Journal of
Nanomedicine & Nanotechnology

Nakamura et al. J Nanomedic Nanotechnol 2011, 2:1
DOI: 10.4172/2157-7439.1000106

Research Article Open Access

In Vivo Cancer Targeting of Water-Soluble Taxol by Folic Acid Immobilization

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Abstract

Previously, folic acid receptor-targeted Dextran-Taxol-Folic acid (Dex-TXL-FA) has shown the *in vitro* superior and selective antitumor activity against human oral cancer cell line (KB) compared with the absent of folic acid, Dextran-Taxol. Present study is given for further investigation of *in vivo* antitumor efficacy of Dex-TXL-FA in the murine tumor xenograft model. To evaluate the antitumor effect of taxol, tumor bearing mice were prepared by s.c. inoculation of 1.0×10^6 KB cells in the back of nude mice. Seven days after inoculation, the administration of saline, paclitaxel for injection (PTX), Dex-TXL, FA-adsorbed Dex-TXL and Dex-TXL-FA (covalent) was started at a dose of 10mg/kg, by i.v. injection via the lateral tail vein three times (on day 7, 9, and 11) and animal survival rate and tumor sizes were monitored.FA-adsorbed Dex-TXL and Dex-TXL-FA (covalent) showed approximately 3 times greater anticancer effect than that of taxol at the 30th day after tumor implantation. Furthermore, these FA immobilized TXL showed 2-3 month longer animal survival than that of taxol. These results suggest the conjugation with Dex and FA could provide an improvement in the anticancer therapy of taxol.

Introduction

Paclitaxel is an anticancer drug used for lung, breast and ovarian tumors [1]. Due to its poor water solubility, paclitaxel is generally administered as a mixture with poly (oxyethylene) castor oil (Cremophor EL) and dehydrated ethanol [2]. Because Cremophor EL causes serious side-effects, such as irritation, in approximately 30% of patients [3], a steroid drug is required prior to its use to suppress these side effects [4]. To exclude the need for Cremophor EL, paclitaxel conjugation with poly (L-glutamic acid) [5] and albumin [6], and drug delivery systems (DDS) such as liposomes, polymer micelles, and nanoparticles have been studied to enhance its water solubility and anticancer efficacy by systematic delivery [7, 8]. Folic acid (FA), a water-soluble vitamin that plays an important role in cell proliferation, has also been used as targeting molecule in micelle and liposome systems [9, 10]. Overexpression of the FA receptor in some cancer cells such as ovarian and brain carcinomas [11], and a human oral cancer cell line (KB) has been reported [12].

In our previous study, the water solubility of paclitaxel and the *in vitro* targeting activity were remarkably enhanced by the conjugation with dextran and FA, respectively [13]. The anticancer effect was examined *in vitro* by using KB cells which overexpress FA receptors. FA was immobilized with dextran-conjugated paclitaxel (Dex-PXL) to provide cancer targeting to the FA receptor overexpressed on cancer cells. It was found that the water solubility of paclitaxel could be improved by dextran conjugation by as much as 2700 times, and that the cytotoxicity against KB cells could be enhanced 2-3 times by FA conjugation (Dex-PXL-FA) than that observed against a cell line without FA receptor over expression [13].

In vivo studies of DDS drugs such as micelles [14], liposomes [15], polymers [16], and nanoparticles [17] have already been reported. Recently, many studies have used antibodies [18] and peptides [19] as a ligand to target tumors. However, in spite of the specific antigen and antibody reaction for targeting, the presence of similar antigens *in vivo* would suppress the targeting efficacy of the drug [20]. Maeda et al. compared the permeability of immature blood vessels of cancer tissue with that of normal tissue in 1986, and they found that compounds larger than about 100 nm in diameter accumulated to a higher extent in cancer tissues [21]. Therefore, the drugs modified polymers to have a larger size show higher efficacy than those without the modification due to the accumulation of the larger drugs. Furthermore, these studies suggest that research should be focused on designing a drug with an enhanced permeability and retention (EPR) effect. Dextran was reported to have an EPR effect in DDS and to make a contribution to cancer-specific targeting [22]. In particular, a molecular size of 50-200nm in diameter was found to be crucially important for cancer tissue targeting. Even a modified agent is immediately excreted from the kidney when the size is smaller than 50nm [23]. In contrast, if the size of the conjugated drug is larger than 200 nm, it becomes trapped in the liver and is degraded [24].

The present study further examined the *in vivo* antitumor efficacy of Dex-PXL-FA using a murine tumor xenograft model. In addition, the active (FA-targeting) and passive (EPR) effects of Dex-PXL-FA are also discussed in detail with regard to their molecular size evaluation.

Materials and Methods

The paclitaxel used for injection and for chemical modification were kindly donated by NIPPON KAYAKU Co., Ltd (Tokyo, Japan) and by Samyang Genex Corporation (Korea), respectively. Dextran (MW 70,000) was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan). Folic acid (FA), diethyl sulfoxide (DMSO), 1, 1 -carbonyldiimidazole (CDI), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and ethylenediamine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and used without further purification.

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Received December 05, 2010; **Accepted** January 24, 2011; **Published** January 24, 2011

Citation: Nakamura J, Nakajima N, Matsumura K, Hyon SH (2011) *In Vivo* Cancer Targeting of Water-Soluble Taxol by Folic Acid Immobilization. J Nanomedic Nanotechnol 2:106. doi:10.4172/2157-7439.1000106

Cell culture

KB cells (DS Pharma Biomedical Co., Ltd., Osaka, Japan) were cultured in RPMI1640 (folate-free, Invitrogen Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum and 100 g/ml of penicillin-streptomycin at 37°C under 5% CO_2 in a humidified atmosphere. When the cells reached 80% confluence, they were detached by 0.25% (w/v) of trypsin containing 0.02% (w/v) of ethylenediamine tetracetic acid (EDTA) in phosphate buffered saline without calcium and magnesium (PBS(-)) and seeded on a new tissue cuture plate for subculture. The KB cells were used for each experiment within five passages.

Synthesis of amino-Dex, Dex-PXL, FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent)

Dex-PXL, FA-adsorbed Dex-PXL, and Dex-PXL-FA (covalent) were synthesized according to the previous report [13]. Briefly, synthesis method of their complex was shown as follows. Dextran (10 g) was dissolved in 70 ml DMSO and mixed with a solution of 2 g CDI in 5 ml DMSO, and the activation reaction was preceded at 50°C for 15 min. Subsequently, 5 ml of ethylenediamine was added and the mixture was stirred at 50°C for 18 h. After dialysis against running water for 24 h and distilled water (3 L, 1.5 h × 2) with a dialysis membrane (cut-off molecular weight of 14,000 Da), amino-Dex was recovered by air and vacuum drying. To activate the paclitaxel OH group, a solution of 0.7 g paclitaxel in 40 ml DMSO was added to a solution of 0.6 g CDI dissolved in 10 ml DMSO at 50°C for 15 min. Five grams of amino-Dex in 150 ml of DMSO was added to the solution, and the reaction proceeded at 50°C for 18 h. Dex-PXL was recovered by the same purification manner as described above. The yield of Dex-PXL was about 70. Recovered Dex-PXL and FA were dissolved in PBS at a concentration of 800 and 240 g/ml, respectively. Here, a solution of 0.5 ml Dex-PXL was simply mixed with 0.5 ml of FA and stirred at 25°C for 18 h to prepare FA-adsorbed Dex-TXL. A solution of 0.1 g FA and 0.15 g NHS in 20 ml DMSO was mixed with 0.07 g of EDC in 10 ml DMSO, and the activation reaction of the FA COOH groups proceeded at 50°C for 5 min. Then, 0.5 g of Dex-PXL in 10 ml DMSO was added to the mixture and reacted at 50°C for 3 h. Dex-PXL-FA (covalent) was recovered after purification by dialysis against water and drying.

Molecular size evaluation by dynamic light scattering (DLS)

The hydrodynamic molecular size of the conjugates was measured at 25°C by DLS using a DLS-7000 instrument (Otsuka Electronics, Osaka, Japan) equipped with a He–Ne laser. The samples were dissolved in PBS and DMSO (for paclitaxel) at a concentration of 5000ppm, and 5ml of the solution was used for the evaluation.

Animals

Female nude mice (BALB/c nu/nu; 4weeks old and weighing 9-17 g) were purchased from Charles River Laboratories Japan, Inc (Kanagawa, Japan). The nude mice were maintained on a folate-free rodent diet (AIN-93G-based Folate-Deficient Rodent Diet, Oriental Yeast CO., LTD, Tokyo, Japan) on arrival and for the duration of the study. Animal experiments were performed according to the criteria established in the Guidelines of the Committee on Animal Care and Use of Kyoto University.

Acute toxicity

To evaluate the acute toxicity of the conjugates, mice were administered a single i.v. injection of 100μl/mouse containing 140-220μg of paclitaxel. The body weights of mice were recorded for 2 weeks and compared with those of tumor-free nude mice (6 weeks old and weighting 14-22g).

Antitumor effects and survival rates in tumor-bearing mice

To evaluate the antitumor effect of paclitaxel, tumor-bearing mice were prepared by inoculating KB cells S.C onto the back of nude mice (1.0×10^6 cells/mouse). Seven days after inoculation, paclitaxel for injection (PXL), Dex-PXL, FA-adsorbed Dex-PXL, and Dex-PXL-FA (covalent) were administrated by i.v. injection via the lateral tail vein three times (at a dose of 10mg PXL /kg, on days 7, 9, and 11). Tumor size was measured with a digital vernier caliper and the volume was calculated using the formula for a prolate ellipsoid, V (mm^3) = ($a \times b^2$)/2, where a is the longer, and b is the shorter diameter. The survival rate was also monitored at 2 or 3 day intervals.

Tumor-targeting effect of Dex-PXL-FA

To investigate the tumor-targeting effect of FA, fluorescein isothiocyanate (FITC)-labelled FA-adsorbed Dex-PXL (Dex-PXL-FITC) was prepared as follows: In one glass vial, Dex-PXL and FITC were dissolved in DMSO at the concentration of 10 w/v and 0.075 w/v, respectively, and stirred at 50°C for 1 h, followed by re-precipitation of Dex-PXL-FITC with an excess of acetone and vacuum drying. The recovered Dex-PXL-FITC and FA were dissolved in PBS at concentrations of 800 and 240μg/ml, respectively, and 0.5ml of each solution was simply mixed and stirred at 25°C for 18 h to prepare FA-adsorbed Dex-PXL-FITC (Dex-PXL-FITC-FA). The targeting effect was evaluated using the same method as that for the antitumor effects. The tumor tissue was isolated from each mouse on the day after the third injection. Tumor tissues were bisected and placed in the Tissue-Tek® Optimum Cutting Temperature compound (Sakura Ltd. Tokyo, Japan) for cryosection, and the distribution of Dex-PXL-FITC-FA was observed under an optical/fluorescence microscope (Biozero-8000, Keyence, Osaka, Japan).

Statistical analyses

All data are shown as the means ± standard deviation (SD). Data among the groups were compared by Tukey-Kramer multiple comparison test. Differences were considered to be statistically significant at $p < 0.05$.

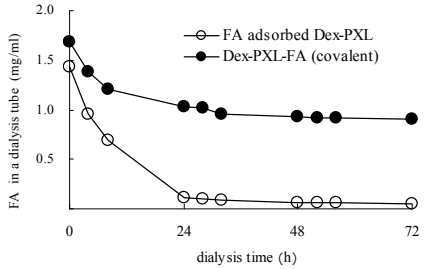

Figure 1: Changes in the FA concentration in the dialysis tube by dialysis against PBS at 25°C.

Sample	Molecular size (nm)
Paclitaxel*	11.2 ± 1.2
Dextran	14.2 ± 3.8
amino-Dex	18.5 ± 1.0
Dex-PXL	97.1 ± 2.2
FA-adsorbed Dex-PXL	93.0 ± 3.2
Dex-PXL-FA (covalent)	89.8 ± 7.1

*Only taxol was dissolved in DMSO and the others were in PBS

Table 1: Average size of samples by DLS measured. (5000 ppm, 25°C).

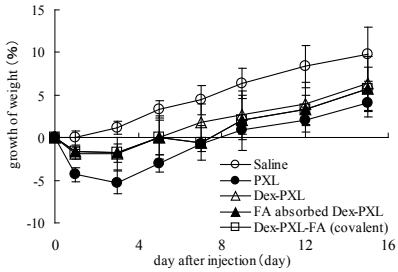

Figure 2: Body weight changes after injection of the conjugates. Female nude mice were given a single i.v. injection of saline, PXL, Dex-PXL, FA-adsorbed Dex-PXL, or Dex-PXL-FA (covalent). Data are the average ± S.D (n=10).

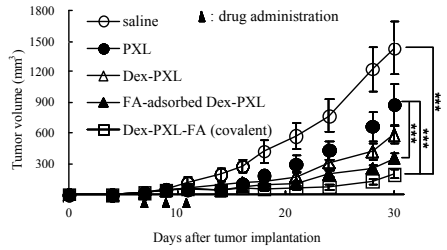

Figure 3: Tumor growth inhibition by PXL, Dex-PXL, FA-adsorbed Dex-PXL, and Dex-PXL-FA (covalent). Nude mice with KB xenograft tumors were treated with a series of three i.v. injections (given every other day, as indicated by the arrow heads) of Dex-PXL containing 10 mg/kg PXL (arrow head showing the day of administration). ***means p < 0.001.

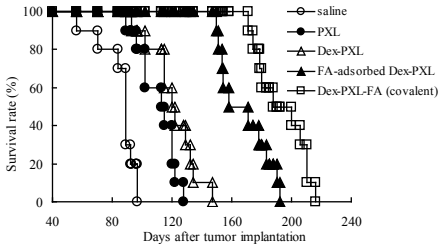

Figure 4: The survival rate of nude mice bearing KB tumors. The mice (n=10/group) were given three i.v. injections, then the tumor volume was evaluated every other day. Saline vs. PXL $P = 0.0008$; PXL vs. Dex-PXL $P = 0.07$; PXL vs. FA-adsorbed Dex-PXL $P = 0.0002$; PXL vs. Dex-PXL-FA (covalent) $P = 0.0002$.

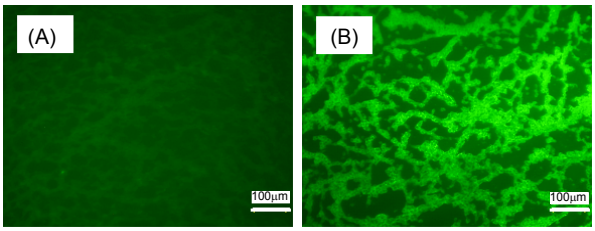

Figure 5: Fluorescence microphotographs of cryosections of tumor tissues. Dex-PXL-FITC (A) and FA-adsorbed Dex-PXL-FA (B). The exposure time was 10 sec in both cases.

Results

FA-conjugation with Dex-PXL

The results of the PBS dialysis of Dex-PXL-FA (covalent) and FA-adsorbed Dex-PXL are shown in Figure 1. In the case of FA-adsorbed Dex-PXL, FA easily detached from the Dex-PXL during PBS dialysis, and FA almost completely disappeared within 24 hours. In contrast, the extent of FA decrease from Dex-PXL-FA (covalent) was far smaller, and 60% of the FA remained after 72 h, confirming the covalent immobilization of FA into Dex-PXL.

Molecular size evaluation by DLS

The average molecular size of each sample measured by DLS is summarized in Table 1. The molecular size of paclitaxel in DMSO and dextran and amino-Dex in PBS were closely arranged, within 10-20 nm. In contrast, Dex-PXL, FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent) were much larger. After paclitaxel conjugation in dextran, the particle size became about 10 times larger due to high hydrophobicity of paclitaxel molecules. The particle size of Dex-PXL, FA-adsorbed Dex-PXL, and Dex-PXL-FA (covalent) suggested an EPR effect *in vivo*.

Acute toxicity

Each sample was administered to intact mice (nude mouse n=5) as a single i.v. injection, and the body weight change was recorded for two weeks. The results are given in Figure 2. The body weight of saline-treated mice increased normally. On the other hand, the weight decreased until the third day, when PXL was administered. Dex-PXL, FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent) led to a smaller weight loss than PXL.

Antitumor effects and survival of tumor-bearing mice

The tumor growth inhibitory activities were evaluated using KB xenograft tumor-bearing nude mice. As shown in Figure 3, the tumors rapidly grew in size when the mice were treated with saline. PXL controlled cancer growth better than saline, and showed remarkable cancer growth inhibition after the 20th day. After the 30th day, the mean tumor volumes in the PXL and Dex-PXL groups were 600 and 900 mm^3, respectively. FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent) further delayed the tumor growth, and almost complete growth suppression was observed around 3 weeks, which suggested an excellent targeting effect of FA.

The effects of the drugs on for the survival of tumor-bearing mice were also evaluated, and the survival data are summarized in Figure 4. The average survival of the saline, PXL, Dex-PXL, FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent) treated mice were 84.7±12.3, 110.7±12.7, 120.9±15.8, 168.3±16.8 and 193.4±16.8 days, respectively. The mice treated with PXL and Dex-PXL showed almost the same survival rate. In contrast, far longer survival (2-3 months) was observed in FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent)-treated mice.

FA targeting effect using FA-adsorbed Dex-PXL-FITC

The targeting effect of the FA-adsorbed Dex-PXL was evaluated by FITC conjugation, and the results are shown in Figure 5. Figures 5A and B show the fluorescent microscopic images of the cancer tissue 12 days after injection of Dex-PXL-FITC and FA-adsorbed Dex-PXL-FITC, respectively. The fluorescence intensity of Dex-PXL-FITC injected tissue was higher than that of Dex-PXL-FITC, thus suggesting the better targeting effect of FA conjugation.

Discussion

In the present study, we evaluated the *in vivo* anticancer effects of water soluble and FA immobilized paclitaxel conjugations. Dex-PXL without FA conjugation showed an improved anticancer effect

compared to PXL, which was due to the EPR effect of the molecule. In addition, the anticancer effect was remarkably enhanced by FA immobilization to Dex-PXL. Both FA-adsorbed and FA-covalently bound Dex-PXLs exerted enhanced antitumor activity against FA receptor over expressing cancer cells [13].

It is likely that the FA immobilized by adsorption might be desorbed into the systemic circulation *in vivo*, and Figure 1 suggests that there is a loss of the anticancer efficacy of FA-adsorbed Dex-PXL during PBS dialysis compared with Dex-PXL-FA (covalent). However, Figure 5B clearly showed that the FA-adsorbed Dex-PXL-FITC remained in the tumor tissue even 11 days after injection, suggesting the rapid accumulation of FA-adsorbed Dex into the tissue before FA desorption. This is in agreement with the fact that the tumor volume in mice treated with FA-adsorbed Dex-PXL after 30 days was smaller than that of Dex-PXL (Figure 4). These results reveal the usefulness of the easy adsorption method for targeting. The Dex-PXLs with FA immobilized by both covalent bonding and adsorption suppressed the tumor growth, however, the survival rate was significantly higher when covalently bonded drug was selected.

The FA receptor is abundantly expressed in a large percentage of human tumors, but it is only minimally distributed in a normal tissue [25-28]. The FA receptor has three isoforms α, β and γ. The isoform is expressed in some normal epithelial cells, and its expression is elevated in certain carcinomas [23]. It was reported that KB [29, 30], coco2 [31], and ovarian carcinomas cells express the FA receptor α on their surface. Therefore, FA-adsorbed and FA-covalently bound Dex-PXL are excellent tumor targeting agents for cells expressing FA receptor α.

In summary, FA-adsorbed Dex-PXL and Dex-PXL-FA (covalent) showed excellent anticancer effects due to their EPR and cancer targeting effects, with the conjugation of FA and Dex providing more effective and safer anticancer therapy with paclitaxel than when the agent is used alone.

Acknowledgements

The authors gratefully express their appreciation to Samyang Genex Corporation, Korea and NIPPON KAYAKU Co., Ltd, Tokyo, Japan for the kindly supplying us with paclitaxel.

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