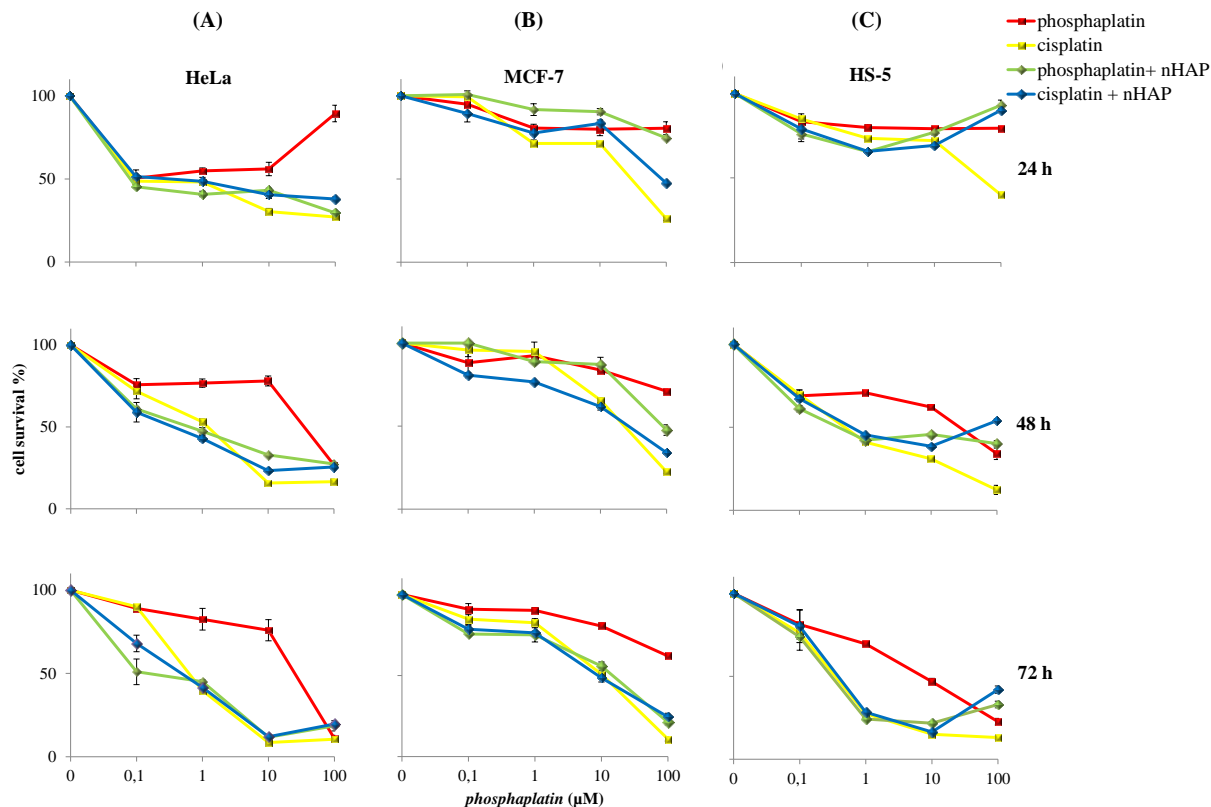
Figure 5. Cytotoxicity of $Na_2\{cis\text{-}[Pt(NH_3)_2(P_2O_7)]\}$ (*phosphaplatin*) and $cis\text{-}[PtCl_2(NH_3)_2]$ (*cisplatin*) in the 0-100 μM concentration range, toward (A) cancerous *cisplatin*-sensitive HeLa (human epithelial carcinoma), (B) *cisplatin*-resistant MCF-7 (human breast adenocarcinoma) and (C) non cancerous bone tissue HS-5 (human bone marrow stromal) cells, compared with the same complexes in the presence of nHAP (Pt/nHAP = 40 mmol/g), after incubation times of 24, 48 and 72 h. In each experiment, the dotted lines indicate the 50% cell survival level. Intersections of dotted lines with continuous lines give the corresponding IC_{50} values after treatment for 24-72 h. Viable cell number was determined by MTT assay; data represent the mean ± SD for three different experiments, each with eight replicates, and are presented as a percentage of the control.
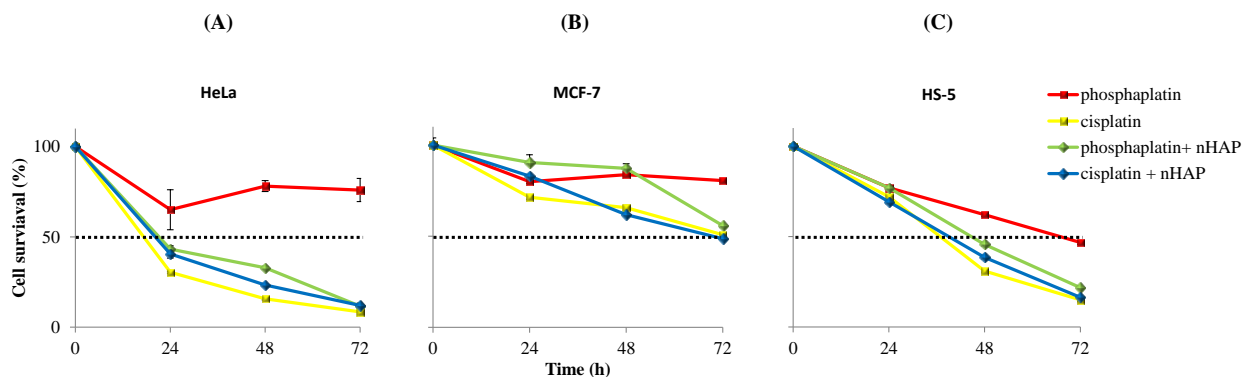
Figure 6. Time-course of the cytotoxic effects of $Na_2\{cis$-$[Pt(NH_3)_2(P_2O_7)]\}$ (*phosphaplatin*) and cis-$[PtCl_2(NH_3)_2]$ (*cisplatin*) at 10 mM toward (A) cancerous *cisplatin*-sensitive HeLa (human epithelial carcinoma), (B) *cisplatin*-resistant MCF-7 (human breast adenocarcinoma) and (C) non cancerous bone tissue HS-5 (human bone marrow stromal) cells, compared with the same complexes in the presence of nHAP (Pt/nHAP = 40 mmol/g), after incubation times of 24, 48, and 72 h. Dotted lines indicate the 50% cell survival level. Intersections of dotted lines with continuous lines give the corresponding IT_{50} values at 10 μM treatment. Viable cell number was determined by MTT assay; data represent the mean ± SD for three different experiments, each with eight replicates, and are presented as a percentage of the control.
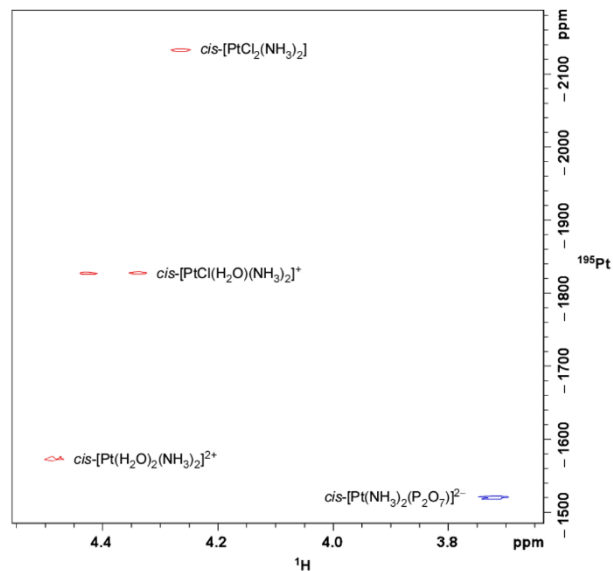
Figure 7. ^{1}H{^{195}Pt} HSQC NMR spectrum, collected at 300K, after three days, for a mother solution of Na$_2$\{*cis*-[Pt(NH$_3$)$_2$(P$_2$O$_7$)]\} (*phosphaplatin*) ($\approx$ 1 mM) and NaCl ($\approx$ 3 mM), dissolved in H$_2$O/D$_2$O = 90/10, at pH $\approx$ 5.5 (blue cross peak). The same mother solution of *phosphaplatin* in the presence of nHAP (250 mg/L) shows, after three days, the formation of *cis*-[PtCl$_2$(NH$_3$)$_2$] (*cisplatin*) and related hydrolytic species, *cis*-[Pt(NH$_3$)$_2$Cl(H$_2$O)]$^+$ and *cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ (red cross peaks). The solid nHAP suspension was dissolved, before acquisition, by addition to the NMR tube of the minimum amount of HNO$_3$.
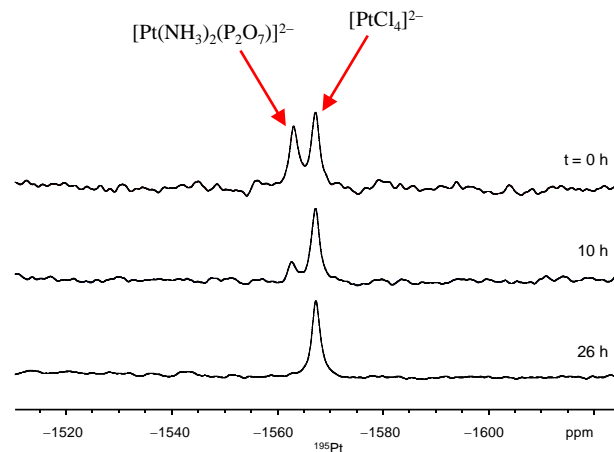
Figure 8. Kinetic of the hydrolysis of $Na_2[Pt(NH_3)_2(P_2O_7)]$ (*phosphaplatin*) (≈ 10 mM) in a mother solution containing KCl (≈ 100 mM) and nHAP (≈ 10 g/L) in $H_2O/D_2O = 90/10$, at pH $\approx$ 6.5. The [195]Pt NMR spectra were taken at different time intervals (hours, as indicated on each spectrum). The hydrolysis of *phosphaplatin* produces the observed progressive reduction of its [195]Pt NMR signal with respect to the $[PtCl_4]^{2-}$ reference signal (see Experimental). Immediately before the [195]Pt NMR acquisition the minimum amount of HCl was added to the NMR tube, in order to dissolve the solid nHAP suspension. These data permitted the calculation of a half-life of about 8 h.