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Enhancement of Chemosensitivity by Tyrphostin AG825 in High-p185^{neu} Expressing Non-Small Cell Lung Cancer Cells[1]

Chun-Ming Tsai,[2] Alexander Levitzki, Li-Hwa Wu, Kuo-Ting Chang, Chia-Chien Cheng, Aviv Gazit, and Reury-Perng Perng

Department of Medicine, School of Medicine, National Yang-Ming University [C-M. T., R-P. P.], Taipei, Taiwan 11217, Republic of China; Section of Thoracic Oncology [C-M. T., K-T. C., C-C. C.], Chest Department [R-P. P], and Section of General Surgery, Department of Surgery [L-H. W], Veterans General Hospital-Taipei, Taipei, Taiwan 11217, Republic of China; and Departments of Biological Chemistry [A. L., A. G.] and Organic Chemistry [A. G.], Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel

ABSTRACT

The HER-2/neu gene product, p185^{neu}, is a membrane-bound receptor with tyrosine kinase activity. High levels of p185^{neu} is correlated with intrinsic chemoresistance of non-small cell lung cancer (NSCLC) cell lines. We investigated the effects of tyrphostin AG825, a selective tyrosine kinase inhibitor preferentially inhibiting HER-2/neu kinase, on the chemosensitivities and on the drug-induced cell cycle changes of NSCLC cell lines that expressed different levels of p185^{neu}. Compared to the low-p185^{neu} expressing cell lines, we found that the high-p185^{neu} expressing cell lines were more resistant to doxorubicin, etoposide, and *cis*-diamminedichloroplatinum(II) but more sensitive to AG825. AG825 was able to significantly enhance the chemosensitivities of the high-p185^{neu} expressing cell lines, whereas it had little effect on the chemosensitivities of the low-p185^{neu} expressing cells, with a few exceptions in which minor antagonistic effects were observed. Although high concentrations of AG825 could reduce the drug-induced G_2 arrest that was accompanied by the activation of phosphorylated p34^{cdc2}, we failed to find any remarkably differential effects of AG825 on drug-induced G_2 arrest and the accompanying phosphorylation status of p34^{cdc2} of the high- and the low-p185^{neu} expressing cell lines. In summary, tyrphostin AG825 can enhance chemosensitivity in high- but not in low-p185^{neu} expressing NSCLC cell lines. This differential effect cannot be explained by the alterations of drug-induced cell cycle changes by AG825. Our results provide a rationale to develop p185^{neu}-specific tyrphostin and to test them in combination with anticancer agents *in vivo* and in clinical trials.

INTRODUCTION

The *HER-2/neu* gene encodes a transmembrane glycoprotein (p185^{neu}), which is a member of the EGF[3] receptor subgroup of the protein tyrosine kinase superfamily (1, 2). Amplification and/or overexpression of the *HER-2/neu* gene have been detected in subpopulations of many types of human cancers and have been linked to the presence of extensive disease and poor patient survival (3–7). In lung cancer, overexpression of the *HER-2/neu* gene is encountered in a subgroup of all types of NSCLC but not in small cell lung cancer (5–7). A high level of p185^{neu} has been linked to shortened survival in adenocarcinomas (6, 7). Using a panel of NSCLC cell lines as an experimental model, we have demonstrated that the intrinsic chemoresistance of NSCLC cells correlates well with the expression of p185^{neu} (8, 9). By transfection of *HER-2/neu* cDNA into a human NSCLC cell line expressing a very low level of p185^{neu}, we (10) have demonstrated that increasing the expression of p185^{neu} significantly enhances the chemoresistance to doxorubicin, cisplatin, mitomycin C, and etoposide in the transfected clones. The levels of the increased chemoresistance in the *HER-2/neu* transfectants correlates well with the level of p185^{neu} as well as the level of p185 phosphotyrosine. Since increased tyrosine kinase activity of p185^{neu} has been implicated in the oncogenic signal transduction (11, 12), it is possible that multiple drug resistance of NSCLC cells is also conferred by the increased tyrosine phosphorylation activity of p185^{neu}. In addition, we (13) demonstrated previously that caffeine enhances the chemosensitivities of high-p185^{neu} expressing NSCLC cell lines to anticancer agents and that the magnitude of enhancement correlates with the level of p185^{neu}. Caffeine has been shown to override the drug-induced cell cycle arrest at checkpoints, in particular G_2-M (14, 15). It is generally accepted that G_2 arrest results from the inactivation (phosphorylation) of p34^{cdc2} kinase, allowing the cells to repair DNA lesions prior to mitosis, thus preventing lethal chromosome aberrations (16, 17). Caffeine exposure during G_2 arrest inhibits the activity of tyrosine kinase that phosphorylates p34^{cdc2}, thus inducing premature mitosis, leading to enhanced cell killing (14, 18). Our findings (13), therefore, raise the possibility that high levels of p185^{neu} may enhance chemoresistance by potentiating DNA repair either by enhanced phosphorylation of p34^{cdc2} and/or by enhanced enzyme activity (or activities) for DNA repair.

To further investigate whether the *HER-2/neu* gene expression is correlated to the chemoresistance of NSCLC cells and examine whether p185^{neu} is involved in the regulation of p34^{cdc2} phosphorylation during drug exposure, we have applied a novel approach to inhibit specifically the tyrosine kinase activity of p185^{neu}. We intended to monitor the effects of p185^{neu} blockade on the chemosensitivity, drug-induced cell cycle changes, and alterations of p34^{cdc2} phosphorylation in the high- and the low-p185^{neu} expressing NSCLC cell lines. To achieve that goal, we have used a protein tyrosine kinase inhibitor, tyrphostin AG825, which has been shown to selectively block p185^{neu} kinase in cell-free systems (19, 20). In this study, we investigate the differential effects of tyrphostin AG825 on the chemosensitivities for three commonly used anticancer drugs, doxorubicin, *cis*-diamminedichloroplatinum(II) (cisplatin), and etoposide, of six NSCLC cell lines that expressed a wide range of p185^{neu}. One high- and one low-p185^{neu} expressing cell line were also selected to further monitor the effect of AG825 on the etoposide-induced cell cycle changes and the accompanying alterations of the phosphorylation status of p34^{cdc2}.

MATERIALS AND METHODS

Cell Lines. Six NSCLC cell lines, five adenocarcinomas NCI-H23, NCI-H1155, NCI-H1355, NCI-H1435, and NCI-H1437, and one large cell carcinoma, NCI-H1299, were studied. All lines were established and characterized from previously untreated patients (9, 21) and expressed a minimal level of EGF receptor (data not shown). All these cell lines contain point mutations in the *p53* gene except the NCI-H1299 cell line, which contains an intragenic deletion in the gene (22). All the lines had been maintained in RPMI 1640

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: EGF, epidermal growth factor; NSCLC, non-small cell lung cancer; cisplatin, *cis*-diamminedichloroplatinum(II); CI, combination index.

supplemented with 5% heat-inactivated fetal bovine serum for at least 6 months before being tested.

Quantitative Measurement of the p185^{neu} Protein. The nearly confluent cells were harvested by scraping, followed by centrifugation to form a cell pellet. Cells were incubated in lysis buffer [10 mM Tris-HCl (pH 7.6), 1.5 mM EDTA, 10% glycerol, and 0.1% sodium azide] and Dounce homogenized. After homogenization, the proteins were extracted in detergent. After centrifugation, supernatants were collected, and protein concentrations were determined. The immunoassay for detection and quantitation of p185^{neu} was a sandwich assay utilizing monoclonal antibodies NB-3 (coated onto microtiter wells) and TA-1 (biotin labeled) (human neu assay kits; Oncogene Science, Inc., Uniondale, NY; Ref. 23). The biotin-labeled TA-1 was detected using a streptavidin-horseradish peroxidase conjugate in PBS (pH 7.4), 1% BSA, and 0.1% chloroacetamide. After the addition of *O*-phenylenediamine substrate, the color change was measured at 490 nm using a microplate reader. A standard curve was generated by using standard solutions. The concentration of p185^{neu} was determined by interpolation of the sample absorbance from the standard curve. Results were expressed as human neu units/μg of protein. The experiments were performed in triplicate. Each experiment was performed in duplicate wells.

Anticancer Agents and Tyrphostin AG825. Three commonly used DNA-damaging anticancer agents, doxorubicin, etoposide, and cisplatin, as well as a tyrphostin of the benzylidene malononitrile family, AG825, were tested. The three anticancer drugs were dissolved in PBS to 1 mM, and AG825 was dissolved in DMSO to 40 mM. All the agents then were subsequently diluted with the culture medium before addition to the cells. The culture medium containing the equivalent amounts of DMSO was also included to serve as vehicle controls and had no obvious effects on cell growth of the six cell lines tested. The absorbance determined by the tetrazolium colorimetric assay were 98.8 ± 1.0%, 97.2 ± 1.9% and 92.6 ± 3.3% of the control wells at DMSO 0.1%, 0.2%, and 0.3% exposure for 96 h, respectively.

Experimental Design of the Drug Testing and Identification of the Combination Effects. *In vitro* drug combination testing was designed and performed using the tetrazolium dye colorimetric assay with application of classical isobole methods (24) as described previously (25, 26). The cytotoxic effects of each single agent, doxorubicin, etoposide, cisplatin, and AG825, as well as the combination effects of AG825 plus doxorubicin, AG825 plus etoposide, and AG825 plus cisplatin, were tested. AG825 was tested over a 2-log range (7 concentrations, in a 1/3 log increment, within 1–100 μM), and the anticancer drugs were tested over a 3-log range (7 concentrations, in a 1/2 log increment) to cover the entire dose-effect curves. Anticancer drug was added immediately after the addition of AG825, and the duration of the exposure was 96 h. For single agents tested, the results reported as IC_{50}s were the means of three independently performed assays. The CI at the 50% effect level was used to represent the combination effects of AG825 plus drug. CI = (dose of AG825/IC_{50} of AG825)+(dose of drug/IC_{50} of drug). The mean values of the survival fractions of three independently performed experiments (each experiment was done in four replicate wells at the same time) were used to generate a set of CI values (data points) for a particular cell line and AG825-drug combination because of the fact that there are multiple combinations within the assay range that achieve the same isoeffect. Each set of CIs was used to construct the isobole. The mean CI for this set was reported as the summary measure. Mean CIs >1.05 or <0.95 were interpreted as being suggestive of antagonism or synergism, respectively. Sign tests were performed on each set of CIs to formally evaluate whether antagonism or synergism was evident for a particular cell line and AG825-drug combination. CI_{TD}, CI_{TV}, and CI_{TC} were designated for the mean CIs for AG825+doxorubicin, AG825+etoposide, and AG825+cisplatin, respectively. The Spearman rank correlation was used to analyze the correlations between the levels of p185^{neu} and the CIs of the tyrphostin-containing regimens. Significance was assumed if $P < 0.05$.

Preparation of Asynchronous and Synchronized Cells and Cell Cycle Phase Fractions Study. The low-p185^{neu} expressing NCI-H1299 cell line and the high-p185^{neu} expressing NCI-H1355 cell line were selected for cell cycle and biochemical studies. For experiments on asynchronous cells, 24 h after cells being plated at the appropriate seeding density, they were exposed to etoposide over a 2-log range (5 concentrations in a 1/2 log increment, 0.1–10 μM) for 24 h, and the optimal concentration that resulted in maximal cell accumulation in G_2 was determined. AG825 at a concentration of 0, 10, 20, 40, 60, or 80 μM and the culture medium containing the equivalent concentrations of DMSO (0.025–0.20%), serving as vehicle controls in the absence or concurrent presence of etoposide at the optimal concentration, were tested. After 24-h incubation, cells were collected for analyses of the cell cycle phase fraction, p185^{neu}, and the phosphorylation status of p34^{cdc2} kinase. For experiments on synchronized cells, cells were synchronized at G_1-S by serum starvation (for a period of cell doubling time × 2: H1299, 32 h; H1355, 72 h) followed by aphidicolin (2 μg/ml) treatment for 24 h. Immediately after being released from the G_1-S boundary, cells were treated with no drugs or treated with etoposide at the optimal concentration (3 μM; see "Results") for 1/18 doubling time (*i.e.*, H1299 for 50 min and H1355 for 2 h). After removal of etoposide by washing twice with PBS, AG825 (40 μM) was added in the tested dishes for continuous exposure. Samples of etoposide-untreated and -treated cells without and with AG825 exposure were collected before treatment and for various time periods, starting from the beginning of etoposide exposure. Asynchronous and synchronized cells were stained with propidium iodide (50 μg/ml) plus 0.1% Triton X-100 and RNase (180 units/ml), and DNA fluorescence distributions and the proportion of cells in G_1, S, and G_2-M of the cell cycle were analyzed by flow cytometry as described previously by Chiu *et al.* (27).

Immunoblotting. Immunoblot analyses were performed to detect the expression of p185^{neu} and the effects of AG825 on the expression of p185^{neu} and p34^{cdc2} phosphorylation in the absence and concurrent presence of etoposide. Cell extracts were prepared, and 100 μg of protein from each sample were electrophoresed and Western blotted against the appropriate antibody. The primary monoclonal antibodies used for immunoblot analyses were Ab-3 (1:250) against p185^{neu} protein (Oncogene Science, Inc.; Ref. 28) and anti-cdc2 HS (1:200) recognizing human cdc2 homologue (Upstate Biotechnology, Inc., Lake Placid, NY; Ref. 29).

RESULTS

Effects of AG825 on the Expression of p185^{neu}. The levels of p185^{neu} of the six cell lines were determined by immunoblottings as well as antibody-sandwich ELISAs as shown in Fig. 1. The cell lines H1155, H23, and H1299 were classified as the low-p185^{neu} expressors, and the cell lines H1437, H1435, and H1355 were classified as the high-p185^{neu} expressors. The levels of p185^{neu} of the high-p185^{neu} expressing H1355 were reduced by AG825 exposure at the concentrations ≥60 μM for 24 h. (Fig. 2*A*). The addition of the optimal etoposide concentration at 3 μM (see below) itself had little

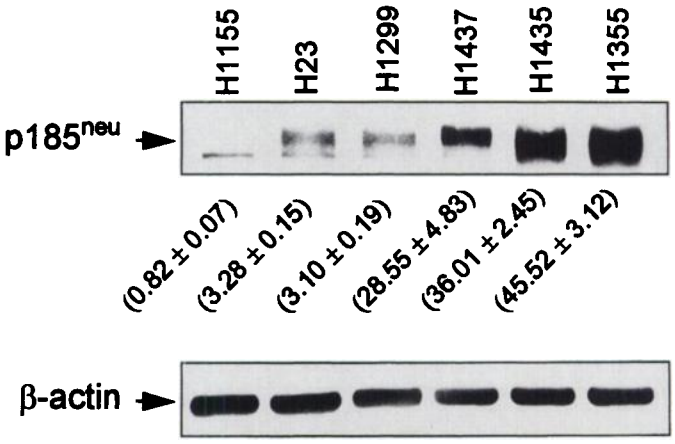

Fig. 1. Immunoblot of p185^{neu} of the six cell lines tested. The nearly confluent cells in logarithmic growth phase were harvested. Cell lysates (100 μg/lane) were immunoblotted with anti-p185^{neu} (Ab-3) antibody. The different mobilities of p185^{neu} in the different cell lines is likely due to protein phosphorylation, which has been shown to produce heterogeneous protein bands in similar immunoblots (41). Numbers in parentheses are the levels of p185^{neu} determined by ELISAs using NB-3 (coated onto microtiter wells) and TA-1 (biotin-labeled) monoclonal antibodies. The results are the mean (± SE) values of three replicate experiments (each experiment was performed in duplicate wells), which are expressed as a human neu unit/μg protein.

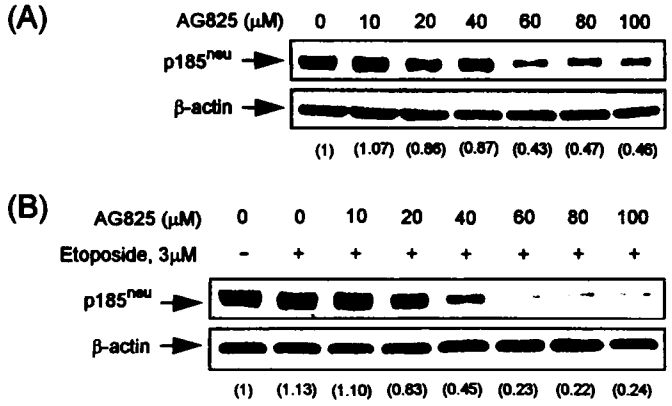

Fig. 2. *A*, effects of AG825 on the expression of p185^{neu} of the high-p185^{neu} expressing H1355 cell line. After being plated at the appropriate seeding density for 24 h, cells were incubated without (*Lane 1*) or with AG825 (10–100 μM; *Lanes 2–7*) for 24 h; then cell lysates (100 μg/lane) were immunoblotted with anti-p185^{neu} antibody (Ab-3). AG825 at the concentration ≥60 μM reduced the levels of p185^{neu}. *B*, effects of AG825 treatment for 24 h on the expression of p185^{neu} in the absence or concurrent presence of 3 μM etoposide. The addition of etoposide had little effect on the expression of p185^{neu} (*Lane 2*). However, in the presence of etoposide, AG825 suppressed the expression of p185^{neu} in a dose-dependent manner in the range of 10–60 μM (*Lanes 3–6*). Numbers in parentheses are the levels of p185^{neu} quantitated by soft laser densitometry (Model SLR-2D/1D; Biomed, Fullerton, CA), which are expressed in arbitrary units relative to the value of the control cells, to which a value of 1 has been assigned. p185^{neu} levels were also normalized by densitometry of the β-actin signal.

effect on the expression of p185^{neu} (Fig. 2*B*, *Lane 2*) but appear to facilitate the inhibitory effect of AG825 on the expression of p185^{neu}. In the presence of etoposide, AG825 reduced the expression of p185^{neu} in a dose-dependent manner in the range of 10 to 60 μM (Fig. 2*B*).

Cytotoxic Effects of Doxorubicin, Cisplatin, Etoposide, and AG825. Anticancer agents doxorubicin, etoposide, and cisplatin were tested for their cytotoxic effects on the designated NSCLC cell lines. Their dose-effect curves are shown in Fig. 3. The three high-p185^{neu} expressing cell lines (Fig. 3, *A-C, solid lines*) were apparently more resistant to the chemotherapeutic agents than the three low-p185^{neu} expressing cell lines (Fig. 3, *A-C, dotted lines*). When tyrphostin AG825 was tested for its cytotoxic effect, the IC_{50}s were very similar (34.1 to 44.3 μM) for the cell lines tested. However, AG825 alone at low concentrations (2.15–21.5 μM) had a greater inhibitory effect on the high- than on the low-p185^{neu} expressing cell lines (Fig. 3*D*).

Effects of Tyrphostin AG825 on the Chemosensitivities of High- and Low-p185^{neu} Expressing Cell Lines. Mean CIs was signified to represent the interaction between AG825 and the anticancer agents. The mean CI at the 50% effect level of the three AG825-containing regimens against the six cell lines are depicted in Fig. 4. AG825 significantly enhanced the chemosensitivities for doxorubicin and etoposide of the three high-p185^{neu} expressing cell lines (all Ps < 0.05) and significantly enhanced the chemosensitivity for cisplatin ($P = 0.012$) of the high-p185^{neu} expressing H1355 cell line. But AG825 had little effect on the cytotoxicity of cisplatin against the other two high-p185^{neu} expressing cell lines. In contrast, AG825 had no statistically significant effects on the chemosensitivities of the three low-p185^{neu} expressing cell lines, with the exceptions that AG825 significantly attenuated the cytotoxicities of doxorubicin and cisplatin against H1299 (CI_{TD}, 1.103 ± 0.036; CI_{TC}, 1.231 ± 0.086; Ps < 0.005) and the cytotoxicity of etoposide against H23 (CI_{TV}, 1.093 ± 0.023; $P < 0.05$). Our results clearly demonstrate that AG825 enhances the chemosensitivities of the high-p185^{neu} expressing cell lines, whereas AG825 had only a small effect on the chemosensitivities of the low-p185^{neu} expressing cell lines, with a few exceptions that minor antagonistic effects were observed (Fig. 4). There was a statistically significant correlation between the level of p185^{neu} and CI_{TD} ($r = -0.943$, $P = 0.035$). The correlations between the level of p185^{neu} and CI_{TV} and between the level of p185^{neu} and CI_{TC} were marginally significant ($r = -0.771$, $P = 0.085$ and $r = -0.829$, $P = 0.064$, respectively). These findings suggest that the

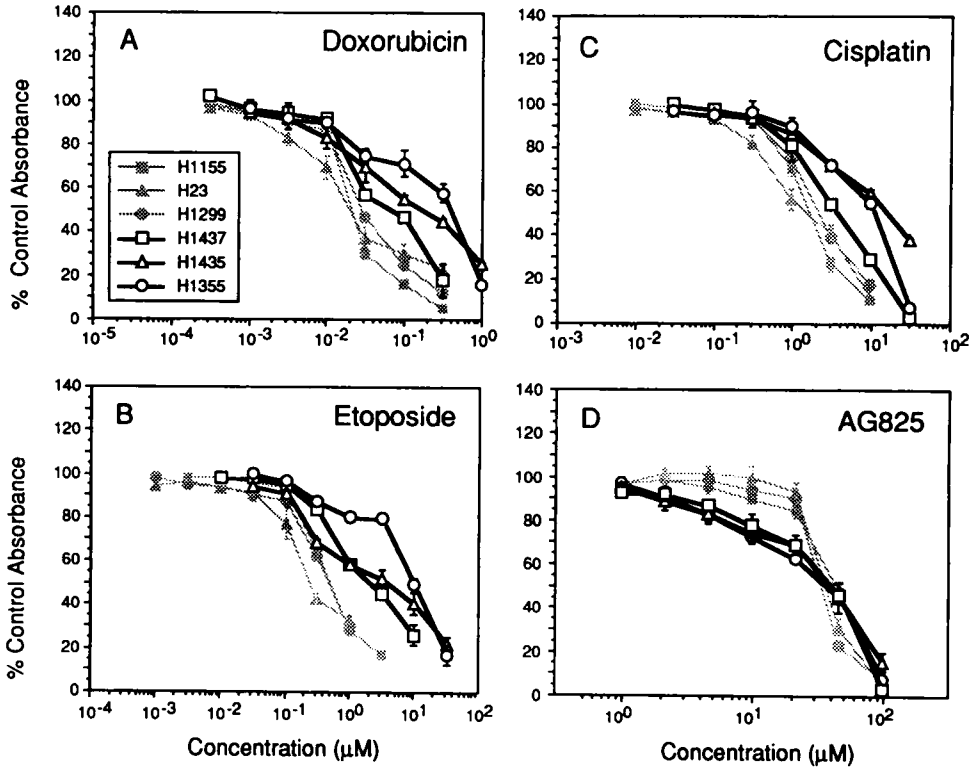

Fig. 3. Dose-response curves of three single anticancer agents, doxorubicin (*A*), etoposide (*B*), and cisplatin (*C*), and the tyrphostin AG825 (*D*) of the low-p185^{neu} expressing cell lines H1155, H23, and H1299 (*dotted lines*) and the high-p185^{neu} expressing cell lines H1437, H1435, and H1355 (*solid lines*). *In vitro* chemosensitivities were determined by the tetrazolium assays, with drug exposure for 96 h. The percentage of control absorbance was considered to be the surviving fraction of cells. The mean surviving fractions of three separate experiments are demonstrated; *bars*, SE.

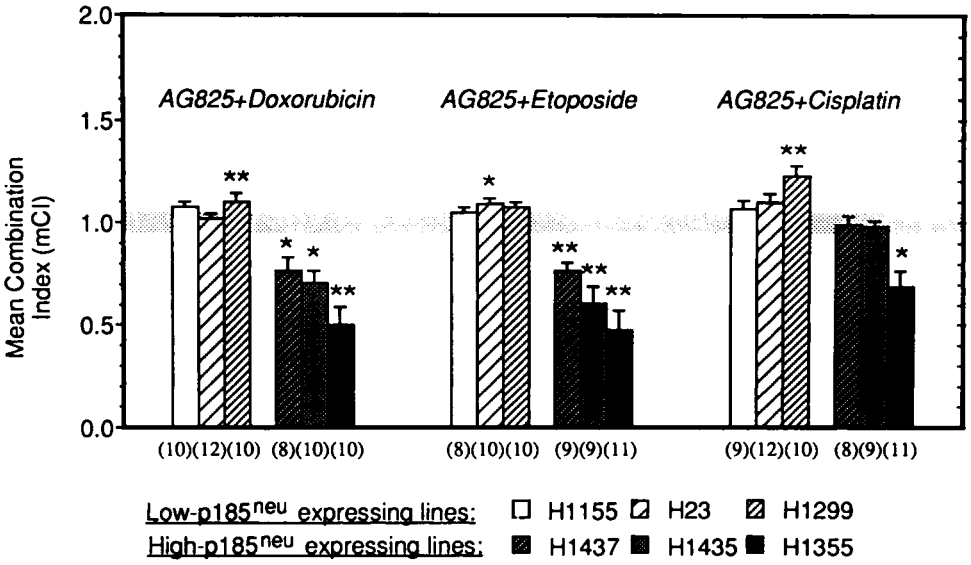

Fig. 4. The combination effects of AG825 plus doxorubicin, AG825 plus etoposide, and AG825 plus cisplatin, which were expressed as the mean values of CI at the 50% effect level (see "Materials and Methods"). The mean values of the survival fractions of three independently performed tests were used to generate a set of CIs (data points) and to construct the isobole at the 50% effect level for a particular cell line and AG825-drug combination. The mean CI (± SE) for this set was reported as the summary measure. The numbers in parentheses is the number of data points. *Shaded horizontal band*, the area of additivity based on null interval of 0.95–1.05. CI >1.05 or <0.95 were interpreted as being suggestive of antagonism or synergism, respectively. Sign tests were performed to formally evaluate whether antagonism or synergism was evident for a particular cell line and drug combination. *, $P < 0.05$; **, $P < 0.005$. The effects of AG825 on the cytotoxicities of the anticancer agents noticeably paralleled the levels of $p185^{neu}$ expressed by the cell lines (Fig. 1).

more the cells express $p185^{neu}$, the greater the chemosensitivities might be enhanced by AG825 (Figs. 1 and 4).

Effects of AG825 on the Cell Cycle Progression of Asynchronous Cells in the Absence or Presence of Etoposide. Since the cytotoxic effect of etoposide was enhanced by AG825 to the greatest degree in our panel of high-$p185^{neu}$ expressing cell lines as compared to the other two anticancer agents (Fig. 4), we selected etoposide for further cell cycle analyses and biochemical studies of the effects of AG825 on the H1299 and H1355 cell lines. H1299 possesses a homozygous deletion of the *p53* gene (27) and expresses low levels of $p185^{neu}$ (Fig. 1). H1355, which contains an endogenous mutant *p53* and expresses $p185^{neu}$ at a steady high level (Fig. 1), yielded the most significant responses to the AG825-containing regimens (Fig. 4). Continuous exposure to 3 μM etoposide for 24 h resulted in a maximal G_2 arrest to these two cell lines (Fig. 5, *A* and *B*). Etoposide at the concentration of 3 μM was, therefore, selected as the optimal concentration for the succeeding cell cycle and biochemical experiments. Although AG825 itself had little effect on cell cycle progression of the H1299 and the H1355 cell lines (Fig. 5, *C* and *D*), AG825 reduced the etoposide-induced G_2 arrest of both H1299 and H1355 in a dose-dependent fashion. Accompanying the reduction of G_2 fraction, both cell lines demonstrated a greater increase of the G_1 fraction than the S fraction at the high concentrations (≥60 μM) of AG825 (Fig. 5, *E* and *F*). However, at the low concentrations (≤40 μM) of AG825, the reduction of G_2 fraction was accompanied by a predominant increase of the S fraction in H1299 (Fig. 5*E*) but a predominant increase of the G_1 fraction in H1355 (Fig. 5*F*). These results suggested the possibility that AG825 at low concentrations delayed S-phase progression of the etoposide-treated H1299 cells and prevented them from reaching G_2. In contrast, AG825 throughout the tested concentrations might circumvent etoposide-induced G_2 arrest in a dose-dependent fashion in H1355. The differential effects of low concentrations of AG825 on the etoposide-induced cell cycle changes of H1355 and H1299 cells in a 24-h time period might, however, simply result from the inherently different proliferating activities (doubling times) of these two cell lines. Experiments, therefore, were carried out with synchronized cells to examine this possibility (see below).

Effects of AG825 on Phosphorylation of $p34^{cdc2}$ in the Presence of Etoposide. As noted, etoposide-induced G_2 arrest was associated with the accumulation of phosphorylated $p34^{cdc2}$ kinase that was independent of the level of $p185^{neu}$ expression of the cell lines (Fig. 6, *A* and *B*, *Lane 2*). With the addition of AG825, a marked change in the etoposide-treated H1355 and H1299 cells was a shift in $p34^{cdc2}$ from the slower-migrating (phosphorylated) isoform to the faster-migrating (dephosphorylated) isoform of $p34^{cdc2}$ at the high concentrations of AG825 (Fig. 6, *A* and *B*, *Lanes 6–8*). In contrast, the etoposide-induced G_2 arrest and hyperphosphorylation of $p34^{cdc2}$ of both cell lines remained little affected at the low doses of AG825 (Fig. 6, *A* and *B*, *Lanes 3–5*). These findings were compatible with the findings of the cell cycle study (Fig. 5, *E* and *F*), indicating that AG825 at higher concentrations could reduce etoposide-induced G_2 arrest through dephosphorylation (activation) of phosphorylated $p34^{cdc2}$. However, AG825 throughout the tested concentrations may not have remarkably differential effects on the phosphorylation regulation of the etoposide-induced G_2 arrest of the high- and the low-$p185^{neu}$ expressing cell lines.

Effects of AG825 on the cell cycle progression of synchronized cells with or without etoposide treatment. Cells were synchronized as described in "Materials and Methods." In both H1355 and H1299, AG825 (40 μM) alone delayed S-phase progression to a minimal degree. In the etoposide-treated cells, however, the addition of AG825 further enhanced the etoposide-induced S-phase delay (Fig. 7, *A* and *B*). Following the slow progression of S phase, the etoposide-treated cells progressed into and arrested at G_2-M in the absence or presence of AG825 (Fig. 7, *C* and *D*). In H1299 and H1355, the time-S-phase fraction curve as well as the time-G_2-M phase fraction curve of the etoposide+AG825-treated cells paralleled those of the etoposide-treated cells, with a marked progress delay of the etoposide+AG825-treated cells. In the presence of AG825, no reduction of the etoposide-induced G_2 arrest could be detected in these two cell lines. Our findings indicated that AG825 (40 μM) had no effect on the etoposide-induced G_2 arrest, irrespective of the level of $p185^{neu}$ expressed by the cells.

DISCUSSION

The chemosensitivity of neoplastic cells to DNA-damaging agents may depend on the fidelity of cell cycle checkpoints (30). DNA damaged cells delay cell cycle progress at checkpoints to allow DNA repair (31). Evidence revealed that p53 is involved in the control of the G_1-S checkpoint. Following DNA damage, p53 acts to suppress growth by inducing cell cycle arrest at G_1 while the cell attempts

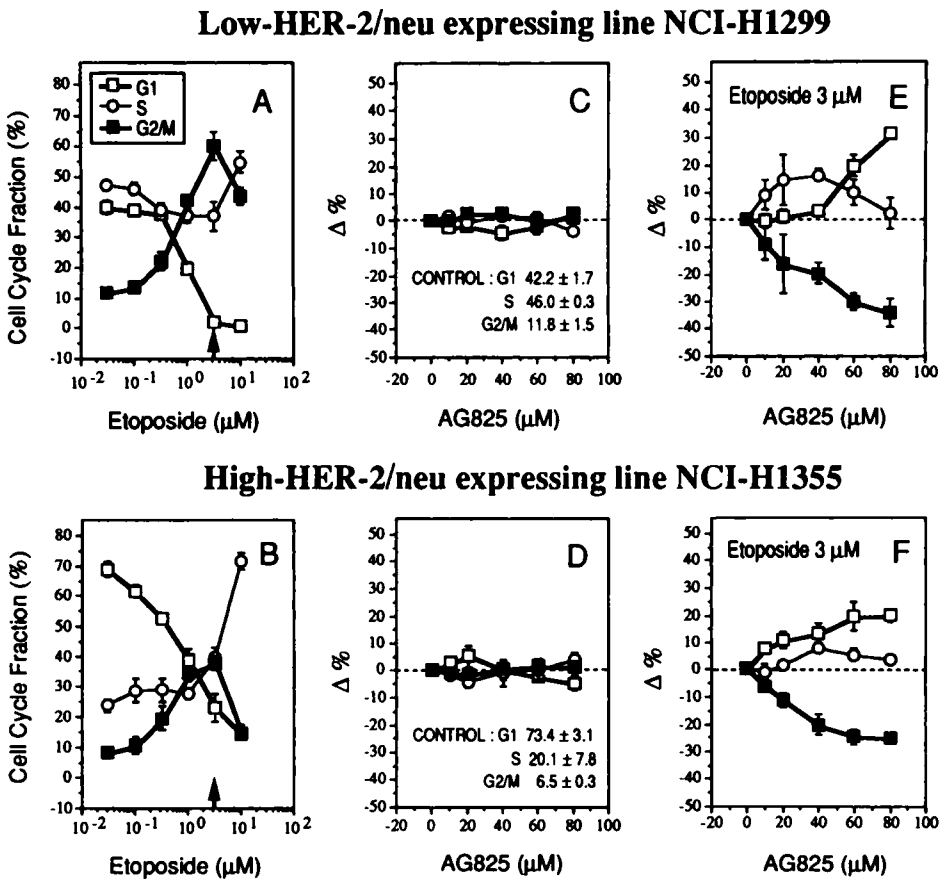

Fig. 5. Effects of 24 h treatment of etoposide alone (0.1–10 μM; *A* and *B*) and AG825 (10–80 μM) in the absence (*C* and *D*) or concurrent presence (*E* and *F*) of etoposide (3 μM) on the cell cycle fractions of the asynchronous H1299 (*upper panels*) and H1355 (*lower panels*) cell lines (see "Materials and Methods"). Etoposide at 3 μM was the optimal concentration that resulted in maximal cell accumulation in G_2-M, and no etoposide-induced G_1 arrest could be detected in these two lines (*A* and *B*). The effects of AG825 on the cell cycle fractions were calculated by using the cells with no tested agent exposure as control (*C* and *D*). The combination effects of AG825 plus etoposide *versus* etoposide were calculated by using the etoposide-treated cells as control (*E* and *F*). Results were the mean values of three separate experiments; *bars*, SE.

DNA repair or to promote apoptosis if optimal repair is impossible (32, 33). In lung cancer, introduction of the wild-type *p53* gene into a human p53-null NSCLC cell line was able to enhance chemosensitivity (34). Cell cycle arrest in G_2 for DNA repair is also a universal response to DNA damage (16, 17). Nevertheless, a much longer G_2 phase in the presence of severe DNA damage may lead to cell death. Cells with normal *p53* genes usually respond to DNA damage by a delay in both G_1 and G_2 phases, whereas tumor cells with deficient *p53* arrest only at G_2 (32, 35, 36). Caffeine has been shown to override cell cycle checkpoints, particularly the G_2-M checkpoint (14, 15). We have reported previously that caffeine enhances the chemosensitivities of NSCLC cells that overexpress $p185^{neu}$ to a greater degree as compared to low-$p185^{neu}$ expressing cells (13). Mutation of the *p53* gene was a common event (14 of 16) in the cell lines selected for the study. This finding led us to propose that high-$p185^{neu}$ expressing, chemoresistant NSCLC cells might utilize the repair machinery at the G_2-M checkpoint more effectively.

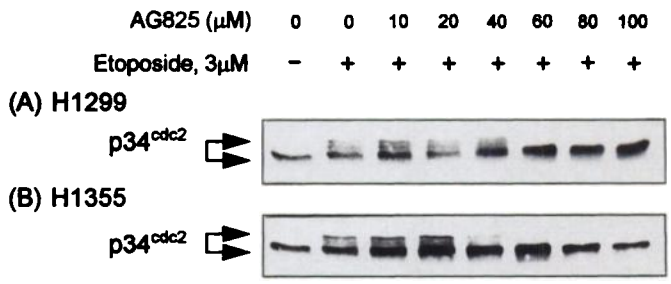

Fig. 6. Effects of 24-h treatment of AG825 on cell cycle-regulated protein $p34^{cdc2}$ in etoposide-induced G_2 arrest cells. After being plated for 24 h, asynchronous H1299 (*A*) and H1355 (*B*) cells were treated without (−) or with etoposide 3 μM (+). AG825 at a concentration of 0, 10, 20, 40, 60, 80, or 100 μM was added immediately before the addition of etoposide, and the incubation concurrently continued for 24 h. Then cell lysates (100 μg/lane) were immunoblotted with anti-$p34^{cdc2}$ monoclonal antibody.

In the present study, we have selected the cell lines with defective *p53* and examined the modulation of the action of DNA-damaging agents by the selective $p185^{neu}$ inhibitor, tyrphostin AG825 (19, 20). Our results show that treatment with AG825 reduces the levels of $p185^{neu}$. In the presence of etoposide, the negative regulation of AG825 was augmented and behaved in a dose-dependent fashion in the high-$p185^{neu}$ expressing H1355 cell line. By testing the effects of AG825 against six NSCLC cell lines that expressed minimal EGF receptor protein (data not shown), we show that AG825 alone or in combination with doxorubicin, etoposide, or cisplatin has enhanced cytotoxic effects against high-$p185^{neu}$ expressing cell lines as compared to low-expressing lines (Fig. 3). The magnitude of AG825-induced enhancement parallels the level of $p185^{neu}$ expression. AG825 had little effect on the cytotoxicities of DNA-damaging agents on the low-$p185^{neu}$ expressors, with a few exceptions in which minor antagonistic effects were observed (Fig. 4). We also found that high concentrations of AG825 were able to reduce the etoposide-induced G_2 arrest, which was accompanied by the activation of $p34^{cdc2}$; however, this effect was not $p185^{neu}$ specific. On the other hand, AG825 at low concentrations markedly retarded the etoposide-induced S-phase delay, whereas it had no definite effect on the etoposide-induced G_2 arrest in both high- and low-$p185^{neu}$ expressing cell lines. It seems, therefore, that $p185^{neu}$ is not involved in the phosphorylation regulation of $p34^{cdc2}$ kinase during drug exposure.

The finding that AG825 enhances chemosensitivity of high-$p185^{neu}$ expressing cells further supports the view that $p185^{neu}$ plays a role in determining the chemoresistance of NSCLC cells. Although the mechanism by which $p185^{neu}$ confers multiple chemoresistance remains unclear at this time, several lines of evidence have emerged to

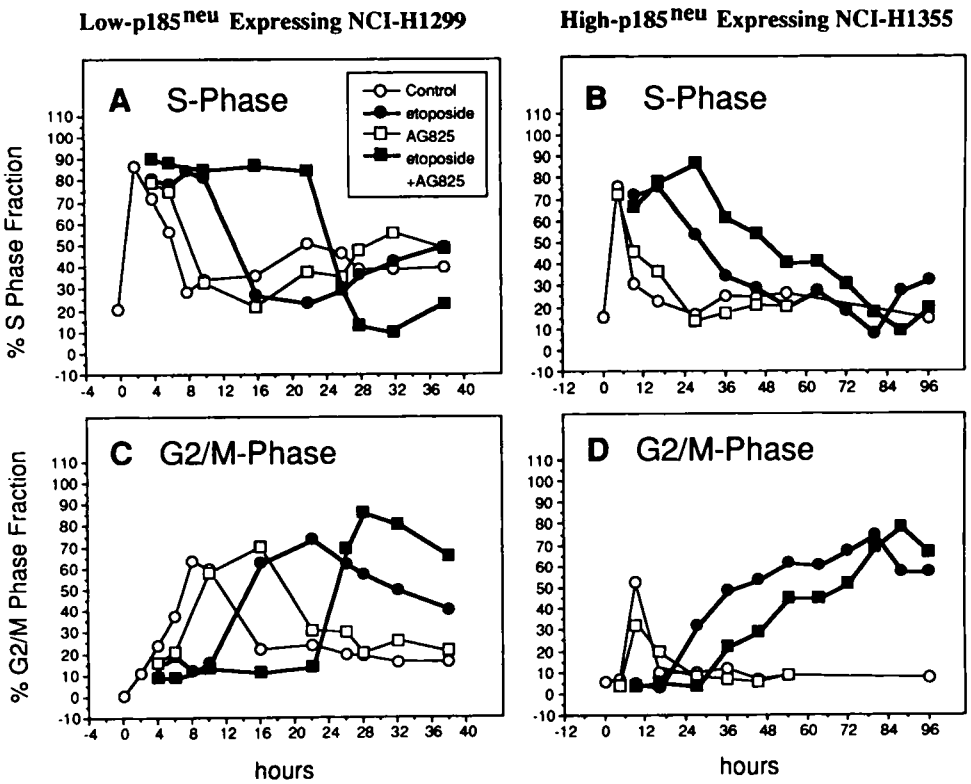

Fig. 7. Flow cytometric analysis of the effects of etoposide (3 μM), AG825 (40 μM), and etoposide + AG825 on S phase (*A* and *B*) and G_2-M phase (*C* and *D*) distribution. H1299 (*left panels*) and H1355 (*right panels*) cells were synchronized at G_1-S. Immediately after being released from the G_1-S boundary, cells were treated with no drug or 3 μM etoposide for 1/18 of cell doubling time (H1299 for 50 min and H1355 for 2 h). After removal of etoposide, AG825 was added in the tested dishes and incubated continuously. Samples of etoposide-untreated and etoposide-treated cells without and with AG825 exposure were collected at various time interval for analysis. Data points were the mean values of two separate experiments.

suggest that high levels of p185^{neu} promote DNA repair (13, 37–39). Two recent reports (38, 39) have shown that p185^{neu}-specific antibodies may enhance the cytotoxicity of cisplatin against the high-p185^{neu} expressing human breast and ovarian cancer cells by interfering with DNA repair. We have demonstrated that caffeine (13) and gemcitabine (37) enhance the chemosensitivities to a greater degree in high- than in low-p185^{neu}-expressing NSCLC cells. Caffeine is known to enhance chemosensitivity of cancer cells by shortening the time for DNA repair (14, 15, 18). Gemcitabine is a novel pyrimidine analogue that has been shown to be able to inhibit DNA synthesis and repair (40). It seems that high-p185^{neu} expressing cancer cells possess a more effective DNA repair system and that this survival advantage can be overcome by caffeine, gemcitabine, and p185^{neu}-specific antibodies, as well as p185^{neu}-specific tyrphostins. The possible role of p185^{neu} in modulating DNA repair is in accordance with the finding of its localization in the nucleus (41).

Drug resistance is one of the major obstacles to successful chemotherapy and is a major challenge for cancer researchers and therapists. At the time of diagnosis, NSCLC tumors usually are more refractory to chemotherapy than small cell lung cancer tumors. Aberration of the *HER-2/neu* gene has been detected in 30% or more of NSCLC (5–7). Because activation of the *HER-2/neu* gene may contribute to metastatic (42) and chemoresistant (8–10) malignant phenotypes and normal critical tissues show little or undetectable expression of the gene (43), the *HER-2/neu* gene and its protein product are attractive targets for therapeutic approach. Inhibition of the activity of p185^{neu} by p185^{neu}-specific tyrphostins, in combination with DNA-damaging agents, could be of potential clinical utility for the treatment of tumors that express high levels of p185^{neu}. Our findings provide a rationale to test p185^{neu}-specific, tyrphostin-containing regimens *in vivo* and in clinical trials. The p185^{neu}-specific tyrphostins can also be utilized to explore the mechanism that is responsible for the enhancement of chemoresistance in high-p185^{neu} expressing tumor cells.

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REFERENCES

1. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science (Washington DC), *230:* 1132–1139, 1985.
2. Parsons, J. T., and Parsons, S. J. Protein-tyrosine kinases, oncogenes, and cancer. *In:* V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Important Advances in Oncology, pp. 3–18. Philadelphia: J. B. Lippincott Co., 1993,
3. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. Science (Washington DC), *244:* 707–712, 1989.
4. Kameda, T., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Ito, M., Ito, H., and Tahara, E. Expression of ERBB2 in human gastric carcinomas: relationship between p185ERBB2 expression and the gene amplification. Cancer Res., *50:* 8002–8009, 1990.
5. Schneider, P. M., Hung, M. C., Chiocca, S. M., Manning, J., Zhao, X., Fang, K., and Roth, J. A. Differential expression of the *c-erbB-2* gene in human small cell and non-small cell lung cancer. Cancer Res., *49:* 4968–4971, 1989.
6. Kern, J. A., Schwartz, D. A., Nordberg, J. E., Weiner, D. B., Greene, M. I., Torney, L., and Robinson, R. A. p185^{neu} expression in human lung adenocarcinomas predicts shortened survival. Cancer Res., *50:* 5184–5191, 1990.
7. Tateishi, M., Ishida, T., Mitsudomi, T., Kaneko, S., and Sugimachi, K. Prognostic value of c-erbB-2 protein expression in human lung adenocarcinoma and squamous cell carcinoma. Eur. J. Cancer, *27:* 1372–1375, 1991.
8. Tsai, C. M., Chang, K. T., Perng, R. P., Mitsudomi, T., Chen, M. H., Kadoyama, C., and Gazdar, A. F. Correlation of intrinsic chemoresistance of non-small cell lung cancer cell lines with *HER-2/neu* gene expression but not with *ras* gene mutations. J. Natl. Cancer Inst., *85:* 897–901, 1993.
9. Tsai, C. M., Chang, K. T., Wu, L. H., Chen, J. Y., Gazdar, A. F., Mitsudomi, T., Chen, M. H., and Perng, R. P. Correlations between intrinsic chemoresistance and *HER-2/neu* gene expression: *p53* gene mutations and cell proliferation characteristics in non-small cell lung cancer cell lines. Cancer Res., *56:* 206–209, 1996.
10. Tsai, C. M., Yu, D., Chang, K. T., Wu, L. H., Perng, R. P., Ibrahim, N. K., and Hung, M. C. Enhanced chemoresistance by elevation of the levels of p185^{neu} in the HER-2/neu transfected human lung cancer cells. J. Natl. Cancer Inst., *87:* 682–684, 1995.

11. Padiella, A., Lehvaslaiho, H., Magni, M., Alitalo, K., and Meldolesi, J. Activation of an EGFR/neu chimeric receptor: early intracellular signals and cell proliferation responses. Oncogene, *4:* 1299–1305, 1989.
12. Peles, E., Ben-Levy, R., Or, E., Ullrich, A., and Yarden, Y. Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase Cγ. EMBO J., *10:* 2077–2086, 1991.
13. Tsai, C. M., Perng, R. P., Chen, M. M., Jan, Y. H., Hung, M. C., Ku, T. Y., and Chang, K. T. Greater enhancement of chemosensitivity by caffeine in high $p185^{neu}$ expressing human non-small cell lung cancer cell lines. J. Natl. Cancer Inst., *86:* 1018–1020, 1994.
14. Schlegel, R., and Pardee, A. Caffeine induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science (Washington DC), *232:* 1264–1266, 1986.
15. Fingert, H. J., Chang, J. D., and Pardee, A. B. Cytotoxic, cell cycle, and chromosomal effects of methylxanthines in human tumor cells treated with alkylating agents. Cancer Res., *46:* 2463–2467, 1986.
16. Rao, A. P., and Rao, P. N. The cause of G_2-arrest in Chinese hamster ovary cells treated with anticancer drugs. J. Natl. Cancer Inst., *57:* 1139–1143, 1976.
17. Lock, R. B., Ross, W. E., and Mattern, J. Inhibition of $p34^{cdc2}$ kinase activity by etoposide or irradiation as a mechanism of G_2 arrest in Chinese hamster ovary cells. Cancer Res., *50:* 3761–3766, 1990.
18. Smythe, C., and Newport, J. W. Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates $p34^{cdc2}$. Cell, *68:* 787–797, 1992.
19. Osherov, N., Gazit, A., Gilon, C., and Levitzki, A. Selective inhibition of the epidermal growth factor and HER-2/neu receptors by tyrphostins. J. Biol. Chem., *268:* 11134–11142, 1993.
20. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. Science (Washington DC), *267:* 1782–1788, 1995.
21. Gazdar, A. F., Kadoyama, C., Venzon, D., Park, J. G., Tsai, C. M., Linnoila, R. I., Mulshine, J. L., Ihde, D. C., and Giaccone, G. The effects of histological type and neuroendocrine differentiation on drug sensitivity of lung cancer cell lines. Natl. Cancer Inst. Monogr., *13:* 191–196, 1992.
22. Mitsudomi, T., Steinberg, S. M., Nau, M. M., Carbone, D., D'Amico, D., Bodner, S., Oie, H. K., Linnoila, R. I., Mulshine, J. L., Minna, J. D., and Gazdar, A. F. *p53* gene mutations in non-small cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene, *7:* 171–180, 1992.
23. Carney, W. P., Hamer, P. I., and Petit, D. Detection and quantitation of the human neu oncoprotein. J. Tumor Marker Oncol., *6:* 53–72, 1991.
24. Berenbaum, M. C. Synergy, additivism and antagonism in immunosuppression: a critical review. Clin. Exp. Immunol., *28:* 1–18, 1977.
25. Tsai, C. M., Shiao, S. H., Frey, C. M., Chang, K. T., Perng, R. P., Gazdar, A. F., and Kramer, B. S. Combination cytotoxic effects of *cis*-diamminedichloroplatinum(II) and 5-fluorouracil with and without leucovorin against human non-small cell lung cancer cell lines. Cancer Res., *53:* 1079–1084, 1993.
26. Tsai, C. M., Gazdar, A. F., Perng, R. P., and Kramer, B. S. Schedule dependent *in vitro* combination effects of methotrexate and 5-fluorouracil in human tumor cell lines. Int. J. Cancer, *47:* 401–407, 1991.
27. Chiu, J. H., Kao, H. L., Wu, L. H., Chang, H. M., and Lui, W. Y. Prediction of relapse or survival after resection in human hepatomas by DNA flow cytometry. J. Clin. Invest., *89:* 539–545, 1992.
28. Yu, D., Suen, T. C., Yan, D. H., Chang, L. S., and Hung, M. C. Transcriptional repression of the *neu* protooncogene by the adenovirus 5E1A gene products. Proc. Natl. Acad. Sci. USA, *87:* 4499–4503, 1990.
29. Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T., and Beach, D. Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. Nature (Lond.), *336:* 738–744, 1988.
30. O'Connor, P. M., and Kohn, K. W. A fundamental role for cell cycle regulation in the chemosensitivity of cancer cells. Semin. Cancer Biol., *3:* 409–416, 1992.
31. Hartwell, L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell, *71:* 543–546, 1992.
32. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.
33. Lowe, S. W., Ruley, H. E., Jacks, T., and Houseman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, *74:* 957–967, 1993.
34. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Zhang, W. W, Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene. Cancer Res., *54:* 2287–2291, 1994.
35. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. Wild-type p53 is a cell cycle checkpoint determinant following radiation. Proc. Natl. Acad. Sci. USA, *89:* 7491–7495, 1992.
36. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell, *71:* 587–597, 1992.
37. Tsai, C. M., Chang, K. T., Chen, J. Y., Chen, Y. M., Chen, M. H., and Perng, R. P. The cytotoxic effects of gemcitabine-containing regimens against human non-small cell lung cancer lines which express different levels of $p185^{neu}$. Cancer Res., *56:* 794–801, 1996.
38. Arteaga, C. L., Winnier, A. R., Poirier, M. C., Lopez-Larraza, D. M., Shawver, L. K., Hurd, S. D., and Stewart, S. J. $p185^{c\text{-}erbB\text{-}2}$ signaling enhances cisplatin-induced cytotoxicity in human breast carcinoma cells: association between an oncogenic receptor tyrosine kinase and drug-induced DNA repair. Cancer Res., *54:* 3758–3765, 1994.
39. Pietras, R. J., Fendly, B. M., Chazin, V. R., Pegram, M. D., Howell, S. B., and Slamon, D. J. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene, *9:* 1829–1838, 1994.
40. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., and Plunkett, W. Action of 2′,2′-difluoro-2′-deoxycytidine on DNA synthesis. Cancer Res., *51:* 6110–6117, 1991.
41. Kiyokawa, N., Yan, D. H., Brown, M. E., and Hung, M. C. Cell cycle-dependent regulation of $p185^{neu}$: a relationship between disruption of this regulation and transformation. Proc. Natl. Acad. Sci. USA, *92:* 1092–1096, 1995.
42. Yu, D., Wang, S. S., Dulski, K. M., Tsai, C. M., Nicolson, G. L., and Hung, M. C. c-erbB2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. Cancer Res., *54:* 3260–3266, 1994.
43. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the *HER-2/neu* proto-oncogene in normal human adult and fetal tissues. Oncogene, *5:* 953–962, 1990.