Significant Growth Inhibition of Human Lung Cancer Cells Both in Vitro and in Vivo by the Combined Use of a Selective Cyclooxygenase 2 Inhibitor, JTE-522, and Conventional Anticancer Agents

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ABSTRACT
This study reports that a selective COX-2 inhibitor JTE-522 inhibits both in vitro and in vivo growth of human lung cancer cells as a single agent. Furthermore, the adjunct use of JTE-522 is shown to significantly enhance treatment efficacy of conventional anticancer drugs not only in vitro but also in vivo without causing any noticeable side effects. Indeed, IC₅₀ of various anticancer agents in vitro were reduced by up to 70%, whereas the combination therapy of JTE-522 with docetaxel and vinorelbine inhibited tumor growth in vivo by 65 and 55%, respectively. Taken together, these findings suggest that the use of a selective COX-2 inhibitor in the treatment of lung cancer may be promising, especially because of its enhancement of the treatment efficacy of conventional anticancer agents without compromising quality of life.

INTRODUCTION
Lung cancer currently claims >50,000 lives annually and has recently become the leading cause of cancer deaths in Japan (1). Despite aggressive approaches made in the treatments of lung cancer in the past decades, the 5-year survival rate for lung cancer remains <15% (2). Surgery, chemotherapy, and radiation have been generally unsatisfactory, especially in the treatment of advanced diseases. New strategies based on better understanding of the biology are thus clearly needed to improve the treatment efficacy of this fatal disease.

Recent studies suggested that an increase in the expression of COX-2, a key inducible enzyme involved in the production of prostaglandins and other eicosanoids, may play a significant role in carcinogenesis in addition to its well-known role in inflammatory reactions (3–8). Although previous studies have been largely confined to colorectal tumorigenesis, we have shown that a significantly increased expression of COX-2 is also frequently present in lung cancers (9). In addition, we found that increased COX-2 expression was associated with a shortened survival of patients who underwent surgical resection of early stage adenocarcinoma (10), whereas several lines of in vitro and in vivo evidence, including that reported by us, suggest its potential role in invasion and metastasis (11–14). Our previous in vitro study also showed that a selective COX-2 inhibitor nimesulide could inhibit proliferation of lung cancer cell lines in culture (15).

In this study, we show that a selective COX-2 inhibitor JTE-522 can exert not only in vitro but also in vivo antitumor effects when used alone or in combination with anticancer agents in the treatment of human lung cancer cell line.

MATERIALS AND METHODS
Cell Lines and Animals.
ACC-LC-319, an adenocarcinoma cell line established in our laboratories at Aichi Cancer Center (16), has been maintained in RPMI 1640 supplemented with streptomycin (100 µg/ml), penicillin (100 units/ml), and 5% FCS. HPL1D, a human epithelial cell line derived from normal peripheral lung, was also established in our laboratories (17), whereas BEAS2B, a human bronchial epithelial cell line, was kindly donated by Dr. Curtis C. Harris (National Cancer Institute; Ref. 18). Five-week-old female athymic nude mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and maintained under specific pathogen-free conditions.

Agents.
JTE-522, a selective COX-2 inhibitor, was from Japan Tobacco, Inc., Tokyo, Japan (19–21). Amrubicin and its
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H9262 sensitivity of NSCLC cells whether adjunct use of JTE-522 could enhance the chemosensitivity of neoplastic cells of the lung. We next investigated HPL1D, were significantly more resistant to the growth inhibition of prostaglandin E2 production at the concentration of 10 μM in ACC-LC-319. In this regard, JTE-522 inhibited 90% of prostaglandin E2 dependent inhibition of the proliferation of ACC-LC-319 (Fig. 1). In this experiment, JTE-522 inhibited 90% of prostaglandin E2 production at the concentration of 10 μM in ACC-LC-319 (data not shown). The normal lung epithelial cell lines, BEAS2B and HPL1D, were significantly more resistant to the growth inhibitory effect. Each data point represents the mean of quadruplicate determinations in a representative experiment; bars, SD. Similar results were obtained in four independent experiments.

**Colorimetric Cell Proliferation Assay for Chemosensitivity.** For the evaluation of chemosensitivity, a colorimetric cell proliferation assay was performed using the Cell Titer 96 kit (Promega Corp., Madison, WI). Briefly, cells were plated in 96-well plates and exposed continuously for 4 days to a range of concentrations of JTE-522 and/or anticancer agents. At least three independent experiments were carried out in quadruplicate.

**In Vivo Study.** ACC-LC-319 cells (1 × 10⁶) in 100 μl of serum-free RPMI 1640 medium were injected into the s.c. tissue of the left abdominal wall of 7-week-old female nude mice. The mice received either a conventional diet or one containing 0.5% JTE-522 from the day of injection of tumor cells. Two weeks after inoculation, the mice were randomly divided into two groups, each consisting of six mice within either JTE-522-treated or nontreated groups. They were then administered a single dose of either saline or 5 mg/kg docetaxel, vinorelbine, or amrubicin from their tail veins. On the basis of the daily consumption of the diet and body weight of mice, the average doses of JTE-522 could be calculated as 100 mg/kg/day. Two weeks after injection of anticancer agents, each mouse was sacrificed, and the tumor weight was determined. In addition, frozen and paraffin-embedded samples were prepared.

Microvessels in a tumor were counted after immunostaining with a rat antimonoclonal antibody (PharMingen, San Diego, CA). For microvessel assessment, specimens were examined under a light microscope, and 5–10 areas of high-power view (690 × 460 μm) were selected, scanned, and analyzed. The mean value for 5–10 fields was regarded as the microvessel density for each tumor and expressed as the number of vessels/mm².

**RESULTS**

Inhibition of Lung Cancer Cell Growth in Vitro by JTE-522 as a Single or Adjunct Agent of Conventional Anticancer Drugs. The colorimetric cell proliferation assay was first performed to examine the *in vitro* antitumor effects of a COX-2 inhibitor JTE-522 as a single agent showing dose-dependent inhibition of the proliferation of ACC-LC-319 (Fig. 1). In this regard, JTE-522 inhibited 90% of prostaglandin E₂ production at the concentration of 10 μM in ACC-LC-319 (data not shown). The normal lung epithelial cell lines, BEAS2B and HPL1D, were significantly more resistant to the growth inhibitory effect, suggesting possible importance of COX-2 in the growth of neoplastic cells of the lung. We next investigated whether adjunct use of JTE-522 could enhance the chemosensitivity of NSCLC cells *in vitro*. The inhibitory effects of JTE-522 at 0, 3, 6, or 9 μM were evaluated in combination with docetaxel, vinorelbine, or amrubicin-13-OH, which are known to be effective to a certain extent in the management of NSCLC. Significant reduction of IC₅₀ was observed in ACC-LC-319 (Fig. 2A). The use of 9 μM JTE-522 in combination with docetaxel and vinorelbine resulted in the reduction of IC₅₀ by 70 and 63%, respectively. A 48% reduction of IC₅₀ was observed in ACC-LC-319 treated with amrubicin-13-OH as a result of the addition of 9 μM JTE-522, whereas the use of 3 μM JTE-522 in combination with docetaxel, vinorelbine, and amrubicin-13-OH resulted in the reduction of IC₅₀ by 39, 36, and 34%, respectively. In addition, the use of 6 μM JTE-522 as an adjunct yielded 39–62% reductions in IC₅₀.

An isobologram was constructed based on the dose-response curves for each individual agent, including JTE-522, to examine its synergistic effects with various anticancer agents in ACC-LC-319. It was clearly shown that supra-additive effects could be obtained using docetaxel or vinorelbine in combination with JTE-522 (Fig. 2B). The additive effects were observed in the combination of amrubicin-13-OH with 6 or 9 μM JTE-522, whereas a marginal supra-additive effect was observed in combinational use with 3 μM JTE-522. These findings indicated that the chemosensitivity to docetaxel and vinorelbine could be synergistically enhanced in ACC-LC-319 cells *in vitro* by the adjunct use of JTE-522.

**In Vivo Antitumor Effects of JTE-522 as a Single Agent or in Combination with Anticancer Agents.** We next investigated, using a xenografted ACC-LC-319, whether the antitumor effects of JTE-522 on lung cancer cell growth *in vivo*, an
effects that had not been reported previously, could also be observed. Treatment with JTE-522 alone inhibited tumor growth by 36% compared with the nontreatment group (P < 0.005 by Mann-Whitney U test) without showing any observable toxicity such as weight loss or gastrointestinal bleeding (Fig. 3). We additionally assessed whether JTE-522 was able to enhance the effects of the anticancer agents including docetaxel, vinorelbine, and amrubicin. As shown in Fig. 3, treatment with JTE-522 in conjunction with docetaxel significantly reduced tumor growth by 65% (P < 0.0001) when compared with no treatment group, whereas 55% inhibition of tumor growth was observed in the combination treatment with JTE-522 and vinorelbine (P < 0.0005). It was noted that significantly enhanced effects of the anticancer agents were detected by the addition of JTE-522 when compared with treatment with either docetaxel (53% reduction; P < 0.0001) or vinorelbine (49% reduction; P < 0.05) alone. In contrast, adjunctive use of JTE-522 did not enhance growth inhibition by amrubicin. Therefore, JTE-522 alone appeared to be able to inhibit tumor growth in vivo, and a marked enhancement could be attained by its adjunct use with docetaxel or vinorelbine.

To gain an insight into the molecular mechanisms of the in vivo antitumor effects of JTE-522, the antiangiogenic effect of JTE-522 was examined by counting microvessels (Fig. 4). Tumors were immunohistochemically stained with an anti-CD31 monoclonal antibody reactive with endothelial cells. The mean microvessel counts/mm² was significantly reduced in both JTE-522-treated mice (29.6 ± 6.0; P < 0.05) and docetaxel plus JTE-522-treated mice (32.9 ± 5.5; P < 0.05) when compared with that in control mice (40.8 ± 2.4). Treatment with docetaxel resulted in a modest increase in the microvessel count, although the difference was not statistically significant. In this regard, the ELISA examination showed that treatment of ACC-LC-319 in vitro with docetaxel increased COX-2 expression by 53% (data not shown).

**DISCUSSION**

Treatment with novel drugs that selectively interfere with an important pathway controlling cancer cell survival, proliferation, and/or metastasis in combination with conventional anticancer treatment such as chemotherapy has generated enormous clinical interest (22–25). This study shows for the first time that a selective COX-2 inhibitor JTE-522 inhibits both in vitro and in vivo growth of human lung cancer cells as a single agent. Although JTE-522 directly inhibited lung cancer cell growth as shown in vitro, this study suggests that JTE-522 may also affect tumor growth in vivo, in part, by inhibiting tumor angiogenesis.
The numbers of microvessels in a xenograft tumor treated with JTE-522 and/or docetaxel. The number of microvessels is significantly fewer in the JTE-522-treated groups with and without docetaxel (32.9 ± 5.5 and 29.6 ± 6.0/mm², respectively) compared with the nontreatment group (40.8 ± 2.4/mm²; *P < 0.05). The Mann-Whitney U test was used to assess statistical differences.

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angiogenesis either by augmenting the release of angiogenic peptides such as vascular endothelial growth factor by the tumor cells or by directly increasing production of prostaglandins (26, 27). The inhibition of COX-2 in endothelial cells by COX-2 inhibitor resulted in the inhibition of endothelial cell spreading and migration in vitro and the suppression of fibroblast growth factor-2-induced angiogenesis in vivo (28). Selective COX-2 inhibitors are generally used in the long-term treatment of various chronic inflammatory diseases such as rheumatic arthritis, whereas the maximum tolerated dose of JTE-522 within a short period of time in the treatment of cancer patients has not been explored. However, it is encouraging that we did not observe any noticeable side effects such as weight loss or gastrointestinal bleeding at the present dose (100 mg/kg/day), consistent with the previous report on lack of any serious side effects even at 300 mg/kg/day in rats (19).

Furthermore, the combined use of JTE-522 with conventional anticancer drugs was shown to exhibit significant enhancement in the treatment efficacy not only in vitro but also in vivo. Although the potential mechanism of this enhancement needs to be studied more in depth, the IC₅₀ₐ₆ of various anticancer agents were indeed reduced by up to 70% in vitro, and the combination therapy of JTE-522 with docetaxel and vinorelbine was shown to inhibit in vivo tumor growth by 65 and 55%, respectively. It was noted that JTE-522 appeared to reduce tumor angiogenesis, whereas treatment with docetaxel resulted in a modest increase in the microvessel count. This may account for the finding that combinatorial use of JTE-522 and docetaxel was more effective in vitro than in vivo.

In summary, the present findings suggest that the use of a selective COX-2 inhibitor may be a promising therapeutic approach in the treatment of lung cancer because previous studies by us and others indicated the presence of increased COX-2 expression in NSCLC (9, 29), which is notoriously resistant to chemotherapy. Although additional studies are necessary to generalize the present findings, it is especially encouraging that it may be possible to enhance the anticancer activity by treatment without compromising the quality of life with this type of selective inhibitor.


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