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Self-assembled Green Tea Polyphenol-based Coordination Nanomaterials to Improve Chemotherapy Efficacy by Inhibition of Carbonyl Reductase 1

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Abstract

Nanomedicine has become a promising approach to improve cancer chemotherapy. It remains a major challenge how to enhance anti-drug efficacy and reduce side effects of anti-cancer drugs. Herein, we report a self-assembled nanoplatform (FDEP NPs) by integration of doxorubicin (DOX) and epigallocatechin-3-O-gallate (EGCG) with the help of coordination between Fe³⁺ ions and polyphenols. The EGCG from FDEP NPs could inhibit the expression of carbonyl reductase 1 (CBR1) protein and thereby inhibit the doxorubicinol (DOXOL) generation from DOX both *in vitro* and *in vivo*, thus the efficacy of DOX to cancerous cells is improved significantly. More importantly, the FDEP NPs could reduce cardiac toxicity and the DOX mediated toxicity to blood cells due to the repression of DOXOL production. Moreover, the blood half-life of FDEP NPs is longer than 23 h as determined by positron emission tomography (PET) imaging of biodistribution

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Author contributions

L.S., Y.D. and X.C. conceived the designed the experiments. L.S., Y.D., Z.Y. and K.Z. synthesized the nanomaterials, L.S., G.G., W.W., W.T., W.F. and G.Z. did the cell experiment, L.S. and Y.D. performed the animal experiment. Z.W. and O.J. performed the PET experiment. L.S., Y.D. and X.C. wrote the paper. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interest.

Additional information

Supplementary data to this article can be found online.

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of radiolabelled NPs and HPLC measurement of plasma level of DOX, ensuring high tumor accumulation of FDEP NPs by enhanced permeability and retention (EPR) effect. The FDEP NPs also exhibited much improved antitumor effect over free drugs. Our work sheds new light on the engineering of nanomaterials for combination chemotherapy and may find unique clinical applications in the near future.

Keywords

chemotherapy; epigallocatechin-3-O-gallate (EGCG); positron emission tomography; doxorubicin; carbonyl reductase 1 (CBR1); polyphenols

1. Introduction

Traditional chemotherapy is still the leading approach in current cancer treatment to suppress tumor proliferation and prolong patient survival by eliminating or inhibiting cancer cells through anticancer drugs.[1, 2] The anthracycline, doxorubicin (DOX), a widely used anticancer drug, can intercalate duplex DNA and inhibit the action of topoisomerase II which blocks DNA repair.[3, 4] However, its therapeutic power is curtailed owing to drug resistance of tumor cells, dose-dependent irreversible cardiotoxicity, short blood circulation time and ineffective tumor delivery.[5–7] Nowadays, major challenges remain in providing safe and effective DOX therapy in the clinic. Doxorubicinol (DOXOL), the major metabolite of DOX, formed by reducing C13-keto groups of DOX into the C13-alcohol, has been reported to be responsible for drug resistance and cardiotoxicity.[8] Recent studies have also demonstrated that carbonyl reductase 1 (CBR1) plays an essential role in reducing DOX into DOXOL.[9–11] Additionally, the activity of CBR1 is relatively high in brain tumor compared to normal tissues.[12] To avoid DOX reduction, inhibition of the activity of CBR1 offers a promising pathway to enhance the chemotherapeutic efficacy of DOX and attenuate cardiotoxicity.

Polyphenols are promising candidates in the human diet with additional therapeutic effects to prevent pathological diseases, such as inflammation, cancer, and cardiovascular diseases.[13–17] Metal-polyphenol networks (MPNs) are an emerging class of smart materials by exploiting the coordination between various metals and phenolic ligands for biological applications thanks to the unique advantages (*e.g.* rapid preparation, tuneable size, and excellent biocompatibility).[18–23] Epigallocatechin-3-O-gallate (EGCG), a type of polyphenol, is the main constituent of green tea catechins with potential anticancer and antioxidant properties.[24–26] Interestingly, the EGCG could be employed as a promising inhibitor of CBR1 to enhance the efficacy of DOX.[12] However, EGCG can also bind to biomacromolecules nonspecifically and accumulate in main organs rapidly *in vivo*, which is undesirable for tumor specific delivery.[27] Poly(ethylene glycol) (PEG) has become an essential polymer to reduce mononuclear phagocytic system clearance and thereby improve the biodistribution and pharmacokinetic performances of nanomaterials.[28, 29] The ability to incorporate EGCG and DOX into a nanoformula with PEG modification based on MPNs outlines a new approach for engineering therapeutic systems for cancer therapy.

Herein, we developed a self-assembled nanoplatform by making use of the coordination chemistry between ferric (Fe^{3+}) ions and polyphenols. The nanoplatform combined both DOX and EGCG and encapsulated them in high molecular weight PEG. The as-prepared nanoplatform had prolonged circulation in the blood and excellent accumulation in the tumor site. The uppsala 87 malignant glioma (U87MG) xenograft model was used in this study as this cell line expresses high level of CBR1 protein. The optimal nanoformula was found to be able to effectively inhibit U87MG tumor growth.

2. Methods

2.1. Fabrication of EGCG@DOX Fe nanoparticles (FDEP30 NPs, FDEP50 NPs and FDEP80 NPs)

Firstly, doxorubicin hydrochloride (DOX HCl, 3 mg, 0.005 mmol, 1 eq.) was mixed with triethylamine (TEA, 7.7 μL , 0.05 mmol, 10 eq.) in dimethylformamide (DMF, 1 mL) overnight to prepare the hydrophobic DOX (0.005 mmol, 1 eq.). The hydrophobic DOX (50 μL , 0.005 mmol, 1.25 eq.) was added into the EGCG aqueous solution (100 μL , 0.002 mmol, 1eq.). PEG-polyphenols aqueous solution (20 μL , 50 mg/mL) was further added to the mixture with stirring. Then, the iron(III) chloride (FeCl_3 , 30 μL , 50 μL , or 80 μL , 3 mg/mL) aqueous solution was slowly added into the mixture to obtain the FDEP30 NPs, FDEP50 NPs or FDEP80 NPs. The mixture was stirred for 30 min under room temperature. Finally, DMF in the reaction mixture was removed by dialysis (8000 MWCO) overnight. The concentrations of DOX and EGCG were evaluated by HPLC. The FDEP NPs were concentrated by centrifugal filter tubes. Then, the concentration of FDEP NPs can be measured by drying a certain volume of FDEP NPs in the oven overnight. Both the FDEP30 NPs and FDEP50 NPs were employed for further *in vitro* and *in vivo* experiments.

2.2. *In vitro* investigation of intracellular CBR1 protein activity

The U87MG cells and human embryonic kidney 293 cells (293T cells) were seeded at 6-well plate with a density of 2×10^5 /well and cultured for 24 h at 37 °C. Then, fresh medium with DOX (6.8 μM), EGCG (10.2 μM), DOX + EGCG (DOX concentration: 6.8 μM , EGCG concentration: 10.2 μM), FDEP30 NPs (DOX concentration: 6.8 μM) and FDEP50 NPs (DOX concentration: 6.8 μM) were added to U87MG and 293T cells, respectively. The cells were incubated for another 24 h. After incubation, all the cells were handled by the anti-CBR1 antibody manufacturer's instructions and measured by flow cytometry.

2.3. *In vitro* assessment of DOX and DOXOL

293T cells and U87MG cells were seeded in 6-well plate at a density of 2×10^5 cells/well. Fresh medium with DOX (6.8 μM), EGCG (10.2 μM), DOX + EGCG (DOX concentration: 6.8 μM , EGCG concentration: 10.2 μM), FDEP30 NPs (DOX concentration: 6.8 μM) or FDEP50 NPs (DOX concentration: 6.8 μM) were added to U87MG and 293T cells, and cells were incubated for another 24 h. All cells were centrifuged at 3000 rpm for 20 min, and the supernatants were removed, extraction solution (4 mL) containing chloroform and methanol (4 : 1) was added, and the mixture was centrifuged at 3000 rpm for 20 min and then the supernatants were collected and dried by N_2 . Finally, all samples were dissolved and measured using HPLC.

2.4. Pharmacokinetics studies

The normal Balb/C mice were used to investigate the pharmacokinetics of FDEP30 NPs and FDEP50 NPs *in vivo*. Mice (average body weight 21 ± 0.8 g) were divided into four groups: free DOX, FDEP30 NPs, FDEP50 NPs and FDEP80 NPs ($n = 4/\text{group}$). The mice were tail-vein injected with free DOX (200 μL , 2 mg/kg), FDEP30 NPs (DOX dose of 2 mg/kg), FDEP50 NPs (DOX dose of 2 mg/kg) or FDEP80 NPs (DOX dose of 2 mg/kg). After injection, the whole blood samples were collected at 10 min, 0.5, 1, 2, 4, 24 and 48 h postinjection from the submandibular vein. The blood (100 μL) was collected and mixed with physiological buffered saline (PBS, 900 μL) containing 10 mM EDTA anticoagulant. All blood samples were centrifuged for 20 min, then extract solution (4 mL) containing chloroform and methanol (4 : 1) was added, the mixture was centrifuged at 3000 rpm for 20 min and the supernatants were collected and dried by N_2 . Finally, each sample was dissolved in 300 μL methanol and the DOX amount was measured by HPLC. The blood time-activity curve was fitted based on a two-compartment pharmacokinetic model.

3. Results

3.1. Self-assembly and characterization of coordination based nanoplatform and drug release *in vitro*

As illustrated in Fig. 1a, the Fe^{3+} ions coordinate with hydrophobic DOX in DMF to form the core of nanoparticles; EGCG and PEG polyphenol aqueous solutions are further assembled with Fe^{3+} to yield the Fe^{3+} -DOX@EGCG-PEG nanoparticles (denoted as FDEP NPs). In this FDEP NPs system, the DOX and EGCG are encapsulated in the core of FDEP NPs and a physical barrier is achieved between drugs and external environment by PEG polyphenols. Upon internalization by cells, the release of EGCG from the FDEP NPs inhibits the activity of CBR1 overexpressed in glioma cells, which therefore improves the potency of DOX against cancerous cells (Fig. 1b). Furthermore, the reduced activity of CBR1 ameliorates DOX-induced cardiotoxicity by blocking the reduction of DOX to DOXOL. Thanks to the high PEG ratio in the FDEP NPs, the nanoparticles exhibit reasonably long circulation half-life and high tumor accumulation, which are beneficial for tumor delivery and therapy.

First, the PEG polyphenols were prepared by a reported method,[15] and the structure was identified by ^1H NMR spectroscopy (Fig. S1). The hydrophobic DOX in DMF was mixed with three different volumes of FeCl_3 (30, 50 and 80 μL) at the same concentration (3 mg/mL). Then, the EGCG and PEG polyphenols aqueous solutions were further added into the above solution and dialyzed against H_2O to obtain three types of FDEP NPs (denoted as FDEP30 NPs, FDEP50 NPs, and FDEP80 NPs with 30, 50, and 80 μL of FeCl_3 , respectively). *The components of three kinds of FDEP NPs have been further measured by ICP-OES for Fe ions and HPLC for DOX and EGCG, respectively. The ratios of PEG polyphenols in the three kinds of FDEP NPs were given in Table S1. It can be seen that PEG polyphenols were the main components in all the FDEP NPs.* The TEM images of the three kinds of FDEP NPs were given in Fig. 1c. Interestingly, all the three as-prepared FDEP NPs exhibited excellent monodispersity and rod-like morphology. The size of FDEP NPs was dependent on the amount of FeCl_3 . The hydrodynamic sizes of FDEP30 NPs, FDEP50 NPs,

and FDEP80 NPs were 77.4 ± 13 , 126 ± 24 , and 296 ± 19.7 nm as measured by dynamic light scattering (DLS). The DLS results matched well with the TEM observations. The zeta potentials of three kinds of FDEP NPs have been measured and provided in Fig. S2. All three NPs are negatively charged (~ 20 mV) owing to the polyphenols (EGCG and PEG polyphenols) in the nanoparticles, which was consistent with the previous report.[15] The high concentration Fe^{3+} (FDEP80 NPs) could coordinate with more polyphenols and increase the zeta potential.

The drug release curves in vitro were provided in Fig. S3, revealing that FDEP NPs exhibited a pH-controlled release behaviour for both DOX and EGCG. All the FDEP NPs were quite stable at pH 7.4. Less than 20% of DOX or EGCG released at pH 7.4 within 48 h. In contrast, DOX and EGCG release amounts at pH 5.5 were 45.3% or 56.5% after 48 h, which were significantly higher than those at pH 7.4 (13.0% of DOX or 16.6% of EGCG). The above release phenomenon can be attributed to the pH dependent coordination between metal and polyphenols. At low pH, most of the polyphenol hydroxyl groups were protonated, leading to the rapid disassembly of the FDEP NPs and release of both DOX and EGCG.

3.2. Cell uptake, CBR1 expression, and DOXOL generation from two different cell lines

The cell uptake of AF488-labelled FDEP30 NPs and FDEP50 NPs were evaluated by confocal microscopy and flow cytometry. The U87MG cells were incubated with FDEP30 NPs and FDEP50 NPs for 1, 4, and 8 h separately. As seen in Fig. 2a and b, the efficient cell uptakes were observed from both FDEP30 NPs and FDEP50 NPs by confocal microscopy images. Moreover, flow cytometry data indicated that a longer incubation time led to relatively higher cell association (Fig. S4). When the incubation time was prolonged from 1 to 8 h, the fluorescence of DOX could be observed in the cell nucleus (Fig. 2a and b), suggesting the release of DOX from the FDEP NPs in the cytoplasm. The changes in U87MG cell morphology after the incubation of FDEP30 and FDEP50 NPs were observed under confocal microscopy. The relative CBR1 expression from two kinds of cells (U87MG cancerous cells and 293T normal cells) were investigated by flow cytometry. As shown in Fig. 2c and d, the results indicated that the CBR1 level in U87MG cancerous cells is much higher than that in 293T normal cells. Therefore, inhibition of CBR1 in U87MG cells by EGCG from FDEP NPs may significantly reduce the DOXOL generation and promote DOX toxicity. Subsequently, the CBR1 inhibitory effect of FDEP30 NPs and FDEP50 NPs was further evaluated by flow cytometry (Fig. 2c and d) and Western blot (Fig. 2e and f). The level of CBR1 protein expression decreased in both U87MG and 293T cells after incubation with DOX + EGCG, EGCG, FDEP30 NPs, and FDEP50 NPs. Most importantly, the FDEP NPs exhibited stronger inhibition effect than the free EGCG, which can be attributed to higher concentration of EGCG release from FDEP NPs after internalization into cells by endocytosis.[30] Furthermore, the DOXOL generation from both U87MG and 293T cells were evaluated by HPLC after various treatments (Fig. 2g and h). Both the FDEP30 NPs and FDEP50 NPs could inhibit DOXOL production in the U87MG and 293T cells.

3.3. Cytotoxicity and cell apoptosis studies of FDEP30 NPs, FDEP50 NPs and FDEP80 NPs

In vitro cytotoxic effect of FDEP30 NPs and FDEP50 NPs were further tested by two cell lines (U87MG and 293T cells). The concentrations of DOX and EGCG were determined by HPLC (Fig. S5 and S6). As given in Fig. 3a and b, all the drug formulas presented dose-dependent toxicity against the two cell lines. Especially, in the U87MG tumor cells, FDEP30 NPs and FDEP50 NPs both showed inhibition ratios greater than 90%. By contrast, DOX and DOX + EGCG inhibited a maximum of 70% and 75% of U87MG cells, respectively. In addition, negligible toxicity was found for free EGCG against both U87MG and 293T cells. The half maximal inhibitory concentration (IC_{50}) of free DOX was higher than the two FDEP NPs, indicating the excellent synergistic combination effect of FDEP NPs (Fig. 3c and d). In contrast, the two FDEP NPs exhibited less potency against 293T normal cells than U87MG cancerous cells, which should be attributed to the relatively low expression of CBR1 in 293T normal cells.[31] We also investigated the cytotoxicity of FDEP80 NPs (Fig. S7). The IC_{50} of FDEP80 NPs was 6.2 μ M against 293T cells, which was lower than both FDEP30 NPs (15.6 μ M) and FDEP50 NPs (19.5 μ M). The FDEP80 NPs exhibited more cytotoxic effect against normal 293T cell line than the two other NP formulas. The IC_{50} value of FDEP80 NPs against U87MG cells was 1.76 μ M, higher than those of FDEP30 NPs (0.21 μ M) and FDEP50 NPs (0.25 μ M).

The U87MG cell apoptosis study was further investigated by a dead cell apoptosis kit. As given in Fig. 3e, f and S8, the quantitative flow cytometry results indicated the cells undergoing early apoptosis (Q2) to late apoptosis (Q4). The percentage of apoptotic cells increased with incubation time. The FDEP30 NPs displayed the highest percentage of apoptosis after 48 h incubation. Then, Western blot analyses were performed in U87MG cells (Fig. 3g and S9). Compared with free EGCG and DOX, both FDEP30 and FDEP50 NPs significantly increased the p53 protein level, which further confirmed the apoptotic effect of FDEP NPs.[32] The above results suggested that the two FDEP NPs had low cytotoxicity to normal cells and high cytotoxicity to U87MG cancerous cells overexpressing CBR1 protein.

We further investigated the penetration behavior of FDEP30 NPs and FDEP50 NPs by multicellular spheroids (MCS) model *in vitro*. The MCS with a size of about 500 μ m were prepared by our reported method.[18] Both the Ce6-labelled FDEP30 NPs and Ce6-labelled FDEP50 NPs were co-cultured with MCS for 24 h. As given in Fig. S10 and S11, the strong red fluorescence can be seen from the core of the Ce6-labelled FDEP30 NPs and Ce6-labelled FDEP50 NPs, indicating the effective penetration of FDEP30 NPs and FDEP50 NPs into MCS. The mean fluorescence intensity of Ce6-labelled FDEP30 NPs was higher than that of Ce6-labelled FDEP50 NPs (Fig. S12), which may be because of the relatively smaller size of the FDEP30 NPs.

3.4. PET and optical imaging for biodistribution and pharmacokinetic studies

Further biodistribution and pharmacokinetic studies were evaluated in detail. First, the whole-body PET imaging was performed by both the ^{89}Zr labelled FDEP30 and FDEP50 NPs. The *p*-isothiocyanatobenzyl-desferrioxamine (DFO) was conjugated to the PEG

polyphenols and coordinated with ^{89}Zr . [33] The ^{89}Zr labelled FDEP30 and FDEP50 NPs were injected into the U87MG tumor-bearing mice intravenously. The mice were scanned at various time points post-injection. The quantitative PET imaging results were shown in Fig. 4a, both FDEP30 NPs and FDEP50 NPs quickly accumulated at the tumor site within 4 h. The PET signals reached a peak at 24 h post-injection with 7.73 ± 0.35 %ID/g (FDEP30 NPs) and 6.89 ± 0.29 %ID/g (FDEP50 NPs). Even after 72 h post-injection, the tumor uptakes from FDEP30 NPs and FDEP50 NPs were still as high as 5.00 ± 0.57 %ID/g and 4.69 ± 0.68 %ID/g, respectively. All the mice were euthanized after PET scanning at 72 h post-injection. The blood and main organs were collected to measure the radioactivity level by a gamma counter (Fig. S13), which matched well with the PET results. In addition, Ce6-labelled FDEP30 NPs and FDEP50 NPs were intravenously injected to U87MG tumor mice for fluorescence imaging. [34] The major organs and tumors were collected at 48 h post-injection for *ex vivo* imaging (Fig. 4b). Strong signals could be seen from tumor in both Ce6-labelled FDEP30 NPs and FDEP50 NPs groups. Indeed, the signal from tumor was much stronger than that from all the other organs in the FDEP30 NPs group. Compared with the FDEP50 NPs, the FDEP30 NPs exhibited better tumor accumulation due to the relatively smaller particle size. Moreover, the pharmacokinetics study of FDEP30 NPs, FDEP50 NPs, and free DOX were further performed by measuring plasma DOX levels at various time points after intravenous injection (Fig. 4c). As given in Fig. 4c, Fig. S14, and Table S2, the blood circulation half-lives of FDEP30 NPs, FDEP50 NPs, FDEP80 NPs and free DOX were 26.05, 23.9, 6.92, and 0.69 h, respectively. The promising circulation profiles of FDEP NPs ensured the high tumor accumulation. The half-lives of both FDEP30 NPs and FDEP50 NPs were more than 36-fold higher than DOX. According to the PET imaging results, the pharmacokinetics was further tested by quantitative analysis of heart signals at various time points. [35] The half-life of FDEP NPs by measuring DOX level in plasma was consistent with that by ^{89}Zr -PET (Fig. 4d and Table S3). As the above two methods were based on the measurement of different compositions from the FDEP NPs, we can conclude that the FDEP NPs were quite stable during circulation.

3.5. Blood biochemistry study and cardiac toxicity

In this study, FDEP NPs were designed by utilizing the coordination between Fe^{3+} ions and polyphenols, which aimed to decrease the conversion from DOX to DOXOL, thereby reducing cardiotoxicity and enhancing chemotherapeutic effect at the same time. The acute toxicity of FDEP30 NPs and FDEP50 NPs was tested *in vivo*, and some important blood biochemistry parameters were measured after administration of the two FDEP NPs and free DOX at the same doses. The blood from the above groups were collected and analyzed at 1, 3, and 7 days post-injection. As shown in Fig. 4e-h, serious hepatotoxicity and cardiotoxicity were found from the free DOX treatment group with significantly higher levels of AST, ALT, CK, and CK-MB. Meanwhile, LDL was slightly higher from the DOX treatment group (Fig. S15). However, the blood parameters of mice from the two FDEP NPs were within the normal range. The above results suggested that the two EGCG-loaded FDEP NPs could significantly reduce the toxicity of DOX to blood cells. The cardiac toxicity was evaluated by H&E staining of heart tissue after euthanizing the mice 5 days post-injection (Fig. 4e). Compared with the FDEP30 and FDEP50 NP groups, the free DOX showed slight damage

to the heart. Moreover, neither free DOX nor FDEP NPs had obvious histological signs of liver damage (Fig. S16).

3.6. The antitumor activity of FDEP30 NPs and FDEP50 NPs

The tumor inhibition effect of FDEP30 NPs and FDEP50 NPs was further evaluated in U87MG tumor mice. All the mice were randomly divided into six groups and administered various formulas. The tumor size and body weight were measured every other day. As given in Fig. 5a and S17, compared with the free drug group, both the FDEP30 NPs and FDEP50 NPs could significantly delay tumor growth thanks to the long circulation time and high tumor accumulation of the NPs. The FDEP30 NPs exhibited the most prominent tumor growth inhibition (TGI) rate (74.38%), followed by FDEP50 NPs (69.75%), DOX (40.08%), DOX + EGCG (44.13%) and EGCG (18.02%) (Fig. 5b). Moreover, the FDEP NPs groups could significantly prolong the mouse survival (Fig. 5c). There is no statistically significant difference between the control group and free drug groups (DOX and DOX+EGCG groups). In addition, there was no obvious body weight loss from the FDEP NPs groups (Fig. 5d). In contrast, the body weight of mice decreased with free DOX administration, which was attributed to severe side effects and systemic toxicity of DOX. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stained tumor section images were shown in Fig. 5e, with high percentages of green fluorescence that could be observed from FDEP30 NPs and FDEP50 NPs groups. As given in Fig. 5f, the percentages of apoptosis/necrosis from the TUNEL staining images were 3.2, 10.3, 25.4, 27.9, 45.06, and 48.7% in the groups of control, EGCG, DOX, DOX + EGCG, FDEP30 NPs, and FDEP50 NPs, respectively, as calculated by the Image-pro plus 6.0 software (Media Cybernetics, Inc., MD, USA). The representative tumor tissue sections from various treatment groups were given in Fig. S18. Compared with the other free drug administration groups, the tumor tissues from the FDEP30 NPs and FDEP50 NPs groups exhibited high populations of dead cells, intercellular blank spots, and necrosis, indicating effective tumor therapy. The heart and liver tissues of mice from all the treatment groups were excised and further evaluated by histopathological examination after the treatment (Fig. S19 and S20). There were no distinct pathological changes in the heart and liver of the mice administered with DOX + EGCG, FDEP30 NPs, or FDEP50 NPs. However, the heart and liver sections from the free DOX administration group showed slight myocardial necrosis (Fig. S19c and S20c). That result suggested that free EGCG or FDEP NPs through EGCG coordination could decrease the cardiotoxicity effectively because the EGCG could inhibit the activity of CBR1 and thereby reduce the DOXOL generation in cells. To confirm the above mechanism, the expression levels of CBR1 and p53 were measured from the tumor tissues by Western blot analyses (Fig. 5g and 5h). Free EGCG and EGCG + DOX could reduce the expression levels of CBR1 to a certain degree. However, the expression levels of CBR1 from FDEP30 NP and FDEP50 NP groups significantly decreased. The above results could be attributed to the high tumor accumulation of FDEP30 NPs and FDEP50 NPs, which can block the CBR1 expression by EGCG release. Meanwhile, the p53 protein levels of the FDEP30 NPs and FDEP50 NPs groups increased, indicating effective DNA damage in tumor cells. As the CBR1 expression was inhibited by EGCG from the FDEP30 NPs and FDEP50 NPs, the lower activity of CBR1 could reduce DOX reduction to DOXOL and thereby improve the anti-tumor efficacy of DOX.

Discussion

While nanoplatform-based cancer theranostics have been investigated for many years, there are still many issues remaining that hinder their clinical translations. In this study, we exploited both natural polyphenols (EGCG) and their derivatives (PEG polyphenols) and designed the FDEP NPs by a tailor-made process. The therapeutic agents and PEG are the main components without the need for additional delivery vehicles, which can reduce the unwished side effects of drug carriers.

Three kinds of rod-like FDEP NPs including FDEP30 NPs, FDEP50 NPs, and FDEP80 NPs were prepared. The size of FDEP NPs can be tuned by varying the added amount of FeCl_3 . It is necessary improve therapeutic efficacy while reducing the side effects and risks imposed by both the nanovehicles and cargoes and our system does just that. Polyphenols, compounds found in our diet, can help prevent degenerative diseases such as cancer and cardiovascular diseases. EGCG can inhibit the CBR1 protein expression, which reduces DOX to DOXOL, a main cause of drug resistance and cardiotoxicity. Here, we demonstrate that EGCG in FDEP NPs could effectively overcome cardiotoxicity of DOX and enhance the cancer treatment efficacy. Glioma is the most common malignant primary tumor of the brain in adults, accounting for 81% of malignant brain tumors. The U87MG, a classic glioma cell line which can overexpress the CBR1 protein, was employed as the tumor model for cancer treatment. Significantly, the toxicity of FDEP NPs is relatively low against normal cells with low CBR1 protein expression, possibly opening a new avenue for specific killing of only CBR1 protein overexpressing cancer cells.

The long blood circulation time of drug-loaded nanomaterial is essential to guarantee the enhanced drug accumulation in the tumor region by the enhanced permeability and retention (EPR) effect. In this work, high ratio of PEG (65 % by weight) with high molecule weight in the nanoparticles could significantly enhance the blood circulation time and tumor accumulation. The blood circulation half-lives of FDEP30 NPs and FDEP50 NPs were 20.5 h and 25 h, respectively, which were higher than most reported nanoparticle formulas in the literature. Another interesting finding was the NIR fluorescence signal of FDEP30 NPs in tumor was higher than all the other main organs at 24 h post-injection, indicating highly effective accumulation of nanoparticles in the tumor. The stability of nanomaterials is another important factor, which influences the safety and efficacy of the nanomaterials. The as-prepared FDEP30 NPs and FDEP50 NPs were stable during the blood circulation as confirmed by both PET imaging and pharmacokinetic studies. Interestingly, both FDEP30 NPs and FDEP50 NPs could effectively reduce CBR1 protein expression *in vivo* and inhibit tumor growth. In addition, the reduced activity of CBR1 could ameliorate the DOX-induced cardiotoxicity by blocking the reduction of DOX to DOXOL.

4. Conclusions

In this research, we have exploited the coordination between Fe^{3+} and polyphenols to encapsulate DOX and EGCG in the nanoplatform by self-assembly. The EGCG not only improves the DOX efficacy, but also reduces cardiotoxicity by inhibiting CBR1 expression and further DOXOL production. The long blood circulation time leads to high tumor

accumulation of FDEP NPs. Furthermore, compared with free drugs groups, both the FDEP30 NPs and FDEP50 NPs could inhibit tumor growth and prolong survival of tumor mice. We believe that this therapeutic nanoplatform integrating DOX and EGCG with high efficacy, negligible cardiotoxicity and effective tumor delivery, represents an advance in nanomedicine and has great potential for clinical cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Formulation and characterization of nanoparticles and proposed mechanism of enhanced anticancer effect.

(a) Schematic illustration of the procedures for self-assembly of FDEP NPs. (b) The mechanism of FDEP NPs in the intracellular environment. Upon internalization by cells, EGCG from FDEP NPs inhibits the activity of CBR1, thereby promoting the efficacy of DOX and ameliorating DOX-induced cardiotoxicity by preventing the reduction of DOX to DOXOL. (c) TEM images and DLS of FDEP30 NPs (left), FDEP50 NPs (middle), and FDEP80 NPs (right).

Fig. 2. Cell uptake, CBR1 expression and DOXOL generation from various cell lines. (a-b) The confocal microscopy images of U87MG cell uptake and DOX release from FDEP30 NPs (a) and FDEP50 NPs (b) following various incubation times. Both the FDEP30 NPs and FDEP50 NPs were labelled with AF488 (green). The blue and red fluorescence were from the cell nucleus (DAPI) and DOX, respectively. Scale bar: 25 μ m. (c-d) The flow cytometric analyses of CBR1 expression from U87MG cells (c) and 293T cells (d). (e-f) The CBR1 expression from 293T cells and U87MG cells by Western blotting after various treatments. (g-h) The HPLC results of DOXOL generation from cells by incubating U87MG cells (g) or 293T cells (h) with FDEP30 NPs, FDEP50 NPs and free drug.

Fig. 3. Cell viability, apoptosis, and Western blot analysis after treatment with FDEP30 NPs and FDEP50 NPs.

(a-b) Cell viability of 293T cells (a) and U87MG cells (b) following incubation with free drug, FDEP30 NPs, and FDEP50 NPs for 48 h. (c-d) The IC₅₀ values of free drug, FDEP30 NPs, and FDEP50 NPs for 293T cells (c) and U87MG cells (d). (e-f) The cell apoptosis of U87MG cells after incubation with FDEP30 NPs and FDEP50 NPs for 24 and 48 h (e), and the apoptosis cycle percent in Q2 and Q4 zones (f). (g) Western blot results of p53 expression from U87MG cells after 48 h incubation with free drug, FDEP30 NPs, and FDEP50 NPs.

Fig. 4. In vivo PET and optical imaging, pharmacokinetic study, blood biochemistry and cardiac toxicity analysis.

(a) Whole-body PET images of ^{89}Zr -labelled FDEP30 NPs and FDEP50 NPs in U87MG xenograft mice. White dash circles indicate the tumor location. (b) *Ex vivo* NIR fluorescence images of major organs and tumor at 48 h post-injection of Ce6-labelled FDEP30 NPs and FDEP50 NPs. (c) Pharmacokinetics of free DOX, FDEP30 NPs, and FDEP50 NPs by measuring plasma DOX concentration in mice ($n = 4/\text{group}$). (d) Pharmacokinetics of ^{89}Zr -labelled FDEP30 NPs and FDEP50 NPs through quantitative analysis of heart signals at various time points by PET imaging. (e-h) Blood biochemistry tests of AST (e), ALT (f), CK (g), and CK-MB (h) after various treatments ($n = 4/\text{group}$). $*P < 0.05$; $**P < 0.01$. (i) H&E stained images of heart after various administrations. Scale bar: 100 μm .

Fig. 5. *In vivo* antitumor evaluation.

(a-d) U87MG tumor growth curves (a), tumor growth inhibition rates (b), survival curve (c), and body weight changes (d) after various treatments (*n* = 5/group). **P* < 0.05; ***P* < 0.01. (e-f) Tumor tissue images (e) and apoptosis/necrosis ratio (f) after TUNEL staining assay. Scale bar: 200 μm. (g-h) The tumor tissue Western blot results of CBR1 and p53 expression under various treatments. **P* < 0.05; ***P* < 0.01.