# Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells

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## Abstract

**The oncogenic epidermal growth factor receptor (EGFR) pathway triggers downstream phosphatidylinositol 3-kinase (PI3K)/RAS-mediated signaling cascades. In transgenic mice, glioblastoma cannot develop on single but only on simultaneous activation of the EGFR signaling mediators RAS and AKT. However, complete blockade of EGFR activation does not result in apoptosis in human glioblastoma cells, suggesting additional cross-talk between downstream pathways. Based on these observations, we investigated combination therapies using protein kinase inhibitors against EGFR, platelet-derived growth factor receptor, and mammalian target of rapamycin, assessing glioblastoma cell survival. Clinically relevant doses of AEE788, Gleevec (imatinib), and RAD001 (everolimus), alone or in combinations, did not induce glioblastoma cell apoptosis. In contrast, simultaneous inactivation of the EGFR downstream targets mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase and PI3K by U0126 and wortmannin triggered rapid tumor cell death. Blocking EGFR with AEE788 in combination with sublethal concentrations of the microtubule stabilizer patupilone also induced apoptosis and reduced cell proliferation in glioblastoma cells, accompanied by reduced AKT and ERK activity. These data underline the critical role of the PI3K/AKT and the RAS/RAF/mitogen-activated protein/ERK kinase/ERK signaling cascades in the cell-intrinsic survival program of sensitive glioblastoma cell lines. We conclude that drug combinations, which down-regulate both ERK and protein kinase B/AKT activity, may prove effective in overcoming cell resistance in a subgroup of glioblastoma. [Mol Cancer Ther 2007; 6(2):773–81]**

## Introduction

Glioblastoma multiforme is the most frequent malignant neoplasm of the human central nervous system. Surgery can only control the highly proliferative component of the disease, whereas widespread tumor cell infiltration into normal brain areas resists radiotherapy and chemotherapy (1, 2). Amplification and overexpression of the gene encoding the epidermal growth factor (EGF) receptor (EGFR) are detected in ~50% of glioblastomas and are mainly associated with disease progression (3, 4). EGFR is a member of the ErbB family of receptor tyrosine kinases (5). Small molecular weight compounds with EGFR protein kinase inhibitory (PKI) activity, such as PKI-166 or AEE788, have a cytostatic effect *in vitro* on tumor cells that overexpress EGFR (6, 7). In addition, treatment of non–small cell lung cancer with the EGFR PKI imatinib (gefitinib) resulted in tumor growth control in 10% of patients (8). In fact, tumors that responded to gefitinib specifically carried specific mutations in the EGFR tyrosine kinase domain (8–10). Although responses to gefitinib have also been observed in a limited number of glioblastoma cases (11), a specific molecular profile is associated with response that differs from the lung signature (12–15). To date, several small molecular weight inhibitors, such as Gleevec or erlotinib/gefinitib, applied as monotherapies for the treatment of gliomas, only resulted in limited effectiveness (16). This has supported the hypothesis that combination of drugs would be a more appropriate treatment for glioma.

EGF mediates signaling via phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways (17–19), which are redundantly stimulated by other activated growth factor receptors (e.g., the insulin-like growth factor receptor-I; ref. 20). In at least 50% of glioblastoma, PI3K is activated either by loss of function of the tumor suppressor PTEN (21) or by gain-of-function mutations in the *PIK3CA* gene, which encodes the p110 catalytic subunit of PI3K (22). Interestingly, activated protein kinase B (PKB)/AKT cooperated with RAS in the induction of malignant gliomas in a murine brain tumor model (23). Furthermore, activation of PKB/AKT has been detected in several types of human cancers and found to be associated with poor clinical outcome (24–26) and resistance to chemotherapy and radiotherapy (24, 27, 28).

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Although no RAS mutations have been detected in glioblastoma, high levels of RAS-GTP have been documented in high-grade astrocytomas (29, 30). It has been suggested that ERK plays a critical role in cellular transformation and resistance to apoptosis (31). Evidence for a cooperation between AKT and RAS has been proven by a transgenic mouse model in which both oncogenes are ectopically expressed in normal astrocytes, giving rise to glioblastoma, whereas neither AKT nor RAS alone was sufficient for tumor induction (23).

Several examples of combined therapies have recently undergone advanced clinical trials in cancer treatment. For example, lapatinib with tamoxifen has been tested together in breast cancer (32), and in xenografted tumors (32), PKI AEE788 has been associated with the rapamycin-derivative RAD001 for the treatment of glioblastoma (33–35). Successful drug combinations allow the use of lower doses, possibly reducing toxicity and limiting the degree of acquired drug resistance. Given the limited efficacy of PKI to induce cell death in rapidly proliferating tumor cells, these inhibitory drugs may depend on the coadministration of a cytotoxic drug for the induction of cell death rather than on the coadministration of a second cytostatic compound. The principle of cytotoxicity of patupilone (epothilone B, EPO906) relies on the inhibition of microtubule depolymerization, which is lethal for all dividing cells. The antitumor activity of patupilone has been proven *in vitro* and *in vivo* in lung, breast, colon, and prostate cancers (36). Patupilone also shows clinical activity in a range of solid tumors (37) and is now in phase III clinical development.

In the present study, we examined the induction of cell death on glioblastoma lines *in vitro* by targeting EGFR/ErbB2 by the receptor tyrosine kinase inhibitor AEE788 alone or in combination with the cytotoxic compound patupilone. We further evaluated the activation status of the EGFR downstream signaling mediators PKB/AKT and ERK following combined treatment with patupilone and AEE788. Our study points to a critical role of both PI3K and ERK in glioblastoma cell survival.

## Materials and Methods

### Cell Culture

All cell lines were grown at 37°C in a 5% $CO_2$ humidified atmosphere. DMEM culture medium was supplemented with 10% FCS and standard antibiotics. Cells were detached with 1 mL trypsin-EDTA (1×) for 5 min at 37°C. The genetic status of all cell lines at *p53, p16, p14ARF*, and *PTEN* tumor suppressor genes has been previously reported (38, 39).

### Pharmacologic Inhibitors

AEE788, RAD001, and patupilone (provided by Novartis Pharma AG, Basel, Switzerland) and U0126 (Promega, Madison, WI) were dissolved in DMSO as 10 mmol/L stocks and stored as aliquots at −20°C. Wortmannin (Sigma, St. Louis, MO) was prepared as a 1 mmol/L stock solution in DMSO and stored at 4°C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; LC Laboratories, Woburn, MA) was prepared as a 2 mmol/L stock solution in DMSO, aliquoted, and stored at −20°C. Treatment of the cells with TPA, U0126, or wortmannin consisted of daily additions without replacing the medium.

### Measurement of Cell Proliferation and Cell Cycle Profile

In experiments shown in Fig. 1B, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Bromodeoxyuridine (BrdUrd) was added 1 h before cell harvesting to a 10 μmol/L final concentration. Fluorescence-activated cell sorting analysis was done according to the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ). The cell proliferation assays presented in Figs. 2C and 3B were done with the Biotrak ELISA System version 2 (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's instructions. Cells ($5 \times 10^3$) were seeded in 96-well plates and grown for 24 h, and drugs were applied for an additional 24 h. BrdUrd incorporation was allowed during the last 2 h of treatment. In experiments shown in Fig. 1D, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Cell DNA content and apoptosis were analyzed on a FACSCalibur. Cells were trypsinized and fixed in 70% ice-cold ethanol for 1 h and stained with 50 μg/mL propidium iodide for fluorescence-activated cell sorting analysis. The percentages of cells in the cell cycle phases are reported (results from three independent experiments).

### Measurement of Apoptosis

Expanding cells were subjected to drug treatment for 24 h, briefly washed with PBS, and trypsinized. Cell suspension was pelleted at 800 rpm for 5 min, fixed in ice-cold 70% ethanol, and kept at 4°C for 30 min. Cells were resuspended in a 1× PBS solution containing 50 ng/mL propidium iodide and 50 μg/mL RNase. Percentage of cells in sub-$G_1$ for apoptosis was determined by flow cytometry.

### Protein Extraction and Western Blot Analysis

Cells were washed with PBS, resuspended in 1× SDS sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 50 mmol/L DTT), and boiled at 95°C during 5 min; aliquots were stored at −20°C. Proteins (30 μg) were separated by size on SDS-PAGE gels (10%) and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Western blot analysis was done using antibodies against the following proteins: phosphorylated AKT (Upstate Biotechnology, Lake Placid, NY), ERK1/2, phosphorylated ERK, phosphorylated EGFR ($Tyr^{1173}$), and EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Sigma). The anti-AKT antibody was a gift from Dr. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland). Western blots were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL).

## Results

### Specific Targeting of the EGF Signaling Pathway by AEE788

Nearly 50% of primary glioblastomas exhibit amplification of the *EGFR* gene (3, 4), which is also associated with

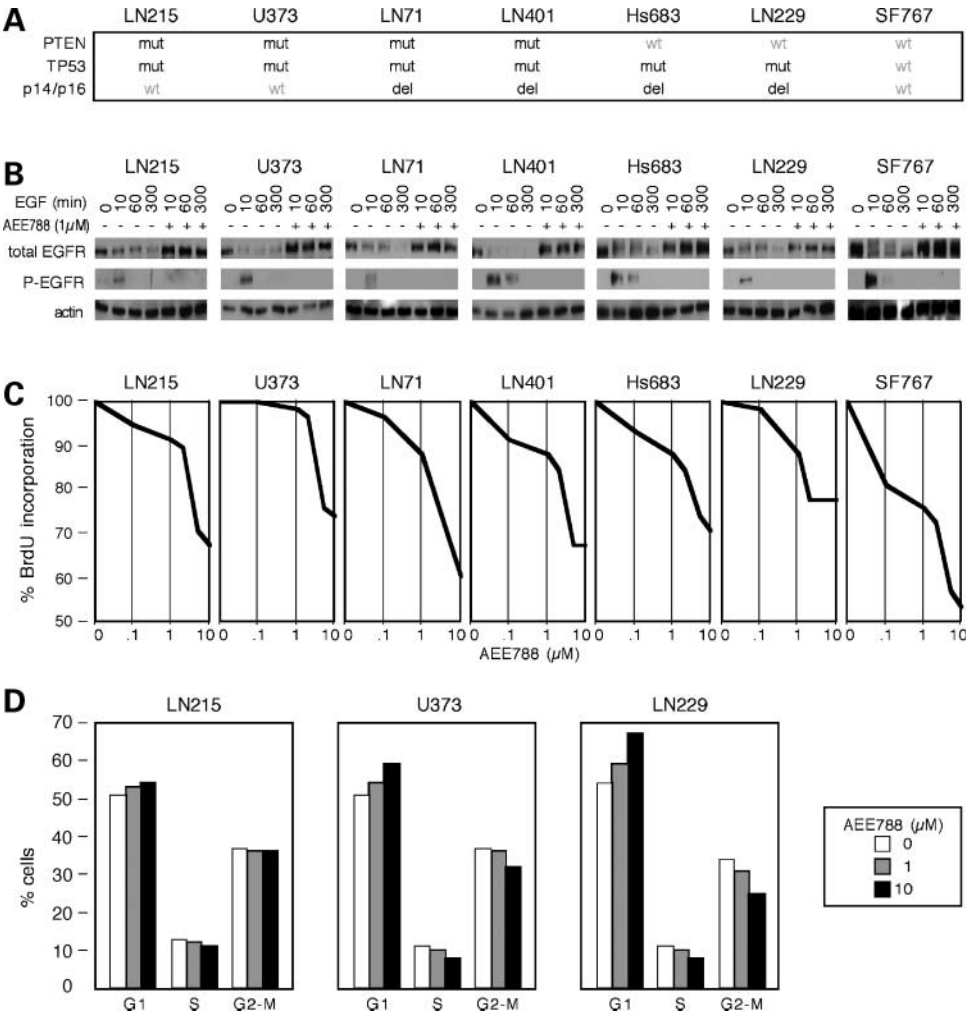


**Figure 1.** Treatment of glioblastoma cell lines with EGFR inhibitor. **A,** genotyping of used glioma cell lines by Ishii et al. (39). **B,** cells were preincubated in the presence or absence of 1 μmol/L of the EGFR inhibitor AEE788 during 30 min and stimulated with 100 ng/mL EGF for 10, 60, and 300 min. EGFR, phosphorylated EGFR ($Tyr^{1173}$), and actin were detected by Western blot analysis of cell lysates. **C,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Proliferation was measured by BrdUrd and flow cytometry. Experiments were done in triplicate. *Points,* average of three independent determinations; *bars,* SD. **D,** cell cycle phase analysis on glioblastoma cell lines on 1 and 10 μmol/L of AEE788 treatment.

poor prognosis. For this reason, the inhibitor of EGFR and of ErbB receptor protein kinase activities AEE788 has been considered to be a potent drug to induce cell death in glioblastoma cell lines. AEE788 was tested for its ability to induce cell growth arrest and apoptosis in various cell lines derived from glioblastoma, for which the genetic status of established cancer genes had been previously defined (Fig. 1A; refs. 38, 39). For a preliminary determination of the minimal drug concentration needed to fully prevent EGFR activation by EGF, increasing concentrations of AEE788 were applied to the LN229 glioblastoma cell line, and EGFR/ErbB receptor activation status was monitored by the presence of EGFR phosphorylated at $Tyr^{1173}$. To completely block receptor phosphorylation, 1 μmol/L was needed (data not shown). Further time course experiment done with 1 μmol/L AEE788 showed a strong inhibition of the transient phosphorylation of $Tyr^{1173}$ as well as of the degradation of the receptor protein in all seven glioblastoma cell lines tested (Fig. 1B).

Increasing concentrations of AEE788 were applied to the glioblastoma cell lines, and proliferation was assayed by integration of BrdUrd after 1 day. At a 1 μmol/L concentration, AEE788 gives up to 20% of proliferation reduction compared with the control cell population (Fig. 1C). Cell cycle analysis after 24 h of 1 and 10 μmol/L of AEE788 treatment shows an increase of cell population in the $G_1$ phase (Fig. 1D).

Cell viability was assayed by flow cytometry after 24, 48, and 96 h. Very low or no apoptosis was induced at drug concentrations <2 μmol/L as late as 96 h (Fig. 2A). High levels of apoptosis were observed at unphysiologically high concentration (10 μmol/L) in five of the seven cell lines (SF767, LN215, U373, LN229, and LN71). Under the same condition, LN401 and Hs683 did not show significant levels of apoptosis. Because AEE788 was not capable of inducing strong apoptosis in glioblastoma cells when used as a single drug, we tested whether a specific double or a triple combination of compounds would significantly increase the induction rate of apoptosis. For this purpose, we combined suboptimal doses of AEE788 (0.5 μmol/L) with two additional biological drugs: Gleevec (imatinib, 0.5 μmol/L) and RAD001 (everolimus, 20 nmol/L). In gliomas, Gleevec is being explored as a PKI of platelet-derived growth factor receptor (34), which is activated in

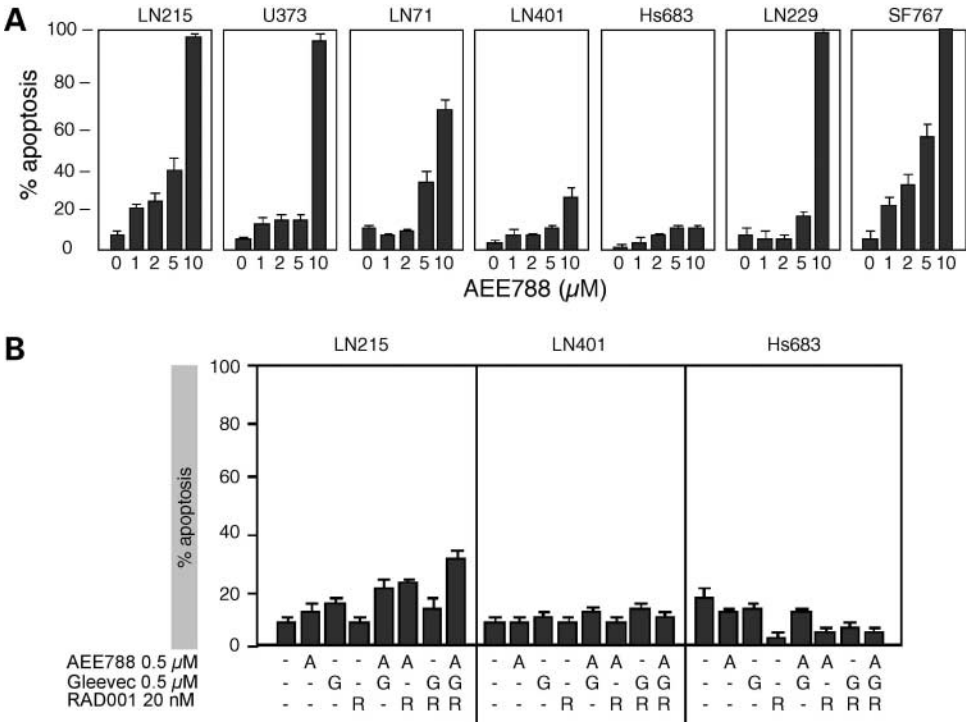


**Figure 2.** Survival of glioblastoma cell lines on treatment with EGFR inhibitor and in combination with Gleevec and RAD001. **A,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Apoptosis was measured by flow cytometry. Experiments were done in triplicate. *Columns,* average of three independent determinations; *bars,* SD. **B,** combinatorial study of biological drugs AEE788 (A), Gleevec (G), and RAD001 (R) on glioma cell survival.

primary tumors (40, 41). Platelet-derived growth factor receptor phosphorylation levels correlated with sensitivity to Gleevec in glioma primary cultures (42). RAD001, a derivative of rapamycin, inhibits mammalian target of rapamycin, leading to inactivation of ribosomal S6K1 and inhibition of cap-dependent translation (43).

The application of the combined drugs to 10 glioblastoma cell lines for 4 days revealed that there was a combination-specific and a cell line–specific response with regard to induction of cell death (Fig. 2B). The cell lines could be divided into three categories. One group (SF767, LN18, and LN215; Fig. 2B, *left*) showed a modest additive induction of apoptosis by all of the three drugs, reaching a maximum of 35% of cell death with the triple combination. A second group of glioblastoma cell lines (LN401, LN71, LN229, and LN319; Fig. 2B, *middle*) was basically insensitive to all drug combinations, showing the same rate of apoptosis as untreated cells. The third group (Hs683, U373, and U343; Fig. 2B, *right*) exhibited a relatively high level of basal apoptosis (15–20%), which was, however, decreased by the three drugs applied together. Especially, RAD001 exhibited an antiapoptotic effect, alone or in combination. No differences in apoptosis rate were evidenced between PTEN-mutant (LN215, LN401, LN71, and U373) and wild-type (Hs683, SF767, and LN229) cell lines on RAD001 application. Although, in theory, RAD001 was expected to overcome the constitutive AKT activation resulting from loss of PTEN activity.

Taken together, the results indicated that neither single nor combined application of the three biological drugs, at physiologic concentration, was able to strongly induce cell death in glioblastoma cell lines.

### Induction of Apoptosis in Glioblastoma Cell Lines by Patupilone

Because neither the single nor the combined application of the three biological drugs led to a consistent induction of apoptosis, we tested whether the combination of AEE788 with a cytotoxic drug could result in a more cooperative induction of apoptosis. Patupilone is a member of the group of epothilones, which represent a new class of low molecular weight compounds that target microtubules by inhibiting their depolymerization and therefore impairs cell division (44). Patupilone prevents chromosome alignment at metaphase and drives cells to undergo apoptosis (45). It has been shown to exhibit antitumor activity *in vitro* and *in vivo* (36, 37). As a result of inhibition of microtubule depolymerization, after addition of patupilone, dramatic changes in cell morphology occurred within 18 h (Fig. 3A, *right*). To investigate its ability to induce apoptosis, glioblastoma cells were treated with increasing concentrations of patupilone in the picomolar and nanomolar concentration range for 4 days (Fig. 3A, *left*). Although 100% apoptosis was induced in all cell lines when the highest drug concentration (35 nmol/L) was applied, levels of apoptosis induced by 0.7 nmol/L patupilone strongly varied between the cell lines, from Hs683 and LN401, the most resistant, to LN229, the most sensitive one.

### Combined Treatment with AEE788 and Patupilone Strongly Induced Apoptosis and Reduced Proliferation of Glioblastoma Cells

AEE788 (1 μmol/L) was applied together with increasing concentrations of patupilone on cell lines LN71, LN229, Hs683, and SF767, and cell survival was determined after 4 days (Fig. 3B). The combined drugs induced apoptosis in

a cooperative manner in LN71 and SF767 but not in Hs683 and LN229 cell lines. In LN71 and SF767 lines, the patupilone concentration required to induce apoptosis in 50% of cells ($AC_{50}$) revealed that AEE788 acted as a sensitizer, reducing the patupilone concentration from a nanomolar to a picomolar concentration range. Thus, addition of AEE788 shifted down the amount of patupilone required to induce apoptosis in 50% of cells, up to 2 orders of magnitude in SF767 cells. It is noteworthy that the synergistic effect of patupilone on those glioma cell lines seemed independent of their respective sensitivities to AEE788. Consistent with the absence of synergy of both compounds on LN229 cells, also proliferation was not affected either by AEE788 or patupilone or by the combination of both drugs. In contrast, SF767 cells, on which the AEE788/patupilone synergized the best, had a significant reduction of proliferation up to 80% ($P < 0.001$) after each individual or combined drug application (Fig. 3C).

### Simultaneous Inhibition of Both ERK1/2 and AKT Kinase Activities Parallels the Induction of Apoptosis

Both signaling pathways, PI3K/AKT and RAS/MEK/ERK, are under the control of EGF-directed activation of ErbB receptors. To investigate the activation of these two pathways, phosphorylation status of the kinases AKT and ERK was chosen as readouts. AEE788 (1 μmol/L) and patupilone (0.7 nmol/L), alone and in combination, were applied for 24 and 72 h to the four glioblastoma cell lines that differed remarkably in their sensitivities toward those drugs. In contrast to LN229 and Hs683, LN71 and SF767

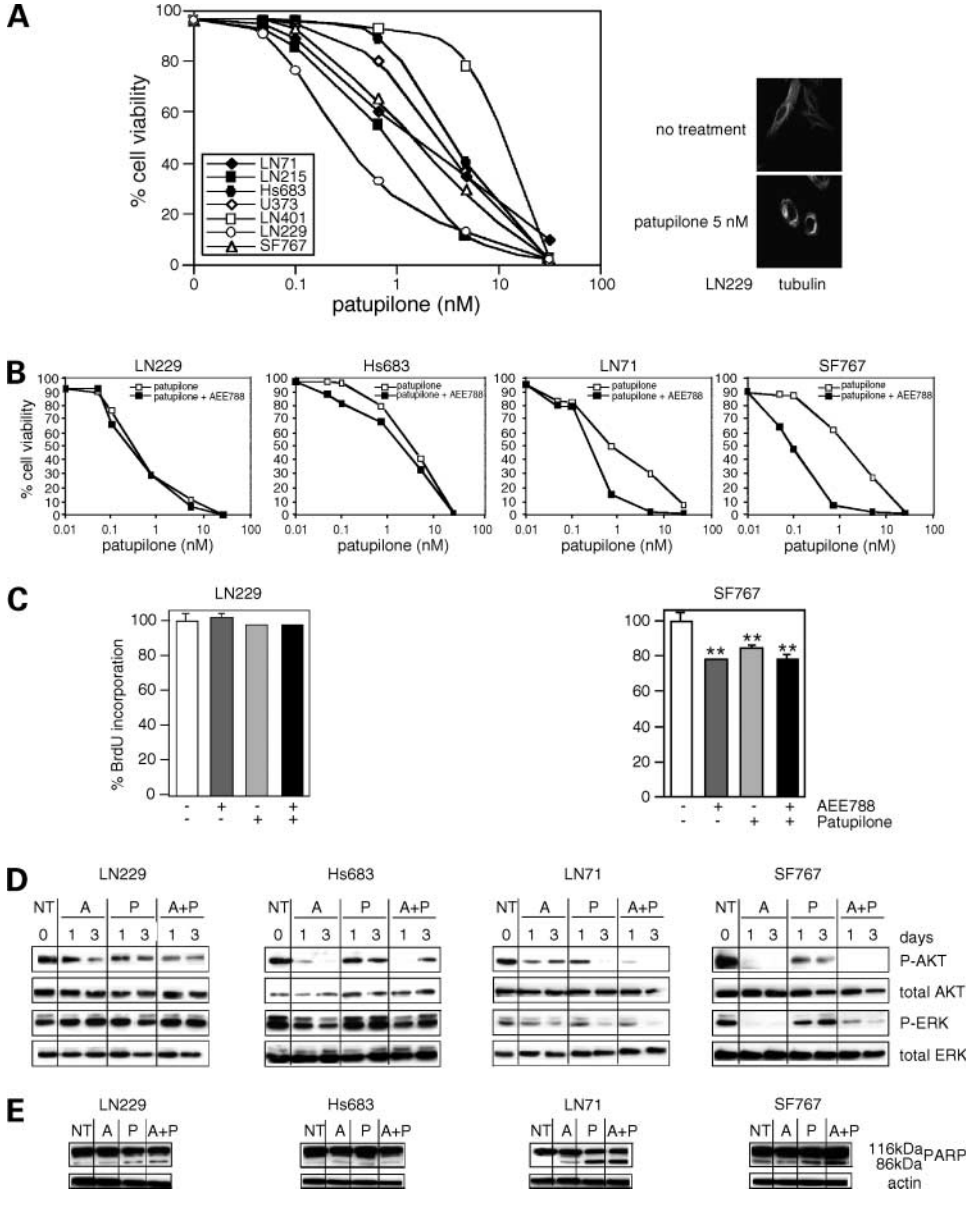


**Figure 3.** Combined treatment of glioblastoma cells stabilizing microtubules and blocking EGFR. **A,** increasing concentrations of patupilone (0.05, 0.1, 0.7, 5, and 35 nmol/L) were applied to seven cell lines for 4 d and the percentage of viable cells was determined using flow cytometry. *Points,* average of three independent determinations; *bars,* SD. Confocal microscopy of LN229 cells after 18 h of patupilone treatment. **B,** cells were treated for 4 d either with increasing concentrations of patupilone alone or in combination with 1 μmol/L AEE788. Cell survival was measured by flow cytometry. *Points,* average of three independent experiments; *bars,* SD. **C,** cells were treated with 1 μmol/L AEE788 and/or 0.7 nmol/L patupilone and assayed for proliferation with BrdUrd by ELISA. **D,** *top,* cells were incubated during 24 h and 3 d with patupilone (0.7 nmol/L), AEE788 (1 μmol/L), or both. Western blot analysis was done on cell lysates. *NT,* no treatment; *A,* AEE788; *P,* patupilone. *Bottom,* poly(ADP-ribose) polymerase (*PARP*) cleavage after 24 h.

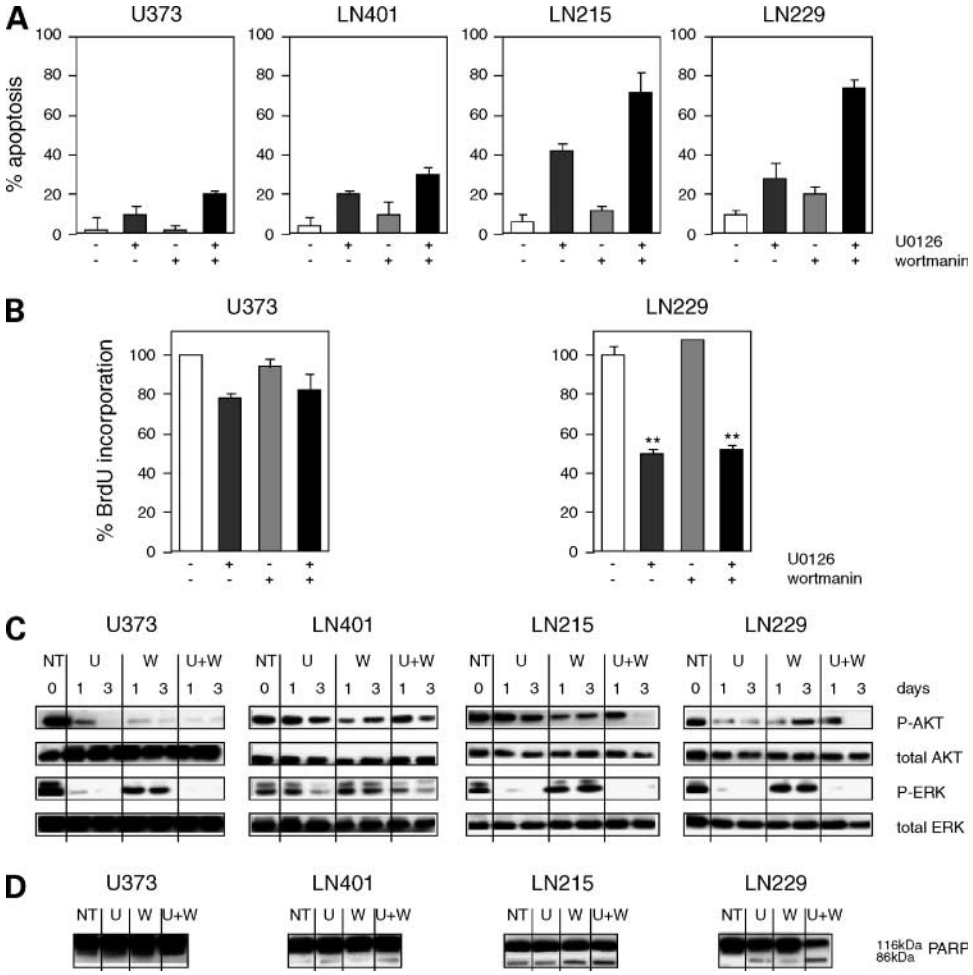


**Figure 4.** Combined treatment of glioblastoma cells blocking PI3K and MEK. **A,** cell lines were treated with the MEK inhibitor U0126 (20 μmol/L) and/or the PI3K inhibitor wortmannin (1 μmol/L) for 3 d, whereby the addition of both drugs was repeated every day. Percentage of apoptotic cells was measured using flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD. **B,** cells were treated with 20 μmol/L U0126 and/or 1 μmol/L wortmannin and assayed for proliferation with BrdUrd by ELISA. **C,** Western blot analysis was done to determine the relative phosphorylation levels of ERK1/2 and AKT after 3 d of treatment. *U,* U0126; *W,* wortmannin. **D,** poly(ADP-ribose) polymerase (PARP) cleavage after 24 h.

showed full down-regulation of phosphorylation of both protein kinases after 3 days of combined treatment (Fig. 3D), accompanied by widespread apoptosis between 85% and 95% of cells. In contrast, LN229 and Hs683 still retained strong phosphorylation of the two protein kinases and showed much lower levels of apoptosis. These results suggested a link between the simultaneous inhibition of the two EGF-dependent signaling pathways and the induction of apoptosis triggered by the combination of patupilone and AEE788. To support this hypothesis, we analyzed the cleavage status of poly(ADP-ribose) polymerase, which triggers caspase-dependent apoptosis (46, 47). The cleaved form of poly(ADP-ribose) polymerase was indeed detected in glioblastoma cells undergoing apoptosis (Fig. 3E).

### Simultaneous Inhibition of AKT and MEK Cooperatively Induces Apoptosis in a Subgroup of Glioblastoma Cell Lines

To test whether sensitivity of LN71 and SF767 cells to the AAE788-patupilone combination is due to low phosphorylation levels of AKT and/or ERK, inhibitors of PI3K (wortmannin) and MEK (U0126) were used to abrogate activation of AKT and ERK pathways. The potential to induce cell death was tested by applying single or combined inhibitors to four glioblastoma cell lines and measuring the proportion of apoptotic cells (Fig. 4A). When used as a single compound, U0126 was able to induce a significant level of apoptosis in LN215 and LN229 cells but to a much lesser extent in U373 and LN401 cells, whereas wortmannin alone had no or very little effect. However, strong induction of apoptosis was caused by the combination of the two inhibitors. On U0126 application, proliferation was significantly decreased up to 50% in LN229 cell line ($P < 0.001$), whereas U373 cells showed a 20% reduction. Again, wortmannin alone had no effect on reduction of proliferation on both LN229 and U373 cells, and no synergistic effect was present after drug combination (Fig. 4B).

U373 and LN401 generally exhibited much less sensitivity toward the two inhibitors. To examine the long-term effect of the inhibitors on phosphorylation of AKT and ERK, the two most sensitive and the two much less sensitive cell lines were treated for 24 h as well as for 3 days by applying fresh doses of the compounds daily. The activation status of AKT and ERK was determined as a readout for drug efficiency (Fig. 4C). In response to the combination of U0126 and wortmannin, a decrease of

phosphorylated ERK and phosphorylated AKT occurring in all glioblastoma lines confirmed the activity of the two drugs used. Unexpectedly, on treatment with the MEK inhibitor, U0126, U373, and LN229 cell lines show a decrease of phosphorylated AKT levels, suggesting a cross-talk between the RAS/MEK/ERK and PI3K/AKT pathways.

In the sensitive cell lines LN215 and LN229, simultaneous inactivation of protein kinases AKT and MEK was associated with poly(ADP-ribose) polymerase cleavage and apoptosis (Fig. 4D). However, in the resistant cell lines U373 and LN401, AKT and MEK inactivation was not sufficient to trigger apoptosis. There was no correlation between glioblastoma cell line sensitivity or resistance to apoptosis and a given genotype (Fig. 1A).

### Phorbol Ester TPA Restores Activation of MEK and Inhibits Induction of Apoptosis

Phorbol ester TPA activates protein kinase C and ERK1/2, thereby modifying mitogenic signaling pathways (44, 48, 49). When exposed to 20 or 100 nmol/L of TPA, LN215 and LN229 cells showed a strong increase of phosphorylated ERK levels (Fig. 5A). Treatment of the cell lines with combined U0126 and wortmannin for 4 days in the presence of TPA (20 or 100 nmol/L) led to a substantial increase of activated ERK1/2, whereas phosphorylation was completely down-regulated without TPA (Fig. 5A). In parallel, the extent of the induction of apoptosis was determined after 4 days under the same conditions, revealing that the addition of TPA to both glioblastoma cell lines substantially decreased the induction of apoptosis caused by the two inhibitors (Fig. 5B). Taken together, the results further supported the model of a correlation between down-regulation of the two signaling pathways and induction of apoptosis.

## Discussion

We report that the combination of the inhibitor of EGFR kinase AEE788 and of the microtubule depolymerization inhibitor patupilone synergistically induced death of glioblastoma cells. Of high interest is that this synergy occurred at drug concentrations that were not effective to induce cell death when each drug was applied alone. AEE788 alone at 1 μmol/L only induced low levels of apoptosis in glioblastoma cells *in vitro*. Its main antitumor activity consists of inhibition of cell growth and motility (50). Consistently, survival of animals bearing intracranial tumors had been extended by giving AEE788 at concentration below the maximally tolerated dose (34) and confirmed a role of AEE788 as an antitumor agent. However, lasting responses cannot be obtained with a cytostatic effect but require induction of tumor cell death, which may only be achieved by drug combinations. Therefore, we designed a strategy to trigger glioblastoma cell apoptosis by combining the PKI AEE788 with patupilone, a compound with strong cytotoxic potential against various cancer types and also against multidrug resistance cancer cell lines (51). Glioblastoma cell death could be triggered in all tumor cell lines *in vitro* by 35 nmol/L patupilone, a dose that can already be toxic *in vivo*. Interestingly, synergistic induction of apoptosis was observed in glioblastoma cells when using 1 μmol/L AEE788 in combination with only 0.1 nmol/L patupilone. Of interest, the genetic backgrounds of the cell lines that responded the best to the combination therapy with AEE788 and patupilone were different with regard to the three main glioma pathways: TP53, PTEN, and p14/p16 (Fig. 1A). Whereas SF767 cells are wild-type at all three loci, LN71 cells are either mutated or null (39), suggesting that such a combination could be applied to a wide spectrum of gliomas. When analyzing the activation status of EGFR, PKB/AKT, and ERK as readouts for critical pathways in the glioblastoma signaling network, we found that synergistic induction of apoptosis by combining patupilone and AEE788 was paralleled by inactivation of PKB/AKT and ERK. Conversely, the two critical cell signaling mediators remained active in glioblastoma cells that did not show this drug synergism. Although AEE788, patupilone, and U0126, applied individually, reduced glioblastoma cell growth, AEE788/patupilone and U0126/wortmannin combinations

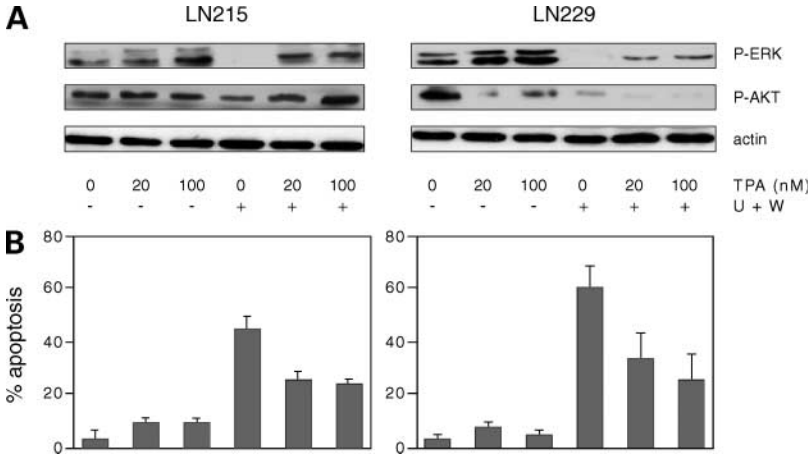


**Figure 5.** Phorbol ester TPA antagonizes induction of apoptosis and maintains AKT and ERK1/2 activities. **A,** cells were treated with 20 and 100 nmol/L of TPA in the presence or absence of wortmannin (W) and U0126 (U) for 3 d, whereby the additions were renewed every day. Cell extracts were prepared by the addition of SDS sample buffer, and Western blot analysis was done. U0126, 20 μmol/L; wortmannin, 1 μmol/L; TPA, 20 or 100 nmol/L. **B,** cells were treated in the same way for 4 d and the percentage of dead cells was determined by flow cytometry.

did not affect cell growth in a synergistic manner, suggesting that these processes are driven by distinct pathways. The importance of the two signaling pathways PI3K/PKB and RAS/RAF/MEK/ERK for glioblastoma signaling is further supported by the finding that PI3K is activated in the majority of human glioblastoma either by loss of PTEN function (38, 39) or by activating mutations of PI3KCA (22), whereas growth factor stimulation causes activation of RAS in glioblastoma (52). Further evidence stems from a murine transgenic glioblastoma model, in which simultaneous activation of RAS and PKB gives rise to malignant gliomas (53, 54). Glioma animal models have further shown that the combination of ectopic expression of activated RAS and AKT in the glial lineage (23) led to spontaneous induction of murine gliomas, whereas an activated allele of either RAS or AKT alone failed to induce tumor formation. In analogy to this animal model and the effects on signaling induced by the described synergism, we hypothesized that direct inhibition of signaling mediators downstream of EGFR within the PI3K/PKB and RAS/RAF/MEK/ERK pathways will overcome glioblastoma cell resistance. Therefore, we specifically blocked activity of both MEK and PI3K, which induced strong apoptosis in a subgroup of glioblastoma cell lines, indicating a critical role of these two pathways in glioblastoma signaling. Combining the MEK inhibitor U0126 and the PI3K inhibitor wortmannin led to synergistic induction of apoptosis in LN215 and LN229 cell lines. Interestingly, although phosphorylation of ERK1/2 and PKB/AKT was decreased in all cell lines, it was not associated with apoptosis in resistant lines, suggesting additional defects in the proapoptotic machinery. Activation of protein kinase C and ERK1/2 by TPA counteracted U0126- and wortmannin-dependent apoptosis, confirming the specificity of drug effect.

An unresolved question is at which cellular level the intervention has to take place. Our data argue that combined blocking of signaling mediators downstream of growth factor receptors, interfering with the signaling cross-talk, may be more effective than inhibition of a single cell surface receptor (e.g., EGFR; ref. 13). This view is supported by other findings in glioblastoma cell lines, where inhibition of PKB/AKT could be counteracted by a stimulation of insulin-like growth factor receptor-I, resulting in sustained activation of PI3K (19, 20).

In conclusion, our results indicate that the induction of apoptosis in glioblastoma cell lines requires drug combination, which down-regulate distinct pathways. Blocking EGFR activation alone did not induce apoptosis unless complemented with the microtubule stabilizer patupilone. If PKIs were targeted without the help of cytotoxic drugs, combined inhibition of MEK and PI3K was found to be the most efficient combination to induce glioblastoma cell death. Drug effects were independent of mutation statuses at the major glioblastoma pathways (Fig. 1A). Additional studies are needed to develop other target for treatment of resistant glioblastoma cells to directly down-regulate key members of the critical pathways.

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