Synergistic Augmentation of Antimicrotubule Agent-induced Cytotoxicity by a Phosphoinositide 3-Kinase Inhibitor in Human Malignant Glioma Cells

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ABSTRACT

Because the aberrantly activated phosphoinositide 3-kinase (PI3K)/Akt pathway renders tumor cells resistant to cytotoxic insults, including those related to anticancer drugs, inhibition of the pathway may possibly restore or augment the effectiveness of chemotherapy. Using the human malignant glioma cell lines U87, A172, LN18, and LN229, we examined effects of the PI3K inhibitor LY294002 on both apoptosis and cytotoxicity induced by chemotherapeutic agents, including antimicrotubule agents vincristine and paclitaxel, an alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea, a topoisomerase II inhibitor etoposide, and a DNA cross-linking agent cisplatin (cis-diamminedichloroplatinum), and we compared the LY294002-induced enhancement of effects of those agents. Ten to 20 μM LY294002 augmented both apoptosis and caspase 3-like activity caused by antimicrotubule agents to a larger extent than induced by 1,3-bis(2-chloroethyl)-1-nitrosourea, etoposide, and cisplatin in all four malignant glioma cell lines examined. The same doses of LY294002 enhanced cytotoxicity more efficiently with antimicrotubule agents than with other chemotherapeutic agents. Quantitative analyses using a modified isobologram and median effect plot method revealed that enhancement by LY294002 of vincristine- or paclitaxel-induced cytotoxicity was synergistic, whereas enhancement by the PI3K inhibitor of the other chemotherapeutic agent-induced cytotoxicity was additive. Our study indicates that the synergistic augmentation of the cytotoxicity by LY294002 occurs specifically with antimicrotubule agents, at least partially through an increase in caspase 3-dependent apoptosis, and we suggest that inhibitors of the PI3K/Akt pathway in combination with antimicrotubule agents may induce cell death effectively and be a potent modality to treat patients with malignant gliomas.

INTRODUCTION

Drug resistance has been mostly explained by molecular changes, such as overexpression of p-glycoprotein or O6-methylguanine-DNA-methyltransferase, which interfere with drug actions on the targets (1, 2). Because most anticancer agents, regardless of their diverse targets, exert effects by inducing apoptosis via activation of apoptotic pathways common to many cellular stresses, any antiapoptotic changes disrupting the common intrinsic pathways to execute physiological cell death can also make malignant cells resistant to chemotherapy (3). Such alterations opposing apoptosis are routinely observed in malignant tumors, including both the functional loss of tumor suppressors, such as Ras, Bcl-2, and PI3K (3).

In tumor cells with an aberrantly activated PI3K/Akt survival pathway, the increased antiapoptotic signals overcome apoptotic signals of anticancer drugs, confer drug resistance on the tumor cells, and result in a limited effectiveness of chemotherapy (3). Thus, inhibition of the PI3K/Akt survival pathway should decrease drug resistance of tumor cells and sensitize them to chemotherapeutic agent-induced cytotoxicity by increasing apoptosis. Inhibition of the pathway in combination with chemotherapeutic agents may be a potent therapeutic modality for patients with tumors, especially those harboring aberrantly activated PI3K/Akt.

The poor outcome for patients with malignant glioma is mainly attributed to two characteristics of the tumor. One is invasiveness of the tumor into adjacent normal brain parenchyma, which makes total resection less feasible, and the other is drug resistance of the tumor, resulting in unsuccessful chemotherapy (4, 5). In malignant glioma, amplification of the epidermal growth factor receptor gene and mutation or deletion of the PTEN tumor suppressor gene, both of which could activate the PI3K/Akt pathway, occur at 40–50 and 30–40% of glioblastomas, respectively (6). In addition to the mechanisms proposed previously, these genetic alterations frequently found in human glioblastomas may aberrantly augment the survival signal and thus contribute to the drug-resistant nature of malignant gliomas (1, 3). We have found that pharmacological inhibition of the PI3K/Akt pathway by a PI3K inhibitor LY294002 increases anticancer drug-induced cytotoxicity in malignant glioma cells, under conditions where the survival pathway is stimulated (7). However, what type of chemotherapeutic agent the PI3K inhibitor should be combined with to ensure cell death remained to be determined. Here, we quantitatively analyzed effects of LY294002 on apoptosis and growth inhibition of human malignant glioma cells induced by several chemotherapeutic agents with different pharmacological actions, and we found that LY294002 synergistically increases antimicrotubule agent-induced cytotoxicity.

MATERIALS AND METHODS

Materials. LY294002, BCNU, and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO), etoposide was from Calbiochem (Nottingham, United Kingdom), and vincristine and paclitaxel were from Wako (Osaka, Japan) and Sigma Chemical Co., respectively. These reagents were dissolved in DMSO. The final concentrations of DMSO in culture media for glioma cells did not exceed 0.2%. Origins of human malignant glioma cell lines and culture conditions of these cells were as described (7).

Apoptosis and Cell Growth. For caspase 3-like activity, cells harvested were lysed with a buffer containing 10 mM HEPES (pH 7.4), 2 mM EDTA, 5 mM DTT, and 0.5% NP40 and centrifuged at 18,000 × g for 10 min. The supernatants (50–100 μg of protein) were incubated at 23°C with 0.1 mM acetyl-t-aspartyl-t-glutamyl-t-valyl-t-aspart-7-amino-4-methylcoumarin (Peptide Institute, Inc., Osaka, Japan), 100 mM HEPES (pH 7.4), 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride in a total volume of 50 μL. Increase in the amount of the reaction product amino-4-methylcoumarin was monitored by measuring the fluorescence (excitation: 380 nm, emission: 460 nm), and the initial velocity of the caspase reaction was determined. Detection of oligonucleosomal DNA fragmentation, determination of apoptotic indices and growth inhibition, and cytotoxicity assays were done as described (7).

Isobologram and Median Effect Plot Analyses. Interaction between LY294002 and each chemotherapeutic agent was analyzed using the isobologram method modified by Steel and Peckham (8, 9). On the basis of the dose-response curves using MTT assay for glioma cells treated with each chemotherapeutic agent alone or LY294002 alone for 72 h, three isoeffect

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2 The abbreviations used are: PI3K, phosphoinositide 3-kinase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CI, combination index; cisplatin, cis-diamminedichloroplatinum; MTT, 3(4,5-dimethylthiazol-2-yl)(2,5-diphenyltetrazolium bromide.

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curves at 50% decrease in relative number of viable cells were drawn. The total area enclosed by these three lines represents additive response and is called an envelope of additivity (see Fig. 3A). The data point of IC_{50} was determined with doses of drug X and Y to achieve a 50% suppression in growth in combination and IC_{50} values of the individual drugs. These experiments were done at least twice in duplicate. In median plot analyses, fractional survival (f) was determined using MTT assay. Data were subsequently analyzed by the median effect plot method by Chou and Talalay (10, 11). In this study, calculation of CI was done based on the assumption that action of the two drugs was mutually nonexclusive for the strict detection of synergism.

**Statistical Analysis.** Unpaired Student’s or Welch’s t tests were used for comparison between the two groups. Differences were considered statistically significant at a P < 0.05.

**RESULTS AND DISCUSSION**

**Enhancement by LY294002 of Antimicrotubule Agent-induced Apoptosis.** We examined the effects of a PI3K inhibitor LY294002 on anticancer drug-induced apoptosis in LN18 human malignant glioma cells. Oligonucleosomal DNA fragmentation was slight in the cells treated with either LY294002 or each anticancer drug alone (Fig. 1A). However, when the cells were treated with vincristine or paclitaxel combined with LY294002, the DNA fragmentation remarkably increased and was much greater than when the cells were treated with each reagent alone (Fig. 1A). In contrast, etoposide, cisplatin, and BCNU in combination with LY294002 did not induce increased DNA fragmentation when compared with the extent of DNA fragmentation caused by each reagent alone (Fig. 1A). The drastic increase in DNA fragmentation specifically by the combinations of LY294002 and antimicrotubule agents was also observed when the additional three malignant glioma cell lines were examined (data not shown).

Treatment with 2 nM vincristine and 10 nM paclitaxel in combination with 10 μM LY294002 induced apoptosis ∼3.8- and 1.8-fold, respectively, over that caused by each antimicrotubule agent without LY294002 (Fig. 1B). However, 200 μM BCNU in combination with the same concentration of LY294002 did not induce any remarkably increased apoptosis when compared with a level of apoptosis caused by BCNU alone (Fig. 1B). Similarly, treatment with 5 nM vincristine in combination with 20 μM LY294002 induced apoptosis ∼3.2-fold over that caused by vincristine alone, whereas at the same concentration, LY294002 did not markedly enhance the apoptosis caused by 20 μM etoposide or 5 μM cisplatin (Fig. 1B). The drastic increase in apoptotic indices specifically by combinations of LY294002 and antimicrotubule agents was also observed when the other three malignant glioma cell lines were examined (data not shown).

Caspase 3-like activity induced by vincristine in combination with LY294002 was 20-fold over that induced by vincristine alone (Fig. 1C). However, treatment with etoposide in combination with LY294002 induced caspase 3-like activity only 5-fold over that caused by treatment with etoposide alone (Fig. 1C). LY294002 tended to increase caspase 3-like activity induced by paclitaxel but not that caused by BCNU or cisplatin (data not shown). In A172, LN18, and U87 cells, LY294002 augmented caspase 3-like activity together with vincristine but not with etoposide (data not shown). From these results, we conclude that LY294002 specifically augments antimicrotubule agent-induced apoptosis, at least in part by increasing caspase 3-like activity in malignant glioma cells.

**Enhancement by LY294002 of Antimicrotubule Agent-induced Growth Inhibition.** Although vincristine at 1 nM reduced the viability percentage of LN229 cells only slightly; the addition of 10 μM LY294002 increased the reduction to 50% (Fig. 2A). LY294002 also enhanced the decrease in cell viability by another antimicrotubule agent, paclitaxel (Fig. 2B), whereas LY294002 did not affect the dose-response curves of viability percentage with DNA-damaging anticancer drugs etoposide, cisplatin, and BCNU (Figs. 2, C–E). In three other human glioma cell lines, U87, A172, and LN18, LY294002 also effectively augmented vincristine- and paclitaxel-induced growth inhibition but did not enhance those induced by anticancer drugs other than antimicrotubule agents (data not shown). These findings indicate that LY294002 preferentially increases the growth inhibitory effects of antimicrotubule agents in malignant glioma cells.

**Synergistic Interaction of LY294002 with Antimicrotubule Agents.** To determine whether effects of LY294002 on chemotherapeutic agent-induced cytotoxicity are synergistic or additive, isobolograms at IC_{50} and a median effect plot were made. When LN229 cells were treated with either vincristine (Fig. 3B) or paclitaxel (Fig. 3C) in combination with LY294002, data points in the isobologram fell on the left side of the envelope of additivity. However, in combinations of etoposide (Fig. 3D), cisplatin (Fig. 3E), or BCNU (Fig. 3F) with LY294002, data points were in the
envelope of additivity. Similar results were obtained when A172, LN18, and U87 cells were tested (data not shown). Thus, interactions between LY294002 and vincristine or paclitaxel are synergistic, whereas those between LY294002 and etoposide, cisplatin, or BCNU are additive at IC50.

With the median effect plot analysis, CI values for A172 and LN229 cells treated with vincristine or paclitaxel in combination with LY294002 were ≤1 at IC50 (data not shown). However, CI values for cells treated with BCNU, cisplatin, or etoposide in combination with LY294002 were in the vicinity of or exceeded 1 at IC50 (Table 1). Similarly, CI values for U87 and LN18 cells treated with combinations of LY294002 and vincristine or paclitaxel were ≤1, but those for cells treated with combinations of LY294002 and cisplatin, BCNU, or etoposide were in the vicinity of or >1 at IC50 (data not shown). Thus, the median effect plot method revealed that interactions between LY294002 and vincristine or paclitaxel are synergistic, whereas those between LY294002 and BCNU, cisplatin, or etoposide are additive or subadditive at IC50 in human malignant glioma cells. Both of two different analyses indicate that interactions between LY294002 and antimicrotubule agents at IC50 are synergistic for all four human glioma cell lines.

Fig. 2. Enhancement of anticancer drug-induced growth inhibition by LY294002 in LN229 cells. Viability percentage of cells treated with each anticancer drug (solid lines) or with each anticancer drug in combination with LY294002 (broken lines) was determined, using MTT. * and **, significance of P < 0.05 and <0.01, respectively, in comparison between the values with and without LY294002. Data are expressed as mean ± SD of two to three independent experiments done in triplicate.

Fig. 3. A, an isobologram for the combination of drugs X and Y. An envelope of additivity (dotted area) is surrounded by two of three lines, Mode I, II (A), and II (B) determined by dose-response curves for drugs X and Y. In cases of data points (triangles) on the left side of the envelope of additivity, interaction between the two agents was interpreted as being synergistic. Data points in and on the right side of the envelope of additivity were considered to be indicative of additivity and antagonism, respectively. B–F, isobolograms for combinations of LY294002 and anticancer drugs in LN229 cells. Interactions between LY294002 and indicated anticancer drugs at the point of IC50 were analyzed, using the isobologram method. Triangles are data points of IC50 determined by combinations of reagents that achieved IC50. Representative data from two independent experiments done in duplicate are presented.

Table 1 CI at IC50 for each anticancer drug combined with LY294002 in malignant glioma cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>CI at IC50 (±SD)</th>
<th>CI at IC50 (±SD)</th>
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<tr>
<td>Vincristine</td>
<td>0.67 ± 0.18</td>
<td>0.74 ± 0.042</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.84 ± 0.59</td>
<td>0.41 ± 0.14</td>
</tr>
<tr>
<td>BCNU</td>
<td>1.24 ± 0.25</td>
<td>1.15 ± 0.63</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.21 ± 0.28</td>
<td>1.12 ± 0.14</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.96 ± 0.23</td>
<td>1.23 ± 0.085</td>
</tr>
</tbody>
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Data are expressed as mean ± SD of two independent experiments done in duplicate. CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.
**Possible Mechanisms of the Selective Synergism of LY294002 with Antimicrotubule Agents.** The selective augmentation of antimicrotubule agent-induced cell killing by LY294002 implies a distinctive relationship between the PI3K/Akt pathway and tubulin-microtubule equilibrium. Two possible signaling molecules mediating antimicrotubule agent-induced apoptosis, c-Jun-NH$_2$-terminal kinase and extracellular signal-regulated kinase, do not seem to be responsible for the specific augmentation of the cytotoxicity by the PI3K inhibitor, because we found no marked decrease or increase in the phosphorylation of extracellular signal-regulated kinase or c-Jun-NH$_2$-terminal kinase specific to treatment with the combinations of LY294002 and antimicrotubule agents. Although molecular basis of the synergism remains to be elucidated, our study does suggest that antimicrotubule drugs may be more sensitive to the PI3K survival signal than are other chemotherapeutic drugs. The implication may aid in tailoring chemotherapy in which chemotherapeutic agents will be rationally selected based on genetic profiling (3). The PI3K/Akt pathway in other tumor cells is also often abrogated to more highly activated states (3, 12). Ovarian cancer cells with increased PI3K levels have been reported to be efficiently suppressed with the combination of LY294002 and paclitaxel (13). Thus, the synergistic interaction may also occur in other tumor cells with activated PI3K/Akt signaling.

**Clinical Implications of PI3K Inhibitors.** Neither vincristine nor paclitaxel has served as standard chemotherapeutics for either glioblastoma or astrocytoma (4, 14). However, our results do suggest that the combination of an antimicrotubule agent with a PI3K inhibitor provides a novel therapeutic regimen for patients with PI3K/Akt-activated malignant gliomas. The concentrations of vincristine and paclitaxel used in this study have been shown to be achievable in cerebrospinal fluid of patients who are given therapeutic doses of the antimicrotubule agents (15, 16). The inhibition of PI3K may augment the cytotoxicity induced by clinically permitted doses of the antimicrotubule agents and/or allow for reasonable clinical results at lower doses of the drugs with decreased side effects. The hydrophobic property and low molecular weight (307.4) of LY294002 mean that the reagent crosses the blood–brain barrier (17). Although LY294002 has not been given to humans, i.p. administered LY294002 decreases both phosphatidylinositol 3,4,5-trisphosphate and phosphorylated Akt in mouse brain tissue (18). Additional investigations, including in vivo studies using intracerebral or s.c. tumor models with implanted human malignant glioma cells (19) to examine not only antitumoral effects but also toxicity in normal tissues, are required.

In summary, we quantitatively compared effects of the PI3K inhibitor LY294002 on cytotoxicity and apoptosis induced by five different anticancer drugs in four glioma cell lines and found that the synergistic enhancement of cytotoxicity associated with apoptosis by the PI3K inhibitor LY294002 occurs specifically with the antimicrotubule agents. These results suggest that the combination of LY294002 and an antimicrotubule agent seems to be effective therapeutic strategy and that the PI3K/Akt pathway differentially influences chemotherapeutic agent-induced cytotoxic effects.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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*3 T. Shingu et al., unpublished observation.*

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