DOI: https://doi.org/10.1002/anie.202114250

ACCEPTED VERSION

^{19}F NMR Allows to Investigate the Fate of Platinum(IV) Prodrugs in Physiological Conditions

Siming Yuan,[a] Yang Zhu,[a] Yi Dai,[a] Yu Wang,[a] Duo Jin,[a] Manman Liu,[a] Liqin Tang,*[a] Fabio Arnesano,[b] Giovanni Natile*[b] and Yangzhong Liu*[a]

[a] Dr. S. Yuan, Dr. Y. Zhu, Dr. Y. Dai, Y. Wang, D. Jin, M. Liu, Prof. L. Tang, Prof. Y. Liu
Department of Pharmacy, the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine; Department of Chemistry, University of Science and Technology of China, Hefei, Anhui, China
E-mail: liuyz@ustc.edu.cn, tangliqin@ustc.edu.cn

[b] Prof. F. Arnesano, Prof. G. Natile
Dipartimento di Chimica, Università di Bari "A. Moro", via E. Orabona 4, 70125 Bari, Italy
E-mail: giovanni.natile@uniba.it

Supporting information for this article is given via a link at the end of the document.

Abstract: Pt(IV) prodrugs can overcome resistance and side effects of conventional Pt(II) anticancer therapies. By ^{19}F-labeling of a Pt(IV) prodrug (Pt-FBA, FBA = *p*-fluorobenzoate), the activation under physiological conditions could be investigated. It is found that, unlike single-electron reductants, multi-electron agents can efficiently promote the two electrons reduction of Pt(IV) to Pt(II). Moreover, the activation of Pt-FBA in cell lysate is highly dependent upon the type of cancer cells. When administered to *E. coli*, Pt-FBA is reduced intracellularly and free FBA can shuttle out of the cell. Interestingly, the reduction rate greatly increases by inducing metallothionein overexpression and is lowered by addition of Zn(II) ions. Finally, when injected into mice, Pt-FBA undergoes fast reduction in the bloodstream accompanied by metabolic degradation of FBA; nevertheless, unreduced Pt-FBA can accumulate to detectable levels in liver and kidneys. The proposed ^{19}F NMR approach has the advantage of avoiding the interference of all background signals.

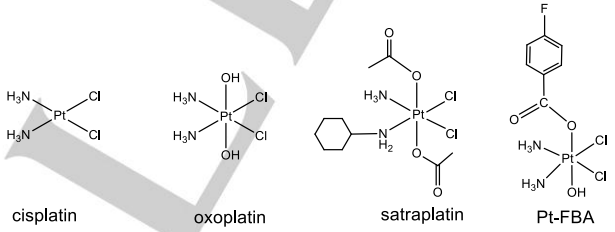
Introduction

Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, Scheme 1) is a widely used anticancer drug for the treatment of a wide variety of human malignancies. Prospective alternatives to cisplatin in cancer treatment are the more inert, six-coordinated Pt(IV) prodrugs (*cis,cis,trans*-$[PtCl_2(NH_3)_2(X)(Y)]$ complexes) which could overcome the drawbacks of conventional Pt(II) drugs stemming from non-specific reactions with other physiological substrates.[1] A first example was satraplatin (Scheme 1), an orally available Pt(IV) drug that has entered phase III clinical trial.[2] Moreover, the two additional axial ligands of Pt(IV) prodrugs provide the possibility to introduce tumor targeting and/or additional pharmacologically active entities that could further enhance drug efficacy. Therefore, a large variety of Pt(IV) agents have been synthesized and proven to be endowed with prominent antitumor efficacy.[1a, 1b, 3] However, Pt(IV) prodrugs must be reduced to active Pt(II) counterparts just prior to interacting with the target(s),[4] so that the simultaneously released axial ligands can perform additional functions at the tumor site and achieve synergistic effects. Although it has been proposed that glutathione and ascorbic acid may be the cellular reducing agents for the activation of Pt(IV) prodrugs, it is still a challenge to elucidate the reduction process occurring under physiological conditions.

Some remarkable attempts have been made in recent years. For instance, X-ray fluorescence microtomography and X-ray absorption near edge spectroscopy (XANES) have been applied to analyze subcellular localization and reduction of Pt(IV) prodrugs,[5] however these techniques cannot be used on living cells. By coordinating a fluorescent ligand in the axial position of the Pt(IV) agent, it was possible to monitor the reduction process by fluorescence imaging (the release of the fluorophore during reduction of the Pt(IV) prodrug is typically accompanied by a change in fluorescence).[6] Although this method offers high sensitivity and good resolution, the fluorophore molecule, typically bulky and hydrophobic, can affect the cellular uptake and reduction process. More recently, fluorescent probes have been designed for selective reaction with the resulting Pt(II) species, so that the reduction of Pt(IV) complexes can be detected by the fluorescence or the FRET response.[7] This procedure, however, can only analyze the reduction product since the Pt(IV) prodrug is not detectable. Therefore, it is still challenging to design a probe capable of simultaneously monitoring the Pt(IV) precursor and the Pt(II) reduction product under physiological conditions.

NMR is a non-invasive method that can provide atomic-level resolution of biomolecules and give information on dynamic biochemical processes that occur under physiological conditions.[8] However, the crowd of biomolecules in a cell can prevent the direct observation of the molecule under investigation with classical NMR methods. The ^{15}N or ^{13}C isotope labeling strategy is generally used for the in-cell NMR, as it allows detection of the molecule of interest by ^{1}H-^{15}N or ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectroscopy. However, the crowded cellular environment and the occurrence of molecular interactions often preclude the detection of relevant NMR signals. Another possibility is to use ^{19}F-labeled molecules. The ^{19}F isotope is NMR sensitive with a large chemical shift range, and its chemical shift is sensitive to molecular and conformational changes occurring in the labeled molecule.[9] ^{19}F NMR has been employed to investigate the pharmacokinetics and pharmacodynamic of 5-fuorouracil in patients.[10] In addition, incorporation of a fluorine atom usually causes minimal effects on the structure and dynamics of a molecule.[11] Finally, there is practically no background ^{19}F signal in biological systems, therefore ^{19}F-labeling can be a very effective strategy for in-cell NMR detection.[9, 12]

In this work, a F-labeled prodrug of cisplatin was prepared by coordinating *p*-fluorobenzoic acid (FBA) in an axial position of a Pt(IV) complex, thus generating the *cis,cis,trans*-$[PtCl_2(NH_3)_2(OH)(FBA)]$ complex (Pt-FBA, Scheme 1). The reduction of Pt-FBA, while generating cisplatin, is accompanied by the release of a free FBA ligand. Therefore, the ^{19}F chemical shift of FBA provides a convenient probe for monitoring the pre- and post-reduction of Pt-FBA in biological systems of increasing complexity.



Scheme 1. Chemical structures of cisplatin, oxoplatin, satraplatin, and Pt-FBA.

Results and Discussion

Synthesis and characterization of the ^{19}F-labeled Pt(IV) Complex. The desired Pt(IV) compound (Pt-FBA, Scheme 1) was prepared following a literature method.[13] Briefly, after oxidation of cisplatin to the di-hydroxido intermediate *cis,cis,trans*-$[PtCl_2(NH_3)_2(OH)_2]$ (oxoplatin, Scheme 1) by H_2O_2, one hydroxido ligand of oxoplatin was substituted by FBA using the 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy-succinimide (EDC/NHS) method. Pt-FBA was purified by ether/acetone precipitation and fully characterized by high-performance liquid chromatography (HPLC), electrospray mass spectrometry (ESI-MS), and ^{1}H and ^{19}F NMR spectroscopy (Figures S1 and S2).

The stability of Pt-FBA in solution was assessed by ^{19}F NMR spectroscopy and HPLC analysis. The signal of Pt-FBA falling at −107.7 ppm remained unchanged for 24 h in phosphate-buffered saline (PBS, pH 7.4) at 37 °C, with no sign of reduction product (free FBA) (Figure S3A). This result was confirmed by HPLC analysis (Figure S3B) also proving the stability of Pt-FBA in PBS. The stability of Pt-FBA was also tested in fetal bovine serum (FBS) and in Dulbecco's modified eagle medium (DMEM) containing 10% FBS. In both media, ^{19}F NMR spectroscopy showed that no free FBA is released after incubation for 24 h at 37 °C,

while a minor peak slowly grows close to the signal of Pt-FBA (Figure S4). The chemical shift value of the new peak suggests that FBA is still bound to Pt(IV) and that, most likely, it originates from an interaction of the Pt-FBA complex with FBS. These results indicate that the ^{19}F chemical shift remains nearly constant in Pt(IV) species and therefore it is suitable for monitoring the reduction process of Pt(IV) complexes. Moreover, to test the sensitivity of 1D ^{19}F NMR with respect to the classical 2D ^{1}H-^{15}N HSQC NMR, an equimolar mixture of ^{15}N-labeled cisplatin (containing two $^{15}NH_3$) and Pt-FBA (containing a single F) was dissolved in PBS or in cell lysate, and 1D ^{19}F and 2D ^{1}H-^{15}N HSQC spectra were recorded using the same acquisition time. In PBS the signal-to-noise ratio (SNR) calculated for three different concentrations (0.5, 0.1, and 0.02 mM), was 188.08, 49.11, and 11.86 for 1D ^{19}F NMR, and 77.83, 23.72, and 8.98 for 2D ^{1}H-^{15}N HSQC NMR, respectively. The signals of both Pt-FBA and cisplatin at 20 μM concentration could be detected in 10 min (Figure S5). Interestingly, in the cell lysate, the signal of 20 μM Pt-FBA could be clearly detected (SNR = 5.48), while the signal of 100 μM cisplatin was about the noise level (Figure S6). The sensitivity of ^{19}F NMR was also tested at a shorter acquisition time. The signal of 20 μM Pt-FBA could be clearly detected with an acquisition time of 2.58 min in both PBS buffer and cell lysate (Figure S7). Altogether, these results indicate that the sensitivity of the two types of measurements are comparable and that it may be advantageous to use ^{19}F NMR in the case of complex physiological matrices.

Reduction of Pt-FBA *in vitro*. Two well-known cellular reducing agents, glutathione (GSH) and ascorbic acid (AsA), which exist in high concentration in cells (1-10 mM for GSH and 1-5 mM for AsA), were initially used to investigate the reduction of Pt-FBA.[14] Treatment of Pt-FBA with 2 molar equivalents of AsA in PBS at 37 °C showed, after 12 h incubation, the complete disappearance of the NMR signal of Pt-FBA at −107.7 ppm and appearance of the free FBA signal at −110.1 ppm (Figure 1A). This result confirms that the reduction of Pt-FBA by AsA is accompanied by the release of free FBA. The same experiment, monitored at different incubation times, allowed to estimate the half-life for reduction ($t_{1/2}$ ~31 min, Figures 1C and 1D and Figure S8), whose value is comparable with those reported in the literature for Pt(IV) prodrugs.[15] Unlike AsA, GSH causes only a negligible reduction of Pt-FBA after 12 h incubation (Figure 1A). Similarly to GSH, cysteine (Cys) also causes a minimal reduction of Pt-FBA (Figure S9). These results further support the notion that Pt(IV) prodrugs can be reduced by AsA much more efficiently than by isolated thiol groups (GSH and Cys), which is in agreement with the results reported for various Pt(IV) agents.[15-16] HPLC analysis fully confirmed the results obtained by ^{19}F NMR spectroscopy: complete disappearance of the peak of Pt-FBA (~12 min retention time) and appearance of the peak of free FBA (15.9 min retention time) observed after 12 h incubation only in the case of AsA, while the peak of Pt-FBA remained nearly intact after 12 h incubation with GSH (Figure 1B).

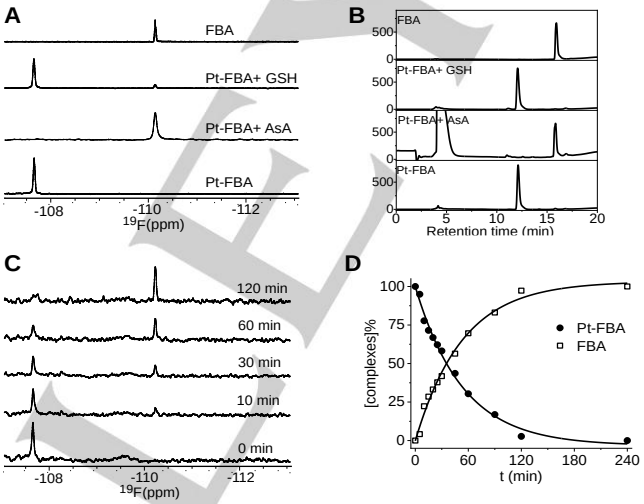


Figure 1. Reaction of Pt-FBA with physiological, low molecular weight, reducing agents. 1 mM Pt-FBA was incubated with 4 molar equivalents of GSH or 2 molar equivalents of AsA in PBS (pH 7.40) at 37 °C. (A) ^{19}F NMR spectra after 12 h incubation. (B) HPLC profiles after 12 h incubation. (C) Reduction of Pt-FBA (0.5 mM) by AsA (1.0 mM) monitored by ^{19}F NMR spectroscopy at different incubation times. (D) Plots of % Pt-FBA and % FBA at different incubation times in the reaction of Pt-FBA with AsA.

In addition to small molecular weight reactants, also some biomacromolecules, such as bovine serum albumin (BSA) and metallothionein (MT), have been reported to reduce Pt(IV) complexes.[16c, 17] Therefore, we have monitored the corresponding reactions by ^{19}F NMR spectroscopy. NMR measurements indicate that Pt-FBA is totally inert to the action of BSA (no detectable reduction after 12 h incubation) (Figure S9); in contrast, a fast reduction of Pt-FBA is observed in the case of reaction with MT (complete reduction of Pt-FBA with release of free FBA after 4 h incubation, Figure 2A). Monitoring of the MT reaction progress as a function of time provided a half-life ($t_{1/2}$) for reduction of ~35 min (Figures 2B and 2C and Figure S8). Furthermore, since MT is a metal storage protein that can bind zinc ions in cells, the effect of zinc concentration on the reducing activity of MT was also investigated. Interestingly, the presence of Zn^{2+} ions decreases the reducing activity of MT, which becomes about half of its original value at an MT:Zn^{2+} ratio of 1:5 (Figure 2A), thus indicating that the Zn^{2+}-coordination to thiol groups attenuates the reducing activity of MT. This result clearly indicates that cellular zinc levels may influence the activation of Pt(IV) prodrugs. It is noteworthy the broadening of the Pt-FBA peak observed in the presence of BSA which, most likely, stems from a non-specific interaction between prodrug and protein, as also observed in cells (see below).

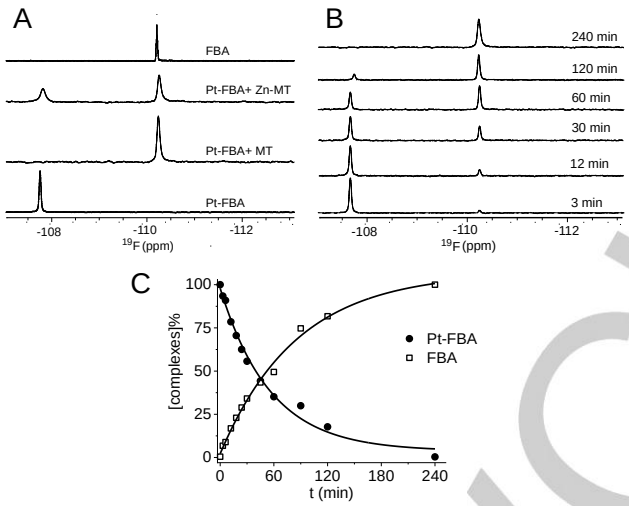


Figure 2. Reduction of Pt-FBA with MT. (A) 1 mM Pt-FBA was incubated with 2 mM apo-MT or Zn-MT in PBS (pH 7.40) in the presence of 4 molar equivalents of GSH at 37 °C for 4 h. Zn-MT was prepared by incubating apo-MT with 10 molar equivalents of Zn^{2+} and 20 molar equivalents of GSH in PBS (pH 7.40); the excess Zn^{2+} and GSH were removed by ultrafiltration. The ^{19}F NMR spectra were recorded at 25 °C. (B) Reduction, as a function of time, of Pt-FBA (0.5 mM) by apo-MT (1.0 mM) at 37 °C under nitrogen protection. The ^{19}F NMR spectra were recorded at 37 °C. (C) Plots of % Pt-FBA and % FBA as a function of the incubation time in the reaction of Pt-FBA with apo-MT.

Reduction of Pt-FBA in the lysate of cancer cells. Having investigated the *in vitro* reduction of Pt-FBA by small molecular weight as well as macro biomolecules, our interest was directed to the activation of the prodrug in conditions closer to the cellular environment. To this end, Pt-FBA was incubated in the lysate of four different cancer cell lines: epidermoid carcinoma A431, HeLa cervical carcinoma, lung carcinoma epithelial A549, and cisplatin-resistant A549R cells. The results are shown in Figure 3. After incubation at 37 °C for 4 h, a complete disappearance of the ^{19}F signal of Pt-FBA (-107.7 ppm) with concomitant appearance of the free FBA peak (-110.1 ppm) was observed in the case of A431 and A549 cells. In contrast, only a partial reduction of Pt-FBA (~40%) occurred in the lysate of HeLa cells, while Pt-FBA was found to be very stable in the lysate of A549R cells (almost no reduction product detected after 4 h of incubation). Interestingly, this result reveals that the reduction of Pt(IV) complexes largely depends on the type of cancer cells.

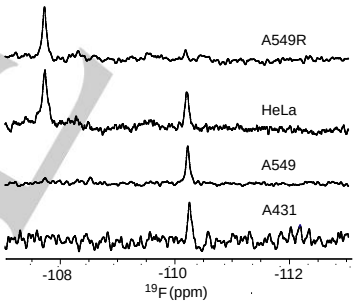


Figure 3. Influence of cancer cell type on the reduction of Pt-FBA. 2×10^7 living cancer cells were trypsinized and collected into a 1.5 mL centrifuge tube. Cells were lysed through sonication and then incubated with 0.5 mM of Pt-FBA in PBS at 37 °C for 4 h.

Reduction of Pt-FBA inside living cells. Encouraged by the results obtained with the lysate of cancer cells, we explored the possibility of using the ^{19}F probe to monitor the reduction of Pt-FBA directly in living cells. To this end, 0.25 mM Pt-FBA was incubated with *E. coli* cells at 37 °C for 3 h, then the cells were separated from the culture medium and the two fractions were subjected to NMR analysis. No ^{19}F NMR signals could be observed in the fraction of living cells even after cell lysis (neither Pt-FBA, nor FBA) (Figure 4A).

Since the ICP-MS analysis confirmed sufficient uptake of platinum drug by the cells, we conclude that, most likely, non-specific interactions with cellular proteins (or possible metabolic degradation) prevent the detection of the ^{19}F signal inside bacterial cells. We recall that the presence of the BSA protein alone could remarkably broaden the peak of Pt-FBA (Figure S9). Unlike the cellular fraction, ^{19}F NMR signals could be observed in the fraction of the culture medium (LB supernatant). The results showed that Pt-FBA was partially reduced resulting in ~45% Pt-FBA and ~55% FBA (Figure 4A, spectrum 2). We propose that Pt-FBA can be reduced inside cells and released free FBA can shuttle out of the cells. The latter hypothesis is supported by the observation that Pt-FBA, incubated in the medium alone (after removal of the cultured *E. coli* cells), showed no signs of reduction (Figure 4A, spectrum 4), thus confirming that Pt-FBA was not reduced by any component of the medium, including cell secretions.

Since MT can reduce Pt-FBA *in vitro*, we wanted to investigate the effect of cellular MT on the reduction of Pt(IV) prodrugs inside cells. Therefore, after overexpression of MT in *E. coli* cells, Pt-FBA was added to the cell culture and incubated for 3 h at

37 °C. Then the culture medium was separated from the cells and analyzed by ^{19}F NMR. As shown in Figure 4B (first spectrum from the bottom), under these conditions only the reduction product (free FBA) could be detected. If we compare this result with the half reduction observed in the analogous experiment performed on regular *E. coli* cells (Figure 4A, spectrum 2), we can conclude that, indeed, overexpression of cellular MT can remarkably enhance the reduction rate. Furthermore, since MT is confined inside the cells, this experiment confirms the occurrence of the Pt-FBA reduction inside the cells and the trafficking of free FBA out of the cells. The effect of cellular zinc levels on Pt-FBA reduction was assessed by adding extra Zn^{2+} ions (1 and 5 mM) to the culture medium during the induction of MT expression. The results (Figure 4B, spectra 2 and 3 from the bottom) clearly indicate that a high concentration of Zn^{2+} ions can decrease the reduction of Pt-FBA, thus confirming that cellular MT participates in the reduction of Pt-FBA and that zinc coordination to MT can diminish its reducing activity.

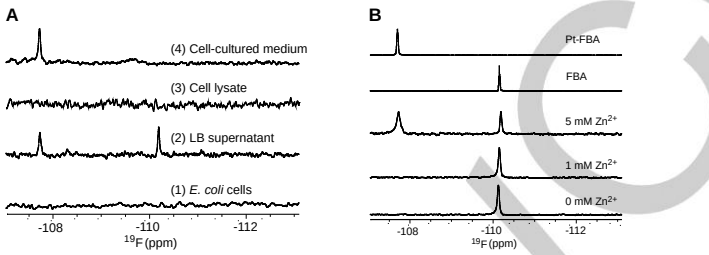


Figure 4. Reduction of Pt-FBA in *E. coli* cells. (A) Pt-FBA (0.25 mM) was incubated with *E. coli* cells at 37 °C for 3 h. After incubation, the cells were separated from the Luria-Bertani (LB) culture medium and both fractions were subjected to ^{19}F NMR detection. Then the *E. coli* cells were lysed and the lysate was also analyzed by ^{19}F NMR: (A1) fraction of living cells washed 3 times; (A2) LB supernatant; (A3) cell lysate; (A4) ^{19}F NMR spectrum of Pt-FBA incubated (3 h) in the medium where *E. coli* cells had been cultured. (B) Reduction of Pt-FBA (0.25 mM) incubated with *E. coli* cells overexpressing the GB1-MT protein in the presence of different concentrations of added $ZnCl_2$ (0, 1, and 5 mM). After incubation at 37 °C for 3 h, the culture medium was separated from the cells and subjected to ^{19}F NMR detection. All ^{19}F NMR spectra were recorded at 25 °C.

The experiment performed on *E. coli* cells was replicated on A431 cancer cells (Figure S10). Surprisingly, only a very small reduction of Pt-FBA was observed after 9 h incubation at 37 °C. We argued that this difference could be a peculiarity of the cancer cells used; therefore we tried the same experiment on another type of eukaryotic cells and, in particular, on red blood cells (RBCs). RBCs, collected from mice blood, were incubated with Pt-FBA in 0.9% NaCl containing 20 U/mL heparin sodium.

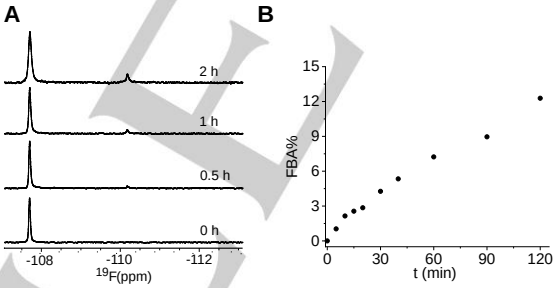


Figure 5. Reduction of Pt-FBA incubated with RBCs. (A) ^{19}F NMR spectra of Pt-FBA incubated with mice RBCs in 0.9% NaCl containing 20 U/mL heparin sodium (the incubation time is reported on each spectrum). The spectra were recorded at 25 °C. (B) Plot of the % of free FBA versus time in the reaction of Pt-FBA with RBCs.

The ^{19}F NMR spectra showed that also with RBCs the reduction of Pt-FBA is very slow and only a weak signal of free FBA could be detected after 2 h incubation (Figure 5). Most likely, the rate of reduction depends on the uptake of Pt-FBA by A431 or RBCs, and this process may be slower in eukaryotes than in bacteria.

Reduction of the Pt-FBA prodrug in mice whole blood and plasma. It has been reported that satraplatin is rapidly metabolized in the blood ($t_{1/2}$ = 6.3 min), while it is much more stable in fresh plasma ($t_{1/2}$ = 5.3 h).[18] For this reason we have investigated by ^{19}F NMR spectroscopy the metabolism of Pt-FBA in mice whole blood and plasma. Incubation with mice whole blood caused, as expected, a decrease of the ^{19}F signal of Pt-FBA with concomitant increase of the signal of free FBA with a $t_{1/2}$ of ~2 h (Figure 6A). However, unexpectedly, a similar reduction rate was observed in the incubation of Pt-FBA with mice plasma, which also caused a gradual decrease of the ^{19}F signal of Pt-FBA and the concomitant growth of the signal of free FBA (Figure 6B). Thus this experiment indicates that plasma alone can cause the biotransformation of Pt(IV) prodrugs and that red blood cells (shown above to have a very little reducing activity) play only a marginal role in the biotransformation that occurs in whole blood. It should be noted, however, that the reduction of Pt-FBA in plasma was observed only when the sample was handled under argon atmosphere.

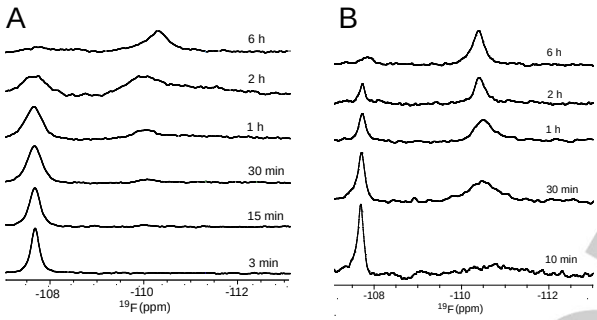


Figure 6. Reduction of Pt-FBA incubated with mice whole blood and plasma. (A) ^{19}F NMR spectra of Pt-FBA (0.5 mM) incubated with mice whole blood (containing 20 U/mL heparin sodium) at 37 °C (the incubation time is reported on each spectrum). (B) ^{19}F NMR spectra of Pt-FBA (0.5 mM) incubated with mice plasma under argon protection at 37 °C for different time intervals. All spectra were recorded at 37 °C.

Metabolism of the Pt-FBA prodrug in living mice. Encouraged by the results obtained in mice blood, we wanted to see if ^{19}F NMR spectroscopy could be used for monitoring the metabolism of Pt-FBA in living mice. Therefore, Pt-FBA (300 μL of a 30 mM solution) was administered to mice via the tail vein, then blood samples were taken at different time intervals from the mice orbital sinus, diluted with 2 times volume of 0.9% NaCl containing 30 U/mL heparin sodium, and subjected to ^{19}F NMR detection at 10 °C. The signal of Pt-FBA decreased rapidly (complete disappearance in about 25 min), meanwhile only a small peak assignable to free FBA could be observed in the early stages of the experiment (1.5 and 6 min, Figure 7A). Unexpectedly, a rather intense peak, not present in the reaction performed in whole blood *ex vivo*, was observed at ~-108.3 ppm, whose intensity was greatest in the blood sample taken 6 min after injection and then gradually decreased and disappeared completely within one hour.

Since the peak at ~-108.3 ppm was not observed in the incubation of Pt-FBA in mice blood *ex vivo*, we speculated that this signal could come from a metabolic degradation of Pt-FBA or FBA. It was quite straightforward to perform a similar experiment but administering to the mice free FBA instead of Pt-FBA. The ^{19}F spectra showed that the signal of free FBA decreases rapidly with time and is accompanied by the temporary appearance of a new peak at ~-108.3 ppm (Figure 7B) coinciding with that observed in the case of mice treatment with Pt-FBA. Also the timescale was similar in the two cases (complete disappearance of the peak at -108.3 ppm in about one hour). Although the metabolite resonating at ~-108.3 ppm has not been identified due to its instability, its downfield shift, compared to free FBA, could be in accord with an oxidation product. This result indicates that Pt-FBA is cleared from the bloodstream of living mice ($t_{1/2}$ ~6 min) faster than in whole blood *ex vivo*; moreover, the free ligand FBA is rapidly metabolized (complete disappearance in about one hour) while it was stable in whole blood *ex vivo*. The faster clearance of Pt-FBA from the bloodstream of living mice could be due to its faster reduction and/or delivery to other tissues and organs. To clarify this point, 300 μL of 30 mM Pt-FBA was administered intravenously to the mice via the tail vein and, one hour after administration, several organs were dissected and incubated with CH_3CN/H_2O (1:1, v/v) at 4 °C for 10 min. Then the supernatant was collected by centrifugation (15000 rpm) and analyzed by ^{19}F NMR. The spectra showed that Pt-FBA could be detected in the liver and kidneys (Figure S11). This result is quite interesting since it indicates that *in vivo,* after one hour incubation, the Pt-FBA prodrug can survive in some organs while it is completely cleared from the bloodstream. Furthermore, this experiment clearly shows how different the metabolic processes that take place *in vitro*, in *ex vivo* tissues, and *in vivo* systems can be.

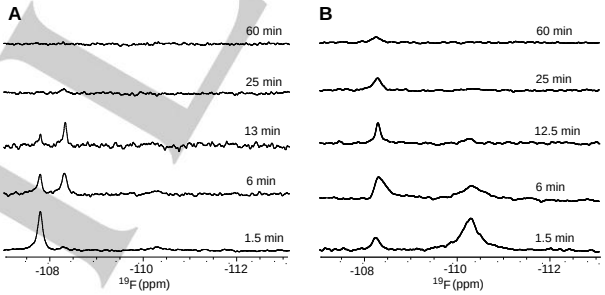


Figure 7. Metabolism of Pt-FBA (A) and FBA (B) in the blood of living mice. 300 μL of 30 mM Pt-FBA or FBA were administered to mice *via* tail vein. Then blood samples were taken at different time intervals from the mice orbital sinus, diluted with 2 times volume of 0.9% NaCl containing 30 U/mL heparin sodium, and analyzed by ^{19}F NMR. The ^{19}F NMR spectra were recorded at 10 °C.

Conclusion

The use of Pt(IV) prodrugs has been shown to be a very promising strategy to circumvent the limitations of conventional Pt(II) drugs; however, since many biomolecules can influence the reaction in cells as well as in the bloodstream, unraveling the dynamic reduction process under physiological conditions remains a challenge. NMR spectroscopy offers a non-invasive method to monitor the alteration of a ^{19}F-labeled Pt(IV) prodrug in complex physiological environments, avoiding the interference of all background

signals. Moreover, labeling with a single F-atom avoids large structural modifications of the prodrug; as a consequence, the results are more reliable and provide a confident picture of the whole Pt(IV) prodrug activation process.

This investigation has revealed how the reduction of Pt(IV) to Pt(II), which requires the overall transfer of two electrons, is performed much better by multi-electron reducing agents, such as AsA and MT, than by one-electron reductants such as GSH and Cys.

A remarkable difference was found between the behavior of bacterial cells, such as *E. coli*, and eukaryotes, such as A431 cancer cells and red-blood cells. Bacteria appear to be much more permeable to Pt(IV) prodrugs than eukaryotes. In both cases the reduction appears to take place inside the cells and the reduction product (free FBA) is able to shuttle out of the cells.

A striking difference was also found between the lysates of different tumor cells, indicating that the cellular environment can deeply influence the activation rate of Pt(IV) prodrugs.

No difference was found between the rate of reduction in mice whole blood and in mice plasma, indicating that the cellular systems play only a marginal role in the reduction of Pt(IV) prodrugs as compared to the action of other reductants present in solution.

Finally it is noteworthy that, when injected in living mice, the Pt(IV) drug, although rapidly degraded in the bloodstream, could significantly accumulate in organs such as liver and kidneys. Another unexpected feature was the rapid degradation of FBA, which was observed only in the experiment performed on living mice but not in the experiment performed on whole blood *ex vivo*.

Collectively, this investigation provides a comprehensive view of the various actors that can take part in the activation of Pt(IV) prodrugs and shows how different can be the metabolism of a prodrug in the physiological system compared to a simplified or even simply manipulated system (as in the case of bloodstream in living mice and mice whole blood *ex vivo*).

Experimental Section

The materials and methods used in this work, together with Figures S1-S11 are reported in the Supporting Information.

Acknowledgements

This work was supported by the National Key R&D Program of China (2017YFA0505400, 2020YFA0710700) and the National Science Foundation of China (22177109, 21877103, 52021002). We also thank the Italian Ministero dell'Istruzione, dell'Università e della Ricerca [PRIN 2017WBZFHL], the University of Bari and the Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici (CIRCMSB) for support. A portion of this work was performed on the Steady High Magnetic Field Facilities, High Magnetic Field Laboratory, CAS.

Keywords: platinum • prodrugs • fluorine NMR • antitumor agents • metabolism *in vivo*

[1] a) T. C. Johnstone, K. Suntharalingam, S. J. Lippard, *Chem. Rev.* **2016**, *116*, 3436-3486; b) C. Jia, G. B. Deacon, Y. Zhang, C. Gao, *Coordin. Chem. Rev.* **2021**, *429*, 213640; c) X. Hu, F. Li, N. Noor, D. Ling, *Sci. Bull.* **2017**, *62*, 589-596; d) D. Gibson, *Dalton Trans.* **2016**, *45*, 12983-12991; e) D. Gibson, *J. Inorg. Biochem.* **2021**, *217*, 111353; f) S. van Zutphen, J. Reedijk, *Coordin. Chem. Rev.* **2005**, *249*, 2845-2853; g) E. J. Anthony, E. M. Bolitho, H. E. Bridgewater, O. W. L. Carter, J. M. Donnelly, C. Imberti, E. C. Lant, F. Lermyte, R. J. Needham, M. Palau, P. J. Sadler, H. Shi, F. Wang, W. Zhang, Z. Zhang, *Chem. Sci.* **2020**, *11*, 12888-12917.

[2] a) A. O. Sartor, D. P. Petrylak, J. A. Witjes, W. R. Berry, G. S. Chatta, D. J. Vaughn, J. Ferrero, T. Demkow, J. C. Eymard, C. N. Sternberg, *J. Clin. Oncol.* **2008**, *26*; b) H. Choy, C. Park, M. Yao, *Clin. Cancer Res.* **2008**, *14*, 1633-1638.

[3] a) Z. Wang, Z. Deng, G. Zhu, *Dalton Trans.* **2019**, *48*, 2536-2544; b) H. Y. Shi, C. Imberti, P. J. Sadler, *Inorg. Chem. Front.* **2019**, *6*, 1623-1638.

[4] C. Loup, A. T. Vallina, Y. Coppel, U. Letinois, Y. Nakabayashi, B. Meunier, B. Lippert, G. Pratviel, *Chem. - Eur. J.* **2010**, *16*, 11420-11431.

[5] a) M. D. Hall, C. T. Dillon, M. Zhang, P. Beale, Z. Cai, B. Lai, A. P. J. Stampfl, T. W. Hambley, *J. Biol. Inorg. Chem.* **2003**, *8*, 726-732; b) R. A. Alderden, H. R. Mellor, S. Modok, M. D. Hall, S. R. Sutton, M. G. Newville, R. Callaghan, T. W. Hambley, *J. Am. Chem. Soc.* **2007**, *129*, 13400-13401; c) M. D. Hall, H. L. Daly, J. Zhang, M. Zhang, R. A. Alderden, D. Pursche, G. J. Foran, T. W. Hambley, *Metallomics* **2012**, *4*, 568-575.

[6] a) E. J. New, R. Duan, J. Zhang, T. W. Hambley, *Dalton Trans.* **2009**, 3092-3101; b) Y. Song, K. Suntharalingam, J. S. Yeung, M. Royzen, S. J. Lippard, *Bioconjugate Chem.* **2013**, *24*, 1733-1740; c) Y. Yuan, Y. Chen, B. Tang, B. Liu, *Chem. Commun.* **2014**, *50*, 3868-3870.

[7] a) D. Montagner, S. Q. Yap, W. H. Ang, *J. Biol. Inorg. Chem.* **2014**, *19*, 765-765; b) J. X. Ong, C. S. Q. Lim, H. V. Le, W. H. Ang, *Angew. Chem. Int. Ed.* **2019**, *58*, 164-167; c) D. Montagner, S. Q. Yap, W. H. Ang, *Angew. Chem., Int. Ed.* **2013**, *52*, 11785-11789.

[8] a) E. Luchinat, L. Banci, *Acc. Chem. Res.* **2018**, *51*, 1550-1557; b) F. Arnesano, *Curr. Opin. Chem. Biol.* **2021**, *61*, 214-226; c) Y. Kasherman, S. Sturup, D. Gibson, *J. Med. Chem.*, **2009**, *52*, 4319-4328; d) K. A. Tacka, D. Szalda, A.-K. Souid, J. Goodisman, J. C Dabrowiak, *Chem. Res. Toxicol*., **2004**, *17*, 1434-1444.

[9] H. Chen, S. Viel, F. Ziarelli, L. Peng, *Chem. Soc. Rev.* **2013**, *42*, 7971-7982.

[10] W. Wolf, V. Waluch, C. A. Presant, *NMR Biomed.* **1998**, *11*, 380-387.

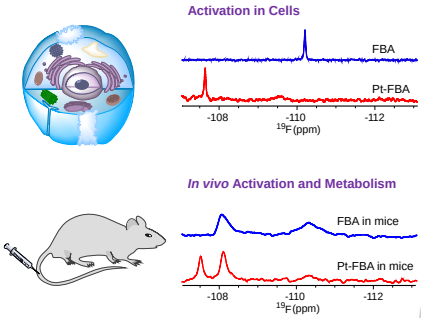
[11] C. Li, E. A. Lutz, K. M. Slade, R. A. S. Ruf, G. Wang, G. J. Pielak, *Biochemistry* **2009**, *48*, 8578-8584.

[12] C. Dalvit, M. Veronesi, A. Vulpetti, *J. Biomol. NMR* **2020**, *74*, 613-631.

[13] a) Z. Xu, W. Hu, Z. Wang, S. Gou, *Eur. J. Med. Chem.* **2017**, *141*, 211-220; b) L. Fang, X. Qin, J. Zhao, S. Gou, *Inorg. Chem.* **2019**, *58*, 2191-2200.

[14] a) J. M. May, A. Jayagopal, Z. Qu, W. H. Parker, *Biochem. Biophys. Res. Commun.* **2014**, *452*, 112-117; b) T. Du, H. Zhang, J. Ruan, H. Jiang, H. Chen, X. Wang, *ACS Appl. Mater. Interfaces* **2018**, *10*, 12417-12423.
[15] Q. Cheng, H. Shi, H. Wang, Y. Min, J. Wang, Y. Liu, *Chem. Commun.* **2014**, *50*, 7427-7430.
[16] a) D. Corinti, M. E. Crestoni, S. Fornarini, E. Dabbish, E. Sicilia, E. Gabano, E. Perin, D. Osella, *J. Biol. Inorg. Chem.* **2020**, *25*, 655-670; b) M. Ravera, E. Gabano, S. Bianco, G. Ermondi, G. Caron, M. Vallaro, G. Pelosi, I. Zanellato, I. Bonarrigo, C. Cassino, D. Osella, *Inorg. Chim. Acta* **2015**, *432*, 115-127; c) J. L. Carr, M. D. Tingle, M. J. McKeage, *Cancer Chemother. Pharmacol.* **2006**, *57*, 483-490.
[17] a) A. Lasorsa, O. Stuchlikova, V. Brabec, G. Natile, F. Arnesano, *Mol. Pharmaceutics* **2016**, *13*, 3216-3223; b) N. A. Kratochwil, P. J. Bednarski, *Arch. Pharm.* **1999**, *332*, 279-285; c) B. Xing, H. Zhu, Y. Shi, W. Tang, *Biometals* **2001**, *14*, 51-57.
[18] J. L. Carr, M. D. Tingle, M. J. McKeage, *Cancer Chemother. Pharmacol.* **2002**, *50*, 9-15.

Entry for the Table of Contents



By ^{19}F-labeling of a Pt(IV) prodrug (Pt-FBA, FBA = *p*-fluorobenzoate), the activation *in vitro*, in living cells and in mice could be investigated by NMR spectroscopy while avoiding the interference of all background signals and allowing the simultaneous detection of pre- and post-activation species.