Integrated into Lipid Microspheres against Human Ovarian Carcinoma Cells
Resistant to Cisplatin in Vivo1

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Abstract

One of the Δ7-prostaglandin A1 derivatives with unique antitumor activities, 13,14-dihydro-15-deoxy-Δ7-prostaglandin-A1-methyl ester, was integrated into lipid microspheres (Lipo-TEI9826) and examined for its antitumor effect in vitro and in vivo. The in vitro relative resistance of human ovarian cancer, A2780<sup>CP</sup>, to cisplatin (CDDP) and Lipo-TEI9826 was 27.3 and 2.0, respectively, compared with A2780, the parent cell line of A2780<sup>CP</sup>. In in vitro experiments, when A2780<sup>CP</sup> and the parent cell line A2780 were inoculated into nude mice, A2780<sup>CP</sup> grew two times more rapidly than did A2780. The growth of A2780<sup>CP</sup> tumor was not suppressed by CDDP, whereas that of the A2780 tumor was significantly suppressed. Nevertheless, the growth of both the A2780 and the A2780<sup>CP</sup> inoculated tumors was significantly inhibited by treatment with Lipo-TEI9826 at any time after the initial treatment, compared with the lipid microspheres only. These results show that Lipo-TEI9826 may be an effective antitumor agent and capable of overcoming CDDP resistance.

Introduction

The series of PGs<sup>3</sup> A and J, members of a unique category of PG, the cyclopentenone PGs, exhibit potent antitumor, antiviral, and differentiation activities, including activities affecting cell cycle arrest at the G<sub>1</sub> phase (2–5). Recent reports have unveiled molecular mechanisms of such PGs and their physiological roles in adipogenesis (6, 7). One of the antitumor mechanisms of such PGs is an induction of p21, which does not depend on p53, resulting in G<sub>1</sub> arrest in tumor cells, i.e., the compound might mimic p53 in the cells lacking p53 (8–11). In the previous study, we demonstrated in vitro antitumor activity of Δ7-PG<sub>I</sub> and Δ7-PG<sub>A</sub> against a human ovarian cancer cell line (12). These PGs displayed very little cross-resistance to either CDDP or doxorubicin. Although Δ7-PG<sub>I</sub> is active in vivo in various tumor systems (4, 13, 14), the half-life of this type of PG in sera is very short, because it is converted to an inactive form (15). These facts prompted us to examine the antitumor activity of 13,14-dihydro-15-deoxy-Δ7-PG<sub>A</sub> which is stable in sera as Δ12-PG<sub>J</sub> (16, 17). TEI9826, an analogue of Δ12-PG<sub>J</sub>, has a novel structure and exhibits unique antitumor profiles by the COMPARE program with 38 human ovarian strains in vitro (18). Here we report the antitumor activity of lipid microsphere-integrated TEI9826 in vitro and in vivo with human ovarian cancer cells both sensitive and resistant to CDDP.

Materials and Methods

Cell Lines. Two cell lines derived from human ovarian adenocarcinoma were used in this study. A2780 cells and A2780<sup>CP</sup> cells were kindly provided by Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The two cell lines were grown at 37°C in RPMI 1640 supplemented with 10% FCS, 2 mm l-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin and were maintained in a humidified atmosphere of 5% CO<sub>2</sub>.

Chemicals. Fetal bovine serum was purchased from FLOW Laboratories (North Ryde, Australia). CDDP (Briplatin; 10 mg/ml) was obtained from Bristol-Myers Squibb Co. Ltd. (Tokyo, Japan). TEI9826, the chemical structure of which is as shown in Figure 1, was the product of Teijin Ltd. (Tokyo, Japan). TEI9826 was dissolved at a concentration of 10 mg/ml in 99.9% ethanol and stored in −20°C. The stock solution was diluted with fresh RPMI 1640 at the appropriate concentrations just before use in vitro. Each equivalent solvent solution was added to control dishes such that the final concentration of ethanol was <0.56% (v/v) in all experiments on PGs. 1-Chloro-2,4-dinitrobenzene was obtained from Aldrich Chemical Co. and was diluted before use in vitro. Lipid microsphere-integrated TEI9826 was prepared as described below. Purified egg lecithin (PL100E) was purchased from QP Co. Ltd. (Tokyo, Japan); the content of phosphatidyl choline is ~80%, and the content of phosphatidyl ethanolamine is ~20%. This lecithin is suitable for clinical use. Purified, clinical grade soybean oil was purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan) for use in the formulation of fatty lipid emulsion.

Preparation of Lipid Microsphere-integrated TEI9826 (Lipo-TEI9826)

We have been examining Lipid microspheres as a dosage formulation for antitumor PG (14, 16, 18–20), because these PGs are highly lipophilic substances and cannot be administered in aqueous solutions. TEI9826 is a liquid at −20°C and is miscible at any ratio with vegetable oils such as soy bean oils, which is the oil phase of fatty lipid emulsion in clinical use. Therefore, preparation of lipid emulsion containing TEI9826 is easy by the procedure for commonly used fatty lipid emulsion in laboratory scale and industrial scale. Egg yolk lecithin (1.2 g) was dispersed in injection-grade water (85.8 g) containing glycerin (2.5 g) at 60°C. The mixture (clear oily liquid) of TEI9826 (0.5 g) and soybean oil (9.5 g) was introduced into the lecithin dispersion solution (total volume, 100 ml) and emulsified well with a high-pressure homogenizer (Microfluidizer) at a pressure of 20,000 psi. Oil droplet size was measured with a dynamic light scattering particle sizer (Zeta-Plus; Brookhaven Instrument Co.) and was ~180 nm. TEI9826 content in the formulation was 5.0 mg/ml. Lipo-TEI9826 is freely soluble in 5% glucose and stable for years at 4°C. Control lipid microspheres were prepared by the same method without TEI9826.

Measurement of Growth Inhibition. The two cell lines in log-phase, grown as a monolayer in 100-cm<sup>2</sup> culture flasks, were trypsinized and suspended in 0.2 ml of the complete growth medium. The suspended cells were seeded (1.5 × 10<sup>4</sup> cells for A2780 and 1.75 × 10<sup>4</sup> cells for A2780<sup>CP</sup>) into each well of a 96-well tissue culture plate (Costar, Cambridge, MA) and incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 24 h. Then, a 10-μl quantity of medium containing various concentrations of TEI9826 (0.1–10 μg/ml), Lipo-TEI9826...
(0.1–10 μg/ml), control lipid microspheres, and CDDP (0.024–4 μg/ml) was put into each well, and the plate was incubated for 96 h at 37°C in a CO₂ incubator. The number of cells adhering to the bottom of each well was calculated by a modified indirect colorimetric method with an automatic photometer (Dynatech Laboratories, Inc., Tokyo, Japan) as described before (21). Simply, the medium was discarded, and the adherent cells were fixed with methanol for 2 min, then left at room temperature. Each well was filled with 200 μl of 3% hydrochloric acid with the dissolved dye. The absorbance of the hydrochloric acid solution was measured with an automatic photometer (Dynatech Laboratories, Inc.) at 660 nm. Growth inhibition was calculated by:

\[
\% \text{ growth inhibition} = \frac{1 - A_{660} \text{ (treated cells)}}{A_{660} \text{ (control cells)}} \times 100
\]

A660 (treated cells) indicates the absorption intensity of a methylene blue solution obtained from cells treated with a drug. A660 (control cells) indicates a methylene blue solution obtained from the control cells (4.8 × 10^4 cells). All experiments were performed in triplicates, and each point was obtained from three to four separate experiments. IC₅₀, implying the concentrations necessary for 50% inhibition of cell growth, were calculated from the growth inhibition curves.

**Nude Mice.** Female nude mice (BALB/c, nu/nu, −5 weeks of age) were obtained from Japan Clea laboratories (Tokyo, Japan) and maintained in a pathogen-free environment. The mice were cared for daily, and body weights were recorded to monitor the side effects of the drugs. When necessary, the animals were killed and dissected, and the tumor tissues were fixed in formalin for histological examinations. Distant metastases were not observed during the experimental periods.

**In Vivo Treatment.** To examine the in vivo antitumor activities of CDDP and PGs on the growth of both the A2780 and A2780CP tumors, 2 × 10^6 cells of each tumor type were separately inoculated s.c. into the nude mice. After a tumor grew to about 5 mm in diameter, 80 mg/kg/day Lipo-TEI9826 was administered i.p. three times a week for 2 weeks, up to six times. Ten mice were divided into two groups: five mice treated with 80 mg/kg/day of Lipo-TEI9826 and another five with 5% glucose aqua including the vehicle, i.e., lipid microspheres only as a control group. Tumor sizes (both longitudinal and latitudinal width) of the two groups were measured with a scale.

To investigate CDDP resistance of A2780 tumors in vivo, 3.5 mg/body weight (kg/day) of CDDP was administered i.p. once a week for 3 weeks into five nude mice bearing A2780CP tumors. No significant growth inhibition was shown for either A2780 treated with saline or A2780CP treated with saline or CDDP.

**Results**

The Antitumor Activities of TEI9826, Lipo-TEI9826, Lipid Microspheres, and CDDP on A2780 and A2780CP Cell Growth in Vitro. Fig. 2 shows growth inhibition curves of A2780 and A2780CP cells treated with CDDP, TEI9826, Lipo-TEI9826, and control lipid microspheres. IC₅₀ of A2780 and A2780CP were 0.072 and 1.7 μg/ml for CDDP, 0.70 and 1.4 μg/ml for TEI9826, and 0.70 and 1.4 μg/ml for Lipo-TEI9826, respectively. Lipid microspheres alone exerted little growth inhibition on either 2780 cells or 2780CP cells. These results demonstrate that Lipo-TEI9826 inhibited the growth of both A2780 and A2780CP cells to about the same extent as TEI9826 did in vitro.

**Growth Inhibition of Human Ovarian Carcinoma Cells, A2780, and A2780CP, Inoculated s.c. into Nude Mice Treated with CDDP.** Fig. 3 demonstrates the growth inhibition of A2780 and A2780CP tumors treated with CDDP. The doubling time for tumors of A2780 and A2780CP were roughly 6 and 3 days, respectively. CDDP significantly inhibited the growth of the A2780 tumor, compared with that of the A2780CP tumor. On the contrary, no significant growth inhibition was shown for either A2780 treated with saline or A2780CP treated with saline or CDDP.

**Antineoplastic Activity of PGA1 Analog**

**In Vivo Antitumor Activity of PGA1 Analog.**

**In Vitro Antitumor Activity of PGA1 Analog.**

**Fig. 1. Chemical structure of TEI9826.**

**Fig. 2. Growth inhibition curves of A2780 and A2780CP cells treated with CDDP, TEI9826, Lipo-TEI9826, or control lipid microspheres alone.**

**Fig. 3. Growth inhibition of A2780 and A2780CP tumors treated with CDDP.**
Effect of Lipo-TEI9826 on Growth Inhibition of A2780 and A2780 CP Tumors Inoculated s.c. into Nude Mice in Vivo. Fig. 4A shows growth curves of the A2780 tumor treated with Lipo-TEI9826 or vehicle alone (control). There was a significant difference in growth inhibition between the Lipo-TEI9826 treated and control groups. Fig. 4B shows growth inhibition curves for A2780 CP tumor cells treated with Lipo-TEI9826 or control. Lipo-TEI9826 significantly inhibited the growth of the CDDP-resistant tumor, whereas lipid microspheres did not.

Effect of Lipo-TEI9826 Treatment on Body Weights of Tumor-bearing Nude Mice. The changes in body weights of nude mice bearing the A2780 tumor treated with Lipo-TEI9826 or vehicle were compared with those of nude mice bearing the A2780 CP tumor treated with Lipo-TEI9826 or vehicle. Body weights of Lipo-TEI9826 treated mice did not significantly change during the experiments. Body weights of nude mice bearing the A2780 tumor and treated with vehicle increased gradually to the 10th day, followed by no significant changes from the 10th to 15th day. Body weight gains of nude mice bearing the A2780 CP tumor were observed up to the 15th day unrelated to treatment, and there were no significant differences in weight gains between the experimental group and the controls.

Discussion

A novel species of antitumor agent, TEI9826, was demonstrated to suppress CDDP-resistant human ovarian cancer cells inoculated into nude mice s.c. The human ovarian cancer cell line A2780 CP is 24 times more resistant to CDDP than is its wild-type A2780 in vitro. When A2780 CP cells were inoculated into nude mice s.c., the developing tumor grew two times more rapidly than did that of the wild type in terms of doubling time, and CDDP could not suppress the tumor growth under the conditions where the drug is effective against the wild type. TEI9826 effectively inhibited proliferation of the CDDP-resistant cells in vitro and showed very little cross-resistance to CDDP (IC_{50} were 0.7 μg/ml for A2780 and 1.4 μg/ml for A2780 CP). TEI9826 showed significant suppression of tumor growth under the conditions of our study, with the drugs administered in this dose and schedule. The Lipo-TEI9826 effects on the cell cycle are dependent on its concentration. At the inhibitory concentration 50% (IC_{50}) of Lipo-TEI9826, cells accumulate in G_1 phase. This effect is partially reversible, because the time dependency occurred when the exposure time of Lipo-TEI9826 was prolonged (4). At an IC_{90} level of Lipo-TEI9826, cells accumulate in G_2-M phases and then irreversibly begin cell death mechanisms (4). When the tumors transplanted in nude mice were removed after treatment with Lipo-TEI9826 and then analyzed, fractions in the cell cycle, both G_1-phase cell accumulation and decrease in S-phase cell fraction, were found (4). This suggests that the in vivo mechanism of this compound may be cyto-
static via G1 arrest in the cell cycle (4). In this experimental condition, no toxicity was found, whereas weight gain in the experimental mice during the observation period was slightly lower than that observed in the controls (statistically not significant). These results strongly suggest that TEI9826 may overcome CDDP resistance at least partly in some tumors. Furthermore, Δ12-PGJ2, a physiological alkylidene-cyclopentenone PG, is reported to display synergistic activity with CDDP in vitro in various cell lines, such as Tamoxifen-resistant 289 TAM6 melanoma cells (22, 23) and the colon cancer cell lines, LS174T and COLO201 (24). Although we did not examine the synergism of TEI9826 with CDDP, it should be confirmed by in vivo experiments in the future with the above-mentioned cell lines. We reported before that 2008DDP cells, which showed increased intracellular GSH levels as a major mechanism for their resistance to CDDP, had some cross-resistance to PGs (12). This cross-resistance might be caused by delayed incorporation of PGs into nuclei. On the contrary, however, Lipo-TEI9826 inhibited the growth of both A2780cis and A2780 human ovarian cancer cells, despite increased intracellular GSH levels in A2780cis cells, compared with A2780 cells (25). These results suggest that GSH-related detoxification mechanisms might not necessarily have an important role in the acquired resistance to PG in these cell lines. In contrast to intracellular GSH levels, MRPG/S-X pump overexpression is identified as a candidate for conferring resistance to both CDDP and Δ12-PGJ2 in HL60/R-cp cells, and very recently it was shown that KT-5908, a specific inhibitor of the MRPG/S-X pump, dose dependently enhanced the cellular sensitivity of the cells to Δ12-PGJ2 methyl ester, where cell growth inhibition was linked with cell cycle arrest in G1 phase (26, 27). Because TEI9826 showed a very unique profile of tumor cell growth inhibition for a panel of 38 human cancer cell lines and the COM-PARE program for this PG indicated no similarity to other known antimutator agents (18), it would be quite reasonable to develop this compound toward clinical use.

References

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