A daidzein–daunomycin conjugate improves the therapeutic response in an animal model of ovarian carcinoma

Dalia Somjen, Sara Katzburg, Nava Nevo, Batya Gayer, Richard P. Hodge, Misty D. Renevey, Vyacheslav Kalchenko, Asher Meshorer, Naftali Stern, Fortune Kohen

1. Introduction

Although anthracycline antibiotics (e.g., daunomycin, adriamycin, etc.) are highly effective chemotherapeutic agents, cardiotoxicity of these drugs limits their therapeutic potential. In addition, the concentration needed to kill tumour cells is close to drug levels which produce severe toxicity to normal cells in the body. To circumvent some of these problems anthracycline antibiotics have been conjugated to carriers such as peptide or steroidal hormones, which are recognized by homologous either membranal or nuclear associated steroid receptors present in tumour cells [1–6]. Although some of the receptor mediated cytotoxic drug conjugates appeared promising in vitro, their use in vivo was generally ineffective. More recently nanoparticle drug delivery systems such as lipid or polymer based nanoparticles were designed to improve the pharmacological and therapeutic properties of cytotoxic drugs [7–9]. One such system consisting of PEG-stabilized liposomal doxorubicin has been approved for clinical evaluation in refractory ovarian and breast cancer [9].

Multiple studies have indicated that oestrogenic compounds (e.g., plant oestrogens like the isoflavones genistein, daidzein) can mimic the genomic and non-genomic actions of oestrogen and act as growth modulators in various normal and cancerous cell types [10]. Based on these reports in a preliminary study we exploited the oestrogenic/anti-oestrogenic activities of carboxy derivatives of isoflavones and used them as carriers for affinity drug targeting to H295R adrenal cortical carcinoma cells which express a general abundance of oestrogen receptor (ER)β to ERα mRNA expression [11]. At concentrations ranging from 0.3 to 3 nM these conjugates were 10–30 times more potent than the cytotoxic drug to which they were linked, daunomycin [11].

As a continuation of our work on affinity targeting, we now explore whether a carboxymethyl derivative of daidzein, prepared in our laboratory [12] can act as carrier for daunomycin for site directed chemotherapy of a human model of ovarian cancer in vivo. We chose daidzein as the tag since it was reported that...
Daidzein reduces proliferation in ovarian cancer cells via, at least in part, oestrogen receptor pathway [13]. Moreover, a recent study indicates that daidzein and not genistein interacts with phospholipids present in the cell membrane, [14] suggesting that it may facilitate internalization of the cytotoxic drug–daidzein conjugate. In additional studies, it was also pointed out that in contrast to genistein, daidzein possessed chemopreventive effects against rat mammary carcinogenesis [15]. The results shown in this paper indicate that 7-(O)-carboxymethyldaidzein–daunomycin (cDaid–Dau) conjugate provides an effective cytotoxic tool and holds promise as a potential novel anti-cancer drug with a high therapeutic index, allowing minimal toxicity to normal cells in vivo.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. Chemicals were purchased from Sigma (St. Louis, MI). Daunomycin hydrochloride was purchased from Davos Chemical Corporation (Upper Saddle River, NJ). Neowater solubilization enhancer was obtained from Do-Coop Technology (Or-Yehuda, Israel). Methyl-[3H]-thymidine (5 Ci/mmmole) was obtained from New England Nuclear (Boston, MA).

2.2. Cells

MLS human epithelial ovarian cancer cells, expressing mRNA only for ERβ [16] were kindly provided by Prof. M. Neeman from the Weizmann Institute of Science, Rehovot, Israel and were cultured in a DMEM supplemented with 10% FCS and antibiotics. Cells were grown to subconfluence and then treated with various hormones or agents as indicated.

2.3. Synthesis of 7-(O)-carboxymethyldaidzein–daunomycin conjugate

7-(O)-Carboxymethyldaidzein (100 mg, 0.32 mmoles), synthesized according to Bennetau-Pelissero et al. [17], was dissolved in 5 mL anhydrous dioxane under argon in a 50 mL round bottom flask containing a magnetic stir bar and sealed with a rubber septa. Dicyclohexylcarbodiimide (99 mg, 0.48 mmoles) was warmed to 40°C and added via a warmed syringe to the stirring 7-(O)-carboxymethyldaidzein and allowed to mix for 15 min. N-Hydroxy succinimide (NHS) (111 mg, 0.96 mmole) was then added all at once under argon and the mixture was allowed to stir sealed under argon for 3 h at room temperature. TLC analysis (90:9:1; CHCl3:MeOH:AcOH) showed the product at RF 0.7, along with urea by product and traces of left over reactants. The crude product was filtered of urea under a blanket of argon, washed with 2 mL of anhydrous dioxane and used directly in the next step.

Daunomycin (170 mg, 0.32 mmoles) was dissolved in 10 mL 0.1 M sodium bicarbonate in a 50 mL plastic tube containing a stir bar. Filtered 7-carboxymethyldaidzein NHS ester (approx. 10 mL, crude above) was added in 1 mL increments drop wise to the stirring daunomycin followed by 100 µL 0.1 M sodium bicarbonate after the first and second increments to keep the pH at 8.0. After addition of the 7-carboxymethyldaidzein N-hydroxysuccinimide ester was complete, the reaction was covered in aluminum foil and allowed to stir overnight at room temperature in the dark. The resulting redish paste was then filtered and washed with 100 mL 0.1 M HCl. The aqueous HCl wash was extracted with 100 mL ethyl acetate and the ethyl acetate layer combined with the filtered solid to give 285 mg of the crude product. TLC analysis (90:9:1; CHCl3:MeOH:AcOH) showed the product spot at Rf 0.58 with two other products at Rf 0.78 and 0.09. The product was purified by silica gel chromatography using a stepwise gradient from 0% to 4% MEOH in CHCl3 containing 0.1% AcOH. 100 mL Fractions were collected with product coming off in fractions 9–18 to give 91 mg (34.4% yield) of pure daunomycin–7-carboxymethyldaidzein conjugate (Fig. 1). MS, m/z 822.5 (M+). 1H NMR (δ, d6-acetone): δ 8.48 ppm (br s, 1H, NH), 8.10 (s, 1H), 8.03 (d, J = 8.7), 7.83 (m, 1H), 7.54 (d, J = 8.1), 7.41 (d, 2H, J = 8.7), 7.32 (d, 1H, J = 8.4), 6.99 (s, 1H), 6.84 (d, 2H, J = 8.7), 5.61 (d, 1H, J = 4.5), 5.44 (br s, 1H), 5.17 (br s, 1H), 4.85 (br s, 1H), 4.63 (s, CH2), 4.30 (m, 2H), 3.99 (s, CH3), 3.78 (br s, 1H, NH), 8.10 (br s, 1H, NH), 8.03 (d, J = 8.7), 7.83 (m, 1H), 7.54 (d, J = 8.1), 7.41 (d, 2H, J = 8.7), 7.32 (d, 1H, J = 8.4), 6.99 (s, 1H), 6.84 (d, 2H, J = 8.7), 5.61 (d, 1H, J = 4.5), 5.44 (br s, 1H), 5.17 (br s, 1H), 4.85 (br s, 1H), 4.63 (s, CH2), 4.30 (m, 2H), 3.99 (s, CH3), 3.78 (br s, 1H), 3.67 (br s, 1H), 2.60 (s, 1H), 2.37 (s, CH3), 2.14 (s, CH2), 1.82 (d, 1H, J = 12.3), 1.29 (s, 4H), 1.20 (s, CH3).

2.4. Assessment of DNA synthesis

Cells were grown until subconfluence using conditions previously described [11] and then treated with various hormones or agents for 48 h as indicated. At the end of incubation, [3H]-thymidine was added for 2 h. Cells were then treated with 10% D. Somjen et al. / Journal of Steroid Biochemistry & Molecular Biology xxx (2008) xxx–xxx

Fig. 1. Structure of 7-(O)-carboxymethyldaidzein–daunomycin conjugate.
ice-cold trichloroacetic (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 mL of 0.3 M NaOH, aliquots were taken for counting radioactivity, and [3H]-thymidine incorporation into DNA was calculated. The cytotoxic index was determined according to Varga et al. [5]:

\[
\text{Cytotoxic index} = \frac{\text{mean counts in vehicle treated culture}}{\text{mean counts in treated culture}} - 1
\]

2.5. Xenograft studies

Female CD1 nude mice (8 weeks old) were inoculated sc with MLS human ovarian epithelial carcinoma cells (1.5 × 10^6/mouse). Ten days later when the tumours were palpable, the mice were randomized into eight to ten mice per group. The various groups were treated iv every other day with daunomycin (8 mg/kg) (Dau), 7-(O)-carboxymethyldaidzein–daunomycin conjugate (dose equivalent to daunomycin moiety in the conjugate) (cDaid–Dau) or vehicle (Neowater). During treatment body weight was recorded to monitor toxicity of the treatment, and the tumours were measured with an external caliper. The tumour volume was calculated using the formula length × width × height × π/6. The statistical significance was analyzed using Student’s t-test, and the differences were considered significant at p < 0.05. All animals were handled according to the policies set by the Veterinary Animal Services, Weizmann Institute of Science and all procedures were approved by the Institutional Animal Care and Use Committee, Weizmann Institute of Science.

2.6. Fluorescence imaging studies

The tumour bearing mice were injected iv with cDaid–Dau (0.3 mg in 100 µL of Neowater) or Dau (0.2 mg in 100 µL of Neowater). The fluorescence signal due to the daunomycin signal in the whole animal was monitored at 24 h on an IVIS 100 system (Xenogen inc., USA).

2.7. Histological examination

Samples of heart tissues were fixed by immersion in 4% formaldehyde; sectioned serially at 4 µm thickness and stained for haematoxylin–eosin.

2.8. Statistical analysis

The significance of differences between the mean values obtained from experimental groups and controls were evaluated by the unpaired two-tailed Student’s t-test and by analysis of variance (ANOVA).

3. Results

3.1. Effects of oestrogen and daidzein derivatives on [3H]-thymidine incorporation in vitro in MLS ovarian cells

Oestrogen (E2) at the concentration of 30 nM stimulated [3H]-thymidine incorporation in these cells. Higher concentrations of E2 (>30 nmol/L) had no significant effect on DNA synthesis (Fig. 2). At a concentration range of 30–3000 nM 7-(O)-carboxymethyldaidzein stimulated DNA synthesis whereas daidzein stimulated DNA synthesis only at 30–300 nM. At a concentration of 3000 nM daidzein had no effect on DNA synthesis (Fig. 2).

3.2. Growth inhibition studies of 7-(O)-carboxymethyl daidzein–daunomycin conjugate in cultured ovarian carcinoma cells

The cytotoxicity of 7-(O)-carboxymethyl daidzein–daunomycin conjugate (cDaid–Dau) was tested following 48 h of incubation with cultured MLS ovarian cancer cells using incorporation of [3H]-thymidine as a proliferation marker. As depicted in Fig. 3, the conjugate was 2–3 fold more toxic than daunomycin at the low concentration range (0.3–3 nM). At the concentration range of 30–3000 nM, the conjugate showed the same toxicity as daunomycin.

3.3. In vivo cytotoxicity studies

The efficacy of the cytotoxic effect of cDaid–Dau on tumour growth of MLS xenografts was compared with an equivalent dose of free daunomycin. For this purpose three groups of CD1 female nude mice with eight mice in each group were inoculated sc...
with MLS ovarian cancer cells ($1 \times 10^6$ cells/mouse). As soon as the tumours became palpable mice were injected iv every other day with cDaid–Dau ((0.3 mg/mouse equivalent to 0.198 mg of daunomycin) in 0.1 mL of vehicle, daunomycin (0.2 mg/mouse in 0.1 mL of vehicle, a dose slightly below the IC$_{50}$) or vehicle (Neowater). Body weight of the mice was monitored throughout the experiment as a marker of systemic toxicity. Loss of weight was observed only in the daunomycin treated group, which showed ~10% weight reduction during the treatment. Additionally, two mice in this group died during the treatment, suggesting toxicity of the drug. Further, the daunomycin treated group showed signs of distress and weakness. The remaining two groups, treated with either vehicle or cDaid–Dau, gained weight, thus suggesting that the conjugate did not hamper normal growth. Moreover, the mice in these two groups were active, and did not show signs of distress. When the dose of the conjugate was compared to an equivalent dose of daunomycin, both compounds caused a decrease in tumour volume, indicating that the conjugate was effective. The decrease in tumour volume on day 17 was significant only in the conjugate treated group ($p < 0.049$ control vs. conjugate treated; Fig. 4), again indicating that the conjugate had a therapeutic effect. The efficacy of lower doses (0.2 mg/mouse and 0.1 mg/mouse) of conjugate was tested in CD1 mice bearing MLS xenografts and compared to the efficacy of the therapeutic dose (0.3 mg/mouse). During treatment, treated and as well as control mice gained weight suggesting that the conjugate at varying doses did not cause toxicity. At a dose of 0.3 mg/mouse/48 h, a decrease in tumour volume was achieved. Lower doses, however, (0.2 mg and 0.1 mg) failed to inhibit tumour growth (data not shown).

3.4. Fluorescence studies

Daunomycin can be excited at 500 nm, and the emitted light can be measured at 575 nm by fluorescence. Since the IVIS system is capable of in vivo imaging of mice, tumour bearing mice were injected iv with Dau or cDaid–Dau. While in vivo imaging of the fluorescence signal generated by Dau, indicated uptake of both cDaid–Dau and Dau by the tumour, the intensity of fluorescence was higher in the cDaid–Dau treated mice than in the Dau treated mice, thus suggesting specific delivery of the drug to the tumour (Fig. 5).

3.5. Histological examination

Photomicrographs from sections of myocardium from control and cDaid–daunomycin treated mice indicated the heart of these mice was normal. On the other hand, left ventricular section of myocardium of daunomycin treated group showed changes indicative of myocardial cell toxicity (Fig. 6).

![Fig. 5. Imaging of the fluorescence signal generated by daunomycin with an IVIS system in vivo. Ovarian tumour bearing mice were injected iv with (left mouse) c-Daid–Dau (0.3 mg) or (right mouse) Dau (0.2 mg). The control untreated mouse is shown in the center. 24 h later the fluorescence signal due to the daunomycin moiety was visualized in the tumour after removal of the skin, since it caused autofluorescence.](Image 5)

4. Discussion

Our main goal in this study was to synthesize and evaluate the biological activity of anti-cancer drug derivatives with a high therapeutic index and minimal toxicity to normal cells in vivo, since currently used cytotoxic anthracycline alkaloids are toxic to cancer as well as to normal cells in the body. Linking the affinity tag daidzein to daunomycin may circumvent some of these problems, presumably through some unique properties of this isoflavone. Daidzein and not the structurally related isoflavone genistein is known to interact with the lipid interface on the cell surface [14], thus facilitating endocytosis. Further, acting as a weak oestrogen, daidzein may recognize a putative plasma membrane oestrogen receptor [18], a membrane located ERβ-related protein [19] or a nuclear oestrogen receptor of the β-type [20]. Based on these
observations we prepared a reactive daidzein derivative, 7-(O)-carboxymethyldaidzein, and conjugated it to the primary amino group of the sugar moiety of daunomycin to yield cDaid–Dau (see Fig. 1 for the structure). The MLS ovarian cancer cells were subsequently chosen in our studies since these cells were sensitive to daidzein, estradiol and 7-(O)-carboxymethyldaidzein (see Fig. 2).

The cytotoxicity of cDaid–Dau was tested in MLS ovarian cancer cells (Fig. 3). The conjugate was 2–3 fold more cytotoxic than daunomycin at the low concentration range (0.3–3 nM). At the concentration range of 30–3000 nM, the conjugate showed the same cytotoxicity as daunomycin. When the efficacy of the cytotoxic effect of cDaid–Dau on tumour growth of MLS xenografts was compared with an equivalent dose of free daunomycin, the conjugate was as effective as daunomycin (see Fig. 4). In contrast to daunomycin treated mice, conjugate treated mice did not lose weight, indicating that the conjugate was less toxic than daunomycin.

While in vivo imaging of the fluorescence signal generated by Dau, indicated uptake of both cDaid–Dau and Dau by the tumour, the intensity of fluorescence was higher in the conjugate treated mice than in the Dau treated mice, thus suggesting specific delivery of the drug to the tumour (Fig. 5). This enhanced uptake by tumour tissue in vivo contrasted with the apparent lesser cardiotoxicity afflicted by the cDaid–Dau. As shown in Fig. 6, only the myocardium of daunomycin treated group, but not that of cDaid–Dau treated mice showed toxic myocardial changes and loss of striation (Fig. 6).

In summary, binding of carboxy derivatives of daidzein to ovarian cancer cells capable of expressing yet undefined binding sites for this isoflavone, can be utilized to deliver the cytotoxic drug directly to the cell, thereby enhancing cytotoxic potency beyond what is expected based on the concentration of the cytotoxic drug alone. While binding of the cytotoxic conjugates to normal cells in vivo cannot be avoided, non-tumour tissue toxicity of cytotoxic drugs such as daunomycin can be substantially reduced. Indeed, the results indicate that targeting of Dau via cDaid can elicit suppression of tumour growth, while markedly reducing Dau’s toxicity in an animal model of ovarian cancer. Utilization of isoflavone derivatives in these ovarian tumours for affinity targeting of daunomycin forms the basis for the broader use of cancer type-specific and hormone receptor specific therapy.

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References


