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REVIEW

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Progress in preclinical and clinical studies for the development of new anticancer drugs in Japan, with emphasis on taxanes

Key words Taxane · Anticancer drugs

Introduction

The development of new treatments, especially effective anticancer drugs, is essential for improvement of the survival of cancer patients. Many new molecular targets of anticancer drugs have been identified by studies in molecular and biochemical pharmacology, and microtubules are considered to be one of the most important targets for cancer chemotherapy.

Microtubular structures such as spindles and cytoplasmic microtubules show dynamic interconversion during the mitotic cycle. Tubulin is one of the major microtubular components, and its polymerization and depolymerization regulate microtubular dynamics. Other microtubular components such as microtubule-associated proteins (MAPs), actin, and intermediate and microfilaments have also been demonstrated to be involved in microtubular dynamics.

MAPs are proteins that frequently copurified with tubulin and have been shown to promote microtubule assembly in vitro. Recent evidence suggests that the functions of MAPs and filaments in microtubule assembly are regulated by phosphorylation, which is catalyzed by mitogen-activated protein kinase and/or $p34^{cdc2}$ kinase.

Drugs that act on microtubules can be divided into two categories: taxanes and vinca alkaloids. Taxanes such as paclitaxel and docetaxel promote polymerization of microtubules and enhance microtubule stability. In contrast, vinca alkaloids and rhizoxin inhibit microtubule polymerization. Accordingly, microtubule functions such as cell motility, intracellular transport, and mitosis are disturbed. Factors that influence the effects of drugs that act on tubulin include intracellular accumulation of the drug, genetic alterations in tubulin, tubulin synthesis, the drug-binding capacity of tubulin, metabolic inactivation of the drugs, and alterations in MAPs (MAP2).

In this review the following topics are discussed:

1. Effects of paclitaxel on the interaction between MAP2, tubulin, and mitogen-activated protein kinase
2. Mechanisms of resistance to paclitaxel and docetaxel
3. Reversal of vindesine resistance by rhizoxin
4. Clinical trials of paclitaxel, docetaxel, and vinorelbine
5. Pharmacokinetic/pharmacodynamic analysis of paclitaxel

Effects of paclitaxel on the interaction between MAP2, tubulin, and mitogen-activated protein kinase

Taxanes bind to tubulin at a specific site, promote microtubule assembly, enhance microtubule stability, and disturb microtubule functions such as cell motility, intracellular transport, and mitosis. To analyze the effect of paclitaxel treatment on tubulins, MAP2 was evaluated in synchronized cells [7].

The lung-adenocarcinoma cell lines PC-9 and PC-14 were synchronized at the G1/S phase border by thymidine double-block. After release from the G1/S phase border, the cells were treated for 2 hours with paclitaxel 2 n*M* or phosphate-buffered saline (PBS), and their subsequent pro-

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gress through the cell cycle was evaluated by flow cytometry. Proteins were stained for between 2 and 14 h with anti-α-tubulin, anti-β-tubulin, and anti-MAP2 antibodies and were detected using immunoblotting.

The α- and β-tubulin protein content of PBS-treated control and paclitaxel-treated cells did not differ significantly at any stage of the cell cycle. There was also no difference between several bands detected using anti-MAP2 antibodies in paclitaxel-treated and control cells [7].

The effect of paclitaxel treatment on the interaction between tubulin and MAP2 was evaluated in synchronized PC-14 cells. PC-14 cell samples precipitated with anti-tubulin antibodies were loaded onto 12.5% polyacrylamide gels and reblotted with the anti-MAP2 antibody. Two MAP2 isoproteins were detected, and increased amounts of MAP2 were detected in α- and β-tubulin precipitates from paclitaxel-treated cells as compared with PBS-treated cells [7].

The effect of paclitaxel treatment on mitogen-activated protein kinase activity was evaluated by the "in gel" kinase assay [6] in synchronized cells. Synchronized PC-14 cells were treated with 2 n*M* paclitaxel for 2 h. After each 2- to 48-h period, the mitogen-activated protein kinase activity was evaluated. Two mitogen-activated protein kinases with molecular masses of 42 and 44 kDa were detected. After 8 h of incubation, the mitogen-activated protein kinase activity of paclitaxel-treated samples was lower than that of PBS-treated control cells.

The dose-dependent effects of paclitaxel on mitogen-activated protein kinase activity were also evaluated in the H69 small-cell lung-cancer (SCLC) cell line. Synchronized and unsynchronized H69 cells were exposed to 0.1–100 n*M* paclitaxel for 6 h, and mitogen-activated protein kinase activity was detected using the in gel kinase assay. Paclitaxel produced decreases in the mitogen-activated protein kinase activity of synchronized H69 cells; these decreases were dose-dependent. In contrast, few changes in mitogen-activated protein activity were observed in unsynchronized H69 cells.

The effects of paclitaxel on mitogen-activated protein kinase activity in crude cellular homogenates of PC-14 cells and in the purified MAP-rich tubulin fraction were also evaluated and compared. In the purified MAP-rich tubulin fraction, mitogen-activated protein kinase activity was not affected by the addition of paclitaxel. In contrast, the mitogen-activated protein kinase activity of crude cellular homogenates of PC-14 cells decreased dose-dependently in the presence of paclitaxel. These results suggest that paclitaxel treatment of PC-14 cells reduces their mitogen-activated protein kinase activity. However, the effect of paclitaxel is indirect; other factors directly influence mitogen-activated protein kinase activity.

Myelin basic protein and MAP2 have been used as substrates in mitogen-activated protein kinase assays, but there has been speculation that these proteins are also phosphorylated by $p34^{cdc2}$ kinases [1]. Therefore, we examined the effect of paclitaxel on the $p34^{cdc2}$ kinase activity of PC-9 and PC-14 cells. $p34^{cdc2}$ kinase activity in untreated PC-9 and PC-14 cells synchronized at the G1/S-phase border was increased at 2–4 h after the initiation of cell-cycle progression. This observation agreed with previous reports [7] that $p34^{cdc2}$ kinase activity increases during the G2/M phase. No increase in the $p34^{cdc2}$ kinase activity of paclitaxel-treated PC-9 or PC-14 cells was observed. These results suggest that paclitaxel prevents $p34^{cdc2}$ kinase activation and inhibits cell-cycle progression.

From these studies we conclude that paclitaxel dose-dependently inhibits mitogen-activated protein kinase and $p34^{cdc2}$ kinase in cellular homogenates of lung-cancer cell lines. This biochemical effect may be responsible for the increased affinity between MAP2 and tubulins that results in promotion of microtubule polymerization.

Mechanisms of paclitaxel and docetaxel resistance

One of the main reasons why chemotherapy fails is believed to be the emergence of cellular drug resistance. Therefore, it is important that the mechanisms of drug resistance be identified to improve the clinical efficacy of chemotherapy. Mechanisms of drug resistance can be divided into three categories. The first is multidrug resistance (MDR) mediated by P-glycoprotein (PGP). The second is non-PGP-mediated multidrug resistance, which is related to MRP (multidrug resistant protein) and topoisomerase II. The third is drug-specific resistance; in this case the mechanism of drug resistance differs from drug to drug [14]. Resistance to paclitaxel involves the MDR phenotype produced by the *mdr-1* gene product PGP. However, other mechanisms may also be important in resistance to paclitaxel. We attempted to establish a paclitaxel-resistant human SCLC cell line that did not overexpress PGP.

A paclitaxel-resistant cell line was established by stepwise exposure of the human SCLC cell line H69 to increasing concentrations of paclitaxel; this cell line was called H69/Txl [11]. H69 and H69/Txl cells (2×10^5/ml) were seeded and cultured in the presence or absence of 30 n*M* paclitaxel without changing of the medium for 11 days. Cell proliferation curves showed that in the absence of paclitaxel the H69/Txl cell growth rate was 38% less than that of cells exposed to paclitaxel. In contrast, the growth rate of the parental H69 cells was not increased in the presence of paclitaxel. Therefore, the growth of H69/Txl cells was partially dependent on paclitaxel. It should also be noted that the H69/Txl cells were small and their protein content was low as compared with that of parental H69 cells and that the doubling time of H69/Txl cells was prolonged in the absence of paclitaxel.

The growth-inhibitory effects of paclitaxel and vinca alkaloids on H69 and H69/Txl cells were evaluated by tetrazolium blue (MTT) assay [11], and the 50% inhibitory concentrations of the cell lines were determined. The relative resistance of H69/Txl cells to paclitaxel was 4.73-fold that of H69 cells. H69/Txl cells also showed higher sensitivity to vinca alkaloids such as vindesine, vincristine, and vinblastine than did the parental H69

cells. Therefore, the resistance pattern of H69/Txl cells appeared to differ from the typical MDR phenotype.

Next, the difference in [^{3}H]-paclitaxel accumulation in H69 and H69/Txl cells was evaluated. The intracellular paclitaxel concentration increased in a dose-dependent manner; however, there was no significant difference in the intracellular accumulation of paclitaxel between the two cell lines. Therefore, altered intracellular paclitaxel accumulation is not responsible for the resistance of H69/Txl cells to paclitaxel.

To confirm that H69/Txl cells did not show the PGP-mediated MDR phenotype, the expression of *mdr-1* mRNA was examined in H69/Txl cells by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Etoposide-resistant H69/VP cells, typical *mdr-1* gene-expressing cells, were used as a positive control in these experiments. In H69/VP cells, increased expression of *mdr-1* mRNA was detected. In contrast, *mdr-1* mRNA was not detected in the parental H69 and H69/Txl cell lines.

Total and polymerized α- and β-tubulin contents were evaluated in H69 and H69/Txl cells; the polymerized tubulin ratio was also examined. There was no significant difference in either the α- and β-tubulin content or the polymerized tubulin ratio of H69 and H69/Txl cells. Therefore, to determine whether alterations in tubulin were a cause of resistance, we analyzed the tubulin in H69 and H69/Txl cells by isoelectric focusing and Western blotting with anti-α- and anti-β-tubulin antibodies. In H69 cells, two α-tubulin isoforms were observed, whereas three were observed in H69/Txl cells; two of these comigrated with the isoforms detected in H69 cells, and the other was more acidic. In contrast, there was no detectable difference in β-tubulin from H69 versus H69/Txl cells. Acetylation of α-tubulin in parental H69 and H69/Txl cells was also evaluated. This showed that whereas H69/Txl cell α-tubulin was acetylated, that from H69 cells was not. We believe that the acetylation of α-tubulin in H69/Txl cells may be responsible for the paclitaxel resistance of these cells.

Finally, the effect of paclitaxel on mitogen-activated protein kinase activity was compared in H69 and H69/Txl cells. The mitogen-activated protein kinase activity of synchronized H69/Txl cells was not inhibited by high paclitaxel concentrations, but that of H69 cells was inhibited dose-dependently. These results suggest that the lack of inhibition of mitogen-activated protein kinase by paclitaxel in H69/Txl cells might be related to the mechanism of paclitaxel resistance.

In conclusion, the results of these experiments show that:

1. H69/Txl is a paclitaxel-resistant human SCLC line, the growth of which is partially dependent on paclitaxel.
2. The resistance of H69/Txl cells to paclitaxel is 4.7-fold that of the parental H69 cells, and H69/Txl cells are more sensitive to vinca alkaloids such as vindesine, vincristine, and vinblastine than are H69 cells.
3. There is no significant difference in the intracellular [^{3}H]-paclitaxel content of H69 and H69/Txl cells, and *mdr-1* mRNA is not overexpressed in H69/Txl cells as determined by RT-PCR.
4. There is no difference in the total and polymerized tubulin content of H69 and H69/Txl cells, although the mobility of one of the three α-tubulin isoforms observed in H69/Txl cells is altered and α-tubulin acetylation is increased.
5. The mitogen-activated protein kinase activity of H69/Txl cells is not inhibited by paclitaxel.

Docetaxel is a semisynthetic analogue of paclitaxel, which is obtained from an extract of the needles of the European yew tree. Like paclitaxel, docetaxel stabilizes polymerized tubulin in microtubule bundles within the cell by preventing depolymerization. Its activity as a promoter of tubulin polymerization is stronger and its inhibitory effect on cell proliferation in vitro is more potent than that of paclitaxel. To elucidate its mode of action more precisely, we tried to establish a docetaxel-resistant human non-small cell lung-cancer cell line. A docetaxel-resistant cell line (PC-14/TXT) was established by exposure of the human lung adenocarcinoma cell line PC-14 to increasing concentrations of docetaxel [2]. The diameter of PC-14/TXT cells was similar to that of PC-14 cells, as was their protein contents. However, the PC-14/TXT cell-doubling time (34.2 h) was significantly longer than that of PC-14 cells (23.3 h).

PC-14/TXT cells showed a 6.8-fold increase in docetaxel resistance as compared with the parental PC-14 cell line. This resistance was completely abolished by continuous exposure to 10 μ*M* verapamil for 3 h before the addition of docetaxel. PC-14/TXT cells showed cross-resistance to paclitaxel (14.1-fold that of PC-14 cells) and vindesine (8.2-fold); however, their resistance to doxorubicin (1.7-fold) and etoposide (1.8-fold) was not significantly higher.

To determine whether PGP and mdr were expressed in PC-14/TXT cells, their expression was evaluated by flow cytometry and RT-PCR. PGP overexpression was detected in PC-14/TXT cells by both methods. In addition, MRP was not overexpressed in this cell line.

Intracellular paclitaxel and docetaxel accumulation was determined by the addition of 10 n*M* tritium-labeled paclitaxel or docetaxel to the culture medium for 3 h. Intracellular radioactivity of both PC-14 and PC-14/TXT cells increased in parallel with the concentration of both drugs in the medium. The intracellular [^{3}H]-paclitaxel content of PC-14/TXT cells was lower, amounting to about 60% of that of PC-14 cells at each time point. In contrast, the intracellular [^{3}H]-docetaxel content did not differ significantly between these cell lines. Time-dependent efflux of [^{3}H]-paclitaxel and [^{3}H]-docetaxel was also evaluated. Efflux of both drugs was greater from PC-14/TXT cells than from PC-14 cells and had almost reached a plateau after 180 min.

We also evaluated differences in the total tubulin content and the polymerized tubulin ratio between parental and resistant cells. The total tubulin content of the cell lines did not differ; however, the polymerized tubulin ratio was lower in PC-14/TXT cells as assessed using anti-α- and anti-β-tubulin antibodies.

The results of these studies indicate that:

1. In PC-14/TXT cells, low-grade expression of PGP may be partially responsible for docetaxel resistance.
2. Other mechanisms such as decreased tubulin polymerization contribute to docetaxel resistance.

Reversal of vindesine resistance by rhizoxin

The use of drugs to which cells do not show cross-resistance is the most promising means of overcoming drug resistance, e.g., cells resistant to vinca alkaloids or cisplatin are not resistant to taxanes. Rhizoxin is a 16-membered macrocyclic lactone isolated from the pathogenic fungus *Rhizopus chinensis.* Studies using purified pig-brain tubulin have demonstrated that rhizoxin binds to a binding site different from that of vinca alkaloids, inhibits the polymerization of tubulin, and blocks cells in the G2/M phase of the cell cycle [19]. Rhizoxin was selected for clinical evaluation on the basis of its antitumor activity against several human tumor cell lines. It is of particular interest in that it could act on *mdr-1*-positive cells.

We evaluated the antitumor activity of rhizoxin against various cell lines, including drug-resistant sublines. Cell lines resistant to vindesine and paclitaxel did not show cross-resistance to rhizoxin. However, SBC-3/ADM100, a typical MDR cell line, was resistant to rhizoxin. On the basis of these in vitro results, in vivo studies were conducted in severe combined immunodeficiency (SCID) mice bearing H69 and vindesine-resistant H69/VDS cells [9, 10].

H69 and H69/VDS cells (1×10^7) were inoculated into SCID mice; 2 weeks later, when the resulting tumor had reached 100–400 mm^3 in size, rhizoxin at 0.5 or 1 mg/kg was injected intraperitoneally on days 1, 8, and 15. The growth of H69 and H69/VDS cells in the presence and absence of rhizoxin was monitored. The growth of not only H69 but also H69/VDS cells was inhibited by rhizoxin. These data confirm that cells resistant to vinca alkaloids are not cross-resistant to rhizoxin. We are now analyzing the molecular basis of this phenomenon.

Clinical trials of paclitaxel, docetaxel, and vinorelbine

In Japan, many clinical trials have been conducted using paclitaxel, docetaxel, and vinorelbine. In the case of paclitaxel, late phase II studies in ovarian, lung, and breast cancer have been completed, and early phase II studies in stomach cancer are under way [12, 20]. A phase I study in non-small cell lung cancer (NSCLC) using a combination regimen including cisplatin is in progress. In addition, two late phase II studies using paclitaxel given at 210 mg/m^2 by 3-h infusion in NSCLC have been conducted. In both studies, 60 patients were accrued and the response rates were 38.3% and 31.6%, respectively; the mean response rate was 35.0%. Paclitaxel has also shown activity against ovarian and breast cancers.

Late phase II studies of docetaxel in breast cancer, NSCLC, ovarian cancer, gastric cancer, and head and neck cancer have also been completed, and early phase II studies in leukemia/lymphoma and pancreatic cancer are under way. Docetaxel has shown significant antitumor activity against all the tumor types tested [4, 8, 13, 15–18, 21]. In NSCLC, two late phase II studies using docetaxel given at 60 mg/m^2 by 1-h infusion have been conducted. A total of 75 patients were enrolled in each study, and the response rates were 24% and 18.7%, respectively for a mean response rate of 21.3% [4, 13]. Severe fluid retention was not observed at this dose.

Vinorelbine has been demonstrated to have reproducible antitumor activity against NSCLC [3] and is available commercially in the United States and in France. In both countries, vinovelbine + cisplatin is considered to be a standard regimen in NSCLC. In Japan, late phase II studies of vinorelbine against NSCLC and breast cancer have been completed and have demonstrated that this drug has significant activity against both tumor types. Comparative phase II studies of vinovelbine and vindesine in NSCLC have been conducted by Furuse and colleagues [3]. These studies have demonstrated that navelbine is more effective in treating NSCLC. A study of the combination regimen of mitomycin C + cisplatin + vinovelbine is in progress, but preliminary results show that this combination has excellent activity against NSCLC [5].

Pharmacokinetic/pharmacodynamic analysis of paclitaxel

In phase I studies of 24- and 3-h infusions of paclitaxel, the maximum tolerated dose was 180 and 240 mg/m^2 and the recommended dose was 150 and 210 mg/m^2, respectively. Granulocytopenia was more pronounced in patients receiving the 24-h infusion of paclitaxel, and we therefore tried to evaluate the factors that determine the myelotoxicity of paclitaxel. When pharmacokinetic parameters were compared in patients receiving the same dose of paclitaxel, area under the curve (AUC) values were significantly higher in patients receiving the 3-h infusion than in those receiving the 24-h infusion. The percentage of decrease in granulocyte count did not correlate with the dose, maximum concentration, or AUC of paclitaxel. Dr. Tomoko Ohtsu, National Cancer Center Hospital East, Chiba, has demonstrated that the time during which the paclitaxel concentration >0.05 μM is the most important pharmacokinetic parameter in predicting granulocytopenia, and this fitted the sigmoid E-max model.

Conclusions

Methodology in experimental cancer chemotherapy changes rapidly in line with progress in molecular biology and in molecular and biochemical pharmacology. As new molecular targets for cancer chemotherapy are identified, the development of interesting compounds that act against

them is expected. Over the past 5 years, the methodology for clinical trials has been improved in Japan. Thus, clinical trials of new anticancer drugs are mainly conducted by pharmaceutical companies on the basis of good clinical practice (GCP). The Japanese Ministry of Health and Welfare has provided a series of guidelines for clinical trials that are compatible with those used in the United States and European Community countries. After the Third International Conference on Harmonization, which was held in Yokohama, Japan, from November 29 to December 1, 1995, GCP in Japan is expected to be modified in accordance with the idea of international harmonization, and all clinical trials should be performed in accordance with the new GCP guidelines. This will require an administrative oncology-group office, a data manager, funding, and a board of pathology, among other administrative needs.

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