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Inhibition of BCL2 expression and activity increases H460 sensitivity to the growth inhibitory effects of polyphenon E†

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Abstract

The anti-cancer properties of the green tea-derived mixture Polyphenon E (Poly E) have been demonstrated in a variety of cell culture and animal models. We recently discovered that the H460 lung cancer cell line is markedly resistant to the growth inhibitory effects of Poly E compared with SW480 colon and Flo-1 esophageal cancer cells. We investigated the mechanism of H460 resistance by comparing gene expression profiles of Poly E-sensitive and -resistant cells. Unsupervised hierarchical clustering revealed that Poly E-sensitive cells clustered separately from Poly E-resistant cells, and 6,242 genes were differentially expressed between the two groups at the 0.01 level of significance. We discovered that BCL2 gene and protein expression were significantly higher in H460 cells compared with SW480 and Flo-1 cells (10.60-fold higher gene expression; $P < 0.0001$). Inhibition of BCL2 expression and activity, using siRNA and the small molecule inhibitor HA14-1 respectively, restored sensitivity to Poly E and induced BCL2-related apoptosis by decreasing mitochondrial membrane potential and inducing PARP cleavage. Our results suggest that increased BCL2 expression may contribute to H460 resistance to the growth inhibitory effects of Poly E. If validated in additional laboratory and clinical models, BCL2 could ultimately be used as a marker of Poly E resistance.

Keywords

Polyphenon E; BCL2; green tea; EGCG; resistance

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INTRODUCTION

Population studies have demonstrated an inverse relationship between green tea consumption (mainly in Asian populations) and standardized incidence and mortality rates for a variety of human malignancies, including those of the breast, esophagus, gastric, and hematologic system (1, 2). In addition, there is an extensive literature demonstrating the anti-cancer activity of several green tea-derived compounds or catechins, most notably epigallocatechin gallate (EGCG), the major biologically active component of green tea, in a wide variety of cell culture and animal models (1, 3). These reports have demonstrated significant relationships between catechin dose and relevant cancer endpoints, including inhibition of cell proliferation and tumor growth, induction of apoptosis, induction of G1-phase cell cycle arrest, inhibition of tyrosine kinase (including epidermal growth factor receptor (EGFR) and HER2) signaling, and reduction of tumor multiplicity and volume in animals (4-10). This strong preclinical evidence supported the development of multiple previous and ongoing Phase I and II clinical trials of green tea catechins for the prevention and treatment of human malignancy. Two recently reported trials have demonstrated 70% response rates and significant reductions in tumor incidence in patients with precancerous lesions of the cervix and prostate, respectively (11, 12).

Polyphenon E (Poly E) is a standardized botanical drug substance containing a defined mixture of catechins that are extracted from green tea leaves. The main component is EGCG, which comprises 50-75% of the material. Poly E and EGCG have been formulated into capsules by the Chemoprevention Agent Development Research Group at the National Cancer Institute (NCI) and are undergoing active clinical investigation for the prevention and treatment of a variety of cancers. Biomarkers of both resistance and response would be useful for designing clinical trials involving these compounds, identifying patients who would most likely benefit from treatment, and ultimately for managing patients with these agents in either the prevention or therapeutic setting.

In the present study, we used the Poly E-resistant H460 cell line and two Poly E-sensitive cell lines, SW480 and Flo-1, to investigate the growth inhibitory effects of Poly E. We conducted these studies with Poly E, rather than EGCG, since Poly E is the green tea-derived compound that is being most commonly studied in NCI-sponsored early-phase prevention and therapy trials. We compared gene expression profiles of untreated cells to identify potential markers of Poly E resistance. We also analyzed gene expression profiles of cells treated with Poly E to identify potential markers of Poly E response and activity. We demonstrated that resistance to Poly E in H460 cells is largely driven by increased expression of the anti-apoptotic protein BCL2. If validated in additional cell lines and *in vivo* models, BCL2 could ultimately serve as a marker of resistance to the anti-cancer properties of Poly E.

MATERIALS AND METHODS

Compounds, Antibodies, and Cell Lines

Poly E and EGCG were supplied in powder form by the Division of Cancer Prevention (NCI, Bethesda, MD) and were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) and

stored at −20°C in 50 mg/mL aliquots wrapped in foil. HA14-1 (ethyl 2-amino-6-bromo-4-1-cyano-2-ethoxy-2-oxoethyl-4H-chromene-3-carboxylate; Alexis Biochemicals, San Diego, CA) was dissolved in DMSO (Sigma-Aldrich) and stored at −20°C in 200 mM aliquots. Compounds were added directly to cell culture media at a final concentration of 0.05-0.1% DMSO. Primary antibodies were obtained from the following companies: (a) BCL2 (100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (b) P53 (1C12; Cell Signaling Technology, Inc., Danvers, MA); (c) cleaved PARP (Asp 214; Cell Signaling Technology, Inc.); and (d) actin (Sigma-Aldrich). Human H460 lung carcinoma, SW480 colon adenocarcinoma, and Flo-1 esophageal adenocarcinoma cell lines were generously provided by Dr. David Beer (University of Michigan, Ann Arbor, MI) and were grown in DMEM supplemented with 10% fetal bovine serum (FBS). HCT116 and HT29 colon cancer cells were provided by Dr. Seiji Adachi (Columbia University, New York, NY); MeWo melanoma cells were provided by Dr. Giannicola Genovese (Columbia University); MCF7 and MDA MB231 cells are commercially available (American Type Culture Collection, Manassas, VA). HCT116, HT29, and MCF7 cells were grown in 10% FBS-DMEM; MDA-MB-231 cells were grown in 10% FBS-MEM; and MeWo and HCE7 human esophageal squamous carcinoma (13) cells were grown in 10% FBS-RPMI 1640. All cell culture media and FBS were obtained from Life Technologies, Inc. (Grand Island, NY). All cells were maintained at 37°C in a 5% CO_2 atmosphere.

Cell Proliferation Assays

Cell proliferation was measured using the MTT Cell Proliferation Kit I (Boehringer Mannheim, Indianapolis, IN), which colorimetrically measures a purple formazan compound produced only by viable cells. Cells were plated in flat-bottomed, 96-well microtiter plates (2.0×10^3 cells/6.4-mm-diameter well). After 24 h, cells were treated with DMSO (0.1%) or increasing doses of Poly E. For co-treatment assays, HA14-1 was added 24 h or 48 h after plating. After 72 h, cells were treated with 10 μl of MTT reagent for 4 h at 37°C and then treated with 100 μl of solubilization solution at 37°C overnight. The quantity of formazan product was measured using a spectrophotometric microtiter plate reader (Dynatech Laboratories, Alexandria, VA) at 570 nm wavelength. Results were expressed as a percentage of growth, with 100% representing control cells treated with DMSO alone. All experiments were performed in duplicate.

Cell Growth Assays

Cells were plated in 10 cm culture dishes at concentrations to yield 60-70% confluence within 24 h. Cells were then treated with either media alone or Poly E (25 μg/ml). The numbers of cells were measured using a Coulter Counter (Coulter Electronics, Inc., Beckman Coulter Co., Fullerton, CA).

RNA Extraction and Labeling

H460, SW480, and Flo-1 cells were plated in 10-cm culture dishes at concentrations determined to yield 60–70% confluence within 24 h. All cells were treated with either 10% FBS-DMEM alone (untreated) or Poly E (25 μg/mL). The H460 Poly E-resistant cell line was also treated with an additional Poly E treatment dose (100 μg/mL) based on the higher IC_{50} value of this cell line. Following treatment for 6 h and 24 h, adherent cells were

harvested; untreated cells were collected during the 6 h treatment group. cRNA preparation and array hybridization were conducted in collaboration with the Columbia University Microarray Project. Total cell RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and cleaned and precipitated with the Qiagen RNeasy Cleanup kit (Qiagen, Valencia, CA). The integrity of extracted RNA was checked by agarose gel electrophoresis using 36 μl of total RNA. Comparable RNA quality across the various cell lines and treatment conditions was confirmed by identifying sharp 28S and 18S major rRNA bands (*data not shown*). RNA was reverse transcribed into double-stranded cDNA using Superscript II (Invitrogen) and then cleaned with phase lock gels-chloroform extraction (Qiagen). Biotin-labeled cRNAs were generated by *in vitro* transcription (Enzo BioArray HighYield RNA Transcript Labeling Kit, Enzo Life Sciences, Farmingdale, NY), fragmented by heating at 94°C, and then hybridized onto the Affymetrix GeneChip oligonucleotide microarray, Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA). (Quality of labeled cRNA and fragmented cRNA was determined by gel electrophoresis prior to array hybridization; *data not shown*.) Slides were washed and scanned using a confocal laser scanner to generate fluorescence intensities. All treatment conditions were performed in duplicate.

Gene Expression Analysis

Gene expression signal intensities were normalized using robust multichip analysis. Unsupervised hierarchical clustering was performed using a total sampling of 2000 genes – 500 genes with the highest variability within each of 4 quantiles of expression distribution – low, moderately low, moderately high, and high. Differences in gene expression profiles between Poly E-sensitive (SW480, Flo-1) and Poly E–resistant (H460) cells were evaluated using the moderated t statistic integrated into the Linear Models for Microarray Data (LIMMA) package and based on an empirical Bayes approach (14). A gene was considered significantly differentially expressed if its corresponding unadjusted p-value was less than 0.01. This resulted in a false discovery rate (FDR) of less than 6%. A working set of “interesting” genes was created by screening this set into those with a fold change of 2 or greater.

The working set was analyzed further using FatiGO, a web-based software application (http://babelomics.bioinfo.cipf.es/EntryPoint?loadForm=fatigo), which can identify Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) terms that are overrepresented among the working set of genes (i.e., differentially expressed between resistant and sensitive cell lines), as compared with the entire 54,613-gene genome. Four databases were considered – GO Biological Process, GO Molecular Function, GO Cellular Component, and KEGG. Terms in levels 6 or higher of the GO databases and all terms in the KEGG database with a corresponding adjusted p-value less than 0.01 were explored more closely for potentially interesting patterns.

Protein Extraction and Western Blotting

The methods for protein extraction and Western blot analysis have been described previously (15). Briefly, cells were treated with 10% FBS-DMEM (negative control), Poly E alone (25 μg/mL), increasing concentrations of HA14-1 in combination with Poly E (25 μg/ mL), or 5 nM or 10 nM *bcl2*-specific siRNA with Poly E (25 μg/mL). After treatment, cell lysates

were prepared, and 30–60 μg of protein were separated by SDS-PAGE (8-15%). After transfer to nitrocellulose membranes (Millipore, Bedford, MA), blots were blocked with 5% milk protein, incubated for 1 h or overnight at 4°C with the indicated primary antibody, and then reincubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein-antibody complexes were detected by the enhanced chemiluminescence system (Amersham, Piscataway, NJ). Immunoblotting for actin was performed to verify equivalent amounts of loaded protein.

siRNA Transfections

The siRNA ON-TARGET plus SMARTpool (pool of four designed siRNA duplexes which target the *bcl-2* gene) and individual siRNA duplexes (Duplex 1 (D1) catalogue # J-003307-16, Duplex 2 (D2) catalogue # J-003307-19) were purchased from Dharmacon Research Inc. (Chicago, IL) and dissolved in RNAse-free ddH2O. Stock solution aliquots (200 μM) were stored at −20°C. siRNA transfections were performed the same day cells were seeded using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's “Reverse Transfection” instructions. For each reaction, siRNA-transfection reagent complexes were prepared by mixing RNA oligonucleotides with transfection reagent at the indicated dilution and incubating the complexes for 10 min in serum-free media at room temperature. Final concentrations of siRNA were between 1 and 100 nM. Transfections were performed in 1 ml of 10% FBS-DMEM for 24 h; fresh 10% FBS-DMEM was replaced 24 h following transfection; and cells were assayed 24 to 96 h post-transfection. For each assay, siControl pool RNA (Dharmacon) was used as a negative (i.e., non-targeting) control.

Analysis of Mitochondrial Membrane Potential (MMP)

MMP was measured using the JC-1 staining assay (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were adjusted to a density of 1×10^6/ml, harvested using trypsin, washed with PBS, resuspended in 1 ml of 10% FBS-DMEM, and stained with 5 μg/mL JC-1 mitochondrial membrane dye for 15-20 minutes at 37°C with 5% CO_2 in the dark. (JC-1 dye was dissolved in DMSO (Sigma-Aldrich), and a 2X working solution was prepared in FBS-free DMEM medium (Life Technologies, Inc.) and stored at −20°C.) Cells were then washed twice with PBS and resuspended in 0.5 ml 10% FBS-DMEM. Positive control cells were resuspended in DMEM containing the depolarizing compound CCCP (1 μL/mL). Cells were analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA). All experiments were performed in duplicate.

Statistical Analyses

Data are expressed as mean +/− standard deviation. Comparisons between untreated control (i.e., 0.1% DMSO and Poly E 0 μg/ml) cells and treated (e.g., Poly E-, Poly E plus siRNA-, or Poly E plus HA 14-1-treated) cells were made using the unpaired t-test. Differences between groups of $p < .01$ or $p < .05$ were considered statistically significant.

RESULTS

H460 cells are resistant to the growth inhibitory effects of Poly E

We investigated the effects of Poly E on cell growth in four human cancer cell lines – SW480, H460, Flo-1, and HCE7. Exponentially dividing cells were treated with increasing concentrations of Poly E (0–100 μg/mL) for 72 h. Statistically significant reductions in cell viability were seen after treatment with 20, 30, and 50 μg/ml Poly E in the HCE7, Flo-1, and SW480 cell lines, whereas significant inhibition in the H460 cells occurred only with treatment of Poly E at doses of 60 μg/ml or higher (Fig. 1). In the SW480, HCE7, and Flo-1 cell lines, Poly E caused marked growth inhibition, in a dose-dependent fashion, with IC_{50} values in the range of 15-25 μg/mL (Fig. 1). However, the H460 cells were markedly resistant to growth inhibition, and the IC_{50} value was 100 μg/mL, approximately four-fold higher than for the Poly E-sensitive cell lines. Of note, H460 cells were also resistant to the growth inhibitory activity of EGCG, the major component of Poly E (*data not shown*). To investigate the mechanism of H460 cell resistance, we compared gene expression profiles between Poly E-resistant and Poly-E sensitive cells. Unsupervised hierarchical clustering was performed using a sampling of 2000 genes and revealed that untreated SW480 and Flo-1 cells clustered separately from untreated H460 cells (Fig. 2). Cells that were treated with Poly E (25μg/mL, 100 μg/ml.) for 6 h and 24 h also clustered according to cell line (*data not shown*).

BCL2 gene expression is higher and P53 gene expression is lower in Poly E-resistant H460 cells compared with Poly E-sensitive SW480 and Flo-1 cells

We used the LIMMA approach to identify genes whose expression levels were significantly different between Poly E-resistant and Poly E-sensitive cells. Of the 54,613 genes contained in the genechip, the expression levels of 6,242 were significantly different at the 0.01 level of significance with a corresponding FDR < 6% (*data not shown*). We created a working genelist using the following criteria: 1) genes with a fold-change of 2 or greater; and 2) genes that were categorized with the GO term “apoptosis”. We chose this term to screen genes, because the mechanism of action of many anticancer agents, including chemotherapy drugs and Poly E, can be attributed to the induction of apoptosis. Of the 6,242 significantly different genes, 2,929 had a fold change ≥ 2 (*data not shown*), including 62 “apoptosis”-related probe sets (Table 1A). In the resistant H460 cells, expression levels were significantly increased in 46 gene probe sets, including BCL2 (10.60-fold higher, $P < 0.0001$), and significantly decreased in 16 gene probe sets, including two P53 probe sets (2.68-fold lower, $P = 0.0067$; 2.57-fold lower, $P < 0.0001$). Three additional BCL2 probe sets were increased in H460 cells, although they did not satisfy the statistical criteria used in our analysis (*data not shown*). Both BCL2 overexpression and P53 gene mutations have been implicated in resistance to many drugs, including many chemotherapeutic agents (16, 17). The “apoptosis” gene signature included other genes whose relative expression could explain drug resistance, including increased expression of the anti-apoptotic *mcl-1* and *bag-2* genes and reduced expression of the pro-apoptotic *bid* gene (Table 1A). The contribution of increased expression of the pro-apoptotic *fas-1, caspase 7*, and *caspase 8* genes to Poly E resistance in H460 cells is less clear. Moreover, there were marked differences in gene

expression in other less studied genes, including BCL2 interacting protein 3, synuclein, alpha, and osteopontin.

We also investigated whether any biological or molecular or cellular processes were over-represented among the 6,242 differentially expressed genes and explored if these processes could suggest additional mechanisms of Poly E resistance. Using the FatiGO application, we explored terms in levels 6 or higher of three GO databases (Biological Process, Molecular Function, Cellular Component) and all terms in the KEGG database that were over-represented when compared with the 54,613-gene genome. We identified multiple significant terms that were over-represented with a corresponding p-value less than 0.01 (Table 1B). There were no significant over-represented terms in the KEGG analysis. We then explored the inclusion of BCL2 and P53 in each significant process by determining the prevalence of each gene, by searching for each of 4 BCL2 and 2 P53 probe sets. For example, 1 of the 4 BCL2 and both P53 probe sets were present in the “cell death” pathway that was identified in level 6 of the GO Biological Process database. Interestingly, this analysis could suggest that decreased P53 expression may have a greater impact than increased BCL2 expression in determining Poly E resistance, since it was included more frequently in the over-represented terms.

Treatment with Poly E did not induce significant changes in gene expression in any cell line

We investigated whether treatment with Poly E induced changes in gene expression in Poly E-resistant cells that were different from those induced in Poly E-sensitive cells in an effort to identify potential markers of Poly E response or activity. We used the LIMMA approach to compare gene expression between untreated and treated samples within the Poly E-resistant (H460) cell line and within the Poly E-sensitive (SW480, Flo-1) cell lines and performed an overall comparison between untreated and treated samples across all three cell lines. We did not detect any significant differences in expression between treated and untreated samples – the fold-changes for all genes before and after treatment were ≤ 1.5 at the 0.001 level after adjusting for multiple comparisons (*data not shown*). Because only duplicate samples were run for each treatment condition, this experiment was likely not powered to detect significant treatment effects, given the inherent variability in expression in individual genes.

BCL2 protein expression is increased and P53 protein expression is absent in untreated H460 cells

To confirm our findings of increased BCL2 gene expression and decreased P53 gene expression in H460 cells (Table 1A), we examined the levels of protein expression in untreated cells. Immunoblotting demonstrated that basal BCL2 expression is significantly increased in untreated H460 cells compared with untreated SW480 and Flo-1 cells (Fig. 3A). Similarly, P53 protein expression is present in untreated SW480 and Flo-1 cells and virtually absent in untreated H460 cells. Therefore, these results confirm the changes in BCL2 and P53 gene expression identified from the initial microarray studies (Table 1A).

We next investigated a panel of cell lines with varying degrees of BCL2 and P53 protein expression (Fig. 3B). Human MeWo melanoma and MCF7 breast cancer cells displayed high BCL2 and low P53 protein expression, which is similar to the profile seen in H460 cells. Conversely, there was relatively higher P53 and lower BCL2 protein expression in HT29 colon cancer and MDA MB231 breast cancer cells. All cell lines were then treated with 25 μg/ml Poly E for 48-72 h, which is in the range of the IC_{50} value of the Poly E-sensitive SW480 and Flo-1 cells. Unfortunately, preliminary studies did not demonstrate a correlation between BCL2 protein expression and/or P53 protein expression and growth inhibition (as measured by the Coulter Counter method) – MeWo and MCF7 cells were the most sensitive; HCT 116 and HT29 cells were moderately sensitive; and MDA MB231 cells were the least sensitive; *data not shown*. Therefore, triplicate MTT experiments were not pursued, and IC_{50} values were not formally determined. Thus, the importance of BCL2 in determining Poly E resistance is cell-line dependent.

We also determined that levels of BCL2 protein expression were similar in untreated H460 cells and H460 cells that were treated for 24 h with Poly E (25 μg/mL) (*data not shown*). This is consistent with our microarray analysis, which revealed no significant differences in the expression of *bcl-2* or other genes between Poly E-treated and untreated cells. We did not determine whether treatment with Poly E for different durations affected BCL2 protein expression in H460 or SW480 or Flo-1 cells. From these studies, we demonstrated that both increased expression of the anti-apoptotic protein BCL2 and decreased expression of the pro-apoptotic protein P53 could contribute to Poly E resistance in H460 cells. To further study the effects of these proteins on resistance, we could either restore expression (or induce over-expression) of wild-type P53 or inhibit or knock down the activity or expression of BCL2. Previous studies have demonstrated that restoring wild-type P53 expression in *p53* −/− cells can often result in cell death (18), which would make additional *in vitro* analyses difficult. On the other hand, *bcl-2* gene silencing and BCL2 inhibition can be accomplishment quite effectively using RNA interference (RNAi) and small molecule inhibitors, respectively (19, 20). Furthermore, the latter approach is now being studied in the clinical setting (21, 22).

BCL2 knockdown can decrease H460 resistance to the growth inhibitory effects of Poly E

To investigate the dose- and time-dependent effects of *bcl-2*-specific siRNA on BCL2 protein expression, we initially transfected H460 cells with 5nM and 10nM of SMARTpool siRNA and measured expression after 24h, 48h, and 72h of treatment. Previous reports have demonstrated that transfection of cells with *bcl-2*-specific siRNA at concentrations ≥100 nM frequently lead to nonspecific off-target effects, and concentrations of 10–20 nM minimize off-target binding and exert fewer nonspecific effects (19). Western blot analysis revealed a significant reduction in BCL2 protein expression 24 h after transfection with 5 nM and 10 nM SMARTpool siRNA in a dose-dependent manner compared with untreated cells (Fig. 4A). These effects were increased after 48 h, but some recovery in expression occurred after 72 h of treatment (5 *nM, 72 h treatment not shown*). We also demonstrated significant inhibition of protein expression 24 h, 48 h, 72 h, and 96 h after transfection with 10 nM of two individual siRNA duplexes (D1 and D2; Fig. 4B, *72 h data not shown*). The maximum decrease in BCL2 protein expression occurred at 72 h and 96 h using 10 nM D2 siRNA.

To examine the effects of decreased BCL2 protein expression on sensitivity to Poly E, H460 cells were plated in 96-well plates under the following conditions – no siRNA transfection (untreated cells), cells transfected with negative siControl RNA or 10 nM D1 siRNA or 10 nM D2 siRNA. Twenty-four hours after plating, exponentially dividing cells were treated with increasing concentrations of Poly E (0–100 μg/mL) for 72 h. Untreated cells (i.e., Poly E alone) and cells transfected with negative siControl RNA were relatively resistant to Poly E, and significant reductions in cell viability occurred only at the Poly E 100 μg/ml dose (Fig. 4C). In contrast, D1- and D2-transfected cells were extremely sensitive to Poly E with IC_{50} values in the 5-8 μg/mL range. Poly E treatment doses as low as 10 μg/ml and 5 μg/ml led to significant reductions in cell viability in D1- and D2-transfected cells, respectively. Therefore, reduced BCL2 expression increased H460 sensitivity to the growth inhibitory effects of Poly E.

BCL2 inhibition by HA14-1 can decrease H460 resistance to the growth inhibitory effects of Poly E

To examine the effects of BCL2 inhibition on sensitivity to Poly E, H460 cells were co-treated with increasing doses of Poly E and the small molecule BCL2 functional antagonist, HA14-1 (20). HA14-1 binds to the BCL2 surface pocket and interferes with its function by disrupting the interaction with its target proteins, including BAX. H460 cells were initially plated in 96-well plates and treated after 24 h under the following conditions – increasing doses of Poly E (0–100 μg/mL) alone; increasing doses of HA14-1 (0-50 μM) alone; increasing doses of Poly E (0–100 μg/mL) and 5 μM HA 14-1; increasing doses of Poly E (0–100 μg/mL) and 25 μM HA14-1. After 72 h, H460 cells co-treated with 5 μM and 25 μM HA14-1 were sensitive to Poly E with IC_{50} values of 25 μg/mL (Fig. 4D), which are similar to the IC_{50} values of Poly E-sensitive (SW480, Flo-1) cells (Fig. 1). In fact, there were significant reductions in cell viability in H460 cells that were co-treated with either dose of HA14-1 and all doses of Poly E (5-100 μg/ml), except the HA 14-1 (5 μM)/Poly E (10 μg/ml) combination – this latter dose treatment likely did not reach significance due to the larger standard deviation. Treatment with HA14-1 alone also led to growth inhibition, especially at concentrations above 25 μM, which is consistent with previous reports (20). Therefore, both doses of HA14-1 were equally effective in decreasing Poly E resistance, although treatment with the higher 25 μM dose alone resulted in a higher degree of toxicity. By western blotting, we also determined that treatment of H460 cells with HA14-1 (5 μM and 25 μM) with and without Poly E (25 μg/mL) did not significantly affect the level of BCL2 protein expression (*data not shown*), which is consistent with HA14-1's ability to inhibit BCL2 activity and not expression (20). Therefore, inhibition of BCL2 activity increased H460 sensitivity to the growth inhibitory effects of Poly E.

Loss of MMP and increased PARP cleavage occur after treatment with Poly E, HA14-1, and bcl2-specific siRNA in H460 cells

Because inhibition of BCL2 expression and activity attenuated resistance to Poly E in H460 cells, we investigated whether BCL2 inhibition interfered with downstream effects of BCL2 signaling – maintenance of MMP and prevention of apoptosis. Downregulation or inhibition of BCL2 disturbs the mitochondrial membrane, leading to a loss of mitochondrial membrane

integrity and a loss of MMP. Cytochrome c is released, and apoptosis is induced as demonstrated by the cleavage of PARP (20, 23).

Using the JC-1 mitochondrial membrane dye and flow cytometry, we measured the MMP in H460 cells after treatment under the following conditions: no treatment; 10 nM siControl RNA (negative control), 10 nM D1 siRNA, 10 nM D2 siRNA, 10 nM D2 plus Poly E (25 μg/mL), 10 nM D2 plus Poly E (25 μg/mL) plus HA14-1. Values were normalized to untreated cells (value 1.0). Transfection with 10 nM D1 and 10 nM D2 led to 25% and 45% reductions in MMP, respectively, compared with untreated cells (Fig. 5A). Transfection with 10 nM D2 and Poly E (25 μg/mL) decreased MMP by 35% compared with untreated cells, and transfection with 10 nM D2 and Poly E (25 μg/mL) and HA 14-1 resulted in an 85% decrease in MMP. The MMP of cells transfected with a positive control (CCCP, a disrupter of electron transport) also decreased by 85%, while the MMP of cells transfected with 10 nM negative siControl RNA was virtually identical to that of untreated cells. Statistically significant reductions in MMP occurred in all D2-transfected cells – D2 alone, D2 plus Poly E, and D2 plus Poly E plus HA 14-1. Similarly, treatment with 10 nM D1 siRNA, 10 nM D2 siRNA, 10 nM D2 plus Poly E (25 μg/mL, 24 h), and 10 nM D2 plus Poly E (25 μg/mL, 48 h) led to increased apoptotic death as demonstrated by PARP cleavage (Fig. 5B). Of note, compared with D1 siRNA, transfection of D2 siRNA led to greater Poly E sensitivity (i.e., lower $IC_{50;}$ Fig. 4C), greater loss of MMP (Fig. 5A), and greater PARP cleavage (Fig. 5B).

Thus, H460 cells that were transfected with *bcl-2*-specific siRNA and co-treated with HA14-1 and Poly E demonstrated increased BCL2-related apoptotic death, as demonstrated by PARP cleavage and decreased MMP.

DISCUSSION

This is the first report identifying BCL2 as a potential marker of resistance to the growth inhibitory activity of Poly E. BCL2 has been known to confer resistance to chemotherapeutic agents in a variety of human cancers (for review, please refer to (17)). Resistance occurs at a distal point in the apoptotic process, involving the pro- and anti-apoptotic BCL2 family member proteins which operate at the convergence of multiple pathways leading to the major types of cell death, including apoptosis, necrosis, and autophagy. BCL2 has also been shown to confer resistance to chemotherapy in the clinical setting, and both BCL2 overexpression and *bcl-2* gene rearrangements have been associated with chemoresistance and worsened prognosis in cancers, such as non-Hodgkin lymphoma (24). There is one previous report demonstrating BCL2-induced resistance to non-chemotherapeutic anti-cancer compounds. In a leukemia cell line model, overexpression of BCL2 led to resistance against FLT3 inhibitors, which could explain the limited clinical efficacy of FLT3 inhibitors in the treatment of AML (25). While induction of the pro-apoptotic BCL2 family member BIM has been shown to play a role in apoptosis induced by the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib in lung cancer, upregulation of anti-apoptotic BCL2 family member proteins has not yet been implicated in EGFR TKI resistance (26, 27).

A previous report demonstrated the ability of EGCG and other green tea-derived catechins to bind strongly to the hydrophobic grooves of both BCL2 and BCL-$_{XL}$ (28). The authors proposed that direct inhibition of these proteins could explain the cancer prevention activity of EGCG, although they did not correlate the degree of binding with effects on apoptosis or cell growth. We did not measure the BCL2 binding affinity of the catechins contained in Poly E. However, we did demonstrate that co-treatment of the resistant H460 cells with Poly E and the BCL2 inhibitor HA14-1 restored sensitivity (Fig. 4D) and decreased MMP (Fig. 5A) and that treatment with Poly E and *bcl-2*-specific siRNA was more effective than siRNA alone in causing growth inhibition (Fig. 4C) and apoptosis (Fig. 5B). Although these studies do not provide direct evidence that BCL2 is a molecular target of Poly E, they do suggest that BCL2 inhibition could provide an approach for overcoming resistance. Of course, the mechanism of Poly E resistance is likely to be cell line-dependent and will not always be determined by the relative expression of BCL2 and P53. Conversely, high BCL2 expression will not always lead to Poly E resistance as in the case of MeWo and MCF7 cells that are quite sensitive to Poly E (Fig. 3B).

In our studies, treatment with Poly E did not decrease BCL2 gene or protein expression (*data not shown*); although these assays were performed using a limited number of time points. Interestingly, in the previously mentioned report of BCL2 and resistance to FLT3 inhibitors, treatment with the inhibitor also did not affect BCL2 expression (25). On the other hand, other investigators have shown that EGCG-mediated apoptosis is associated with decreased BCL2 expression in human melanoma, sarcoma, osteosarcoma, prostate cancer, and breast cancer cell lines (29-33).

We also demonstrated that P53 gene and protein expression were significantly reduced in H460 cells (Table 1A and Fig. 3A). P53 gene mutations have also been implicated in chemotherapy resistance (16, 34), and previous studies have demonstrated the development of EGCG resistance after knockout of p53 in both mouse JB6 and human prostate cancer cells (35-37). The “resistant” phenotype is often characterized by alterations in multiple and synergistic resistance pathways (16), and several factors may be contributing to H460 resistance to Poly E. However, despite the multiple potential genetic changes in H460 cells that could account for their resistance to Poly E – increased expression of *bcl-2, mcl-1* and *bag-2* and reduced expression of *p53* and *bid* (Table 1A), correction of the BCL2 increase alone (i.e., reduced expression and activity) was sufficient to overcome Poly E resistance (Fig. 4C and Fig. 4D). Thus, BCL2-induced resistance in H460 cells appears to occur independently of p53-mediated regulation of pro- and anti-apoptotic genes. For example, the activity of BCL2 family proteins can also be modulated by post-translational modifications, including phosphorylation (38).

There have been few reports investigating the mechanism of resistance to EGCG, Poly E, or other green tea-derived catechin. In a series of experiments similar to ours, and coincidentally also in a lung cancer cell line, Kweon, et. al., demonstrated that A549 cells were markedly resistant to apoptosis induction by EGCG (100 μM) (39). Resistance was attributed to Nrf2-mediated overexpression of heme oxygenase-1 (HO-1), a gene that is induced by stress stimuli and may also be associated with chemoresistance. Both inhibition of HO-1 and gene silencing restored sensitivity of these cells to apoptosis induction by

EGCG. Although A549 lung cancer cells express BCL2 protein at levels that are comparable to H460 cells (40), we did not investigate the activity of Poly E in or repeat our series of experiments using this cell line. In fact, HO-1 induction has been shown to induce BCL2 overexpression and resistance to apoptosis in other models of cellular stress and inflammation (41-43). Thus, we would expect that inhibition of BCL2 could also restore sensitivity to EGCG and therefore Poly E. In another study, resistance to EGCG was shown to develop with activation of the MAPK pathway in HER2-positive NF639 breast cancer cells (44). EGCG has previously been shown to inhibit MAPK signaling by inhibiting ERK and MEK activation (45). The level of BCL2 expression in NF639 cells was not reported. In a series of human breast cancers, BCL2 expression was associated with hormone receptor but not HER2 expression (46).

We did not detect significant differences in gene expression between Poly E-treated and untreated cells. Several previous studies were able to identify changes in the expression of specific genes after treatment of cancer cell lines with EGCG or other green tea preparations (for review, please refer to (47)). These discordant results between studies are likely due to multiple confounders and variables, including differences in cell lines, doses (e.g., EGCG range 10-100 μg/mL), green tea preparations (e.g., EGCG, Poly E, GTC, GTP), treatment durations, and assays used for analysis. The largest confounder in our study relates to the small sample size, and increasing the number of replicates for each treatment condition would increase the power to detect significant gene expression differences between Poly E-treated and untreated cells. We could also evaluate more cell lines and additional treatment time-points, in case we are simply missing the time-point at which many gene changes occur.

CONCLUSION

We conducted comparative microarray studies to identify BCL2 as a potential marker of Poly E resistance and demonstrated that inhibition of BCL2 expression and activity restore sensitivity of H460 cells to the growth inhibitory effects of Poly E. Early-phase clinical trials are currently investigating the efficacy of Poly E in preventing or treating a variety of human malignancies. BCL2 expression could be assayed in the human specimens that are being collected in these trials and correlated with the various response and efficacy endpoints used in these trials to further investigate its predictive value as a marker of resistance. BCL2 expression could ultimately be used to design future prospective clinical trials (i.e., for biomarker validation and subsequently as an eligibility criterion) and identify patients who are more likely to respond to Poly E. Moreover, approaches that combine Poly E with BCL2 inhibitors might be more effective in managing precancerous lesions and cancers with high BCL2 expression.

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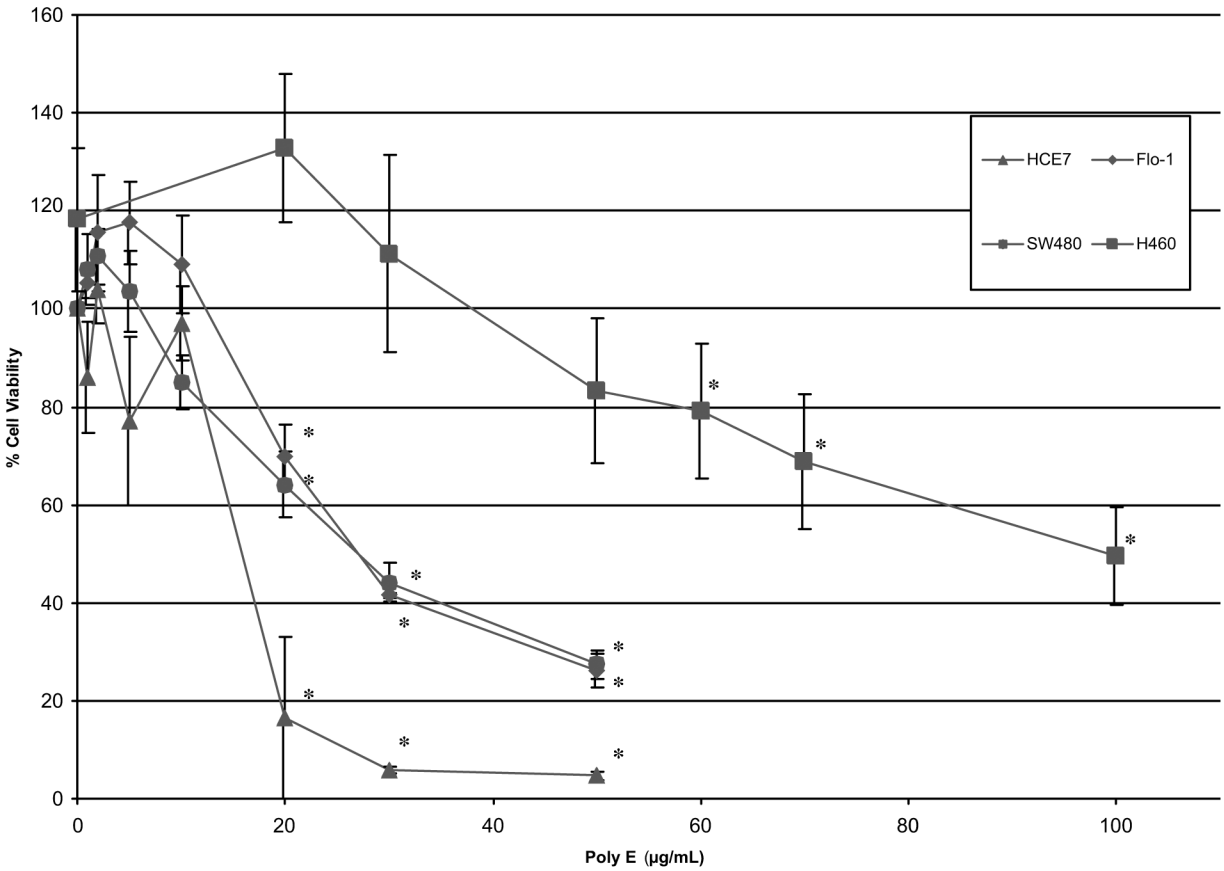


Figure 1.

H460 cells are resistant to the growth inhibitory effects of Polyphenon E. Exponentially dividing cells were treated with increasing concentrations of Poly E for 72 h. Cell viability was determined using the MTT assay. The percentage of growth was calculated, with 100% representing control cells treated with 0.1% DMSO alone. The results are the means ± SDs from quadruplicate experiments (*, $p < .01$).

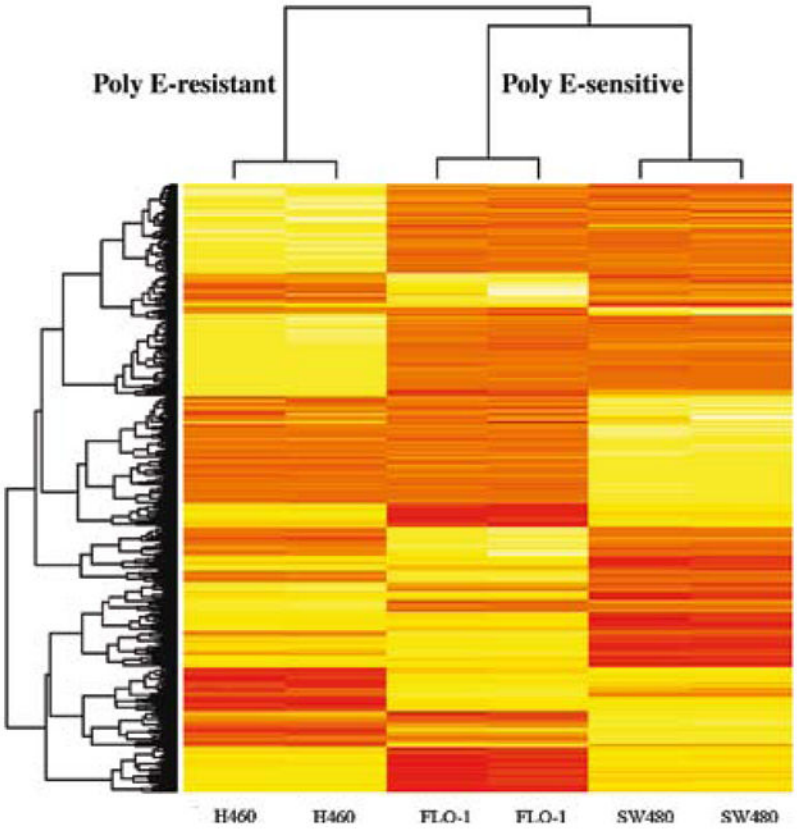


Figure 2.

Poly E-sensitive cells (SW480, Flo-1) cluster separately from Poly E-resistant cells (H460). Gene expression profiles of duplicate samples of untreated H460, Flo-1, and SW480 cells were analyzed using the Affymetrix Human Genome U133 Plus 2.0 array. Unsupervised hierarchical clustering was performed using a sampling of 2000 genes - 500 genes with the highest variability within each of four quantiles of expression distribution.

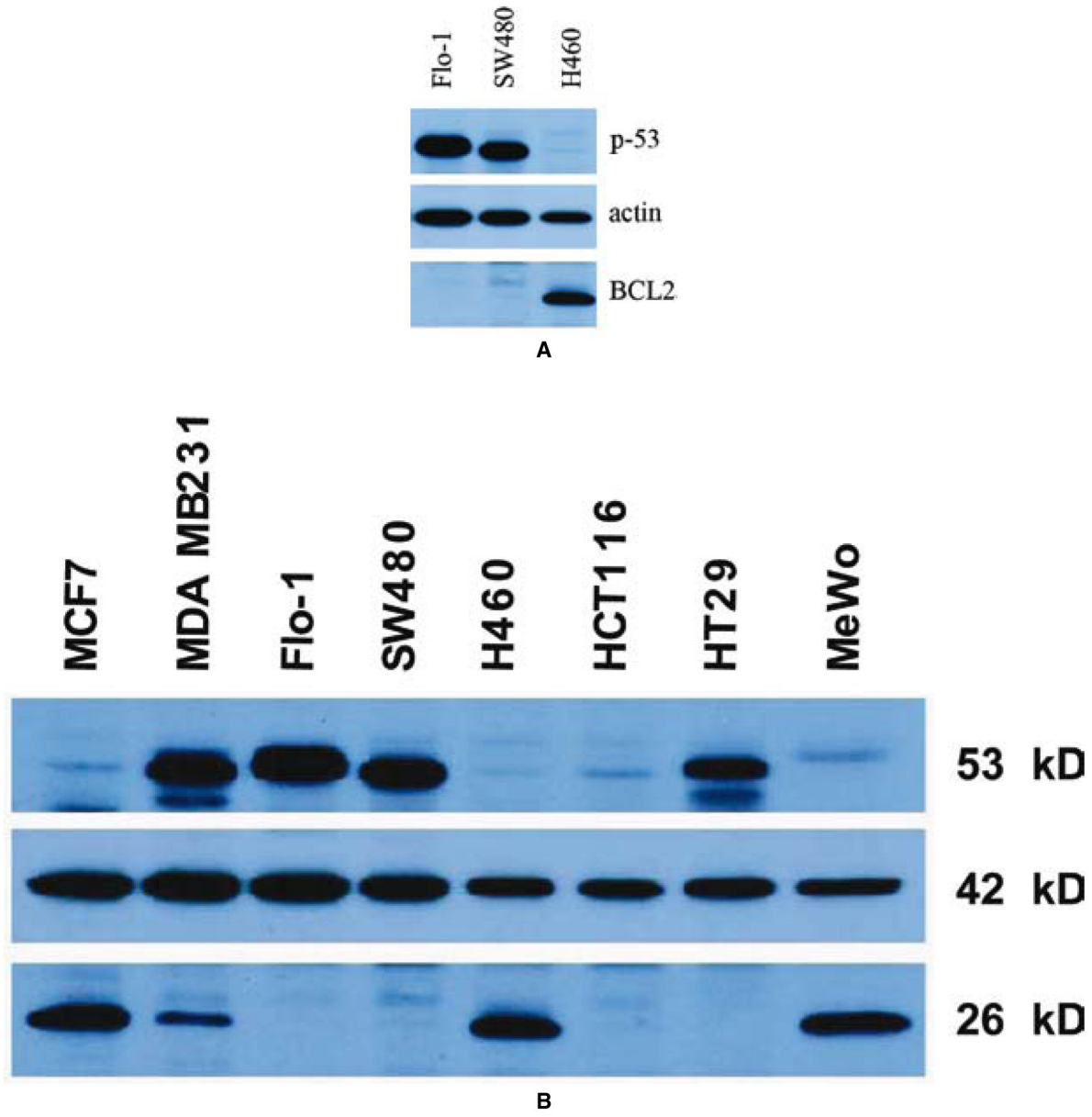
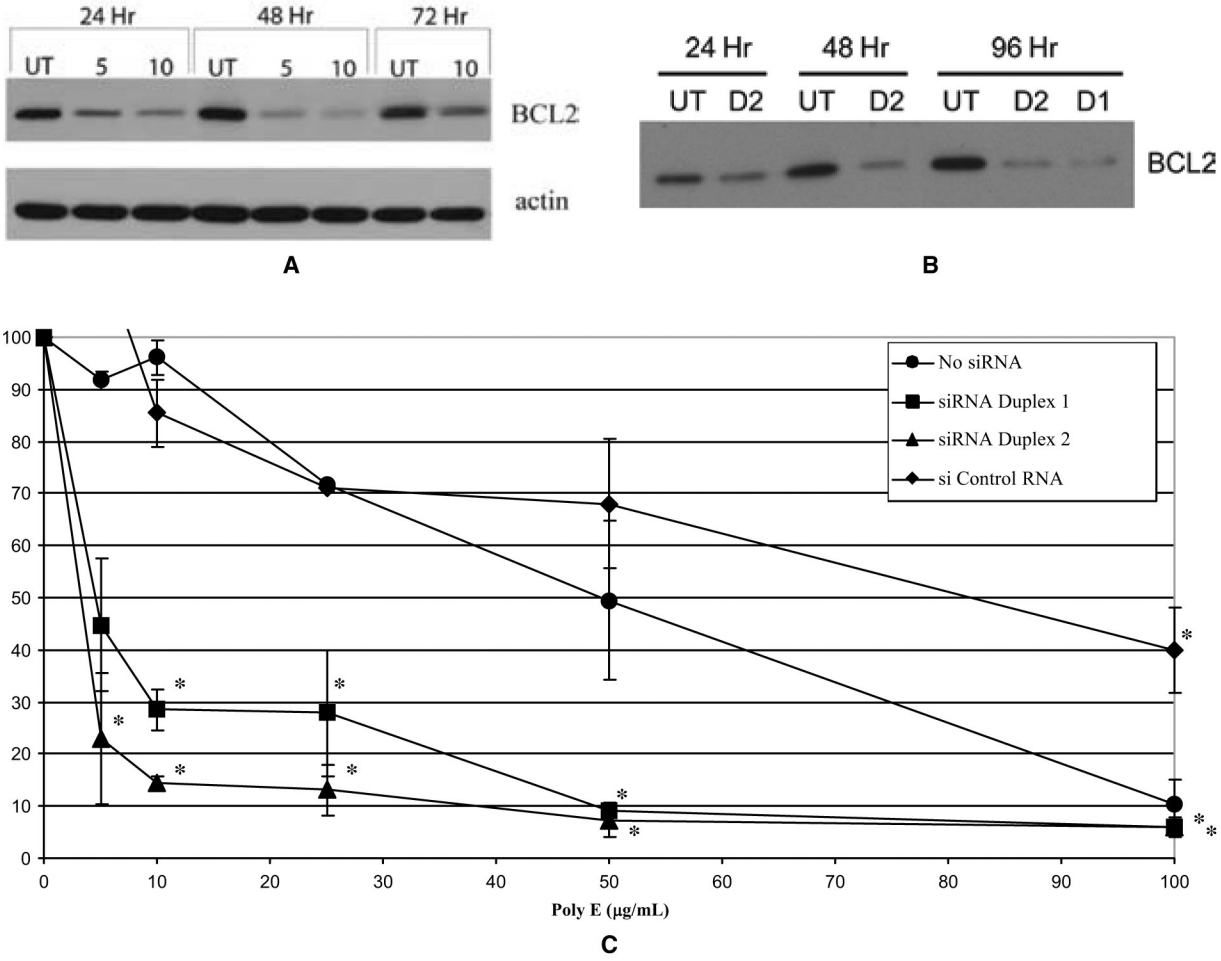


Figure 3.

A, BCL2 protein expression is increased and P53 protein expression is absent in untreated H460 cells. B, Levels of BCL2 and P53 protein in a series of human cancer cell lines – MCF7, MDA MB231, Flo-1, SW480, H460, HCT116, HT29, MeWo. Cells were plated in 10% FBS-DMEM/MEM/RPMI. After 48 h, cell lysates were evaluated for levels of BCL2 and P53 protein by Western blotting. Immunoblotting for actin was performed to verify equivalent amounts of loaded protein.



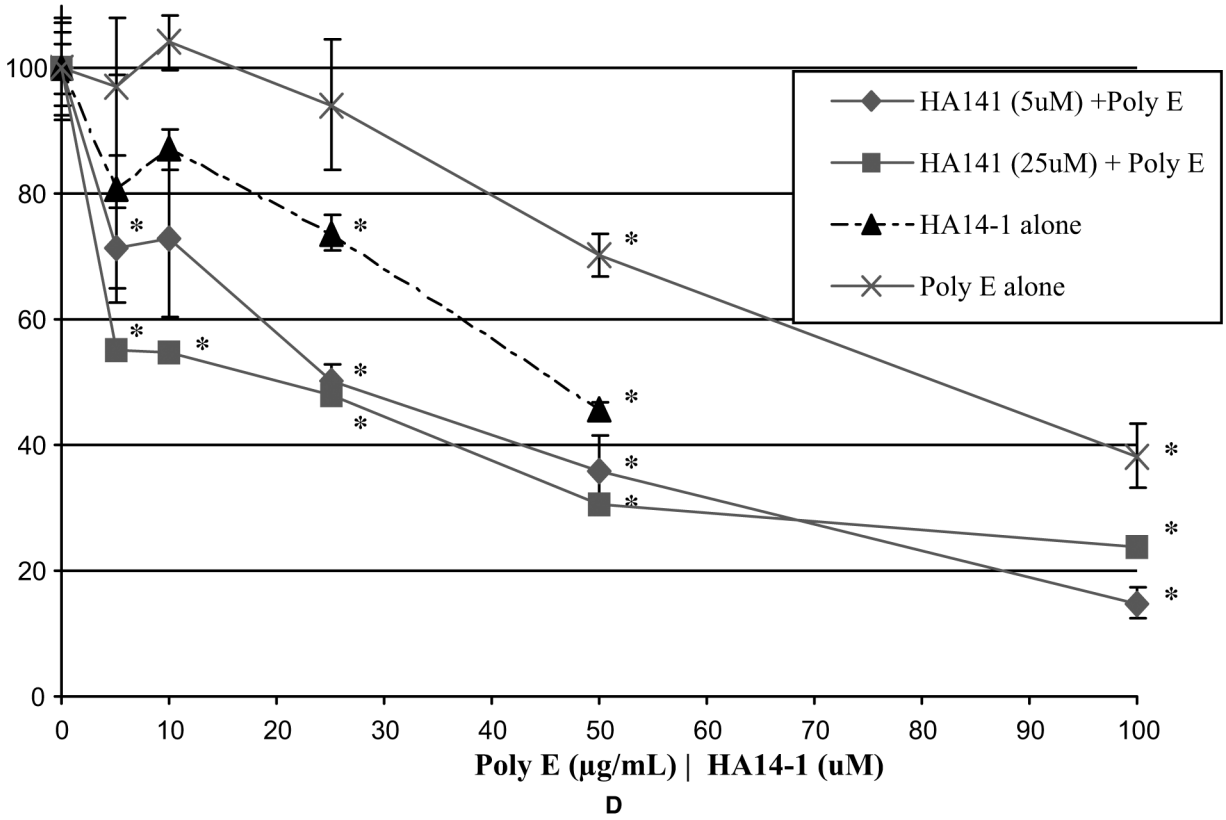


Figure 4.

Inhibition of BCL2 expression and activity attenuates H460 cell resistance to the growth inhibitory effects of Poly E. A, H460 cells were transfected with 5 nM and 10 nM of SMARTpool *bcl-2*-specific siRNA for 24 h, 48 h, and 72 h (*5 nM sample not shown*). Cell lysates from untreated (UT) and transfected cells were collected after each time point and evaluated for BCL2 protein expression by western blotting. Immunoblotting for actin was performed to verify equivalent amounts of loaded protein. B, H460 cells were transfected with 10 nM of two individual *bcl-2*-specific siRNA duplexes (D1 – 96 h; D2 – 24 h, 48 h, and 96 h). Cell lysates from UT and transfected cells were collected after each time point and evaluated for BCL2 protein expression by western blotting. C, exponentially dividing cells were transected with 10 nM of Duplex 1 or Duplex 2 *bcl-2*-specific siRNA or (negative) non-targeting siControl RNA for 24 h and then treated with increasing concentrations of Poly E for 72 h. “No siRNA” cells were not transfected with any siRNA. Cell viability was determined using the MTT assay. The percentage of growth was calculated, with 100% representing control cells treated with 0.1% DMSO alone. The results are the means ± SDs from triplicate experiments (*, $p < .01$). D, exponentially dividing cells were treated under the following conditions: increasing doses of Poly E alone, increasing doses of HA14-1 alone, increasing doses of Poly E and 5 μM HA14-1, increasing doses of Poly E and 25 μM HA14-1. Cell viability was determined after 72 h using the MTT assay. The percentage of growth was calculated, with 100% representing control cells treated with 10% FBS-DMEM alone. The x-axis indicates the doses of Poly E (μg/ml) and/or HA14-1

(μM) that were used in each treatment condition. The results are the means ± SDs from triplicate experiments (*, $p < .05$).

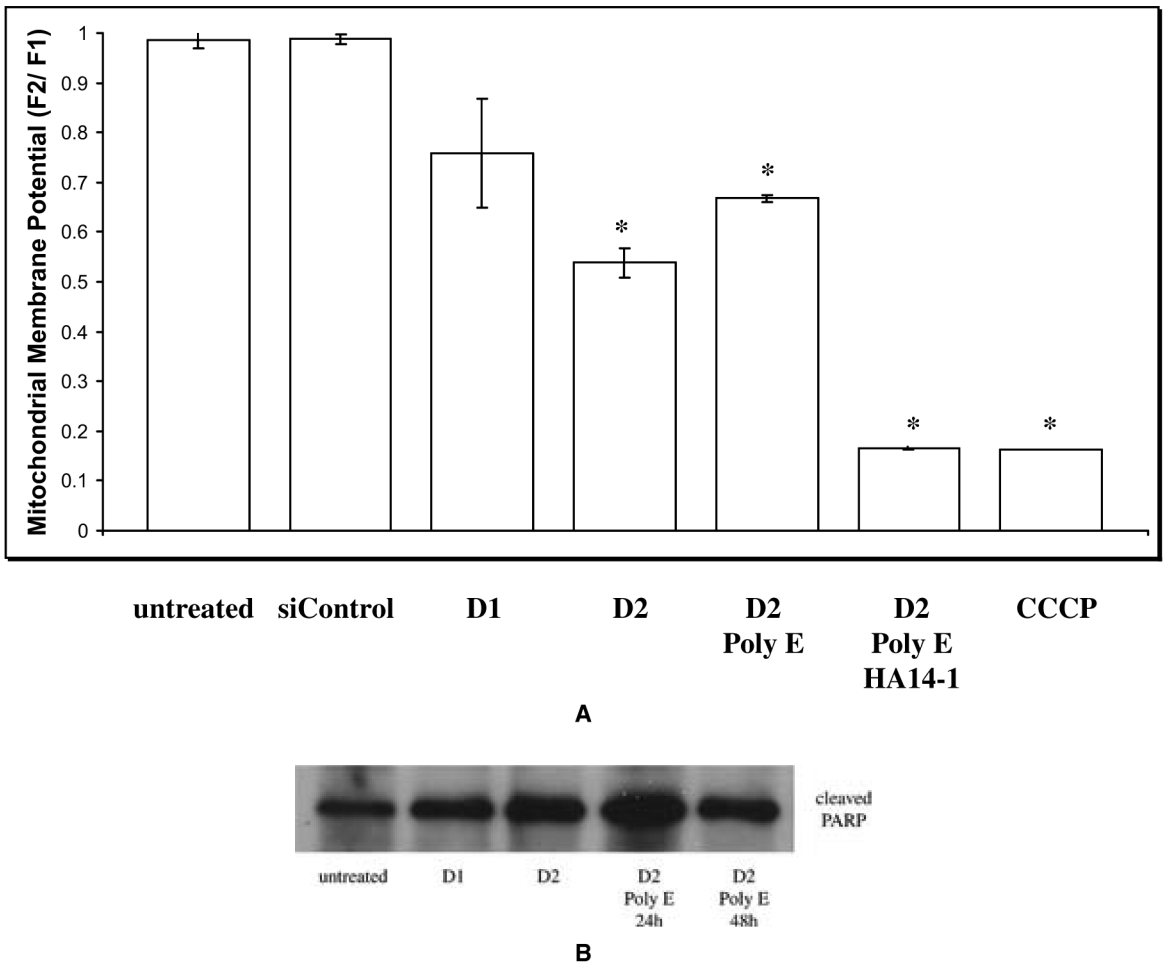


Figure 5.

Treatment of H460 cells with Poly E, *bcl-2*-specific siRNA, and/or HA14-1 decreases MMP and increases PARP cleavage. A, H460 cells were treated under the following conditions: no treatment, 10 nM siControl RNA (negative control), 10 nM D1 siRNA, 10 nM D2 siRNA, 10 nM D2 plus Poly E (25 μg/mL), 10 nM D2 plus Poly E (25 μg/mL) plus HA14-1, CCCP (positive control). MMP was measured using the JC-1 staining assay and flow cytometry. Values were normalized to untreated cells (value 1.0).The results are the means +/− standard deviations from duplicate experiments (*, $p \leq .01$). B, H460 cells were treated under the following conditions: 10 nM D1 siRNA, 10 nM D2 siRNA, 10 nM D2 plus Poly E (25 μg/mL, 24 h), and 10 nM D2 plus Poly E (25 μg/mL, 48 h). Cell lysates were collected and evaluated for cleaved PARP by western blotting. Immunoblotting for actin was performed to verify equivalent amounts of loaded protein (*data not shown*).

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Table 1A.

62 apoptosis-related gene probe sets are differentially expressed between Poly E-resistant (H460) and Poly E-sensitive (SW480, Flo-1) cells

Gene Title	Gene Symbol	Probe Set	Fold Change Higher in H460 Cells	Unadjusted P-value
BCL2-associated athanogene 2	BAG2	209406_at	2.18	0.0001
B-cell CLL/lymphoma 2	BCL2	203685_at	10.60	< .0001
baculoviral IAP repeat-containing 7 (livin)	BIRC7	220451_s_at	2.36	0.002
BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	201848_s_at	106.35	< .0001
BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	201849_at	227.23	< .0001
caspase recruitment domain family, member 6	CARD6	224414_s_at	5.38	<.0001
caspase 7, apoptosis-related cysteine protease	CASP7	207181_s_at	4.63	< .0001
caspase 8, apoptosis-related cysteine protease	CASP8	207686_s_at	4.01	0.0001
caspase 8, apoptosis-related cysteine protease	CASP8	213373_s_at	3.90	0.0004
collagen, type IV, alpha 3 (Goodpasture antigen)	COL4A3	222073_at	3.39	< .0001
engulfment and cell motility 1 (ced-12 homolog, C. elegans)	ELMOl	204513_s_at	4.34	0.0002
fem-1 homolog b (C. elegans)	FEM1B	212367_at	2.48	0.003
forkhead box O1A (rhabdomyosarcoma)	FOXOIA	202724_s_at	3.43	0.0063
G protein-coupled receptor 65	GPR65	214467_at	17.66	< .0001
HIV-1 Tat interactive protein 2, 30kDa	HTATIP2	209448_at	2.30	< .0001
microtubule-associated protein tau	MAPT	203928_x_at	3.25	< .0001
microtubule-associated protein tau	MAPT	203929_s_at	4.19	0.0003
microtubule-associated protein tau	MAPT	20640l_s_at	3.05	<.0001
myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	200796_s_at	2.37	<.0001
myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	200798_x_at	2.23	<.0001
nerve growth factor receptor (TNFRSF16) associated protein 1	NGFRAP1	217963_s_at	25.63	0.0092
neuregulin 2	NRG2	208062_s_at	2.20	0.0008
PRKC, apoptosis, WT1, regulator	PAWR	204004_at	2.22	<.0001
PRKC, apoptosis, WT1, regulator	PAWR	204005_s_at	2.52	0.002
pleiomorphic adenoma gene-like 1	PLAGL1	209318_x_at	11.72	<.0001
pleiomorphic adenoma gene-like 1	PLAGL1	207002_s_at	5.27	<.0001
pleiomorphic adenoma gene-like 1	PLAGL1	207943_x_at	3.04	<.0001

Gene Title	Gene Symbol	Probe Set	Fold Change Higher in H460 Cells	Unadjusted P-value
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	PPP2R1B	202883_s_at	3.70	0.0032
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	PPP2R1B	202884_s_at	2.93	0.0001
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	PPP2R1B	202883_s_at	3.70	0.0032
phosphatidylserine receptor	PTDSR	212722_s_at	2.16	0.0002
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	204614_at	88.86	<.0001
seven in absentia homolog 1 (Drosophila)	SIAH1	229663_at	2.29	0.0004
synuclein, alpha (non A4 component of amyloid precursor)	SNCA	204466_s_at	125.60	<.0001
synuclein, alpha (non A4 component of amyloid precursor)	SNCA	204467_s_at	18.54	<.0001
synuclein, alpha (non A4 component of amyloid precursor)	SNCA	207827_x_at	6.40	<.0001
synuclein, alpha (non A4 component of amyloid precursor)	SNCA	211546_x_at	6.80	<.0001
secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)				
signal transducer and activator of transcription 1, 91kDa	STAT1	209969_s_at	2.77	0.0085
serine/threonine kinase 17a (apoptosis-inducing)	STK17A	202693_s_at	4.56	<.0001
tumor necrosis factor, alpha-induced protein 3	TNFAIP3	202643_s_at	10.77	0.0002
tumor necrosis factor, alpha-induced protein 3	TNFAIP3	202644_s_at	8.67	0.0004
tumor necrosis factor receptor superfamily, member 6	TNFRSF6	216252_x_at	6.84	<.0001
tumor necrosis factor receptor superfamily, member 6	TNFRSF6	204780_s_at	6.80	<.0001
tumor necrosis factor receptor superfamily, member 6	TNFRSF6	204781_s_at	4.78	<.0001
tumor necrosis factor receptor superfamily, member 6	TNFRSF6	215719_x_at	9.58	<.0001

Gene Title	Gene Symbol	Probe Set	Fold Change Lower in H460 Cells	Unadjusted P-value
apoptosis-associated speck-like protein containing a CARD	ASC	221666_s_at	8.16	<.0001
BH3 interacting domain death agonist	BID	204493_at	2.36	0.0022
caspase recruitment domain family, member 10	CARD 10	210026_s_at	18.01	<.0001
death-associated protein 6	DAXX	201763_s_at	2.17	0.0054
immediate early response 3	IER3	201631_s_at	8.32	<.0001
hypothetical protein MGC13096	MGC13096	224467_s_at	2.61	0.0051
hypothetical protein MGC13096	PHLDA2	209803_s_at	4.74	<.0001
RelA-associated inhibitor	RAI	218849_s_at	2.36	0.0025
sphingosine-1-phosphate lyase 1	SGPL1	212321_at	2.72	0.003

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Gene Title	Gene Symbol	Probe Set	Fold Change Lower in H460 Cells	Unadjusted P-value
sphingosine-1-phosphate lyase 1	SGPL1	212322_at	2.19	0.0033
tumor necrosis factor receptor superfamily, member 21	TNFRSF21	218856_at	3.13	<.0001
tumor necrosis factor receptor superfamily, member 21	TNFRSF21	214581_x_at	3.31	0.0002
tumor protein p53 (Li-Fraumeni syndrome)	TP53	201746_at	2.68	0.0067
tumor protein p53 (Li-Fraumeni syndrome)	TP53	211300_s_at	2.57	<.0001
WW domain containing oxidoreductase	WWOX	219077_s_at	3.26	0.0003
WW domain containing oxidoreductase	WWOX	223868_s_at	2.87	0.0078

Table 1B.

Over-representation analysis of 6,242 differentially expressed genes between Poly E-resistant and Poly E-sensitive cells compared with the 54,613-gene genome

Database	Level	Term	BCL2*	P53*
GO biological process	6	cell death	+	++
		intracellular transport	+	++
		regulation of progression through cell cycle	+	++
		water-soluble vitamin biosynthetic process	−	−
		small GTPase mediated signal transduction	−	−
		positive regulation of cellular metabolic		++
		cellular secretion	−	−
		dephosphorylation	−	−
	7	negative regulation of progression through cell cycle		++
		programmed cell death	+	++
		intracellular protein transport	−	++
		secretory pathway	−	−
GO molecular function	6	phosphoric monoester hydrolase activity	−	−
		GTP binding	−	−
	7	cyclic-nucleotide phosphodiesterase activity	−	−
		phosphoprotein phosphatase activity	−	−
		lipid phosphatase activity	−	−
GO cellular component	6	cytoplasm	+	++
		intracellular organelle	+	++
		anchoring collagen	−	−
		sheet-forming collagen	−	−
	7	intracellular membrane-bound organelle	+	++
		cytoplasmic part	+	++
		collagen type IV	−	−
		intracellular organelle part	−	++
		FACIT collagen	−	−

*Each “plus; +” or “minus; −” notation indicates presence or absence, respectively, of 1 of 4 BCL2 probe sets or 1 of 2 P53 probe sets in the over-represented term.

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