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Design and Delivery of Camplatin to Overcome Cisplatin Drug Resistance

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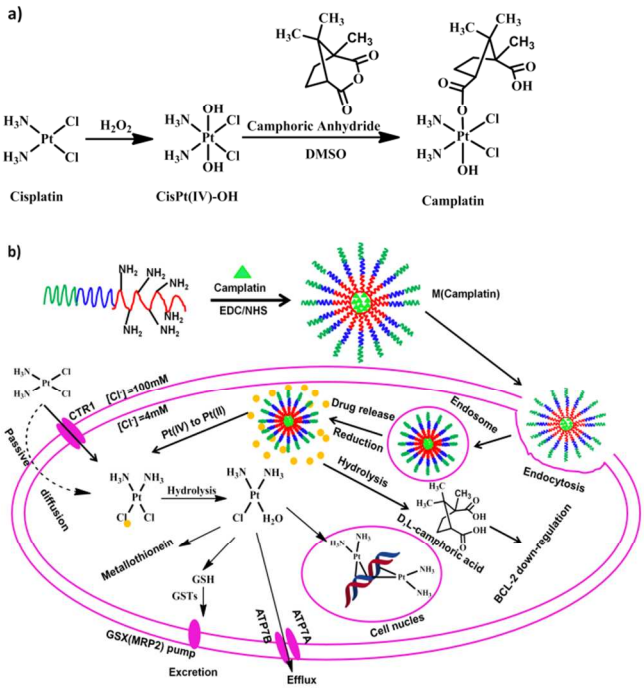
Camplatin, a prodrug formed via coining camphoric anhydride and cisplatin was delivered in biodegradable nanoparticles. This camphoric acid and cisplatin co-delivery system exhibited enhanced anticancer activity compared to cisplatin and successfully overcome cisplatin drug resistance.

Nowadays, ovarian cancer is the most common cause of gynecologic neoplasm and is the fifth cause of cancer mortality in women [1]. The five-year survival of women with stage III and IV epithelial ovarian cancer (EOC) is approximately 45% even with the most advanced surgical techniques and treatment options [1,2]. In common, platinum(II) based drugs such as cisplatin and carboplatin are the most effective drugs for ovarian cancer [3,4]. However, the biggest drawback of present preferred ovarian cancer chemotherapeutic regimen containing platinum(II) drugs is the development of drug resistance[5-7]. Mechanism of platinum drug resistance of ovarian cancer can be ascribed to reduced drug accumulation and altered cell apoptotic signalling pathway [5-7]. Another major drawback of platinum (II) drugs is that they often cause great side effects such as kidney toxicity, nausea, hearing loss as well as irreversible peripheral nerve damage[8]. Therefore, there exists a great incentive to develop much safer alternative strategies to effectively deliver platinum drugs to circumvent the cellular uptake and apoptotic cellular pathway to overcome the drug resistance [9, 10].

Camphor, which was found in old plant camphor laurel is a traditional medicine. It has long acted as slight local anesthetic and antimicrobial substance [12]. Moreover, camphor may also be administered orally in small quantities for minor heart symptoms and fatigue. Therefore, it has been used in ancient Sumatra to treat sprains, swellings, and inflammation and also be a potential anticancer drug in modern world [13].

Encoded Bcl-2 and Bax are the founding member of the cell apoptosis regulator proteins. These proteins can regulate cell apoptosis through the binding into Bcl-Bax dimer which can induce the cell apoptosis [14]. Moreover, cisplatin-mediated cell death is through an apoptotic pathway. Inhibition of this pathway by genes such as increasing the Bcl-2/Bax ratio can lead to drug resistance [15]. Actually, it is found that most cisplatin resistant cancer cells have over-expressed Bcl-2 genes but with reduced Bax genes, which reduces the drug efficiency [16]. Here, we found combination of cisplatin and camphoric acid can work in a concerted way to down-regulate the Bcl-2 levels in the resistant ovarian cancers. To further deliver the drugs in a ratiometric way, we thus designed a hybrid platinum(IV) prodrug, camplatin (Scheme 1), originated from cisplatin(II) and camphoric anhydride. Camplatin was characterized systematically and confirmed by 1H NMR, IR and ESI-MS (Figure S1-S3). The camplatin prodrug was further linked to amine groups in

the biodegradable polymers methoxyl-poly(ethylene glycol)-block-poly(ε-caprolactone)-block-poly(L-lysine) (MPEG-b-PCL-b-PLL, P) as previously described[10, 11] The as-prepared polymer-camplatin conjugate can self-assemble into micelles M(camplatin) (Scheme S1) with a mean diameter of 110 nm and 135 nm as determined by TEM and DLS, respectively. We hypothesized that the polymer micelles mediated delivery of camplatin via endocytosis can circumvent the cellular uptake pathway of cisplatin itself via passive diffusion [6], therefore enhance the drug accumulation in cancerous cells. Consequently, the camplatin internalized in the cancer cells can be fast reduced to highly toxic cisplatin and camphoric acid upon intracellular reduction and hydrolysis of the polymer chains. The released camphoric acid can down-regulate the Bcl-2 levels and cisplatin can chelate with cellular DNA. This concerted way of action makes resistant cell lines much more sensitive to clinic gold standard compound cisplatin.



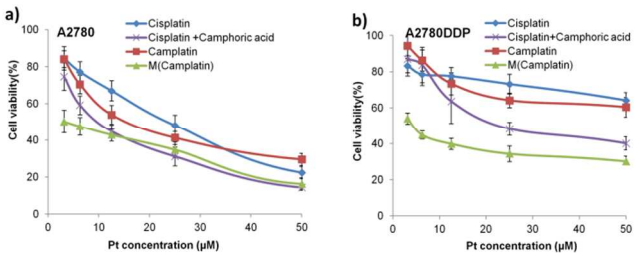
Scheme 1. Design and deliver of the hybrid cisplatin(IV) prodrug, camplatin to the cancer cells for overcoming drug resistance. Camplatin was synthesized by fusing camphoric anhydride with cisplatin (a).

Conjugation of camplatin to biodegradable polymer MPEG-b-PCL-b-PLL to prepare the polymer-drug conjugates and self-assembling the drug conjugates thereafter makes micelles M(camplatin). Cisplatin is majorly internalized by passive diffusion [6] and actively transported by copper transporter 1(Ctr1) which is a key a cell membrane protein for cisplatin uptake [7]. Approximately 1% of cisplatin will eventually bind to DNA, while most of the drugs will be detoxified by intracellular glutathione (GSH) and metallothioeins (MTs) or pumped out by ATP7A and ATP7B [6]. Whereas camplatin was internalized via endocytosis, the loaded camplatin can be reduced to cisplatin(Figure S4), leaving the axial camphoric acid with the polymer chains. Due to various reductive agents such as ascorbic acid and GSH and lower pH values in the tumor cells, camphoric acid can be eventually released. Although ascorbic acid is also used as reductants for mimicking the reduction of platinum(IV) drugs, GSH is more frequently and recongnized by the researchers around the world. Here only GSH is used to be a reductant for platinum(IV) drug activation [17,18]. In this way, camphoric acid down-regulates Bcl-2 mRNA levels and up-regulates Bax mRNA levels and eventually initiates caspase cascade reaction [6].

Representative dose dependant cell viability curves versus drug concentrations of cisplatin, camplatin and M(camplatin) are shown in Figure 1a and Figure 1b on ovarian cancer cells A2780(cisplatin sensitive) and A2780DDP cells(cisplatin resistant), respectively. Compared with A2780, A2780DDP cells display very inert response to cisplatin. Even the cisplatin concentration goes as high as 50 μM, more than 60% of cells are alive for A2780DDP, while this was ~ 20% for A2780. As shown in Figure 1c, the IC50 values of cisplatin on A2780 and A2780DDP were 23.2 and 80.1 μM, respectively, indicating a cisplatin resistance fold of ~ 3.5 for A2780DDP. Mixture of cisplatin and camphoric acid at 1:1 ratio displays IC50 values of 10.2 μM and 23.1 μM towards A2780 and A2780DDP, respectively. Compared to cisplatin, the mixture of cisplatin and camphoric acid has a much lower resistant fold of 2.3, making it rational to use the two drugs in a concerted way. As mentioned, cisplatin can cause great side effects such as kidney toxicity, nausea, hearing loss as well as irreversible peripheral nerve damage [8]. Pt(IV) drugs are much more attractive because they gain much more

chemical inertness thus have less possibility of causing serious side effects.

To make a tandem Pt(IV) prodrug that has both the mild sensitizer camphoric acid and the toxic cisplatin, camplatin was prepared. Camplatin was slightly more effective than cisplatin on A2780 (IC_{50} = 16.2 μM), but it is slightly less effective than cisplatin on A2780DDP (IC_{50} = 88.2 μM). As far as the resistance fold is concerned, it increased to even higher (~5.4 fold). This is possibly due to the incomplete uptake of a relatively larger molecule of camplatin. Moreover, incomplete reduction of Pt(IV) in camplatin to Pt(II) as cisplatin and detoxification by GSH and MTs may contribute to this. . However, M(camplatin) demonstrated highest efficacy both on A2780 (IC_{50} = 3.2 μM) and A2780DDP (IC_{50} = 5.9 μM) with a resistance fold of only ~ 1.8. M(camplatin) reverses cisplatin resistance on A2780DDP by 13.6 fold and sensitizes A2780 cells by 7.3 fold. Considering the camphoric anhydride, camphoric acid and polymer itself exhibited little toxicity to both A2780 and A2780DDP (Figure S5-S7), the results above clearly demonstrated that M(camplatin) showed great ability to both sensitize A2780 and overcome the drug resistance of A2780DDP.



c)

Drug / Cell lines	Cisplatin	Camplatin		M(Camplatin)	
		IC_{50}	Fold	IC_{50}	Fold
A2780	23.2	16.2	1.4	3.2	7.3
A2780/DDP	80.1	88.2	0.9	5.9	13.6
Resistant factor	3.5	5.4		1.8	

Fig. 1 *In vitro* evaluation of camplatin and M(camplatin) on A2780 and A2780DDP ovarian cancer cell lines. (a) Dose dependant cell viability curve of cisplatin, camplatin and M(camplatin) on A2780; (b) dose dependant cell viability curve of cisplatin, camplatin and M(camplatin) on A2780DDP; (c) IC_{50} values of cisplatin, camplatin and M(camplatin). Cisplatin is used as a positive control. For A2780, the fold in the table is called sensitizing fold; For A2780DDP, this fold is called resistance reversal fold. This fold is calculated as IC_{50} of cisplatin / IC_{50} of camplatin or M(camplatin).

Pt(II) based drugs are believed to passively diffuse into the cancer cells and then bind to cellular biomolecules (amino acid, proteins, RNA and DNA, etc) after its subsequent intracellular dissociation[19]. Recent study also revealed that Ctr1 can also actively transport cisplatin to the cancer cells [20]. Resistant cells are shown lack of this key membrane protein Ctr1 thus drug resistance arises. Whatever the uptake pathway is, it is generally believed that the efficacy of the Pt(II) drugs is majorly determined by its intracellular drug amount and the ultimate Pt-DNA adducts[20]. Considering that internalization of Pt drugs would be the first step and the most important parameter for determining drug efficacy, the cellular uptake of cisplatin, camplatin and M(camplatin) were measured. A2780DDP is reported with lower level of Ctr1 (a key cell membrane protein that regulates cisplatin uptake) than A2780 on the cell membrane [7]. Hence, less small molecules based Pt drugs (cisplatin and camplatin) would be uptaken for A2780DDP cell lines. As shown in Figure 2a, this is the case for cisplatin and camplatin (cisplatin vs. camplatin: 559 vs. 342 ng Pt/mg protein at 1 h and 1344 vs. 511 ng Pt/mg protein at 4 h for A2780 cells; 110 vs. 65.6 ng Pt/mg protein at 1 h and 191 vs. 121 ng Pt/mg protein at 4 h for A2780DDP). The results revealed that for cisplatin and camplatin, A2780DDP uptook 1/5 (1 h) and 1/7 (4 h) of cisplatin and 1/5 (1 h) and 1/4 (4 h) of camplatin that A2780 did. On the contrary, comparable amounts of M(camplatin) were internalized by A2780 and A2780DDP (1553 and 1383 ng Pt/mg protein at 1 h; 7259 and 6583 ng Pt/mg protein at 4 h). The results above suggested that the internalization of Pt drugs for M(camplatin) has no dependent on the level of Ctr1 in A2780 (high) and A2780DDP(low) as reported[7]. M(camplatin) were micellar nanoparticles thus could be internalized via endocytosis rather than via passive diffusion and Ctr1 mediated active transportation. Therefore, the cellular uptake pathway of cisplatin is circumvented by M(camplatin). Moreover, the Pt-DNA

adduct formation was also tested by measuring the amount of Pt per milligram of total genomic DNA in these two cell lines. Similar results were found on these two cancer cell lines. M(camplatin) formed much more Pt-DNA adducts than cisplatin and camplatin did. What's more the difference between the two cell lines for M(camplatin) was smaller than for cisplatin and camplatin (Figure 2b). Taken together, compared with A2780, the difference in biological characteristics (lower level of membrane protein copper transporter 1, higher level of intracellular glutathione and DNA-repair gene and proteins) of the resistant A2780DDP makes them internalize less Pt drugs and hence less Pt-DNA adducts are formed. These results indicated that the uptake and drug efficiency on drug resistance A2780 DDP cell line were significantly improved by M(camplatin).

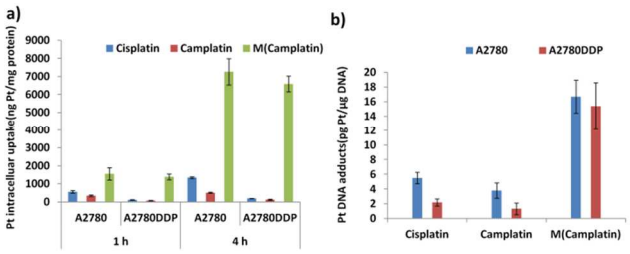


Fig. 2 (a) intracellular uptake of drugs cisplatin, camplatin and M(camplatin) after 1 h and 4 h incubation of A2780 and A2780DDP cell lines with the drugs, and (b) Pt-DNA adducts formation of cisplatin, camplatin, M(camplatin) at a final Pt concentration of 10 μM after 24 h incubation.

To elaborate how the camphoric anhydride or its acid form along with cisplatin can down-regulate cellular Bcl-2 levels in ovarian cancer cell lines and help cisplatin to overcome drug resistance, the mRNA levels of human pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) factors were traced by real-time PCR in both A2780 and A2780DDP. As shown in Figure 3, A2780DDP exhibited 2.4 fold higher Bcl-2 expression and 44% less Bax expression than A2780, which accounts for the cisplatin drug resistance of A2780DDP cell lines as previously reported [16]. Cisplatin, camphoric acid itself, cisplatin plus camphoric acid at 1:1 ratio, and camplatin down-regulate the Bcl-2 mRNA level to 86, 94, 44, and 41% of the untreated A2780DDP cells (control), respectively. However, after treatment with M(camplatin), A2780DDP cells only expressed 28% of the anti-apoptotic gene Bcl-2 with respect to the untreated A2780DDP cells (Figure 3a). On the other hand, after the treatment of M(camplatin), expression of the Bax mRNA is 2 times high compared to the control A2780DDP (Figure 3b). As previously described, the ratio of Bcl-2/Bax is the key factor of anti-apoptosis index. As shown in Figure 3c, the ratio of Bcl-2/Bax for A2780DDP without any treatment is 5.5 times higher than that of A2780, indicating the drug resistance capacity of A2780DDP cells. After treated with cisplatin, camphoric acid itself, cisplatin plus camphoric acid at 1:1 ratio, camplatin, and M(camplatin), this ratio became 2.3, 3.3, 3.1, 3.3, and 1.2, respectively. These data implied that M(camplatin) was the most effective in reversing the drug resistance of A2780DDP. Notably, the treatment with M(camplatin) just exhibited the ratio of Bcl-2/Bax for 1.8 times higher than its treatment with regular A2780, which means to overcome the drug resistance to a greater extent. Overall, the hybrid camplatin prodrug can efficiently down-regulate the anti-apoptosis Bcl-2 gene and maintain the pro-apoptosis Bax gene expression, which decrease the Bcl-2/Bax ratio to convert and induce its apoptosis.

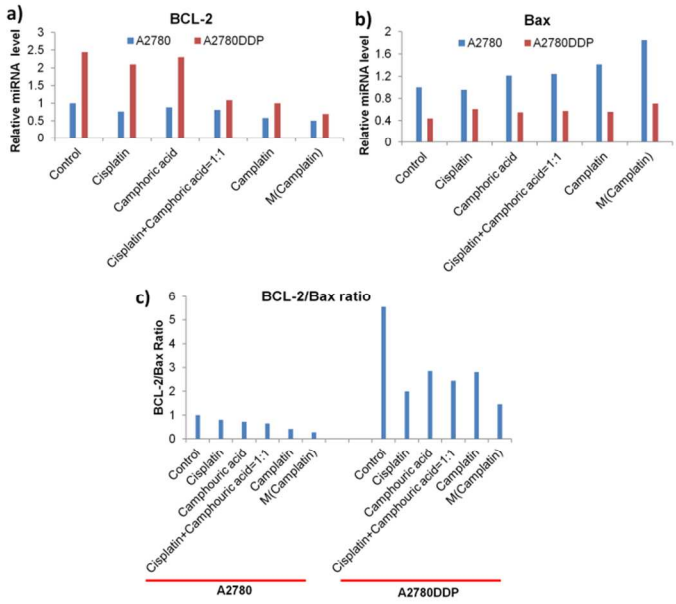


Fig. 3 (a) Bcl-2 mRNA, (b) Bax mRNA expression and (c) the ratio of the Bcl-2/Bax in A2780 and A2780DDP cell lines, respectively, after treated with cisplatin, camphoric acid cisplain+camphoric acid(1:1), camplatin and M(camplatin) at an equivalent Pt concentration of 10 μM for 24 h on A2780 and A2780DDP cell lines respectively.

Conclusions

In summary, we constructed a hybrid drug camplatin from old plant medicine camphor and a common anti-cancer drug cisplatin. The camplatin was conjugated onto the amphiphilic biodegradable polymer MPEG-b-PCL-PLL to form macromolecular prodrug M(camplatin). It displayed enhanced cytotoxicity as compared to cisplatin both on cisplatin sensitive and cisplatin resistant cell lines due to the high endocytosis rate of M(camplatin). More importantly, the hybrid camplatin and M(camplatin) efficiently down-regulated anti-apoptotic gene Bcl-2 and showed little effect on pro-apoptotic gene Bax and decrease the Bcl-2/Bax ratio, that implied the diminishment or reversal of the drug resistance to cisplatin of the A2780DDP cancerous cells. These studies are expected to yield further novel combined therapy with drug resistance cancer in clinic.

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Notes and references

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† Electronic Supplementary Information (ESI and biology experiments details) available: Experimental sections. See DOI:

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