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ctc-[Pt(NH$_3$)$_2$(cinnamate)(valproate)Cl$_2$] is a highly potent and low-toxic triple action anticancer prodrug†

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Pt(IV) prodrugs have gained tremendous attention due to their indisputable advantages compared to cisplatin. Herein, new Pt(IV) derivatives with cinnamic acid at the first axial position, and inhibitor of matrix metalloproteinases-2 and -9, histone deacetylase, cyclooxygenase or pyruvate dehydrogenase at the second axial position are constructed to develop multi-action prodrugs. We demonstrate that Pt(IV) prodrugs are reducible and have superior antiproliferative activity with IC_{50} values at submicromolar concentrations. Notably, Pt(IV) prodrugs exhibit highly potent anti-tumour activity in an *in vivo* breast cancer model. Our results support the view that a triple-action Pt(IV) prodrug acts *via* a synergistic mechanism, which involves the effects of CDDP and the effects of axial moieties, thus jointly leading to the death of tumour cells. These findings provide a practical strategy for the rational design of more effective Pt(IV) prodrugs to efficiently kill tumour cells by enhancing their cellular accumulation and tuning their canonical mechanism.

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Introduction

Platinum drugs constitute a major class of DNA-targeted antitumour agents, represent some of the most successful chemotherapeutics in the clinic, and nearly half of all cancer patients who require chemotherapy are treated with platinum drugs.[1] Importantly, they have significantly improved the survival rates of cancer patients.[2] Although cisplatin (CDDP) is considered to be a "milestone" of modern chemotherapy and continues to play a pivotal role in cancer therapeutic regimens,[3] its clinical outcomes are restricted mainly owing to severe dose-limiting toxic side effects arising from the lack of cancer cells selectivity. This limited applicable dosage likely prevents the whole cancer tissue from being exposed to sufficient drug concentration, eventually leading to cancer recurrence and metastasis.[4] Drug resistance is another major issue. The molecular mechanisms underlying the development of CDDP drug resistance are multi-factorial and undoubtedly complex. A combination of CDDP and other drugs that act by different mechanisms against different cellular targets in the cancer cells is an attractive chemotherapeutic protocol. The main clinical aim of this approach is to more efficiently kill all the cancer cells and reduce toxicity thereby overcoming drug resistance. The major issues in the combination of drugs administered as a mixture of single agents include the definitive exposure to the targets of interest, biodistribution parameters, and individual pharmacokinetics along with the desired ratio and dose. These factors are hard to control when drugs are individually administered. Notably, construction of a single prodrug containing a drug combination to hit multiple biological targets can potentially overcome these issues.[5]

As regards the square planar Pt(II) drugs including CDDP, carboplatin and oxaliplatin, they bind to the N7 position of the purine bases of nuclear DNA and distort its double strand structure, thereby triggering cellular responses that result in apoptosis.[6–8] The semi-labile Pt(II) complex can be oxidized to a more inert octahedral Pt(IV) complex, typically such as satraplatin (Scheme 1a) that has been applied for phase III clinical trial against metastatic castration-resistant prostate cancer.[9] Based on the advantageous properties of the octahedral Pt(IV) complex, the development of the Pt(IV) complex enables the conjugation of anticancer drugs and/or biologically active ligands in axial positions. After the accumulation of these Pt(IV) complexes inside the cells, they are reduced by intracellular

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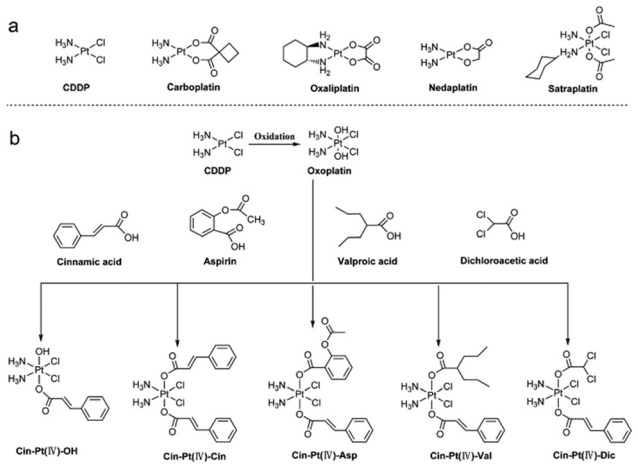

Scheme 1 (a) Three approved Pt(II) anticancer drugs in FDA (CDDP, carboplatin, and oxaliplatin), one approved in Japan (nedaplatin) and Pt (IV) satraplatin. (b) Schematic representation of Pt(IV) derivatives of CDDP with axial ligands which are enzyme inhibitors that exhibit significantly improved antiproliferative potency in comparison to CDDP.

reducing molecules, such as high concentration of ascorbic acid (AsA) and glutathione (GSH) in the cancer cells, to release the conjugated anticancer drugs and bioactive ligands simultaneously with a DNA damaging Pt(II) moiety, upon which the released components can work synergistically with the Pt(II) drugs to kill cancer cells by different mechanisms against different cellular targets in the tumour cells. This makes Pt(IV) complexes ideal prodrugs because they are stable outside the cancer cells, but reducible inside the cancer cells. Particularly, the Pt(IV) complexes are able to bring into the cancer cells along with the molecules of Pt(II) moiety, the molecules of other anticancer drugs or bioactive ligands,[14] therefore obtaining multi-action prodrugs that can overcome resistance.[10,11] Previous studies have verified that these multi-action Pt(IV) prodrugs are effective chemotherapeutic agents both *in vitro* and *in vivo*.[11–15]

Cinnamic acid (Cin) is composed of a phenyl ring substituted with an acrylic acid group, a naturally occurring phytochemical with a variety of pharmacological activities including antibacterial, anti-inflammatory, antioxidant[16,17] and anti-tumour activities *in vitro* against various human solid tumours at doses without significant influence on normal cells. It is found that Cin induces tumour cell apoptosis and differentiation, regulates gene expressions involving the control of tumour growth and immunogenicity, and inhibits invasive growth and metastasis by suppressing the activities of matrix metalloproteinases (MMP)-2 and -9, accompanied by the induction of cell cycle arrest and cytoskeleton disruption in malignant tumour cell lines.[18–22] In addition, Cin has also been demonstrated to influence the differentiation of sphere-derived cancer stem cells (CSCs), making them more sensitive to CDDP by apoptosis[23] and preventing normal cells from the toxic effects of CDDP. Therefore, it has been utilized to develop highly potent Pt(IV) prodrugs, which made the CSCs more sensitive to killing by the CDDP part of the complex and efficiently overcome the resistance of CSCs.[13,14] Recently, several dual-action Pt(IV) derivatives of CDDP by conjugation of specific cyclooxygenase (COX) inhibitor (aspirin, Asp),[15,24] histone deacetylase (HDAC) inhibitor (valproic acid, Val)[25,26] or pyruvate dehydrogenase kinase (PDK) inhibitor (dichloroacetic acid, Dic),[27,28] have been reported. Results suggested that these Pt(IV) complexes not only had encouraging *in vitro* activities but also exhibited better resistance factors than CDDP, high selectivity indices, and improved *in vivo* anti-tumour activity.

Until now, no examples of a triple-action Pt(IV) complex bearing a Pt(II) moiety and Cin and Val axial ligands, which synergistically acts by DNA damage, inhibiting MMP-2 and -9 activity to block tumour cell invasion and metastasis and suppressing HDAC activity to increase the accessibility of DNA to Pt(II) moiety by decondensing chromatin, consequently potentiating the anti-tumour activity of Pt(II) moiety, have been reported. With the challenges in mind, herein we report the synthesis, characterization, and chemical and biological characteristics of the Pt(IV) complexes (Scheme 1b) of CDDP by conjugation of Cin at one axial position and Val, Asp or Dic at the second axial position using hydroxyl groups to prepare triple-action prodrugs. Our results show that the strategy based on the Pt(IV) derivatives of CDDP with Cin and other biologically active moieties can synergistically enhance the anti-tumour activity.

Results and discussion

Synthesis and characterization of Pt(IV) complexes

The rational design and development of multi-action Pt(IV) complexes has already demonstrated huge potential.[5] Due to the difference in their geometry and reactivity between the two oxidation states Pt(II) and Pt(IV), the inert octahedral Pt(IV) complexes are prone to be synthesized by oxidation from semi-labile square planar Pt(II). We employed CDDP-based Pt(IV) as a template and introduced Cin (an MMP-2 and -9 inhibitor), Asp (a COX inhibitor), Val (an HDAC inhibitor) and Dic (a PDK inhibitor) into the axial position to form multi-action Pt(IV) complexes (Scheme 1b). In brief, CDDP was first oxidized with H_2O_2 to give oxoplatin $[Pt(NH_3)_2(OH)_2Cl_2]$ according to the reported procedures.[15] Oxoplatin was then reacted with 1.5 equivalents of Cin anhydride in DMSO to obtain non-symmetric monocarboxylated Cin-Pt(IV)-OH. Subsequently, the second axial hydroxo group was carboxylated using different amounts of Asp, Val or Dic anhydride in DMF or DCM to give Pt(IV) complexes, owing to the fact that the second hydroxo group in oxoplatin is less prone to carboxylation. The synthesis routes are outlined in Scheme S2.† The purified Pt(IV) complexes were characterized using ^{1}H-NMR, ^{13}C-NMR, elemental analysis and HRMS techniques (Fig. S1–S5†).

Reduction by ascorbate and glutathione

It is worthy to note that Pt(IV) complexes safely travel in the bloodstream, but are easily reduced to release Pt(II) drugs

inside cancer cells with a much higher concentration of glutathione (GSH) and ascorbic acid (AsA) in the cytoplasm than in extracellular environments.[15] Thus, we monitored the reduction of the Cin-Pt(IV)-Val complex (1 mM) using GSH or AsA (10 equiv.) as a reductant by HPLC analysis. In all cases, we observed a reduction in the time of the peak of the Cin-Pt(IV)-Val complex that is accompanied by a concomitant increase in the peak of free Cin (at 4.3 min) in the HPLC chromatogram as it has an observable chromophore (Fig. S6†). But the peaks of CDDP and Val were not detectable, in agreement with the reported data,[11] demonstrating that the Cin-Pt(IV)-Val complex was easily reduced thereby releasing two axial ligands. Subsequently, we used cyclic voltammetry to determine how the Cin-Pt(IV)-Val complex undergoes reduction easily to its Pt(II) equivalent in phosphate buffer-0.1 M KCl (Fig. S7†). By mimicking the narrow pH range of cancer cells, it was found that the reduction potentials were −0.010 V at pH 7.4 and −0.256 V at pH 6.4, revealing that the Cin-Pt(IV)-Val complex might be easily reduced at pH 6.4 by the microenvironment of tumor cells than at pH 7.4 by the microenvironment of the normal cells. The stability of the Cin-Pt(IV)-Val complex was measured by analytical HPLC at different times, demonstrating that the Cin-Pt(IV)-Val complex was stable in PBS or a cell culture medium containing ACN for at least 48 h (Fig. S8†), which at least met a period corresponding to the span of cellular experiments. Together, these results confirmed that the Cin-Pt(IV)-Val complex was reducible notably and simultaneously exhibited the dual response characteristics of pH and redox in the cancer cells, thus indicating its potential safety as an anticancer drug.

Antiproliferative activity

A panel of six cancerous cell lines including human lung carcinoma (A549), breast carcinoma (MCF-7), hepatocellular carcinoma (HepG-2) and bladder carcinoma 5637, mice bladder carcinoma MB49 and breast carcinoma 4T1 was employed to assess the antiproliferative activity of the as-prepared Pt(IV) complexes and compare it with the activity of parental CDDP. As shown in Table 1 and Fig. S9,† the IC_{50} values of CDDP were in the range of 4.65–12.60 μM; however, the investigated Pt(IV) complexes inhibited cell proliferation with IC_{50} values ranging from sub-micromolar to micromolar levels. Particularly, the Cin-Pt(IV)-Val complex, bearing Cin and Val as ligands, was shown to be extremely effective against all tested cell lines. The complex exhibited 50% inhibition of the proliferation of HepG-2 cells and A549 cells at a concentration as low as 70 nM and simultaneously exhibited 103.04-fold more potency than the parental CDDP against HepG-2 cells and the lowest potency of 29.06-fold against 4T1 cells, respectively.

The antiproliferative activity of the Cin-Pt(IV)-Val complex was further compared to the activity of CDDP, Cin and Val, and the physical mixtures of CDDP, Cin and Val in 1 : 1 : 1 molar ratios, all three representing free constituents possibly released after the activation of Cin-Pt(IV)-Val in the cellular reducing environment. It is interesting to know how active these complexes are. As shown in Table 2 and Fig. S10,† free Cin and Val were almost nontoxic (IC_{50} > 200 μM) to these cell lines under the same conditions, in agreement with the previously reported data.[13,14] Notably, the physical mixtures exhibited 34–61 fold less cytotoxicity than the Cin-Pt(IV)-Val complex, but similar to that of CDDP, demonstrating that the introduction of Cin and Val ligands in the axial position of the investigated Pt(IV) derivatives of CDDP resulted in enhanced activity in selected tumor cell lines. Based on the cytotoxic profiles and our preliminary experiment about cell migration, we chose bladder carcinoma MB49 cells to perform the following biological assay.

Cell migration

The metastatic spread of primary tumors leads to approximately 90% of all cancer deaths.[29] Enzymes produced by tumor cells digest the various structural components of the extracellular matrix and hence have long been viewed as essential for tumor invasion and metastasis. Among them, the matrix metalloproteinase (MMP) family is the prime candidate and their high expression is responsible for these activities.[30] Therefore, a chemotherapeutic agent capable of intervening the migration and metastasis of tumor cells by inhibiting MMP expression, particularly MMP-2 and MMP-9, has a profound impact on improving clinical outcomes. We then investi-

Table 1 IC_{50} mean values (μM) of the investigated complexes evaluated by using MTT assay

	A549	HepG-2	4T1	MCF-7	MB49	5637
CDDP	4.85 ± 0.57	7.22 ± 0.71	4.65 ± 0.33	10.45 ± 1.71	12.60 ± 2.23	5.75 ± 1.10
Cin-Pt(IV)-OH	0.76 ± 0.06	0.86 ± 0.04	3.39 ± 0.18	3.91 ± 0.05	6.30 ± 0.74	4.02 ± 0.22
Fold increase[a1]	6.38	8.40	1.37	2.67	2.00	1.43
Cin-Pt(IV)-Cin	0.14 ± 0.04	0.31 ± 0.07	0.28 ± 0.03	1.94 ± 0.10	1.31 ± 0.11	0.62 ± 0.09
Fold increase[a2]	34.64	23.29	16.61	5.39	9.62	9.27
Cin-Pt(IV)-Asp	0.49 ± 0.08	0.41 ± 0.09	0.41 ± 0.09	0.64 ± 0.10	0.37 ± 0.09	0.46 ± 0.06
Fold increase[a3]	9.90	17.61	11.34	16.33	34.05	12.50
Cin-Pt(IV)-Val	0.07 ± 0.02	0.07 ± 0.01	0.16 ± 0.03	0.25 ± 0.06	0.18 ± 0.02	0.09 ± 0.03
Fold increase[a4]	69.29	103.14	29.06	41.80	70.00	63.89
Cin-Pt(IV)-Dic	1.61 ± 0.26	1.60 ± 0.30	1.76 ± 0.35	6.60 ± 0.72	2.85 ± 0.47	2.07 ± 0.40
Fold increase[a5]	3.01	4.51	2.64	1.58	4.42	2.78

a1–a5: IC_{50} ratio of CDDP/complexes. The drug-treatment period was 48 h.

Table 2 IC_{50} mean values (μM) of Cin-Pt(IV)-Val, CDDP, Cin, and Val and the physical mixture of CDDP, Cin and Val in 1 : 1 : 1 molar ratios after 48 h incubation

	HepG-2	4T1	MB49
CDDP	7.22 ± 0.71	4.65 ± 0.33	12.60 ± 2.23
Cin-Pt(IV)-Val	0.07 ± 0.01	0.15 ± 0.03	0.18 ± 0.02
Cin	>200	>200	>200
Val	>200	>200	>200
Cin : CDDP : Val = 1 : 1 : 1	4.29 ± 0.57	5.47 ± 0.80	10.83 ± 1.88

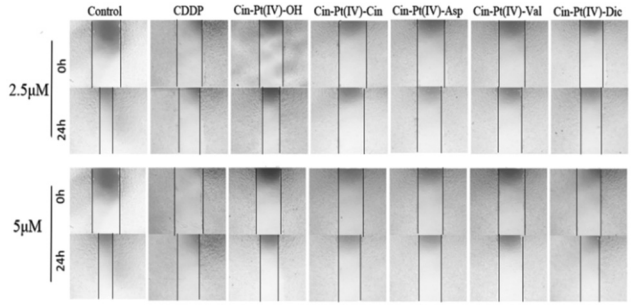

Fig. 1 Migration inhibition (wound-healing assay) of MB49 cells untreated or treated with the tested complexes for 24 h. Typical images were taken at 0 and 24 h.

gated the effect of Pt(IV) complexes on the migration of MB49 cells by a wound-healing assay. As shown in Fig. 1 and Fig. S11,† the gap between the two edges of a scratch decreased after 24 h at 2.5 μM of the complex, and the migration rates were 46.0%, 24.9%, 22.8%, 17.2%, 12.3%, 9.5%, and 7.1% for the control, CDDP, Cin-Pt(IV)-OH, Cin-Pt(IV)-Dic, Cin-Pt(IV)-Cin, Cin-Pt(IV)-Asp and Cin-Pt(IV)-Val complexes, respectively. When the concentration was elevated to 5.0 μM, the migration rate of all complexes was significantly enhanced. As excepted, Cin-Pt(IV)-Val efficiently delayed the migration of MB49 cells with a 2.2% of migration rate, implying its improved anti-tumor activity by interdicting the metastasis process.

Cellular accumulation

The accumulation of low-molecular-mass Pt-based chemotherapeutics in cancer cells plays a key role in the antiproliferative activity because the cellular uptake or efflux is the initial step in the mechanism of their anti-tumor actions.[11,31] Therefore, we examined first the platinum content in MB49 cells after a 12 h treatment with 5 μM of Pt(IV) complexes by ICP-MS. As shown in Fig. 2a and Table S2,† the Pt amount in whole cells rapidly increased during the 12 h incubation with Pt(IV) complexes, reaching 1–26 fold higher than those of CDDP, respectively, following the order of Cin-Pt(IV)-Val > Cin-Pt(IV)-Cin > Cin-Pt(IV)-Asp > Cin-Pt(IV)-Dic > Cin-Pt(IV)-OH > CDDP. Notably, the platinum content in whole cells treated with the Cin-Pt(IV)-Val complex was 369.63 ng Pt per 10^6 cells, with the maximal cellular uptake 26-fold higher than that for the same dose of CDDP. In a general case, the lipophilic property (log *P*) of a compound positively correlates with its cellular uptake

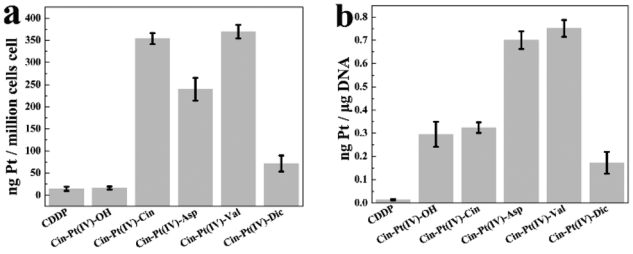

Fig. 2 Platinum content (a) and DNA platination (b) of MB49 cells for 12 h were determined by ICP-MS. Values were given as means ± SDs from at least three independent experiments.

through passive diffusion. The log *P* values for Pt(IV) complexes and various ligands were then predicted using the online Molinspiration software.[32] As listed in Table S2,† the predicted log *P* value of CDDP was −2.83, close to its determined value (−2.3),[11] whereas those of Cin and Val were 1.91 and 2.80, respectively, indicative of lipophilicity. The complex Cin-Pt(IV)-Val had a maximum log *P* value of 2.61, implying that it could more efficiently penetrate the cytoplasmic membrane. Together with the previously reported results,[11,33,34] it demonstrated that the Pt(IV) complex was transported into the cells by passive diffusion depending on their lipophilicity.

The enhanced cellular accumulation of platinum from the more hydrophobic Pt(IV) complexes often contributes to the increase in their antiproliferative activity in tested cells as compared to hydrophilic CDDP.[11] However, our finding suggested that the platinum content of Cin-Pt(IV)-Val in MB49 cells was higher than that of CDDP by *ca.* 26-fold, while its antiproliferative activity in MB49 cells was higher than that of CDDP by *ca.* 70-fold (Table 1). Thus, the higher antiproliferative activity of the Cin-Pt(IV)-Val complex was not only a consequence of enhanced cellular accumulation but also yet another factor was responsible for its improved antiproliferative activity compared to that of CDDP.

DNA platination

Nuclear DNA is a major therapeutic target of traditional platinum anticancer drugs which is responsible for their pharmacological activities. To verify the ability of the Pt(IV) complexes to trigger cell death by damaging the cellular DNA mediated mechanism, we determined the amount of platinum bound to DNA in MB49 cells treated with Pt(IV) complexes using ICP-MS. The results shown in Fig. 2b and Table S2† suggested that under these experimental conditions, 172–751 pg of platinum was associated with 1μg of DNA. The efficiency of the Pt(IV) complex in platinating DNA differed, and the trend was Cin-Pt(IV)-Val > Cin-Pt(IV)-Asp > Cin-Pt(IV)-Cin > Cin-Pt(IV)-OH > Cin-Pt(IV)-Dic > CDDP, with 52–12 fold higher than those of CDDP, respectively. Significantly, the platinum amount of DNA in cells treated with the Cin-Pt(IV)-Val complex was 751 pg Pt per ng DNA, which is 52-fold higher than that in cells treated with the same dose of CDDP, mainly ascribing to a consequence of its higher cellular accumulation. In addition, we also found that Cin-Pt(IV)-Dic exhibited lower cell accumu-

lation, DNA platination, and antiproliferative activity than Cin-Pt(IV)-Asp, Cin-Pt(IV)-Val and Cin-Pt(IV)-Cin, and was similar to Cin-Pt(IV)-OH. Cin-Pt(IV)-Dic underwent about 42% degradation after 48 h of incubation in PBS at 37 °C, suggesting that Dic was hydrolyzed from the axial position due to the electron-withdrawing power of chlorides of Dic,[11] which reduced the lipophilicity of Cin-Pt(IV)-Dic, subsequently leading to lower accumulation and DNA platination, and consequently lower antiproliferative activity. However, enhancement of the cytotoxicity (IC_{50} value shown in Table 1) of Cin-Pt(IV)-Val compared to CDDP was obviously higher than the enhancement of the amount of platinum bound to nuclear DNA in MB49 cells (Table S2†). In general, the Pt(IV) complexes do not bind to DNA owing to their inertness.[14] Based on their intracellular reducing character, we hypothesised that the Cin-Pt(IV)-Val complex upon entering the cells undergoes reduction, subsequently releasing DNA-targeting CDDP together with biologically active molecules of Cin and Val, thus exerting a specific anti-tumor activity through DNA damage by the released CDDP. Simultaneously, other processes induced by the released biologically active axial ligands, such as Cin and Val (HDAC inhibitor), likely potentiated their antiproliferative effects by suppressing MMP-2 and -9 activities[13] and increased the accessibility of DNA to CDDP by decondensing chromatin, respectively.[26] Therefore, we chose the Cin-Pt(IV)-Val complex to perform the following biological assays *in vitro* and *in vivo*.

The ability to inhibit HDAC and MMP activity

CDDP kills cancer cells by forming a chimeric adduct with nuclear DNA, which induced DNA damage, subsequently triggering cellular processes that led to apoptosis. However, only less than 10% of CDDP binds covalently to DNA.[7] In the nucleus, DNA is noncovalently associated with histones to form the nucleosomes which constitute chromatin subunits, suggesting that DNA does not exist as a naked structure. Notably, HDAC inhibitors such as Val, which can induce the hyperacetylation of histone complexed with DNA, increased the accessibility of DNA within chromatin and consequently potentiated the anti-tumor activities of CDDP even when it was incorporated into Pt(IV) prodrugs as axial ligand.[11,25] Meanwhile, it is known that highly invasive and metastatic malignant tumors often accompany the over-expression of MMP enzymes, especially MMP-2 and -9.[35] Cin, as MMP-2 and -9 inhibitor, has been previously reported to inhibit the migration and invasion of tumor cells by modulating AP-1 and NF-κB and the downstream of the MAPK pathway.[20,21] Therefore, we set out to investigate whether the Cin-Pt(IV)-Val complex, designed to release Cin and Val upon entering the cells, has the ability to inhibit HDAC and MMP-2 and -9 activities using a corresponding commercial colorimetric enzyme activity ELISA assay kit. MB49 cells were incubated with 1 μM Cin-Pt(IV)-Val complex for 24 h. The treated cells and the conditioned media were collected as samples, respectively. Fig. 3 shows that the Pt(IV) complex containing Val and Cin in the axial positions significantly increased HDAC and MMP-2 and -9 inhibitory activities compared to CDDP. The treatment of

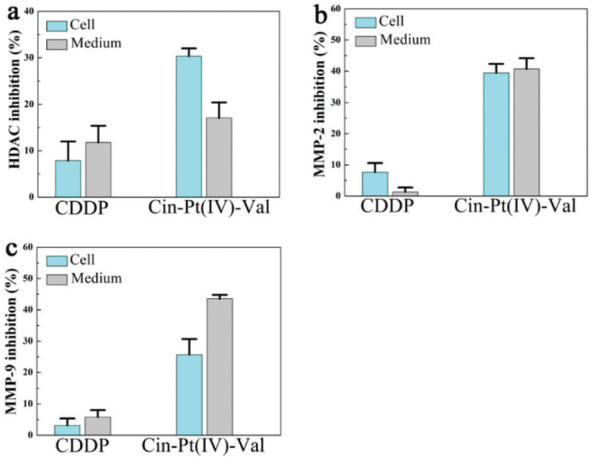

Fig. 3 Enzyme inhibitory activity, (a) HDAC, (b) MMP-2 and (c) MMP-9. After incubation of 24 h, the inhibitory activity was determined using an ELISA kit. Data are the means of at least three independent experiments, and error bars indicate SD.

MB49 cells with the tested complex (Val as an axial ligand) induced two-fold higher HDAC inhibitory activity than that of the conditioned media (Fig. 3a), whereas Cin in another axial position was about equally effective at inhibiting MMP-2 activity (Fig. 3b). Notably, in contrast to the treated cells, the conditioned media exhibited a higher MMP-9 inhibitory activity (Fig. 3c), implying that the tested Cin-Pt(IV)-Val complex efficiently prevented MMP-2 and -9 proteinases from digesting the various structural components of the extracellular matrix, thereby blocking tumor cell invasion and metastasis. The HDAC and MMP-2 and -9 inhibitory activities of Cin or Val released upon the intracellular reduction of the Cin-Pt(IV)-Val complex along with CDDP, which was bound directly to nuclear DNA, consequently enhanced the extent of DNA damage by platinum adducts and in this way potentiated the anticancer activity.

Cell cycle distribution and apoptosis

To further elucidate the cellular mechanism of the Cin-Pt(IV)-Val complex, the cycle distribution of MB49 cells treated with Pt(IV) complexes was analyzed by flow cytometry using untreated cells and CDDP-treated cells as controls. As shown in Fig. 4a and Fig. S12,† exposure to CDDP led to the accumulation of cells mainly in the S phase with a simultaneous increase in G2/M populations, in accordance with the previously published results.[36] After treatment with Pt(IV) complexes, the cells were arrested in the S-phase similar to those of CDDP. However, the population in the S phase decreased compared to that after treatment with CDDP, such that a significantly higher amount of cells were able to proceed and stop in the G2/M phase, except those cells treated with the Cin-Pt(IV)-Cin complex without significant population change. Notably, it has been demonstrated that free Cin also reduced the population in the S-phase inducing G2/M arrest.[14] Therefore, the investigated Pt(IV) complex suggested the effect

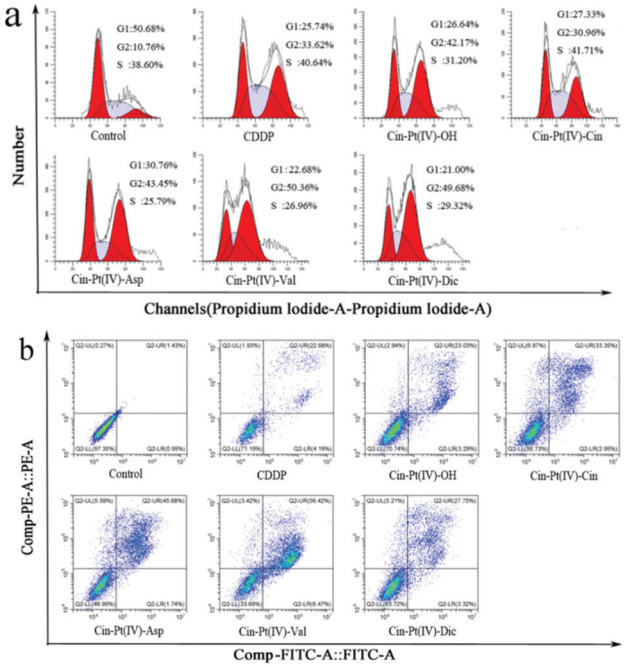

Fig. 4 (a) Cell cycle distribution, (b) cell apoptosis analysis using Annexin V-FITC/PI staining. MB49 cells were treated with 3 μM CDDP and Pt(IV) complexes for 24 h.

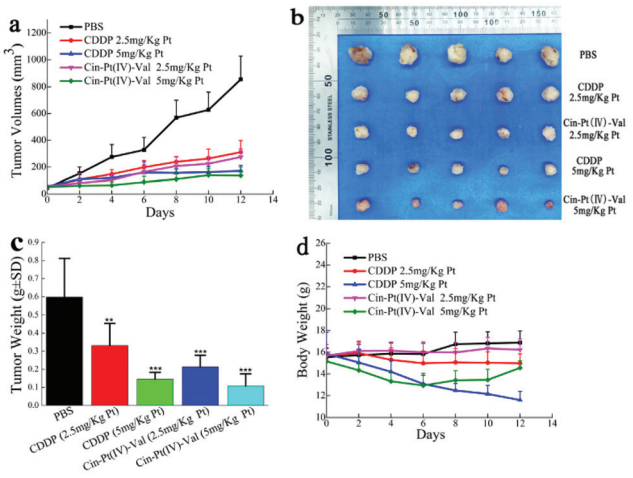

Fig. 5 *In vivo* anti-tumor activity on 4T1 tumor xenografts. (a) Tumor volumes, (b) the tumor images, (c) the tumor weight, and (d) the body weight of mice. ***P < 0.001 and **P < 0.01 compared to the PBS group.

on the cell cycle combines, to some extent, the attributes of both parental CDDP, Cin and Val, supporting the hypothesis that the Cin-Pt(IV)-Val complex acts by a mechanism that was entirely different from that of parental CDDP and involves the effects of the released biologically active axial ligands.

MTT assay based on cells cannot often distinguish the cytostatic and cytotoxic effects of the tested agents.[37] Therefore, we used an annexin V-FITC/propidium iodide (PI) double staining assay to investigate whether the treatment of MB49 cells with the Pt(IV) complex could also induce cell death. As shown in Fig. 4b and Fig. S13,† we found that the treatment of cells with the Pt(IV) complex induced an increase in the apoptotic cell population not only in the early period but also in the late period compared to those of CDDP, indicating apoptosis as a predominant mode of cell death. Notably, the Cin-Pt(IV)-Val complex induced 56.42% of MB49 cells to enter late apoptosis, while CDDP induced 22.68% of the cells to enter late apoptosis, thus verifying the strong proapoptotic ability of the tested Cin-Pt(IV)-Val complex.

In vivo anti-tumor activity

The anti-tumor efficacy of the Cin-Pt(IV)-Val complex *in vivo* was further assessed on female BALB/c mice models bearing 4T1 tumor xenografts. The mice were randomly divided into 5 groups: (1) PBS; (2) CDDP (2.5 mg per kg Pt); (3) Cin-Pt(IV)-Val (2.5 mg per kg Pt); (4) CDDP (5 mg per kg Pt); and (5) Cin-Pt(IV)-Val (5 mg per kg Pt) and were administered intravenously with the above formulations once every 3 days for 4 times. The tumor volumes were measured for 12 days consecutively every 2 days. As shown in Fig. 5a, among the all tested samples, the Cin-Pt(IV)-Val (5 mg per kg Pt) group suppressed the growth of tumor xenografts by 89.5% compared to the PBS group, leading to superior anti-tumor activity. The dissected tumor tissues with the indicated treatments were weighted and photographed (Fig. 5b and c). After 12-day treatments, the tumor size and weight suggested that the Cin-Pt(IV)-Val group could significantly relieve the disease burden by delaying tumor growth. Notably, the Cin-Pt(IV)-Val (2.5 mg per kg Pt) group was hardly affected by the bodyweight and the swing of the mood of the mice. When the dose increased to 5 mg per kg Pt, the body weights of tumor-bearing mice decreased before 6 days but then kept growing, implicating the presence of a very low systemic toxicity (Fig. 5d). In contrast, the CDDP group, particularly treated with 5 mg per kg Pt, appeared to suffer from significant weight loss, marked lethargy and hematochezia. It is notable that systemic toxicities of CDDP, such as hepatotoxicity and nephrotoxicity, are likely associated with Pt accumulation in normal tissues.[32] Therefore, we determined that Pt accumulates in normal tissues after treatment with CDDP and the Cin-Pt(IV)-Val complex using the same dose (5 mg per kg Pt) by ICP-MS. The results showed that the Pt content of the Cin-Pt(IV)-Val complex was lower than that of CDDP in the heart, spleen and kidneys, especially in the kidneys, demonstrating that the Cin-Pt(IV)-Val complex efficiently alleviated the nephrotoxicity caused by CDDP (Fig. S14†). A relatively high Pt accumulation in the liver was observed, which was in agreement with the previously reported results.[32,38] Inspiringly, it was found that Pt accumulation in the tumor tissue was 7.0-fold higher than that of CDDP, thereby contributing to improve the anti-tumor activity of CDDP.

The toxicity was further evaluated by the histological images of major organs including the heart, liver, spleen, lung, kidneys and tumor treated with varied complexes *via* H&E staining on the 12th day. The major organs showed no appreciable abnormality or noticeable tissue damage after treatment with the Cin-Pt(IV)-Val complex (Fig. 6). This might

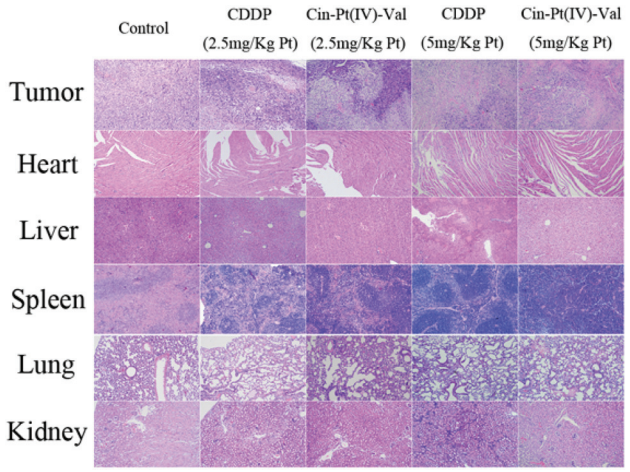

Fig. 6 The H&E stained images of paraffin sections from the heart, liver, spleen, lung, kidneys and tumor tissues of various treatment groups. Image magnification: 100×.

be related to the fact that Cin provided protection to normal cells from the toxic effects of CDDP.[11] Serious damage to the kidneys was observed in mice treated with different doses of CDDP, in agreement with the loss of body weight. The relatively visible morphology changes of tumor cells such as necrosis were also observed in both the Cin-Pt(IV)-Val and CDDP-treated groups. Taken together, the conjugation of two biologically active moieties to CDDP might provide a promising strategy for the development of efficient and low-toxic multi-action Pt-based anticancer drugs.[39]

Mechanism of action

Based on the results obtained in this study, we supposed that the Cin-Pt(IV)-Val complex exerted anti-tumor effects by a synergistic mechanism as shown in Fig. 7. The tested Cin-Pt(IV)-Val complex, upon entering the cancer cells, was reduced by intracellular reductants such as GSH and AsA, thereby releasing the Pt(II) moiety along with biologically active ligands (Cin and Val) that synergistically act with the Pt(II) moiety in tumor cells. Owing to the high lipophilicity, platinum from the Cin-Pt(IV)-Val complex was taken up by the MB49 cells more efficiently than the platinum from CDDP. Inside the tumor cells, the Pt(II) moiety covalently binds to nuclear DNA to form a chimeric adduct, which induced DNA damage, thus leading to tumor cells apoptosis. Meanwhile, the released Val moiety inhibited HDAC activity, thereby increasing the accessibility of DNA to Pt(II) moiety by decondensing chromatin[25] and consequently potentiating the anti-tumor activity of the Pt(II) moiety. In addition, the released Cin moiety inhibited MMP-2 and 9 activities, and subsequently preventing them from digesting the various structural components of the extracellular matrix, thus blocking tumor cell invasion and metastasis. Concurrently, Cin also protects normal cells from the toxic effects of CDDP.[14] Obviously, the Cin-Pt(IV)-Val complex is a multi-action prodrug. The *in vitro* and *in vivo* experiments support the view that the investigated triple-action Cin-Pt(IV)-Val complex act *via* a synergistic mechanism, which involves the effects of CDDP as well as the effects of biologically active axial moieties, thus jointly leading to the death of tumor cells.

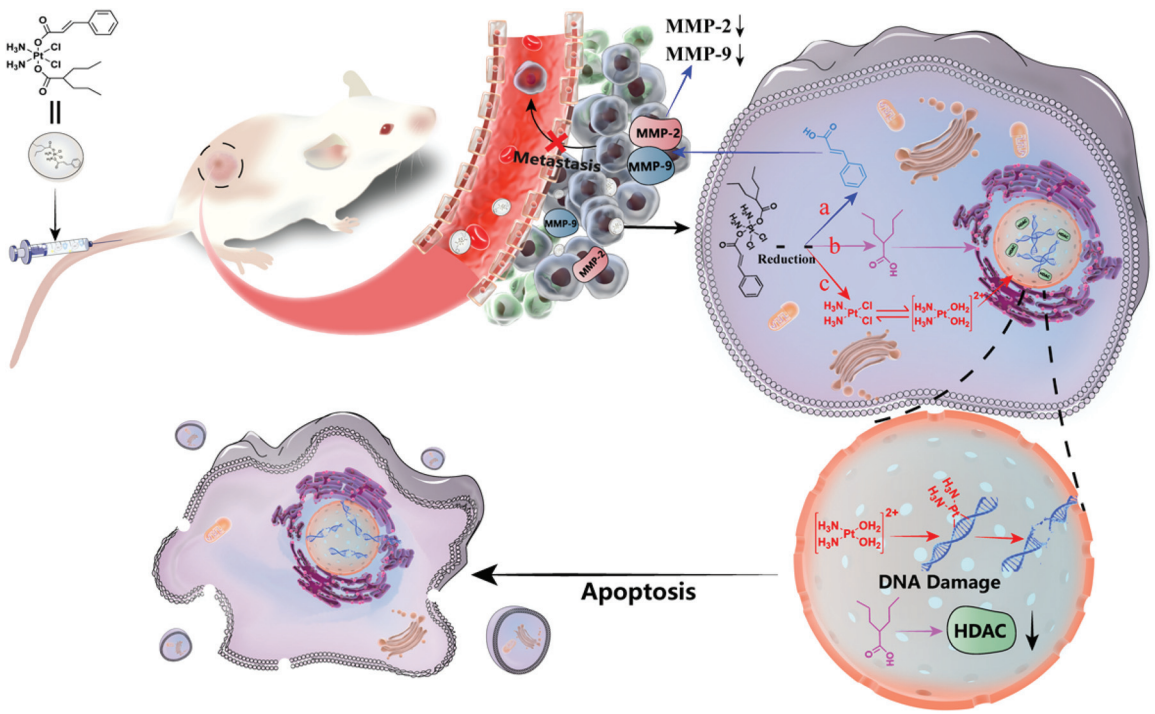

Fig. 7 The proposed synergistic anti-tumour mechanism of the Cin-Pt(IV)-Val complex.

Conclusions

In this study, we present new Pt(IV) derivatives of cisplatin bearing valproic acid and cinnamic acid as axial ligands. These Pt(IV) complexes were designed to prepare prospective multi-action Pt-based drugs efficiently for killing tumour cells. The *in vitro* biological assay demonstrated the superior antiproliferative activity of these Pt(IV) derivatives against a panel of cancer cell lines of various origins, particularly in the case of the Cin-Pt(IV)-Val complex, which exhibited antiproliferative activity at sub-micromolar concentrations. The Cin-Pt(IV)-Val complex was confirmed to cause tumour cell arrest at the G2/M phase of the cell cycle, and specifically inhibit the HDAC and MMP-2 and -9 activities as well as increase lipophilicity that enhances cellular accumulation. *In vivo*, the Cin-Pt(IV)-Val complex significantly inhibited tumour growth and efficiently overcame serious kidney injuries of CDDP. These results obtained in this study also supported the hypothesis that the investigated Cin-Pt(IV)-Val complex, upon entering the cancer cells, was reduced by intracellular reductants, thereby releasing the DNA-damaging Pt(II) moiety along with biologically active and synergistic acting ligands and exemplified the mode of action of the multi-action Cin-Pt(IV)-Val complex, suggesting that all three moieties contributed to the killing of the tumour cells and not just one dominant component. Owing to its improved anti-tumour efficacy, the Cin-Pt(IV)-Val complex has the potential to be further developed as a potential chemotherapeutic agent instead of combined chemotherapy.

Author contributions

Conceptualization: Yang Li, Shan Shi, Wei Li. Data curation: Yang Li and Shan Shi. Formal analysis: Xudong Zhao and Zongjie Gan. Funding acquisition: Wei Li. Investigation: Shurong Zhang, Xin Wang, and Yijian Zhu. Methodology: Yang Li, Shan Shi, and Xin Wang. Project administration: Wei Li. Resources: Xudong Zhao, Shurong Zhang, and Wei Li. Software: Yang Li, Shan Shi, and Xin Wang. Supervision: Yang Li, Shan Shi, and Wei Li. Validation: Xin Wang, Meiting Cao, and Xiaoyue Wang. Roles/writing – original draft: Yang Li and Shan Shi. Writing-review and editing: Wei Li.

Conflicts of interest

The authors declare no conflict of interest.

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