Antitumor Effect of CGP41251, a New Selective Protein Kinase C Inhibitor, on Human Non-Small Cell Lung Cancer Cells

Yuri Ikegami, Seiichi Yano and Kenzo Nakao

Drug Discovery Research Unit, Pharmaceutical Division, Ciba-Geigy Japan Ltd., 10-66, Miyuki-cho, Takarazuka 665, Japan

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ABSTRACT—The antitumor effect of CGP41251 (4'-N-benzoyl staurosporine), a selective protein kinase C (PKC) inhibitor, was examined on two kinds of human non-small cell lung cancer (NSCLC) cell lines (adenocarcinoma: A549 and squamous cell carcinoma: NCI-H520). CGP41251 at 0.5 or 1.0 μM inhibited the proliferation of these tumor cell lines significantly; however, at 0.1 μM, it did not show any significant inhibition. Cell cycle analysis indicated that CGP41251 at 0.5 or 1.0 μM arrested the cell cycle progression at the G2/M phase up to 24 hr, but 0.1 μM did not. It seems that the antiproliferative action of CGP41251 against human NSCLC is related to G2/M accumulation. In NCI-H520, CGP41251 caused DNA re-replication without mitosis. In a nude mice xenograft, CGP41251 at a dose of 200 mg/kg showed antitumor activity against these cell lines. Histopathologically, expansion of central necrosis was observed, although no destruction of tumor nests was seen by CGP41251 administration. In both tumor tissues, the PKC activity of the particulate fraction was significantly decreased by CGP41251 treatment. From these results, it is thought that the antitumor activity of CGP41251 against human NSCLC is accompanied by the decrease of PKC activity in the particulate fraction. Moreover, the G2/M arrest of the cell cycle induced by CGP41251 might be important for the growth inhibitory action of this compound.

Keywords: CGP41251, Protein kinase C inhibitor, Human non-small cell lung cancer, Cell cycle, G2/M arrest

Protein kinase C (PKC) is a critical mediator of signal transduction from stimulated growth factor receptors that lead to cell proliferation, and it induces many cellular responses. PKC is normally activated by diacylglycerol (DG), which is formed by the hydrolysis of membrane inositol phospholipids in response to extracellular signals (1, 2). It is also well-known that this enzyme is a major target of phorbol ester, a potent tumor promoter (1, 2). PKC is expressed in mammalian systems as a family of diverse serine-threonine kinases, consisting of at least ten isoforms differing in both substrate specificity and dependence upon Ca2+ availability (3–6).

To date, many studies have suggested the importance of PKC in tumor promotion or tumor cell growth. In fibroblasts transformed with the PKC-1 gene, enhanced tumorigenicity was expressed (7, 8). The ras- or src-transformed fibroblasts exhibited a steady elevation of DG level and/or increased membrane-bound PKC activity (9). In p21ras or pp60src transfected human colonic cells, the enhanced tumorigenecity and PKC-α expression were demonstrated (10). Moreover, enhanced PKC activity correlated with a rapid proliferation rate was observed in malignant glioma (11). These data suggest that the increased activity of PKC may result in a tumor-promoting effect and growth regulation in tumor cells.

CGP41251, 4'-N-benzoyl staurosporine, exerts a high degree of selectivity for cPKC subtype inhibition and exhibits antitumor activity (12, 13). Moreover, our previous data indicated that CGP41251 showed a broad antitumor spectrum against human tumors, including lung cancer, in nude mice xenografts (14).

In the present study, to clarify the mechanism of the antitumor action by CGP41251, we investigated the effects of CGP41251 on the cell proliferation and cell cycle distribution of human NSCLC cell lines simultaneously. In addition, we also examined the relationship between the antitumor effects of CGP41251 and PKC activity in tumor tissues in the same cell lines.
MATERIALS AND METHODS

Chemicals

CGP41251 was obtained from the Pharmaceuticals Division, Research Department, Ciba-Geigy, Basle, Switzerland. CGP41251 formulated as 18% (w/w) in Gelucire 44/14 was prepared by Ciba Pharmaceutical Development, Horsham, UK, according to a defined procedure of melting the Gelucire and then dissolving the active substance in the melt. For the in vitro study, the compound was dissolved in dimethylsulfoxide (DMSO) as a stock solution (2.5 mM) and diluted to the required concentrations in tissue culture medium. For the in vivo study, prior to administration, the waxy formulation of CGP41251 was added to sterile water, and then the mixture was sonicated for 10 min, resulting in a milky suspension.

Cell culture

Human non-small cell lung cancer cell line, A549, was supplied from Japanese Cancer Research Bank, Tokyo; and NCI-H520 was obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. The cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium supplemented with 100 U/ml of penicillin G, 100 μg/ml of kanamycin and 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The medium was changed to fresh medium every 2–3 days.

Antiproliferative assay

Cells were precultured in appropriate medium supplemented with 10% fetal bovine serum for 24 hr in 96-microwell plates. Drugs were added to each plate at various concentrations and incubated for another 24–72 hr. Each well was added with 5 mM WST-1 (Dojindo, Kumamoto) and 0.2 mM 1-methoxy PMS (Dojindo), and the absorbance at 450 nm was measured by a Microplate Reader (Sankojyunyaku, Tokyo).

Cell cycle analysis

Cells were plated at a density of 5 x 10⁵ cells/5 ml medium in 100-mm diameter dishes. One day after the inoculation, drugs were added to each plate. Cells were removed at the indicated time (24–72 hr) from culture dishes by trypsinization and centrifugation. After washing with PBS(−), cells were suspended in PBS(−) containing 0.1% Triton X-100 to prepare nuclei. After 0.1% ribonuclease A (Sigma Chemical Co., St. Louis, MO, USA) and 50 μg/ml propidium iodide (PI) (Sigma) were added, the cell suspension was filtered through 40-μm mesh. The DNA contents in PI-stained nuclei were analyzed by flow cytometry (Coulter, Hialeah, FL, USA). The population of cells in a particular phase in the cell cycle was estimated by the Multicycle program (Coulter).

Animals

Male athymic nude mice (BALB/c nu/nu, 4 weeks of age) were purchased from Charles River Co., Ltd., Yokohama. Animals were maintained under standard laboratory temperature and humidity. The diet for the athymic nude mice consisted of γ-ray-irradiated food (CL-2; Nippon Clea Co., Ltd., Tokyo) and autoclaved water. All experiments were initiated after 1 week acclimatization.

Antitumor activity

The passaged tumors were excised and rinsed with PBS(−). After removing outer membranes and necrotic portions, the tumors were cut into pieces, 2–3 mm in diameter. Then these tumor fragments from each tumor were transplanted s.c. into the back of nude mice using a trocar. When the tumor volume reached about 100 mm³, drug administration was started. CGP41251, formulated as 18% in Gelucire, was administered p.o. for 3 consecutive weeks. The maximum length (L) and the perpendicular width (W) of the each tumor growing in nude mice were measured twice a week with a slide caliper, and the tumor volume (TV) was calculated by the following formula.

\[ TV (mm^3) = L \times W^2 \times \frac{1}{2} \]

The antitumor activity was determined by comparing the mean TV of the test group with that of the control group and expressed as T/C (%) as follows:

\[ T/C (%) = \left( \frac{TV \text{ of the test group}}{TV \text{ of the control group}} \right) \times 100 \]

At the end of the experiment, the mice were sacrificed and tumors were dissected out. The tumor samples were weighed and stored in 10% neutral buffered formaldehyde solution for the histopathological study or at −80°C for the PKC assay.

Histopathological study

The histopathological evaluation was performed in accordance with the method of Shimosato et al. (15) according to the following scoring system: 0: Ineffective, no morphological change is observed; I: Slightly effective, Ia: If necrosis is present in the cells, it is small in amount with only slight degenerative changes, Ib: Necrosis and/or disappearance of the tumor cells are prominent, but less than 2/3 of the entire lesion in amount; II: Moderately effective, Ila: Necrosis and/or disappearance of the tumor cells are more than 2/3 of the entire lesion in amount, IIB: Nearly the entire lesion is displaced by necrosis and/or fibrosis, but a small number of viable...
tumor cells are still present; III: Markedly effective, no visible tumor cells can be seen.

**PKC assay**

PKC activity was measured according to the method of Hatada et al. (16) with slight modifications. The tissues were cut into small pieces and homogenized in 2 ml of Buffer A (25 mM Tris/HCl (pH 7.5), 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM ethyleneglycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 15 mM 2-mercaptoethanol, and 0.25 M sucrose) on ice. The homogenate was centrifuged at 1000 × g for 10 min to remove the unhomogenized tissue. The supernatant was centrifuged at 100,000 × g for 1 hr at 4°C. The supernatant thus obtained was stored at 4°C for use as the cytosolic fraction. The pellet was solubilized in Buffer A containing 1% Triton X-100 by continuous stirring for 1 hr at 4°C. The solubilized pellet was centrifuged at 100,000 × g for 1 hr at 4°C, and the resulting supernatant was used as the solubilized particulate fraction. The cytosolic and particulate fractions were further purified by DEAE-Sepharose CL-6B (0.5 ml bed volume) to remove inhibitors of PKC or phosphoprotein phosphatase. Each fraction was applied to a 0.5-ml DEAE-Sepharose column equilibrated with Buffer B (25 mM Tris/HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol). The column was washed with 5 ml of Buffer B, and then PKC was eluted with 2 ml of Buffer B containing 0.3 M NaCl. PKC activity was assayed by the Protein Kinase C enzyme assay system (Amersham, Aylesbury, UK). The total PKC activity is defined as the sum of cytosolic and particulate PKC activity.

**Statistical analyses**

The statistical significance of differences were analyzed by one-factor ANOVA followed by Scheffe’s F-procedure. P < 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Antiproliferative activity**

The antiproliferative effects of CGP41251 on human NSCLC cell lines, A549 and NCI-H520 were examined. The concentration dependence of CGP41251 on cell growth inhibition of A549 and NCI-H520 is shown in Fig. 1. CGP41251 at 0.5 and 1.0 μM caused the statistically significant inhibition of cell proliferation for 48–72 hr. However, it did not have any significant antiproliferative action at 0.1 μM on either of the cell lines.

**Cell cycle distribution**

To investigate the effects of CGP41251 on cell cycle progression of A549 and NCI-H520, the DNA contents of nuclei were measured by flow cytometry. In A549 cells, after exposure to CGP41251 at 0.5 or 1.0 μM for 24–72

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**Fig. 1.** Effect of CGP41251 on proliferation of human NSCLC cell lines, A549 (a) and NCI-H520 (b). ○, Control; ▲, 0.1 μM CGP41251; ■, 0.5 μM CGP41251; ●, 1.0 μM CGP41251. Results are each shown as a mean ± S.E. of 4 experiments. *P < 0.05, **P < 0.01, by Scheffe’s F-procedure.
hr, the population of G1 cells were decreased and those of G2/M cells were increased (Figs. 2 and 3). When cells were treated with 1.0 μM CGP41251 for 72 hr, very few G1 cells were observed and the percentage of G2/M cells reached approximately 80% (Figs. 2 and 3). S phase cells were also reduced up to 24 hr by the treatment of CGP41251 at 0.5 or 1.0 μM (Fig. 3). In the NCI-H520 cells, after exposure to CGP41251 at 0.5 or 1.0 μM for 24 hr, the populations of G2/M cells were increased; however, in cells given this treatment for 48–72 hr, G2/M cells were decreased and the cells with 8C DNA content were increased (Figs. 2 and 3). CGP41251 at 0.1 μM did not affect the cell cycle progression in either of the cell lines.

**Antitumor activity**

The antitumor effects of CGP41251 on A549 and NCI-H520 transplanted into nude mice were evaluated. CGP41251 formulated as 18% (w/w) in Gelucire was administered p.o. at doses of 100 and 200 mg/kg once daily for three weeks. CGP41251 dose-dependently inhibited the growth of both lung cancers, compared with that of the gelucire-treated group (Fig. 4). On the final day, CGP41251 at a dose of 200 mg/kg exhibited a statistically significant inhibition of tumor growth, and the T/C % values against A549 and NCI-H520 were 38.6% and 34.3%, respectively (Table 1). The maximum tolerable dose (MTD) of CGP41251 was 750 mg/kg (p.o.); In this study, no notable adverse effects were observed. In the

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Fig. 2. DNA histograms of human NSCLC cell lines, A549 (a) and NCI-H520 (b), treated with 1.0 μM CGP41251. Cells were seeded on day 0 and CGP41251 was added at day 1. After 24, 48 or 72 hr from the drug addition, DNA histograms of the cells were obtained from flow-cytometric analysis.
Fig. 3. Effect of CGP41251 on the cell cycle kinetics of human NSCLC cell lines, A549 (a) and NCI-H520 (b). ○, control; ▲, 0.1 μM CGP41251; ■, 0.5 μM CGP41251; ●, 1.0 μM CGP41251. The percentages of G₁, G₂, S and >G₂ were obtained from DNA histograms by quantitative analysis. Data represent the means of 2 experiments.
Fig. 4. Effect of CGP41251 on the growth of human lung tumor xenograft, A549 (a) and NCI-H520 (b). Drugs were orally administered for three weeks. The values of the relative tumor volume are each given as a mean ± S.E. of 7 animals (A549) or 8 animals (NCI-H520). ○, Control; ▲, 100 mg/kg CGP41251; ●, 200 mg/kg CGP41251. *P < 0.05, by Scheffe's F-procedure.

Table 1. Antitumor effect of CGP41251 on human NSCLC in nude mice xenograft

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Histology</th>
<th>CGP41251 Dose (mg/kg/day)</th>
<th>Schedule</th>
<th>T/C (%)</th>
<th>Histological grade</th>
</tr>
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<tbody>
<tr>
<td>A549</td>
<td>Adenocarcinoma</td>
<td>100</td>
<td>6/W x 3</td>
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</tr>
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<td></td>
<td></td>
<td>200</td>
<td></td>
<td>38.6</td>
<td>Ib</td>
</tr>
<tr>
<td>NCI-H520</td>
<td>Squamous cell</td>
<td>100</td>
<td>7/W x 3</td>
<td>73.1</td>
<td>I</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td>200</td>
<td></td>
<td>34.3</td>
<td>I (Ia)</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of CGP41251 on PKC activity in human NSCLC, A549 (a) and NCI-H520 (b). □, total fraction; ▼, particulate fraction; ▲, cytosolic fraction. Results are each shown as a mean ± S.E. of 3-4 animals. *P < 0.05, by Scheffe's F-procedure.
histopathological study, the dose-dependent expansion of central necrosis and vacuolar degeneration of tumor cells were observed by the administration of CGP41251, although no destruction of tumor nests was seen (Table 1).

**PKC activity in tumor**

PKC activity was analyzed in the cytosolic and particulate fractions extracted from tumor tissues of A549 and NCI-H520 grown in nude mice. In both tumor tissues, the PKC activity of the particulate fraction was higher than that of the cytosolic fraction (Fig. 5). The particulate PKC activity was significantly reduced by administration of CGP41251 in a dose-dependent manner (Fig. 5). On the other hand, the cytosolic PKC activity was not affected by CGP41251.

**DISCUSSION**

In the present study, we demonstrated that CGP41251 showed growth inhibitory effects on human NSCLC cell lines, A549 adenocarcinoma and NCI-H520 squamous cell carcinoma, both in vitro and in vivo. Exposure to 0.5 or 1.0 μM of CGP41251 for 48–72 hr significantly inhibited cell proliferation. At the concentration of 0.1 μM, it did not affect the cell growth. Cell cycle analysis demonstrated that CGP41251 at 0.5 or 1.0 μM blocked cell cycle progression in the G$_2$/M phase for up to 24 hr, but not do so at 0.1 μM. It seems that the antiproliferative effects of CGP41251 are due to a G$_2$/M block of the cell cycle in both human NSCLC cell lines. Several groups have reported that staurosporine, a non-selective PKC inhibitor, could arrest the cell cycle progression in either the G$_1$ or G$_2$ phase, depending on its concentration (17, 18). At a lower concentration, staurosporine arrested the cells at the G$_1$ phase, and at higher concentration, it induced G$_2$ phase arrest. Bruno et al. reported that staurosporine showed different effects on the cell cycle of normal and leukemic lymphocytes (18). In the normal lymphocytes, staurosporine arrested the cells in the G$_1$ phase at a low concentration and in G$_2$, at high concentration; However, it arrested the leukemic lymphocytes in the G$_2$ phase regardless of the concentration. Crissman et al. also described that staurosporine preferentially arrested normal cells in G$_1$ and transformed cells in G$_2$ (19). The effect of CGP41251 on cell cycle progression in human NSCLC cell lines seems to resemble that of staurosporine in transformed cells. In yeast cells, the PKC-1 gene product was shown to participate in cell cycle regulation from the G$_2$ to M phase (20). However, little is known about whether cPKC plays a role in the cell cycle regulation of mammalian cells. In NCI-H520 cells, CGP41251 caused G$_2$/M arrest up to 24 hr and then induced the formation of many polyploid cells with 8C DNA content from 48–72 hr. Staurosporine or other staurosporine analogues, K-252a and RK-286C, were also found to cause DNA re-replication without an intervening mitosis (21). Our data indicate that CGP41251 also induce DNA replication without mitosis in NCI-H520 cells. Our results suggest that CGP41251 sensitive kinases would be involved either in the exit from the G$_2$ phase or in the entry into mitosis, so that the antiproliferative effect of this compound occurred in human NSCLC cell lines.

On the other hand, UCN-01, which is also a staurosporine analogue and a selective PKC inhibitor, was shown to induce the G$_1$ block in human epidermoid carcinoma (22) or human breast cancer (23). It seems that the target of CGP41251 may be different from that of UCN-01.

The human NSCLC cell growth inhibition by CGP41251 was also observed in the nude mice xenograft model. CGP41251 at a dose of 200 mg/kg showed significant antitumor activity against A549 and NCI-H520. It was reported that staurosporine can inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-caused skin tumor promotion (24). However, it exhibited less tolerability and less antitumor activity than CGP41251 (12). These data suggest that the selectivity for cPKC inhibition may be important for antitumor activity and decrease in some toxicity in vivo. In addition, we measured the PKC activity in the cytosolic and particulate fractions of these tumor tissues to examine the relationship between PKC activity and antitumor activity in vivo. It is well-known that the activated PKC is translocated from the cytosol to the plasma membrane (25, 26). Furthermore, the increased membrane-bound PKC activity was found in several types of tumors (9, 16). The PKC activity in human lung tumors were reduced in the particulate fraction by the administration of CGP41251. It is suggested that the reduction of PKC activity in the particulate fraction may be important for the antitumor activity of CGP41251 in vivo.

In conclusion, our results show that CGP41251 has antitumor activity against human NSCLC, which is relative to the decrease of PKC activity in the particulate fraction. Moreover, its antitumor effect against these cell lines is thought to be due to the G$_2$/M block or deficiency of mitosis.

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REFERENCES

4 Stabel S and Parker P: Protein kinase C. Pharmacol Ther 51, 71 – 95 (1991)
19 Crissman HA, GaddoS DM, Tobey RA and Bradbury EM: Transformed mammalian cells are deficient in kinase-mediated control of progression through the G2 phase of the cell cycle. Proc Natl Acad Sci USA 88, 7580 – 7584 (1991)