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A Carrier-Free Nanostructure Based on Platinum(IV) Prodrug Enhances Cellular Uptake and Cytotoxicity

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Abstract

Flurbiprofen, a hydrophobic COX inhibitor, was coordinated axially with oxoplatin to form a new conjugate, *cis,cis,trans*-[Pt(IV)(NH₃)₂Cl₂(flurbiprofen)₂]. The successful synthesis of this new conjugate was confirmed by ¹H, ¹³C, and ¹⁹⁵Pt NMR. The potential of this conjugate being reduced to cisplatin and subsequently exerting its DNA cross-linking ability was verified using cyclic voltammetry (CV), HPLC, and mass spectrometry (MS). This conjugate showed markedly higher cytotoxicity on many cancer cell lines than cisplatin, flurbiprofen, and their physical mixture (mole ratio, cisplatin:flurbiprofen = 1:2). This is consistent with the result of an apoptosis-inducing assay. This conjugate spontaneously assembles carrier-free nanoparticles in aqueous solution, which is confirmed by DLS, TEM, SEM, and AFM, and thus facilitates cellular uptake and markedly improves its cytotoxicity and apoptosis-inducing ability *in vitro*.

Keywords

cisplatin; nanostructure; carrier-free; cyclooxygenase inhibitor; cellular uptake

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.molpharmaceut.8b00070](https://doi.org/10.1021/acs.molpharmaceut.8b00070). Materials and methods, ¹H, ¹³C, and ¹⁹⁵Pt NMR and ESI-MS confirmation of platin-FP, redox activity, reduction, and COX-2 inhibitory property of platin-FP, and other experiments (PDF)

The authors declare no competing financial interest.

INTRODUCTION

The relationship between cancer and inflammation has been extensively researched for tens of years.¹ Approximately 20% of human cancers are related to chronic inflammation.² Cyclo-oxygenases (COX), which are key mediators in generating inflammatory responses, comprise two COX isoforms, COX-1 and COX-2.³ COX-1 can be found in most tissues, while COX-2 is expressed at low level in most cells.^{4,5} At inflammatory sites COX-2 can be upregulated by cytokines and bacterial products. Moreover, COX-2 is reported to be overexpressed in many tumor tissues. Nonsteroidal anti-inflammatory drugs (NSAIDs) can inhibit COX-1 and COX-2, or inhibit COX-2 specifically. Previous work reported that aspirin and other NSAIDs can prevent colorectal cancer possibly through COX inhibition.⁶ Prior studies have confirmed that some NSAIDs can enhance chemotherapeutic efficiency of antitumor drugs or decrease cisplatin-induced side effects.^{7–11}

Inspired by the enhancement in antitumor efficiency of cisplatin by NSAIDs,^{8,9,12} some conjugates of COX inhibitors with cisplatin have been established. Asplatin, a prodrug of aspirin and cisplatin, presented anti-inflammatory ability similar to that of aspirin,¹³ and showed enhanced cytotoxicity in cisplatin-resistant cell lines.¹⁴ Indomethacin and ibuprofen are axially coordinated with *cis,cis,trans*-[Pt(IV)-(NH₃)₂Cl₂(OH)₂]¹⁵ and (SP-4-2)-(*trans*-*R,R*-cyclohexane-1,2-diamine)dihydroxo(oxalato)platinum(IV) [Pt(OH)₂(ox)-(DACH)]¹⁶ to give two conjugates, which both showed a markedly increased cytotoxicity compared with cisplatin or oxaliplatin. It is assumed that the mechanism of these complexes executing their cytotoxicity is COX-independent, and the increased lipophilicity by hydrophobic moiety (COX inhibitor) and kinetic inertness of platinum(IV) complexes seem to be the principal reasons for enhanced cytotoxicity.¹⁶

In order to elucidate the mechanism of enhancing cytotoxicity of cisplatin by NSAID conjugations, a hydrophobic COX inhibitor, flurbiprofen, was coordinated axially with *cis,cis,trans*-[Pt(IV)(NH₃)₂Cl₂(OH)₂] to form a new conjugate in this work. The cytotoxicity and apoptosis-inducing ability of this conjugate is markedly enhanced in all tested cancer cell lines including cisplatin-resistant cell lines. It is confirmed by DLS, TEM, SEM, and AFM that this *cis,cis,trans*-[Pt(IV)-(NH₃)₂Cl₂(flurbiprofen)₂] conjugate spontaneously assembles carrier-free nanoparticles in aqueous solution. This facilitates cellular uptake of platinum complex and thus improves cytotoxicity. As far as we know, this is the first report about carrier-free nanostructure of cytotoxic platinum(IV) prodrug of cisplatin in aqueous solution.

RESULTS AND DISCUSSION

cis,cis,trans-[Pt(NH₃)₂Cl₂(flurbiprofen)₂] (platin-FP), whose structure is shown in Figure 1, was synthesized by reaction of *cis,cis,trans*-[Pt(NH₃)₂Cl₂(OH)₂] with the excess acyl chloride derivative of flurbiprofen (Scheme S1). The ¹H NMR spectrum of flurbiprofen chloride is shown in Figure S1. In the ¹H NMR spectrum of platin-FP presented in Figure S2, compared with free flurbiprofen, the peak of –COOH (12.43 ppm) disappeared, a new peak referred to Pt→NH₃ (6.56 ppm) appeared, and the –CH₃ near the newly formed ester bond shifted from 1.41 to 1.38 ppm, which confirmed the formation of platin-FP. The

structure was further confirmed by ^{13}C and ^{195}Pt NMR spectra and ESI-MS (Figure S3–S5). Platin-FP shows an irreversible reduction analyzed by the cyclic voltammetric method (Figure S6). The Pt(IV)/Pt(II) couple is about -0.68 V vs Ag/AgCl at pH 7.4. This reduction potential means that platin-FP can be reduced in a reductive intracellular environment.¹⁷ To mimic the intracellular reduction, platin-FP was incubated with ascorbic acid, and the release of flurbiprofen from platin-FP was monitored using high-performance liquid chromatography (HPLC, Figure S7a). As time increases, the peak of platin-FP diminishes and a new peak appears and increases, while platin-FP itself is stable when incubated without ascorbic acid (Figure S8). The new peak is assigned as flurbiprofen according to the chromatography of a pure sample of flurbiprofen. At the mean time of flurbiprofen releasing upon reduction, cisplatin was formed. Cisplatin is known as coordinating with the N7 position of guanine bases of DNA to exert its antitumor activity. In order to detect the antitumor activity of this reduction adduct, 2'-deoxyguanosine 5'-monophosphate sodium salt hydrate (5'-GMP) was incubated with platin-FP in the presence of ascorbic acid, and the final products were analyzed by ESI-MS (Figure S7b). It is showed that a bifunctional adduct of Pt(II)/GMP is formed, whose formula is $[\text{Pt}(\text{NH}_3)_2(5'\text{-GMP})_2]^+$ (observed $m/z = 922.46$). The fate of platin-FP in reductive environment is illustrated in Figure S7c. This means that platin-FP can release cisplatin upon reduction and exert its antitumor activity. Platin-FP also exhibits COX-2 inhibition ability similar to that of flurbiprofen (Figure S9).

The cytotoxicity of platin-FP was analyzed by the MTT assay using several sensitive and cisplatin-resistant tumor cells. Cisplatin, flurbiprofen, and cisplatin/FP (physical mixture of cisplatin and flurbiprofen, mole ratio 1:2) groups were used for comparison. Results are shown in Table 1 and Figure S10. In all of the tested cisplatin-sensitive and resistant cancer cells, platin-FP exhibited a much lower IC_{50} value than cisplatin alone and cisplatin/FP. In SW480 cancer cells, platin-FP is more than 80-fold as effective as cisplatin. The cytotoxicity of platin-FP toward cisplatin-resistant cancer cells is comparable to that of platin-FP toward their parent sensitive cancer cells. This means that platin-FP exhibits obviously enhanced cytotoxicity against both cisplatin-sensitive and resistant cancer cells. Cisplatin showed similar cytotoxicity as cisplatin/FP in all tested cell lines, indicating that the cytotoxicity of flurbiprofen at this dosage has negligible contribution to that of platin-FP. To evaluate the apoptosis in cancer cells induced by platin-FP, an annexin V/propidium iodide (PI) staining assay was performed in BEL7404, BEL7404-CP20 (cisplatin-resistant cell lines derived from BEL7404), and SW480 cancer cells using a flow cytometer. Results are presented in Figure 2 and Figure S11. In all three kinds of cell lines, the groups treated with platin-FP showed much higher level of apoptosis than those treated with cisplatin/FP or cisplatin alone. This indicates that the high ability of inducing apoptosis is due to intrinsic property of platin-FP rather than the cisplatin or flurbiprofen part. The conjugation of flurbiprofen with cisplatin remarkably enhances its cytotoxicity and apoptosis inducing ability.

Cellular uptake experiments were performed to study the quantity and distribution of platinum species in cancer cells. BEL7404, BEL7404-CP20, and SW480 cells were incubated with $10\text{ }\mu\text{M}$ platin-FP, cisplatin/FP, or cisplatin for 6 h, and the platinum amount taken by cells and coordinating with nuclear DNA were measured by ICP-MS. As shown in Figure 3, in all three, even cisplatin-resistant cancer cells, platin-FP exhibited at least 21 and 16 times higher cellular uptake than cisplatin and cisplatin/FP. The platinum amount

coordinating with nuclear DNA in platin-FP is at least 5- and 4-fold higher than that in cisplatin and cisplatin/FP. The higher level of Pt/DNA benefits from the higher cellular uptake of platin-FP by cells, and it explains the stronger cytotoxicity of platin-FP since cisplatin-based drugs exert their anticancer ability through cross-linking with DNA.

The reason that platin-FP showed high cellular uptake needs to be investigated. As Figure 4a shows, the stock solution of platin-FP exhibited opalescence and the Tyndall effect. DLS study showed that in stock solution platin-FP formed nanoparticles with a diameter of 115.3 nm, PDI of 0.094. The nanoparticle was further confirmed by SEM, TEM, and AFM (length $\times$ width $\times$ height: about 200 nm $\times$ 200 nm $\times$ 30 nm, see Figures 4b, 4c, and S12). The size measured by SEM and TEM is different from that determined by DLS. This difference is attributed to the mechanism of DLS measurements, which is based on the assumption of isotropic particles.¹⁸ When DLS is used to characterize an anisotropic nanostructure, like a nanorod or nanosheet, it will give an equivalent spherical hydrodynamic diameter, which is some-times different from the three dimension parameters measured by TEM.^{19,20} These results indicate that platin-FP can form nanoparticles in H₂O. The formation mechanism of platin-FP nanoparticles is due to supersaturation and nucleation in poor solvent using reprecipitation method, which is similar to reports of organic nanocrystals.^{21–23} It is well-known that nanoparticles can increase cellular uptake. This explains the much higher level of platinum in cells treated with platin-FP than cisplatin and cisplatin/FP. A possible mechanism of action of platin-FP is shown in Figure 5. The detailed cellular uptake mechanism of platin-FP nanoparticles and the antitumor efficiency *in vivo* are under further study.

The previous reports related to cytotoxic platinum nanoparticles are mainly focused on the following categories: (1) cisplatin, oxaliplatin, or Pt(IV) prodrugs encapsulated in, incorporated in, or conjugated with delivery nanocarriers;^{24–31} (2) Pt(IV) prodrugs modified with a hydrophobic part and a hydrophilic part, e.g., polyethylene glycol or hydrophilic peptides, then self-assembled nanoparticles;^{32–36} (3) platinum metal nanoparticles that can release cytotoxic Pt(II) in intracellular acid environment.^{37–39} To our knowledge, this is the first report about self-assembled carrier-free nanoparticles of Pt(IV) prodrug in aqueous solution.

CONCLUSION

In summary, platin-FP self-assembles into carrier-free nanostructure in aqueous solution and exhibits significantly enhanced cytotoxicity compared to cisplatin. This is the first report about carrier-free nanostructure of cisplatin-derived cytotoxic Pt(IV) prodrug. Platin-FP can be reduced to form cisplatin, cross-linking with DNA and exerting its anticancer ability. Platin-FP spontaneously forms carrier-free nanostructure in aqueous solution. This increases the cellular uptake of platinum and Pt/DNA cross-links in cancer cells and, thus, improves cytotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of flurbiprofen and platin-FP.

Figure 2. Apoptosis ability of cisplatin, mixture of cisplatin and flurbiprofen (cisplatin/FP), platin-FP, and flurbiprofen in different cancer cell lines measured by flow cytometry.

Figure 3.

Platinum content accumulated in cells (a, b, c) and coordinated with nuclear DNA (d, e, f) in different cancer cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.
Characterization of nanostructure of platin-FP by DLS (a), SEM (b), and TEM (c).

Figure 5.
Proposed mechanism of action of platin-FP.

Table 1.

Comparison of IC₅₀ (μ M) values for Cisplatin, Mixture of Cisplatin and Flurbiprofen (Cisplatin/FP), Platin-FP, and Flurbiprofen in Different Cancer Cell Lines

cancer cell lines	type	cisplatin	cisplatin/FP	platin-FP	flurbiprofen
SW480	colon	49.2 $\pm$ 1.1	29.6 $\pm$ 1.0	0.6 $\pm$ 1.1	48.8 $\pm$ 1.1
PC-3	prostate	21.2 $\pm$ 1.1	22.4 $\pm$ 1.1	3.4 $\pm$ 1.0	>50
PANC-1	pancreas	14.4 $\pm$ 1.1	11.1 $\pm$ 1.1	3.4 $\pm$ 1.1	>50
A549	lung	7.4 $\pm$ 1.0	7.1 $\pm$ 1.0	2.7 $\pm$ 1.1	>50
A549-DDP	lung	20.3 $\pm$ 1.1	21.1 $\pm$ 1.2	2.5 $\pm$ 1.1	>50
BEL7404	liver	14.7 $\pm$ 1.1	18.1 $\pm$ 1.0	1.4 $\pm$ 1.1	>50
BEL7404-CP20	liver	>50	>50	3.1 $\pm$ 1.1	>50