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RESEARCH ARTICLE

Evaluation of the tumor targeting of a FAP α -based doxorubicin prodrug

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Abstract

Fibroblast activation protein- α (FAP α) is a tumor-associated antigen uniquely expressed by reactive stromal fibroblasts in the majority of human epithelial tumors. FAP α also possesses both post-prolyl peptidase and endopeptidase activities. Consequently, FAP α is increasingly considered as a potential pan-tumor target for designing tumor-targeted prodrugs. We previously conjugated Doxorubicin (Dox) with a FAP α -specific dipeptide (Z-Gly-Pro) to develop a FAP α -targeting prodrug of Dox (FTPD). The aim of current work was to validate the tumor targeting of this targeted-delivery strategy. The results demonstrated that FTPD could effectually release Dox upon the hydrolysis of FAP α as well as the incubation with tumor homogenate of FAP α -positive tumor (4T1 tumor), while it was highly stable in mouse plasma and a variety of tissue homogenates including heart, liver, and so on. And the FAP α -cleaved FTPD exhibited significantly higher cytotoxicity against 4T1 cells *in vitro* than the uncatalyzed prodrug. Additionally, FTPD produced similar antitumor efficacy in 4T1 tumor-bearing mice to free Dox without obvious cardiotoxic effect. Moreover, subsequent study indicated that the accumulation of FTPD reduced significantly in the heart compared to free Dox. These findings suggest that such FAP α -based prodrug strategy is promising to achieve targeted delivery of antitumor agents.

Keywords: Fibroblast activation protein- α , prodrug, doxorubicin, targeting, delivery

Introduction

In clinical chemotherapy of cancers, the majority of therapeutic drugs used are cytotoxic agents and unfortunately fail to achieve expected efficacy mainly due to the toxic side effects (Chen & Hu, 2009). Doxorubicin (Dox) is a well known representative of those chemotherapeutic drugs. In spite of the potent antitumor activity, Dox is usually restricted in clinical application because of the severe toxicities to normal tissues, especially the heart (Zhou et al., 2001; Carvalho et al., 2009). Actually, the toxic side effects of cytotoxic agents are primarily attributable to the lack of selectivity toward tumor. Hence, the targeted delivery of antitumor agents is indispensable for the tumor therapy.

Currently, the strategy of tumor-targeting prodrugs is being regarded as a promising approach to reduce side effects of current anticancer agents. So far, a number of prodrug strategies have been proposed and some effective anticancer prodrugs have been reported (Atkinson et al., 2008; Chen & Hu, 2009; Law & Tung, 2009). Most of them are based on some specific enzymes such as cytochrome P450, carboxypeptidase and prostate-specific antigen (PSA; Atkinson et al., 2008; Chen & Hu, 2009; Law & Tung, 2009). Generally, the design of anticancer prodrugs principally rests on the special features related to tumors, and should be selectively activated by a specific enzyme or a metabolic pathway, either of which is only present or predominantly present in targeted tumor site

SH and RF contributed equally to this work.

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Abbreviations

AFC	7-amino-4-trifluoro-methylcoumarin
Dox	doxorubicin
DPPIV	dipeptidyl peptidase IV
FAP α	fibroblast activation protein- α
FTPD	FAP α -targeting prodrug of doxorubicin

FBS	fetal bovine serum
HPLC	high-performance liquid chromatography
Rt	retention time
TAF	tumor-associated fibroblasts
Z-GP-AMC	<i>N</i> -carbobenzoxy-glycyl-L-proline-4-methylcoumaryl-7-amide

(Denny, 2001; Papot et al., 2002; Tanabe et al., 2007). In other words, the attainment of such prodrug is to some extent dependent upon the target which it bases on.

Lately, tumor-associated fibroblasts (TAF), termed reactive stromal fibroblasts, have increasingly become one noticeable target site of tumor as a whole. These reactive stromal fibroblasts constitute the predominant cellular component of the tumor stroma (Elenbaas & Weinberg, 2001; Tlsty, 2001; Mueller & Fusenig, 2004). More attractively, the surface of TAF is characterized by high expression of fibroblast activation protein- α (FAP α ; Rettig et al., 1988; Garin-Chesa et al., 1990; Rettig et al., 1993; Scanlan et al., 1994). Studies have indicated that FAP α is highly restricted to TAF in over 90% of common human epithelial carcinomas, but is not detected in normal adult tissues except tissues of healing wound (Rettig et al., 1993; Scanlan et al., 1994; Ariga et al., 2001; Chen & Kelly, 2003). FAP α also shows essential functions to regulate the growth and development of tumor (Ariga et al., 2001; Cheng et al., 2002; Chen & Kelly, 2003; Huang et al., 2004). Consequently, FAP α has been treated as a tumor-associated antigen. And FAP α is a type II transmembrane serine protease belonging to the post-prolyl peptidase family. As we know, the most homologous family member of FAP α is dipeptidyl peptidase IV (DPPIV), and both of FAP α and DPPIV have the capability of cleaving *N*-terminal post-prolyl aminos such as Ala-Pro-7-amino-4-trifluoro-methylcoumarin (AFC), Gly-Pro-AFC and Lys-Pro-AFC (Morimoto & Schlossman, 1998; Iwata & Morimoto, 1999; Levy et al., 1999; Park et al., 1999; Ajami et al., 2003). In contrast to DPPIV, FAP α exclusively exhibits endopeptidase activity toward *N*-blocked amino acid substrates (Collins et al., 2004). Aertgeerts et al. (2005) substantiated that FAP α , but not DPPIV, could specifically hydrolyze the *N*-terminal benzyloxycarbonyl (Z)-blocked peptides, such as substrate Z-GP-AMC. Therefore, on the basis of the highly selective expression and the unique proteolytic activity, FAP α would be utilized as a tumor-associated protease for selective delivery of anticancer agents. Recently, a relevant study reported that a FAP α -activated promelittin protoxin was constructed and produced a significant inhibitory effect on both human breast and prostate cancer xenografts with minimal toxicity to the host animal (LeBeau et al., 2009).

In an earlier time, Firestone et al. proposed a considerable strategy to design FAP α -activated antitumor compounds (Firestone et al., 2003), and conducted a

series of preliminary studies. Nevertheless, whether this approach of the FAP α -based prodrug would definitely improve the targeting of antitumor agents? And whether could it indeed reduce the toxic side effect as expected? And even whether could it be ultimately utilized as a treatment for a wide range of cancers? Up to now, such queries still demand more proofs to elaborate and validate.

Previously, we had developed a FAP α -targeting prodrug of Dox (FTPD), which was incorporated with a FAP α -cleavable bond between Dox and the specific dipeptide Z-Gly-Pro (Figure 1A). The aim of our current research was to evaluate the tumor targeting of FTPD so as to provide further evidence to substantiate the feasibility of such FAP α -based prodrug strategy.

Experimental designs and methods

Chemicals and materials

Doxorubicin and daunorubicin were purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents were purchase from Merck (Darmstadt, Germany). Recombinant human FAP α (rhFAP α) was purchased from R&D Systems (Minneapolis, MN). All other chemicals used were at least reagent grade and obtained from Sigma-Aldrich or Merck and were used without further purification. The buffers used were vacuum-filtered through a 0.22 μ m membrane. Cell culture media, supplements and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD). All culture flasks were obtained from Corning (Corning, NY).

Synthesis of FTPD

FAP α but not DPPIV displays endopeptidase activity toward *N*-terminal benzyloxycarbonyl (Z)-blocked peptides, such as substrate Z-Gly-Pro-AMC. On the basis of these facts, we used the specific Z-Gly-Pro dipeptide to decorate Dox for the synthesis of a FTPD. The synthetic route for preparing the FTPD-conjugate was depicted in Figure 1B. The conjugate has an approximately 834 of molecular weight (834 g/mol). The purity of the conjugate used in this research was over 99%.

Chromatography

High-performance liquid chromatography (HPLC) assay for cleavage of FTPD by FAP α was performed with Agilent 1200 series (Karlsruhe, Germany); UV-detection at 495 nm; column: ZirChrom Separations Inc. (Anoka,

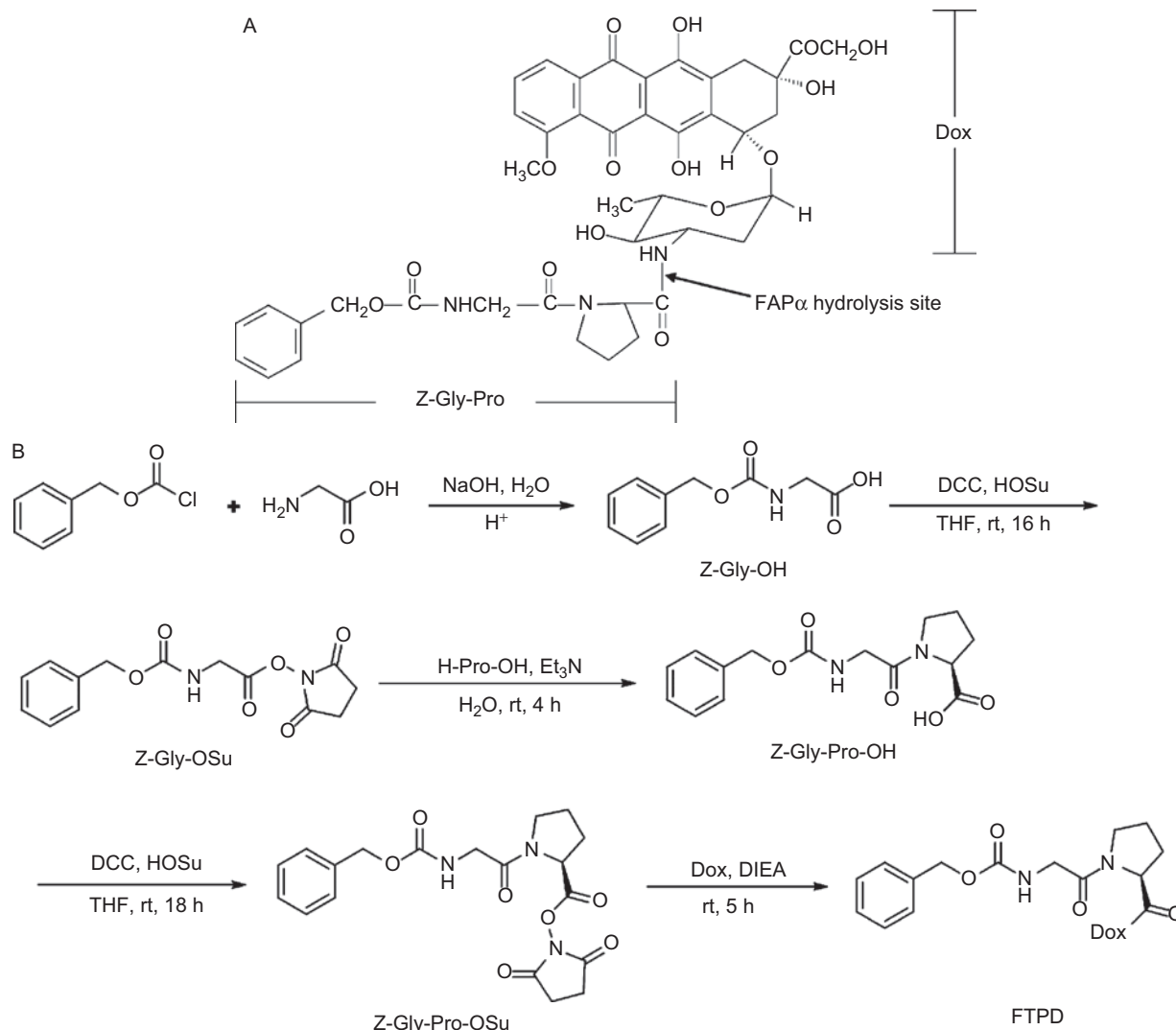


Figure 1. Chemical information of the fibroblast activation protein- α (FAP α)-targeting prodrug of Dox (FTPD). (A) Molecular structure of the FTPD conjugate. FAP α -cleavage site is indicated. (B) Synthetic scheme of the FTPD conjugate. Rt, retention time; Z-Gly-Pro, FAP α -specific dipeptide.

MN), reverse-phase C18 (4.6 $\times$ 250 mm²) with precolumn; chromatography conditions: flow: 1.0 mL/min; mobile phase A: water added with 0.1% trifluoroacetic acid (TFA, v/v); mobile phase B: acetonitrile; gradient: 0–40 min from 70% A to 40% A; injection volume: 20 μ L.

HPLC assay for stability of FTPD incubated with plasma and tissue homogenates and the measurement of drug distribution was performed with Agilent 1200 series (USA); UV-detection at 495 nm and 234 nm; column: ZirChrom Separations Inc., reverse-phase C18 (4.6 $\times$ 250 mm²) with precolumn; chromatography conditions: flow: 1.0 mL/min; mobile phase A: water added with 0.1% TFA (v/v); mobile phase B: acetonitrile; gradient: 0–40 min from 65% A to 60% A; injection volume: 20 μ L.

Cleavage of FTPD by FAP α

FTPD was incubated in Tris-buffer (pH 7.4) with rhFAP α (2 μ g/mL or 5 μ g/mL) at a final concentration of 30 μ M at 37°C for 1, 2, 4, 8, 16, and 24 h. Subsequently, the sample of each time point was collected and analyzed by HPLC.

Cell line and culture

Murine breast cancer cells (4T1) were obtained from China Center for Type Culture Collection (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute 1640 culture medium, supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 U/mL streptomycin. Cells were incubated at 37°C with 5% CO₂. Culture medium was routinely changed every 2 days. For subculture or experiments, cells were released from the flasks by treatment with 0.25% trypsin/ethylenediaminetetraacetic acid. For experiments, cells were used during the logarithmic growth phase.

Measurement of cytotoxicity *in vitro*

Cell viability assays were performed using Cell Counting Kit-8 (CCK-8) (WST-8, Dojindo, Japan) according to the literature protocol. Briefly, 100 μ L of 4T1 cell suspension (approximately 5000 cells) was dispensed in each well of a 96-well culture plate and the cells were allowed to adhere for 24 h. Subsequently, the 4T1 cells were

exposed, respectively to FTPD or Dox at various drug concentrations (0.01 ~ 10 μ M) for 48 h. Then, CCK-8 solution (10 μ L) was added to each well of the plate. After an incubation time of 4 h at 37°C, the absorbance was measured at a wavelength of 450 nm using a Microreader (Bio-Rad Laboratories Inc., Hercules, CA).

FTPD was incubated with rhFAP α (5 μ g/mL in Tris-buffer) at a final concentration of 30 μ M at 37°C. After 0 h, 16 h, or 24 h, samples were collected, respectively and filtered (0.22 μ m), and the release of Dox was confirmed. Subsequently, approximately 5000 4T1 cells in 100 μ L of medium were planted into each well of 96-well culture plate. After adherence for 24 h, the 4T1 cells were exposed, respectively to 4 μ L of above samples collected at each time point for 48 h. Cell viability was determined using the methods as mentioned above.

All experiments were performed in five wells on three separate occasions ($n=3$). Cell viability was expressed as a percentage viability of control group.

Establishment of FAP α -positive tumor-bearing model

BALB/c female mice (6–8 weeks) were purchased from Animal Experimental Center of Sun Yat-sen University (Guangzhou, China) and housed in a specific pathogen-free facility. All animal experiments were conducted in accordance with National Institutes of Health Animal Care and Use Committee guidelines. Cultured 4T1 cells were harvested from monolayer cultures, washed twice with serum-free medium, resuspended and injected subcutaneously (5×10^6 cells, 0.1 mL) into the right inguinal mammary gland of BALB/c mice. Tumor size was determined by caliper measurement according to the formula:

$$V = \text{Length} \times \text{Width}^2 \times 0.5$$

All of the animal experiments were performed using these 4T1 tumor-bearing model mice, which have been reported to be FAP α -positive (Lee et al., 2005).

Fifteen days after implantation, tumor tissues as well as blood plasma and five normal organs including heart, liver, spleen, lung, and kidney were, respectively obtained from the tumor-bearing mice for the subsequent study.

Cleavage of FTPD with tumor homogenate

Tumor tissue was obtained from 4T1 tumor-bearing model mice and cut into small pieces. Every 200 mg of tumor tissue was added to 800 μ L of homogenates buffer [50 mM Tris-HCl buffer (pH 7.4)] to prepare homogenate. FTPD was incubated with the tumor homogenate at a final concentration of 50 μ M. The samples were collected at selected time points and processed and measured using HPLC methods. Briefly, each sample (400 μ L) was added to tube containing 4 mL of a 4:1 mixture of methylene dichloride and acetonitrile. The tube was vigorously shaken, and the organic layer was collected by centrifugalization. After solvent evaporation and dissolution of the residue in 150 μ L of methanol, the sample was filtered (0.22 μ m) and analyzed by HPLC.

Evaluation of the stability of FTPD in mouse plasma and normal tissue homogenates

Samples of blood plasma and five normal organs including heart, liver, spleen, lung, and kidney were taken from tumor-bearing model mice, and organ homogenates were prepared with the method described above. Subsequently, FTPD was added into plasma and homogenates at a final concentration of 50 μ M for incubation at 37°C. The samples were collected, respectively at selected time points and processed and measured using HPLC.

Assessment of antitumor activity *in vivo*

The 4T1 tumor-bearing mice were randomized into control and treatment groups ($n=8$). When the tumors were palpable, mice in each treatment group were treated with Dox (at a dose of 8 mg/kg) or FTPD (at a dose of 12.2 mg/kg, Dox equivalent), respectively by intravenous administration via tail vein twice per week. The control groups received only normal saline.

The volume of administration was 0.2 mL/20 g body weight. Tumor size was measured three times weekly. Also, the body weight of mice was measured three times weekly. The mice were sacrificed after three weeks of administration, and tissue samples including heart, liver, spleen, lung, and kidney were collected immediately for pathological analysis.

Pathological analysis

Heart tissues obtained as described above were fixed in 10% neutral-buffered solution containing about 4% formaldehyde for 24 h and paraffin-embedded. Five sections of each tissue were evaluated using standard protocols for routine hematoxylin and eosin staining.

Drug distribution of FTPD in 4T1 tumor-bearing mice

Fifteen days after transplantation, 4T1 tumor-bearing mice were randomized into two experimental groups. Dox (at a single dose of 8 mg/kg) and FTPD (at a single dose of 12.2 mg/kg, Dox equivalent) were administered, respectively to the mice by intravenous injection. Mice in each group ($n=5$) were sacrificed, respectively 1 h, 4 h, or 12 h after administration. Tissues including heart and tumor were collected at the selected time points, immediately blotted on filter paper, trimmed of extraneous fat and connective tissue, weighed, and homogenized. The homogenates were stored at -80°C prior to use.

The total quantity of FTPD or Dox was determined by HPLC. Briefly, tissue homogenate (350 μ L) was mixed with 50 μ L of internal standard (IS) daunorubicin dissolved in methanol (20 μ g/mL). The mixture was added to a tube containing 4 mL of a 4:1 mixture of methylene dichloride and acetonitrile. The tube was vigorously shaken, centrifuged, and the organic layer was collected. After solvent evaporation and dissolution of the residue in 150 μ L of methanol, the sample was filtered (0.22 μ m) and analyzed by HPLC method. Concentration of drug was calculated based on calibration curves. Calibration was done using standards in a range of 0.5–50 μ g/mL and

linear regression analysis was used to generate best fit line by which the ratio of drug/IS was converted to concentration of drug.

Statistics

Values were presented as mean $\pm$ the standard deviation (SD). Statistical significance of differences was tested using the two-tailed Student's *t*-test, and a *P*-value below 0.05 was considered significant.

Results

Release of Dox from the prodrug by FAP α -cleavage

To confirm whether and how the FTPD conjugate could be specifically cleaved by FAP α , 30 μ M of the conjugates were incubated with rhFAP α (2 μ g/mL or 5 μ g/mL respectively) and chromatograms were recorded at 495 nm using reverse-phase HPLC. As shown in Figure 2A, after incubation with FAP α (5 μ g/mL) at 37°C for 24 h, the initial peak of FTPD (retention time $\sim$ 20.5 min) gradually dwindled and completely disappeared after 24 h, while a new distinct peak of free Dox was observed at 10 min and rose along with the peak of the conjugate falling. According to Figure 2B, the higher the concentration of FAP α was, the faster free Dox was released. Hydrolysis of FTPD incubated with 5 μ g/mL of

FAP α reached $85.36 \pm 3.66\%$, but that of FTPD-treated with 2 μ g/mL of FAP α showed merely $59.72 \pm 5.2\%$ in the same period, indicating that the release of Dox was dramatically FAP α -dependent. The results demonstrated that FTPD could be effectively cleaved by FAP α *in vitro* as expected.

Further, it is equally imperative to investigate whether the conjugate is effectually hydrolyzed in the FAP α -positive tumor tissue. Since 4T1 tumor-bearing model had been proved to be FAP α -positive (Lee et al., 2005), the 4T1 tumor tissue homogenate, therefore, was used to test the cleavage of FTPD. As illustrated in Figure 2C and 2D, approximately 50% of the conjugates (50 μ M) were cleaved to release free Dox after 4 h of incubation, and the cleavage was almost complete after 12 h, suggesting that Dox was efficaciously released from FTPD in the FAP α -positive tumor tissue.

On the other hand, to know the stability of FTPD in plasma and normal organ is likewise necessary, as the release of Dox from prodrug is anticipated to primarily present in tumor site *in vivo*. Therefore, FTPD was tested to be incubated *in vitro*, respectively with mouse plasma and normal organ homogenates including heart, liver, spleen, lung, and kidney at 37°C for 12 h. In Figure 3, the results showed that small amounts of free Dox were released after 12 h of incubation under each condition and < 20% of degradation was observed either in plasma

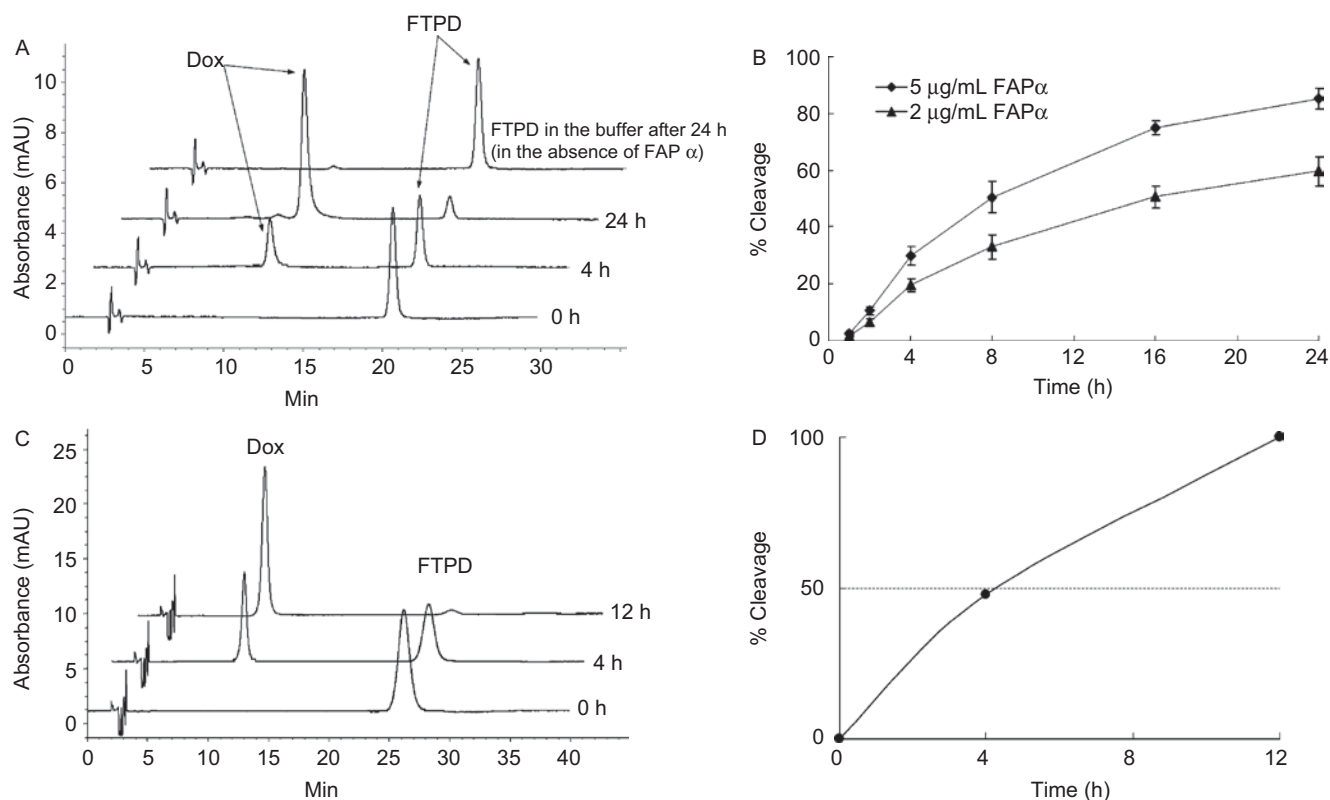


Figure 2. Fibroblast activation protein- α (FAP α)-based cleavage of FAP α -targeting prodrug of Dox (FTPD). (A) Chromatograms of incubation studies of FTPD (30 μ M) with rhFAP α (5 μ g/mL) for 24 h at 37°C. (B) Release of Dox from the FTPD conjugate (30 μ M) during the incubation with rhFAP α (5 μ g/mL or 2 μ g/mL) at 37°C (mean $\pm$ SD and *n* = 3). (C) Chromatograms of incubation studies of FTPD with 4T1 tumor homogenate at 37°C for 12 h. The concentration of FTPD was 50 μ M. (D) Release of Dox (50 μ M) from FTPD with 4T1 tumor homogenate at 37°C in the duration of 12 h.

or in normal organs, implying that the conjugate is relatively stable under the physiological condition. From above studies, FTPD was proved to be specifically hydrolyzed by FAP α to release free Dox.

FAP α -dependent restoration of cytotoxic effect of FTPD

Aside from knowing that FTPD could be specifically cleaved by FAP α , in like manner, it is indispensable to validate the assumption that the cytotoxicity of Dox would be largely reduced through the decoration with Z-Gly-Pro but could be restored due to the FAP α -cleavage. Accordingly, the cytotoxicity of both FTPD and the cleaved FTPD was assessed in 4T1 cell line by using CCK-8 assay. As depicted in Figure 4A, FTPD exhibited extremely low cytotoxicity against 4T1 cells, and even at a high concentration of 10 μ M, it merely killed $\sim$ 13% of cells; whereas Dox showed potent cytotoxicity against

4T1 cells with IC₅₀ value of $\sim$ 0.58 μ M. This evidence suggests that modification with the Z-Gly-Pro dipeptide could decrease cytotoxicity of Dox. Furthermore, the conjugate incubated with FAP α for 0 h, 16 h, or 24 h were tested to investigate the difference of cytotoxicity between the prodrug and the FAP α -incubated prodrug. The results (see Figure 4B) reflected that the cleaved conjugate produced a significant antitumor effect in comparison with the uncatalyzed conjugate ($P < 0.05$), and the cytotoxicity was enhanced along with the prolonging incubation during 16–24 h ($P < 0.05$). The above findings implied that the conjugate activated by FAP α would restore the cytotoxicity of the parent drug Dox.

FTPD producing similar antitumor efficacy to Dox in FAP α -positive tumor-bearing mice

On account of the evidence that the modification with the specific dipeptide could lead to cytotoxicity reduction

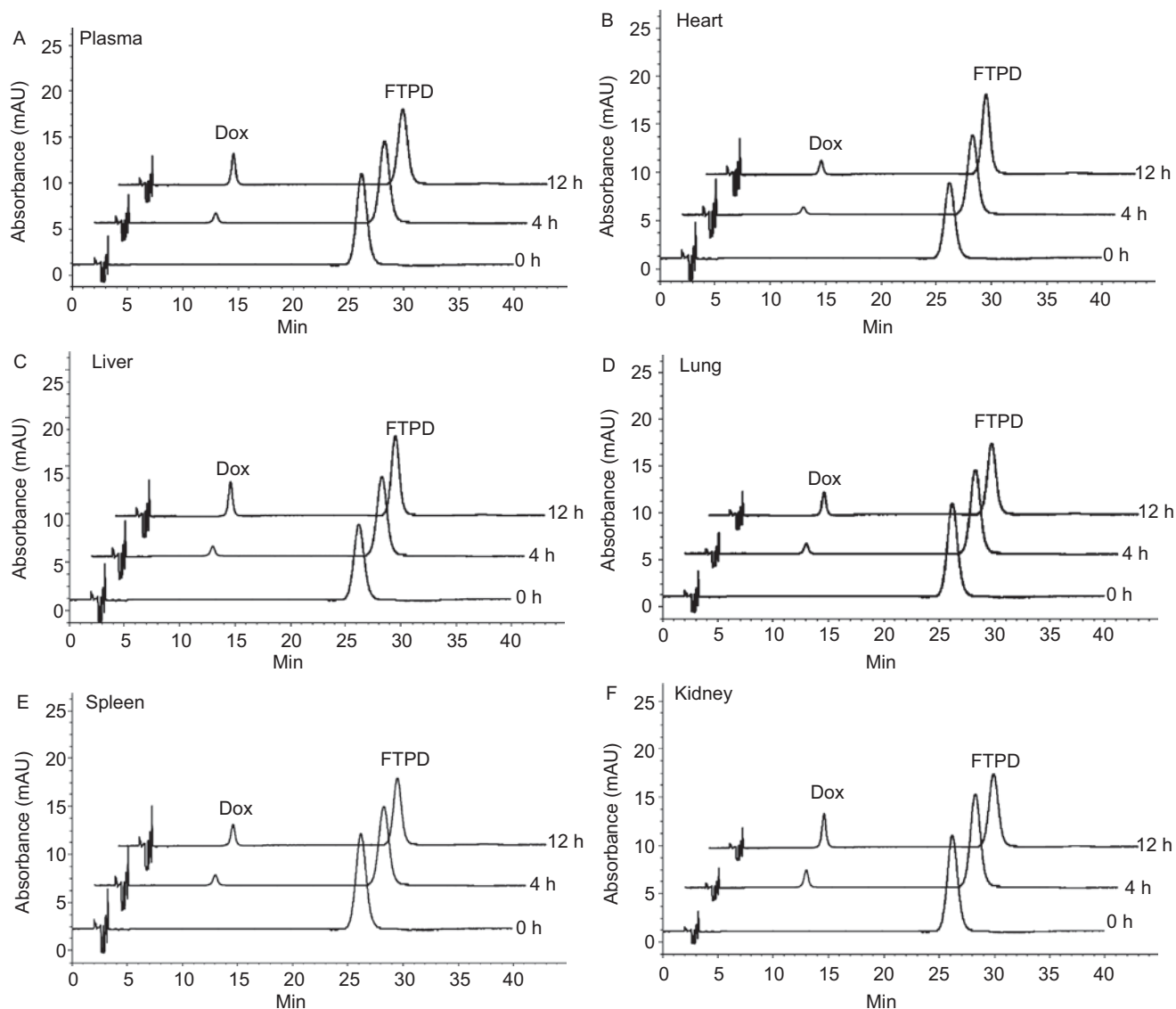


Figure 3. Chromatograms of stability studies of fibroblast activation protein- α -targeting prodrug of Dox (FTPD) under the condition of mouse plasma or normal tissue homogenates at 37°C for 12 h. Concentration of the FTPD conjugate was 50 μ M equally. (A) Plasma; (B) heart; (C) liver; (D) lung; (E) spleen; (F) kidney.

of Dox, it should be essential to find out whether FTPD would similarly produce effective antitumor activity *in vivo* like its pristine drug. Hereby, the antitumor efficacy of FTPD was evaluated in 4T1 tumor-bearing mice that are FAP α -positive. According to the study *in vivo* (see Figure 5), Dox showed obvious inhibitory effect on 4T1 tumor at the dose of 8 mg/kg which also caused a notable decrease of body weight present after 6 days of treatment compared to control group ($P < 0.05$). Meanwhile, a Dox equivalent dose of FTPD (12.2 mg/kg) was administered to 4T1 tumor-bearing mice. It was demonstrated (see Figure 5) that the treatment with FTPD at 12.2 mg/kg exhibited a similar antitumor activity to free Dox without a significant decrease of body weight. These results suggest that FTPD would produce superior therapeutic effect to the parent drug in FAP α -positive tumor-bearing mice.

FTPD revealing low cardiac toxicity

It is well known that severe cardiac toxicity is the most dominating adverse effect of Dox, which largely limits its application. Therefore, whether or not the cardiac toxicity of Dox would be decreased through such modification is a critical issue to be investigated. Pathological analysis indicated that cardiac muscle of mice treated by Dox

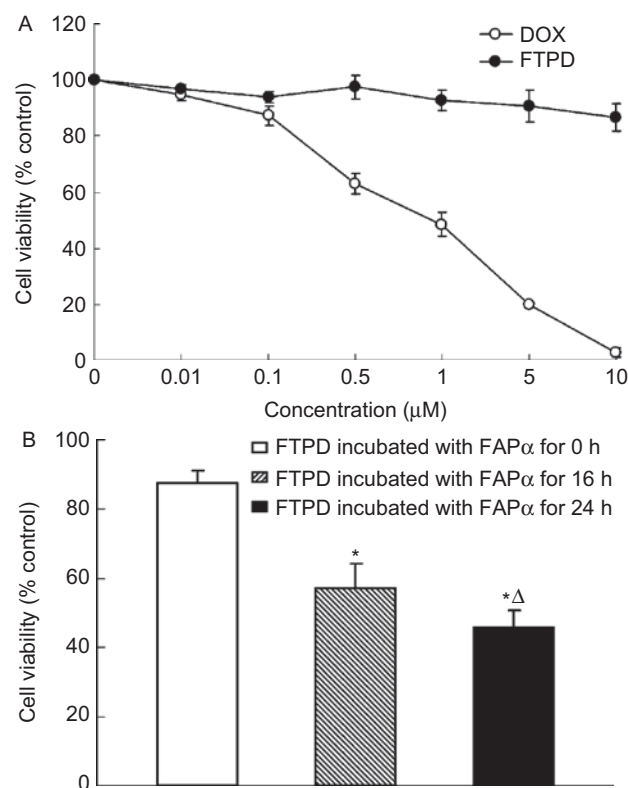


Figure 4. Measurement of antitumor activity *in vitro*. (A) The cytotoxicity of Dox and fibroblast activation protein- α (FAP α)-targeting prodrug of Dox (FTPD) against 4T1 cells was assessed, respectively by Cell Counting Kit-8 (CCK-8) assay over 48 h (mean $\pm$ SD and $n=3$). (B) Change in cytotoxicity of FTPD after being incubated with FAP α for 0 h, 16 h, or 24 h was determined in 4T1 cells over 48 h by CCK-8 assay (mean $\pm$ SD and $n=3$). * $P < 0.05$ versus 0 h; Δ : $P < 0.05$ versus 16 h.

presented extensive cytoplasmic vacuolization, whereas almost no vacuolization was obviously found in the FTPD-treated group (see Figure 6A). Besides, it was also observed that FTPD exhibited no significant toxic effect on other normal organs such as liver, lung, spleen and kidney (data not shown). Furthermore, the biodistribution property of FTPD *in vivo* was analyzed. Given a Dox equivalent dose of 14.7 μ mol/kg, the drug level of FTPD or Dox in tumor and heart was, respectively detected at 1, 4, and 12 h. As illustrated in Figure 6B and 6C, the concentration of FTPD both in heart and tumor were detected to reach the peak value at 4 h after administration, which were delayed compared to Dox whose highest drug levels were found at 1 h. And FTPD displayed significantly lower accumulations in heart than Dox at any of three time points ($P < 0.05$) (see Figure 6B). As for drug level in tumor (see Figure 6C), Dox showed a higher level at 1 h while FTPD possessed higher accumulations at both 4 h and 12 h. What is more, the drug distribution study also revealed that FTPD demonstrated a higher ratio of tumor/heart than Dox at all selected time points (see Figure 6D). From this perspective, such a favorable ratio

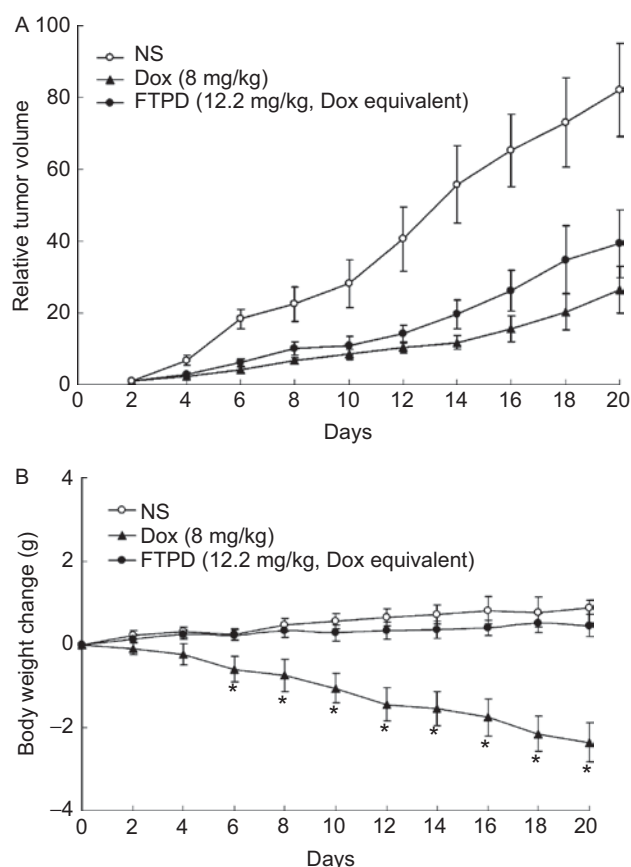


Figure 5. Antitumor efficacy of fibroblast activation protein- α -targeting prodrug of Dox (FTPD) in 4T1 tumor-bearing mice. (A) Changes in relative average tumor volume of mice from each experimental group were measured over 3 weeks. Data represents mean $\pm$ SD of tumor volumes ($n=8$). (B) Changes in body weight of mice from each experimental group were determined over 3 weeks. Data represents mean $\pm$ SD of tumor volumes ($n=8$). * $P < 0.05$ versus control group.

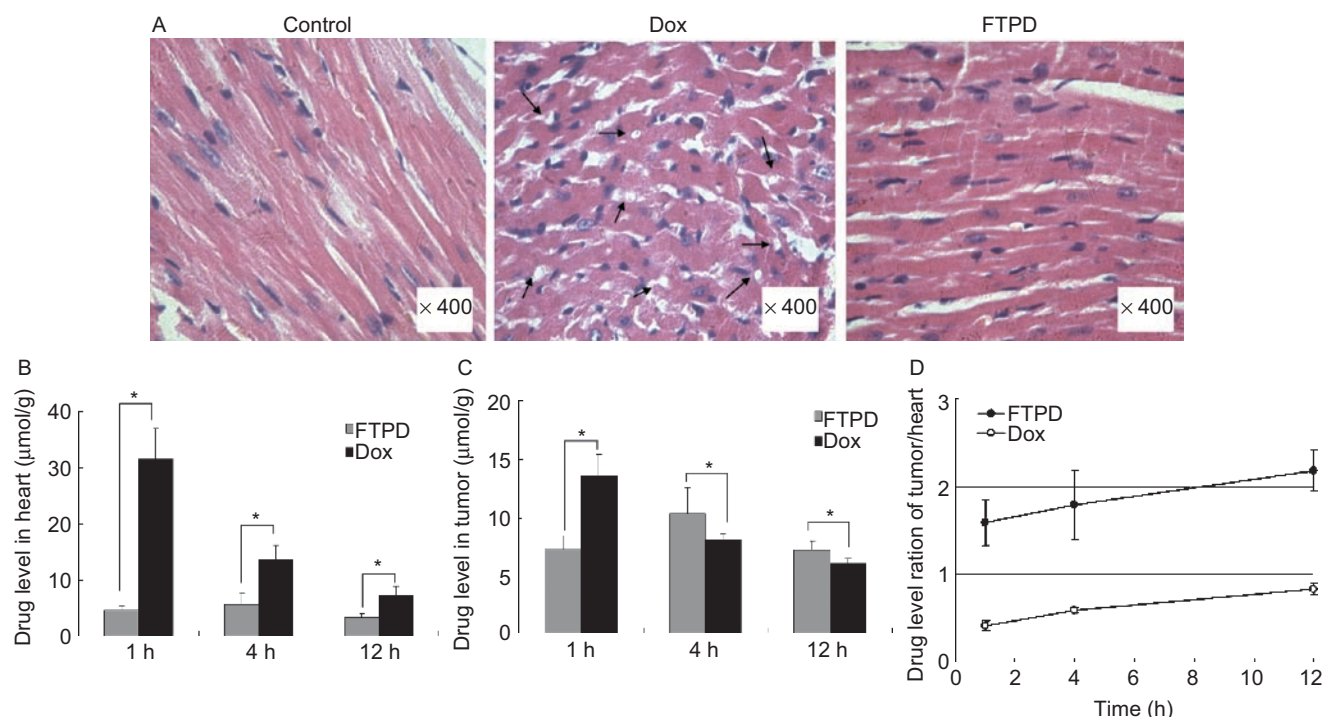


Figure 6. Evaluation of the tumor targeting of the fibroblast activation protein- α -targeting prodrug of Dox (FTPD) conjugate. (A) Histopathological detection of heart from experimental groups. Control cardiac myocytes showing normal morphology ($\times 400$); cardiac myocytes from Dox-treated group showing extensive cytoplasmic vacuolization (arrows indicate the vacuolization in myocytes); cardiac myocytes from the FTPD-treated group showing no obvious vacuolization ($\times 400$). (B), (C), and (D) Drug distribution of Dox or FTPD at an equivalent dose of $14.7 \mu\text{mol/kg}$ in the 4T1 tumor-bearing mice after intravenous administration. (B) Drug level in heart. The data represents mean $\pm$ SD ($n=5$; $*P<0.05$). (C) Drug level in tumor. The data represents mean $\pm$ SD ($n=5$; $*P<0.05$). (D) Drug level ratio of tumor/heart. The data represents mean $\pm$ SD ($n=5$).

of tumor/heart seemed to be an indicator of potent targeting effect. This evidence reveals that the decoration with the Z-Gly-Pro dipeptide would effectually reduce the cardiac toxicity of Dox via the targeted delivery of Dox to tumor tissue.

Discussion

One of the most critical purposes to design antitumor prodrugs is to improve the targeting effect of cytotoxic agents and thereby to reduce their systemic side effects. Over the past two decades, remarkable progresses have been made in the development of anticancer prodrugs. A number of prodrug strategies were proposed to selectively deliver antitumor agents to tumor cells. Most of these specific prodrugs were principally developed to be cleaved by tumor-expressing enzymes such as PSA (Kratz et al., 2005), human glandular kallikrein 2 (Janssen et al., 2006) and cathepsin B (Warnecke et al., 2007). As indicated in these studies, some of them produced favorable therapeutic efficacy toward tumors *in vivo*. However, since the expression of those tumor-expressing enzymes and their activities might vary during different progression and between individuals, advanced knowledge of enzyme structure, mechanisms, regulation, and distribution *in vivo* should be further understood in order to attain the ultimate goal (Rooseboom et al., 2004; Atkinson et al., 2008; Law & Tung, 2009).

Currently, we evaluated a new type of prodrug strategy based on FAP α uniquely expressed by the stromal fibroblasts of tumor. Such tumor stroma-mediated prodrug would provide at least two potential advantages. First of all, FAP α is specifically expressed by TAF in the majority of common human epithelial carcinomas including breast, lung, colorectal, and ovary, so on and so forth. And the reactive stromal fibroblasts constitute the predominant cellular component of the tumor stroma. In this way, FAP α could be treated as a pan-tumor target protease to develop prodrugs for the treatment of a wide range of solid tumors. In the second place, such FAP α -activated prodrug approach could also overcome the problems originating from the heterogeneity and mutability of tumors themselves which would possibly result in the unstable production of the tumor-expressing proteases. Additionally, in comparison with tumors, the genome of the stromal fibroblast might be more stable as we know. Considering these factors, such FAP α -based prodrug strategy would possess more favorable features for tumor therapy.

In this study, we observed *in vitro* that the cytotoxicity of Dox against 4T1 tumor cells was dramatically decreased by the chemical decoration while it could be restored through the enzymolysis of FAP α . And experiments *in vivo* indicated that FTPD produced similar antitumor efficacy to its parent drug at an equivalent dose without a noteworthy decrease of body weight. Also,

the pathological study showed that FTPD would not lead to toxic effects on heart as free Dox. Furthermore, FTPD was proved to demonstrate a favorable ratio of tumor/heart. All the results implied that this FAP α -based prodrug strategy could be feasible to improve the targeting effect of Dox.

Admittedly, a possible limitation of the current FAP α -targeting prodrug, found in this study, was that it could also be slightly cleaved by nonspecific hydrolysis within the normal organs and plasma. However, such nonspecific enzymatic degradation has been reported in several case of prodrugs (Law & Tung, 2009). The main reason of this problem might be that the enzyme-cleavable bond between the parent drug and the peptide could not be absolutely stable under the condition of plasma or normal tissues. To overcome such limitation, one potential solution is through the modification of peptide to make the bond more stable under the nonenzymatic condition, which is also needed to be solved in our further study.

To investigate the scope of application of such prodrug strategy, what we will do next is to confirm the antitumor activity of the FAP α -cleavable conjugate toward other types of solid tumors, such as liver cancer, melanoma, and colon carcinoma. And the comprehensive pharmacokinetic properties of the conjugate are also required to be figured out so as to further understand the difference between the prodrug and its parent drug.

Conclusion

To sum up, through modification with Z-Gly-Pro dipeptide, the FAP α -based prodrug of Dox was shown to achieve desired targeting effect in 4T1 tumor-bearing model. Along with the advances in the knowledge of tumor microenvironment as well as profound understanding of the enzyme/prodrug interaction, this FAP α -activated prodrug strategy would be promising to effectively improve therapeutic efficacy of cytotoxic agents in the treatment of cancer.

Declaration of interest

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References

- Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, Zhang Y, Kraus ML, Salakian S, Sridhar V, Wijnands R, Tennant MG. (2005). Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha. *J Biol Chem*, 280, 19441–19444.
- Ajami K, Abbott CA, Obradovic M, Gysbers V, Kähne T, McCaughan GW, Gorrell MD. (2003). Structural requirements for catalysis, expression, and dimerization in the CD26/DPIV gene family. *Biochemistry*, 42, 694–701.
- Ariga N, Sato E, Ohuchi N, Nagura H, Ohtani H. (2001). Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast. *Int J Cancer*, 95, 67–72.
- Atkinson JM, Siller CS, Gill JH. (2008). Tumour endoproteases: the cutting edge of cancer drug delivery? *Br J Pharmacol*, 153, 1344–1352.
- Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS, Moreira PI. (2009). Doxorubicin: the good, the bad and the ugly effect. *Curr Med Chem*, 16, 3267–3285.
- Chen WT, Kelly T. (2003). Seprase complexes in cellular invasiveness. *Cancer Metastasis Rev*, 22, 259–269.
- Chen Y, Hu LQ. (2009). Design of anticancer prodrugs for reductive activation. *Med Res Rev*, 29, 29–64.
- Cheng JD, Dunbrack RL Jr, Valianou M, Rogatko A, Alpaugh RK, Weiner LM. (2002). Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal model. *Cancer Res*, 62, 4767–4772.
- Collins PJ, McMahon G, O'Brien P, O'Connor B. (2004). Purification, identification and characterisation of seprase from bovine serum. *Int J Biochem Cell Biol*, 36, 2320–2333.
- Denny WA. (2001). Prodrug strategies in cancer therapy. *Eur J Med Chem*, 36, 577–595.
- Elenbaas B, Weinberg RA. (2001). Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res*, 264, 169–184.
- Firestone RA, Rettig WJ, Lenter M, Peters S, Garin-Chesa P, Mack J, Leipert D, Park JE, Telan LA. (2003). FAP-activated anti-tumor compounds. *U.S. Pat*, 6, 613–879.
- Garin-Chesa P, Old LJ, Rettig WJ. (1990). Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci USA*, 87, 7235–7239.
- Huang Y, Wang S, Kelly T. (2004). Seprase promotes rapid tumor growth and increased microvessel density in a mouse model of human breast cancer. *Cancer Res*, 64, 2712–2716.
- Iwata S, Morimoto C. (1999). CD26/dipeptidyl peptidase IV in context. The different roles of a multifunctional ectoenzyme in malignant transformation. *J Exp Med* 190, 301–306.
- Janssen S, Rosen DM, Ricklis RM, Dionne CA, Lilja H, Christensen SB, Isaacs JT, Denmeade SR. (2006). Pharmacokinetics, biodistribution, and antitumor efficacy of a human glandular kallikrein 2 (hK2)-activated thapsigargin prodrug. *Prostate*, 66, 358–368.
- Kratz F, Mansour A, Soltan J, Warnecke A, Fichtner I, Unger C, Dreys J. (2005). Development of albumin-binding doxorubicin prodrugs that are cleaved by prostate-specific antigen. *Arch Pharm (Weinheim)*, 338, 462–472.
- Law B, Tung CH. (2009). Proteolysis: a biological process adapted in drug delivery, therapy, and imaging. *Bioconjug Chem*, 20, 1683–1695.
- LeBeau AM, Brennen WN, Aggarwal S, Denmeade SR. (2009). Targeting the cancer stroma with a fibroblast activation protein-activated promelittin protoxin. *Mol Cancer Ther*, 8, 1378–1386.
- Lee J, Fassnacht M, Nair S, Boczkowski D, Gilboa E. (2005). Tumor immunotherapy targeting fibroblast activation protein, a product expressed in tumor-associated fibroblasts. *Cancer Res*, 65, 11156–11163.
- Levy MT, McCaughan GW, Abbott CA, Park JE, Cunningham AM, Müller E, Rettig WJ, Gorrell MD. (1999). Fibroblast activation protein: a cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis. *Hepatology*, 29, 1768–1778.
- Morimoto C, Schlossman SF. (1998). The structure and function of CD26 in the T-cell immune response. *Immunol Rev*, 161, 55–70.
- Mueller MM, Fusenig NE. (2004). Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer*, 4, 839–849.
- Papot S, Tranoy I, Tillequin F, Florent JC, Gesson JP. (2002). Design of selectively activated anticancer prodrugs: elimination and

- cyclization strategies. *Curr Med Chem Anticancer Agents*, 2, 155-185.
- Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. (1999). Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem*, 274, 36505-36512.
- Rettig WJ, Garin-Chesa P, Beresford HR, Oettgen HF, Melamed MR, Old LJ. (1988). Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. *Proc Natl Acad Sci USA*, 85, 3110-3114.
- Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Ozer HL, Schwab M, Albino AP, Old LJ. (1993). Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. *Cancer Res*, 53, 3327-3335.
- Rooseboom M, Commandeur JN, Vermeulen NP. (2004). Enzyme-catalyzed activation of anticancer prodrugs. *Pharmacol Rev*, 56, 53-102.
- Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, Old LJ, Rettig WJ. (1994). Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc Natl Acad Sci USA*, 91, 5657-5661.
- Tanabe K, Zhang Z, Ito T, Hatta H, Nishimoto S. (2007). Current molecular design of intelligent drugs and imaging probes targeting tumor-specific microenvironments. *Org Biomol Chem*, 5, 3745-3757.
- Tlsty TD. (2001). Stromal cells can contribute oncogenic signals. *Semin Cancer Biol*, 11, 97-104.
- Warnecke A, Fichtner I, Sass G, Kratz F. (2007). Synthesis, cleavage profile, and antitumor efficacy of an albumin-binding prodrug of methotrexate that is cleaved by plasmin and cathepsin B. *Arch Pharm (Weinheim)*, 340, 389-395.
- Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB. (2001). Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. *Cancer Res*, 61, 771-777.