Synergistic Effects of New Chemopreventive Agents and Conventional Cytotoxic Agents against Human Lung Cancer Cell Lines

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ABSTRACT

Non-small cell lung cancer (NSCLC) cells have constitutively high expression of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase (COX) 2. These NSCLC cells also have increased prostaglandin expression. Many lung cancers also express 12-lipoxygenase RNA and 12-lipoxygenase protein and biosynthesize 12(S)-hydroxyeicosatetraenoic acid, which correlates with their metastatic potential. Several studies have demonstrated that COX-1 and COX-2 inhibitors could inhibit the in vitro growth of human lung cancer cell lines. In this report, we evaluated the growth-inhibitory effects of sulindac sulfide, a COX-1 and COX-2 inhibitor; exisulind (sulindac sulfone), a novel proapoptotic agent that does not inhibit COX enzymes; and nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor on human lung cancer cell lines. We compared these effects with those of 13-cis-retinoic acid, a chemoprevention agent, and with the cytoxic chemotherapeutic agents paclitaxel and cisplatin, alone or in combination. Our goal was to develop new chemoprevention and treatment strategies. Each of the six agents tested inhibited the in vitro growth of three NSCLC and three SCLC cell lines at the highest concentration. Paclitaxel was the most potent agent (IC50 = 0.003–0.150 μM); sulindac sulfide, NDGA, and 13-cis-retinoic acid had intermediate potency (IC50 = 4–80 μM), and cisplatin and exisulind were the least potent (IC50 = 150–500 μM). Combination studies showed synergetic interactions for sulindac sulfide, exisulind, and NDGA with paclitaxel, cisplatin, and 13-cis-retinoic acid, regardless of drug-resistance phenotype. At high concentrations, the combination of 13-cis-retinoic acid and each of the five other drugs resulted in a strong synergistic effect. These studies provide a rationale for chemoprevention (exisulind ± retinoic acid ± NDGA) and therapeutic (exisulind ± paclitaxel ± cisplatin) studies in patients at risk for, or with, lung cancer.

INTRODUCTION

Lung cancer is the third most common cancer in the United States (178,000 new cases in 1998) and the leading cause of cancer death (160,400 deaths in 1998; 1). The mortality is high because there are no known effective screening procedures, there is a high propensity for early spread, and systemic therapies do not cure metastatic disease. More than 85% of cases develop in current or former tobacco smokers, and more than one-half of new cases develop in former smokers, which indicates a need for both new chemopreventive and treatment agents (2, 3).

Understanding lung cancer biology opened the opportunity for new treatment strategies. Increased levels of eicosanoids have been reported in both SCLCs3 and NSCLCs, which indicates that they may play a role in tumorigenesis (4, 5). In SCLC cells, neuropeptide autocrine growth factors stimulate arachidonic acid release through activation of phospholipase A2 (5). In NSCLC cells, especially those with ras mutations, there is constitutively high expression of the M185,000 cytosolic phospholipase A2 and COX-2 (6). The arachidonic acid metabolic pathway generates bioactive lipids that modulate physiological and pathological responses involved in tumor growth and promotion (5). LOXs generate various hydroperoxides (such as HETES), which tend to promote invasion and metastases (5, 7). Several LOX inhibitors inhibit the growth of human lung cancer cell lines and inhibit lung tumor carcinogenesis (7, 8). COX-1 and COX-2 generate prostaglandins and thromboxanes. COX-1 is a constitutive enzyme present in most cells, whereas COX-2 is inducible and is often up-regulated in tumors (5, 9, 10).

Classical NSAIDs (such as sulindac) inhibit both of the COX enzymes and were shown to inhibit tumor formation in animal models (11–13). However, the inhibition of COX-1 has serious side effects such as peptic ulcer formation and renal dysfunction. Selective COX-2 inhibitors and exisulind were developed to avoid these toxicities (14–16). Exisulind lacks the inhibitory effects on COX-1, COX-2, and LOX but induces apoptosis and inhibits tumor growth in studies involving rodent models of chemically induced mammary and colon carcinogenesis (14–16). Exisulind was also reported to inhibit lung tumor carcinogenesis in mice (17). A recent study reported that exisulind inhibits cGMP phosphodiesterases, which leads to apoptosis (18).

The combination of COX and LOX inhibitors produced synergistic growth inhibition against Lewis lung carcinomas when used together and in combination with cisplatin (4). In another study, both exisulind and sulindac sulfide produced synergistic cytotoxicity with doxorubicin and VP-16 against the lung cancer cell line A549, which overexpresses MRP. These synergistic cytotoxicities did not occur with cell lines overexpressing MDR-1P190(19). On the basis of these results, we chose to evaluate the effects of a COX-1 and -2 inhibitor (sulindac sulfide), a non-COX inhibitor (exisulind), a LOX inhibitor (NDGA), 13-cis-retinoic acid, cisplatin and paclitaxel on the growth of human lung cancer cells (both SCLC and NSCLC) alone and in combination. Our goal was to determine whether combinations of these agents would produce synergistic activity which would open the potential for combination-chemoprevention and therapeutic strategies.

MATERIALS AND METHODS

Cell Lines. Human SCLC line NCI-H345 and human NSCLC lines NCI-H157 and NCI-H460 were kindly provided by Drs. Bruce Johnson (National Cancer Institute, Bethesda, MD) or John Minna and Adi Gadzar (Simmons Cancer Center, Dallas, TX). The SCLC cell line SHP77 was obtained from Dr. Aurelia Koros at the University of Pittsburgh (Pittsburgh, PA). The SCLC line NCI-H69 and the NSCLC line A549 were obtained from American Type Culture Collection (Manassas, VA). All of the cell lines had overexpression of p16 or p53 mutations except for H157, which has wild-type p53 expression (20, 21). The cell lines were maintained in RPMI 1640 with 10% fetal bovine

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5The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-SCLC; NDGA, nordihydroguaiaretic acid; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; MDR, multidrug-resistant/multidrug-resistance; MDR-1, multidrug resistant gene 1; MRP, multidrug resistance-associated protein; LRF, lung cancer resistance-associated protein; CI, combination index; MTT, modified tetrazolium salt; NSAID, nonsteroidal anti-inflammatory drug; PGE, prostaglandin expression; IL, interleukin; CDK, cyclin-dependent kinase; Rb, retinoblastoma.
The cell viability was assayed in triplicate using the MTT growth assay. Cell viability was determined as the percentage of cells that reduce the tetrazolium salt, MTT (Sigma Chemical Co.), dissolved in RPMI 1640, to a blue formazan product in the bottom of the wells. The reduced MTT product was measured using an automated plate reader. The data were analyzed using a SlideWrite program to determine the IC50 of each drug alone. The CI-isobologram by Chou and Talalay (23) was used to analyze the drug combination assays. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate). CI > 1, CI = 1, and CI < 1 indicate antagonism, additive effect, or synergy, respectively.

**Cell Cycle Analysis.** SHP77 cells were added to each well of a 6-well plate (Corning Glass Works, Corning NY) at a concentration of 1 × 105 cells/well and incubated overnight. Drugs, at various concentrations, were added to each well the following day. Cells were then incubated for various times—24, 48, 72, and 120 h. On the scheduled day, 15 μl of a dye made of propridium iodide (1 mg/ml) and Hoechst (1 mg/ml) at a ratio of 6:1 were added to each well and incubated for 1 h. Cells were then removed from the plates using EDTA (0.01 M) and spun at 1000 RPM. The supernatant was removed, and the precipitate containing the cells was placed on a slide. Cells were viewed on a fluorescent microscope (Zeiss Axioskop) under ×40 with oil immersion, and 200 cells were scored for the percent of apoptotic cells.

**RESULTS**

**In Vitro Cytotoxicity.** All of the six agents completely inhibited the growth of the 6 cultured lung-cancer cell lines (Table 2 and Fig. 1). Between the two chemotherapeutic agents tested, paclitaxel was the most potent with an IC50 ranging from 3 to 50 nM in all of the cell lines with the exception of the MDR-1-positive SHP77 cell line, which, as expected, required a higher concentration (0.15 μM) to inhibit 50% of control cell growth. NDGA was the next most potent drug with an average IC50 of 28 μM in the SCLC cell lines and 52 μM in the NSCLC cell lines. Sulindac sulfide had cytotoxicities similar to those of NDGA in five of the cell lines tested. The SCLC cell line H69 was more sensitive to NDGA. The SCLC cell line H345 was the cell line most sensitive to four of the drugs (excepting exisulind and cisplatin) and was particularly sensitive to NDGA (IC50 = 10 μM), sulindac sulfide (IC50 = 8 μM), and 13-cis-retinoic acid (IC50 = 4 μM). Like sulindac sulfide and NDGA, 13-cis-retinoic acid had a similar effect on all of the cell lines with the exception of H345, which was the most sensitive (IC50 = 4 μM). Exisulind, was the least potent of the
NSAID-related compounds (IC\(_{50}\) = 175–225 μM). The inhibitory effects of exisulind were similar in both NSCLC and SCLC cell lines. Cisplatin was found to be the least potent drug tested (IC\(_{50}\) = 200–500 μM). Like exisulind, cisplatin produced similar effects on NSCLC and SCLC cell lines. Overall, no relationship between p53 status, the presence of MDR-1, MRP, or LRP, and drug cytotoxicity were observed with the exception of paclitaxel resistance in the MDR-1 positive SHP77 cell line.

### Cell Cycle Analysis

As shown in Fig. 2 and Table 3, each of the six drugs produced strikingly different effects in the SCLC line SHP77 on cell cycle distribution and apoptosis. These differences are consistent with different mechanisms of action. As previously reported, paclitaxel resulted in a block in G2-M with a shift in cell population from G0-G1 to G2-M (see Table 3). The peak effect was evident after 72 h of drug exposure, but increases in G2-M were seen as early as after 24 h, the first time point evaluated. Paclitaxel’s cell cycle effects lasted through 120 h, the last time point evaluated. The only other drug that produced an increase in the G2-M fraction was cisplatin which led to a maximum of 52% of cells in G2-M at 96 h (in contrast to 23% of control cells). A striking finding was the increase in G2-M fraction in exisulind-treated cells. These differences are evident after 24 h, the first time point evaluated. Paclitaxel’s cell cycle effects lasted through 120 h, the last time point evaluated. The only other drug that produced an increase in the G2-M fraction was cisplatin which led to a maximum of 52% of cells in G2-M at 96 h (in contrast to 23% of control cells). A striking finding was the increase in G2-M fraction in exisulind-treated cells. This effect was seen as early as 24 h, peaked at 96 h, and was still present at 120 h. The reasons for this previously unreported accumu-
Fig. 3. Combination drug effects of exisulind plus paclitaxel or cisplatin and combination effects of paclitaxel plus cisplatin on the NSCLC cell line A549. In these studies, both drugs were added simultaneously to the cell line. After a 5-day incubation, MTT was added, and the plates were harvested 4 h later. The effects of exisulind on the cytotoxicity of paclitaxel and cisplatin are shown in A and C, respectively. The interaction between cisplatin and paclitaxel is shown in E. The CIs for exisulind + paclitaxel and exisulind + cisplatin are shown in B and D, respectively. The CI for paclitaxel + cisplatin is shown in F. A CI equal to 1 indicates an additive effect; CI < 1 indicates synergy between the two drugs; and CI > 1 indicates antagonism between the two drugs. The CIs were determined using the index-isobologram method based on the median-principle developed by Chou and Talalay (23). Variable ratios of drug concentrations and mutually exclusive equations were used to determine the CI. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate).
SYNERGISTIC EFFECTS OF AGENTS AGAINST HUMAN LUNG CANCER

Apoptosis Analysis. Paclitaxel produced the greatest increase in apoptotic cells in all of the three cell lines tested (A549, SHP77, and H460). The results for the MDR+ SCLC line SHP77 are shown in Table 3. A greater than 10-fold increase in apoptotic cells was seen at the highest paclitaxel concentration. This effect was evident after 48 h of drug exposure with early evidence of apoptosis seen on fluorescent microscopy. Although cisplatin was the least potent drug for growth inhibition, it produced the second highest percentage of apoptotic cells (44% in SHP77 cells), a 9-fold increase compared with controls. This effect was observed as early as 72 h, peaked at 96 h and persisted through 120 h. Exisulind, sulindac sulfide, and NDGA produced similar degrees of apoptosis in the SHP77 line. A maximum of 25% of cells was apoptotic at 96 h with NDGA, 22% with sulindac sulfide, and 19% with exisulind. 13-cis-retinoic acid, produced the lowest maximal percentage (17%) of apoptotic cells.

Growth Inhibitory Effects of Drug Combinations. Paclitaxel and cisplatin are frequently used in combination to treat both SCLC and NSCLC patients. Modest synergy (CI ≥ 2 +) was noted between these agents. Peak synergy (2 +) with these two cytotoxic agents occurred in the two NSCLC cell lines at moderate concentrations. Greater synergy was achieved when several of the inhibitors of arachidonic acid metabolism were combined with the cytotoxic agents. For example, exisulind at the highest concentration produced synergy with paclitaxel and additive or synergistic effects with cisplatin in all three of the cell lines tested. (Fig. 3 and Table 4). Exisulind combined with NDGA or 13-cis-retinoic acid also produced 2 + or greater synergy in each of the three cell lines tested. The combination of exisulind and sulindac sulfide produced synergy in the two NSCLC cell lines but antagonism in the SCLC cell line SHP77. The above synergies occurred most often at the highest concentration of exisulind tested. The synergy between these agents is consistent with the differing mechanism of actions of these agents.

The most consistent synergy was observed with 13-cis-retinoic acid combinations. Combinations of 13-cis-retinoic acid with sulindac sulfide, exisulind and NDGA resulted in moderate to strong synergy at high concentrations. No significant difference was noted between NSCLC and SCLC cell lines. When combined with cisplatin, better synergy was seen in the SCLC cell line SHP77, compared with the NSCLC cell lines. When 13-cis-retinoic acid was combined with paclitaxel, strong synergy was seen in the NSCLC cell lines.

The synergy of the noncytotoxic agents suggests that combination-chemoprevention strategies are warranted, and the synergy with cytotoxic agents suggests that preclinical and clinical trials of these combinations in advanced stages are indicated. The SCLC cell line SHP77 has strong expression of MDR-1 and is more resistant to cytotoxic agents including paclitaxel compared with other lung cancer cell lines. In this cell line, these inhibitors of arachidonic acid metabolism produced greater synergy than the combination of cytotoxic agents.

DISCUSSION

In this report, we showed that three inhibitors of arachidonic acid metabolism inhibited the growth of both SCLC and NSCLC cell lines at clinically achievable concentrations. The growth inhibition seemed to be due to different mechanisms of action because the inhibitors produced different effects on cell cycle and were synergistic with one another (although there was less synergy between sulindac sulfide and NDGA). These differences are consistent with the differences in the effects of the agents on arachidonate metabolism (4). These agents were also synergistic with 13-cis-retinoic acid, which is being evaluated as a chemoprevention agent for lung cancer, and with paclitaxel and cisplatin, which are used routinely in lung cancer therapy (25). This suggests the possible clinical use of these agents in combination-chemoprevention strategies. Such combination strategies have not,
heretofore, been studied. The results also suggest that studies of therapeutic combinations should be conducted.

Abnormalities in arachidonic acid metabolism are present in both NSCLC and SCLC (4, 5). The generation of bioactive lipids, from this metabolic pathway modulates physiological and pathological responses involved in both tumor growth and promotion. LOX and COX inhibitors have been shown to inhibit the growth of human lung cancer cell lines in vitro and in animal models (7, 8). LOX inhibitors have been shown to prevent lung carcinogenesis in a xenograft model as well as in A/J mice given the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Ref. (7). Similarly, sulindac, a potent COX inhibitor, has also been shown to inhibit tumor formation in animal models (11–13). The mechanism of growth inhibition of sulindac sulfide has been attributed to the inhibition of COX. One study noted the markedly up-regulated COX-2 expression in about one-third of atypical adenomatous hyperplasia and carcinoma in situ specimens and a significant increase in COX-2 expression in 70% of invasive adenocarcinomas (9). In contrast, small cell carcinomas showed virtually negligible expression, and squamous cell carcinomas showed infrequent and low expression (9). In the present study, sulindac sulfide showed similar growth inhibitory effects on all of the lung cancer cell lines except for the SCLC line H345, which was more sensitive.

In a study involving A549 NSCLC cells, Huang et al. (26) demonstrated increased PGE2 production in response to IL-1β. Tumor-derived PGE2 promotes lymphocyte and macrophage IL-10 induction while simultaneously inhibiting macrophage IL-12 production, thus, altering the cytokine balance in the lung cancer microenvironment. They further demonstrated that specific inhibition of COX-2 abrogated the capacity of IL-1β-stimulated A549 cells to induce IL-10 in lymphocytes and macrophages as well as reversing the tumor-derived PGE2-dependent inhibition of macrophage IL-12 production. An effect on cell cycle regulation may be involved in tumor growth. Aspirin, a COX inhibitor, was shown to induce cell cycle arrest with a decrease in the proportion of cells in G0-G1 and a relative increase in the percentage of cells in S phase and G2-M when given in high concentrations (27). In the present study, however, no effect on the cell cycle was seen with the addition of the COX inhibitor sulindac sulfide. Higher concentrations may, thus, be needed to see this effect.

In a study involving colon cancer cells, Tsuji et al. (28) demonstrated an inhibitory effect of NS-398 (a selective COX-2 inhibitor) on endothelial migration and tube formation, which suggested a possible role of COX in endothelial cell migration and angiogenesis. Thus, it remains unclear whether the inhibition of COX is necessary for growth inhibition.

The sulindac metabolite, exisulind, which is devoid of inhibiting prostaglandin synthesis activity, was the least potent of the NSAIDs but produced a similar apoptotic rate to NDGA and sulindac sulfide. Unlike NDGA or sulindac sulfide, exisulind seemed to cause cell cycle arrest in G0-G1 with a significant proportion of the cell population remaining in G0-G1. This effect was evident by 48 h of drug exposure and lasted up to 120 h. Cell cycle regulation involves multiple factors known as cyclins and CDKs. The phosphorylation of Rb by CDKs results in the release of E2F, which drives the cell into completing the cell cycle. The activity of the CDKs requires cyclin binding, and this activity is suppressed by the inhibitors p16ink4B and p15ink4A. Exisulind may affect the cell cycle by either indirectly or directly inhibiting cyclin D1 or stimulating p16 and thus regulating the effect of Rb on the cell cycle in NSCLC. A similar effect was seen in the SCLC lines that lack Rb. Thus, the mechanism by which exisulind blocks cell cycle in G0-G1 remains to be elucidated. Other possible targets for the effects of exisulind may be the reduction of B-catenins, which are also known to regulate the levels of cyclin D1.

Piazza et al. (14), also studied the mechanism of growth inhibition of both exisulind and sulindac sulfide and showed that it was produced by the induction of apoptosis, independent of p53 status in comparison to 5-fluorouracil, an agent that requires p53 to induce apoptosis. Apoptosis by exisulind has been shown to occur via a COX-independent pathway in multiple tumor cell lines (29). Recently, Thompson et al. (18) reported that exisulind induced apoptosis in cultured tumor cells via the inhibition of cyclic GMP phosphodiesterase. Phosphodiesterases are responsible for the hydrolysis of cyclic AMP and cyclic GMP, which act as intracellular second messengers in a variety of cellular functions.

The cure rate for lung cancer is a low 14%. More than 85% of cases are found in current and former smokers. One-half of the newly diagnosed cases develop in former smokers, which indicates a need for chemopreventive agents. 13-cis-retinoic acid is a vitamin-A derivative that has been shown as an effective chemopreventive agent. Two small studies suggested a benefit of 13-cis-retinoic acid in the prevention of secondary malignancies in high-risk individuals (30, 31). A clinical trial of 13-cis-retinoic acid as a chemopreventive agent for second malignancies in patients with completely resected NSCLC has been completed, and the final results are eagerly awaited. A smaller trial evaluating 13-cis-retinoic acid in combination with α-tocopherol is currently in progress for patients with longstanding tobacco abuse and evidence of sputum dysplasia. The present study showed that the addition of sulindac sulfide, exisulind, or NDGA to 13-cis-retinoic acid produced synergistic growth-inhibitory effects. These data suggest that clinical combination-chemoprevention trials will be warranted.

Their side effects and the development of drug resistance limit the use of conventional cytotoxic chemotherapeutic agents for treating patients with lung cancers. Continuous usage of cisplatin and paclitaxel often results in severe peripheral neuropathy, myelosuppression, and resistance to multiple cytotoxic chemotherapeutic agents. In the present study, the addition of exisulind or sulindac sulfide to cisplatin and paclitaxel resulted in synergistic growth inhibition, especially at high concentrations, in all of the cell lines including SHP77, which overexpresses MDR-1 and is resistant to many chemotherapeutic agents. The addition of sulindac sulfide, exisulind, or NDGA to paclitaxel and cisplatin may increase effectiveness, limit toxicity, and overcome drug resistance. Sulindac sulfide at high concentrations can result in serious gastrointestinal and renal toxicity because of its effect on prostaglandins. Exisulind, being devoid of any antiapoptaglandin activity, would be a logical candidate for combination studies with present chemotherapeutic agents. The combination of the sulfide metabolites with either cisplatin or paclitaxel is presently being evaluated in animal models and should be evaluated also in a clinical setting.

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