Co-delivery of docetaxel and retinoic acid by poly (ethylene glycol)-retinoic acid conjugates based micelles for synergistic prostate cancer therapy

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Abstract
In the present work, docetaxel (DTX) and retinoic acid (RA) co-loaded micelles were developed for synergistic prostate cancer therapy. PEG-RA and RA-PEG-RA were synthesized and subsequently used for loading of DTX and RA. The as prepared DTX/RA@RA-PEG-RA micelles exhibit smaller size and higher drug loading capacity as compared to DTX/RA@PEG-RA micelles. The in vitro drug release study shows that DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit similar drug release behaviours in which DTX exhibits fast release and RA exhibits sustained release. Nile red (NR) was loaded into PEG-RA and RA-PEG-RA micelles to investigate the cellular uptake of the micelles by fluorescence microscope and flow cytometry. The results show that NR@RA-PEG-RA micelles exhibit slightly enhanced cellular uptake as compared to NR@PEG-RA micelles. The cellular uptake of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles determined by HPLC also show similar results. In in vitro cytotoxicity against C4-2 and 22Rv1 cells, DTX in combination with RA exhibit synergistic antitumor effect, and DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit enhanced cytotoxicity against C4-2 cells and similar cytotoxicity against 22Rv1 cells as compared to DTX/RA. DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles show significant potential for prostate cancer therapy.

1 | INTRODUCTION

Prostate cancer is the most common cancer and second leading death from cancer among males in United States [1]. Numerous studies have shown that prostate cancer is an androgen-dependent cancer [2]. For decades, androgen deprivation therapy (ADT) has been the primary therapeutic option for patients with prostate cancer. However, almost all patients who exhibit an initial response to ADT will relapse within 3 years and progress into a castration-resistant stage which is fatal [2, 3].

Docetaxel (DTX) is a semisynthetic chemotherapeutic agent which exhibits antitumor activity in a broad range of tumour types [4]. DTX exerts the cytotoxic effects on microtubules. DTX can also regulate cell signalling and expression of certain genes, inducing apoptosis [5, 6]. DTX based chemotherapy has been the most used therapeutic option after castration-resistant prostate cancer develops [7–10]. Available studies show that treatment of prostate cancer cells with DTX induces phosphorylation of Bcl-2 and abrogates the normal anti-apoptotic function of Bcl-2, resulting in apoptosis [11, 12]. However, there are also some problems associated with DTX based chemotherapy, such as development of drug resistance [13–15], serious adverse effects [16, 17], which significantly affect its therapeutic outcomes.

Retinoic acid (RA), a natural derivative of vitamin A, plays important roles in a variety of cell activities such as growth, differentiation, morphogenesis, and induction of apoptosis [18]. RA mainly exhibits its effect by binding to nuclear retinoic acid receptors [19]. Numerous studies have shown that RA exhibits anticancer activity against a spectrum of cancers such as head and neck cancer, breast cancer, liver cancer, lung cancer, and prostate cancer [20–23]. It has been also reported that...
RA can enhance the cytotoxicity of chemotherapeutic agents against some cancer cells [24, 25]. For instance, Kucukzeybek et al. reported that RA can enhance DTX-induced cytotoxicity and apoptosis by down-regulation of surviving (BIRC5), MCL-1, and LTbeta-R in hormone- and drug-resistant prostate cancer cell line DU-145 [26]. Nehmé et al. reported that RA can enhance the cytotoxicity of DTX against DU145 and LNCaP prostate cancer cells though inhibition of DTX-induced phosphorylation of cdc2 and DTX-induced activation of MAPK [27]. Despite that these studies demonstrated the synergistic antitumor effect of DTX and RA against DU145 and LNCaP prostate cancer cells, the combinational therapeutic effect of DTX and RA against other prostate cancer cell lines is still unknown. In addition, DTX and RA exhibit poor water solubility and enhancing DTX solubility by Cremophor EL may cause serious allergic reactions in patients. Therefore, it is highly desirable to investigate the combined therapeutic effect of DTX and RA against other prostate cancer cell lines and develop novel drug delivery systems to enhance the solubility of DTX and RA in order to better suppress the prostate cancer.

In our previous work, mPEG-RA micelles were developed for loading of dimethylcurcumin and show a series of advantages including enhanced water solubility, enhanced cellular uptake, and good biocompatibility [28]. Inspired from this, PEG-RA and RA-PEG-RA micelles were developed here for DTX and RA co-delivery. DTX and RA co-loaded micelles were prepared by thin-film hydration method. The drug loading capacity, size distribution, morphology, in vitro drug release, cellular uptake, and in vitro cytotoxicity against castration-resistant prostate cancer cells C4-2 and 22Rv1 of the prepared DTX and RA co-loaded micelles were then investigated.

2 | MATERIALS AND METHODS

2.1 | Materials

Polyethylene glycol (PEG, M.W. 2000), Retinoic acid (RA, 98 %), N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 98%), 4-dimethylaminopyridine (DMAP, 99 %), Nile red (NR, 95 %), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 98 %) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Docetaxel (DTX, 99 %) were purchased from Jiangsu Yew Pharmaceutical Co., Ltd (China). All other chemicals were of HPLC grade or analytical grade and used as received.

2.2 | Synthesis of PEG-RA and RA-PEG-RA

Briefly, RA (30.0 mg, 0.1 mmol) and EDCI (76.7 mg, 0.4 mmol) were dissolved in 5 mL of dichloromethane. To the solution, DMAP (12.2 mg, 0.1 mmol) and PEG (200.0 mg for PEG-RA, 100.0 mg for RA-PEG-RA) were added and the resulting mixture was vigorously stirred at 40 °C for 2 days. Then, the solvent was evaporated under reduced pressure and the resulting mixture was suspended in deionized water. The suspension was dialyzed against deionized water for 3 days (MWCO: 3500 Da) and freeze dried. The structure of the synthesized PEG-RA and RA-PEG-RA was characterized by 1H NMR and FT-IR. The molar ratios (MR) of RA to PEG in the synthesized products were determined by 1H NMR and calculated as

$$MR = \left( \frac{I_{RA}}{6} \right) / \left( \frac{I_{PEG}}{181.8} \right)$$

Where $I_{RA}$ is the integral for the protons of two methyl groups (a, b) in retinoic acid between 0.95 and 1.13 ppm (Figure 2(a),(b)), and $I_{PEG}$ is the integral for the protons of methyl groups in PEG between 3.40 and 3.80 ppm.

2.3 | Preparation of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles

Briefly, 10 mg of DTX, 10 mg of RA, and 100 mg of PEG-RA or RA-PEG-RA were dissolved in 10 mL of dichloromethane and the resulting mixture was stirred at room temperature for 1 h. Then, the solvent was evaporated under reduced pressure and the resulting mixture was suspended in deionized water under sonication. After sonication for 5 min, the suspension was centrifuged at 8000 rpm for 10 min and the supernatant was collected and freeze dried. The DTX and RA loading content of the prepared DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles were measured by high performance liquid chromatography (HPLC, 1260 Infinity, Agilent) by using a standard curve. Briefly, the freeze dried DTX/RA@PEG-RA or DTX/RA@RA-PEG-RA micelles were dissolved in methanol and measured by HPLC. For the determination of DTX loading content, the injection volume was 10 µL, the mobile phase consists of acetonitrile (60%, v/v) and deionized water (40%, v/v), the flow rate was 1 mL/min, and the detection wavelength was 227 nm. For the determination of RA loading content, the injection volume was 10 µL, the mobile phase consists of methanol (90%, v/v) and acetic acid-ammonium acetate buffer (10 mM, pH 4.0, 10%, v/v), the flow rate was 1 mL/min, and the detection wavelength was 320 nm.

2.4 | Characterization

1H NMR spectra were recorded on a nuclear magnetic resonance spectrometer (AVANCE II 500 MHz, Bruker) using tetramethylsilane as an internal reference. FT-IR spectra were recorded on a flourier transform infrared spectrometer (SS50, Thermo). The hydrodynamic diameter and zeta potential of the prepared micelles were measured by dynamic light scattering (DLS, Nano ZSE, Malvern). The morphology of the prepared micelles was imaged by transmission electron microscopy (TEM, JEM-2100, JEOL) operated at an accelerating voltage of 200 KV. The samples for TEM imaging were prepared by placing a small drop of the prepared micelle suspension onto a carbon-coated copper grid and dried at room temperature followed by negatively stained with phosphotungstic acid at a concentration of 2% (w/w) for 3 min.
2.5 In vitro drug release

The DTX and RA release behaviours of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles were studied by a dialysis method in pH 7.4 and pH 5.0 PBS buffer (10 mmol/L, Tween 80 0.5%). Briefly, 200 µL of DTX/RA@PEG-RA or DTX/RA@RA-PEG-RA micelles was placed into a dialysis tube and immersed in 30 mL of release media, and shaken at a speed of 180 rpm under 37 °C. At desired intervals, 2 mL release media was taken out and replenished with 2 mL fresh media. The DTX and RA release amount was determined by HPLC. The experiments were conducted in triplicate.

2.6 Cell culture

Human prostate cancer cells C4-2 and 22Rv1 were cultured in RPMI1640 medium supplemented with 10% foetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C under 5% CO₂ atmosphere. C4-2 and 22Rv1 cells are androgen-responsive and androgen-independent human prostate cancer cell lines, which express endogenous AR.

2.7 Cellular uptake

NR-loaded PEG-RA and RA-PEG-RA micelles were prepared to study the cellular uptake of PEG-RA and RA-PEG-RA micelles. NR@PEG-RA and NR@RA-PEG-RA micelles were prepared by the same method as DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles. Briefly, C4-2 and 22Rv1 cells were seeded on 12-well plates at a cell density of 1 × 10⁵ cells per well. After treatment with NR@PEG-RA and NR@RA-PEG-RA micelles for 2, 4, and 6 h, the medium was removed and the cells were washed by PBS buffer (pH 7.4, 6.7 mmol/L) for three times. The cells were fixed with paraformaldehyde and observed with fluorescence microscope. The cellular NR fluorescence intensity was quantified by flow cytometry. Briefly, C4-2 and 22Rv1 cells were seeded on 12-well plates in triplicate at a cell density of 1 × 10⁵ cells per well. After treatment with NR@PEG-RA and NR@RA-PEG-RA micelles for 2, 4, and 6 h, the medium was removed and the cells were washed by PBS buffer (pH 7.4, 6.7 mmol/L) for three times. Then, the cells were tripinisized and centrifuged (1000 rpm, 5 min). The obtained cells were re-suspended in PBS buffer (pH 7.4, 6.7 mmol/L) and measured by flow cytometry.

The cellular uptake of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles was quantified by HPLC. Briefly, C4-2 and 22Rv1 cells were seeded on 12-well plates in triplicate at a cell density of 1 × 10⁵ cells per well. After treatment with DTX/RA, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles (10 µg/mL as DTX, 8 µg/mL as RA) for 2 and 6 h, the medium was removed and the cells were washed by PBS buffer (pH 7.4, 6.7 mmol/L) for three times. Then, the cells were tripinisized for cell number counting followed by ultrasonication. The intracellular DTX and RA was extracted with methanol and determined by HPLC.

2.8 In vitro cytotoxicity against C4-2 and 22Rv1 cells

The in vitro cytotoxicity of free DTX, free RA, DTX/RA, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles against C4-2 and 22Rv1 cells were assessed by MTT assays. Briefly, C4-2 or 22Rv1 cells were seeded on 96-well plates in quintet at a cell density of 5 × 10³ cells per well. After treatment with different drugs for 48 and 72 h, 20 µL MTT (5 mg/mL) was added to each well and incubated for 4 h. Then, the medium was removed and 150 µL of DMSO was added to each well to dissolve the formed crystals. The absorbance was measured at 492 nm by a microplate reader. Cells incubated with culture medium alone were used as reference for 100 % viability.

2.9 Statistical analysis

Data are presented as the means ± SDs. The statistical significance of differences between two groups of data was analysed by paired t-test and p values < 0.05 were considered as significant.

3 RESULTS AND DISCUSSION

PEG-RA and RA-PEG-RA were synthesized from PEG and RA by esterification reactions (Figure 1). The synthesized PEG-RA and RA-PEG-RA were characterized by ¹H NMR and FT-IR. As shown in Figure 2a and b, the characteristic signals of RA at δ_H (ppm) 1.02 (a, b), 1.44 (c), 1.58 (d), 1.69 (f), 2.00 (m), 2.11 (e), 2.31 (n), and 5.00–9.00 (g–l) appear in the ¹H NMR spectra.
of PEG-RA and RA-PEG-RA. The molar ratios of RA to PEG in the synthesized PEG-RA and RA-PEG-RA calculated by $^1$H NMR are 1.25 and 2.05, respectively, suggesting that about one RA molecule was conjugated with one PEG chain for PEG-RA and two RA molecules were conjugated with one PEG chain for RA-PEG-RA. The FT-IR spectra of PEG-RA and RA-PEG-RA show the characteristic band of the ester bond at 1710 cm$^{-1}$ (Figure 3), suggesting that RA is conjugated onto PEG through ester bond. Taken together, these results indicate that PEG-RA and RA-PEG-RA are successfully prepared.

The assembly of the synthesized PEG-RA and RA-PEG-RA in deionized water was investigated by DLS. As shown in Table 1, PEG-RA assembles into micelles with hydrodynamic diameter of 121.7 nm and zeta potential of 0.17 mV, while RA-PEG-RA assembles into micelles with hydrodynamic diameter of 16.1 nm and zeta potential of 0.04 mV. Interestingly, the size of RA-PEG-RA micelles is much smaller than PEG-RA micelles. The DTX and RA co-loaded micelles were prepared by thin-film hydration method. After loading of DTX and RA, the hydrodynamic diameters and zeta potentials of the resultant DTX/RA@PEG-RA ($\bar{D}_h$ 130.4 nm, zeta potential 3.74 mV) and DTX/RA@RA-PEG-RA micelles ($\bar{D}_h$ 22.6 nm, zeta potential 3.52 mV) increase slightly. The PDI of the prepared micelles are all less than 0.3 and the sizes of the prepared micelles show log-normal distribution in DLS analysis (Table 1, Figure 4a), indicating that all prepared micelles show good polydispersity. The morphologies of the prepared DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles were observed with TEM. As shown in Figure 4b and c, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles are homogeneously dispersed as individual particles with near-spherical morphologies and the sizes of which are in accordance with those determined by DLS. The drug loading content (DLC) and encapsulation efficiency (EE) of DTX and RA were determined by HPLC. DTX/RA@RA-PEG-RA micelles exhibit higher DLC and EE of DTX (DLC 4.9 %, EE 53.8 %) and RA (DLC 4.1 %, EE 45.1 %) as compared to DTX/RA@PEG-RA micelles (DTX: DLC 4.0 %, EE 44.0 %; RA: DLC 3.4 %, EE 37.4 %). RA-PEG-RA exhibits enhanced proportion of hydrophobic segment as compared to PEG-RA, which is beneficial for the hydrophobic interactions between the hydrophobic drugs and RA-PEG-RA. This may be the reason for that DTX/RA@RA-PEG-RA micelles exhibit enhanced DLC and EE of DTX and RA as compared to DTX/RA@PEG-RA micelles. The in vitro DTX and RA release of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA

**TABLE 1** DLS characters of the prepared micelles

| Sample           | $\bar{D}_h$ (nm) | PDI            | Zeta potential (mV) |
|------------------|------------------|----------------|---------------------|
| PEG-RA           | 121.7 ± 4.8      | 0.282 ± 0.012  | 0.17 ± 0.20         |
| RA-PEG-RA        | 16.1 ± 1.6       | 0.291 ± 0.021  | 0.04 ± 0.21         |
| DTX/RA@PEG-RA    | 130.4 ± 8.5      | 0.240 ± 0.044  | 3.74 ± 0.51         |
| DTX/RA@RA-PEG-RA | 22.6 ± 4.7       | 0.148 ± 0.061  | 3.52 ± 0.17         |

$\bar{D}_h$, hydrodynamic diameter. PDI, polydispersity index.
Characterization of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles. (a) Size distribution of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles determined by DLS (b) TEM image of DTX/RA@PEG-RA micelles (c) TEM image of DTX/RA@RA-PEG-RA micelles

Figure 4

micelles were studied in pH 7.4 and pH 5.0 PBS buffer (10 mmol/L, Tween 80 0.5%) by a dialysis method. As shown in Figure 5, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit similar drug release behaviours in pH 7.4 and pH 5.0 PBS buffer. DTX is released fast with over 80% of total DTX released within 8 h, whereas RA is released sustainably with less than 50% of total RA released within 24 h. Collectively, these results show that DTX/RA@RA-PEG-RA micelles exhibit smaller size and higher DLC and EE of DTX and RA as compared to DTX/RA@PEG-RA micelles, and DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit similar drug release behaviours in which DTX exhibits fast release and RA exhibits sustained release.

Fluorescence probe NR was loaded into PEG-RA and RA-PEG-RA micelles to study the cellular uptake of the micelles. The cellular uptake of the prepared NR@PEG-RA and NR@RA-PEG-RA micelles by human prostate cancer cell lines C4-2 and 22Rv1 was studied by fluorescence microscope and flow cytometry. As shown in Figure 6, the cellular uptake

Figure 5

In vitro drug release profiles of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles in pH 7.4 and pH 5.0 PBS buffer (10 mmol/L, Tween 80 0.5%). (a) DTX release profiles (b) RA release profiles

Figure 6

Cellular uptake of NR@PEG-RA and NR@RA-PEG-RA micelles. (a) Fluorescence images of C4-2 cells after treatment with NR@PEG-RA and NR@RA-PEG-RA micelles for 2, 4, and 6 h (b) Fluorescence images of 22Rv1 cells after treatment with NR@PEG-RA and NR@RA-PEG-RA micelles for 2, 4, and 6 h (c) Mean cellular fluorescence intensity of C4-2 cells determined by flow cytometry, * p < 0.05, ** p < 0.01 (d) Mean cellular fluorescence intensity of 22Rv1 cells determined by flow cytometry
FIGURE 7  Cellular uptake of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles in (a) C4-2 cells, *p < 0.01 compared to DTX/RA, ***p < 0.001 compared to DTX/RA, ##p < 0.01 compared to DTX/RA@PEG-RA (b) 22Rv1 cells, *p < 0.05 compared to DTX/RA, **p < 0.01 compared to DTX/RA, ***p < 0.001 compared to DTX/RA.

FIGURE 8  In vitro cytotoxicity of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles against (a) C4-2 cells after treatment for 48 h, *p < 0.05, **p < 0.01, ***p < 0.001 (b) C4-2 cells after treatment for 72 h, *p < 0.05 (c) 22Rv1 cells after treatment for 48 h (d) 22Rv1 cells after treatment for 72 h

of NR@PEG-RA and NR@RA-PEG-RA micelles increases with the increasing incubation time. Notably, NR@RA-PEG-RA micelles exhibit slightly enhanced cellular uptake as compared to NR@PEG-RA micelles. The size of NR@RA-PEG-RA micelles is much smaller than NR@PEG-RA micelles, which may lead to different cellular uptake pathways and result in the difference in cellular uptake amount.

The cellular uptake of the prepared DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles was determined directly by HPLC. As shown in Figure 7, the cellular uptake of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles increases with increasing incubation time from 2 to 6 h and DTX/RA@RA-PEG-RA micelles exhibit slightly enhanced cellular uptake as compared to DTX/RA@PEG-RA micelles. These results are in accordance with the cellular uptake results of NR loaded micelles. The cellular uptake amount of RA is higher than DTX for DTX/RA, whereas the cellular uptake amount of RA is close to DTX for DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles, suggesting that the encapsulated DTX and RA are internalized simultaneously with the micelles. DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit significantly enhanced intracellular DTX amount as compared to DTX/RA after incubation with C4-2 and 22Rv1 cells for 2 and 6 h. Although the micelles exhibit similar and reduced intracellular RA amount as compared to DTX/RA after incubation with C4-2 and 22Rv1 cells for 2 h, respectively, the intracellular RA amount of the micelles increases significantly and is higher than DTX/RA when the incubation time is increased to 6 h. These results indicate that DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit enhanced cellular uptake as compared DTX/RA and RA@PEG-RA micelles exhibit slightly enhanced cellular uptake as compared to DTX/RA@PEG-RA micelles.

The in vitro cytotoxicity of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles against C4-2 and 22Rv1 cells was studied by MTT assays. As shown in Figure 8, both DTX and RA exhibit cytotoxicity against C4-2 and 22Rv1 cells in a time- and dose-dependent manner. DTX in combination with RA (DTX/RA) exhibit enhanced cytotoxicity against C4-2 and 22Rv1 cells as compared to DTX alone and RA alone. To evaluate the combined antitumor effect of DTX and RA against C4-2 and 22Rv1 cells, the combination index (CI) values for DTX/RA were calculated by CalcuSyn 2.0 software. The CI values define the synergism...
Almost all calculated CI values are less than 1, indicating that DTX and RA exhibit synergistic antitumor effect against C4-2 and 22Rv1 cells. Notably, DTX/RA@RA-PEG-RA micelles exhibit significantly enhanced cytotoxicity against C4-2 cells as compared to DTX/RA after incubation for 48 and 72 h, and DTX/RA@PEG-RA micelles also exhibit significantly enhanced cytotoxicity against C4-2 cells as compared to DTX/RA after incubation for 48 h. DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit apparently enhanced cellular uptake as compared to DTX/RA on C4-2 cells, accounting for their enhanced cytotoxicity against C4-2 cells. In comparison, the micelles exhibit similar cytotoxicity against 22Rv1 cells as compared to DTX/RA after incubation for 48 and 72 h, which is ascribed to the limited cellular uptake of the micelles on 22Rv1 cells. These results indicate that DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit enhanced cytotoxicity against C4-2 cells as compared to DTX/RA and similar cytotoxicity against 22Rv1 cells as compared to DTX/RA.

4 CONCLUSION

In summary, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles were prepared for synergistic prostate cancer therapy. DTX/RA@RA-PEG-RA micelles exhibit smaller size and higher DLC and EE of DTX and RA as compared to DTX/RA@PEG-RA micelles. DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit similar drug release behaviours in which DTX exhibits fast release and RA exhibits sustained release. NR-loaded PEG-RA and RA-PEG-RA micelles were prepared to investigate the cellular uptake of the micelles. The results show that NR@RA-PEG-RA micelles exhibit slightly enhanced cellular uptake as compared to NR@PEG-RA micelles. The cellular uptake of DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles were determined directly by HPLC and the results are in accordance with the cellular uptake of NR-loaded micelles. Notably, DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit enhanced cellular uptake as compared to DTX/RA. The in vitro cytotoxicity against C4-2 and 22Rv1 cells shows that DTX in combination with RA result in synergistic antitumor effect, and DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles exhibit enhanced cytotoxicity against C4-2 cells and similar cytotoxicity against 22Rv1 cells as compared to DTX/RA. The DTX/RA@PEG-RA and DTX/RA@RA-PEG-RA micelles developed in the present work show significant potential for co-delivery of DTX and RA for treatment of prostate cancer.

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