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COMMUNICATION

Monochalcoplatin: an actively transported, quickly reducible, and highly potent Pt(IV) anticancer prodrug

Lili Ma, *Na Wang*,‡ *Rong Ma*,‡ *Cai Li*, *Zoufeng Xu*, *Man-Kit Tse*, and *Guangyu Zhu**

Abstract: Recently, Pt(IV) prodrugs have attracted much attention as the next generation of platinum-based antineoplastic drug candidates. Here we report the discovery and evaluation of monochalcoplatin, a monocarboxylated Pt(IV) prodrug that is among the most cytotoxic Pt(IV) prodrugs to date. Compared with its dicarboxylated counterpart chalcoplatin, monochalcoplatin astonishingly effectively and rapidly accumulates in cancer cells, which is not ascribed to its lipophilicity. The prodrug is quickly reduced, causes DNA damage, and induces apoptosis, resulting in superior cytotoxicity with IC_{50} values in the nanomolar range in both cisplatin-sensitive and -resistant cells, which is up to 422-fold higher than cisplatin. A detailed mechanistic study reveals that monochalcoplatin actively enters cells through a transporter-mediated process. Moreover, monochalcoplatin shows significant antitumor activity in an *in vivo* colorectal tumor model. Our study implies a practical strategy for the design of more effective Pt(IV) prodrugs to conquer drug resistance by tuning both cellular uptake pathways and activation processes.

Among non-conventional platinum-based anticancer agents that do not obey the original "structure-activity relationships" of cisplatin, Pt(IV) prodrugs have recently shown significantly promising antineoplastic activity.[1] The octahedral geometry of the Pt(IV) center endows the metal with inertness before entering cells, and the reducing environment within cancer but not normal cells allows for the efficient activation of the prodrugs, releasing the reduced Pt(II) moiety to induce DNA damage.[2] These properties make these prodrugs able to have reduced side effects, to achieve oral availability, and to overcome cisplatin resistance. Previous inspiring work on Pt(IV) prodrugs mainly focused on the modification of axial ligands to tune lipophilicity or kinetics, the application of drug carriers to target cancer cells, and the generation of dual- or multi-targeting Pt(IV) complexes using bioactive moieties to achieve enhanced pharmacological effects.[2c, 3]

In the process of obtaining novel platinum anticancer agents, we have synthesized and biologically evaluated chalcoplatin, a "dual-targeting" Pt(IV) prodrug containing two moieties of chalcone, which is an inhibitor of p53-MDM2 interaction, in the axial position (Figure 1).[4] Compared to cisplatin, chalcoplatin shows elevated cytotoxicity in the micromolar range in p53 wild-type human cancer cells, and this Pt(IV) prodrug enters cells more efficiently than cisplatin, likely due to its lipophilicity. Chalcoplatin significantly stabilizes p53 and triggers downstream apoptotic pathways. The prodrug displays a distinctive mode of action, further indicating the role of the p53 agonist.

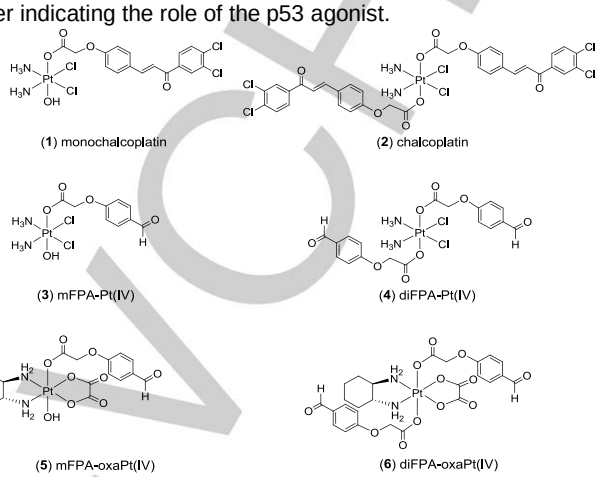

Figure 1. Chemical structures of compounds **1**-**6**.

Although different Pt(IV) prodrugs with bioactive axial ligands have been prepared, few efforts have been put forward to design Pt(IV) prodrugs with transporter-mediated cellular uptake mechanisms and instant activation processes. Here we report our discovery that monochalcoplatin, a monocarboxylated Pt(IV) prodrug, but not chalcoplatin or other analogues, is actively/facilitated transported into cells and rapidly reduced, which leads to significant cell death in a short time frame. Our finding points to the importance of designing small-molecule non-functionalized Pt(IV) prodrugs with unique structures that can be actively transported into cells and subsequently quickly activated, which lies in the first two steps of their mechanism of action. By tackling these factors, downstream processes including DNA damage and cellular responses are strengthened and the anticancer efficacy especially in cisplatin-refractory tumor is dramatically enhanced.

We first synthesized monochalcoplatin as well as several other chalcoplatin analogues (Figures 1). As the building block of chalcone, 4-formylphenoxyacetic acid was used as the axial ligand as well. To synthesize monocarboxylated Pt(IV) compounds **1**, **3**, and **5**, the carboxyl groups of ligands were activated by 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDCI)/*N*-hydroxysuccinimide (NHS) to yield NHS esters, which subsequently reacted with *c,c,t*-$[Pt(NH_3)_2Cl_2(OH)_2]$ or $[Pt(DACH)(ox)(OH)_2]$ (Figures S1 and S2).[2b] The dicarboxylated Pt(IV) compounds **4** and **6** were obtained as well (Figure S2). These compounds were fully characterized by ^1H, ^{13}C, ^{195}Pt NMR, ESI-MS, and CHN elemental analysis (Figures S4-S22). The stability of compounds **1**–**6** in PBS was confirmed by RP-HPLC (Figure S23).

Cytotoxicities of these Pt(IV) compounds were first screened in A2780 cisplatin-sensitive and A2780cisR cisplatin-resistant ovarian human cancer cells (Table S1). Oxaliplatin-based Pt(IV) compounds are significantly less active than their corresponding

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cisplatin-based ones. For cisplatin-based scaffolds, the dicarboxylated Pt(IV) complexes are less potent than their monocarboxylated counterparts, no matter the axial ligand is chalcone or 4-formylphenoxyacetic acid. Among these compounds, monochalcoplatin displays the highest cytotoxicities. We, therefore, focused on this monocarboxylated Pt(IV) complex in the following tests.

A detailed cytotoxicity test of cisplatin, chalcoplatin, and monochalcoplatin was performed. Besides A2780 and A2780cisR cells, other p53 wild-type human cancer cells including A549, MCF-7, HCT116, and HeLa were utilized. Cisplatin-resistant A549cisR cells were also included. The half maximal inhibitory concentration (IC_{50}) values of cisplatin for 72 h treatment are in the typical micromolar range in the cisplatin-sensitive cell lines, and the drug is not significantly active in the resistant cell lines (Table 1). Chalcoplatin is more active than cisplatin in most of the cells tested, although its IC_{50} values are still in the micromolar range. Monochalcoplatin is significantly more active than both cisplatin and chalcoplatin, and the IC_{50} values of monochalcoplatin are in the nanomolar range in all the cells tested. For example, the IC_{50} values of chalcoplatin in A2780 and A549 cells are 830 and 1900 nM, respectively, while those of monochalcoplatin are as low as 10 and 80 nM, respectively. Compared with cisplatin, monochalcoplatin displays up to a 422-fold increase in cytotoxicity in the cells tested. Thus, monochalcoplatin is among the most cytotoxic Pt(IV) prodrugs to date. Furthermore, monochalcoplatin is able to overcome cisplatin resistance, and the IC_{50} values are 70 and 140 nM in A2780cisR and A549cisR cells, respectively. In A549 and A549cisR cells, the resistant factors (RF), defined as the ratio of the IC_{50} value in cisplatin-resistant cells to that in cisplatin-sensitive cells, is 4.3 for cisplatin, and the value decreases to 1.8 for monochalcoplatin. A similar trend is also found in A2780 and A2780cisR cells (Table 1). Although monochalcoplatin shows striking cytotoxicities in cancer cells, the selectivity index (SI) of monochalcoplatin is comparable to that of cisplatin. Additionally, monochalcoplatin exhibits excellent cell-killing effect in a 3-D spheroid model generated from MCF-7 cells (Figure S24).

The ability of monochalcoplatin to induce apoptosis and to bind to nucleotides was subsequently examined. Monochalcoplatin, but not cisplatin and chalcoplatin, was able to induce apoptosis after 6 h (Figure S25).[5] After the treatment of 10 μM monochalcoplatin for 1 h, the Pt levels in genomic DNA are 13 ± 3 and 19 ± 3 ng Pt/10^6 cells in A2780 and A2780cisR cells, respectively. To further reveal whether monochalcoplatin was able to cause DNA damage within a short time, the expression levels of γH2A.X were examined by immunoblotting (Figure S27). H2A.X plays a key role in DNA repair, and phosphorylation of Ser 139 will occur to form γH2A.X rapidly after the formation of DNA double strand breaks (DSBs).[6] Compared to cisplatin, monochalcoplatin evokes a remarkably increased level of γH2A.X in A2780 cells after 4 h, and a similar tendency was observed after 6 h.

Table 1. IC_{50} values (nM) of Pt compounds. Cells were treated for 72 h and cell viability was determined by MTT assay.

Cell lines	cisplatin	chalcoplatin	mono-chalcoplatin	FI[a]
A2780	1320 ± 490	830 ± 570	10 ± 3	132
A2780cisR	17425 ± 4375	1590 ± 250	70 ± 10	249
RF[b]	12	1.9	7.0	----
A549	1380 ± 160	1900 ± 700[d]	80 ± 10	17
A549cisR	5880 ± 420	1600 ± 500[d]	140 ± 10	42
RF	4.3	0.84	1.8	----
MCF-7	18020 ± 400	4800 ± 1000[d]	240 ± 8	75
HCT116	9703 ± 793	800 ± 400[d]	23 ± 6	422
HeLa	9080 ± 3200	1500 ± 800[d]	180 ± 10	50
MRC-5	2760 ± 420	9000 ± 400[d]	120 ± 20	23
WI38	3220 ± 800	620 ± 200	50 ± 20	64
SI[c]	2	4.7	1.5	----

[a] FI (fold increase) is defined as IC_{50}(cisplatin)/IC_{50}(monochalcoplatin)
[b] RF (resistant factor) is defined as IC_{50} in A2780CisR/IC_{50} in A2780 or as IC_{50} in A549cisR/IC_{50} in A549
[c] SI (selectivity index) is defined as IC_{50} in MRC-5/IC_{50} in A549
[d] Data were cited from *Chem. Commun.*, 2015, **51**, 6301

The cell-based assays mentioned above confirm that monochalcoplatin quickly induces apoptosis and is significantly cytotoxic but they do not reveal the reason. We next examined reduction processes because such properties may significantly influence the cytotoxicity of Pt(IV) prodrugs. Cyclic voltammetry measurement shows that monochalcoplatin and chalcoplatin have E_p values of -0.85 V and -0.73 V (vs. Fc^+/Fc= +0.05 V), respectively (Figure S28).[4] The similar and moderately low reduction potentials of monochalcoplatin and chalcoplatin imply that they are not easily reduced before their cellular entrance.

Although chalcoplatin and monochalcoplatin have similar reduction potentials, their activation process may not be exactly the same. Indeed, the reduction speed is also a factor mediating the anticancer activity of Pt(IV) prodrugs.[7] We assume that monochalcoplatin may be quickly reduced from Pt(IV) to Pt(II) inside the cells to execute its DNA-damaging function. To corroborate our hypothesis, the Pt(IV) prodrugs were incubated in and without the presence of sodium ascorbate (NaAs), a cellular reducing agent, and the fractions of intact Pt(IV) prodrug were quantified by RP-HPLC (Figures 2 and S29). Both complexes are stable without NaAs, and chalcoplatin is slowly reduced in the presence of NaAs. In striking contrast, monochalcoplatin is reduced very quickly under this condition. After only 2 h, the percentage of Pt(IV) form remained drops to 71%, and the value decreases to 50% after 6 h. This instant activation process may contribute to monochalcoplatin's quick mode of action, although there is a risk of reduction in the blood stream.

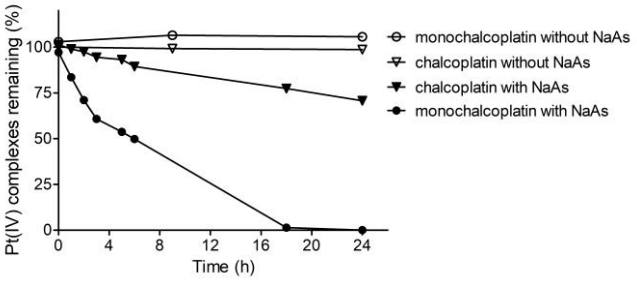

Figure 2. RP-HPLC analysis of remaining Pt(IV) complexes. 50 μM Pt(IV) complexes were incubated in and without the presence of 2 mM sodium ascorbate in a solution of PBS/MeOH (9/1, V/V, pH 7.4) at 37 °C in dark.

Another common reason for improved cytotoxicity of a Pt drug is increased cellular uptake. The Log$P_{o/w}$ values of chalcoplatin and monochalcoplatin are determined to be 1.39 ± 0.06 and 1.29 ± 0.03, respectively. The monocarboxylated Pt(IV) prodrug is slightly less lipophilic than its dicarboxylated counterpart due to

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monosubstitution and the remaining hydroxyl group on the Pt(IV) moiety,[8] indicating that the cellular entrance of monochalcoplatin by just passive diffusion may not be as efficient as that of chalcoplatin. Next, Pt accumulation in A2780 and A2780cisR cells upon treatment with 10 μM Pt compounds for 6 h was studied (Figure 3A, B). Cisplatin treatment results in 5.2 and 2.1 ng Pt/10^6 cells in A2780 and A2780cisR cells, respectively, and the numbers increase to 18.1 and 9.9 ng Pt/10^6 cells, respectively, for chalcoplatin-treated cells. These results are consistent with our previous work under the same condition.[4] In stark contrast, monochalcoplatin accumulates much more efficiently. The levels of Pt are 189 and 229 ng Pt/10^6 cells in A2780 and A2780cisR cells, respectively, showing a 36 and 111-fold increase over cisplatin. A similar trend is also observed in A549 cells (Figure S30), and the concentration of Pt inside the cells is estimated to be 2.4 mM based on the cell volume,[9] which is much higher than the treatment concentration, indicating an active transport process. These levels are among the highest for currently known Pt(IV) prodrugs in the same cell lines in a similar time frame.[2c, 10] This greatly enhanced accumulation of monochalcoplatin might be partially related to the rapid reduction and subsequent aquation processes that lead to Pt trapping. This significantly elevated cellular levels of Pt are believed to be one of the major reasons for monochalcoplatin's remarkable cytotoxicity.

Further investigation was undertaken to reveal the possible cellular entrance pathways of monochalcoplatin. The compound shares similar lipophilicity with chalcoplatin but the accumulation is astonishingly higher, showing that monochalcoplatin may enter cells via energy-dependent pathways including active transport and endocytosis. To investigate these possibilities, A2780 and A2780cisR cells were treated with 10 μM chalcoplatin or monochalcoplatin for 1 h at either 37 °C or 4 °C and Pt levels were assessed. Statistically significantly decreased Pt levels were found at 4 °C compared to 37 °C, indicating the role of energy-dependent transport.[11] For example, the Pt levels in monochalcoplatin-treated A2780cisR cells are 86 ± 23 and 5.6 ± 1.0 ng Pt/10^6 cells at 37 °C and 4 °C, respectively (Figure 3C, D). The possibility of endocytosis and macropinocytosis was subsequently determined, as these processes are responsible for the uptake of certain types of metal complexes.[12] Pretreatment with wortmannin (inhibition of endocytosis and macropinocytosis by blocking phosphoinositide 3-kinase)[13] does not significantly alter the level of Pt accumulation for chalcoplatin or monochalcoplatin. These results indicate that active transport but not passive diffusion or endocytosis/macropinocytosis is the primary uptake route for monochalcoplatin. Our initial experiment using copper transporter 1 (Ctr1)-knockout cells has shown that Ctr1 is not responsible for the uptake of monochalcoplatin (Figure S31), although it plays roles in the cellular accumulation of Pt in cisplatin.[14] We also measured the cellular accumulation of oxaliplatin-based Pt(IV) compounds bearing one or two chalcone axial ligands (compounds **7** and **8**, Figure S3). These mono- and di-carboxylated Pt(IV) compounds have similar levels of Pt accumulation in A2780 cells, which are significantly lower than that of monochalcoplatin (Figure S32). Thus, the remarkable cellular accumulation of monochalcoplatin is more likely due the unique structure of the entire molecule.

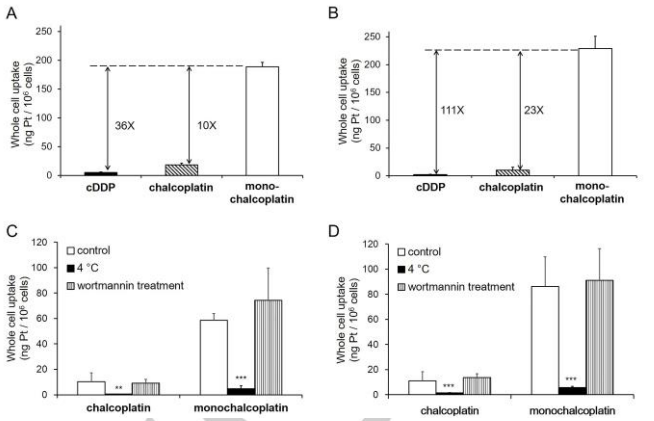

Figure 3. Cellular accumulation of cDDP, chalcoplatin, and monochalcoplatin. (A) A2780 cells, 6 h treatment; (B) A2780cisR cells, 6 h treatment; (C) A2780 cells, 1 h treatment; (D) A2780cisR cells, 1 h treatment. **, p< 0.01; ***, p< 0.001.

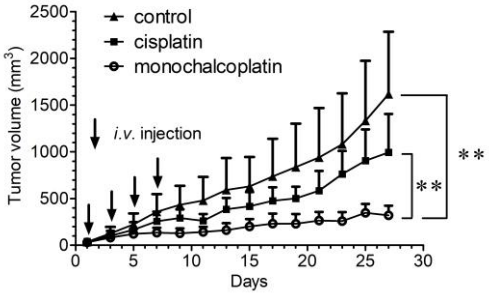

Figure 4. The growth of HCT116 tumor in xenograft model with different treatment. The xenograft tumor model was established in BALB/c nude mice by subcutaneous injecting HCT116 colon carcinoma cells. The indicated compounds were injected intravenously (i.v.) every two days for 4 times totally, including monochalcoplatin (0.72 mg-Pt/kg), cisplatin (0.95 mg-Pt/kg), and vehicle (1% DMF and 1% Tween-80 in 0.9% NaCl) as control. Mean ± SD, n= 5. **, p< 0.01. Student's t-Test.

The antitumor efficacy of monochalcoplatin *in vivo* was further demonstrated. The *in vitro* results show that monochalcoplatin has significantly higher cytotoxicity than cisplatin in HCT116 cells. Therefore, a xenograft tumor model was established in BALB/c nude mice by subcutaneous injecting HCT116 colon carcinoma cells. Each of five HCT116-transplanted mice received vehicle, cisplatin (0.95 mg-Pt/kg), or monochalcoplatin (0.72 mg-Pt/kg) intravenously (i.v.) every two days for total four treatments. Compared to the control group, cisplatin-treated group has no significant difference in tumor volume. On day 27, the average tumor volume is 993.9 ± 367.6 mm^3 upon the treatment of cisplatin, and the value is 1617.0 ± 596.7 mm^3 for the control group. Remarkably, monochalcoplatin effectively suppresses the tumor growth. The average tumor volume in monochalcoplatin-treated group after 27 days is 320.2 ± 91.7 mm^3, which is 32% of that in cisplatin-treated group (p< 0.01), and 20% of that in the control group (p< 0.01) (Figure 4). After termination of the experiments, the tumor of each animal was collected and the weight was recorded (Figure S33). Upon the treatment of monochalcoplatin, the tumor weight decreased significantly, which is 33% and 25%

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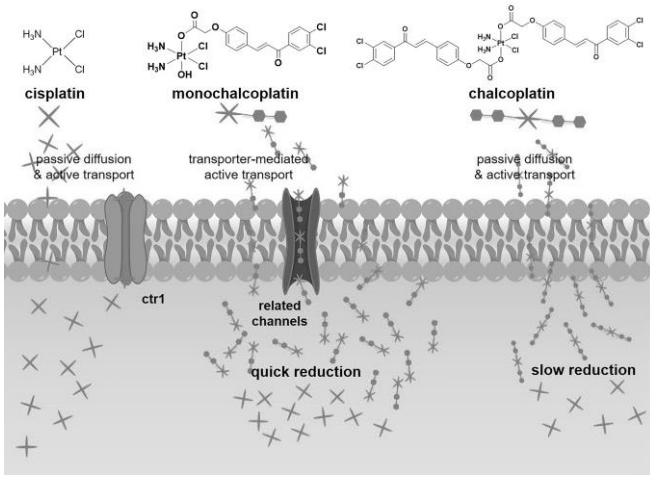
of that in the cisplatin-treated and control groups, respectively. These results clearly indicate that monochalcoplatin is highly efficient in inhibiting tumor growth *in vivo*. Furthermore, the acceptable toxicity of monochalcoplatin was confirmed by the measuring the changes of body weight, which is within 20% of decrease during the experiment (Figure S34).



Scheme 1. Proposed cellular entrance pathway and reduction process of monochalcoplatin and chalcoplatin.

Taken together, our findings suggest that monochalcoplatin enters cancer cells majorly through a transporter-mediated active transport process (Scheme 1). This monocarboxylated Pt(IV) prodrug is then quickly activated, binds to DNA at a high level, and induces p53-independent apoptosis within a short period of time. The significantly elevated cell accumulation and fast activation ultimately result in remarkable cytotoxicity especially in cisplatin-resistant cancer cells. The discovery of the potential role of transporter(s) in the cellular accumulation of a monocarboxylated Pt(IV) prodrug broadens our strategy for the design of more active Pt(IV) prodrugs utilizing transporter-mediated cellular entrance, although the details of monochalcoplatin's active transport process require further exploration. Nevertheless, we provide the first example of a monocarboxylated Pt(IV) prodrug being actively transported into cancer cells, possibly by certain unidentified transporters, and being quickly reduced in a reducing environment with implications for the design of more active Pt(IV) prodrugs to conquer cisplatin-resistance by tuning both cellular accumulation and activation processes.

Acknowledgements

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Keywords: Pt(IV) prodrugs • cisplatin • active transport • antitumor activity • prodrug activation

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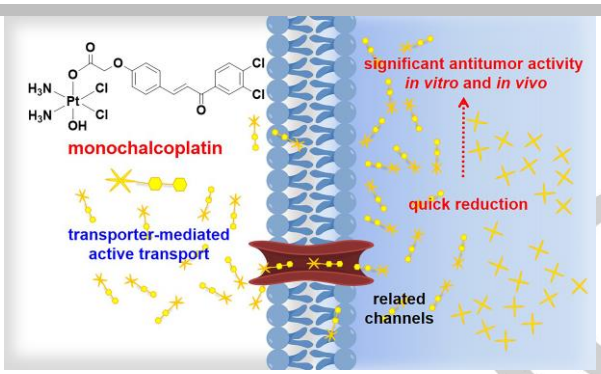
A monocarboxylated Pt(IV) prodrug is actively transported into cells and reduced promptly, resulting in nanomolar range IC_{50} values *in vitro* and effective tumor growth inhibition *in vivo*.



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