Front. Med. 2022, 16(5): 701−713
https://doi.org/10.1007/s11684-022-0951-0

REVIEW

Targeting apoptosis to manage acquired resistance to third generation EGFR inhibitors

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Abstract **A significant clinical challenge in lung cancer treatment is management of the inevitable acquired resistance to third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs), such as osimertinib, which have shown remarkable success in the treatment of advanced NSCLC with EGFR activating mutations, in order to achieve maximal response duration or treatment remission. Apoptosis is a major type of programmed cell death tightly associated with cancer development and treatment. Evasion of apoptosis is considered a key hallmark of cancer and acquisition of apoptosis resistance is accordingly a key mechanism of drug acquired resistance in cancer therapy. It has been clearly shown that effective induction of apoptosis is a key mechanism for third generation EGFR-TKIs, particularly osimertinib, to exert their therapeutic efficacies and the development of resistance to apoptosis is tightly associated with the emergence of acquired resistance. Hence, restoration of cell sensitivity to undergo apoptosis using various means promises an effective strategy for the management of acquired resistance to third generation EGFR-TKIs.**

Keywords acquired resistance; EGFR inhibitor; apoptosis; lung cancer

Background

Lung cancer, which consists of small cell lung cancer and over 80% non-small cell lung cancer (NSCLC), is the leading cause of cancer death in the United States [1] and worldwide [2]. For decades, the 5-year survival rate of lung cancer remained below 20% worldwide despite advances in early diagnosis and various therapeutics. Encouragingly, 5-year survival has recently increased to over 20% for the first time in the United States after decades of effort [1], largely due to the application of effective targeted therapies and immunotherapy.

Treatment of NSCLC patients with epidermal growth factor receptor (EGFR) activating mutations such as exon 19 deletions (19del) and exon 21 L858R point mutation using EGFR-tyrosine kinase inhibitors (EGFR-TKIs) represents the successful targeted therapy of lung cancer. To combat acquired resistance, EGFR-TKIs have developed rapidly from first generation (e.g., gefitinib and erlotinib) to second generation (e.g., afatinib) and currently mutation-selective third generation (e.g., osimertinib/AZD9291) agents over the past two decades.

Received: April 27, 2022; accepted: June 28, 2022
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The third-generation EGFR-TKIs were developed to primarily target the EGFR resistance mutation, T790M, which develops during application of the first and second-generation EGFR-TKIs, in addition to efficacy against other common EGFR activating mutations. Because of limited activity against wild-type (WT) EGFR, these agents are considered mutation-selective EGFR-TKIs [3]. Among them, osimertinib is the first FDA-approved drug for the second-line treatment of EGFR mutant (EGFRm) NSCLCs relapsed from first generation EGFR-TKI therapy due to T790M mutation and for a first-line option for advanced EGFRm NSCLC due to its promising overall clinical efficacy in terms of both progression-free survival (PFS) and overall survival (OS) [4,5]. Aumolertinib (formerly almonertinib; HS-10296) was recently approved in China for the treatment of NSCLC patients harboring EGFR T790M mutation who have relapsed to other EGFR-TKI therapy based on the promising outcomes of the open-label phase II APOLLO study [6,7], thus being second marketed third generation EGFR-TKI.

Despite this promising clinical activity, all patients eventually relapse due to the inevitable emergence of acquired resistance to these third generation EGFR-TKIs,

regardless of whether they are used as a first-line or second-line treatment option [8]. Therefore, there is an urgent clinical need for the effective management of acquired resistance to third generation EGFR-TKIs in order to improve patient survival duration. Through effective management of acquired resistance, patients will have a higher chance to continue to benefit from treatment with third generation EGFR-TKIs.

Apoptosis represents a major form of programmed cell death involved in cancer. Evasion of cell death including apoptosis is considered a key hallmark of cancer [9,10]. Thus, acquisition of apoptosis resistance has become a major mechanism accounting for the emergence of acquired resistance to cancer therapeutic agents. Accordingly, targeting the induction of apoptosis is a valid cancer therapeutic strategy accompanied with the successful development of anticancer drugs that directly target the apoptosis machinery, such as BH3-mimetic drugs [11]. Considering the critical role of apoptosis induction in mediating therapeutic efficacy of third generation EGFR-TKIs, this review will focus on the topic of effective management of acquired resistance to osimertinib and other third generation EGFR-TKIs through targeting induction of apoptosis.

Apoptotic pathways

It is well recognized that there are two major apoptotic pathways: the extrinsic apoptotic pathway that involves signals transduced through cell surface death receptors and the intrinsic apoptotic pathway that relies on a signal from mitochondria (Fig. 1). Both pathways are centered by a coordinated and sequential activation of several cysteine proteases named caspases that in turn cleave cellular substrates, resulting in the characteristic morphological and biochemical changes of apoptosis [12,13].

Extrinsic apoptotic pathway

The key component of the extrinsic apoptotic pathway is the cell surface death receptors, which belong to the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) including Fas (CD95 or Apo1), TNFR1, DR3, DR4 (TRAIL-R1), DR5 (TRAIL-R2), and DR6 [14]. They share similar cysteine-rich extracellular ligand binding domains, transmembrane domains, and intracellular death domains, which enable transmission of death signals after ligand binding or receptor trimerization [14]. There are also other related cell surface receptors called decoy receptors (e.g., DcR1 and DcR2) that contain no death domain or a truncated death domain. These decoy receptors can still bind ligand but cannot transmit death signal, and thus function as antagonists to compete with death ligands for inhibiting death ligand/death receptor-induced apoptosis [15].

Once bound to their own ligands, e.g., FasL or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), trimerization of the death receptors is induced at the cell surface, triggering apoptosis through the recruitment of the adaptor protein, Fas-associated death domain (FADD), and pro-caspase-8 and subsequent formation of the death inducing signaling complex (DISC). Within the DISC, pro-caspase-8 recruited by FADD is converted via cleavage into activated caspase-8, which in turn activates effector caspases such as caspase-3. Activation of the death receptors can also indirectly activate effector caspases by caspase-8-dependent cleavage of Bid, a BH3 only Bcl-2 family protein, facilitating Bax activation, subsequent pore formation in the mitochondrial membrane, release of cytochrome C and Smac/DIABLO, and final activation of caspase-9 (Fig. 1) [14,16]. Hence, a crosstalk between death receptors and activation of the intrinsic mitochondrial apoptotic pathway can occur in certain types of cells.

The extrinsic death receptor-mediated apoptotic pathway is primarily negatively regulated by cellular FLICE-inhibitory protein (c-FLIP), which inhibits caspase-8 activation by preventing FADD recruitment of caspase-8 to the DISC [17,18]. There are multiple splice variants of c-FLIP; however, only two of them, the 26 kDa short form (c-$FLIP_S$) containing two death effector domains and the 55 kDa long form (c-$FLIP_L$) containing an inactive caspase-like domain in addition to the two death effector domains, have been well characterized at the protein levels in human cells [19,20]. Both $FLIP_L$ and $FLIP_S$ are unstable proteins and their levels are modulated by ubiquitin/proteasome-mediated degradation [21–23].

Because immune cells such as cytotoxic T cells and natural killer (NK) cells can generate and secrete death ligands such as FasL and TRAIL, the ligation of these endogenous death ligands with their corresponding death receptors on cancer cells eliminates cancer cells via induction of apoptosis. Thus, activation of the extrinsic death receptor-mediated apoptosis has been recognized as a critical mechanism by which immune surveillance eradicates malignant cancer cells [24–26].

Intrinsic apoptotic pathway

The intrinsic apoptotic pathway is centered on the activation of death signal from mitochondria and is thus also known as the mitochondria-mediated apoptotic pathway. In response to DNA damage and other different stimuli, mitochondrial membrane permeability is altered due to Bax/Bak insertion into mitochondrial membrane, causing release of cytochrome C into the cytoplasm and subsequent formation of a complex named the apoptosome, together with apoptotic protease activating factor 1 (APAF1) and the inactive form of pro-caspase-9.

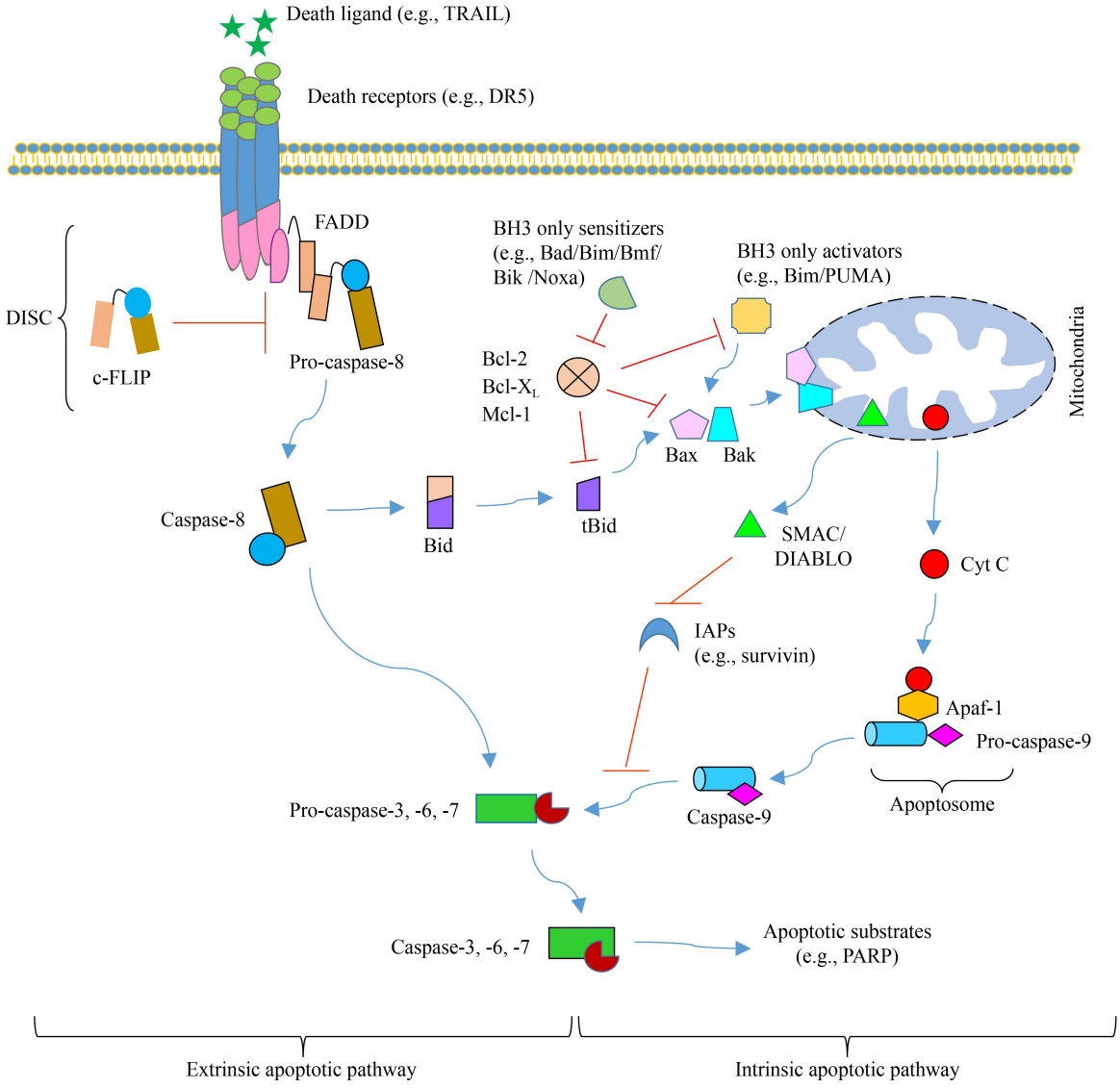

Fig. 1 Schema for two major apoptotic pathways. Ligation of death ligands (e.g., TRAIL) with their receptors (e.g., DR5) or death receptor aggregation induces formation of the death-inducing signaling complex (DISC). In the DISC, pro-caspase-8 is recruited through the death adaptor protein FADD and cleaved to generate activated caspase-8. This process is negatively regulated by c-FLIP, a truncated pseudo-protein of pro-caspase-8. Signals that activate BH3 only proapoptotic proteins and/or inhibit Bcl-2 antiapoptotic proteins facilitate the insertion of Bax and Bak protein into mitochondrial membrane, increasing mitochondrial outer membrane permeabilization (MOMP); this leads to cytochrome C (Cyt C) and Smac/DIABLO release from the mitochondria into the cytosol. The released cytochrome C then activates pro-caspase-9 by forming an apoptosome through binding to Apaf-1. Both activated caspase-8 and caspase-9 further cleave downstream effector caspases including pro-caspase-3, -6, and -7, to generate activated caspase-3, -6, and -7 that cleave a variety of substrate proteins such as PARP and cause eventual cell death. Caspase-8 also cleaves the BH3 only protein, Bid, to generate truncated Bid (tBid) that facilitates insertion of Bax into the mitochondrial membrane. Thus, tBid connects the extrinsic and intrinsic apoptotic pathways together. Inhibitors of apoptosis proteins (IAPs) such as XIAP and survivin can bind to caspase-9 and prevent its effect on cleavage of effector caspases, whereas Smac/DIABLO binds to IAPs, allowing free caspase-9 to activate the effector caspases.

Within this complex, pro-caspase-9 is cleaved to become active caspase-9, which then cleaves and activates the effector caspases-3/6/7, resulting in apoptosis. The mitochondrion also releases another protein named Smac/DIABLO into the cytosol, which indirectly facilitates apoptosis by counteracting the effects of anti-apoptotic proteins called inhibitor of apoptosis proteins (IAPs).

The intrinsic apoptotic pathway is tightly regulated by the group of B cell lymphoma 2 (Bcl-2) protein family proteins, which function as either pro-apoptotic or apoptotic proteins. Bax and Bak, which contain multiple BH domains, function as “effector” pro-apoptotic proteins exclusively responsible for triggering cytochrome C and Smac/DIABLO release from mitochondria via altering mitochondrial membrane permeability by inserting into

mitochondrial membrane. In contrast, other Bcl-2 family proteins primarily function to regulate this process either positively or negatively. BH3-only "activator" proteins including Bim, Bid, and PUMA can bind directly to, and activate Bax and/or Bak. In addition, they also bind to and sequester anti-apoptotic/pro-survival Bcl-2 family proteins such as Bcl-2, Bcl-X_L, and Mcl-1. Other BH3-only proteins (Bad, Noxa, HRK, Bik, and Bmf) lacking the capability to directly engage Bax and Bak can function as "sensitizer" proteins and predominantly act by inhibiting anti-apoptotic proteins. All anti-apoptotic members of the Bcl-2 family (Mcl-1, Bcl-2, Bcl-X_L, and Bcl-W) contain all four BH domains and function to prevent cytochrome C and Smac/DIABLO release from mitochondria by directly binding to and sequestering pro-apoptotic Bcl-1 proteins (Fig. 1) [11,27,28].

Critical role of apoptosis induction in mediating therapeutic efficacy of third generation EGFR-TKIs

Our early work has clearly demonstrated that osimertinib potently induces apoptosis primarily in sensitive EGFRm NSCLC cell lines, leading to decreased cell survival [29]. Mechanistically, osimertinib rapidly inhibits MEK/ERK signaling accompanied by Bim elevation and Mcl-1 reduction. Both Bim and Mcl-1 are phosphorylated by ERK1/2 at S69 and T163, respectively, leading to protein destabilization (Bim) or stabilization (Mcl-1). Osimertinib effectively inhibited phosphorylation of both Bim (S69) and Mcl-1 (T163), leading to enhanced Mcl-1 degradation and compromised Bim degradation. Consequently, Bim levels were elevated while Mcl-1 levels were decreased (Fig. 2). These two events are critical for the induction of apoptosis by osimertinib in EGFRm NSCLC cells since gene knockdown-mediated blockade of Bim elevation or Mcl-1 overexpression in the sensitive EGFRm NSCLC cells attenuated or abolished induction of apoptosis by osimertinib [29]. Therefore, inhibition of ERK1/2-dependent modulation of Bim and Mcl-1 is a key mechanism accounting for osimertinib-induced apoptosis in EGFRm NSCLC cells. Beyond osimertinib, a recent study has demonstrated that aumolertinib also exerts therapeutic efficacy through inducing apoptosis, likely via enhancing ROS production, in EGFRm NSCLC cells [30].

Beyond activation of the intrinsic apoptotic pathway, osimertinib induces the activation of caspase-8 since we detected clear cleavage of pro-caspase-8 accompanied with caspase-3 activation in sensitive EGFRm NSCLC cell lines exposed to osimertinib [29], suggesting the possible activation of the extrinsic death receptor-mediated apoptotic pathway. Indeed, we have demonstrated that osimertinib reduces c-FLIP levels via facilitating its degradation primarily in NSCLC cells with EGFR activating mutations (Fig. 2). Moreover, modulation of c-FLIP expression levels, to some degree, also altered the sensitivities of EGFRm NSCLC cells to undergo osimertinib-induced apoptosis, suggesting that c-FLIP suppression is also an important event contributing to the antitumor activity of osimertinib against EGFRm NSCLC [31].

Although DR4 is generally thought to be a pro-apoptotic protein in transducing death signaling upon binding to its ligand TRAIL, osimertinib as well as other EGFR-TKIs, paradoxically, robustly decreased DR4 levels in EGFRm NSCLC cells and tumors; this effect was tightly associated with induction of apoptosis. This modulation was lost once EGFRm cells became resistant to these inhibitors. Interestingly, increased levels of DR4 were detected in cell lines with acquired osimertinib resistance and in NSCLC tissues relapsed from EGFR-targeted therapy. DR4 knockdown induced apoptosis and augmented apoptosis when combined with osimertinib in both sensitive and resistant cell lines, whereas enforced DR4 expression significantly attenuated osimertinib-induced apoptosis, suggesting that DR4 downregulation is coupled to therapeutic efficacy of osimertinib and other EGFR-TKIs [32]. As discussed above, co-inhibition of MEK/ERK signaling effectively overcomes acquired resistance to osimertinib via enhancing induction of apoptosis [29]. The combination of osimertinib with a MEK inhibitor further augmented reduction of DR4 in osimertinib-resistant cells accompanied with enhanced induction of apoptosis [32]. This finding also supports the connection between DR4 downregulation and induction of apoptosis.

Mechanistically, osimertinib induced MARCH8-mediated DR4 proteasomal degradation and suppressed MEK/ERK/AP-1-dependent DR4 transcription, resulting in DR4 downregulation (Fig. 2) [32]. Unfortunately, we currently do not know the underlying mechanisms accounting for DR4 downregulation-induced apoptosis. Further investigation in this aspect is warranted.

Resistance to apoptosis as a critical mechanism accounting for emergence of acquired resistance to third generation EGFR-TKIs

In agreement with the critical roles of Bim elevation and Mcl-1 reduction in mediating therapeutic efficacies of third generation EGFR-TKIs as discussed above, we found that all EGFRm NSCLC cell lines with osimertinib acquired resistance were resistant to modulation of both Bim and Mcl-1 by osimertinib, suggesting that acquisition of apoptosis resistance is a critical mechanism for EGFRm NSCLC cells to develop acquired resistance [29]. Given the critical role of the MEK/ERK signaling in modulation of Bim and Mcl-1 protein stability (Fig. 2),

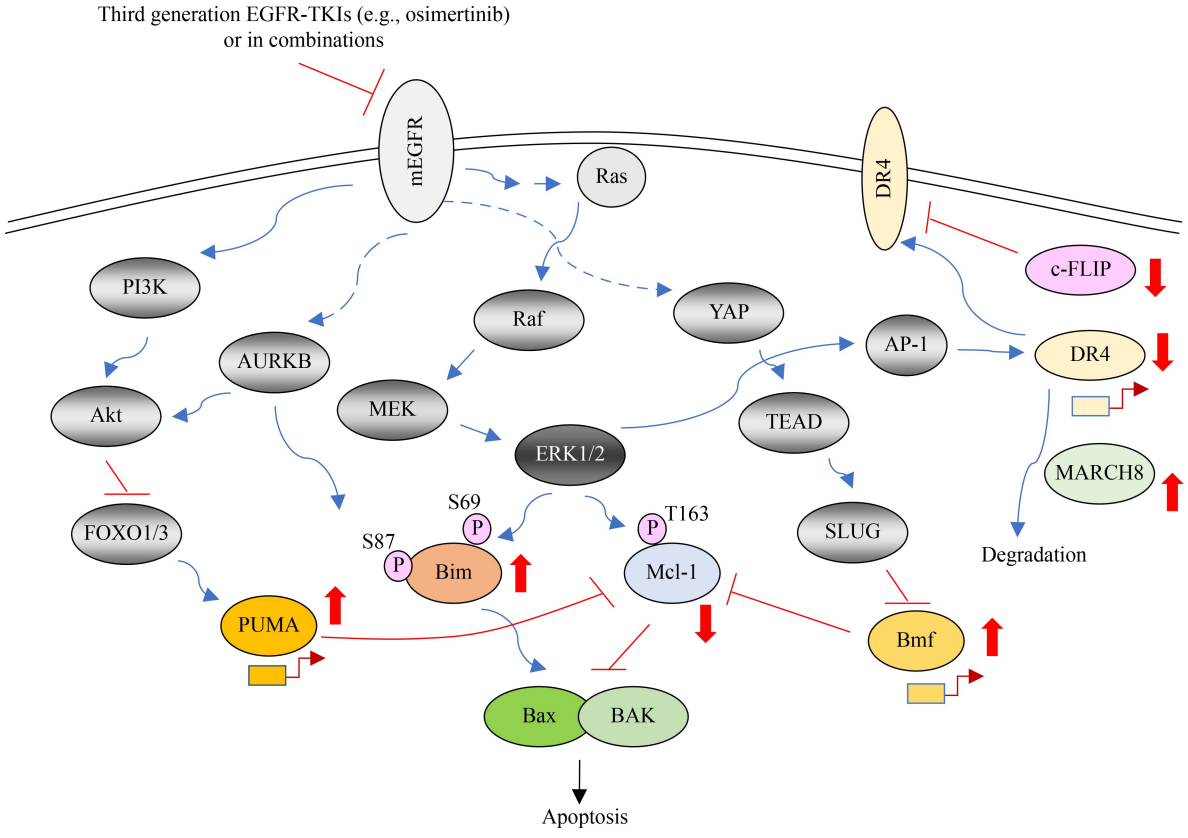

Fig. 2 Summary of potential molecular mechanisms accounting for induction of apoptosis by osimertinib in EGFRm NSCLC cells and by combinations in resistant EGFRm NSCLC cells. The bold red arrows indicate modulations of key apoptosis-regulatory proteins by osimertinib or combinations, which occur at transcriptional and/or posttranslational levels.

enhanced expression of MET, HER2, IGF1R, AXL and/or FGFR and Ras activation, which represent common resistance mechanisms to osimertinib, can all induce bypass activation of the MEK/ERK signaling [33], resulting in apoptosis resistance to osimertinib or other third generation EGFR-TKIs, likely through downregulation of Bim levels and elevation of Mcl-1 levels.

Epithelial-mesenchymal transition (EMT) has been tightly associated with development of acquired resistance to EGFR-TKIs including osimertinib, likely via insufficient expression of BIM, preventing apoptotic cell death [34–39]. While Slug was significantly increased in gefitinib-resistant PC-9/GR compared with parental PC-9 cells, silencing of Slug in gefitinib-resistant cells caused Bim upregulation and caspase activation, restoring the ability of gefitinib to induced apoptosis of the resistant cells [36]. Consistently, ZEB1 and TWIST1, both of which are critical EMT transcription factors, were reported to confer apoptosis resistance to EGFR-TKIs via inhibiting Bim expression by binding directly to the Bim promoter and repressing transcription [35,37]. De-repression of Bim expression, e.g., by depletion of ZEB1 or treatment with the BH3 mimetic ABT-263 enhanced "free" cellular Bim levels, leading to re-sensitization of mesenchymal EGFRm NSCLC cells to EGFR-TKIs [35,37]. These findings are in line with the critical role of Bim elevation in mediating therapeutic efficacy of osimertinib [29].

In a clinical study that detected Bim expression in pre-treatment tumors from patients with EGFRm NSCLC, a lower response rate to EGFR-TKIs in patients with low Bim expression compared to those with high Bim expression was observed, although the difference was not significant [40]. Consistently, the European Tarceva (EURTAC) trial showed that both PFS and OS were significantly shorter in patients with low/intermediate BIM mRNA levels in primary tumors than in those with high mRNA levels [41]. It will be interesting to determine whether the finding is applied for EGFRm patients to osimertinib as well.

It was reported that a common intronic deletion polymorphism in BIM gene was present in 12% of an Asian population although it was absent in individuals from African and European populations and was significantly associated with a shorter PFS in patients receiving EGFR-TKI treatment [42]. This polymorphism switched Bim splicing from exon 4 to exon 3, resulting in increased expression of BIM RNA isoforms lacking the pro-apoptotic BH3 domain, such as BIM-γ [42]. A subsequent pilot study with 33 EGFRm NSCLC patients

treated with gefitinib found that BIM-γ expression was significantly higher in patients with BIM deletion polymorphism than among those without BIM deletion polymorphism inside and patients with BIM-γ had significantly shorter PFS than those without BIM-γ [43]. However, there were also clinical studies that failed to demonstrate the association between BIM deletion polymorphism and the treatment response of EGFR-TKIs [44–46].

An early preclinical study also showed that EGFRm NSCLC cell lines with the BIM deletion polymorphism exhibited apoptosis resistance to osimertinib [47]. In a clinical study with a total of 152 Chinese Han NSCLC patients including 143 T790M-positive and nine T790M-negative patients with osimertinib therapy, BIM deletion polymorphism was detected in 17.5% of T790M-positive patients. Patients with BIM deletion polymorphism had a poorer objective response rate than those without and was associated with a significantly shorter PFS and a moderately shorter OS [48]. A recent clinical study with quantification of BIM mRNA in circulating tumor cells of osimertinib-treated EGFRm NSCLC patients found that the response rate to osimertinib was worse in patients with high than in those with low BIM-γ mRNA expression, but did not see a significant impact of BIM-γ expression on PFS [49]. Nonetheless, these studies also suggest a critical role of Bim in modulation of the response of EGFRm NSCLC to the treatment with osimertinib and possible other third generation EGFR-TKIs.

Although osimertinib potently decreased DR4 levels, including cell surface DR4 levels in sensitive EGFRm NSCLC cells [32], it still enhanced TRAIL-induced apoptosis in these cell lines as we reported [31]. Consistently, transient knockdown of DR4 further enhanced TRAIL-induced apoptosis; this finding is in agreement with our previous finding that DR4 knockdown enhanced apoptosis induced by TRAIL or the combination of TRAIL and GGTI-298 (a geranylgeranyltransferase I inhibitor) in NSCLC cells [50]. Interestingly, osimertinib-resistant cell lines had compromised sensitivities to TRAIL, and even the combination of osimertinib and TRAIL, compared with their corresponding parental cell lines [31], despite the elevated basal levels of DR4 [32]. Endogenous TRAIL/death receptor interaction in TRAIL-resistant cancer cells may activate NF-κB signaling and induce inflammatory cytokine (e.g., CCL2) secretion, which inactivates immune cells and promotes a tumor-supportive immune microenvironment and tumor growth [51,52]. It is possible that, in addition to the direct effects of osimertinib on EGFRm NSCLC cells, including induction of apoptosis, there may also be an indirect effect of osimertinib on enhancing the immune clearance of EGFRm NSCLC cells, contributing to osimertinib's therapeutic efficacy. Accordingly, the acquisition of resistance to extrinsic apoptosis that compromises immune clearance may also contribute to the development of acquired resistance.

Targeting apoptosis to overcome acquired resistance

Since the acquisition of apoptosis resistance represents a key mechanism accounting for acquired resistance to osimertinib and other third generation EGFR-TKIs as discussed above, science-driven means or regimens that can restore cellular sensitivity to undergo apoptosis should have high potential to overcome acquired resistance to third generation EGFR-TKIs.

In the setting of osimertinib-resistant EGFRm NSCLC cells, the inability of osimertinib to modulate Bim and Mcl-1 levels by altering ERK1/2-dependent Bim and Mcl-1 degradation is a key mechanism for the acquisition of osimertinib resistance. This leads logically to the speculation that enforced elevation of Bim levels and/or decrease in Mcl-1 levels via abrogation of ERK1/2-dependent Bim and Mcl-1 phosphorylation with a MEK or ERK inhibitor could restore the sensitivity of osimertinib-resistant EGFRm NSCLC cells to undergo apoptosis, when combined with osimertinib, achieving the goal of overcoming osimertinib acquired resistance. With different MEK inhibitors including trametinib, selumetinib, and mirdametinib (PD0325901), our previous study generated identical results, i.e., osimertinib combined with either MEK inhibitor synergistically decreased the survival and induced apoptosis of osimertinib resistant cell lines irrespective of the underlying resistance mechanisms. This combinatorial strategy also generated promising *in vivo* activity against osimertinib-resistant tumors with both concurrent and intermittent schedules [29]. In agreement, osimertinib also synergized with an ERK inhibitor such as GDC0994 (ravoxertinib) or VRT752271 (ulixertinib or BVD-523) in decreasing the survival and enhancing apoptosis of osimertinib-resistant cell lines with impressive *in vivo* effects on inhibiting the growth of osimertinib-resistant tumors [53]. Activation of MEK5/ERK5, another mitogen-activated protein kinase (MAPK) signaling pathway, also contributes to cell survival, proliferation, epithelial-to-mesenchymal transition (EMT), and radioresistance in lung cancer [54–57]. Our recent study has shown that inhibition of this signaling with either a MEK5 or ERK5 inhibitor in combination with osimertinib synergistically decreased cell survival and enhanced induction of apoptosis in several osimertinib-resistant NSCLC cell lines primarily via upregulation of Bim. Moreover, this combination was also very effective in suppressing the

growth of osimertinib-resistant tumors with increased Bim and apoptosis *in vivo* [58]. These findings thus provide solid preclinical support for overcoming acquired osimertinib resistance by co-targeting MERK5/ERK5 signaling.

Several other studies have shown different strategies for overcoming acquired resistance to osimertinib and other third generation EGFR-TKIs through enhancing induction of apoptosis primarily via modulation of Bcl-1 family members, particularly Bim, despite of aiming to different target. EGFRm NSCLC cell lines with the BIM deletion polymorphism exhibited apoptosis resistance to osimertinib; this resistance could be overcome by combined use with the histone deacetylase (HDAC) inhibitor vorinostat (also known as suberoylanilide hydroxamic acid (SAHA)). It was shown that vorinostat affected the alternative splicing of BIM mRNA in the deletion allele, increased the expression of active Bim protein, and thereby induced apoptosis in osimertinib-treated resistant cells [47]. Our own study with another HDAC inhibitor, LBH589 (panobinostat), has also shown that the combination with osimertinib synergistically decreased the survival of different osimertinib-resistant cell lines, including those harboring C797S mutations and enhanced Bim-dependent induction of apoptosis in osimertinib-resistant cells with enhanced growth suppression of osimertinib-resistant xenograft tumors in nude mice [59].

Bufalin is a natural product and has been shown to restore the sensitivity of osimertinib-resistant cells to osimertinib-induced growth regression and apoptosis *in vitro* and *in vivo*, when combined with osimertinib, by downregulation of Mcl-1 through inhibition of Ku70-mediated Mcl-1 stabilization [60]. Similarly, another natural product, honokiol, when combined with osimertinib synergistically decreased the survival of several osimertinib-resistant cell lines with enhanced induction of apoptosis through enhancing Mcl-1 reduction by facilitating its degradation. Importantly, this combination showed greater growth suppression of osimertinib-resistant xenograft tumors including those with 19del, T790M, and C797S triple mutations in nude mice [61]. In our recent study, the natural product berberine functions as a naturally-existing MET inhibitor and, when combined with osimertinib, synergistically and selectively decreased the survival of several MET-amplified osimertinib-resistant EGFRm NSCLC cell lines with enhanced induction of apoptosis likely through Bim elevation and Mcl-1 reduction. This combination effectively enhanced the growth suppression of MET-amplified osimertinib-resistant xenografts in nude mice and was well tolerated [62].

Acetylsalicylic acid (aspirin) is one of the most commonly used non-steroidal anti-inflammatory drugs and also widely used as an antiplatelet agent to prevent myocardial infarction and stroke. It has been shown that aspirin synergistically enhances the antitumor activity of osimertinib in osimertinib-resistant NSCLC cells and tumors through promoting Bim-dependent apoptosis [63]. Our recent studies have shown that both sterol regulatory element binding protein 1 (SREBP1) activation and c-Myc elevation are associated with acquired resistance to third generation EGFR-TKIs [64,65]. Targeting either SREBP1 or c-Myc with both genetic knockdown and a pharmacological inhibitor sensitized osimertinib-resistant cells and tumors to osimertinib primarily through enhancing Bim-dependent induction of apoptosis [64,65]. Similarly, inhibition of aurora B kinase combined with osimertinib was recently shown to have promising activity in overcoming acquired resistance to osimertinib by enhancing Bim- and PUMA-mediated apoptosis. This is because aurora B inhibition stabilizes Bim protein through reduced Ser87 phosphorylation, and transactivates PUMA expression through removal of Akt-mediated suppression of FOXO1/3 (Fig. 2) [66]. The Src inhibitor, dasatinib, when combined with osimertinib or ASP8273, another third generation EGFR-TKI, showed activity in overcoming EGFR-TKI resistance in T790M-positive NSCLC cells. This combination enhanced induction of apoptosis in T790M-positive resistant cells, involving downregulation of Bcl-X_L [67].

Interestingly, some combination treatments do not always elevate Bim levels accompanied with Mcl-1 reduction. In fact, we found that targeting MEK5/ERK5, c-Myc or SREBP1, when combined with osimertinib, increased Mcl-1 levels while substantially enhancing the levels of Bim [58,64,65]. Given that the combinations still augmented Bim-dependent apoptosis, presenting promising activities in overcoming acquired resistance to osimertinib, it is very likely that Bim elevation plays a dominant role in triggering apoptosis, which can override the potential inhibitory effect caused by Mcl-1 elevation.

Since activation of the intrinsic apoptotic pathway is a key mechanism for the therapeutic efficacy of osimertinib, and acquisition of apoptosis resistance is a critical mechanism of acquired resistance to osimertinib [29], it can be logically speculated that activation of the intrinsic apoptotic pathway through directly inhibiting Mcl-1, activating Bax, or both should sensitize osimertinib-resistant cells to osimertinib, achieving the goal of overcoming acquired resistance to osimertinib and even other third generation EGFR-TKIs. Indeed, our recent study has demonstrated that osimertinib, when combined with Mcl-1 inhibition or Bax activation, synergistically decreased the survival of different osimertinib-resistant cell lines, enhanced the induction of intrinsic apoptosis, and inhibited the growth of osimertinib-resistant tumor *in vivo*. Remarkably, the triple-combination of osimertinib with Mcl-1 inhibition and Bax activation exhibited the

most potent activity in decreasing the survival and inducing apoptosis of osimertinib-resistant cells and in suppressing the growth of osimertinib-resistant tumors. These effects were associated with increased activation of the intrinsic apoptotic pathway evidenced by augmented mitochondrial cytochrome C and Smac/DIABLO release [68]. Our study thus convincingly demonstrates a novel strategy for overcoming acquired resistance to osimertinib and other third generation EGFR-TKIs by directly targeting activation of the intrinsic apoptotic pathway through Mcl-1 inhibition, Bax activation or both, warranting further clinical validation of this strategy.

Another study by Lu *et al.* [69] reported upregulated expression of Bcl-2 and Bcl-X_L in osimertinib resistant HCC827/OR cells. Suppression of Bcl-2 and Bcl- X_L through gene knockdown or using the small molecule inhibitor, ABT-263, re-sensitized HCC827/OR cells to osimertinib treatment with enhanced induction of apoptosis through the mitochondrial apoptotic pathway. Moreover, the combination effectively inhibited the growth of HCC827/OR tumors. Similarly, significant upregulation of Bcl-2 was also observed in AZD9291-resistant H1975 (H1975/AR) cells. The combination of osimertinib with the Bcl-2 inhibitor, ABT263 or ABT199, synergistically decreased the survival of H1975AR with enhanced induction of apoptosis [70].

Targeting apoptosis to delay or prevent the emergence of acquired resistance

The greatest clinical challenge in targeted cancer therapeutics, including EGFR-targeted therapy, is their inability to eradicate all tumor cells to achieve clinical cure. During sustained treatment, the surviving residual cancer cells can serve as sources for the development of acquired resistance to the given targeted therapy. It is generally thought that resistance may arise from selection and expansion of pre-existing resistant clones and/or from so-called dormant drug-tolerant persister cells or drug-tolerant cells (DTCs). These DTCs can often survive the initial phase of treatment, possess reversible feature of drug insensitivity upon drug removal, are typically slow cycling or dormant, and do not carry classical drug resistance driver gene alterations (see reviews [71,72]). Upon sustained treatment, e.g., with an EGFR-TKI, dormant DTCs can eventually acquire resistance through gaining additional genetic mutational or non-mutational mechanisms [71–75]. Hence, any approaches or strategies that can result in maximal removal or elimination of pre-existing resistant clones and/or DTCs should have high potential to substantially improve the outcome of the initial treatment with third-generation EGFR-TKIs via delaying or even preventing the emergence of acquired resistance. To do so, the most effective way is to induce death, particularly apoptosis, of these pre-existing resistant clones and/or dormant DTCs during the initial phase of the treatment.

We found that the combination of osimertinib with the MEK inhibitor trametinib, or the ERK inhibitor ulixertinib (VRT-752271 or BVD-523), synergistically decreased the survival of several pre-existing clones that are intrinsically resistant to osimertinib and were derived from sensitive PC-9 cells via effectively enhancing induction of apoptosis, demonstrating that these combinations have the potential to eradicate pre-existing osimertinib-resistant clones. The combinations were also more effective than either agent alone in decreasing the survival and inducing apoptosis of sensitive EGFRm NSCLC cell lines, suggesting the capacity to eliminate DTCs as well. Indeed, both concurrent and intermittent applications of trametinib and osimertinib combination remarkably delayed the development of osimertinib acquired resistance both *in vitro* and *in vivo* [76]. Intriguingly, some mice were even tumor-free in combination treatment groups with a collective cure rate of 27.8% (5 of 18 mice in total) [76], suggesting that this intervention strategy may achieve long-term remission, which is a clinically meaningful benefit.

Despite these promising results, it has been recently shown that acquired resistance still occurs to this therapeutic strategy of co-targeting MEK/ERK signaling [75]. This is largely because EGFR/MEK co-inhibition can result in the activation of YAP/TEAD survival signaling causing the development of acquired resistance given that single-agent osimertinib treatment can lead to reactivation of both ERK1/2 and YAP/TEAD signaling [75]. The activated YAP/TEAD facilitates EGFRm NSCLC cells to enter a senescence-like dormant state or drug-tolerant state via suppression of apoptosis by regressing the expression of pro-apoptotic *BMF* gene via the EMT transcription factor SLUG (Fig. 1) [75]. Accordingly, co-targeting YAP/TEAD, with either genetic or pharmacological approaches, eliminated dormant DTCs by enhancing EGFR/MEK inhibition-induced apoptosis via Bmf upregulation [75]. Therefore, co-targeting EGFR, MEK/ERK, and YAP/TEAD may be an attractive strategy to enhance the initial treatment efficacy of osimertinib as well as other third generation EGFR-TKIs in EGFRm NSCLC by causing synthetic lethality of pre-existing resistant clones and/or DTCs through enhanced induction of apoptosis. Consequently, prolonged treatment responses or long-term remissions in cancer patients may be achieved. Of course, this strategy needs to be validated in the clinic.

Summary and perspectives

Cancer would be cured if we could eliminate all cancer cells and residual tumors from a patient's body. In

general, apoptosis is known to be a major form of cancer cell death during cancer therapy [12,77,78]. Given the predominant role of apoptosis induction in mediating the therapeutic efficacy of osimertinib as well as other third generation EGFR-TKIs and the acquisition of apoptosis resistance as a key mechanism accounting for the emergence of acquired resistance to third generation EGFR-TKIs, there is strong scientific rationale to manage the challenging issue of acquired resistance through targeting apoptosis. Only by maximally eradicating the source cells (pre-existing resistant clones and/or DTCs) that can become resistant cells during the initial phase of treatment, we will have high likelihood to substantially improve the outcome of initial therapy with third generation EGFR-TKIs via abrogating emergence of acquired resistance. There are some related preclinical studies with promising outcomes. Well-designed clinical trials are thus urgently needed to validate these clinical findings. Currently, there are some ongoing clinical trials that test the efficacies of osimertinib combined with direct apoptosis-inducers or with other agents that lead to enhanced induction of apoptosis either as an initial treatment strategy (overcoming primary resistance and/or delaying emergence of acquired resistance) or a treatment option for overcoming acquired resistance to osimertinib (Table 1).

Although apoptosis has a critical role, there are other types of cancer cell death such as ferroptosis, necroptosis, and pyroptosis. Whether induction of these cell death mechanisms plays a role in mediating therapeutic efficacy of third generation EGFR-TKIs and whether targeting different cell death processes beyond apoptosis can help with overcoming acquired resistance is largely unknown and needs to be studied in the future.

The potential for increased toxicity may be a concern while considering the enhanced therapeutic efficacy of different combinations, particularly for delaying or preventing the emergence of acquired resistance, which would require long-term drug administration. Fortunately, the third generation EGFR-TKIs are mutation-selective drugs with limited effect against WT EGFR. We found that several combinations such as osimertinib combined with a MEK or ERK inhibitor or with an Mcl-1 inhibitor and Bax activator had no enhanced effects on decreasing the survival of human NSCLC with WT EGFR [68,76], strongly suggesting that these combinations essentially enhance the efficacy of osimertinib. These findings may also imply that these combinatorial strategies may not affect the growth of cells or tissues (e.g., normal tissues) with WT EGFR. Indeed, these combinations were well tolerated in mice even after a sustained treatment of over 3 months [68,76], suggesting their safety. These data may provide valuable information to facilitate the clinical validation of these promising preclinical findings.

Acknowledgements

I am thankful to Dr. Anthea Hammond in my department for editing the manuscript. Some of work done in my laboratory were supported by the NIH/NCI R01 CA223220, R01 CA245386, UG1

Table 1 Ongoing clinical trials for osimertinib combined with an apoptosis-inducing agent and an agent that leads to enhanced induction of apoptosis

Clinical trial title	Trial registration ID
Combinations with direct apoptosis-inducers	
A study of APG-1252 plus osimertinib (AZD9292) in EGFR TKI resistant NSCLC patients	NCT04001777
Osimertinib and navitoclax in treating patients with EGFR-positive previously treated advanced or metastatic NSCLC	NCT02520778
Combinations that lead to enhanced induction of apoptosis	
A phase 2 study of osimertinib in combination with selumetinib in EGFR-inhibitor naïve advanced EGFR mutant lung cancer	NCT03392246
Alisertib in combination with osimertinib in metastatic EGFR-mutant lung cancer	NCT04085315
Dasatinib and osimertinib (AZD9291) in advanced non-small cell lung cancer with EGFR mutations	NCT02954523
A study comparing savolitinib plus osimertinib vs. savolitinib plus placebo in patients with $EGFRm^+$ and MET amplified advanced NSCLC	NCT04606771
Osimertinib in combination with alisertib or sapanisertib for the treatment of osimertinib-resistant EGFR mutant stage IIIB or IV non-small cell lung cancer	NCT04479306
Combination of osimertinib and aspirin to treat osimertinib resistance NSCLC	NCT03532698
MRX-2843 and osimertinib for the treatment of advanced EGFR mutant non-small cell lung cancer	NCT04762199
Aurora kinase inhibitor LY3295668 in combination with osimertinib for the treatment of advanced or metastatic EGFR-mutant non-squamous non-small cell lung cancer	NCT05017025
A study of tepotinib plus osimertinib in osimertinib relapsed MET amplified NSCLC (INSIGHT 2)	NCT03940703
Clinical study on savolitinib + osimertinib in treatment of $EGFRm^+/MET^+$ locally advanced or metastatic NSCLC	NCT05009836

The information was obtained from clinicaltrials.gov website.

CA233259 awards and Emory University Winship Cancer Institute lung cancer pilot funds. Shi-Yong Sun is a Georgia Research Alliance Distinguished Cancer Scientist.

Compliance with ethics guidelines

Shi-Yong Sun declares no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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