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ORIGINAL ARTICLE

A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells

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Abstract
Purpose Overexpression of Bcl-2 family members as well as deregulated apoptosis pathways are known hallmarks of lung cancer. Non-small cell lung cancer (NSCLC) cells are typically resistant to cytotoxic chemotherapy and approaches that alter the balance between pro-survival and pro-death Bcl-2 family members have shown promise in preclinical models of NSCLC.
Methods Here we evaluated the effects of a novel pan-Bcl-2 inhibitor GX15-070 on NSCLC survival and when combined with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors as well as traditional cytotoxic agents. GX15-070 is a small molecule agent that binds anti-apoptotic Bcl-2 proteins and interferes with their ability to interact with pro-apoptotic proteins. We evaluated the effect of GX15-070 and correlated the effect on EGFR status as well as Bcl-2 family protein expression.
Results We show that GX15-070 can disrupt Mcl-1:Bak interactions in lung cancer cells. We identified differential sensitivity of a panel of lung cancer cells to GX15-070 and no clear relationship existed between EGFR status or Bcl-2 family protein expression and sensitivity to GX15-070. GX15-070 could induce apoptosis in a subset of lung cancer cell lines and this correlated with the effects on cell viability. GX15-070 combined with gefitinib was synergistic in a cell line dependent on EGFR for survival but GX15-070 could not reverse resistance to gefitinib in cell lines not dependent on EGFR for survival. Finally, we observed synergy between GX15-070 and cisplatin in NSCLC cells.
Conclusions Based on these results, GX15-070 can trigger apoptosis in NSCLC cells and can enhance chemotherapy-induced death. These data suggest that clinical trials with GX15-070 in combination with cytotoxic chemotherapy are indicated.

Keywords Mcl-1 · Bcl-2 · GX15-070 · Lung cancer · Apoptosis · Chemotherapy

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Introduction

Overexpression of anti-apoptotic Bcl-2 family members and deregulation of pathways that regulate pro-apoptotic family members have been observed in lung cancers [1]. It is now the general consensus that the relative ratio of pro-apoptotic to anti-apoptotic family members ultimately dictate the cell's fate toward survival or death [2, 3]. The Bcl-2 family consists of three main subfamilies including Bcl-2, Bcl-xL, and Mcl-1 which inhibit apoptosis, the Bax subfamily consisting of Bax, Bak, and Bok that promote apoptosis, and the BH3-only subfamily consisting of Bid, Bim, Bad, and others that also promote apoptosis [4, 5]. Elevated levels of Bcl-xL and to some extent Bcl-2 have been observed in non-small cell lung cancer (NSCLC) [6–18]. Cellular models have demonstrated a role of Bcl-2 proteins in maintaining lung cancer survival and control of apoptosis in response to cytotoxic chemotherapy [19–24]. Inhibition of Bcl-2 or Bcl-xL

with antisense oligonucleotides can trigger apoptosis in lung cancer cells [19–24]. Similarly, enforced Bcl-2 overexpression can prevent apoptosis resulting from inhibition of the epidermal growth factor receptor (EGFR) [25]. In addition to Bcl-2 and Bcl-xL, Mcl-1 is overexpressed in NSCLC and knockdown of Mcl-1 levels result in apoptosis of lung cancer cells and sensitizes lung cancer cells to apoptosis induced by cytotoxic agents [8, 26].

Because apoptosis is deregulated in lung cancer as well as in many cancers, apoptosis targets such as Bcl-2 proteins are being explored for identification of new cancer drugs that can be used in clinic [27]. For example, development of antisense oligonucleotide therapy directed against Bcl-2 has been evaluated as therapy for patients with advanced lung cancer, as well as other tumor types [28–31]. Clinical studies using antisense oligonucleotides directed against Bcl-2 have been reported in small cell lung cancer and a current trial in NSCLC is comparing single agent docetaxel to docetaxel plus antisense Bcl-2 [32]. An alternative approach to antisense strategies is to develop small molecule inhibitors of pro-survival Bcl-2 family inhibitors [33]. One promising early compound is GX15-070 (2-[5[(3,5-Dimethyl-1H-pyrrol-2-ylmethylene)-4-methoxy-5H-pyrrol-2-yl]-1H-indole), an indole-derivative novel small molecule inhibitor of Bcl-2 proteins (Fig. 1a). GX15-070 binds anti-apoptotic Bcl-2 proteins and interferes with their ability to interact with and negatively regulate pro-apoptotic proteins through the BH3 domain. This disruption of binding allows the pro-apoptotic Bcl-2 proteins induce caspase-dependent apoptosis in cancer cells. GX15-070 can inhibit the interaction between recombinant Bcl-2 and Bax as well as the interaction between Mcl-1 and Bak both in vitro and in vivo. Finally, GX15-070 can lead to phosphorylation of the pro-apoptotic BH3-only protein Bim and can induce Bim translocation to the mitochrondria.

Based on the importance of Bcl-2 proteins in lung cancer, we evaluated the effect of GX15-070 in human NSCLC cells. We used lung cancer cell lines with known EGFR mutational status and sensitivity to EGFR tyrosine kinase inhibitors (TKI). The level of pro-survival and pro-death Bcl-2 proteins were examined in these cell lines and the effect of GX15-070 was evaluated in regard to Bcl-2 family member expression level and ability of GX15-070 to induce apoptosis. GX15-070 in combination with either EGFR TKI or cytotoxic chemotherapy was examined for its effect on NSCLC cells.

Materials and methods

Cell lines and reagents

Human lung cancer cell lines were purchased from ATCC and maintained in RPMI-1640 medium supplemented with

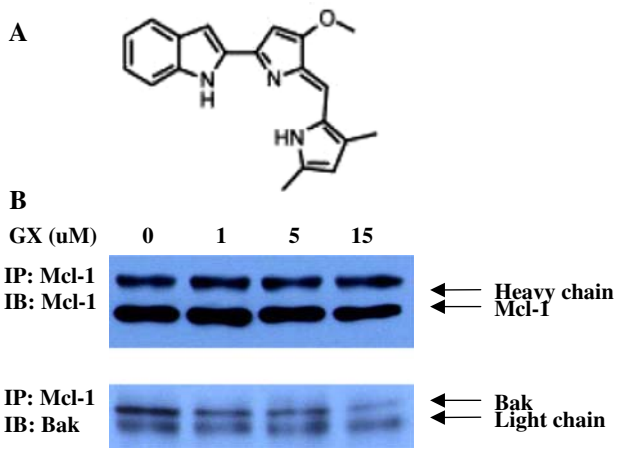

Fig. 1 Effect of GX15-070 on Mcl-1:Bak protein–protein interactions. **a** Chemical structure of GX15-070 **b** PC9 cells were exposed to indicated concentrations of GX15-070 for 6 h before being harvested for total proteins. Protein lysates were immunoprecipitated with anti-Mcl-1 antibody as described and immunoprecipitate was immunoblotted with antibodies that recognize Mcl-1 and Bak. Heavy chain and light chains indicate contaminating IgG from anti-Mcl-1 antibody used for immunoprecipitation. *GX* GX15-070

10% bovine calf serum (BCS, Gibco). H3255 cells were provided by Dr Pasi Janne (Dana Farber, Boston, MA, USA) and grown in ACL-4 media [34]. HCC827, HCC2279, and H4006 cells were a gift of Dr Jon Kurie (MD Anderson, Houston, TX, USA) [35]. Gefitinib-resistant H1650 cells were provided by Dr Jeffrey Settleman (Harvard, Boston, MA, USA) [36]. Stock solutions of gefitinib, paclitaxel, cisplatin, and GX15-070 in 100% DMSO and gemcitabine in sterile PBS were diluted directly into the media to indicated concentrations. Gefitinib was provided by Astra-Zeneca (Wilmington, DE, USA) and GX15-070 by GeminX, Inc. (Malvern, PA, USA). Paclitaxel and cisplatin were purchased from Sigma (St Louis, MO, USA) and gemcitabine was provided by Dr Doug Cress (Moffitt Cancer Center, Tampa, FL, USA).

Immunoprecipitation assays

Whole cell lysates were prepared using an ice-cold IP buffer (10 mM Tris, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 10 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin. 300 μg of total protein was incubated with 1.5 μg of rabbit Mcl-1 antibody (Santa Cruz, CA, USA) overnight at 4°C with rotating, and then incubated with Protein A-agarose beads (Roche, Indianapolis, IN, USA) for 4 h at 4°C. Beads were washed three times with IP buffer containing protein inhibitors and immunoprecipitated proteins were eluted from beads with 30 μl of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer from BIO-RAD (Hercules, CA, USA) at 90°C for 3 min. Proteins

were separated on 12% SDS-PAGE gels and immunoblots were performed on membranes incubated overnight with primary antibody mouse anti-Mcl-1 (Pharmingen, San Diego, CA, USA) and rabbit anti-Bak (Upstate, Lake Placid, NY, USA). Detection of signals was performed using the corresponding secondary HRP-conjugated antibody and enhanced chemiluminescence (ECL) purchased through Amersham (Piscataway, NJ, USA).

Cytotoxicity and apoptosis assays

Cytotoxicity assays (MTT) were performed according the manufacturers recommendations of Cell Proliferation Kit from Roche (Indianapolis, IN, USA). 5×10^3 cells in 5% BCS complete media were placed into single wells in a 96-well plate from FALCON (Franklin Lakes, NJ, USA), exposed to indicate agents, and viability assessed following 72 h. The IC_{50} was defined as the drug concentration that induced a 50% reduction in cell viability in comparison with DMSO controls and was calculated by non-linear regression analyses. Data presented represents three separate experiments with eight data points per condition. Apoptosis was assayed using cleaved PARP antibody From Cell Signaling (Beverly, MA, USA).

For combination cell viability assays, we first used MTT to identify relevant and effective concentrations of inhibitors and cytotoxic agents. For final data analysis and reporting, viable cells were identified and counted using trypan blue exclusion. 5×10^4 cells in 5% BCS complete media were placed into single wells in a 24-well plate, exposed to indicated agents, and viable and non-viable cells were counted using trypan blue solution (Sigma, St Louis, MO, USA) following 72 h. Analysis of synergism between different agents in inducing apoptosis of cells was performed by median dose–effect analysis and calculation of combination indices (CI) using commercially available software (Calcusyn; Biosoft, Ferguson, MO, USA) [38]. According the recommendations of this methodology, the value of CI represents as following: 0.9–1.10 is mildly additive; 0.85–0.9 is slight synergism; 0.7–0.85 is moderate synergism; 0.3–0.7 is synergism; 0.1–0.3 is strong synergism; less than 0.1 is very strong synergism.

Protein expression analysis

Cell lysates were prepared using RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 2 mM Na_3VO_4, 0.25% sodium deoxycholate, 1% NP-40, 1 mM PMSF, 60 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin), were normalized for total protein content (50 μg) and subjected to SDS-PAGE.

Primary antibodies used in these studies consisted of Bcl-2, Bcl-xL, Bim, and Mcl-1 from Santa Cruz (Santa Cruz, CA, USA), cleaved PARP, Bax and Bak from Cell Signaling (Beverly, MA, USA) and β-actin from Sigma (St Louis, MO, USA). The cleaved PARP (Asp214) antibody detects endogenous levels of the large fragment (89 kDa) of human PARP1 produced by caspase cleavage. The antibody does not recognize full length PARP1 or other PARP isoforms. Detection of proteins was accomplished using horseradish-peroxidase conjugated secondary antibodies and ECL purchased through Amersham (Piscataway, NJ, USA) [37].

Results

GX15-070 disrupts Mcl-1:Bak complexes in vivo

To assess the effect of GX15-070 on pro-survival and pro-death Bcl-2 family protein complexes, we evaluated the effect on Mcl-1:Bak complexes using immunoprecipitation assays. We chose to focus on Mcl-1:Bak complexes since Bcl-2 is rarely expressed in NSCLC cells and our previous results as well as results of others suggest the importance of Mcl-1 in the survival of epithelial tumors [26]. PC9 cells were exposed to increasing concentrations of GX15-070 for a total of 6 h and then total proteins harvested for immunoprecipitation. Lysates were immunoprecipitated with a Mcl-1 antibody and pulldowns evaluated for total Mcl-1 as well as co-immunoprecipitated Bak. These results are shown in Fig. 1b and demonstrate the ability of GX15-070 to disrupt Mcl-1:Bak complexes in cells in a concentration-dependent manner. Because re-association of Mcl-1 and Bak proteins into complexes may result secondary to dilution of GX15-070 during the process of creating protein lysates and immunoprecipitation, our results may underestimate the concentrations required to disrupt the complex in whole cells.

Effect of GX15-070 on cell proliferation and apoptosis in NSCLC cell lines

To assess the effects of GX15-070 on NSCLC cell lines, cell lines with distinct EGFR status and sensitivity to EGFR TKI were exposed to increasing concentrations of GX15-070 and cell viability was assayed. We chose to stratify cell lines based on the EGFR status (wildtype vs. mutant) as well as their sensitivity to EGFR tyrosine kinase inhibitors [39–41]. These data are shown in Fig. 2 and the IC_{50} summarized in Table 1. These lung cancer cell lines demonstrated differential sensitivity to GX15-070 and there was no clear relationship between EGFR status and sensitivity to GX15-070. In cells with wildtype EGFR, A549 cells were the most sensitive to GX15-070 with an approximate IC_{50} of 1.3 μM (Fig. 2a). Of the lung cancer cells lines that

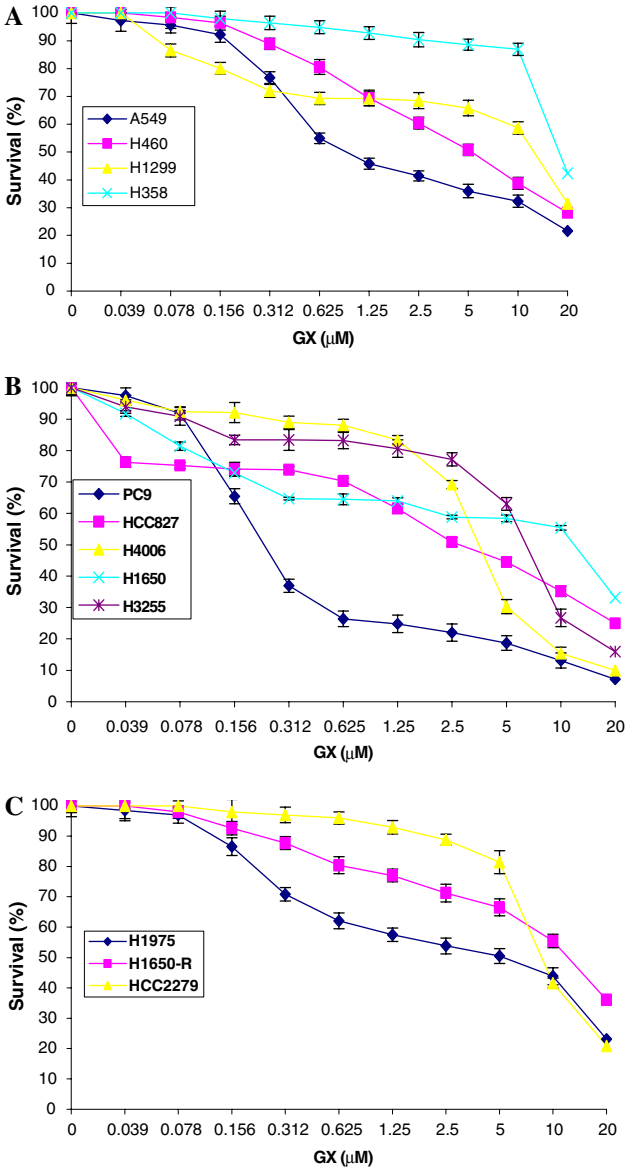

Fig. 2 Effect of GX15-070 on cell viability in lung cancer cell lines. Indicated lung cancer cells were exposed to increasing concentrations of GX15-070 and cell viability assessed after 72 h. Each *bar* represents the standard deviation of three separate experiments. Cell survival was normalized to DMSO-treated control cells. **a** Wildtype EGFR lung cancer cells (A549, H460, H1299, H358), **b** mutant EGFR and gefitinib-sensitive lung cancer cells (PC9, HCC827, H4006, H1650, and H3255), and **c** mutant EGFR and gefitinib-resistant lung cancer cells (H1975, H1650-R, and HCC2279)

show strong dependence on EGFR for survival, the PC9 cell with the deletion EGFR mutation was very sensitive to GX15-070 with an approximate IC_{50} of 0.25 μM (Fig. 2b). All the other cells that have been reported either by us or others to be dependent on EGFR for survival demonstrated $IC_{50} > 1$ μM for GX15-070. Finally, we also explored cells that have mutations in EGFR but are nonetheless insensitive

Table 1 Activity of GX15-070 in human lung cancer cell lines

Cell lines	EGFR status	IC_{50} (μM)
A549	WT	1.33 ± 0.47
H460	WT	3.85 ± 1.60
H1299	WT	7.62 ± 2.80
H358	WT	15.4 ± 2.09
PC9	Mutant, gefitinib-sensitive	0.26 ± 0.12
HCC827	Mutant, gefitinib-sensitive	1.83 ± 0.39
H4006	Mutant, gefitinib-sensitive	2.89 ± 0.75
H1650	Mutant, gefitinib-sensitive	5.99 ± 1.55
H3255	Mutant, gefitinib-sensitive	4.77 ± 1.52
H1975	Mutant, gefitinib-resistant	3.04 ± 1.27
H1650-R	Mutant, gefitinib-resistant	7.78 ± 2.10
HCC2279	Mutant, gefitinib-resistant	7.32 ± 1.90

Mean ± SD ($N = 3$) IC_{50} values for GX15-070 in the evaluated cell lines along with EGFR status are listed

to EGFR TKI. This includes the H1975 cell with a L858R + T790M mutation, H1650 cells that were engineered to be resistant to EGFR TKI, and HCC2279 cells that have an activating EGFR mutation but have reduced ErbB-3 and HER ligand expression [35, 36, 42]. As shown in Fig. 2c, these cells have IC_{50} of greater than 1 μM.

Because GX15-070 prevents Bcl-2:Bax and Mcl-1:Bak interactions, we next examined the expression of both pro-survival and pro-death Bcl-2 proteins using western analysis to determine if a relationship exists between target protein expression and GX15-0970 sensitivity (Fig. 3). We included Bcl-2, Mcl-1, Bcl-xL, Bim, Bax, and Bak in our analysis. Consistent with prior reports, Bcl-2 expression is relatively rare in NSCLC cells with high levels of expression only found in H460 and H1299 cells with no obvious expression in other cells including the cells with activating EGFR mutations. Consistent with our previous studies, the majority of the lung cancer cell lines expressed Mcl-1 protein levels albeit to different levels and this did not correlate with EGFR mutation status [26]. Similarly, Bcl-xL was expressed in the majority of these NSCLC cell lines and again no obvious relationship existed between EGFR mutation status and protein expression. Expression of Bax was identified to different extents in all the cell lines evaluated and Bak expression was seen in most cells but not all. When the expression level of these pro-survival and pro-death Bcl-2 proteins was examined in relation to the sensitivity of the cells to GX15-070, we could not identify an obvious relationship between protein expression and drug sensitivity.

We next examined the effect of GX15-070 on lung cancer cell apoptosis. These data are shown in Fig. 4. In order to assess whether GX15-070 can induce apoptosis directly in these lung cancer cells, we evaluated cleavage of PARP as a

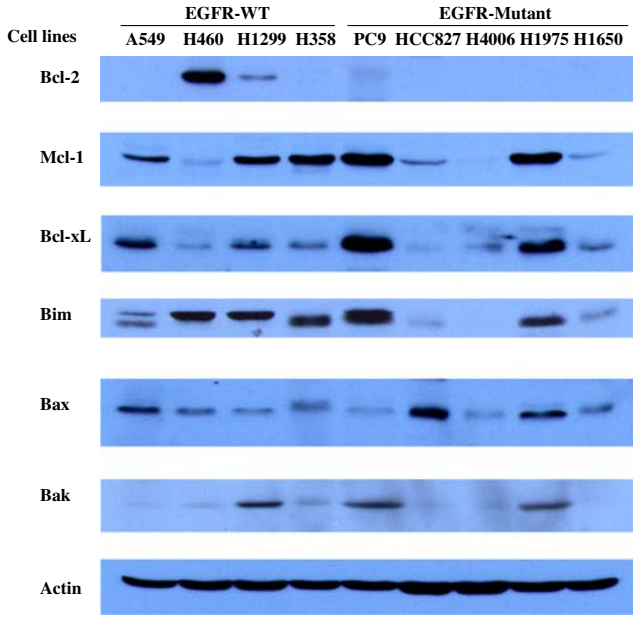

Fig. 3 Bcl-2 family protein expression in lung cancer cell lines. Total protein was collected from lung cancer cells growing in serum and Bcl-2, Mcl-1, Bcl-xL, Bim, Bax, and Bak protein levels evaluated using western analysis. Equal protein loading was confirmed by evaluation of actin

biochemical marker of apoptosis and evaluated the effect of GX15-070 exposure time on PARP cleavage. As shown in Fig. 4a, a 1 μM concentration of GX15-070 induces a time-dependent effect on PARP cleavage in A549 cells. While no PARP cleavage is evident at 24 h, we identified the onset of PARP cleavage starting at 48 h and increasing at 72 h. Similar effects were seen in H460 cells where little PARP cleavage was identified after 24 h (data not shown) but was clearly evident by 48 h (Fig. 4b). In contrast, H1299 and H358 cells demonstrated little to no PARP cleavage above baseline in untreated cells and this observation corresponds to the higher IC_{50} identified in the viability assays in Fig. 2 and Table 1. We next examined apoptosis using PARP cleavage in the cells with EGFR mutation. As shown in Fig. 4c, PC9 cells demonstrate PARP cleavage as early as 24 h and at a concentration of 0.5 μM. Of the other cells that have demonstrated dependence on EGFR for survival, we only identified PARP cleavage at a 1 μM concentration of GX15-070 in the HCC827 cells but not H4006, H3255, or H1650 (Fig. 4d). We again noted the correlation with PARP cleavage with the IC_{50} since PC9 and HCC827 cells have lower IC_{50} that corresponds to the ability of GX15-070 to induce PARP cleavage. Finally, we also evaluated the apoptotic effect of GX15-070 on H1975 cells. Here we observed PARP cleavage at a concentration of 1 μM. From these studies, we conclude that GX15-070 can induce apoptosis in a subset of lung cancer cell lines and the induction of apoptosis based on PARP cleavage correlates with the IC_{50} identified in cell viability assays.

Effect of EGFR TKI and GX15-070 on NSCLC survival

We next examined the effect of combined EGFR tyrosine kinase inhibition and GX15-070 on lung cancer cell survival.

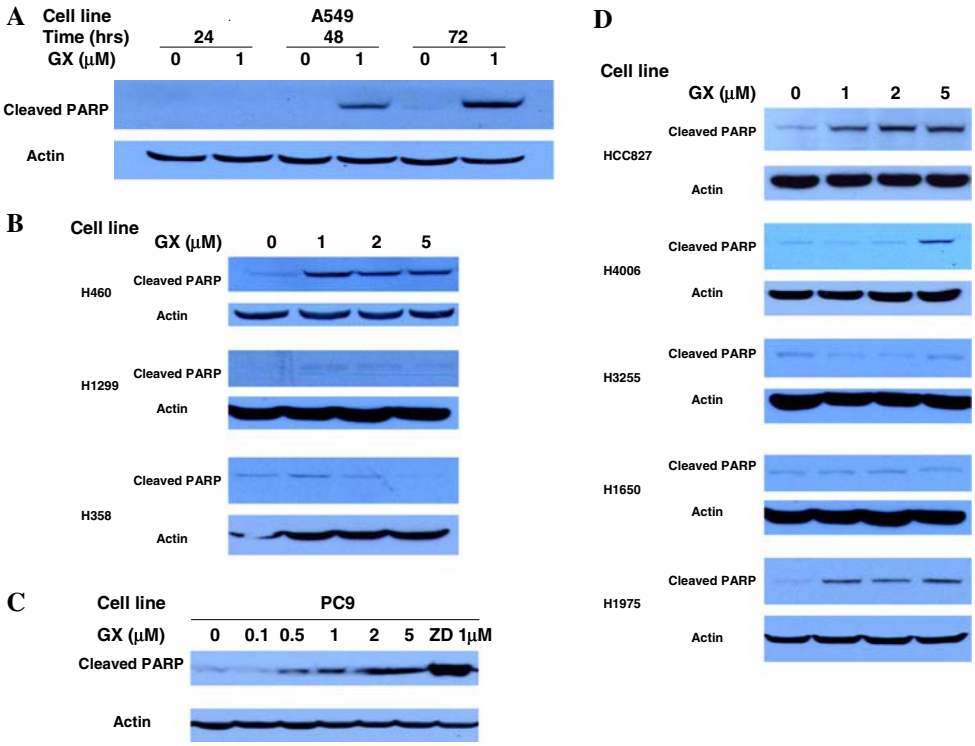

Fig. 4 Effect of GX15-070 on biochemical apoptosis. Lung cancer cells were exposed to GX15-070 at different concentrations and for indicated times. The cells were collected and total protein harvested. PARP cleavage was evaluated using western analysis and equal protein loading confirmed by evaluation of actin. **a** A549 cells were exposed to either control solvent or GX15-070 for indicated times and cleaved PARP assayed. **b** Wildtype EGFR lung cancer cells were assayed for cleaved PARP after 48 h. **c** PC9 cells were exposed to indicated concentrations of GX15-070 for 24 h and cleaved PARP assayed. *ZD* ZD1839 (gefitinib) was used as a positive control for apoptosis in these mutant EGFR cells. **d** PARP cleavage following 48 h exposure to GX15-070 in mutant EGFR lung cancer cells

This was based in part on studies that demonstrated that Bcl-2 or Mcl-1 could inhibit death induced by gefitinib [25, 26]. These data are shown in Fig. 5. First, we examined if combination of gefitinib, an EGFR TKI, with GX15-070 could produce enhanced apoptosis in lung cancer cells with activating EGFR mutation and sensitivity to EGFR TKI. For these experiments, PC9 cells were exposed to GX15-070, gefitinib, or the combination and the effect on cell viability quantified. As shown in Fig. 5a, both GX15-070 and gefitinib result in a concentration-dependent increase in cell death and the combined effect of both agents results in enhanced cell death. Analysis of synergism between gefitinib and GX15-070 in inducing cell death was performed by median dose–effect analysis. Importantly, exposure to the combination of gefitinib and GX15-070 exerted synergistic apoptotic effect in PC9 cells, as determined by the median dose–effect analysis, which revealed combination index values of <1.0 (0.77 ± 0.12).

We next determined if GX15-070 could reverse resistance of lung cancer cells to gefitinib. These data are shown

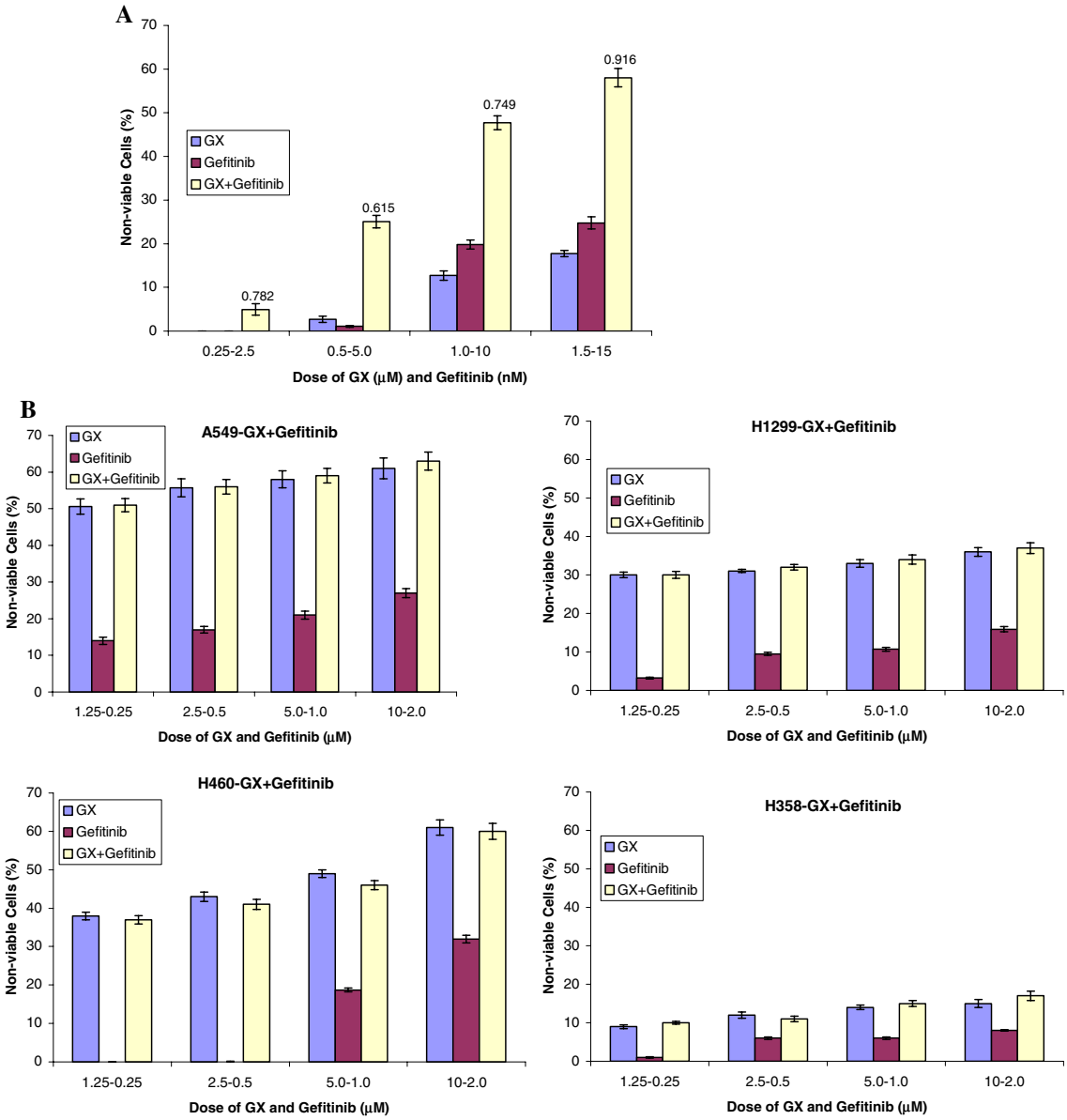

Fig. 5 Combination effect of GX15-070 and gefitinib in lung cancer cells. (**a** PC9 cells were exposed to increasing concentrations of GX15-070, gefitinib, or the combination of both agents in a fixed ratio. Viable cells were assayed by trypan blue exclusion and combination indices were calculated using Calcusyn software using percent of viable cells. *GX* GX15-070, *GEF* gefitinib. **b** A549, H460, H1299, and H358 cells were exposed to increasing concentrations of GX15-070, gefitinib, or the combination of both agents in a fixed ratio. Viable cells were assayed and combination indices (CI) were calculated using Calcusyn software as described. The values above each *bar* represent CI for that dose combination

in Fig. 5b and in Table 2. Lung cancer cell lines that do not have activating EGFR mutations were exposed to increasing concentrations of GX15-070, gefitinib, or the combination of both agents and the effect on cell viability was assessed. Analysis of synergism between gefitinib and GX15-070 in inhibiting cell viability was performed by median dose–effect analysis. In none of the cell studied did we observe the ability of GX15-070 to enhance the anti-proliferative effect of gefitinib. From these studies we conclude that GX15-070 can synergistically enhance apoptosis in lung cancer cells dependent on EGFR for survival but cannot reverse gefitinib-resistance in lung cancer cells with wildtype EGFR.

Effect of cytotoxic chemotherapy and GX15-070 on NSCLC survival

We next examined the effect of GX15-070 on cytotoxic chemotherapy-induced cell death. Lung cancer cell lines that do not have activating EGFR mutations were exposed to increasing concentrations of GX15-070, cytotoxic chemotherapy agents commonly used in clinic for the treatment of advanced lung cancer (cisplatin, gemcitabine, paclitaxel), or the combination of both agents. MTT was used initially to identify dose ranges for further study. The effect on cellular viability was examined by counting viable cells identified with trypan blue staining and analysis of synergism between cytotoxics and GX15-070 was performed by median dose–effect analysis. These data are shown in Fig. 6 and are summarized in Table 3. For A549 cells, combination of cisplatin and GX15-070 resulted in evidence of synergism since all CI were below 1.0 (0.677 ± 0.186) while combination of GX15-070 and either gemcitabine or paclitaxel had CI that averaged around 1.0. Similarly, in H460 cells, cisplatin and GX15-070 led to synergistic cell kill with all CI below 1.0 (0.657 ± 0.139). H1299 cells however demonstrated little effect of combined GX15-070 and cytotoxics and no evidence of synergy on cell viability was identified.

Discussion

One approach to improving the outcome of advanced NSCLC is to alter the balance of pro-apoptotic to anti-apoptotic mechanisms and therefore sensitize cancer cells to apoptosis triggered either endogenously through oncogenic signals that trigger apoptosis or through exogenous stimuli such as exposure to cytotoxic chemotherapy. A previous study demonstrated that another small molecule Bcl-2 inhibitor (ABT-737) could induce regressions in solid tumors [43]. Here we evaluated the effect of a novel pan-Bcl-2 protein inhibitor on lung cancer cell lines with defined EGFR status and evaluated the combination effect with either EGFR TKI or cytotoxic chemotherapy. The major findings include demonstrating that GX15-070 is cytotoxic in some NSCLC cells and the ability to induce apoptosis, based on PARP cleavage, correlates with a lower IC_{50} in cell viability assays. Our results also suggest that prolonged exposure time may result in increased cell death. We identified no obvious relationship between the studied Bcl-2 proteins and the sensitivity of the cells to GX15-070. GX15-070 in combination with gefitinib was synergistic in a cell line with mutant EGFR sensitive to gefitinib. However, in cells with wildtype EGFR that were not dependent on EGFR for survival, GX15-070 cannot reverse gefitinib resistance. Finally, GX15-070 exhibited synergistic effects with cisplatin in two of the three cell lines studied.

To our knowledge this is the first examination of Bcl-2 family members in cells with activating EGFR mutations. Consistent with prior reports suggesting that Bcl-2 protein expression is rare in NSCLC, we found no expression of Bcl-2 protein in cells with activating EGFR mutations while Bcl-xL and Mcl-1 expression were more evident. This suggest that agents targeting Bcl-2 are unlikely to demonstrate efficacy in mutant EGFR cell lines, generally of adenocarcinoma origin, since Bcl-2 protein is not expressed. These current students reiterate our previous work suggesting that Mcl-1 is a viable anti-apoptotic target in lung cancer cells [26].

We were unable to find a clear relationship between Bcl-2 family protein expression and sensitivity to GX15-070. However, the more interesting finding was that cells undergoing apoptosis as evidenced by cleavage of PARP was correlated with lower IC_{50} in cellular viability assays. This suggests one possible biomarker of GX15-070 activity may be early evidence of apoptosis in tumor cells. For example, induction of apoptosis was monitored using

Table 2 Combination effect of GX15-070 and EGFR TKI on cell proliferation

Listed are the cell line studied, dose range of GX15-070 and gefitinib, respectively, and the combination indices (mean ± SD)

Cell line	Combination drug	Dose range (μM)	CI
PC9	GX and gefitinib	0.25–1.5 and 0.0025–0.015	0.765 ± 0.123
A549	GX and gefitinib	1.25–10 and 0.25–2	0.957 ± 0.203
H460	GX and gefitinib	1.25–10 and 0.25–2	3.441 ± 1.247
H1299	GX and gefitinib	1.25–10 and 0.25–2	0.984 ± 0.105
H358	GX and gefitinib	1.25–10 and 0.25–2	1.121 ± 0.211

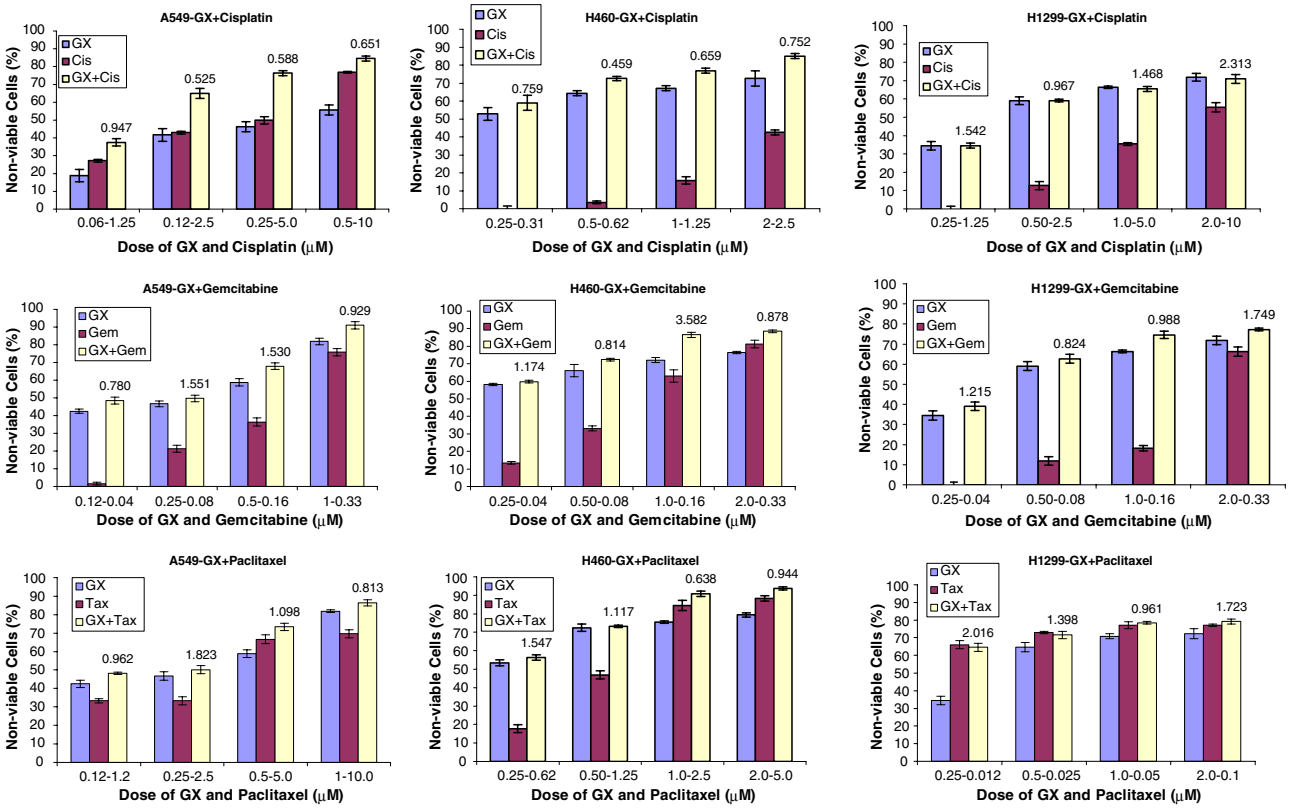

Fig. 6 Combination effect of GX15-070 and chemotherapy in lung cancer cells. A549, H460, and H1299 cells were exposed to increasing concentrations of GX15-070, cytotoxic agents (cisplatin, gemcitabine, paclitaxel), or the combination of both agents in a fixed ratio. Viable cells were counted using trypan blue staining and combination indices (CI) were calculated using Calcusyn software as described. The values above each *bar* represent CI for that dose combination. *GX* GX15-070, *Cis* cisplatin, *Gem* gemcitabine, *Tax* paclitaxel

Table 3 Combination effect of GX15-070 and chemotherapy on cell proliferation

Listed are the cell line studied, compounds, dose range of GX15-070 and cytotoxic, respectively, and the combination indices (mean ± SD)

Cell line	Combination drug	Dose range (μM)	CI
A549	GX and cisplatin	0.06–0.5 and 1.25–10	0.677 ± 0.186
	GX and gemcitabine	0.12–1.0 and 0.04–0.33	1.197 ± 0.400
	GX and paclitaxel	0.12–1.0 and 1.25–10	1.174 ± 0.448
H460	GX and cisplatin	0.25–2.0 and 0.31–2.5	0.657 ± 0.139
	GX and gemcitabine	0.25–2.0 and 0.04–0.33	1.612 ± 1.322
	GX and paclitaxel	0.12–1.0 and 0.62–5.0	1.061 ± 0.379
H1299	GX and cisplatin	0.25–2.0 and 1.25–10	1.572 ± 0.555
	GX and gemcitabine	0.25–2.0 and 0.04–0.33	1.194 ± 0.403
	GX and paclitaxel	0.12–1.0 and 0.012–0.1	1.524 ± 0.452

plasma oligonucleosomal DNA complexes in patients with chronic lymphocytic leukemia treated with GX15-070 [44]. While difficult to perform, another approach could be rapid serial biopsies in patients with accessible solid tumors to assess apoptosis. Alternatively, non-invasive imaging strategies that can evaluate in vivo tumor apoptosis could be an early marker for GX15-070 activity in patients. Further studies are indicated to evaluate this hypothesis.

Based on our studies, the combination of cisplatin and GX15-070 results in some degree of synergistic tumor cell kill while combinations of other cytotoxics does not demonstrate such effect. These studies are obviously limited since the data are generated using cell culture models instead of in vivo models. In addition, we found that GX15-070 cannot reverse gefitinib resistance in lung cancer cells not dependent on EGFR for survival. While previous studies demonstrated that exogenous Bcl-2 or Mcl-1 expression

can prevent gefitinib-induced apoptosis, our results suggest that simply downregulating the activity of these proteins may be insufficient for reversing EGFR TKI sensitivity [25, 26]. This may not be surprising given the numerous parallel pathways that exist in lung cancer cells that can affect apoptosis [1].

Early studies of GX15-070 in humans with advanced cancer are underway and the agent appears to be safe and well tolerated. Based on our results as well as the literature, studies of GX15-070 in combination with cytotoxic chemotherapy are planned for patients with advanced NSCLC.

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References

1. Haura EB, Cress WD, Chellappan S, Zheng Z, Bepler G (2004) Antiapoptotic signaling pathways in non-small-cell lung cancer: biology and therapeutic strategies. Clin Lung Cancer 6:113–122
2. Reed JC (1996) Mechanisms of Bcl-2 family protein function and dysfunction in health and disease. Behring Inst Mitt 97:72–100
3. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci U S A 92:7834–7838
4. Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326
5. Cory S, Huang DC, Adams JM (2003) The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 22:8590–8607
6. Anton RC, Brown RW, Younes M, Gondo MM, Stephenson MA, Cagle PT (1997) Absence of prognostic significance of bcl-2 immunopositivity in non-small cell lung cancer: analysis of 427 cases. Hum Pathol 28:1079–1082
7. Apolinario RM, van der Valk P, de Jong JS, Deville W, van Ark-Otte J, Dingemans AM, van Mourik JC, Postmus PE, Pinedo HM, Giaccone G (1997) Prognostic value of the expression of p53, bcl-2, and bax oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. J Clin Oncol 15:2456–2466
8. Borner MM, Brousset P, Pfanner-Meyer B, Bacchi M, Vonlanthen S, Hotz MA, Altermatt HJ, Schlaifer D, Reed JC, Betticher DC (1999) Expression of apoptosis regulatory proteins of the Bcl-2 family and p53 in primary resected non-small-cell lung cancer. Br J Cancer 79:952–958
9. Fontanini G, Vignati S, Bigini D, Mussi A, Lucchi M, Angeletti CA, Basolo F, Bevilacqua G (1995) Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small-cell lung cancer. Br J Cancer 71:1003–1007
10. Gaffney EF, O'Neil AJ, Staunton MJ (1994) Bcl-2 and prognosis in non-small-cell lung carcinoma. N Engl J Med 330:1757–1758
11. Higashiyama M, Doi O, Kodama K, Yokouchi H, Tateishi R (1995) High prevalence of bcl-2 oncoprotein expression in small cell lung cancer. Anticancer Res 15:503–505
12. Higashiyama M, Doi O, Kodama K, Yokouchi H, Tateishi R (1996) Bcl-2 oncoprotein expression is increased especially in the portion of small cell carcinoma within the combined type of small cell lung cancer. Tumour Biol 17:341–344
13. Higashiyama M, Doi O, Kodama K, Yokouchi H, Nakamori S, Tateishi R (1997) Bcl-2 oncoprotein in surgically resected non-small cell lung cancer: possibly favorable prognostic factor in association with low incidence of distant metastasis. J Surg Oncol 64:48–54
14. Ikegaki N, Katsumata M, Minna J, Tsujimoto Y (1994) Expression of bcl-2 in small cell lung carcinoma cells. Cancer Res 54:6–8
15. Katz HR (1994) Bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 330:221
16. Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, Harris A, Gatter KC, Mason DY (1993) Bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med 329:690–694
17. Ritter JH, Dresler CM, Wick MR (1995) Expression of bcl-2 protein in stage T1N0M0 non-small cell lung carcinoma. Hum Pathol 26:1227–1232
18. Sartorius UA, Krammer PH (2002) Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. Int J Cancer 97:584–592
19. Gautschi O, Tschopp S, Olie RA, Leech SH, Simoes-Wust AP, Ziegler A, Baumann B, Odermatt B, Hall J, Stahel RA, Zangemeister-Wittke U (2001) Activity of a novel bcl-2/bcl-xL-bispecific antisense oligonucleotide against tumors of diverse histologic origins. J Natl Cancer Inst 93:463–471
20. Kataoka M, Wiehle S, Spitz F, Schumacher G, Roth JA, Cristiano RJ (2000) Down-regulation of bcl-2 is associated with p16INK4-mediated apoptosis in non-small cell lung cancer cells. Oncogene 19:1589–1595
21. Koty PP, Zhang H, Levitt ML (1999) Antisense bcl-2 treatment increases programmed cell death in non-small cell lung cancer cell lines. Lung Cancer 23:115–127
22. Leech SH, Olie RA, Gautschi O, Simoes-Wust AP, Tschopp S, Haner R, Hall J, Stahel RA, Zangemeister-Wittke U (2000) Induction of apoptosis in lung-cancer cells following bcl-xL anti-sense treatment. Int J Cancer 86:570–576
23. Ouyang N, Ran P, Qiu Z (2000) [Bcl-2 antisense oligodeoxyribonucleotide increases apoptosis of lung carcinoma cells induced by cisplatin]. Zhonghua Jie He He Hu Xi Za Zhi 23:722–724
24. Ziegler A, Luedke GH, Fabbro D, Altmann KH, Stahel RA, Zangemeister-Wittke U (1997) Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J Natl Cancer Inst 89:1027–1036
25. Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G (2003) Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. Clin Cancer Res 9:2316–2326
26. Song L, Coppola D, Livingston S, Cress D, Haura EB (2005) Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol Ther, 4:267–276
27. Fesik SW (2005) Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 5:876–885
28. Banerjee D (1999) Technology evaluation: G-3139. Curr Opin Mol Ther 1:404–408
29. Banerjee D (2001) Genasense (Genta Inc). Curr Opin Investig Drugs 2:574–580

30. Morris MJ, Tong WP, Cordon-Cardo C, Drobnjak M, Kelly WK, Slovin SF, Terry KL, Siedlecki K, Swanson P, Rafi M, DiPaola RS, Rosen N, Scher HI (2002) Phase I trial of BCL-2 antisense oligonucleotide (G3139) administered by continuous intravenous infusion in patients with advanced cancer. Clin Cancer Res 8:679–683
31. Rudin CM, Otterson GA, Mauer AM, Villalona-Calero MA, Tomek R, Prange B, George CM, Szeto L, Vokes EE (2002) A pilot trial of G3139, a bcl-2 antisense oligonucleotide, and paclitaxel in patients with chemorefractory small-cell lung cancer. Ann Oncol 13:539–545
32. Rudin CM, Kozloff M, Hoffman PC, Edelman MJ, Karnauskas R, Tomek R, Szeto L, Vokes EE (2004) Phase I study of G3139, a bcl-2 antisense oligonucleotide, combined with carboplatin and etoposide in patients with small-cell lung cancer. J Clin Oncol 22:1110–1117
33. Reed JC, Pellecchia M (2005) Apoptosis-based therapies for hematologic malignancies. Blood 106:408–418
34. Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA (2004) Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. Cancer Res 64:7241–7244
35. Fujimoto N, Wislez M, Zhang J, Iwanaga K, Dackor J, Hanna AE, Kalyankrishna S, Cody DD, Price RE, Sato M, Shay JW, Minna JD, Peyton M, Tang X, Massarelli E, Herbst R, Threadgill DW, Wistuba II, Kurie JM (2005) High expression of ErbB family members and their ligands in lung adenocarcinomas that are sensitive to inhibition of epidermal growth factor receptor. Cancer Res 65:11478–11485
36. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, Harris PL, Driscoll DR, Fidias P, Lynch TJ, Rabindran SK, McGinnis JP, Wissner A, Sharma SV, Isselbacher KJ, Settleman J, Haber DA (2005) Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci USA 102(21):7665–7670
37. Song L, Turkson J, Karras JG, Jove R, Haura EB (2003) Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. Oncogene 22:4150–4165
38. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
39. Haura EB, Zheng Z, Song L, Cantor A, Bepler G (2005) Activated epidermal growth factor receptor-stat-3 signaling promotes tumor survival in vivo in non-small cell lung cancer. Clin Cancer Res 11:8288–8294
40. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500
41. Sordella R, Bell DW, Haber DA, Settleman J (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 305:1163–1167
42. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2:1–11
43. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435:677–681
44. O'Brien S, Kipps TJ, Fader S, Crump M, Keating M, Anderson B, Soho C, Bole J, Turner R, Viallet J, Cheson B (2004) A phase I trial of the small molecule pan-bcl-2 family inhibitor GX15-070 administered intravenously (IV) every 3 weeks to patients with previously treated chronic lymphocytic leukemia (CLL). American Society of Hematology (ASH), Washington