17-Allylamo-17-Demethoxygeldanamycin and MEK1/2 Inhibitors Kill GI Tumor Cells via Ca\(^{2+}\)-Dependent Suppression of GRP78/BiP and Induction of Ceramide and Reactive Oxygen Species

Teneille Walker\(^1\), Clint Mitchell\(^1\), Margaret A. Park\(^1\), Adly Yacoub\(^1\), Mohamed Rahmani\(^2\), Dieter Häussinger\(^6\), Roland Reinehr\(^6\), Christina Voelkel-Johnson\(^5\), Paul B. Fisher\(^3\), Steven Grant\(^1\), Paul Dent\(^1\)

**Abstract**

The present studies determine in greater detail the molecular mechanisms upstream of the CD95 death receptor by which geldanamycin heat shock protein 90 inhibitors and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 (MEK1/2) inhibitors interact to kill carcinoma cells. MEK1/2 inhibition enhanced 17-allylamo-17-demethoxygeldanamycin (17AAG) toxicity that was suppressed in cells deleted for mutant active RAS that were nontumorigenic but was magnified in isogenic tumorigenic cells expressing Harvey RAS V12 or Kirsten RAS D13. MEK1/2 inhibitor and 17AAG treatment increased intracellular Ca\(^{2+}\) levels and reduced GRP78/BiP expression in a Ca\(^{2+}\)-dependent manner. GRP78/BiP overexpression, however, also suppressed drug-induced intracellular Ca\(^{2+}\) levels. MEK1/2 inhibitor and 17AAG treatment increased reactive oxygen species (ROS) levels that were blocked by quenching Ca\(^{2+}\) or overexpression of GRP78/BiP. MEK1/2 inhibitor and 17AAG treatment activated CD95 and inhibition of ceramide synthesis; ROS or Ca\(^{2+}\) quenching blocked CD95 activation. In SW620 cells that are patient matched to SW480 cells, MEK1/2 inhibitor and 17AAG toxicity was significantly reduced, which correlated with a lack of CD95 activation and lower expression of ceramide synthase 6 (LASS6). Overexpression of LASS6 in SW620 cells enhanced drug-induced CD95 activation and enhanced tumor cell killing. Inhibition of ceramide signaling abolished drug-induced ROS generation but not drug-induced ceroidal Ca\(^{2+}\) levels. Thus, treatment of tumor cells with MEK1/2 inhibitor and 17AAG induces ceroidal Ca\(^{2+}\) loss and loss of GRP78/BiP function, leading to de novo ceramide synthesis pathway activation that plays a key role in ROS generation and CD95 activation. Mol Cancer Ther; 9(5); 1378–95. ©2010 AACR.

**Introduction**

In the United States, colon cancer is diagnosed in ~150,000 patients per annum with ~50,000 deaths from the disease, with an overall 5-year survival rate of ~60% (1, 2). However, for patients with nonlocalized tumor at diagnosis, the 5-year survival is ~10%. Liver and pancreatic cancers have much lower overall 5-year survival rates than colon cancer, of ~10% and 5%, respectively (1, 2).

The RAF–mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2)–ERK1/2 pathway is frequently dysregulated in the neoplastic transformation of GI tumors, including mutational activation of RAS proteins in colorectal, liver, and pancreatic carcinomas (3–5). There are three widely recognized isoforms of RAS: Harvey RAS (H-RAS), Kirsten RAS (K-RAS), and neuroblastoma RAS. GTP-RAS can interact with multiple downstream effector molecules, including the RAF-1 protein kinase and the phosphatidylinositol 3-kinase (PI3K) lipid kinase. Guanine nucleotide exchange of “RAS” proteins from their GDP-bound state to their GTP-bound form permits RAF-1 and p110 PI3K to associate with the “RAS” protein, resulting in kinase translocation to the plasma membrane environment where activation of these kinases, via complex mechanisms, takes place. RAS contains a GTPase activity that catalyzes exchange of GDP for GTP.
to the plasma membrane environment via the p85 SH2 domain interaction with phosphorylated tyrosine residues on adaptor proteins and growth factor receptors (e.g., GAB2, IRS-1, and ERBB3). RAS proteins also can interact with RAL-GDS; RAL-GDS via RAL small GTPases regulates multiple processes including altered metastatic spread and the regulation of reactive oxygen species (ROS) levels (refs. 6, 7, and references therein).

The MEK1/2-ERK1/2 module comprises, along with c-Jun NH2-terminal kinase 1/2 (JNK1/2) and p38 MAPK, members of the MAPK superfamily. These kinases are involved in responses to diverse mitogens and environmental stresses and have also been implicated in cell survival processes. Activation of the ERK1/2 pathway is often associated with cell survival, whereas JNK1/2 and p38 MAPK pathway signaling often causes apoptosis. Although the mechanisms by which ERK1/2 activation promote survival are not fully characterized, a number of antiapoptotic effector proteins have been identified, including increased expression of antiapoptotic proteins, such as c-FLIP-s (8–11). In view of the importance of the RAF-MEK1/2-ERK1/2 pathway in neoplastic cell survival, inhibitors that have entered clinical trials have been developed, including sorafenib (Bay 43-9006, Nexavar; a Raf inhibitor), AZD6244 (MEK1/2 inhibitor), and 17-allylamino-17-demethoxygeldanamycin (17AAG) and 17-dimethylamino-geldanamycin [heat shock protein 90 (HSP90) inhibitors that facilitate lower expression of Raf kinases and other regulatory proteins; refs. 12, 13].

HSP90 and HSP70 are families of chaperone proteins involved in the proper folding and intracellular disposition of multiple proteins involved in cell signaling and survival (14, 15). Tumor cells generally have higher rates of protein synthesis than nonneoplastic cells and disruption of HSP90 family function in tumor cells, e.g., by benzoquinoid ansamycin antibiotics, such as 17AAG, which has both superior pharmacokinetic and reduced normal tissue toxicity characteristics compared with geldanamycin induces improper folding of diverse proteins, including RAF-1, B-RAF, AKT, ERBB family receptors, among numerous others, culminating in their proteasomal degradation (16–21). These events have been shown to induce apoptosis or, alternatively, to increase the susceptibility of tumor cells to established cytotoxic agents (18, 19). Many studies have argued that inhibition of the PI3K-AKT pathway, rather than the RAF-MEK1/2-ERK1/2 pathway, represents a key component of 17AAG toxicity and sensitization effects in tumor cells (22, 23). Of particular note, however, has been the observation in some cell types that pretreatment of tumor cells with geldanamycins, which leads to a compensatory increase in the expression of HSP70 family chaperone proteins, can protect tumor cells from geldanamycin toxicity (e.g., ref. 24, and references therein). Because of this phenomenon, many laboratories are actively searching for approaches to suppress expression/function of HSP70 family chaperones.

Free plasma concentrations of 17AAG in patients have been noted to be in the low 1 to 5 μmol/L range for up to 12 hours after drug infusion, which is significantly higher than the required concentration of drug to inhibit HSP90 function (25, 26). We recently published that pharmacologically achievable concentrations of 17AAG and MEK1/2 inhibitors interact to kill in hepatocellular carcinoma cells in vitro and in vivo via activation of the CD95 extrinsic apoptotic pathway, concomitant with drug-induced reduced expression of c-FLIP-s that was, in part, due to prolonged inactivation of ERK1/2 and AKT (27). Recent studies in which inhibitors of MEK1/2 and PI3K have been combined to achieve a tumoricidal effect in lung cancer, in principle, also confirm these findings (28). The mechanism by which CD95 was activated in hepatoma cells was dependent on ROS-induced activation of p38 MAPK (27).

The studies in the present manuscript were designed to understand, upstream of CD95, how ROS was generated by the 17AAG + MEK1/2 inhibitor combination; the putative primary target of the drug combination permitting ROS generation; whether other GI tumor cell types could be killed by this drug combination; and to determine if the tumorigenicity of a tumor cell (with specific reference to RAS signaling) effected upon drug combination lethality (27). We found that the drug-induced generation of intracellular Ca2+, the generation of ROS, and ceramide signaling were all causal in CD95 activation. We ordered the sequence of induction of these signaling processes: drug treatment promoted Ca2+ generation, which facilitated ceramide-dependent increases in ROS levels. We noted that the ER resident HSP70 family chaperone GRP78/BiP played a key role in regulating the induction of Ca2+ by drug treatment but that also the drug treatment resulted in lower GRP78/BiP expression in a Ca2+-dependent manner. This argues that GRP78/BiP function/expression is one key primary effector protein upon which the drug combination acts. In prior studies it was shown that deletion of mutant active K-RAS D13 in HCT116 cells abolished their tumorigenicity, and we have noted that introduction of H-RAS V12 into such cells lacking a mutant active RAS restores their tumorigenic potential. Loss of K-RAS D13 expression abolished the lethal interaction between 17AAG and MEK1/2 inhibitors that was restored by expression of H-RAS V12. These data show that tumorigenic cells are selectively killed by the 17AAG + MEK inhibitor drug combination over transformed cells that cannot form tumors.

Materials and Methods

Materials

Total BAX, cleaved caspase-3, phosphorylated/total ERK1/2/5, phosphorylated/total JNK1-3, phosphorylated/total p38 MAPK, anti-S473 AKT, and total AKT antibodies were purchased from Cell Signaling Technologies. Active BAX-specific antibody (6A7) for immunoprecipitation was purchased from Sigma. JNK inhibitor peptide, caspase inhibitors (zVAD, IETD, LEHD), and 17AAG were purchased from Calbiochem.
supplied by Calbiochem as powder, dissolved in sterile DMSO and stored frozen under light-protected conditions at −80°C. Trypsin-EDTA, RPMI medium, and penicillin-streptomycin were purchased from Life Technologies Bethesda Research Laboratories. HuH7, HEPG2, and Hep3B (hepatoma); pancreatic (PANC1, Mia Paca2); and colorectal (SW480, SW620, DLD1, HCT116) cancer cells were obtained from American Type Culture Collection. Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were from Qiagen: CD95 (S102654463; S103118255), ATG5 (S102655310), Beclin 1 (S100055573, S100055587), BAX (S102662401; S102654533), and BAK (S100299376; S102654512). We also made use for confirmatory purposes of the short hairpin RNA construct targeting ATG5 (pLVTHM/Atg5), which was a gift from Dr. Yousefi, Department of Pharmacology, University of Bern. BAK−/−, BAX−/−, BAX+BAK−/−, and fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University). The dominant-negative p38 MAPK and activated MEK1 EE recombinant adenoviruses were kindly provided by Drs. K. Valerie (Virginia Commonwealth University) and J. Moltken (University of Cincinnati), respectively. The plasmids to express LC3-GFP, GRP78/BiP, and Calbindin D28 were from Dr. S. Spiegel (Virginia Commonwealth University), Dr. A. Lee (University of California at Los Angeles), and Dr. Y.J. Oh (Yonsei University), respectively. Other reagents were of the highest quality and commercially available (11, 27, 29–32).

Methods

Cell culture and in vitro exposure of cells to drugs. All established cell lines were cultured at 37°C [5% (v/v) CO2] in vitro using RPMI supplemented with 5% (v/v) FCS and 10% (v/v) nonessential amino acids. For short-term cell killing assays and immunoblotting, cells were plated at a density of 3 × 10^5/cm² and 36 hours after plating were treated with various drugs, as indicated. In vitro small molecule inhibitor treatments were from a 100 mmol/L stock solution of each drug, and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). For adenoviral infection, cells were infected 12 hours after plating, and the expression of the recombinant viral transgene was allowed to occur for 24 hours before any additional experimental procedure. Cells were not cultured in reduced serum media during any study.

Generation of Rho 0 HuH7 cells. HuH7.Ntcp human hepatoma cells (kindly provided by Dr. G. Gores, Mayo Clinic) were cultured in DMEM containing 10% (v/v) FCS. To generate HuH7.Ntcp Rho 0 cells, HuH7.Ntcp cells were cultured in DMEM containing 10% (v/v) FCS, 50 μg/mL uridine, 1 mmol/L sodium pyruvate, and the growth medium supplemented (for Rho 0 cell generation) with 10 μg/mL ethidium bromide. Cells were cultured in this medium or in parallel in growth medium without ethidium bromide for 8 weeks before any further experimentation. Removal of uridine and pyruvate from the growth medium of established HuH7 Rho 0 cells resulted in rapid (~24–48 h) growth arrest and cell death (data not shown).

Cell treatments, SDS-PAGE, and Western blot analysis. Unless otherwise indicated in the figure legends, cells were treated with either vehicle (DMSO) or the combination of MEK1/2 inhibitor PD184352 (PD184, 1 μmol/L) or PD98059 (PD98, 25 μmol/L), as indicated, and geldanamycin (17AAG, 0.1–1.0 μmol/L or 17-dimethylaminogeldanamycin, 0.25 μmol/L) or both agents combined. For SDS-PAGE and immunoblotting, cells were lysed in either a nondenaturing lysis buffer and prepared for immunoprecipitation as described in refs. 27, 29–32 or in whole-cell lysis buffer [0.5 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue], and the samples were boiled for 30 minutes. 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 nitrocellulose and immunoblotted with indicated primary antibodies against the different proteins. All immunoblots were visualized by an Odyssey Infrared imager. For presentation, blots were imported into Adobe Photoshop 8.0, their color was removed, and figures were generated in Microsoft PowerPoint.

Recombinant adenoviral vectors: infection in vitro. We generated and purchased previously noted recombinant adenoviruses to express constitutively activated and dominant-negative MEK1 proteins, dominant-negative p38 MAPK, dominant-negative caspase-9, the caspase-9 inhibitor XIAP, the endogenous caspase-8 inhibitor c-FLIP-s, the polyclonal virus caspase-8 inhibitor CRM A, and mitochondrial protective protein BCL-XL (Vector Biolabs). Unless otherwise stated, cells were infected with these adenoviruses at an approximate multiplicity of infection of 50, which results in >80% infection of tumor cells. As noted above, cells were further incubated for 24 hours to ensure adequate expression of transduced gene products before drug exposures (27). In confirmatory studies and in agreement with published studies using these reagents, we noted that activated and dominant-negative MEK1 proteins activated and reduced ERK1/2 phosphorylation in cells and that dominant-negative p38 MAPK suppressed sorbitol-induced p38 MAPK activation (27).

siRNA transfection in vitro. Approximately 10 nmol/L of a defined prevalidated siRNA (Ambion Technologies) were diluted into 50-μL growth media lacking fetal bovine serum (FBS) and pen-strep. Based on the manufacturer’s instructions, an appropriate amount of Lipofectamine 2000 reagent (usually 1 μL; Invitrogen) was diluted into a separate vial containing media with lacking FBS or pen-strep. The two solutions were incubated separately at room temperature for 5 minutes, then mixed together (vortexed), and incubated at room temperature for 30 minutes. The mixture was added to each well (slide or 12-well plate) containing an appropriate amount (~0.5 mL) of pen-strep–free and FBS-free medium. Cells were incubated
for 2 to 4 hours at 37°C with gentle rocking. Media were then replaced with 1 mL of 1× pen-strep-containing and FBS-containing media. In the figures, we present representative immunoblots showing knockdown of CD95, ATG5, and Beclin 1 in the various cell lines used. Regardless of cell line, knockdown of protein by siRNA was at least 70% in all cell lines used (data not shown; ref. 27).

**Detection of cell death by Trypan blue, Hoechst, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, and flow cytometric assays.** Cells were harvested by trypsinization with Trypsin/EDTA for ~10 minutes at 37°C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1,500 rpm for 5 minutes. 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 done by counting of cells using a light microscope and a hemacytometer. Five hundred cells from randomly chosen fields were counted, and the number of dead cells was counted and expressed as a percentage of the total number of cells counted. For confirmatory purposes, the extent of apoptosis was evaluated by assessing Hoechst and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling—stained cytospin slides under fluorescent light microscopy and scoring the number of cells exhibiting the “classic” morphologic features of apoptosis and necrosis. For each condition, 10 randomly selected fields per slide were evaluated, encompassing at least 1,500 cells. Alternatively, the Annexin V/propidium iodide assay was carried to determine cell viability out as per the manufacturer’s instructions (BD Pharmingen) using a Becton Dickinson FACScan flow cytometer (29, 30).

**Plasmid transfection.** Plasmid DNA (0.5 μg/total plasmid transfected) was diluted into 50 μL of RPMI growth media that lacked supplementation with FBS or with penicillin-streptomycin. Lipofectamine 2000 reagent (1 μL; Invitrogen) was diluted into 50 μL growth media that lacked supplementation with FBS or with penicillin-streptomycin. The two solutions were then mixed together and incubated at room temperature for 30 minutes. The total mix was added to each well (4-well glass slide or 12-well plate) containing 200 μL growth media that lacked supplementation with FBS or with penicillin-streptomycin. The cells were incubated for 4 hours at 37°C, after which time the media was replaced with RPMI growth media containing 5% (v/v) FBS and 1× pen-strep.

**Assessment of ROS generation.** Hepatoma and colon cancer cells were plated in 96-well plates. Cells were preincubated with dihydro-DCF (5 mmol/L for 30 min), which is nonfluorescent in its dihydro form but upon reaction with ROS (peroxide) becomes highly fluorescent. Dihydro-DCF is sensitive to oxidation by hydroxyl radicals and peroxy-nitrite directly and hydrogen peroxide in the presence of oxidases. Fluorescence measurements were obtained 0 to 30 minutes after drug addition with a Vector 3 plate reader. Data are presented corrected for basal fluorescence of vehicle-treated cells at each time point and expressed as a severalfold increase in ROS levels. Each time point represents the mean of six data points per experiment and of a total of three independent experiments.

**Assessment of cytosolic Ca^{2+} levels.** A high-speed wavelength switching fluorescence image analysis system (Vector 3 plate reader) was used to determine [Ca^{2+}]_{i} in the carcinoma cells, seeded in 96-well plates (20,000 cells per well), with fura-2 acetoxymethylester (fura-2) as an indicator. After loading with 10 μmol/L fura-2 at room temperature for 50 minutes, the cells were washed thrice with Hanks’ buffer (pH 7.4). Then vehicle (DMSO), 17AAG, PD184352, or the drug combination was added into the media solution to stimulate the cells, respectively. A fluorescence ratio of excitation at 340 nm to that at 380 nm (F340/F380) was determined after background subtraction. The ratio of fura-2 emissions, when excited at the wavelengths of 340 and 380 nm, was recorded, and an analysis software was used to process and analyze statistical data.

**Microscopic assessment of autophagy.** For autophagy studies, cells transfected in duplicate with LC3-GFP in glass-chambered slides were applied after drug exposure to high-powered light/confocal microscopes (Zeiss LSM 510 metaconfocal scanning microscope; Zeiss HBO 100 microscope with Axio Cam MRm camera) at 40× magnification and 40 cells per well assessed for formation of punctate autophagic vesicles.

**Colony formation assays.** Tumor cells growing in log phase were replated as single cells in 60-mm dishes. Cells were treated 12 hours after plating (250–1,500 per well) in sextuplicate with vehicle (DMSO), 17AAG (μmol/L), or PD184352 (μmol/L), or with both drugs combined, as indicated at a fixed concentration ratio to perform median dose-effect analyses for the determination of synergy. After drug exposure (48 h), the media were changed and cells were cultured in drug-free media for an additional 10 to 14 days. Cells were fixed and stained with crystal violet, and colonies of >50 cells per colony were counted. Colony formation data were entered into the Calcsysyn for Windows program and combination index values determined (Fa, fraction affected). A combination index value of <1.00 indicates synergy.

**Data analysis.** Comparison of the effects of various treatments was done using one-way ANOVA and a two-tailed Student’s t test. Differences with a P value of <0.05 were considered statistically significant. These values were determined using the statistical programing within Sigma Stat and Sigma Plot. Median dose-effect isobologram analyses to determine synergism of drug interaction were done according to the methods of T-C. Chou and P. Talalay using the Calcsyn program for Windows (BIOSOFT). Data points from all experiments shown are the mean of multiple individual data points summated from the stated number of multiple experiments, i.e., (mean data shown = Σn, all data points, ± SEM).
Results

Treatment of pancreatic and liver cancer cells that express mutant active RAS proteins and other membrane-proximal oncogenic signaling proteins with the geldanamycin HSP90 inhibitor 17AAG and the MEK1/2 inhibitor PD184352 resulted in greater than additive increases in tumor cell killing within 48 hours (Fig. 1A). PD184352 and 17AAG were shown to synergistically interact to kill multiple cancer cell types in long-term colony formation assays (Supplementary Table S1). As MiaPaca2, PANC-1, and HEPG2 cells express mutated active K-RAS, K-RAS, and neuroblastoma RAS proteins, respectively, we next examined whether other GI tumor cell types that express mutated active RAS proteins or activated downstream effectors of RAS, such as B-RAF and PI3K, also present with a greater than additive toxic interaction between 17AAG and MEK1/2 inhibitor.

HT-29 colon cancer cells express a mutated active B-RAF protein but do not express a mutated RAS allele. SW620 and SW480 cells are patient-matched colon cancer lines that express two alleles of mutated active K-RAS: SW620 has also been noted to be refractory to death receptor-induced lethality in part by lacking ceramide synthase 6 (LASS6) expression (33). HCT116 cells express a single mutated active allele of K-RAS as well as a mutated active PI3K p110α protein (34). In HCT116, SW480, and HT29 cells, PD184352 and 17AAG interacted in an additive to greater than additive manner to cause tumor cell death (Fig. 1B). However, SW620 cells were refractory to drug-induced killing.

To determine the most important pathways downstream of activated RAS proteins in GI tumor cells that define 17AAG + PD184352 toxicity, we made use of HCT116 cells and variants of HCT116 cells that are genetically deleted for K-RAS D13 and transfected with H-RAS V12 and with effector mutants of H-RAS V12 (27, 35). In short-term viability assays, PD184352 and 17AAG interacted in a greater than additive manner to kill parental HCT116 cells (Fig. 1C). Knockout of K-RAS D13 significantly suppressed drug toxicity and transfection of H-RAS V12 into these cells restored drug lethality. Of note, HCT116 cells lacking K-RAS D13 expression are nontumorigenic (35). Transfection of H-RAS V12 G37 into tumor cells lacking K-RAS D13, a RAS effector domain mutant that specifically activates the RAL-GDS pathway, profoundly enhanced the toxicity of 17AAG and of 17AAG + MEK1/2 inhibitor treatment (Fig. 1C; Supplementary Fig. S1).

Expression of H-RAS V12 G37 enhanced 17AAG-induced and 17AAG + PD184352–induced activation of JNK1/2 and inactivation of ERK1/2 (Fig. 2A, top blots). Inhibition of JNK1/2 signaling suppressed the toxicity of 17AAG + PD184352 in cells expressing H-RAS V12 G37 but not in parental HCT116 cells (Fig. 2A, bottom graph). This is of particular note because, in hepatoma cells, inhibition of p38 MAPK and not that of JNK1/2 was responsible for drug combination lethality (27).

Expression of activated MEK1 significantly suppressed the toxicity of 17AAG and the MEK1/2 inhibitor PD98059 (that does not inhibit MEK1 EE) in both parental cells and in cells expressing H-RAS V12 G37 (Fig. 2B). Knockdown of CD95 in parental HCT116 cells blocked the potentiation of 17AAG lethality by PD184352; however, knockdown of CD95 in cells expressing H-RAS V12 G37 had no effect on the toxicity of the drug interaction (Fig. 2C). In a similar manner to parental HCT116 cells, inhibition of CD95/extrinsic pathway signaling blocked 17AAG + PD184352–induced cell killing in pancreatic and liver cancer cells, and in these cells, the drug combination also caused CD95 activation (Fig. 3A and B; Supplementary Fig. S2). CD95 activation could be mediated by either FAS-L or by ligand-independent processes, e.g., increased ROS or ceramide levels (36). Use of a neutralizing anti-FAS ligand antibody CD95 did not block either drug-induced CD95 activation or drug-induced cell killing (Fig. 3C).

Prior studies from this group have shown in hepatoma cells that agents which quench ROS block 17AAG + MEK1/2 inhibitor toxicity, although in those analyses we did not measure actual alterations in ROS generation (27). Treatment of hepatoma cells with 17AAG weakly increased ROS levels that were significantly enhanced by inhibition of MEK1/2 (Fig. 4A). As CD95 signaling was not responsible for enhanced morbidity of HCT116 cells expressing H-RAS V12 G37, we determined whether a differential induction of ROS comparing parental and H-RAS V12 G37 HCT116 cells could explain the altered levels of cell survival after drug exposure and the CD95-independent nature of drug-induced killing in cells expressing H-RAS V12 G37. As was noted in hepatoma cells, in parental HCT116 cells, PD184352 rapidly enhanced 17AAG-induced ROS levels (Fig. 4B). In cells expressing H-RAS V12 RAL 37,
Figure 2. Signaling by RAL-GDS downstream of H-RAS V12 promotes 17AAG toxicity. A, top blotting, parental HCT116 (WT) cells and HCT116 H-RAS V12 G37 were treated with vehicle (DMSO), 17AAG, PD184352, or 17AAG + PD184352. Cells were isolated 24 and 48 h after exposure, and cell lysates were subjected to SDS-PAGE and immunoblotting to determine the phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 and the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In parallel blots, 24 h after plating, cells were treated with vehicle (DMSO) or JNK-IP (10 μmol/L) and 30 min later with vehicle (DMSO) or 17AAG + JNK-IP (10 μmol/L) and 30 min later with vehicle (DMSO) or 17AAG + PD184352, and cells were isolated 24 h after drug exposure. Bottom graph, parental HCT116 (WT) cells and HCT116 H-RAS V12 G37 were infected with recombinant adenoviruses to express nothing (cytomegalovirus, CMV) or dnp38 MAPK. At 24 h after infection, cells were treated with vehicle (DMSO) or JNK-IP (10 μmol/L) and 30 min later with vehicle (DMSO), 17AAG, PD184352, or 17AAG + PD184352. At 48 h after exposure, cells were isolated. Cell viability was determined 48 h after drug treatment by Trypan blue exclusion (±SEM, n = 3 independent studies; *, P < 0.05, lower amount of cell killing compared with parallel treated WT cells). B, parental HCT116 and HCT116 H-RAS V12 G37 cells were infected with recombinant adenoviruses to express nothing (CMV) or caMEK1. At 24 h after infection, cells were treated with vehicle (DMSO), 17AAG, PD98059 (25 μmol/L), or 17AAG + PD98059. At 48 h after exposure, cells were isolated. Cell viability was determined 48 h after drug treatment by Trypan blue (±SEM, n = 3 independent studies; *, P < 0.05, lower amount of cell killing compared with parallel drug-treated CMV-infected cells).
however, both 17AAG and 17AAG + PD184352 treatment enhanced ROS production, ∼2.5-fold above that observed in parental cells, and in addition prolonged drug-induced ROS generation. Quenching of ROS production blocked CD95 activation and drug-induced cell killing (Fig. 4C; data not shown). Molecular quenching of ROS production in cells expressing H-RAS V12 RAL 37, via overexpression of thioredoxin, suppressed drug-induced JNK pathway activation; JNK pathway signaling plays a key role in drug-induced killing (Supplementary Fig. S3; Fig. 2A).

A significant source of ROS generation induced by this drug combination was dependent on functioning mitochondria, as judged by use of mitochondria-deficient Rho zero HuH7 cells (Fig. 4D). Similar data arguing for mitochondrial-dependent induction of ROS by this drug combination was also obtained in HEPG2 cells (Supplementary Fig. S4). It is known that activation of CD95 can generate ROS via activation of NADPH oxidases; as HuH7 cells lack CD95, our data with 17AAG and PD184352 thus tend to support an NADPH oxidase–independent form of ROS generation (37–39). As previously stated, the small GTP-binding protein RAL is activated by RAL-GDS, one of the effector molecules for RAS. Active RAL binds to a GTPase-activating protein for CDC42 and RAC, and the activity of NADPH oxidase is regulated by RAC. Thus of note was that, whereas inhibition of p47phox-dependent NADPH oxidase in parental HCT116 cells did not alter drug-induced ROS levels, knockdown of p47 phox in HCT116 cells expressing H-RAS V12 RAL 37 significantly reduced the induction of ROS and the enhanced levels of tumor cell killing (Supplementary Fig. S5). Thus, enhanced drug toxicity in H-RAS V12 G37 cells was due to high levels of NADPH oxidase–dependent ROS generation.

In prior studies, we have noted in a stimulus-specific manner that ligand-independent activation of CD95 could be dependent on the actions of acidic sphingomyelinase and/or the de novo ceramide synthesis pathway (29, 30). Knockdown of acidic sphingomyelinase expression or treatment with an inhibitor of the de novo ceramide synthesis pathway blocked 17AAG + PD184352–induced
Figure 3. MEK1/2 inhibitor and 17AAG toxicity in GI cancer cells is mediated by CD95–caspase-8 signaling and does not require FAS-L. A, MiaPaca2 cells were infected with recombinant adenoviruses (empty vector CMV, caspase-8 inhibitor CRM A, caspase-8 inhibitor c-FLIP-s, BCL-XL, dominant-negative caspase-9) at a multiplicity of infection of 50. At 24 h after infection, cells were treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs together. At 48 h after exposure, cells were isolated and stained with Annexin V–propidium iodide and viability was determined by flow cytometry (±SEM, n = 3; *P < 0.05, greater amount of cell killing compared with vehicle (VEH) or individual drug-treated cells). B, top images, MiaPaca2 cells 24 h after plating in glass-chambered slides were treated with vehicle (DMSO), 17AAG, PD184352, or 17AAG + PD184352. At 6 h after exposure, cells were fixed and stained for plasma membrane localization of CD95 (±SEM, n = 2). Bottom graph, HEP3B and MiaPaca2 cells were transfected with either a scrambled siRNA (siSCR, 20 nmol/L) or a siRNA to knock down CD95 expression. At 24 h after transfection, cells were treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs together. At 48 h after exposure, cells were isolated, and cell viability was determined via Trypan blue exclusion (±SEM, n = 3; *P < 0.05, lower amount of cell killing compared with corresponding value in siSCR cells).
Inhibition of acidic sphingomyelinase or the \( \alpha \)-eIF2 expression of LASS6 in SW620 cells significantly contributed to 17AAG + MEK1/2 inhibitor toxicity; however, stable expression of LASS6 in SW620 cells significantly enhanced the lethality of 17AAG + MEK1/2 inhibitor treatment and facilitated drug-induced CD95 activation (Fig. 5B and C).

Treatment with 17AAG causes mitochondrial-dependent ROS generation and also promotes an unfolded protein response/endoplasmic reticulum stress. As Ca\(^{2+}\) homeostasis plays a central role in the functions of both organelles, we next investigated whether drug-induced ROS generation was dependent on changes in the levels of cytosolic Ca\(^{2+}\). Knockdown of acidic sphingomyelinase or inhibition of de novo ceramide synthesis in hepatoma cells did not alter the induction of cytosolic Ca\(^{2+}\) levels by 17AAG + PD184352 treatment (Fig. 5D, left).

However, quenching of cytosolic Ca\(^{2+}\) using Calbindin D28 suppressed drug-induced ROS (Fig. 5D, right). In SW620 cells regardless of LASS6 expression, 17AAG + PD184352 treatment increased cytosolic Ca\(^{2+}\) levels; however, in vector control-transfected SW620 cells, lacking LASS6 expression, drug exposure only weakly increased ROS levels (Supplementary Figs. S6 and S7). Expression of LASS6 restored drug-induced ROS generation in SW620 cells. Hence, 17AAG and MEK1/2 inhibitors increase cytosolic Ca\(^{2+}\) levels that activate ceramide synthetic/regulatory pathways, which in turn lead to the generation of ROS. Increased ceramide and ROS levels play an essential role in promoting mitochondrial dysfunction and CD95 activation.

In addition to linking ceramide in CD95 signaling, we have also shown in several prior studies that activation of CD95 increased autophagic vesicle formation, an event that was “protective” against CD95-induced apoptosis (29, 30). Treatment of hepatoma cells with 17AAG + MEK1/2 inhibitor enhanced autophagic vesicle formation 6 to 24 hours after exposure that was blocked by knockdown of ATG5 or CD95 or by expression of dominant-negative PERK (Fig. 6A). HuH7 hepatoma cells do not express CD95 and did not produce a significant autophagic response following 17AAG and MEK1/2 inhibitor exposure (Fig. 6B). However, expression of CD95-YFP in HuH7 cells increased the number of drug-induced punctate LC3-GFP vesicles and in agreement with CD95 mediating the toxic actions of this drug combination. Knockdown of ATG5 or Beclin1 enhanced 17AAG + MEK1/2 inhibitor toxicity, whereas expression of dominant-negative PERK reduced drug lethality (Fig. 6C). Similar data were obtained in ATG5 null SV40-transformed MEFs (Supplementary Fig. S8). Deletion of PERK or expression of dominant-negative eIF2\(\alpha\) (S51A) in SV40-transformed MEFs suppressed the toxicity of 17AAG + MEK1/2 inhibitor exposure (Fig. 6D).

In addition to promoting phosphorylation of PERK and eIF2\(\alpha\), treatment with 17AAG + MEK1/2 inhibitor increased the levels of CHOP and IRE1 without altering his levels of activating transcription factor 6 and, surprisingly, decreased the levels of the Er resident HSP70 family chaperone GRP78/BiP (Fig. 6C, top inset). Overexpression of GRP78/BiP protected cells from drug-induced killing (Supplementary Fig. S9). Overexpression of GRP78/BiP suppressed drug-induced autophagy, CD95 activation, and drug-induced ROS generation (Supplementary Figs. S10 and S11). Molecular quenching of Ca\(^{2+}\) signaling suppressed basal levels of GRP78/BiP but also inhibited drug-induced loss of GRP78/BiP levels (Supplementary Fig. S12). In contrast, overexpression of GRP78/BiP suppressed drug-induced Ca\(^{2+}\) signaling (Supplementary Fig. S13). Collectively, these findings argue that the primary increase in Ca\(^{2+}\) levels following geldanamycin + MEK1/2 inhibitor exposure is responsible for loss of protective HSP70 family chaperone function facilitating ROS generation and CD95 activation.
Discussion

The present studies were designed to examine in greater detail the molecular mechanisms by which 17AAG and MEK1/2 inhibitors interact to kill GI tumor cells and, in particular, the role RAS mutational status played in regulating drug sensitivity. In short-term viability assays, 17AAG and MEK1/2 inhibitors caused an additive to greater than additive increase in cell death that was dependent on CD95 activation. In long-term colony formation assays, 17AAG and MEK1/2 inhibitor treatment synergized to kill multiple GI tumor cell types with combination index values of ≤0.70, indicating a strong level of synergy.

Based on the concept of oncogene addiction, it could be postulated that expression of a mutated active RAS protein, which in a cell type–dependent fashion constitutively increases ERK1/2 and/or PI3K activities, would in turn facilitate the cytotoxic actions of both 17AAG, whose HSP90 inhibitory actions will tend to reduce signaling through ERK1/2 and AKT, and of MEK1/2 inhibitors, whose actions will reduce signaling through...
ERK1/2. In agreement with this concept, we found that the lethality of 17AAG or the drug combination was suppressed by deletion of K-RAS D13 from HCT116 colon cancer cells that correlated with lower constitutive levels of ERK1/2 or AKT activity. It is known that HCT116 cells deleted for K-RAS D13 are nontumorigenic, which in turn argues that our drug combination will have less toxicity in nontransformed cells. In agreement with this hypothesis in primary rodent hepatocytes, MEK1/2 inhibitors did not enhance 17AAG

**Figure 4. Continued.** C, top, immunohistochemistry; HEPG2, parental HCT116, and HCT116 H-RAS V12 G37 cells 24 h after plating in eight-well chamber slides were treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs combined and where indicated treated in parallel with vehicle (PBS) or MnTBAP (1 μmol/L). Cells were fixed 6 h after drug exposure and stained for surface localization of CD95 (±SEM, n = 3; *, P < 0.05, lower amount of CD95 activation compared with corresponding value in vehicle-treated cells). Bottom graph, HEPG2, MiaPaca2, parental HCT116, and HCT116 H-RAS V12 G37 cells 24 h after plating were treated with vehicle (DMSO) or 17AAG (1 μmol/L) and PD184352 (1 μmol/L) combined and where indicated treated in parallel with vehicle (PBS) or MnTBAP (1 μmol/L). Cells were isolated 48 h after exposure and survival determined by Trypan blue exclusion (±SEM, n = 3; *, P < 0.05, lower amount of cell killing compared with corresponding value in vehicle-treated cells). D, HuH7 parental and Rho zero cells 24 h after plating in 96-well plates were treated with vehicle (DMSO), 17AAG (1 and 5 μmol/L), PD184352 (1 and 5 μmol/L), or both drugs combined. As indicated, cells were treated with vehicle (DMSO), Cyclosporine A (CsA), or Bongkrekic acid (Bong). The generation of ROS in cells was determined 15 min after drug addition using a Vector 3 plate reader (±SEM, n = 3; *, P < 0.05, lower amount of ROS compared with corresponding vehicle-treated cells).
toxicity. We then investigated the relative importance of three of the best-defined pathways downstream of RAS proteins that were likely to be involved in controlling drug toxicity: RAF-MEK1/2 (H-RAS V12 S35), PI3K-AKT (H-RAS V12 C40), and RAL-GDS (H-RAS V12 G37). We have noted using these cells in vitro and in vivo that activation of RAS predicted resistance to PI3K inhibitors even in the presence of activating PI3K mutations.

Figure 5. Ceramide-dependent generation of ROS plays a key role in CD95 activation. A, HEPG2 cells were transfected with scrambled siRNA (siSCR, 20 nmol/L) or an siRNA to knock down acidic sphingomyelinase expression (siASMase). Cells were then plated in 8-well chambered slides for determination of CD95 surface localization and in 96-well plates for determination of ROS levels. Unless otherwise indicated, 24 h after replating, cells were pretreated with vehicle or myriocin (1 μmol/L) and 30 min later treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs combined. For CD95 plasma membrane localization, cells were fixed 6 h after drug exposure and surface CD95 detected by immunohistochemistry in unpermeabilized cells. A representative study from three experiments. The generation of ROS in cells was determined 15 min after drug addition using a Vector 3 plate reader (±SEM, n = 3 independent studies). B, SW620 cells stably transfected with either vector control plasmid or a plasmid to express LASS6, 24 h after plating, were exposed to vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs combined. At 48 h after exposure, cells were isolated and viability was determined by Trypan blue (±SEM, n = 2). *, P < 0.05, greater than corresponding value in empty vector cells. The toxicity of PD184352 did not alter between vector control and LASS6 expressing cells (data not shown).
or loss of PTEN, whereas H-RAS V12 (C40)–induced single activation of PI3K predicted for sensitivity to PI3K inhibitors (ref. 35; Supplementary Fig. S14).

Hence, a priori based on the hypothesis of oncogene addiction and the inhibitory actions of 17AAG on AKT and ERK1/2 signaling, we would have predicted that expression of the “RAF-1 (S35)” or the “PI3K-AKT (C40)” activating RAS mutants would have been linked to elevated drug toxicity. Expression of H-RAS V12 but surprisingly not the S35 or the C40 point effector mutants of H-RAS V12 restored the toxicity of 17AAG and of the drug combination to near those levels observed in wild-type (WT) cells expressing K-RAS D13. In contrast to the other RAS effector mutants, activation of the RAL-GDS pathway (G37 mutant) profoundly enhanced 17AAG lethality and that of the drug combination. This correlated with a large increase in drug combination–induced ROS levels. In HCT116 cells expressing H-RAS V12 G37 that were hypersensitive to 17AAG-induced ROS production and drug-induced lethality, the large enhanced levels of ROS were partially dependent on mitochondrial function and were largely reliant on NADPH oxidase signaling.

There are several studies that have linked RAS signaling to activation of NADPH oxidases, and our data suggest that RAS-dependent activation of the RAL-GDS pathway, in the absence of survival signaling by the RAF and PI3K pathways, plays a key role in RAS-dependent tumor cell killing (40, 41). In other GI tumor cell types, including parental HCT116 cells, the generation of ROS by 17AAG + PD184352 treatment was almost abolished by use of inhibitors of mitochondrial respiration, and mitochondria-deficient Rho zero HuH7 cells lacked an ROS response to 17AAG + PD184352 treatment. As HuH7 cells do not express CD95, our data would argue that drug-induced ROS levels through mitochondrial signaling are truly a “primary” signal and not part of an amplification loop, wherein activated CD95 promotes ROS generation through the actions of, e.g., NADPH oxidase enzymes. Treatment of parental HCT116 cells with 17AAG or with 17AAG + MEK1/2 inhibitor reduced ERK1/2 and increased JNK activity, and treatment of HCT116 cells expressing H-RAS V12 G37 caused even greater activation of JNK1/2 activation and a greater reduction in ERK1/2 signaling, effects that were ROS dependent. These data argue, in agreement with other studies, that oncogenic RAS signaling can both promote cell survival but also, under certain circumstances, play an active role in pro-apoptotic signaling. These findings also emphasize the possibility that a combination of agents that disrupt PI3K and MEK1/2 signaling plus the use of 17AAG may represent a useful approach to treat RAS-dependent/addicted tumors.

7 Mitchell and Dent, unpublished observation.
In prior studies wherein hepatoma and pancreatic cancer cells were treated with the drugs sorafenib and vorinostat, it was shown that cell killing was PERK dependent and CD95 dependent and the induction of protective autophagy was also PERK and CD95 dependent (29, 30). In the present studies with geldanamycin and MEK1/2 inhibitor exposure, we found that drug combination lethality was also CD95 dependent. Furthermore, 17AAG + MEK1/2 inhibitor treatment increased the phosphorylation of PERK and eIF2α in a CD95-dependent fashion and knockout or knockdown of PERK suppressed drug lethality. MEK1/2 inhibitor + 17AAG treatment also promoted LC3-GFP vesiculization in a CD95-dependent and PERK-dependent fashion that was a “protective” signal. Several groups activating the tumor necrosis factor-related apoptosis-inducing ligand receptor have recently shown that death receptor signaling has both proapoptotic components via caspase-8 and caspase-10, and prosurvival components via increased autophagy (42, 43). Our findings in GI tumor cells and primary hepatocytes treated with bile acids, sorafenib + vorinostat and now with 17AAG + MEK1/2 inhibitor, provide further strong evidence to argue that activation of the CD95 death receptor produces...
conflicting survival signals in tumor cells (29, 30). This finding, with respect to CD95 signaling, could represent one mechanism by which tumor cells could escape immune surveillance killing and have a general resistance to toxic therapeutic agents.

In our studies using bile acids and sorafenib + vorinostat, we have shown that ceramide generation plays a central role in the ability of these agents to cause CD95 activation; however, it has also been shown that ceramide generation can alter mitochondrial membrane fluidity.

Figure 6. Continued. C, bottom panel, HEPG2 cells 24 h after plating were transfected with siRNA molecules to knock down expression of ATG5 or Beclin1, with a siScramble control (20 nmol/L), with an empty vector control plasmid, or a plasmid to express dominant-negative PERK. At 24 h after transfection, cells were treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (1 μmol/L), or both drugs combined. Cells were isolated 48 h later, and cell viability determined via Trypan blue exclusion assay (±SEM, n = 3 independent studies). Top inset blot, HEPG2 cells were isolated 6 and 24 h after 17AAG + PD184352 exposure, and immunoblotting was done to determine the expression and phosphorylation of the indicated proteins (n = 2). D, SV40 large T antigen transformed mouse embryonic fibroblasts with specific genomic homozygous deletions (BAK/BAX−/−, BID−/−, PERK−/−) or expressing dominant-negative eIF2α, were, 24 h after plating, treated with vehicle (DMSO), 17AAG (1 μmol/L), PD184352 (2 μmol/L), or both drugs combined. At 48 h after drug exposure, cells were isolated and cell viability was determined via Trypan blue exclusion (±SEM, n = 3 independent studies). Inset, HEPG2 cells were transfected with siRNA molecules to knock down expression of CD95 or with a siScramble control (20 nmol/L). At 24 h after transfection, cells were treated with vehicle (DMSO) or with 17AAG (1 μmol/L) and PD184352 (1 μmol/L). Cells were isolated 6 h after drug exposure, and the phosphorylation of PERK was determined by immunoblotting (n = 2).
and mitochondrial ROS production (44, 45). Inhibition of the acidic sphingomyelinase or the de novo ceramide generation pathways blocked 17AAG + MEK1/2 inhibitor-induced ROS and CD95 activation. These data were confirmed, in part, when LASS6 was expressed in LASS6 null SW620 cells that facilitated 17AAG + MEK1/2 inhibitor-induced ROS generation, CD95 activation, and cell killing. Mitochondrial ROS production has been linked to changes in mitochondrial and cytosolic Ca\(^{2+}\) levels and vice versa (46). Knockdown of acid sphingomyelinase or inhibition of de novo ceramide synthesis did not significantly alter the induction of Ca\(^{2+}\) after drug exposure; however, quenching Ca\(^{2+}\) blocked drug-induced ROS production. These findings argue that 17AAG + MEK1/2 inhibitor treatment induces a primary alteration in cellular Ca\(^{2+}\) fluxes that result in increased ceramide–ROS production. These findings argue that 17AAG + MEK1/2 inhibitor treatment could have profound effects on tumor cell chemosensitivity (27, 51–53). Studies to prove or refute this possibility are beyond the scope of the present manuscript (Supplementary Fig. S14).

In conclusion, the present findings show that RAS-transformed tumor cells are highly susceptible to being killed by a combination of 17AAG + MEK1/2 inhibitor exposure. Killing by activated RAS proteins was facilitated by RAL-GDS signaling to promote ROS-dependent activation of JNK1/2 and ultimately CD95. As many GI cell types also express highly activated WT RAS proteins, e.g., HEP3B, our findings argue that the 17AAG + MEK1/2 inhibitor drug combination could have utility in treating many GI malignancies regardless of RAS mutational status.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Grant Support**

P01-CA104177, R01-CA108325, R01-DK52825, R01-CA63753, R01-CA77141, R01-CA097318, R01-CA098712, and P01-NS031492.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/07/2009; revised 02/09/2010; accepted 03/08/2010; published OnlineFirst 05/04/2010.

**References**


Molecular Cancer Therapeutics

17-Allylamino-17-Demethoxygeldanamycin and MEK1/2 Inhibitors Kill GI Tumor Cells via Ca\(^{2+}\)-Dependent Suppression of GRP78/BiP and Induction of Ceramide and Reactive Oxygen Species

Teneille Walker, Clint Mitchell, Margaret A. Park, et al.

*Mol Cancer Ther* Published OnlineFirst May 4, 2010.

Updated version

Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-09-1131

Supplementary Material

Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/05/04/1535-7163.MCT-09-1131.DC1

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.