In Vivo Antitumor Activity of Novel Water-Soluble Taxoids

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The antitumor activity of newly developed taxoids possessing a sugar moiety (glucose, galactose or mannose) to improve water solubility was evaluated. Galactose-bound taxoid (10-α-GAG-DT) proved superior to all other taxoids in both water solubility and antitumor activity. Therapeutic efficacy of 10-α-GAG-DT in terms of in vivo antitumor activity was found to be approximately equivalent to that of docetaxel, which is known to be superior to that of paclitaxel. The observation that the sugar moiety was gradually hydrolyzed in serum to release docetaxel indicates that 10-α-GAG-DT acts as a prodrug.

Key words antitumor; paclitaxel; docetaxel; derivative; prodrug

Paclitaxel (1, Taxol®), a diterpenoid originally isolated from the bark of the Pacific yew, Taxus brevifolia, is a powerful inhibitor of microtubule assembly in tumor cells and is currently used in the treatment of various kinds of cancers.1) However, poor water solubility (0.4 μg/ml) compromises its use as an anticancer agent. Cremophor EL® (polyoxyethylene castor oil) was therefore designed as a solubilizing agent to enhance water solubility (60 μg/ml), but causes hypersensitivity in patients. Conversely, docetaxel (2, Taxotere®), an analogue of paclitaxel exerting powerful antitumor activity, is more soluble (14 μg/ml) than paclitaxel, but solubility is not greatly improved.

To date, much effort has been devoted to the synthesis of paclitaxel analogues with reduced side effects and improved solubility. For instance, a number of taxoids bound to hydrophilic functionalities such as polyethylene glycols,2–4) amino acids,5) phosphates,6–8) malic acid9) and sugars10,11) have been identified as prodrugs of 1.

Interestingly, a naturally occurring glycoside-bound paclitaxel (7-O-xyllosylpaclitaxel, 312,13)) reportedly shows potent activity in the disassembly of microtubules, which prompted us to export new sugar-bound taxoids.

At an earlier stage of our project, efforts were made to synthesize sugar-bound taxoids enzymatically. However, the attempted glycosylation with a variety of enzymes failed entirely, probably due to both the intrinsically poor water-solubility and reactivity of 1. Accordingly, we turned our attention to the chemical synthesis of sugar-bound taxoids. Two problematic issues exist in the glycosylation of 7-OH of 1. One is the poor solubility of 1 in organic solvent, and the other is the inevitable use of a Lewis acid to activate anomeric functionalities of sugars. We were thus concerned that the baccatin nucleus would be damaged if conventional glycosylation protocols were adopted. In this context, we designed glycosyloxy acetic acid (414) and achieved esterification of 4 to 7-OH of 1.

We have recently reported on the water-solubility and in vitro antitumor activity of taxoids 5a—d and 6a—e, which are esterified with 4 at either 7-OH of 1 or 10-OH of 2.15) We report herein in vivo antitumor activity of these new taxoids.

MATERIALS AND METHODS

Chemicals and Agents Each taxoid (Fig. 3, 5a—d, 6a—e) was synthesized as described previously.15) The quantity of those taxoids was analyzed by high-performance liquid chromatography (HPLC) on a C18 column (Taxil 5 μm, 250×4.6 mm I.D.; MetaChem Technologies, Torrance, CA, U.S.A.) using methanol/water=65/35 as eluent with a flow rate of 0.5 ml/min at 35 °C. Taxoids were detected by absorption at 230 nm using an SPV-10A photodiode array detector (Shimadzu, Kyoto, Japan). Human serum AB type was purchased from Dainippon Pharmaceutical (Osaka, Japan). Rat serum was prepared from Sprague Dawley rats (Nippon

Fig. 1. Taxoids

Fig. 2. Glycosyloxyacetic acid

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Animals and Tumor Cells  Male CDF1 mice were obtained from Nippon SLC. All experiments were performed in 8-week-old mice. P388 murine leukemia cells were kindly provided by Dr. K. Takeda (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan) and maintained by serial intraperitoneal passage in DBA/2 mice (Nippon SLC). All experimental procedures were consistent with those stipulated in the Guide for the Care and Use of Experimental Animals of Kaken Pharmaceutical (Kyoto, Japan).

In Vivo Antitumor Activity against P388 Leukemia Cells  Male CDF1 mice were inoculated intraperitoneally with $1 \times 10^6$ of P388 murine leukemia cells and then administered intraperitoneally with a constant dose (20 mg/kg) or varying dose of each taxoid for 5 d from 1 d after P388 inoculation. For the constant dose, each derivative was prepared with saline/Cremophor EL®/ethanol=97.5/1.25/1.25 (=75.0/12.5/12.5 as to 1 or 2) and an appropriate volume in proportion to mouse body weight was injected. For varying doses, the above solution was diluted to the appropriate concentration with saline and the proper volume was injected. The potency of compounds was evaluated as percentage increase in life span (%ILS) and toxicity (decrease in body weight). The following formula was used to calculate %ILS:

$$\frac{\text{median survival days in drug-treated} - \text{median survival days in vehicle control}}{\text{median survival days in vehicle control}} \times 100.$$  

Vehicle control mice were P388-bearing mice treated with vehicle (saline/Cremophor EL®/ethanol=75.0/12.5/12.5). Although solvent compositions differed between 1, 2 and derivatives, we chose the above vehicle as representative, due to the highest Cremophor EL®/ethanol content. No significant differences in survival days or change in body weight were noted between these solvents. Median survival in vehicle control mice was 7 d in all experiments.

Stability of Taxoids in Human or Rat Serum  Each taxoid (2.5 mg) was dissolved in ethanol (125 µl) and filled with saline to 25 ml. Human or rat serum (7.5 ml each) was added to 7.5 ml of the above solutions and these mixtures were incubated at 37 °C. The solution (2 ml) was taken up over time and extracted with ethyl acetate. This extract was concentrated in vacuo and the residue was dissolved in methanol/water=65/35 (v/v) and analyzed by HPLC. The following formula was used to calculate % remaining of each derivative:

$$\frac{\text{peak area of derivative}}{\text{peak area of derivative} + \text{peak area of docetaxel}} \times 100.$$  

**Fig. 3. Synthesized Taxoid Compounds**

**Fig. 4. In Vivo Antitumor Activity of Taxoids against P388 Murine Leukemia Cells at Constant Dose ($n=10$/Group Except for Paclitaxel, $n=9$)  
*p < 0.001 versus vehicle, *p < 0.01 versus paclitaxel**

**Fig. 5. Changes in Mean Body Weight of Mice Treated with Taxoids ($n=10$/Group)  
○, non-treated; ●, paclitaxel (1); □, 10-α-GAG-DT (6a); ○, 10-α-GAG-DT (6c); ■, 10-β-GAG-DT (6d); □, 10-α-MAG-DT (6e). *p < 0.001 versus non-treated.
highest water-solubility and the most potent antitumor activity, we compared the in vivo antitumor activity of 6c with 2 at various doses (Fig. 6). The antitumor activities of 6c and 2 proved to be dose-dependent. Considering the dose to show equal activity, activity of 6c seemed to be about a half that of 2. In another experiment, the antitumor activities of 1 and 2 were compared at various doses (Fig. 7). Consequently, it was estimated that 2 was approximately 8-fold more active than 1. From these observations, we supposed that 6c was approximately 4-fold more active than 1.

Decreases in body weight can be regarded as a critical side effect of antitumor agents. In this regard, a considerable decrease in body weight was observed for both 6c and 2 in a dose-dependent manner (Fig. 8). The decrease in body weight of 6c proved equal to that of 2 with a two-fold dose. This suggests that the therapeutic efficacy of 6c is approximately comparable to that of 2.

Stability of 6a—e in Human or Rat Serum Stability of 6a—e and 2 in human or rat serum in vitro was surveyed. All of 6a—e gradually degraded to 2 (Fig. 9). Any unknown derivative which possesses taxane skeleton was not detected in the present experiments, and total peak area of each 6a—e and docetaxel was almost equal to peak area of each 6a—e at 0 time. To verify the assumption that glycoside bond fission followed by ester hydrolysis might occur, we synthesized 10-O-hydroxyacetyldocetaxel (7) as shown in Fig. 3 and examined stability in human serum. As a result, 2 was undetectable after 1 h, apparently indicating that ester hydrolysis is faster than cleavage of anomeric oxygen. Compounds 6a, 6c and 6d degraded much faster than 6e (Fig. 9).

**DISCUSSION**

We envisaged the synthesis of highly water-soluble taxoids, as the presently used solubilizing agent, Cremophor EL, is considered to exhibit frequent adverse effects during clinical treatment. To enhance water solubility, several water-
soluble appendages such as polyethylene glycols, amino acids, phosphates and malic acids have been reported to date. We selected sugars as first-rate candidates for two reasons: 1) that sugars would be expected to exert a certain biological activity; and 2) that sugars display an intrinsically highly water solubility. Moreover, we designed a link to a sugar moiety to a baccatin skeleton via an ester bond, for the reason that conventional glycosylation protocols are ineffective, since a Lewis acid as a promoter must cause either skeletal rearrangement or oxetane ring opening.

In an earlier stage, 4 was esterified to the C-2’ position of 1 to give a 2’-ester that was very unstable and hydrolyzed very easily to produce 1 during column chromatographic purification. We therefore next synthesized 7-modified compounds (5a–d) and 10-modified compounds (6a–e). Compounds 6a–d showed almost equal in vitro antitumor activity to 2, although 6e did not, whereas compounds 5a–d exhibited lower antitumor activity than 1. Furthermore, as indicated in the present experiments, 6a, 6c, and 6d were found to show higher in vivo antitumor activity than 1 with constant dose (20 mg/kg/d). The therapeutic efficacy of 6c was equivalent to that of 2, which is superior to that of 1. Examination of the stability of 6a–e in serum revealed that 6a–d easily degraded to 2 within 1 h, except for 6e. From this observation, 6a–d can be considered as prodrugs of 2.

From the observation that 6c was less active than 2, a considerable amount of 6c can be considered to be excreted in the body prior to degradation to 2. The half-life of 2 in rat plasma is reportedly 7 min. Both the concentration of 2 to which 6c degrades in serum and the concentration of 6c thus seem to be drastically decreased after administration, and activity of 6c was inferior to that of 2. Docetaxel derivatives seem likely to need to remain in circulation in serum for a longer time to prevent almost immediate excretion out of the body.

The question arose regarding which bond was cleaved initially when 6c was transformed to 2. Stability of 7 in serum was therefore surveyed. Consequently, 7 turned out to be rather more stable than 6a–d, and no 2 was detected after 1 h (data not shown). Therefore, 6a–d were probably degraded to 2 not via cleavage of a glycosyl bond, but by cleavage of an ester linkage.

From examination of the stability of 6a–d in serum, 7 was not detected by HPLC analysis. As a result, 6a–d were considered to be degraded enzymatically to 2 not at the position of glycosyl linkage, but at an ester linkage. As shown in Fig. 10, we similarly synthesized other docetaxel derivatives in which ω-galactosylxylo carboxylic acids bind to the 10-po-

Fig. 10. Other Synthesized Docetaxel Derivatives (n=2—4, 6, 8)

REFERENCES