A novel therapy for ovarian cancer using real-time imaging

ZHEN ZHANG, ZHAOJIE CHEN, BEIBEI XIE and HAIYAN ZHANG

Department of Gynaecology, Linyi People's Hospital, Linyi, Shandong 276003, P.R. China

Received September 17, 2015; Accepted September 27, 2016

DOI: 10.3892/mmr.2016.6000

Abstract. The present study was designed to develop an activable, dual-targeted theranostic platform combining fluorescent and cytotoxic templates to provide a novel strategy for specific drug delivery and cellular imaging in ovarian cancer cells. Two compounds of a folic acid-prodrug-doxorubicin (Dox) scaffold were synthesized, and their antiproliferative activities were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometric analysis. The process of drug release was investigated using fluorescence emission spectra assay and confocal laser scanning microscopy. The results showed that the synthesized compounds exhibited potent antitumor activities against ovarian cell lines. Among them, compound 1e exhibited the most potent activity demonstrating half maximal inhibitory concentration values of 0.85±0.10, 8.64±0.37 and 0.81±0.03 µM against A2780, A2780/Dox and A2780/cisplatin cell lines. The fluorescence imaging of live cell lines also provided an easy and reliable method to monitor site-specific drug activities through turn-on systems induced by drug release. The results of the present study may assist in the treatment of ovarian cancer cells with strengthened efficiency and real-time imaging, which may be used as a multifunctional system for the optimization of anticancer drugs.

Introduction

Cancer is a class of diseases, in which a group of cells exhibit uncontrolled growth, invasion and sometimes metastasis (1). Ovarian cancer remains the most frequent type of cancer among women, which is a heterogeneous cancer encompassing multiple subgroups with a complicated molecular basis (2,3). Improvements in current understanding of the underlying biology of ovarian cancer may lead to novel therapeutic strategies. Several models of ovarian carcinogenesis have been suggested. One of the models involves the malfunction of tumor suppressors and the occurrence of oncogenes, thereby leading to the hyperproliferation of epithelial cells and/or inactivation of DNA repair genes. Another model suggests the activation or deactivation of chromatin proteins associated with DNA, with resultant effects on different cellular processes (4,5). In reference to these models, the field of DNA-targeted drug development has gained increased attention due to their high sensitivity and specificity.

Furthermore, due to the complicated molecular basis of ovarian cancer, certain clinical results have evaluated and encouraged the utility of combination therapies, including the combination of doxorubicin (Dox) and cisplatin (Cis), which are beneficial in the treatment of cancer (6-8). An optimized therapeutic strategy requires a drug delivery system, which can guide the drug into the targeted cancer cells and improve therapy. It is known that the overexpression of high-affinity folate receptors (FRs) on human cancer cells provides advantages in treating tumor cells without interfering with neighboring normal tissue (9,10). For this reason, the FR is used as an attractive target for cancer treatment, further enforcing the selective active targeting in anticancer therapy (11,12). In addition, a combination therapy is not complete without the real-time monitoring of the drug activity. Therefore, developing a sensitive and accurate platform to evaluate drug activities is critical in scientific investigations and clinical applications.

Inspired by the multifunctional chemotherapy, the present study was performed to design a combination therapy integrating a targeted delivery platform and an activatable real-time sensor. In the design, folic acid (FA) acted as a selective tumor-targeting ligand and a quencher for Dox fluorescence (13-16). A reducible platinum (Pt) (IV) prodrug and its palladium (Pd) analogue were introduced as an activatable ligand with two axial positions linked to FA and Dox. Once this platform enters the tumor cells through FA targeting, the prodrug can be activated via reduction by several extracellular or intracellular reducing agents, including glutathione (GSH), cysteine and ascorbic acid, to achieve effective anti-tumor treatment (17,18), with concomitant breakage of two axial bonds and the release of free Dox. This system showed a turn-on effect on fluorescence, thereby realizing the sensitive and site-specific detection of drugs. This design was based on a combination therapy, which can achieve optimal effectiveness due to their synergic roles in the ovarian cancer environment.

The combining of fluorescence imaging, a targeted delivery platform and combination chemotherapy provides advantages to treat ovarian cancer cells with improved efficiency and
real-time imaging. Supported by the data of the present study, such conjugation provides improved efficiency and bioactivity, which can be used as a multifunctional system for the optimization of ovarian cancer treatment.

Materials and methods

Chemistry. All chemicals and reagents used in the present study were of analytical grade. All electrospray ionisation mass spectrometry (ESI-MS) spectra were recorded on a Mariner System 5304 mass spectrometer [Mariner Systems (UK) Ltd., Wokingham, UK]. Column chromatography was performed using silica gel (200-300 mesh) eluted with ethyl acetate and petroleum ether. Analytical reverse-phase high performance liquid chromatography (HPLC) analysis was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) using the Grace™ Altichrom™ Altima™ C-18 (250x10 mm) column (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a flow rate of 0.5 ml/min for preparation, and a C-18 (250x4.6 mm) column at 1.0 ml/min for analysis. The $^{195}$Pt NMR spectra were recorded on a Bruker A VANCE-400 spectrometer (Bruker Systems, Inc., Billerica, MA, USA). Elemental analyses were performed on a CHN-O-Rapid instrument (Heraeus, Hanau, Germany).

General procedure for FA-prodrug-Dox derivatives (1e-2e). (1) Synthesis of M (NH$_2$)$_2$Cl$_2$ (O$_2$CClH$_2$CH$_2$COH) [O$_2$CClH$_2$CH$_2$CONH-polyethylene glycol (PEG)-FA], Cis-[MCl$_2$(NH$_2$)$_2$] (1 mmol; QianKun Chemistry Technology Co., Ltd., Shanghai, China) was suspended in water (5 ml) and a 10-fold excess of H$_2$O was added. The mixture was stirred for 4 h at 50°C. Recrystallization of Cis-[MCl$_2$(OH)$_2$(NH$_2$)$_2$] was performed in situ and collected. The mixture was washed with cold water, ethanol and ether, and dried in a desiccator. Subsequently, succinic anhydride (4 mmol) was added to a suspension of Cis-[MCl$_2$(OH)$_2$(NH$_2$)$_2$] in dimethylformamide (DMF; 5 ml; QianKun Chemistry Technology Co., Ltd.), and the reaction mixture was stirred at 70°C for 4 h. The resulting solution was dried in vacuo, leaving a dark yellow oil, which was dissolved in a small volume of acetone (5 ml). The addition of ether precipitated a solid, which was collected and dried in vacuo to leave the product as a powder. To a solution of Cis-[MCl$_2$(NH$_2$)$_2$(O$_2$CClH$_2$CH$_2$COH)$_2$] in DMF (10 ml), DMF solution (0.5 ml) containing HATU (1.5 mmol) was added. This mixture was stirred for 10 min at room temperature. To the resulting solution, DMF solution containing PEG linker (0.8 mmol) and N,N-diisopropylethylamine (DIPEA; 1.2 mmol; QianKun Chemistry Technology Co., Ltd.) was added. The mixture was stirred at room temperature for 24 h in the dark. The DMF was then removed under a vacuum and the resulting compound was purified using HPLC. FA (0.8 mmol; QianKun Chemistry Technology Co., Ltd.) was dissolved in 5 ml DMF and coupled with 0.5 mmol of dicyclohexylcarbodiimide (DCC) and 1 mmol of N-hydroxysuccinimide (NHS). The reaction was stirred for 14 h at room temperature in the dark to obtain the folate-NHS ester. The resulting folate-NHS was reacted with M (NH$_2$)$_2$Cl$_2$ (O$_2$CClH$_2$CH$_2$COH) (O$_2$CClH$_2$CH$_2$CONH-PEG-PD) in DMF and purified by reprecipitation.

(2) Synthesis of M (NH$_2$)$_2$Cl$_2$ (O$_2$CClH$_2$CH$_2$CONH-Dox) (O$_2$CClH$_2$CH$_2$CONH-PEG-FA): The synthesis of the final Dox-prodrug-FA conjugates was performed using standard amide coupling reactions. Following the procedure mentioned above, 1.0 equiv prodrug-PEG-FA was reacted with 1.5 equiv 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; QianKun Chemistry Technology Co., Ltd.) coupled with DIPEA (20 µl) in 2 ml DMF at a temperature of 70°C. The resultant mixture was evaporated under pressure and washed with water to remove the remaining EDC, following which 1.0 equiv Dox (QianKun Chemistry Technology Co., Ltd.) was added and the reaction was set at the temperature of 70°C overnight. The product was purified by reverse-phase HPLC with an eluting system consisting of water solution (A) and acetonitrile solution (B) under a linear gradient. The linear gradient stretched over 20 min from t=0 min at 20% solution B to t=20 min at 80% solution B.

FA-Cis (NH$_2$, NH$_2$, Cl, Cl) Pt (IV)-Dox (1). Yellow powders, yield 65%; Rf=0.25 (PE/EtOAc=2:1); ESI-MS calculated for C$_{46}$H$_{48}$N$_{12}$O$_{13}$Pt(M+H)$^+$: 1686.4738; found: 1686.4716. $^{195}$Pt NMR (DMSO-d$_6$): δ1298.21 ppm. Calculated for C$_{46}$H$_{48}$N$_{12}$O$_{13}$Pt: C, 55.55; H, 5.02; N, 9.96; O, 23.70. Found: C, 44.35; H, 5.40; N, 9.83; O, 23.02.

FA-Cis (NH$_2$, NH$_2$, Cl, Cl) Pd (IV)-Dox (2). Yellow powders, yield 62%; Rf=0.25 (PE/EtOAc=2:1); ESI-MS calculated for C$_{46}$H$_{48}$N$_{12}$O$_{13}$Pd (M+H)$^+$: 1597.4182; found: 1597.4163. Calculated for C$_{46}$H$_{48}$N$_{12}$O$_{13}$Pd: C, 48.08; H, 5.30; N, 10.51; O, 25.02; found: C, 47.86; H, 5.11; N, 10.13; O, 24.92.

Antiproliferation assay. Target tumor cell lines were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics. Following dilution to 1x10$^3$ cells ml$^{-1}$ with complete medium, 100 µl of the obtained cell suspension was added to each well of 96-well culture plates. The cells were incubated with FA-prodrug-Dox at 37°C, 5% CO$_2$ for 24 h prior to the cytotoxicity assessments. The samples at pre-set concentrations (1 µg/µl) were added to six wells, and Dox and Cis were co-assayed as positive references. After 48 h exposure at 37°C and 5% CO$_2$, 40 µl of PBS containing 2.5 mg ml$^{-1}$ of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. After 4 h, 100 µl per well extraction solution (10% SDS-5% isobutyl alcohol-0.01 M HCl) was added. Following incubation overnight at 37°C, the optical density was measured at 570 nm on an ELISA microplate reader. In all experiments, three replicate wells were measured for each drug concentration. Each assay was repeated at least three times. The results are summarized in Table I.

Time course of fluorescence emission spectra assay in vitro. In the experiments, incubation was performed in the presence of the reducing agent, GSH. Each FA-prodrug-Dox (1 µM) in PBS (pH 7.4) was incubated with GSH solution (5 mM), and their fluorescence emissions ($\lambda_{ex}=497$; $\lambda_{em}=594$ nm) were measured at different time points. The change in fluorescence emission ($\lambda_{ex}=497$; $\lambda_{em}=594$ nm) was read using a Cary Eclipse fluorometer (Varian Medical Systems, Inc., Palo Alto, CA, USA).

Fluorescence imaging using confocal laser scanning microscopy. A2780 cells (Chuanbo Biotech Co., Ltd., Nanjing, China)
Six FA-prodrug-Dox conjugates was performed using standard amide coupling reactions. Following the procedure mentioned above, 1.0 equiv prodrug-PEG-FA (0.5 mmol) was reacted with 1.5 equiv EDC coupled with DIPEA (20 µl) in 2 ml DMF at the temperature of 70°C, the resulting mixture was evaporated under pressure and washed with water to remove the remaining EDC. Subsequently, 1.0 equiv Dox was added and incubated at a temperature of 70°C overnight. The general formula of the final purified compound was Dox-[Pt(IV)]/PEG-FA.

**Synthesis of Pt(NH$_3$)$_2$Cl$_2$(O$_2$CCH$_2$CH$_2$CONH-Peg-FA)**. To a solution of Cis-[PtCl$_2$(NH$_3$)$_2$] (10 mg) in DMF (5 ml), 1.5 equiv EDC coupled with DIPEA (20 µl) was added at the temperature of 70°C for 4 h. The resulting solution was precipitated by ethanol and ether, and dried in a desiccator. Succinic anhydride (4 mmol) was added to a suspension of Cis-[PtCl$_2$(NH$_3$)$_2$] in DMF (5 ml), and the reaction mixture was stirred at 70°C for 4 h. The resulting solution was precipitated by ether and collected. To a solution of Cis-[PtCl$_2$(NH$_3$)$_2$] (0.5 ml) containing HATU (1.5 mmol) was added. This mixture was stirred for 10 min at room temperature. The resulting solution, DMF solution containing PEG linker (0.8 mmol) and DIPEA (20 µl) was added. The mixture was stirred at room temperature for 24 h in the dark. The DMF was then removed under a vacuum and the resulting compound was purified by column chromatography. FA (0.8 mmol) was dissolved in 5 ml DMF coupled with 0.5 mmol of DCC and 1 mmol of NHS. The mixture was stirred for 14 h at room temperature in the dark to produce folate-NHS ester. The resulting folate-NHS ester was reacted with M(NH$_3$)$_2$Cl$_2$(O$_2$CCCH$_2$CH$_2$CO$_2$H) (O$_2$CCCH$_2$CH$_2$CONH-PEG) in DMF and purified by reprecipitation.

**Synthesis of M(NH$_3$)$_2$Cl$_2$(O$_2$CCH$_2$CH$_2$CONH-Peg-Dox) (O$_2$CCH$_2$CH$_2$CONH-Peg-FA)**. The synthesis of the final Dox-prodrug-FA conjugates was performed using standard amide coupling reactions. Following the procedure mentioned above, 1.0 equiv prodrug-PEG-FA (0.5 mmol) was reacted with 1.5 equiv EDC coupled with DIPEA (20 µl) in 2 ml DMF at the temperature of 70°C, the resulting mixture was evaporated under pressure and washed with water to remove the remaining EDC. Subsequently, 1.0 equiv Dox was added and incubated at a temperature of 70°C overnight. The general formula of the final purified compound was Dox-[Pt(IV)]/PEG-FA.
Figure 1. General synthesis of compounds 1e, and 2e. Reagents and conditions were as follows: (i) H₂O₂, 50°C, 4 h; (ii) succinic anhydride, 70°C, 4 h; (iii) PEG, DCC, room temperature, Folate-NHS, 24 h; (iv) EDC, DIPEA, 70°C, overnight. Dcc, dicyclohexylcarbodiimide; NHS, N-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; PEG, polyethylene glycol; DIPEA, N,N-diisopropylethylamine.

Figure 2. Structures of Folic acid-Pt (IV)-Dox used as novel template for combination chemotherapy with real-time imaging. Dox, doxorubicin; Pt, platinum.
Biological activity. To assess the anticancer activities of the synthesized compounds, the present study evaluated the antiproliferative activities against A2780, A2780/Dox and A2780/Cis cell lines. As shown in Table I, all the compounds showed marked activity against ovarian cancer cells, suggesting that the FA-prodrug-Dox derivatives exhibited improved efficacy, compared with the single drugs of Dox and Cis. In addition, the target compounds exhibited a more potent effect, compared with the additive effect of the two cytotoxins (Dox-Cis), the superiority of the designed compounds were predominantly attributed to the function of FA-targeting. For the compounds, it was observed that compound 1e showed the most potent biological activity, with half maximal inhibitory concentration (IC$_{50}$) values of 0.85±0.10, 8.64±0.37 and 0.81±0.03 µM against the A2780, A2780/Dox and A2780/Cis cell lines, respectively.

In addition, the activity of the assessed compounds was correlated with the core metallic atom variation and ligand modifications (Fig. 3). It was revealed that Pd had a similar antiproliferative activity to Pt, suggesting that Pd derivatives qualified as a novel substitute for Pt as an anticancer drug.

In line with the improved anticancer potency, the present study further investigated the process of drug release in cancer cells with fluorescent imaging. The fluorescence activation was first assessed by treating cells with the FA-prodrug-Dox in reducing conditions and in the presence of GSH. In these experiments, compound 1e (1 µM) in PBS (pH 7.4) was incubated with GSH solution (5 mM), as the intracellular GSH concentration is between 1 and 10 mM. The fluorescence emissions ($\lambda_{ex}=497$; $\lambda_{em}=594$ nm) were measured at different time points. As shown in Fig. 4A, the fluorescence emission of FA-Pt(IV)-Dox increased 2-fold as a result of reduction of the axial bond, reaching a plateau at 1 h. In addition, treatment with FA-Pt(IV)-Dox (1 µM in PBS; pH 7.4) in the absence of GSH (PBS pH 7.4) showed no effect on the fluorescence emission of Dox, suggesting that the observed increase in the fluorescence of Dox was due to GSH-mediated cleavage of the axial bond of the prodrug. The present study further investigated the drug release of FA-Pd(IV)-Dox (2e), which showed a similar trend of fluorescent enhancement with FA-Pt(IV)-Dox (1e), as shown in Fig. 4B, indicating there was no significant difference in the release efficiency between the Pt(IV) and Pd(IV) complexes (Fig. 5).

In conclusion, a series of FA-prodrug-Dox derivatives were synthesized in the present study and were evaluated to further confirm the FA-targeting effect of the designed compounds (Fig. 2).
for multifunctional anticancer therapy. These compounds exhibited potent antitumor activities against ovarian cell lines. Among them, compound 1e demonstrated the most potent activity, with IC₅₀ values of 0.85±0.10, 8.64±0.37 and 0.81±0.03 µM against A2780, A2780/Dox and A2780/Cis cell lines, respectively. The fluorescence imaging of live cell lines also provided an easy and reliable method for monitoring of the site-specific drug activities through turn-on systems induced by drug release. The results of the present study may provide assistance in the treatment of ovarian cancer cells with improved efficiency and real-time imaging, which can be used as a multifunctional system for the optimization of anticancer drugs.

References