Anticancer drugs have been explored by means of random screening and demonstrated to be active against not only hematologic malignancies but also some solid tumors. Recent progress in the field of molecular biology has

Sensitivity to anticancer drugs is influenced by two factors, pharmacokinetics and cellular biology. Pharmacokinetic factors involve the dose, schedule, and absorption of anticancer drugs; drug anabolism; and pharmacokinetic sanctuaries. Cellular factors include drug transport, efflux pump, detoxifying substances, metabolizing enzymes, target enzymes, repair capacity, cell cycle, and amplification of oncogenes. Strategies to overcome drug resistance include the use of non-cross-resistant drugs, modulators of drug resistance, or alternative modalities like radiation therapy or surgery; the introduction of new categories of anticancer drugs; and the application of dose-intensive or high-dose chemotherapy.

Recently, a number of molecular targets for cancer chemotherapy have been identified. Potential biologic approaches to therapy include topo-isomerase I and II, microtubules, cell-cycle regulators, apoptosis, signal-transduction pathways, suppressor genes, oncogenes, and drug transport processes. Compounds specific to these molecules have been isolated, and taxanes and camptothecins, for example, already have been approved for commercial use. Other new compounds are currently under evaluation in preclinical and clinical trials. Interestingly, different anticancer drugs are known to influence the new target molecules via a common pathway. In the present study, preclinical investigations into the mode of action of kinase activators and inhibitors, as well as the antitumor effect of taxanes via signal-transduction pathways, are presented.

Antitumor Activity of Kinase Activation/Inhibition

Several kinase activators are known to inhibit tumor growth. For example, we previously demonstrated that multidrug-resistant cells and cisplatin-resistant cells were sensitized by phorbol ester. Phase I and II studies of bryostatin, an activator of protein kinase C (PKC), are ongoing in the United States. In contrast, kinase inhibitors like staurosporin and UCN-01 also inhibit tumor growth. In addition, the phosphatase inhibitor okadaic acid also has demonstrated antitumor activity and sensitizes multidrug-resistant and cisplatin-resistant cells. This evidence suggests that phosphorylation status may determine sensitivity to anticancer drugs.

Gnidimacrin
We have succeeded in isolating and identifying an antitumor compound, gnidimacrin, from the Chinese plant *Stellera chamaejasme* L.[1] The antitumor activity and mechanism of action of gnidimacrin were examined using human tumor cell lines (such as K562, HL-60, CCRF-CEM, MKN-28, MKN-45, MKN-74, PC-7, PC-9, PC-14, N231, H69, and HLE) and murine tumors (such as P388, L1210, Lewis lung carcinoma, B-16 melanoma, and colon 26 carcinoma) in vitro and in vivo, respectively. In the in vitro assays, gnidimacrin exhibited antiproliferative activity against human leukemia, stomach cancer, and non-small-cell lung cancer. The sensitivity of individual cell lines to gnidimacrin appeared to be independent of their histologic origin, and some cell lines, such as H69 small-cell lung cancer and HLE hepatoma, were refractory. The difference between sensitive and refractory cell lines in their sensitivities to gnidimacrin was more than 10,000-fold. Gnidimacrin existed in a bound form with serum albumin that was stable and retained its antiproliferative activity. In the in vivo assay, gnidimacrin was active against not only murine leukemias P388 and L1210 but also the solid tumors, Lewis lung carcinoma, B-16 melanoma, and colon 26 adenocarcinoma, when administered by intraperitoneal or intravenous injection. In both B-16 and Lewis lung solid tumors, some mice were cured by, respectively, intraperitoneal and intravenous injection of gnidimacrin at a dose as low as 10 to 20 µg/kg/day. Gnidimacrin appears to have no specific effects on the
biosynthesis of DNA, RNA, or protein, based on the incorporation of H-labeled thymidine, uridine, and leucine, respectively, into the acid-insoluble fraction of K562 cells within 2 hours. When Col E1 supercoiled plasmid DNA was incubated in the presence of 10 to 20 µg/mL gnidimacrin for 24 hours at 37°C, no damage to the DNA was observed under the condition where linear and fragmented DNA could be induced by 4 µg/mL bleomycin. Cell-cycle arrest in the S-phase was not observed in the gnidimacrin-treated cells. These findings indicate that gnidimacrin might not directly affect DNA or DNA synthesis-associated enzymes.

In K562 cells, gnidimacrin induced bleb formation within a short time after treatment. Time to bleb formation depended on the concentration of gnidimacrin. These results raise the possibility that gnidimacrin exerts its antitumor action by binding to cell-surface components responsible for the differences in sensitivity of the tumor cell lines. Various daphnane-type diterpene esters are reported to inhibit the specific binding of the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) to protein kinase C in a receptor preparation from mouse brain. Phorbol esters are known to enhance the activity of PKC and cause blebs on the cell membrane of K562 cells. A competitive binding assay and an in vitro assay of PKC isolated from tumor cells indicated that gnidimacrin had the ability to bind with the receptor for phorbol ester and directly activate PKC in sensitive K562 cells, but not in refractory H69 cells. This finding suggests that gnidimacrin acts as a PKC activator in tumor cells and that this function may be associated with its antitumor action.

At present, relatively little is known about the biochemical mechanism behind the growth inhibition exhibited by PKC activators like phorbol esters. Both PKC activity and PKC isozyme expression were reported to be decreased in the bryostatin-resistant subline P388. Therefore, loss of PKC activation in H69 cells might be responsible for their resistance to gnidimacrin. This is further evidence that PKC activation may be the major mechanism responsible for the antitumor activity of gnidimacrin. Thus, PKC will likely become a more important element in cancer chemotherapy, and the activity of gnidimacrin makes it a good choice for studying the mechanism of antitumor effects exerted via PKC activation. To clarify the multiple actions of PKC on various cell lines, it may be necessary to examine differences in the expression of PKC isotypes and signal transduction after PKC activation. The PKC family comprises c-, n- and α-PKC isozymes. The c-PKC isozymes (α, β, γ, and δ) are activated by phorbol esters and are Ca²⁺-dependent and -independent, respectively. The α-PKC (ζ and λ) isozymes are not substantially activated by phorbol esters and are also Ca²⁺-independent. Expression of PKC-α, β, γ, δ, ε, and ζ was studied by Western blot analysis using isozyme-specific antibodies. The sizes of individual PKC isozyme-specific bands were 80 kDa for PKC-α, -β and -γ, and 90 kDa for PKC-ε. The abundantly expressed PKC-ζ isozyme was detected as a doublet of two bands. In completely gnidimacrin-resistant HLE cells, PKC-α is expressed strongly, but PKC-β is not. Strong PKC-β expression was found in gnidimacrin-sensitive HL-60, CCRF-CEM, and especially K562 cells. MCF-7 breast carcinoma, rat 3Y-1 fibroblast, and mouse NIH3T3 fibroblast cells, which express PKC-α, but not PKC-β, were also resistant to gnidimacrin. Murray et al reported that PKC-β is essential for K562 cell proliferation,[2] and Hocevar and Fields reported the selective translocation of PKC-β to the nucleus of HL-60 cells.[3] Based on these findings, PKC-β might have an important role in the antitumor effect of gnidimacrin.

Paclitaxel and Signal-Transduction Pathways

Microtubules are important cyto-skeletal components involved in many cellular processes. Several types of microtubular structures exist, including the spindle and cytoplasmic microtubules that show dynamic interconversion during the mitotic cycle. Tubulin is one of the major microtubular structural proteins. Although tubulin polymerization and depolymerization basically regulate microtubular dynamics, other factors, such as microtubule-associated proteins, actin, and intermediate filaments and microfilaments, which are microtubular components, have been demonstrated to be involved in microtubular dynamics.

The microtubule-associated proteins frequently copolymerize with tubulin and have been shown to promote microtubular assembly in vitro. Several proposed mechanisms that may determine the sensitivity/resistance of cells to antimitotic agents include (1) intracellular accumulation (multidrug and similar forms of resistance),[4] (2) changes in tubulin assembly,[5] (3) changes in tubulin synthesis, (4) decreased drug-binding affinity of tubulin, (5) metabolic inactivation of the drug, and (6) alterations in microtubule-associated proteins. The fifth and sixth mechanisms have not been proven, however, consequently, in this study, we examined the effects of paclitaxel (Taxol) on microtubule-associated proteins.

Elucidation of the effects of paclitaxel on microtubule-associated proteins and on mitogen-activated
protein (MAP) kinases will be important if effective combination regimens of paclitaxel with compounds like estramustine, which act on microtubule-associated proteins and/or MAP kinase, are to be designed.

Paclitaxel, an antimitotic anticancer agent, is active against solid tumors. The inhibition of depolymerization and promotion of microtubular assembly are essential elements of its antitumor activity. Microtubule-associated proteins copolymerize with tubulin and play some roles in microtubular dynamics.[5] We examined the effect of paclitaxel on the interaction between tubulin and microtubule-associated proteins. The human lung cancer cells PC-14 were synchronized to the G/S border by the thymidine double-block technique. After exposure to thymidine was terminated, the cells were treated briefly with 2 nmol/L paclitaxel and the levels of α and β tubulins and microtubule-associated proteins were examined at various times.

Immunoblot analysis of paclitaxel-treated cells showed no changes in the overall expression of α and β tubulins, microtubule-associated protein 2, or other microtubule-associated proteins compared with controls. The samples were immunoprecipitated with anti-α-tubulin and anti-β-tubulin antibodies and rebotted with an anti-microtubule-associated protein antibody, which showed that the amount of coimmunoprecipitated microtubule-associated protein 2 in the synchronized cells was increased by the brief paclitaxel exposure. These results suggest that paclitaxel treatment enhances the interaction between α and β tubulins and microtubule-associated protein 2.

Since the phosphorylation state of microtubule-associated protein 2 regulates its affinity for tubulins, and MAP kinase is considered to be one of the kinases responsible for phosphorylating microtubule-associated protein 2, the effect of paclitaxel treatment on the MAP-kinase activity of synchronized PC-14 cells was examined. Two bands with molecular masses of 42 and 44 kDa were detected by an intra-gel MAP kinase assay using myelin basic protein as the substrate. Paclitaxel treatment inhibited the MAP kinase activity of PC-14 cells, with inhibition maximal at the G/M phase of the cell cycle.

Similar concentration-dependent inhibition by paclitaxel of cellular MAP kinase of the human synchronized small-cell lung carcinoma H69 was observed. Paclitaxel was not observed to inhibit the MAP kinase of the paclitaxel-resistant subline H69/Tx1, suggesting that some change of the MAP-kinase cascade had occurred in these cells. No direct inhibition of the MAP kinase activity by paclitaxel was observed in the cell-free assay (in vitro), suggesting that paclitaxel did not inhibit MAP kinase directly. Since p34-kinase has been speculated to phosphorylate microtubule-associated protein 2, the effect of paclitaxel treatment on the p34-kinase activity of synchronized PC-14 and PC-9 cells was examined. Paclitaxel inhibited P34-kinase activation at the G/M phase. These results suggest that paclitaxel inhibited MAP kinase and p34-kinase in vivo indirectly. These actions of paclitaxel may be responsible for the increased affinity between microtubule-associated protein 2 and tubulins.

**Inhibition of Tumor Growth by MAP Kinase Oligonucleotide**

Based on the speculation that inhibiting the MAP kinase cascade mediates the antitumor effects of taxanes, the effect of a MAP kinase antisense oligonucleotide on cell growth and on paclitaxel-induced growth inhibition was evaluated. EAS1, an antisense oligonucleotide for the MAP kinase ERK1, inhibited the growth of human lung cancer cell lines like PC-14 on exposure to 10- to 10-µmol/L EAS1 with mean effective dose values comparable to those obtained for the inhibition of MAP kinase activity, DNA synthesis, and cell-cycle arrest at the G/M phase followed by apoptosis.[6] NIH3T3 cells transformed by the H-ras gene (PT22-3) showed higher sensitivity to the effects of EAS1. Because MAP kinase activity was activated by H-ras gene transfection in PT22-3, the status of the MAP kinase cascade in cells was the determining factor for the efficacy of EAS1. In addition, digitonin-induced cell permeability enhanced the growth-inhibitory effect of EAS1. Indeed, penetration of the cell membrane by EAS1 is critical to its growth-inhibitory effect. EAS1 augmented the paclitaxel-induced growth-inhibitory effect on NIH3T3 cells, which were minimally sensitive to EAS1 alone. It can be concluded that MAP kinase is an important target for cancer treatment and EAS1 is a potential antitumor oligonucleotide.

**Conclusion**

Data from molecular biology studies on cell-cycle regulators, signal transduction, apoptosis, etc, are critical to our gaining a thorough knowledge of the mechanisms of action behind newer anticancer agents like the taxanes and camptothecins. It is important, therefore, to exploit the recent advances in molecular biology and consider therapeutic strategies in light of the many new molecular targets.
for anticancer therapy that have been identified. Numerous studies of the molecular pharmacology of drug-resistance mechanisms are under way. The importance of identifying the molecular mechanisms of drug resistance cannot be overstressed, as such knowledge is critical to identifying compounds that specifically inhibit target molecules in drug-resistant cells. The techniques of molecular engineering could provide tools that stimulate or inhibit specific functions of molecular targets.

References:


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