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Low-Dose BBR3610 Toxicity in Colon Cancer Cells Is p53-Independent and Enhanced by Inhibition of Epidermal Growth Factor Receptor (ERBB1)-Phosphatidyl Inositol 3 Kinase Signaling

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ABSTRACT

We have examined the mechanisms by which the multinuclear platinum chemotherapeutic BBR3610 kills human colon cancer cells. BBR3610 more efficiently killed HCT116, DLD1, SW480, and HT29 cells than BBR3464, cisplatin, or oxaliplatin. The amount of platinum uptake per cell and its incorporation into DNA were identical for BBR3464 and BBR3610. BBR3610 lethality (IC_{75}) was unaltered comparing HCT116 wild-type and p53−/− cells, was reduced in p21−/− cells, and was enhanced in K-RAS D13 null cells. Small molecule or molecular inhibition of epidermal growth factor receptor (ERBB1) or phosphatidyl inositol 3 kinase (PI3K) enhanced BBR3610 toxicity in HCT116, DLD1, and SW480 cells. Small molecule or molecular inhibition of caspase 8 function abolished the toxicity of BBR3610 and of BBR3610 + ERBB1 inhibitor treatments, whereas inhibition of caspase 9 suppressed the ability of ERBB1 inhibitors to enhance BBR3610 lethality. Treatment with BBR3610 reduced AKT activity; the expression of dominant-negative AKT enhanced and expression of constitutively active AKT suppressed, respectively, the toxicity of BBR3610 and of BBR3610 + ERBB1 inhibitor treatments. Treatment with BBR3610 reduced expression of c-FLIP-s and MCL-1, levels that were maintained in cells expressing constitutively active AKT. Overexpression of c-FLIP-s or loss of BID function suppressed BBR3610 toxicity, whereas overexpression of XIAP or Bcl-xL suppressed the potentiation of cell killing by ERBB1 inhibitors. Collectively, our data argue that BBR3610 promotes cell killing via a caspase 8-dependent mechanism, which can be enhanced by ERBB1/PI3K inhibitors that promote additional BBR3610-dependent cell killing via activation of BAX and caspase 9.

Cisplatin is known to be one of the most active antitumor drugs (Kelland, 1993). In addition to cisplatin a variety of mononuclear platinum compounds have been developed, including carboplatin and oxaliplatin (Harrap, 1985; Wong and Giandomenico, 1999). However, it has also been established that polynuclear platinum compounds, in which two or three platinum coordination units are linked through alkanediamine or polyamine chains, are an important and structurally distinct new class of potential therapeutic agents with greater activity than the first-generation mononuclear plat-

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated extracellular regulated kinase; PI3K, phosphatidyl inositol 3 kinase; −/−, null/gene deleted; MAPK, mitogen-activated protein kinase; PD184352, 2-(2-chloro-4-iodophenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide; JNK, c-Jun NH_2-terminal kinase; FAS, fatty acid synthase; FADD, fatty acid synthase-associating death domain protein; WT, wild type; BBR3464, (SP-4–1)-diamminebis((SP-4–2)-diamminechloroplatinum(π) (μ-hexane-1,6-diamine))platinum tetranitrate; ECL, enhanced chemiluminescence; DMSO, dimethyl sulfoxide; JNK-IP, c-Jun NH_2-terminal kinase inhibitor peptide; ERBB1, epidermal growth factor receptor; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; siRNA, small interfering RNA; MEF, mouse embryonic fibroblast; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; AG1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; PD98059, 2′-amino-3′-methoxyflavone; ABT-737, (*R*)-4-(3-dimethylamino-1-phenylsulfanylmethyl-propylamino)-*N*-{4-[4-(4′-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl}-3-nitro-benzenesulfonamide; BMS-354825, *N*-(2-chloro-6-methylphenyl)-2-((6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-yl)amino)thiazole-5-carboxamide.

inum compounds (Perego et al., 1999, Pratesi et al., 1999, Farrell, 2004; Manzotti et al., 2000). The first multiplatinum compound to undergo human clinical trials, the trinuclear BBR3464, demonstrated objective responses in phase I trials and phase II ovarian cancer and non–small-cell lung cancer (Farrell, 2004; Gourley et al., 2004; Hensing et al., 2006). The toxic effects of BBR3464 were noted to be p53-independent and seem to be mechanistically different from those of cisplatin with respect to their DNA interactions (Kasparkova et al., 2004). The "central" platinum unit in BBR3464 contributes to DNA binding only through hydrogen-bonding and charge effects, which also contribute (Farrell, 2004). The second-generation agent BBR3610 contains two platinum coordination units and was designed to replace the central platinum unit of BBR3464 by an amine but with the same overall length between the platinum-chloride bonds responsible for DNA platination (Farrell, 2004). The dinuclear polyamine-linked compounds in general display activity profiles similar to those of BBR3464, and cytotoxicity is dependent on the nature of the polyamine (Farrell, 2000). BBR3610 treatment of glioma cells caused more killing in vitro on a molar basis than BBR3464. Cell killing correlated with a G_2/M cell cycle arrest, which was in part dependent on enhanced ERK1/2 signaling (Billecke et al., 2006).

The apoptotic cell death threshold of cells is modulated by the activities of multiple signal transduction pathways and the expression, usually controlled by the signaling pathways, of multiple pro- and antiapoptotic proteins (Grant and Dent, 2002; Dent et al., 2003; Valerie et al., 2007). Activation of the epidermal growth factor receptor (EGFR, also called ERBB1) stimulates signaling through both the PI3K-AKT and RAF-ERK1/2 pathways, which have been linked to increased expression of many cytoprotective proteins, including Bcl-2 family members and inhibitor of apoptosis proteins. In colon cancer cells, activation of ERBB1 and expression of mutated active K-RAS proteins has been shown to protect this cell type from a wide variety toxic stresses, including platinum therapeutic agents (De Luca et al., 1997; Janmaat and Giaccone, 2003). In general, PI3K-AKT signaling in tumor cells has been argued to be a greater protective signal than signaling by RAF-ERK1/2, and in contrast to a general perceived role of RAF-ERK1/2 signaling being protective, it should be noted that several studies exist using small-molecule inhibitors of MEK1/2 suggest that platinum agent-induced ERK1/2 signaling plays a key role in promoting drug toxicity, including BBR3610 (Zhuang and Schnellmann, 2006; Singh et al., 2007).

The mechanisms by which cells process the DNA damage of mononuclear drugs cisplatin and oxaliplatin leading to tumor cell death have been investigated extensively (Siddik, 2003; Wang and Lippard, 2005). In a cell type-dependent fashion, cisplatin and oxaliplatin have been shown to promote the activation of caspase 8 (extrinsic apoptosis pathway) or cause mitochondrial dysfunction and activation of caspase 9 (intrinsic apoptosis pathway) (Schneiderman et al., 1999; Lacour et al., 2004; Toyozumi et al., 2004). In a variety of colon cancer cell lines, oxaliplatin has been shown to promote predominantly activation of the extrinsic or, to a lesser extent, intrinsic apoptosis pathways (Arango et al., 2004; Griffiths et al., 2004; Galligan et al., 2005; McDermott et al., 2005; Longley et al., 2006). Cisplatin toxicity can be enhanced by inhibition of cell cycle checkpoints, signaling pathways, and in combination with other therapeutic modalities, such as ionizing radiation. The present studies were performed to understand in greater detail the molecular mechanisms by which the novel dinuclear platinum-containing agent BBR3610 (as the chloride salt) caused colon carcinoma tumor cell death; whether inhibition of ERBB1 function enhanced the lethality of the platinum drug in a synergistic fashion; and the molecular mechanisms by which these events occurred.

Materials and Methods

Materials. Phospho-/total-ERK1/2, phospho-/total-JNK1/2, phospho-/total-p38 MAPK, Anti-S473 AKT and total AKT, caspase 8, c-FLIP, XIAP, MCL-1, and Bcl-xL antibodies were purchased from Cell Signaling Technologies (Danvers, MA). All of the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI3K inhibitor (LY294002), JNK inhibitor peptide (JNK-IP), and ERBB1 inhibitor (AG1478) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at −80°C. Enhanced chemiluminescence (ECL) kits were purchased from GE Healthcare (Chalfont St Giles, Buckinghamshire, UK) and PerkinElmer Life and Analytical Sciences (Waltham, MA). Trypsin-EDTA and Dulbecco's modified Eagle's medium were purchased from Invitrogen (Carlsbad, CA). BAX/BAK−/−, BIM−/−, and BID−/− fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University, Boston, MA). Transformed protein kinase R-like endoplasmic reticulum −/− cells were a kind gift from the Ron Laboratory (Skirball Institute, New York University School of Medicine, New York, NY). The Bcl-xL and activated MEK1 EE adenoviruses were kindly provided by Dr. J. Moltken (University of Cincinnati, Cincinnati, OH). Other reagents were of the highest quality commercially available (Yacoub et al., 2006, 2007; Mitchell et al., 2007).

Culture of Human Colon Cancer Cells and Drug Treatments for Short-Term Viability Assays. Cells were plated (5×10^4/cm^2), and 24 h after plating, cells either were infected with recombinant adenovirus (see below for details) or were treated with either a platinum-containing agent (0–3.0 μM, as indicated) or AG1478/LY294002 /PD184352 (0–10.0 μM) for up to 96 h as indicated.

Cellular Platinum Accumulation Assays. Cells were plated at 2.0×10^6 cells/ml. BBR3464 or BBR3610 was added in 20 μM. After 8 h, cells were harvested and washed twice with phosphate-buffered saline. The cell pellets were then heated in nitric acid followed by the addition of hydrogen peroxide and hydrochloric acid, according to the United States Environmental Protection Agency procedure 3050b (all volumes reduced by 1/10) and diluted with Milli-Q water (Millipore Corporation, Billerica, MA). Platinum analysis was performed on a Vista-MPX simultaneous inductively coupled plasma optical emission spectroscopy at 265 nm (Varian Inc., Palo Alto, CA). Standards and vehicle control were prepared the same as the samples.

Assessment of Platinum Accumulation in DNA. Cells were plated at 2.0×10^6 cells/ml. BBR3464 or BBR3610 was added at 55 μM. After 10 h, cells were harvested and washed twice with phosphate-buffered saline. DNA was then extracted from the cell pellets using a high salt method (Miller et al., 1988; Montagna et al., 2002). In brief, the cell pellets were treated with nuclei lysis buffer, proteinase K, 10% SDS, and 6 M NaCl. DNA was then precipitated using isopropanol and 3 M sodium acetate. DNA was rinsed using 70% ethanol, and the purity of the DNA was measured at an absorbance of 260 nm. The DNA was then harvested for platinum analysis according to the United States Environmental Protection Agency procedure 3050b as explained above.

Culture of Human Colon Cancer Cells and Drug Treatments for Colony Formation Assays. Cells were plated (250–

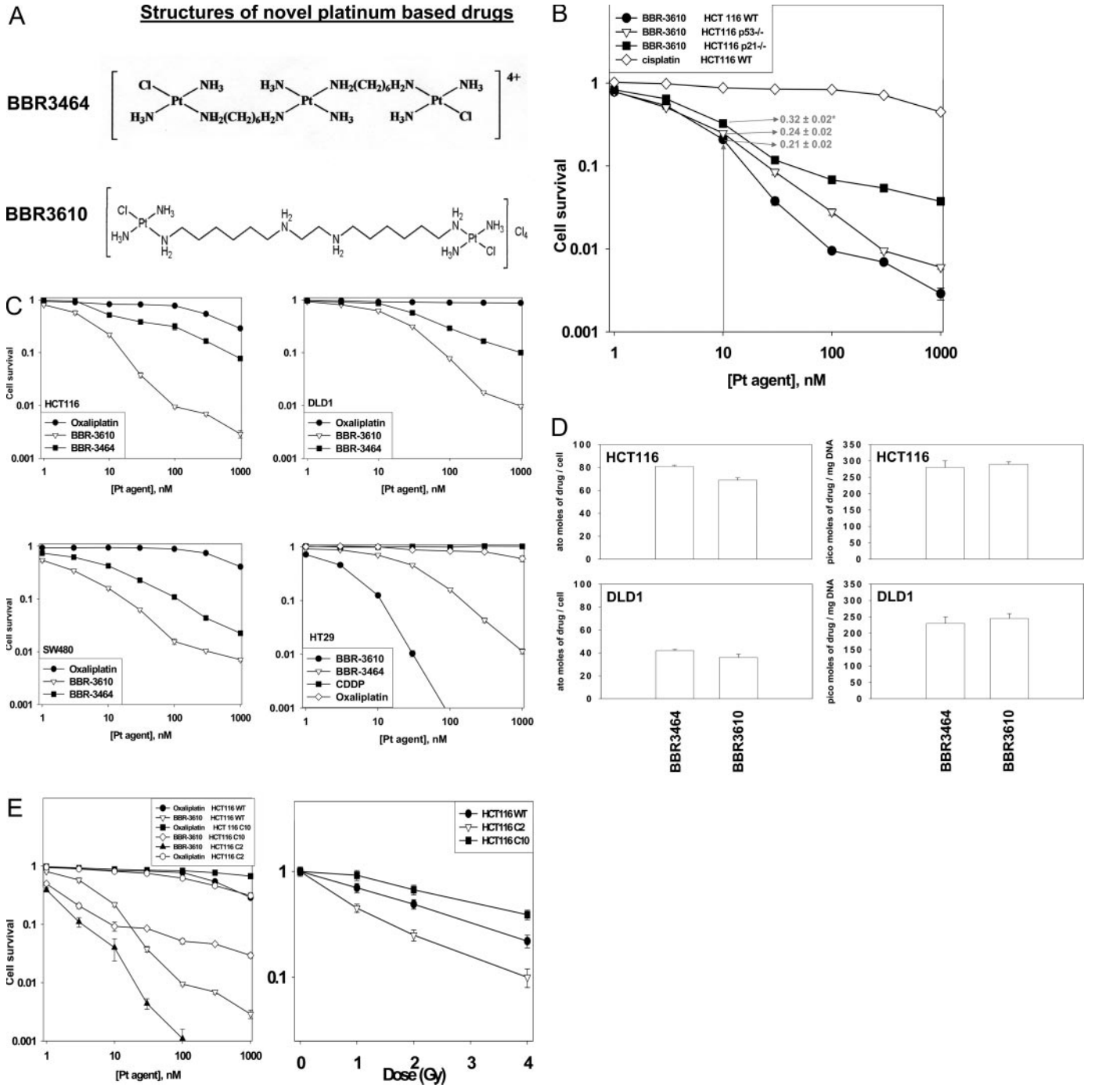

Fig. 1. BBR3610 is a more toxic agent than BBR3464, oxaliplatin, and cisplatin in colon cancer cells. A, chemical structures of BBR3464 and BBR3610. B, HCT116 cells growing in log phase were trypsinized, and single cells were replated in 60-mm dishes (250–2500 cells/dish). Twelve hours after plating, cells were treated with BBR3610 or cisplatin as indicated. Media containing the drug were removed 48 h after treatment, the plates were washed carefully with drug-free media, and then fresh drug-free media were added to the cells. Cells were cultured for 10 to 14 days to permit >50 cell colonies to form. The media were removed, and the cells were fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, and the survival for each condition was calculated. The data are from a representative experiment (n = 2) of six plates per data point (± S.E.M., n = 6). C, HCT116, DLD1, SW480, and HT29 cells growing in log phase were trypsinized, and single cells were replated in 60-mm dishes (250–2500 cells/dish). Twelve hours after plating, cells were treated with BBR3610, cisplatin (CDDP), or oxaliplatin as indicated. Media containing the drug were removed 48 h after treatment, the plates were carefully washed with drug-free media, and then fresh drug-free media were added to the cells. Cells were cultured for 10 to 14 days to permit >50 cell colonies to form. The media were removed, and the cells were fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, and the survival for each condition was calculated. The data are from a representative experiment (n = 2) of six plates per data point (±S.E.M., n = 6). D, uptake and DNA adduct formation of BBR3464 and BBR3610 was measured as described under *Materials and Methods*. Uptake data are expressed as the number of free unbound atomoles of platinum agent within each cell. DNA adduct formation data are expressed as the number of picomoles of platinum drug incorporated per milligram of DNA. Data are in triplicate ± S.E.M. E, HCT116 cells (C2, deleted for the single allele of mutated active K-RAS D13; C10, C2 cells stably expressing low levels of H-RAS V12) were plated as described above for colony-formation assays and either treated with BBR3610 or oxaliplatin or irradiated (Yacoub et al., 2007). Colony formation was determined 10 to 14 days after drug/radiation exposure, and the survival of cells was plotted. Data are from three to six data points per treatment condition ± S.E.M. (n = 2–3).

1500 cells/well of a 6-well plate) and 12 h after plating treated with either platinum agent (0–3.0 μM, as indicated), or AG1478/LY294002/PD184352 (0–10.0 μM) for 48 h as indicated. After 48 h, the drug-containing media were carefully removed, the cells were washed once, and fresh media lacking drugs were added. Colony-formation assays were cultured for an additional 10 to 14 days, after which the media were removed, cells were fixed with methanol, stained with crystal violet, and counted manually.

SDS-PAGE and Western Blot Analysis. Cells were treated with either platinum agent or AG1478/LY294002/PD184352 as indicated. Cells were isolated. For SDS-PAGE and immunoblotting, cells were lysed in either a nondenaturing lysis buffer and prepared for immunoprecipitation or in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromphenol blue), and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole-cell lysis buffer. The boiled samples were loaded onto 10 to 14% SDS-PAGE, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm of nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe PhotoShop 7.0 (Adobe Systems, Mountain View, CA), their color was removed, and figures were generated using MicroSoft PowerPoint (Microsoft, Redmond, WA). Densitometric analysis for ECL immunoblots were performed using a Fluorochem 8800 Image System and the respective software (Alpha Innotech Corporation, San Leandro, CA), and band densities were normalized to that of a total protein loading control.

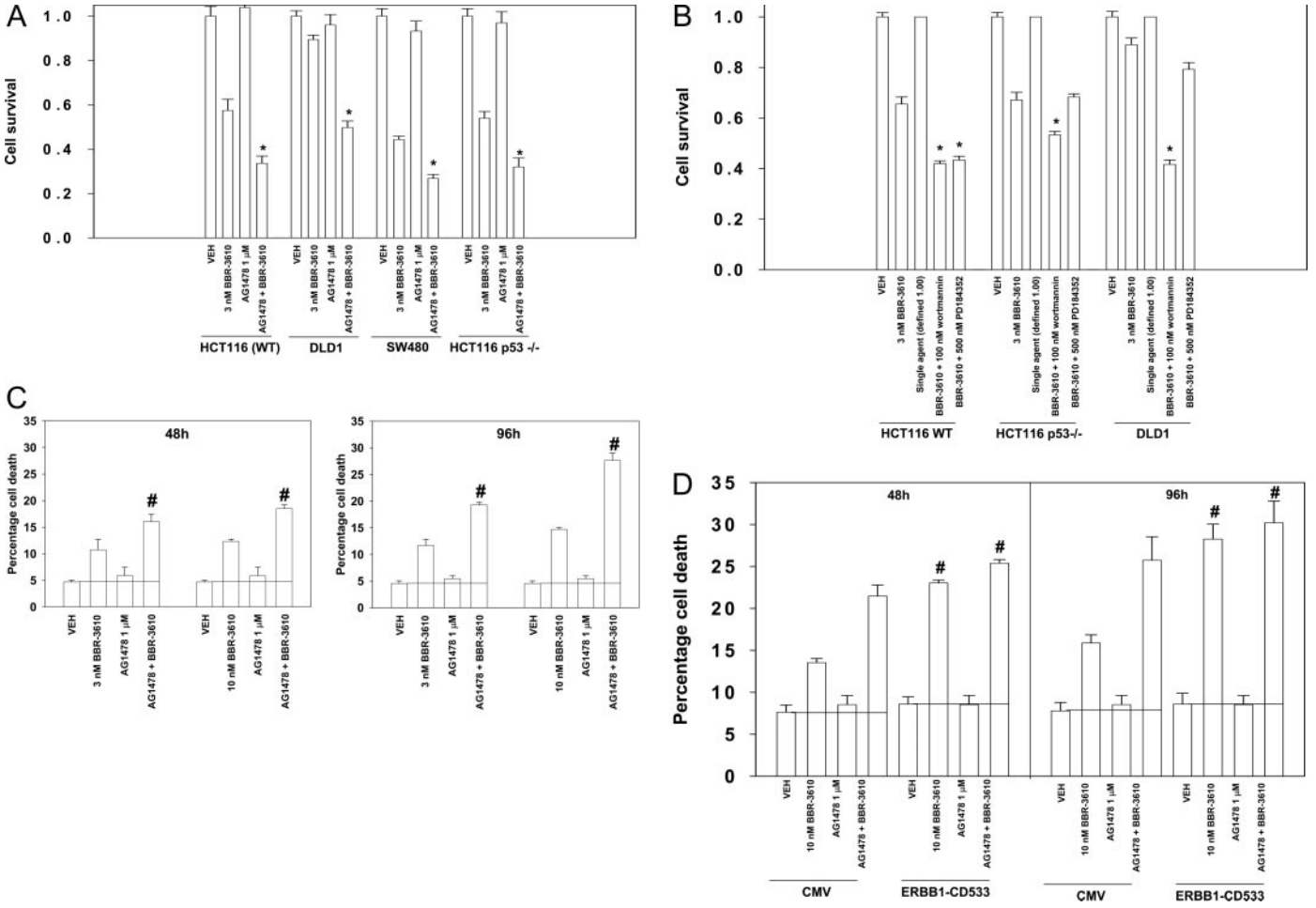

Fig. 2. The lethality of BBR3610 is p53-independent and is enhanced by inhibition of ERBB1 or PI3K signaling. A, HCT116, DLD1, and SW480 cells growing in log phase were trypsinized, and single cells were replated in 60-mm dishes (250–2500 cells/dish). Twelve hours after plating, cells were treated with BBR3610 and AG1478 as indicated. Media containing the drugs were removed 48 h after treatment, the plates were carefully washed with drug-free media, and then fresh drug-free media were added to the cells. Cells were cultured for 10 to 14 days to permit >50 cell colonies to form. The media were removed, and the cells fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, and the survival for each condition was calculated. The data are from a representative experiment ($n = 2$) of six plates per data point (±S.E.M., $n = 6$). B, HCT116 and DLD1 cells growing in log phase were trypsinized, and single cells were replated in 60-mm dishes (250–2500 cells/dish). Twelve hours after plating, cells were treated with BBR3610, PD184352, or wortmannin as indicated. Media containing the drugs were removed 48 h after treatment, the plates were carefully washed with drug-free media, and then fresh drug-free media were added to the cells. Cells were cultured for 10 to 14 days to permit >50 cell colonies to form. The media were removed, and the cells were fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, and the survival for each condition was calculated. The data for single-agent PD184352 or wortmannin treatment is normalized and defined as 1.00 in the figure. The data are from a representative experiment ($n = 2$) of six plates per data point (± S.E.M., $n = 6$). C, DLD1 cells were plated in triplicate and 24 h after plating were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 48 and 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (#, $p < 0.05$ greater than treatment with BBR3610 alone). D, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV) or with a virus to express dominant-negative ERBB1-CD533. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 48 and 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (#, $p < 0.05$ greater than treatment with BBR3610 alone). VEH, vehicle.

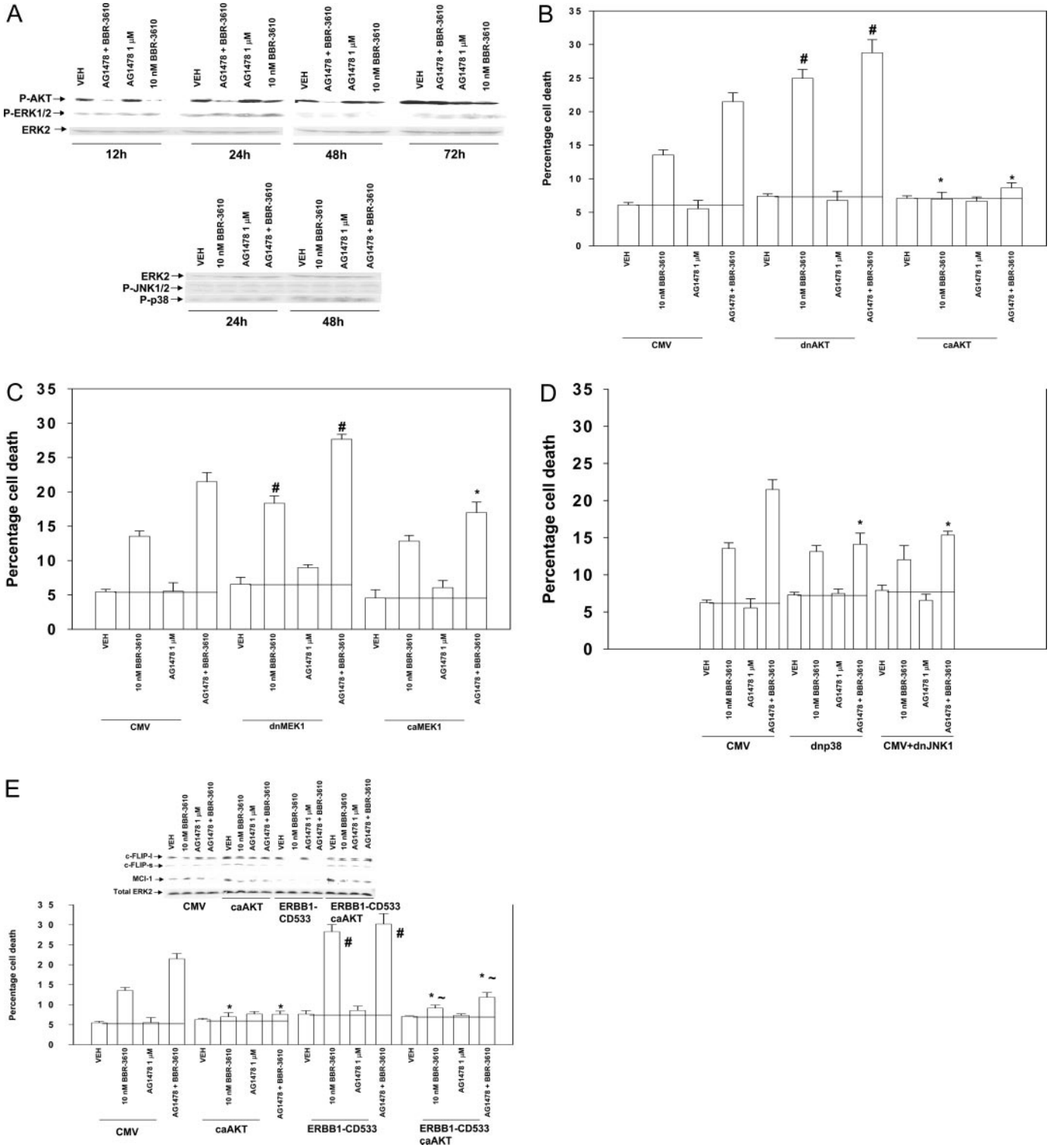

Fig. 3. Activation of AKT suppresses BBR3610 and BBR3610 + ERBB1 inhibition lethality, whereas activation of MEK1 partially suppresses BBR3610 + ERBB1 inhibition lethality: activation of AKT maintains c-FLIP and MCL-1 expression. A, DLD1 cells were plated in 60-mm dishes (10^6 cells) and 24 h after plating were treated with BBR3610 and/or AG1478, as indicated. Cells were isolated at the indicated time points and subjected to SDS-PAGE and immunoblotting then performed to determine the phosphorylation (activity) status of ERK1/2, AKT (Ser473), p38 MAPK, and JNK1/2. Data are from a representative experiment ($n = 2$). B, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV) or with a virus to express dominant-negative AKT or constitutively active AKT. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (*, $p < 0.05$ less than treatment with BBR3610 alone). C, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV) or with a virus to express dominant-negative MEK1 or constitutively active MEK1. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (#, $p < 0.05$ greater than treatment with BBR3610 alone; *, $p < 0.05$

Recombinant Adenoviral Vectors: Generation and Infection in Vitro. We generated and purchased previously noted recombinant adenoviruses to express constitutively activated and dominant-negative AKT and MEK1 proteins, dominant-negative ERBB1 (COOH-terminal 533 amino acid deletion; CD533), dominant-negative caspase 9, CRM A (caspase 8 inhibitor), XIAP, c-FLIP-s, and Bcl-xL (Vector Biolabs, Philadelphia, PA). Colon cancer cells were infected 24 h after plating with these adenoviruses at an approximate multiplicity of infection of 50 for 4 h with gentle rocking, after which time the media were replaced. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures. For transformed mouse embryonic fibroblasts, cells were infected at a multiplicity of infection of 150 as a result of lower CAR levels on these cells.

Detection of Cell Death by Trypan Blue Assays. Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37°C. Because some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1500 rpm for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye-incorporating cells were scored as being dead, was performed by counting of cells using a light microscope and a hemacytometer. Five hundred cells from random fields were counted, and the number of dead cells was counted and expressed as a percentage of the total number of cells counted.

Transfection of DLD1 Cells with Small Interfering RNA Molecules. RNA interference or gene silencing for down-regulating the expression of FADD and CD95 (FAS receptor) was performed using validated target sequences designed by Ambion (Austin, TX). For transfection, 10 nM concentration of the annealed siRNA-targeting FADD or CD95, the positive control doubled-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase, or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines) were used. The siRNA molecules were transfected into cells according to the manufacturer's instructions. Cells were cultured for 48 h after transfection before any additional experimentation.

Data Analysis. Comparison of the effects of various treatments was performed using one-way analysis of variance and a two-tailed Student's t test. Differences with a p value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (±S.E.M.). Characterization of synergistic and antagonistic interactions in cells exposed to a range of BBR3610 and AG1478 concentrations administered at a fixed ratio was done using median dose-effect analysis in conjunction with a commercially available software program (CalcuSyn; Biosoft, Ferguson, MO).

Results

Initial studies examined the toxicity of the novel dinuclear platinum agent BBR3610 in colon cancer cells in comparison with established platinum chemotherapeutic agents and with a multinuclear platinum chemotherapeutic that has undergone phase II trials, BBR3464 (Fig. 1A). BBR3610 was a significantly more toxic agent in colony-formation assays than BBR3464, cisplatin, or oxaliplatin (Fig. 1, B and C). The expression of both p53 and p21 has been linked to the toxicity profiles of cells treated with platinum-containing therapeutic agents (He et al., 2006; Zhou et al., 2006). At approximately the IC_{75} value for BBR3610, loss of p53 function in HCT116 cells did not significantly alter drug toxicity, whereas loss of p21 function modestly suppressed cell killing (Fig. 1B). Although BBR3610 was a more toxic agent than BBR3464, we did not observe significant differences in the uptake of either drug or comparing the incorporation of platinum from either drug into DNA (Fig. 1D). One established mechanism of chemotherapeutic and radio-resistance in colon cancer is due to the high incidence of expression of mutated (active) K-RAS proteins. Deletion of the single K-RAS D13 allele in HCT116 cells markedly enhanced the toxic effects of BBR3610 compared with wild-type cells, an effect that was not readily apparent for oxalipatin (Fig. 1E, on left); expression of H-RAS V12 in HCT116 cells at low BBR3610 doses enhanced BBR3610 toxicity beyond that in wild-type cells, the opposite of the enhanced survival effects of H-RAS V12-expressing cells after irradiation (Fig. 1E, right).

In addition to mutated RAS proteins, colon cancer cells are known to overexpress ERBB receptors and for ERBB receptors to play an important role in colon cancer cell survival. Treatment of HCT116, DLD1, and SW480 cells with low marginally toxic concentrations of an ERBB1 inhibitor, AG1478, enhanced BBR3610 lethality, which was p53-independent (Fig. 2A). Based on median dose-effect analyses using colony-formation assays with a combination index of less than 1, the interaction between BBR3610 and AG1478 was judged to be synergistic in DLD1 and HCT116 cells (Table 1; data not shown). In all lines tested, inhibition of PI3K but not MEK1/2 enhanced the lethality of BBR3610 (Fig. 2B). Be-

TABLE 1

BBR3610 and the ERBB1 inhibitor AG1478 interact in a synergistic fashion to kill colon cancer cells in vitro

DLD1 cells growing in log phase were trypsinized, and single cells were replated in 60-mm dishes (250–2500 cells/dish). Twelve hours after plating, cells were treated with BBR3610 and AG1478 at a fixed ratio. Media containing the drugs were removed 48 h after treatment, the plates were washed carefully with drug-free media, and then fresh drug-free media were added to the cells. Cells were cultured for 10 to 14 days to permit >50 cell colonies to form. The media were removed and the cells were fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, the survival for each condition was calculated, and values were entered into the Calcusyn program. Data shown are from a representative experiment of six plates per data point ($n = 2$). A combination index (CI) value of less than 1.00 indicates synergy.

BBR3610	AG1478	Fa	CI
	μM		
1.5 nM	0.5	0.23	0.32
3 nM	1	0.31	0.52
4.5 nM	1.5	0.40	0.66

Fa, fraction affected.

less than treatment with BBR3610 alone). D, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV) (3 × 8 plates) or with a virus to express dominant-negative p38 MAPK (3 × 4 plates). Twenty-four hours after infection, one portion of CMV-infected cells (3 × 4 plates) were treated with JNK-IP; the other plates were treated with vehicle control (DMSO). Thirty minutes after JNK-IP treatment, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (∗, $p < 0.05$ less than treatment with BBR3610 alone). E, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV) or with a virus to express dominant-negative ERBB1-CD533 or constitutively active AKT, as indicated in the figure. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (#, $p < 0.05$ greater than treatment with BBR3610 alone; ∗, $p < 0.05$ less than treatment with BBR3610 alone; ∼, $p < 0.05$ less than cells expressing ERBB1-CD533 that do not express activated AKT). Inset, cells were infected and treated with drugs in a manner identical with those in the graphic. Cells were isolated 48 h after drug exposure and subjected to SDS-PAGE followed by immunoblotting to determine the expression of c-FLIP proteins, MCL-1, and the total expression of ERK2 ($n = 2$).

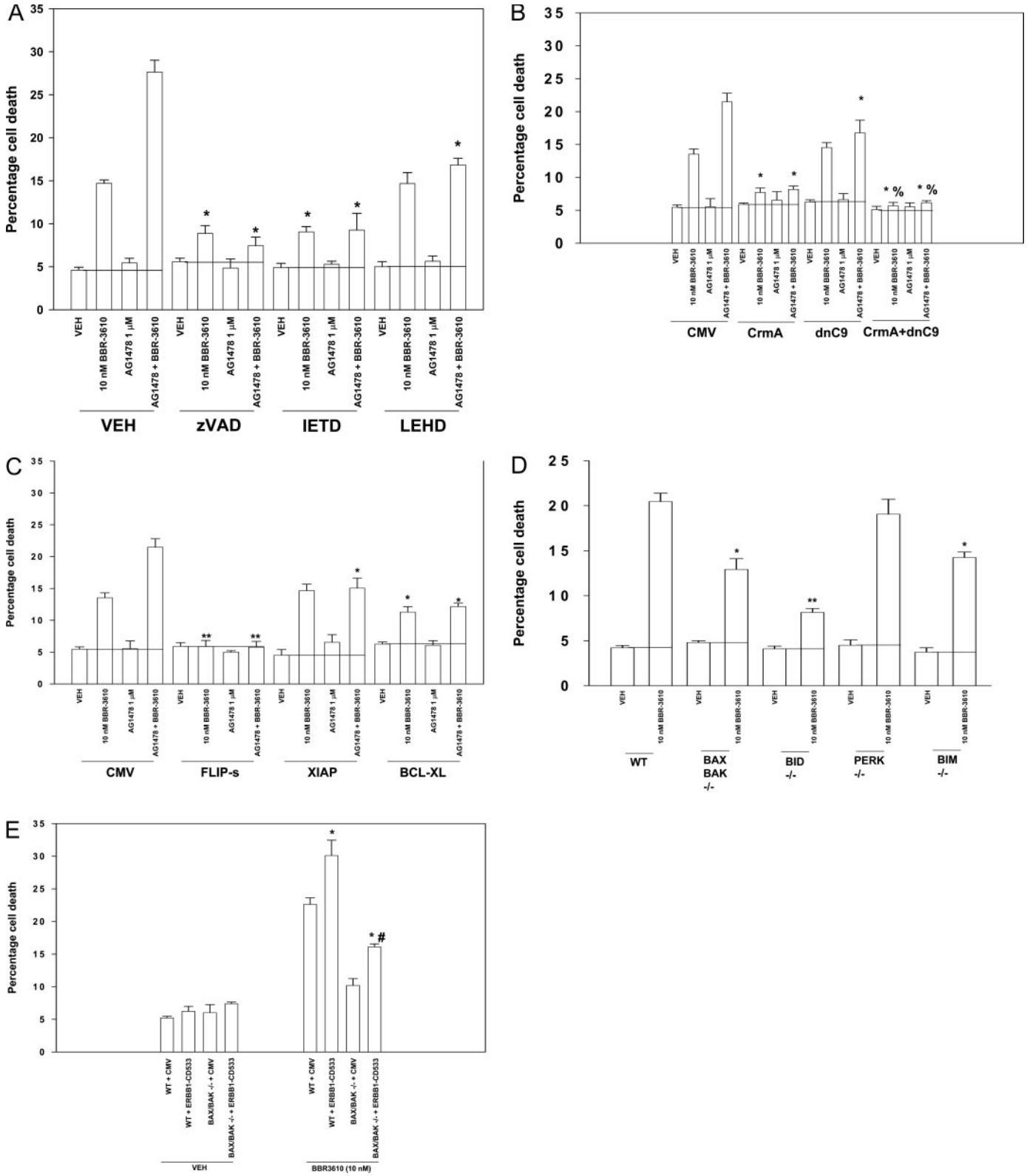

Fig. 4. BBR3610 promotes colon cancer killing by activation of caspase 8; the potentiation of BBR3610 lethality by ERBB1 inhibition is caspase 9-dependent. A, DLD1 cells, 24 h after plating, were treated with BBR3610 and AG1478, as indicated. In parallel, cells were cotreated with vehicle (VEH, DMSO), a pan-caspase inhibitor (benzyloxycarbonyl-Val-Ala-Asp, 50 μM), a caspase 8 inhibitor (IETD, 50 μM), or a caspase 9 inhibitor (LEHD, 50 μM). The caspase inhibitors were resupplemented to the media every 24 h. Cells were isolated by trypsinization 96 h after the addition of BBR3610 and AG1478, and trypan blue assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (*, $p < 0.05$ less than corresponding treatment in VEH-treated cells). B, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV), with a virus to express dominant-negative caspase 9, or with a viral inhibitor of caspase 8, CRM A, as indicated in the figure. Twenty-four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M.

cause the procedures that produced the data presented in Fig. 1 and Table 1 take several weeks to perform, we next determined whether BBR3610 and AG1478 interacted to rapidly promote cell killing within 48 to 96 h of treatment as judged in trypan blue viability assays. AG1478 promoted BBR3610 lethality in DLD1 and in HCT116 tumor cells 48 and 96 h after drug exposure (Fig. 2C; data not shown). In DLD1 cells, expression of a truncated dominant-negative ERBB1 protein (ERBB1-CD533) enhanced BBR3610 toxicity to a similar extent as that induced by the small-molecule ERBB1 inhibitor AG1478 (Fig. 2D).

Chemotherapeutic agents such as Ara C or ionizing radiation promote tumor cell killing by causing the activation of the JNK1/2 and/or p38 MAPK pathways (Carter et al., 1998; Jarvis et al., 1998). Low doses of BBR3610, in the presence or absence of ERBB1 inhibitor, weakly enhanced signaling through the p38 MAPK and JNK1/2 pathways in DLD1 cells (Fig. 3A, bottom). In contrast, BBR3610 significantly reduced the levels of p-AKT and transiently increased p-ERK1/2 levels at 24 h (Fig. 3A, upper section). Inhibition of ERBB1 modestly further suppressed AKT activity 24 to 48 h after exposure. Expression of constitutively active AKT protected DLD1 cells from BBR3610 and BBR3610 + AG1478 toxicity, whereas expression of dominant-negative AKT enhanced the toxicity of BBR3610, AG1478, and BBR3610 + AG1478 (Fig. 3B).

Unlike the relatively specific MEK1/2 inhibitor PD184352, expression of dominant-negative MEK1 enhanced the toxicity of BBR3610, AG1478, and BBR3610 + AG1478 (Fig. 3C). The toxicity of BBR3610 + AG1478 remained significantly greater than the additive individual toxicity of BBR3610 or AG1478, which was in contrast to our observations expressing dominant-negative AKT, in which the greater than additive interaction between BB3610 and AG1478 was lost to the greater enhancement of cell killing (Fig. 3, B and C). Expression of constitutively active MEK1 did not alter BBR3610 toxicity and weakly suppressed the toxicity of BBR3610 + AG1478. Although neither p38 MAPK nor JNK1/2 was strongly activated by any drug treatment, inhibition of either p38 MAPK or JNK1/2 suppressed the ability of ERBB1 inhibition to enhance BBR3610 toxicity (Fig. 3D).

To determine by a molecular approach whether the promotion of BBR3610 toxicity caused by suppression of ERBB1 function was AKT-dependent, we coexpressed dominant-negative ERBB1 and constitutively active AKT and treated cells with BBR3610 and/or AG1478. Expression of constitutively active AKT suppressed BBR3610 toxicity in the presence or absence of dominant-negative ERBB1 (Fig. 3E). We also examined the impact of these genetic manipulations on anti-apoptotic protein expression. Inhibition of ERBB1 did not alter basal c-FLIP expression but enhanced the suppression of c-FLIP levels caused by BBR3610 treatment. Inhibition of ERBB1 suppressed MCL-1 expression. Activation of AKT enhanced basal levels of c-FLIP and MCL-1; AKT activation maintained c-FLIP and MCL-1 levels in cells expressing dominant-negative ERBB1 and when treated with BBR3610 (Fig. 3E, inset). Together, our data argue that BBR3610 suppresses AKT activity, which is causal in cell death, and that inhibition of ERBB1-MEK1/2 signaling, in the presence of already suppressed AKT signaling, further elevates BBR3610 toxicity.

We next examined the molecular pathways by which BBR3610 killed colon cancer cells in vitro. Incubation of DLD1 cells with a pan-caspase inhibitor (benzyloxycarbonyl-Val-Ala-Asp) or a caspase 8 inhibitor (IETD) reduced BBR3610 toxicity, whereas a caspase 9 inhibitor (LEHD) did not alter BBR3610 lethality but abolished the ability of AG1478 to enhance cell killing (Fig. 4A). Similar data were obtained when the caspase 8 inhibitor CRM A and dominant-negative caspase 9 were expressed in DLD1 cells (Fig. 4B). XIAP is an inhibitor of caspase 9, c-FLIP-s is an inhibitor of caspase 8, and Bcl-xL is a protein that in a similar manner to MCL-1 maintains mitochondrial function downstream of caspase 8-BID signaling and from BAX/BAK/BIM-induced cytochrome *c* release. Treatment of cells with BBR3610 increased cleavage of pro-caspase 8 24 and 48 h after exposure (data not shown). Overexpression of c-FLIP-s abolished BBR3610 toxicity (Fig. 4C). Expression of XIAP did not alter BBR3610 lethality but abolished the enhancement of cell killing by ERBB1 inhibition; overexpression of Bcl-xL partially suppressed BBR3610 lethality and abolished the enhancement of cell killing by ERBB1 inhibition (Fig. 4C).

Additional studies were then performed in transformed mouse embryonic fibroblasts (MEFs) lacking key proapoptotic genes, which cause mitochondrial dysfunction. In transformed MEFs, BB3610 lethality was suppressed to a greater extent by loss of BID function than by loss of BAX/BAK or BIM expression (Fig. 4D). The ability of ERBB1 inhibition to enhance BBR3610 lethality was suppressed in cells lacking BAX/BAK function. In addition to caspase 8, BID cleavage and subsequent mitochondrial dysfunction can also be catalyzed by cathepsin B, and loss of cathepsin B expression in cathepsin B−/− MEFs suppressed BBR3610 toxicity (data not shown). Pro-caspase 8 is activated by death receptors via FADD but has also been noted to autocatalyze its own activation independently of FADD when expression of caspase 8 inhibitors such as c-FLIP-s is reduced. Knockdown of FADD and of the FAS death receptor (CD95) suppressed BBR3610

(*, $p < 0.05$ less than treatment with BBR3610 alone; %, $p < 0.05$ less than corresponding value in cells expressing only CRM A). C, DLD1 cells were plated in triplicate and 12 h after plating were infected with either a control empty vector virus (CMV), a virus to express the mitochondrial protective protein Bcl-xL, a virus to express the caspase 9 inhibitor XIAP, or a virus to express the caspase 8 inhibitor c-FLIP-s, as indicated in the figure. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (*, $p < 0.05$ less than corresponding treatment in CMV-infected cells; **, $p < 0.05$ less than corresponding values in all other groups of virally infected cells). D, SV40 transformed mouse embryonic fibroblasts (BAX/BAK−/−, lacking BAX and BAK; BID−/−, lacking BID; PERK−/−, lacking protein kinase R-like endoplasmic reticulum kinase; BIM−/−, lacking BIM), 24 h after plating, were treated with BBR3610. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (*, $p < 0.05$ less than the value in WT cells; **, $p < 0.05$ less than the value in all other fibroblast cell types). E, wild-type and BAX/BAK−/− fibroblasts, 12 h after plating, were infected with either a control empty vector virus (CMV) or with a virus to express dominant-negative ERBB1-CD533, as indicated in the figure. Twenty-four hours after infection, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96 h after drug exposure, and trypan blue cell viability assays were performed. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M.

toxicity in DLD1 cells (Fig. 5). Together, these findings demonstrate that BBR3610 initiates cell killing via a death receptor-dependent activation of pro-caspase 8 that is facilitated by reduced expression of c-FLIP proteins and reduced AKT signaling; inhibition of ERBB1 promotes BBR3610 lethality by facilitating BID- and BAX/BAK/BIM-dependent mitochondrial dysfunction and activation of the caspase 9 pathway.

Discussion

Platinum-containing cancer therapeutic drugs are used in the treatment of many malignancies including testicular, ovarian, lung, and colon cancer. However, the development of resistance to mononuclear platinum agents is common, and the synthesis of new platinum-containing therapeutic drugs that exhibit a greater lethality and activity in tumors historically resistant to the established drugs such as cisplatin is an important developmental goal for better tumor control and patient outcomes.

In vitro, the dinuclear platinum agent BBR3610 is a more toxic agent in multiple colon cancer cell lines compared with the trinuclear platinum agent BBR3464, a differential effect in cell killing that was not initially explained by either drug uptake or drug incorporation into DNA. Furthermore, we have reported previously that BBR3464 and BBR3610 are at least equipotent as antitumor agents in xenograft mouse studies and more potent than cisplatin (Manzotti et al., 2001). Future studies will be required to determine at the molecular level why BBR3463 and BBR3610 have differential in vitro toxicities. In the present studies we have defined how BBR3610 acted to kill colon cancer cells. BBR3610 suppressed AKT activity, which was further enhanced by inhibition of ERBB1 and was correlated with reduced expression of c-FLIP proteins and of Bcl-xL. Expression of activated AKT maintained the expression of c-FLIP proteins and Bcl-xL in cells treated with BBR3610 with or without ERBB1 inhibition. Expression of activated AKT suppressed BBR3610 lethality and the ability of ERBB1 inhibition to promote BBR3610 toxicity, whereas expression of dominant-negative AKT enhanced BBR3610 lethality to an extent that was not further enhanced by inhibition of ERBB1. BBR3610 lethality was dependent on activation of the death receptor-extrinsic/caspase 8 apoptosis pathway as judged by the ability of CRM A, IETD, and knockdown of FADD and CD95 expression to significantly suppress all drug-induced lethality effects. BBR3610 lethality as a single agent was independent of p53 in HCT116 cells, in contrast to the findings of others using oxaliplatin (Arango et al., 2004). The ability of ERBB1 inhibitors to promote BBR3610 lethality was reliant on mitochondrial dysfunction/BAX-BAK/caspase 9 activation and was also p53-independent, as judged by the suppression of this effect by expression of dominant-negative caspase 9 or XIAP. Note that all cell-killing effects were dependent on prior platinum agent-induced activation of caspase 8, as judged by overexpression of the specific caspase 8 inhibitor c-FLIP-s abolishing drug-induced increases in cell death.

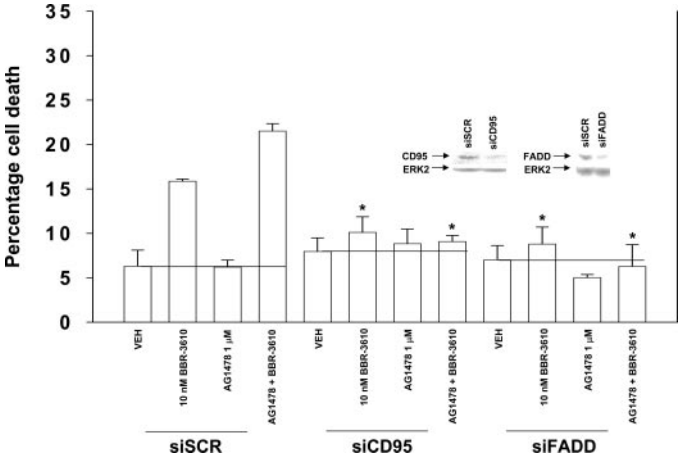

Fig. 5. Knockdown of CD95 or of FADD suppresses the lethality of BBR3610. DLD1 cells 12 h after plating were transfected with siRNA molecules to knock down the expression of FADD or of CD95 (FAS receptor) or with a scrambled siRNA (SCR) as described under *Materials and Methods*. Forty-eight hours after transfection, cells were treated with BBR3610 and/or AG1478. Forty-eight hours after drug treatment, cells were isolated, and cell viability was determined using trypan blue exclusion assays. Data are from a representative experiment ($n = 2$) of triplicate samples ± S.E.M. (*, $p < 0.05$ less than the value in SCR-transfected cells). Inset, 48 hours after transfection, cells from each condition before drug treatment were isolated and subjected to SDS-PAGE followed by immunoblotting to determine the expression of CD95, FADD, and ERK2.

Previous studies using mononuclear platinum agents and BBR3464 have correlated platinum agent-induced ERK1/2 activation to a proapoptotic response, with many of these studies using the MEK1/2/5 inhibitors PD98059 and U0126 (Billecke et al., 2006). Using the more specific MEK1/2 inhibitor PD184352 and molecular approaches, we now show that small-molecule inhibition of the RAF-ERK1/2 pathway at the time of platinum-agent exposure enhanced BBR3610 lethality in HCT116 cells in a p53-dependent fashion. Molecular inhibition of the RAF-ERK1/2 pathway modestly enhanced both BBR3610 and BBR3610 and ERBB1 inhibitor lethality; however, unlike expression of dominant-negative AKT, expression of dominant-negative MEK1 did not abolish the ability of the ERBB1 inhibitor AG1478 to promote BBR3610 lethality. Together, our data argue that treatment of colon cancer cells with low doses of BBR3610 promotes inactivation of AKT that is further enhanced in these cells by inhibition of ERBB receptor function, which lead to suppressed expression of multiple antiapoptotic proteins permitting activation of the intrinsic and extrinsic apoptosis pathways.

The molecular mechanisms by which BBR3610 stimulates both death receptor signaling and inhibition of AKT activity remain to be determined. One potential mechanism by which BBR3610 could cause cell killing, which would correlate with death receptors and with dephosphorylation of AKT, could increase levels of the proapoptotic lipid ceramide. Ceramide has been linked to stimulating both ligand-independent clustering of death receptors and to activation of PP2A isoforms (Ruvolo et al., 2002; Lacour et al., 2004). Cisplatin has been shown to increase ceramide levels in tumor cells, and further studies are required to determine whether BBR3610 significantly alters ceramide levels in colon cancer cells. Because knockdown of CD95 expression abolished BBR3610-induced cell killing, our findings indicate that BBR3610-induced changes in CD95 function are likely to be a primary effector in the chain of proapoptotic events that occur via two pathways: CD95–FADD–caspase 8–caspase 3 and caspase 8–BID–mitochondrial dysfunction–caspase 9–caspase 3.

Prior studies using oxaliplatin have argued that treatment of colon cancer cells with tumor necrosis factor-related ligand or knockdown of FLIP protein expression can enhance the

toxicity of the platinum agent (Galligan et al., 2005; Longley et al., 2006). Of particular note, whereas overexpression of c-FLIP-s protected HCT116 cells from BBR3610 toxicity, overexpression of c-FLIP-l but not c-FLIP-s protected cells from oxaliplatin lethality (Longley et al., 2006). Griffiths et al. (2004) argued in colon cancer cells that Src-induced expression of Bcl-xL and suppression of CD95 function blunted oxaliplatin lethality (Griffiths et al., 2004). Our data argued that BBR3610 as a single agent can suppress MCL-1 levels and those of FLIP proteins. Our findings together with those of other groups argue that BBR3610 lethality has the potential to be enhanced not only by inhibitors of ERBB1-PI3K signaling but also by inhibitors of Src kinases, such as BMS-354825 (dasatinib) or additional mitochondrial protective proteins such as Bcl-2 and Bcl-xL (e.g., ABT-737) (Chen et al., 2007; Nguyen et al., 2007).

Inhibition of ERBB1 enhanced BBR3610 toxicity that was caspase 9-dependent, an effect that was also reliant on the initial BBR3610-dependent activation of caspase 8. Neither BBR3610 nor inhibition of ERBB1 enhanced JNK1/2 or p38 MAPK activity in total cell lysates, but inhibition of either pathway suppressed the ability of ERBB1 inhibition to promote mitochondrial dysfunction and cell killing. A variety of studies have argued that mitochondrial toxic agents can promote activation of BAX and BAK via JNK1/2 and p38 MAPK signaling and loss of BAX/BAK function suppressed BBR3610 toxicity and the ability of ERBB1 inhibition to promote BBR3610 toxicity (Mitchell et al., 2007). Additional studies beyond the scope of this article are required to determine whether selected pools of JNK1/2 and p38 MAPK become activated after BBR3610 and AG1478 drug treatment and play a role in activating BAX and BAK. It will be of interest in future studies to determine whether clinically relevant proprietary inhibitors of ERBB receptor signaling such as cetuximab and lapatinib can also promote BBR3610 lethality in a similar manner to AG1478 and ERBB1-CD533.

Understanding the mechanisms by which a particular agent causes cell killing permits the subsequent rational combination of that agent with other drugs that activate complementary death-inducing processes. With the advent of targeted drugs to the clinic, understanding how they can best be combined with clinically useful cytotoxics is an important developmental goal. The present studies have demonstrated that BBR3610 kills colon tumor cells by activating the extrinsic and, to a lesser extent, intrinsic pathways, and that inhibition of ERBB1 facilitates killing by permitting additional mitochondrial dysfunction to take place. Based on our data, it could be hypothesized that BBR3610 toxicity could also be enhanced by the following: 1) therapeutic agents, which cause further activation of the extrinsic pathway (e.g., tumor necrosis factor-related ligand); 2) therapeutic agents, which cause further DNA damage and mitochondrial dysfunction (e.g., ionizing radiation); and 3) therapeutic agents, which suppress the expression of multiple cytoprotective proteins (e.g., flavopiridol). Further studies will be required to determine whether TRAIL, CDK9 inhibitors, or ionizing radiation can further enhance the lethal actions of (BBR3610 + ERBB1 inhibitor) treatment in human colon cancer cells.

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