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Bcl-2 and Bcl-xL mediate resistance to receptor tyrosine kinase targeted therapy in lung and gastric cancer

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Abstract

Promising clinical efficacy has been seen with receptor tyrosine kinase inhibitors (TKIs) particularly in lung and gastric cancers with mutations or amplifications in the targeted receptor tyrosine kinases (RTKs). However, the efficacy and the duration of the response to these inhibitors are limited by the emergence of drug resistance. Here we report treatment of RTK-dependent lung and gastric cancer cell lines with TKIs increased protein levels of Bcl-2 and Bcl-xL. The combination of the Bcl-2 and Bcl-xL inhibitor ABT-263 and TKIs was superior to TKIs alone in reducing cell viability and capacity of resistant colony formation. Furthermore, resistant cells established with exposing RTK-dependent cells to increasing concentrations of TKIs also express higher levels of Bcl-2 or Bcl-xL compared to their parental cells. The combination of inhibitors of PI3K/AKT, MEK/ERK, and Bcl-2/Bcl-xL effectively reduced viability of resistant cells and inhibited tumor size in a xenograft model derived from resistant cells by inducing apoptosis. Our results define a generalizable resistance mechanism to TKIs and rationalize inhibition of Bcl-2 and Bcl-xL as a strategy to augment responses and blunt acquired resistance to TKIs in lung and gastric cancer.

Keywords

Receptor tyrosine kinase; tyrosine kinase inhibitor; molecularly targeted therapy; Bcl-2; Bcl-xL; lung cancer; gastric cancer

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The authors declare no potential conflicts of interest.

Authors' contributions

JJ contributed to the study concept and design, and data analysis and interpretation, and provided materials. XY and BC performed all the experiments and contributed to data analysis and interpretation. BC wrote the manuscript and contributed to the study concept and design, and study coordination. All authors reviewed and approved the manuscript.

Introduction

The concept of “oncogene addiction” whereby cancer cells become dependent on a specific oncogenic signaling pathway for survival [1] is highlighted by the recent success of molecularly targeted agents [2]. However, the initial enthusiasm over substantial clinical responses has now been tempered by the bedside observation that these responses are not durable because tumors acquire drug resistance [2–4]. The identification of resistance mechanisms is essential to developing a strategy to enhance molecularly targeted therapy and prolong the efficacy of these agents.

Mutations and amplification of receptor tyrosine kinases (RTKs) were nominated as driver events in lung adenocarcinoma and gastric cancer [5, 6]. In RTK mutated or amplified cancers, RTK inhibition dramatically reduces cell viability and invariably leads to downregulation of the PI3K (phosphatidylinositol 3-kinase)/AKT and the MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal-regulated kinase) pathways [3, 7]. Therapies targeting RTKs are part of the arsenal of agents that are used to treat lung, gastric, colorectal and other cancers. Specific drugs used include small molecule tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib (EGFR TKIs), crizotinib (ALK TKI), etc. Despite the initial wide-observed clinical efficacy of these TKIs, resistance invariably develops, typically within 1 to 2 years [3, 4]. Over the past several years, multiple molecular mechanisms of resistance have been identified, and some common themes have emerged [3, 4, 8], which includes: mutation of RTK to a drug-resistant state; activation of a bypass pathway; and histologic transformation to an epithelial-mesenchymal transition [3]. However, a large fraction of clinical cases including both *de novo* and acquired resistance cases are still driven by unknown mechanisms [9, 10]. Novel *de novo* and acquired resistance mechanisms continue to be identified.

The Bcl-2 protein family determines the commitment of cells to apoptosis, an ancient cell suicide program that is important to cancer development and drug response [11]. A common deletion polymorphism of a Bcl-2 family member Bim mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in chronic myeloid leukemia and non-small-cell lung cancer [12]. Genetic or pharmacological inhibition of Bcl-2 increased sensitivity of lung cancer cells to EGFR inhibitors [13, 14].

Here, we examine the role of Bcl-2 and Bcl-xL in the mechanisms underlying acquired resistance to small-molecule TKIs in lung and gastric cancers cells driven by the targeted RTKs. We modeled acquired resistance to various TKIs by exposing RTK-driven cells to increasing concentration of TKIs. Our results suggest a novel common mechanism of resistance to TKIs, potentially leading to new co-treatment strategies.

Materials and Methods

Antibodies and Reagents

The following antibodies were purchased from Cell Signaling Technology: anti-MET (Cat#8198), anti-phospho-MET (Cat#3077), anti-EGFR (Cat#4267), anti-phospho-EGFR

(Cat#3777), anti-HER2 (Cat#4290), anti-phospho-HER2 (Cat#2247), anti-ALK (Cat#3633), anti-phospho-ALK (Cat#12127), anti-AKT (pan, Cat#4691), anti-phospho-AKT (S473, Cat#4058), anti-phospho-S6 (Cat#2215), anti-ERK (Cat#9102), anti-phospho-ERK (Cat#9101), anti-Bcl-2 (Cat#4223), anti-Bcl-xL (Cat#2764), anti-Bim (Cat#2933), and anti-cleaved PARP (Cat#5625). Anti- β -actin (Cat#A3854) and anti- β -tubulin (Cat#T4026) antibodies were purchased from Sigma. Anti-Mcl-1 antibody (Cat#sc-12756) was from Santa Cruz Biotechnology. HRP-linked enhanced chemiluminescence (ECL) mouse (Cat#NA931V) and rabbit IgG (Cat#NAV934V) were purchased from GE Healthcare Life Sciences.

The small molecule inhibitors TAE684 (ALK TKI), Lapatinib (Dual EHER2/EGFR TKI), and ABT-263 (Bcl-2 and Bcl-xL inhibitor) were obtained from Selleck Biochemicals. BEZ235 (Dual PI3K/mTOR inhibitor), GSK1120212 (MEK inhibitor), PHA665752 (MET TKI), Erlotinib (EGFR TKI), Gefitinib (EGFR TKI) were purchased from Cayman Chemical. AZD6094 (MET TKI) was from Chemietek.

Cell culture and transfection

HCC827, PC9, HCC4006, NCI-N87, and MKN45 cells were from American Type Culture Collection. H3122 cells were purchased from Tumor/Cell Line Repository at NCI. EBC-1 cells were from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. All cell lines were authenticated by providers utilizing Short Tandem Repeat (STR) profiling. Cells were used over a course of no more than 3 months after resuscitation of frozen aliquots. Cells were grown in RPMI supplemented with 2 mM Glutamax (Life Technologies) and 10% fetal bovine serum (BioAbChem) at 37°C under 5% CO₂.

Bcl-2 siRNAs (Cell Signaling) were transfected into cells with Lipofectamine 3000 reagents (Thermo Fisher Scientific) according to manufacturer's instructions.

Establishment of RTK TKIs-resistant cells

Cells were exposed to increasing concentrations of TKI every 3 weeks starting from 50 nM until a concentration of 5 μ M was reached at the end of a 5-month period. RTK TKI-resistant cells were successfully expanded in 10% FBS culture medium containing 1 μ M of TKI.

Immunoblotting

Cells were harvested in lysis buffer consisting of 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA. Following 30 min incubation in lysis buffer at 4°C, lysates were cleared by centrifugation at 16,000 $\times g$ for 10 min at 4°C, then protein concentrations were determined by DC Protein Assay (BioRad). Peroxidase conjugated donkey anti-rabbit and sheep anti-mouse (1:10,000; GE Healthcare NA934 and NA931, respectively) antibodies were incubated for 1 h at room temperature. ECL prime kit (GE Healthcare) was used to detect chemiluminescence.

Cell viability assays

Cells were seeded overnight at a density of 3,000 cells per well in 96-well plates in RPMI 1640 containing 10% FBS and then treated with the relevant agents for 3 d. Viable cell numbers were determined using the CellTiterGLO assay kit according to the manufacturer's protocols (Promega). Each assay consisted of three replicate wells and was repeated at least three times. Data was expressed as the percentage of surviving cells compared to control. This was calculated from the absorbance corrected for background.

Animal Experiments

4–6 week old nu/nu nude male mice were obtained from Charles River Laboratories. All studies were performed in compliance with institutional guidelines under an IACUC approved protocol at Medical University of South Carolina. HCC827GR xenograft tumors were established in nude mice by subcutaneously injecting 5×10^6 cells suspended in PBS into the right flank. Mice with well-established tumors were selected and randomized ten days post-implantation (Size > 150mm³). Mice were treated with vehicle, or ABT-263 (25 mg/kg/day), or the combination of BEZ235 (25 mg/kg/day) and GSK1120212 (25 mg/kg/day), or the combination of three chemicals by oral gavage once daily for six days a week. All drugs were dissolved in 0.5% HPMC/0.1% Tween 80. Tumor dimensions were measured with a caliper and tumor volumes calculated (tumor volume (mm³) = (length $\times$ width²)/2.).

Statistical Analysis

The results of quantitative studies are reported as mean $\pm$ SD or Mean $\pm$ SEM. The SEM/SD was calculated based on the number of independent experiments. Differences were analyzed by Student's *t* test. *P* values of < 0.05 were regarded as significant.

Results

Bcl-2 and Bcl-xL are upregulated upon treatment with TKIs in RTK-dependent lung and gastric cancer cell lines

The growth and survival of a number of lung and gastric cancer cell lines have been found to be highly dependent on RTK signaling due to gene mutations or amplifications. These cell lines include lung cancer cell lines HCC827 (*egfr* deletion) [15], PC9 (*egfr* deletion) [16], HCC4006 (*egfr* mutation) [17], EBC-1 (*met* amplification) [18], H3122 (*eml4-alk* fusion) [19], and gastric cancer cell lines MKN45 (*met* amplification) [7], NCI-N87 (*her2* amplification) [20], KATOIII (*fgfr2* amplification) [21]. We have previously shown that treatment of EBC-1 or MKN45 cells with the small-molecule MET TKIs induces the expression of Bcl-2 [22]. To determine whether this is a generalizable phenomenon, we treated HCC827, H3122, and NCI-N87 cells with the EGFR TKI gefitinib, the ALK TKI TAE684, and the dual HER2/EGFR TKI lapatinib, respectively. All inhibitors strongly blocked the RTK signaling they specifically target demonstrated by strongly reduced phosphorylation of EGFR, ALK, or HER2 as well as reduced phosphorylation of the downstream AKT (Fig. 1 A and B). The effectiveness of these inhibitors to induce apoptosis was confirmed by the induction of cleaved PARP, a marker for apoptosis (Fig. 1 A and B). In agreement, downregulation of anti-apoptotic Mcl-1 and upregulation of pro-apoptotic Bim

were seen (Fig. 1). However, we found the expression of anti-apoptotic Bcl-2 and Bcl-xL was upregulated in all of these cells (Fig. 1). Additionally, EGFR inhibition by gefitinib in PC9 cells or a second EGFR inhibitor erlotinib in HCC4006 cells led to increased protein levels of Bcl-2 and Bcl-xL (Fig. 1A) as well.

Combination of TKI and Bcl-2, Bcl-xL inhibitor is superior to TKI alone

Given the well-established anti-apoptotic roles of Bcl-2 and Bcl-xL, we speculated that inhibition of Bcl-2 and Bcl-xL would strengthen the efficacy of TKIs since they caused a feedback upregulation of Bcl-2 and Bcl-xL (Fig. 1). Indeed, ABT-263, a small-molecule Bcl-2, Bcl-xL inhibitor was only minimally active, while the combination of ABT-263 and an EGFR TKI erlotinib significantly decreased cell viability (Fig. 2A) and increased PARP cleavage (Fig. 2A) induced by erlotinib. To confirm this was due to the inhibition of Bcl-2, we applied the Bcl-2 siRNAs and obtained similar results (Fig. S1A). This effect was also seen in ALK-driven lung cancer cell line H3122 treated with the ALK TKI TAE684 and ABT-263 (Fig. 2A) or the Bcl-2 siRNAs (Fig. S1B), and HER2-dependent gastric cancer cell line NCI-N87 treated with the HER2 TKI lapatinib and ABT-263 (Fig. 2A).

ABT-263 blocks the formation of resistant colonies induced by TKIs

The long-term treatment of HCC827 with the small-molecule EGFR TKI erlotinib led to the killing of the majority of these tumor cells but also to the outgrowth of resistant colonies (Fig. 2B). Although ABT-263 had little effect on the growth of these tumor cells, the addition of this inhibitor to erlotinib suppressed the outgrowth of resistant colonies induced by this EGFR inhibitor. Similar results were obtained in H3122 cells treated with TAE684 and NCI-N87 cells treated with lapatinib (Fig. 2B), suggesting the possibility that upregulation of Bcl-2 and Bcl-xL contribute to the emergence of resistance to TKIs.

Resistant cells express higher levels of Bcl-2 and/or Bcl-xL protein

To examine whether Bcl-2 and Bcl-xL levels were also increased in TKI resistant clones, RTK-dependent cancer cells were grown in increasing concentrations of corresponding TKIs, similarly to previously described methods [23, 24]. Cells were then expanded in these TKIs (1 μ M) and designated as the following: HCC827-GR (gefitinib resistant), HCC827-ER (erlotinib resistant), PC9-GR (gefitinib resistant), PC9-ER (erlotinib resistant), H3122-TR (TAE684 resistant), NCI-N87-LR (lapatinib resistant), EBC-1-PR (PHA665752 resistant), EBC-1-AR (AZD6094 resistant), MKN45-PR (PHA665752 resistant), MKN45-AR (AZD6094 resistant). These resistant cells indeed contain elevated levels of Bcl-2 or Bcl-xL (Fig. 3A), but not Mcl-1 (Fig. 3B) as demonstrated by immunoblot assays.

Combination of inhibitors of PI3K/AKT, MEK/ERK, and Bcl-2/Bcl-xL overcomes resistance to TKIs

In RTK-dependent cancers, inhibition of the corresponding RTK leads to suppression of key downstream signaling pathways, such as the PI3K/AKT and the MEK/ERK pathways, resulting in cell growth arrest and death [25, 26]. Indeed, the combination of PI3K/AKT and MEK/ERK inhibitors acted similarly to TKIs in inducing apoptosis in parental cells as indicated by PARP cleavage (Fig. 4). We and others have shown that, in resistant cells, the

PI3K/AKT and the MEK/ERK pathways are no longer regulated by RTK activity [15, 22, 27]. However, these pathways can still be blocked by their own inhibitors in resistant cells (Fig. 4). Nevertheless, the blockade of these key pathways in resistant cells was not enough to induce sufficient apoptosis (Fig. 4, left panels) and reduce cell viability (Fig. 4, right panels) as seen in parental cells, suggesting the function of apoptosis machinery is compromised. We reasoned that increased levels of Bcl-2 or Bcl-xL in resistant cells play an important role. Indeed, when we treated resistant cells with the combination of ABT-263, BEZ235, and GSK1120212, it caused significant apoptosis and dramatic cell death (Fig. 5A).

In addition, we treated the xenograft tumors derived from HCC827 cells with acquired resistance (HCC827-GR) with the BEZ235/GSK1120212/ABT-263 combination and observed marked tumor growth inhibition compared to the control group (Fig. 5B). The treatment also induced cleaved PARP suggesting apoptosis occurred in these tumors (Fig. 5B). This combination appeared to be well tolerated as there was no significant body weight loss or signs of toxicity in the treatment group throughout the experiment (data not shown).

Discussion

All patients who initially benefit from RTK-targeted therapies eventually develop resistance within 1 or 2 years. An increasing understanding of the number and complexity of resistance mechanisms highlights the Herculean challenge of killing tumors that are resistant to TKIs. Our growing knowledge of resistant pathways provides an opportunity to develop new mechanism-based inhibitors and combination therapies to prevent or overcome therapeutic resistance in tumors. In this study, we present a previously unobserved resistance mechanism mediated by Bcl-2 and Bcl-xL which is shared by multiple RTKs. Acute exposure of RTK-dependent lung and gastric cancer cells to their corresponding TKIs lead to increased expression of Bcl-2 and Bcl-xL (Fig. 1). The upregulation of these two molecules is maintained in cells that have acquired resistance through long term TKI exposure (Fig. 3). It would be important to determine whether in specimens of patients who received TKI treatment there is increased expression of Bcl-2 and/or Bcl-xL. Here, we propose two potential uses for Bcl-2 and Bcl-xL inhibitors. First, they may be combined with a TKI to enhance the efficacy of the TKI (Fig. 2A) and to prevent resistance as this combination is significantly better at delaying the onset of resistance than a TKI alone (Fig. 2B). A second potential clinical use for Bcl-2/Bcl-xL inhibitors is to combine with PI3K/AKT and MEK/ERK inhibitors to overcome resistance to TKIs (Fig. 5). To date, in completed clinical trials the activity of single-agent Bcl-2/Bcl-xL inhibitors is limited in solid tumors, suggesting the need to develop novel strategies to utilize these drugs.

Mechanisms of acquired resistance in RTK-driven cancer are broadly divided into two categories. The first involves development of additional genetic alterations in the primary RTK genes, which facilitates continued downstream signaling [25]. This commonly arises through secondary mutations in the kinase target or through gene amplification of the kinase itself. Alternatively, resistance can develop independent of genetic changes in the target. This occurs through activation of downstream signaling pathways, changes in tumor histology, or alterations in drug metabolism [25, 28]. Studies indicate that multiple

resistance mechanisms may operate within an individual tumor to promote acquired resistance [3, 4]. One potential solution to overcome multiple mechanisms of resistance is to target downstream pathways that mediate the balance between survival and apoptosis. A therapeutic strategy that directly activates the machinery that is necessary for apoptosis may circumvent multiple mechanisms of acquired resistance to RTK-targeted therapies and may therefore be a broader approach than aiming to inhibit one resistant mechanism at a time. Given the prominent anti-apoptotic roles of Bcl-2 and Bcl-xL and their upregulation in response to TKI treatment, targeting these molecules in the context of RTK-targeted therapies may have the potential to overcome or prevent acquired resistance resulting from various mechanisms.

It was previously shown that induction of Bim is essential for apoptosis triggered by EGFR TKIs [29]. Our data show that Bim upregulation is present in all RTK-dependent lung and gastric cancer cell lines upon treatment with corresponding TKIs (Fig. 1, [30]) which underscore the importance of RTK signaling for survival. On the other hand, in response to TKI treatment cells trigger a feedback mechanism by increasing the expression of Bcl-2 and Bcl-xL. Augmented transcription of Bim can account for at least part of the increase in Bim protein after treatment with EGFR TKIs in EGFR-dependent lung cancer cells and ERK pathway is responsible for Bim increase [29]. In agreement, our data show that the MEK inhibitor GSK1120212 but not the PI3K/AKT inhibitor BEZ235 induced Bim expression in RTK-dependent cells although the combination of these two inhibitors seems to show further induction of Bim (Fig. 4). This combination not only failed to induce PARP cleavage but also showed reduced Bim induction in resistant cells (Fig. 4), likely resulting from Bcl-2 and/or Bcl-xL upregulation. Indeed, addition of ABT-263 effectively led to significantly better induction of apoptosis (Fig. 5). Studies are underway to determine the molecular mechanisms by which TKIs upregulate Bcl-2 and Bcl-xL expression. Our previous work suggests Pim-1 kinase may be involved in the regulation [30]. This Yin (Bim upregulation) and Yang (Bcl-2 and Bcl-xL upregulation) relationship highlights the opportunity and challenge we are facing in treating these cancers. Although the combination of EGFR TKI and a Bcl-2 inhibitor has been explored before pre-clinically [29] and in clinical trials [31], we for the first time provide a biological basis for targeting Bcl-2 and Bcl-xL and show that this strategy may also work for other TKIs.

To understand the mechanism underlying the acquisition of TKI resistance, we adopted a classical method which is the establishment and characterization of drug-resistant cancer cells selected from drug-sensitive cell lines in vitro following exposure to stepwise increasing doses of the drug [16]. This method has successfully led to identification of many resistance mechanisms to TKIs that are also observed in the clinics [15, 32]. Our data support the further clinical evaluation of Bcl-2/Bcl-xL inhibitors in combination with TKIs. This combination strategy may be extended to other targeted agents that induce apoptosis. Clinical trials are underway to investigate the potential of ABT-263 in combination with the MEK inhibitor trametinib in KRAS mutant tumors or with the BRAF inhibitor dabrafenib in BRAF mutant melanoma [33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

RTK	Receptor Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
EGFR	Epidermal Growth Factor Receptor
ALK	Anaplastic Lymphoma Kinase
PI3K	(phosphatidylinositol 3-kinase
MEK	mitogen-activated protein kinase kinase
ERK	extracellular signal-regulated kinase

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Fig. 1.

Induction of Bcl-2 and Bcl-xL by TKIs in RTK-dependent lung and gastric cancer cell lines. EGFR-dependent lung cancer cell lines HCC827, PC9, and HCC4006 (A), ALK-dependent lung cancer cell line H3122, and HER2-dependent gastric cancer cell line NCI-N87 were treated with gefitinib (1 μ M), gefitinib (0.2 μ M), erlotinib (1 μ M), TAE684 (0.3 μ M), lapatinib (0.1 μ M) respectively for 6, 24, and 48 hrs. Expression of indicated proteins was assessed by immunoblots.

A

B

Fig. 2.

Combination of Bcl-2/Bcl-xL inhibitor ABT-263 and a TKI is superior to the TKI alone. (A) Cell viability was determined after cells were treated as indicated for 72 hrs. Erlotinib, TAE684, Lapatinib, and ABT-263 were used at 1, 0.3, 0.1, and 1 μ M respectively. Results shown are the mean $\pm$ SD. * p < 0.02 compared with DMSO control. (B) Crystal violet staining of cells treated with DMSO or ABT-263 (1 μ M) for 4 days, and TKI (1 μ M erlotinib, 0.3 μ M TAE684, 0.1 μ M Lapatinib), or the combination of TKI and ABT-263 for 4 weeks. Representative results are shown.

Fig. 3. Resistant cells express higher levels of Bcl-2 or Bcl-xL. Expression of Bcl-2, Bcl-xL (A) or Mcl-1 (B) was assessed by immunoblots in parental and resistant cells.

Fig. 4.

Combination of inhibitors of PI3K/AKT and MEK/ERK is effective in parental but not in resistant cells. Cells were treated with indicated inhibitors. Gefitinib, TAE684, Lapatinib, and PHA665752 were used at 1, 0.3, 0.1, and 1 μ M respectively. BEZ235 was used at 0.5 μ M and GSK1120212 was used at 1 μ M. Expression of indicated proteins was assessed by immunoblots (left panels). Cell viability was determined after cells were treated for 72 hrs (right panels). Results shown are the mean $\pm$ SD.

Fig. 5. Combination of inhibitors of PI3K/AKT, MEK/ERK, and ABT-263 is effective in overcoming resistance to TKIs. (A) TKI resistant cells were treated with indicated inhibitors. All inhibitors were used at 1 μ M except that BEZ235 was used at 0.5 μ M. Expression of indicated proteins was assessed by immunoblots (left panels). Cell viability was determined after cells were treated for 72 hrs (right panels). Results shown are the mean $\pm$ SD. * p < 0.02 compared with TKI alone treated cells. (B) Xenograft tumors were established in NU/NU nude male mice by subcutaneous injection. Mice were treated indicated drugs by oral gavage

(n=6 per group). Error bars represent mean $\pm$ SEM. Expression of indicated proteins in xenograft tumors was assessed by immunoblots.