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A cancer specific oxaliplatin-releasing Pt(IV)-prodrug†

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We prepared a Pt(IV)-prodrug, which under cancer specific conditions (elevated concentration of reactive oxygen species, ROS) releases a DNA-binding drug oxaliplatin as well as ROS-amplifying drugs *p*-quinone methide and *N*-alkylferrocenium. Due to the concerted action of these components, an excellent anticancer effect was achieved: IC_{50} = 0.4 ± 0.1 μM for human ovarian carcinoma A2780 cells. Importantly, the prodrug was found to be 45-fold less toxic to normal cells (HDFa).

Cancer is the second-ranked cause of death in developed countries after cardiovascular diseases.[1,2] It is caused by transformed cells, which usually grow quicker and undergo division more often than normal cells. Therefore, these cells rely strongly on gene expression and can be suppressed by replication/transcription inhibitors. The compounds of the latter type are prominently represented in clinically approved anticancer drugs, including *e.g.* Pt(II)-complexes cisplatin, carboplatin and oxaliplatin as well as several regionally approved analogues.[3–5] Despite the excellent activity of the Pt(II) drugs, they also exhibit a range of dose-limiting side effects. That is at least partially caused by their detrimental effects on a set of normal cells, which are quickly growing and often dividing, *e.g.* hair, intestinal and bone marrow cells. Pt(IV)-complexes have emerged as prodrugs, which are reductively activated inside cells with generation of active Pt(II) analogues.[3,6] These complexes have several advantageous properties over the parent drugs including substantial activity against cisplatin-resistant cancer cells, higher stability in the extracellular space and the potential applicability for oral drug delivery.[4–6]

We have recently described a Pt(IV)-based prodrug **1** (Fig. 1), containing protected aminoferrocene (AmFc) moieties as axial ligands.[7] In contrast to the conventional anticancer Pt(IV) prodrugs, **1** is activated specifically in cancer cells in the reaction with ROS, which are usually overproduced in transformed cells. For example, **1** was found to suppress growth and division of human ovarian carcinoma (A2780) cells with IC_{50} = 2.5 μM. However, its activity did not exceed that of cisplatin (IC_{50} 2.1 μM, $p < 0.05$), despite the fact that **1** has better cell membrane permeability and, additionally to cisplatin, releases 2 eq. of toxic $AmFc^+$ ion (analogue of $\mathbf{6}^+$) and 2 eq. of antioxidative system inhibitor (**3**). These data indicate the incomplete intracellular prodrug activation. To achieve the full potential of the recently described concept of cancer-specific anticancer Pt(IV) prodrugs,[7] further studies towards the improvement of the current system are warranted.

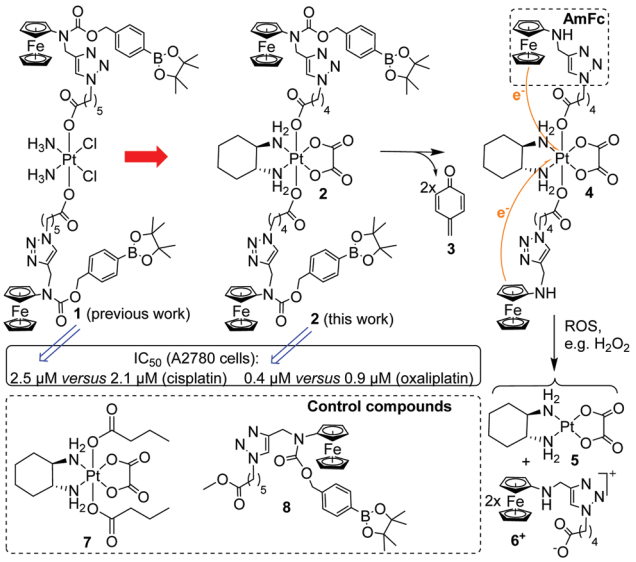

Fig. 1 Structures of known ROS-dependent Pt(IV) prodrug **1**[7] and reported in this work prodrug **2** as well as the mechanism of activation of prodrug **2** under cancer specific conditions (high ROS) with release of oxaliplatin (**5**), cationic compound $\mathbf{6}^+$ (2 eq.), which is precursor for ROS-generating catalyst **6**, and electrophilic *para*-quinone methide **3** (2 eq.). Structures of control compounds used in this work are shown in the inset.

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Herein we describe complex **2**, in which linkers between the switching AmFc units and the Pt(IV) were shortened by one methylene group. This alteration was expected to improve the intramolecular e^--transfer in the activation step (Fig. 1). Moreover, we replaced the cisplatin-generating core for its oxaliplatin analogue. Oxaliplatin (**5**) is a clinically approved drug exhibiting high activity against cisplatin-resistant cells and is safer than cisplatin.[4] Though oxaliplatin-releasing Pt(IV) analogues have been previously reported, they all are not cancer cell specific.[8–10] The mechanism of ROS-induced activation of prodrug **2** is outlined in Fig. 1. In the first step the ROS-sensitive boronic acid pinacol ester fragment is cleaved with formation of 2 eq. *p*-quinone methide **3** and complex **4** containing two AmFc fragments. The latter ones donate two electrons to the Pt(IV) center leading to formation of oxaliplatin.

Synthesis and full characterization of **2** are provided in the ESI (Fig. S1–S3).† The identity of this compound was established by ^{1}H and ^{13}C NMR spectroscopy as well as ESI mass spectrometry, whereas its purity was confirmed by elemental (C, H, N) analysis. We observed that prodrug **2** is soluble up to 48 ± 15 μM in aqueous phosphate-buffered saline (PBS, pH 7) containing 1% *N*,*N*-dimethylformamide (DMF, v/v). The solubility was not affected by addition of fetal bovine serum (FCS, 5%, m/v). Next, we found that **2** is relatively stable both in the mixture of PBS buffer/DMF (99/1, v/v; 90 ± 4% of **2** remains intact after 3 h incubation) and in the same mixture containing glutathione (GSH, 5 mM) and sodium ascorbate (Asc, 1 mM; 73 ± 5% of **2** remains intact after 3 h incubation). In contrast, addition of H_2O_2 was found to facilitate the reaction of the prodrug activation: 37 ± 1% **2** remains intact after 3 h incubation under these conditions (Fig. 2A). Since the latter conditions model the intracellular environment of cancer cells, whereas the former ones – correspondingly the extracellular space (PBS buffer) and the intracellular environment of normal cells (PBS buffer with GSH and Asc), these data indicate that **2** has a potential to target cancer cells selectively over normal ones.

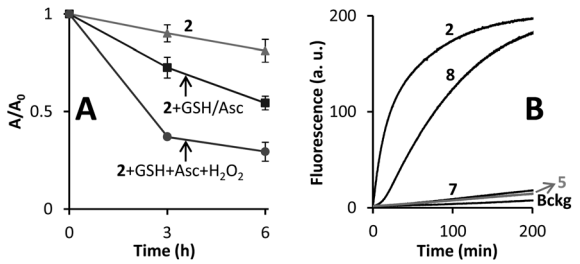

Fig. 2 (A) Changes of absorbance at 347 nm of prodrug **2** (A/A_0, where A is the current absorbance and A_0 is the absorbance of the prodrug immediately after its dissolution) dissolved either in mixture 1 containing *N*,*N*-dimethylformamide (DMF)/phosphate-buffered saline (PBS, pH 7.0 ± 0.2, 1/99, v/v) (▲) or in mixture 2 containing glutathione (GSH, 5 mM), *N*,*N*,*N*′,*N*′-ethylenediaminetetracetic acid (EDTA, 1 mM) and sodium ascorbate (Asc, 1 mM) additionally to mixture 1 (■) or in mixture 3 containing H_2O_2 (200 μM) additionally to mixture 2 (●). (B) Increase of the fluorescence intensity (λ_{ex} = 501 nm, λ_{em} = 531 nm) upon oxidation of 2′,7′-dichlorofluorescin (DCFH, 9.9 μM) by H_2O_2 (9.9 mM) either in the presence of prodrugs/controls (49.5 μM; except of **2** for which 24.75 μM was selected) or in their absence (labeled "Bckg" on the plot). Buffer: 3-(*N*-morpholino)propanesulfonic acid (MOPS, 99 mM, pH 7.5), EDTA (9.9 mM), GSH (5 mM).

According to the suggested mechanism of prodrug **2** activation (Fig. 1), 2 eq. $\mathbf{6}^+$ are released in this process. In the presence of reducing agents (GSH, 5 mM) $\mathbf{6}^+$ can be reduced with formation of the corresponding aminoferrocene derivative **6**. The latter one is able to donate an electron to H_2O_2 with formation of highly reactive HO$^\bullet$. We confirmed generation of HO$^\bullet$ in the mixture of **2** and H_2O_2 by using 2′,7′-dichlorofluorescin (DCFH) as a fluorogenic probe (Fig. 2B). Interestingly, prodrug **2** (24.75 μM or 49.5 μM based on ferrocene) was found to generate more HO$^\bullet$ than the parent prodrug **8** (49.5 μM). As expected, oxaliplatin (**5**) and its Pt(IV) derivative **7** exhibited no catalytic activity. These data corroborate the suggested mechanism of ROS-induced activation of prodrug **2** (Fig. 1).

Furthermore, we investigated the toxicity of **2** and control compounds on human ovarian carcinoma (A2780) cells (Table 1). This cell line was selected, since the toxicity data for previously reported **1** were available.[7] Moreover, cisplatin resistant version of A2780 (A2780cis) was accessible in our laboratory. We were pleased to observe that prodrug **2** (IC_{50} = 0.4 ±

Table 1 Effects of prodrugs and control compounds on viability of representative cancer (A2780, A2780cis) and normal (HDFa) cell lines as well as their selected properties

			IC_{50}[c] (μM)		
Drug	Memb. perm.[a]	log P[b]	A2780	A2780cis	HDFa
1[7]	0.73 ± 0.04	0.3 ± 0.1	2.5 ± 0.5	6 ± 1	>25
CisPt[7]	—	−2.5 ± 0.3[11,12]	2.1 ± 0.3	13 ± 1	41 ± 4
2	0.64 ± 0.09	0.45 + 0.10	0.4 ± 0.1	0.7 ± 0.2	18 ± 3
5	—	−1.39[11,12]	0.9 ± 0.1	3 ± 1	19 ± 3
7	—	0.82 ± 0.08	2 ± 1	4 ± 1	42 ± 1
8[7]	1	0.55 ± 0.05	39 ± 2	>50	>50
7/**8** (2 eq.)	—	—	2 ± 1	4 ± 1	40 ± 5

[a] Membrane permeability determined as described in the ESI. [b] Octanol/water partition coefficient determined as described in the ESI. [c] IC_{50}: concentration of a drug/control, at which half of the cells (relative the cell number in the absence of any drug) remains viable; A2780: human ovarian carcinoma cell line; A2780cis: cisplatin resistant A2780 cell line; time of incubation with prodrugs for these cell lines was 96 h; HDFa: Human Dermal Fibroblasts, adult cell line, incubation time – 48 h.

0.1 μM) was >6-fold more active towards A2780 cells than previously known analogue **1** (IC_{50} = 2.5 ± 0.5 μM). Interestingly, whereas the activity of **1** did not exceed that of cisplatin (2.5 *versus* 2.1 μM correspondingly), prodrug **2** was two-fold more potent than oxaliplatin **5** (0.4 ± 0.1 *versus* 0.9 ± 0.1 μM correspondingly, $p < 0.01$). This indicates that the activation of **2** in cells is more efficient than that of **1**. Furthermore, prodrug **2** was found to be 5-fold more active than both Pt(IV)-prodrug **7** and a mixture of **7** and prodrug **8** (2 eq.) (Table 1). Since **7** could be activated only by endogenous reducing agents or in the mixture with **8** in the intermolecular reduction by the AmFc formed from **8**, these data confirm the importance of the intramolecular reductive activation of Pt(IV) in **2** as outlined in Fig. 1.

We were pleased to observe that the anticancer activity of **2** was reduced only by 1.8-fold in cisplatin resistant cells A2780cis. Comparable resistance patterns were obtained for the Pt(IV)-control **7** (2.0-fold) and previously reported prodrug **1** (2.4-fold),[7] whereas oxaliplatin **5** (3.3-fold) and cisplatin (6.2-fold) were found to be substantially less active towards A2780cis cells. Importantly, prodrug **2** exhibited 45-fold lower activity towards representative normal cells (HDFa).

Finally, we conducted a series of cellular experiments to explore why prodrug **2** is such a potent anticancer agent. For example, we found that **2** is rather lipophilic ($\log P$ = 0.45 ± 0.10) in contrast to the parent drug oxaliplatin ($\log P$ = −1.39). Lipophilicities of Pt(IV) control **7** ($\log P$ = 0.82 ± 0.08), aminoferrocene control **8** ($\log P$ = 0.55 ± 0.08) as well as previously reported prodrug **1** ($\log P$ = 0.3 ± 0.1) were found to be either higher or comparable to that of **2**. Since the toxicity of all these control compounds towards A2780 cells is substantially lower than that of **2** and oxaliplatin (Table 1), we conclude that lipophilicity and correspondingly cell membrane permeability (for **1**, **2** and **8** the membrane permeability was experimentally determined, Table 1) do not correlate with the toxicity in the studied here series of compounds. Next, we determined the ability of the prodrugs and controls to enhance intracellular ROS in A2780 cells. We observed that, similarly to the parent prodrug **1**, prodrug **2** is a strong ROS-inducer (4.7-fold increase of ROS amount relative to that in non-treated cells), followed by aminoferrocene control **8** (77% of the activity of **2**). In contrast, all Pt controls exhibited no effect (Fig. 3A).

Thus, the increased intracellular oxidative stress can contribute to the anticancer activity of **2**. However, it cannot be considered as a dominating factor, since control **8**, which is almost as efficient ROS inducer as **2**, is substantially less toxic (IC_{50} = 39 ± 2 *versus* 0.4 ± 0.1 μM for **2**, $p < 0.0001$). Furthermore, we explored the effect of prodrug **2** and controls on cell cycle in A2780 cells (Fig. 3B). We confirmed that treatment of the cells with oxaliplatin **5** causes their arrest in the S phase (39 ± 2% *versus* 14 ± 1% for non-treated cells, $p < 0.0001$). The Pt(IV)-control **7** exhibited the same tendency, but the effect was substantially weaker (24 ± 2% cells in S phase, $p < 0.001$). In contrast, aminoferrocene **8** did not affect the cell cycle under the applied conditions. Prodrug **2** was similarly effective as oxaliplatin: 44 ± 4% cells in S phase (compare with 39 ± 2 for **5**). In contrast, previously reported prodrug **1** did not reach the effect of its drug cisplatin on the cell cycle: S phase – 29% for prodrug **1** and 38.9% for cisplatin. We observed that the amount of the cells in the S phase caused by the Pt-drugs (**2**, **5** and **7**) correlates with their toxicity towards A2780 cells (R^2 = 0.9954, Table 1, Fig. 3C).

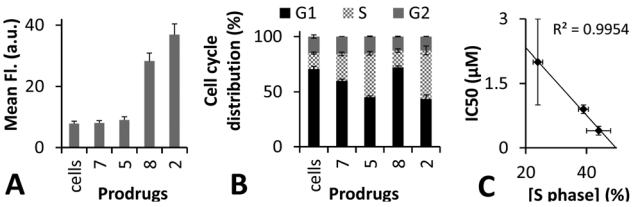

Fig. 3 (A) Mean fluorescence (Mean Fl, a.u. = arbitrary units, λ_{ex} = 488 nm, λ_{em} = 530 nm) of A2870 cells loaded with 2′,7′-dichlorofluorescin diacetate (DCFH-DA, 10 μM) and either non-treated (labelled "cells") or treated with prodrug **7**, oxaliplatin (**5**), prodrugs **8** and **2**. (B) Cell cycle distribution of A2870 cells, which were either non-treated (labelled "cells") or treated with prodrug **7**, oxaliplatin (**5**), prodrugs **8** and **2**. (C) Correlation of IC_{50} values (Table 1) with the amount of cells in the S phase for prodrug **2** and controls **5** and **7**.

In summary, we prepared a Pt(IV)-prodrug **2**, which under cancer specific conditions releases 1 eq. oxaliplatin **5** together with 2 eq. antioxidative system inhibitor **3** and 2 eq. catalyst precursor $\mathbf{6}^+$ able to induce ROS generation. The concerted action of these components leads to the excellent activity towards a representative cancer cell line A2780 (IC_{50} = 0.4 ± 0.1 μM *versus* 2.5 ± 0.5 μM for the previously reported prodrug **1**[7]). Importantly, the activity of **2** retains in cisplatin-resistant cells (A2780cis), but is reduced by 45-fold in normal cells (HDFa). We confirmed that the efficiency of the intracellular activation of prodrug **2** is higher than that of the previously reported **1** and that its anticancer effect relies mainly on the cell cycle arrest in the S phase, which is supported by the increase of the intracellular oxidative stress. The data provided in this paper represent an important milestone that will contribute to achieving a goal of the side effect-free anticancer application of Pt(IV)-based chemotherapeutic agents in the treatment of cancer diseases.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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