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Original Article

The novel BET degrader, QCA570, is highly active against the growth of human NSCLC cells and synergizes with osimertinib in suppressing osimertinib-resistant EGFR-mutant NSCLC cells

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Abstract: Lung cancer remains the leading cause of cancer deaths worldwide despite advances in knowledge in cancer biology and options of various targeted therapies. Efforts in identifying innovative and effective therapies are still highly appreciated. Targeting bromodomain and extra terminal (BET) proteins that function as epigenetic readers and master transcription coactivators is now a potential cancer therapeutic strategy. The current study evaluates the therapeutic efficacies of the novel BET degrader, QCA570, in lung cancer and explores its underlying mechanisms. QCA570 at low nanomolar ranges effectively decreased the survival of a panel of human lung cancer cell lines with induction of apoptosis *in vitro*. As expected, it potently induced degradation of BET proteins including BRD4, BRD3 and BRD2. Moreover, it potently decreased Mcl-1 levels due to transcriptional suppression and protein degradation; this event is critical for mediating apoptosis induced by QCA570. Moreover, QCA570 synergized with osimertinib in suppressing the growth of osimertinib-resistant cells *in vitro* and *in vivo*, suggesting potential in overcoming acquired resistance to osimertinib. These preclinical findings support the potential of QCA570 in treatment of lung cancer either as a single agent or in combination with others.

Keywords: BET, degradation, QCA570, apoptosis, osimertinib, lung cancer

Introduction

Lung cancer, which consists of over 80% non-small cell lung cancer (NSCLC), has remained the leading cause of cancer death in the United States and many other countries. It causes about 20% of all cancer deaths worldwide with a five-year rate of survival of not more than 20% [1-3], despite knowledge gains in cancer biology that have led to successful development of targeted therapies including current immunotherapy. Hence, efforts toward development of novel and effective targeted therapies against lung cancer are highly appreciated.

Bromodomain (BRD) and extra-terminal (BET) proteins are primarily composed of BRD2, BRD3, BRD4, and the testis-specific bromodomain testis-specific protein (BRDT) [4] and function as epigenetic readers and master transcription coactivators. These BET proteins critically regulate the expression of many genes involved in several human diseases, including cancer, at the transcriptional level through binding to acetylated histones and subsequently activating RNA Pol II-driven transcriptional elongation [4, 5]. BET proteins, particularly BRD4, are overexpressed in multiple tumor types and mediate expression of key transcription factors important for cancer development and progres-

sion. BET inhibition displaces BET proteins from super-enhancer regions and blocks expression of certain key oncogenes, such as c-*Myc* [6, 7]. Therefore, targeting BETs has been recognized as a potential cancer therapeutic strategy. Accordingly, a number of selective and potent small-molecule BET inhibitors have been developed, albeit with mixed clinical activities, as cancer therapeutic agents [4, 8].

Using the cutting-edge PROteolysis TArgeting Chimeric (PROTAC) approach, BET degraders that often possess highly potent BET inhibitor activity through induction of BET degradation have been developed. These BET degraders can cause widespread downregulation of gene transcription in tumor cells, exert antitumor activity superior to that of their parent BET inhibitors in preclinical tumor models and are well tolerated in mice [9]. Our recent study has shown that the novel BET degrader, ZBC260, effectively decreases the survival of human NSCLC cells, particularly those with high levels of BET proteins, and inhibits the growth of human NSCLC xenografts and patient-derived xenografts *in vivo* with much better efficacy than the conventional BET inhibitor JQ-1 [10].

QCA570 is a novel pan BET degrader, which was reported to inhibit the growth of human acute leukemia cell lines even at low picomolar concentrations and achieve complete and durable tumor regression in leukemia xenograft models in mice at well-tolerated dose-schedules [11]. Thus, QCA570 is regarded as the most potent and efficacious BET degrader reported to date [11]. In the current study, we focused on evaluating its activity against human NSCLC cells *in vitro* and *in vivo* and understanding its underlying mechanisms.

Material and methods

Reagents

QCA570 was synthesized in Dr. Shaomeng Wang's lab (University of Michigan, Ann Arbor, Michigan) and described previously [11]. JQ-1 was purchased from MedChem Express (Monmouth Junction, NJ). Osimertinib (AZD9-291) was purchased from Active Biochemicals (Maplewood, NJ). Other agents and all antibodies used in this study were the same as described previously [10].

Cell lines and cell culture

Human NSCLC cell lines including Bim knockout (KO) cells lines were the same as we previously reported [10, 12]. Cell lines stably expressing Mcl-1 were established with lentiviruses carrying empty vector or Mcl-1 as described previously [13]. The osimertinib-resistant EGFR mutant (EGFRm) NSCLC cell lines PC-9/AR, PC-9/GR/AR, and HCC827/AR were described previously [15]. These cell lines were cultured in RPMI 1640 medium containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. These NSCLC cell lines, except for H157, were not genetically authenticated. Mycoplasma was detected annually or upon receiving using Nozan MycoAlert Mycoplasma Detection Kit (VWR; Suwanee, GA).

Cell survival and apoptosis assays

Cell numbers in 96-well cell culture plates were determined using sulforhodamine B (SRB) as described previously [14]. Combination index (CI) for drug interaction (e.g., synergy) was calculated using CompuSyn software (ComboSyn, Inc.; Paramus, NJ). Apoptosis was detected with an annexin V/7-AAD apoptosis detection kit (BD Biosciences; San Jose, CA) according to manufacturer's manual. Protein cleavage as an additional indication of apoptosis was determined with Western blot analysis.

Colony formation assays

Cells seeded in 12-well cell culture plates for overnight were exposed to agents tested with three replicate wells per treatment. At the end of treatment, cell colonies were stained with crystal violet as described previously [15, 16].

Western blot analysis

The procedures for preparation of protein lysates and Western blot analysis were described previously [17].

Protein stability assay

Protein stability was determined with cycloheximide (CHX) chase assay as described previously [15, 18].

Cytochrome C (Cyt C) and Smac release assay

The cytosolic proteins released from mitochondria in the cells were prepared with digitonin-

based hypotonic lysis buffer as described in a previous study [19] and detected with Western blotting.

RNA extraction, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and RNA sequencing (RNA-seq)

RNA preparation and RT-qPCR were conducted as we previously described [10]. RNA-seq analysis was performed by MedGenome Inc. (Foster City, CA) as described previously [20]. The expression values for each gene were presented in FPKM (fragments per kilobase per million) units.

Animal xenograft and treatments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and conducted as described previously [10, 15]. In brief, HCC827/AR cells were suspended in sterile PBS at 2×10^6 per mouse and injected (100 µl/mouse) into the flank of 4 week-old female nude mice purchased from Charles River Laboratory [(NU(NCr)-*Foxn1*^{nu}; Charleston, SC]. After 2 weeks, when the average tumor sizes were around 100 mm^2, the mice were divided into 4 groups (6 mice/group) based on equal average tumor volumes and body weights and treatments were started. The treatments included vehicle control, QCA570 alone (0.5 mg/kg; twice/week, ip; 100 µl/mouse), osimertinib alone (5 mg/kg; daily, oral gavage; 100 µl/mouse) and QCA570 and osimertinib combination. Tumor volumes were measured using caliper measurements and calculated with the formula V = (length×*width*^{2})/2. At the end of treatment, mice were euthanized with CO_2 asphyxia for collection of tumors, which were frozen in liquid nitrogen for further analyses.

Statistical analysis

The statistical significance of differences between two groups was analyzed with two-sided unpaired Student's *t* tests when the variances were equal using GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA). Data were examined as suggested by the same software to verify that the assumptions for use of the *t* tests held. Results were considered to be statistically significant at $P<0.05$.

Results

QCA570 potently decreases the survival of human NSCLC cells with induction of apoptosis

We first determined the activity of QCA570 on the growth of human NSCLC cells. As shown in **Figure 1A**, QCA570 very effectively decreased the survival of the 10 human NSCLC cell lines tested, with IC_{50}s ranging from 0.3 nM to 100 nM. Among these cell lines, H1975, H157 and Calu-1 were the most sensitive while H1299 and EKVX were relatively the least sensitive to QCA570. At 5 or 10 nM, QCA570 increased annexin V-positive cell population (**Figure 1B**) and cleavage of PARP, caspase-8 and caspase-3 evidenced by decrease in the proforms of these proteins and/or appearance of cleaved forms (**Figure 1C**), indicating induction of apoptosis in NSCLC cells. In our previous study, we detected baseline levels of BRD4, BRD3 and BRD2 in these 10 cell lines [10]. By correlating the baseline levels of each BET protein with QCA570 at 1 nM on cell number reduction (CNR), we found that increased CNR was significantly ($P<0.01$) correlated with higher baseline levels of each BET protein (r = -0.779, -818 and -869 for BRD4, BRD3 and BRD2, respectively; **Figure 1D**), suggesting that high levels of these BET proteins confer high sensitivity to QCA570.

QCA570 effectively decreases the levels of BET proteins accompanied with reduction of Mcl-1 in human NSCLC cells

Given that QCA570 was designed for degrading BET proteins, we next determined the effects of QCA570 on BET protein levels and on BET-regulated proteins, particularly those associated with regulation of apoptosis. In the 3 tested cell lines including H157, H1975 and Calu-1, QCA570 effectively decreased the levels of BRD4, BRD3 and BRD2 (**Figure 2A**). The reduction of these BET proteins occurred quickly likely before 3 h post treatment (**Figure 2B**) and could be achieved by QCA570 even at 1 nM (**Figure 2C**). Hence, QCA570 indeed potently and rapidly decreased the levels of BET proteins in NSCLC cells. c-Myc expression is known to be regulated by BET inhibition although with varied effects. QCA570 effectively decreased the levels of c-Myc in H157 and minimally H1975, but not in Calu-1 cells (**Figure 2A**). Time-course analysis showed that QCA570

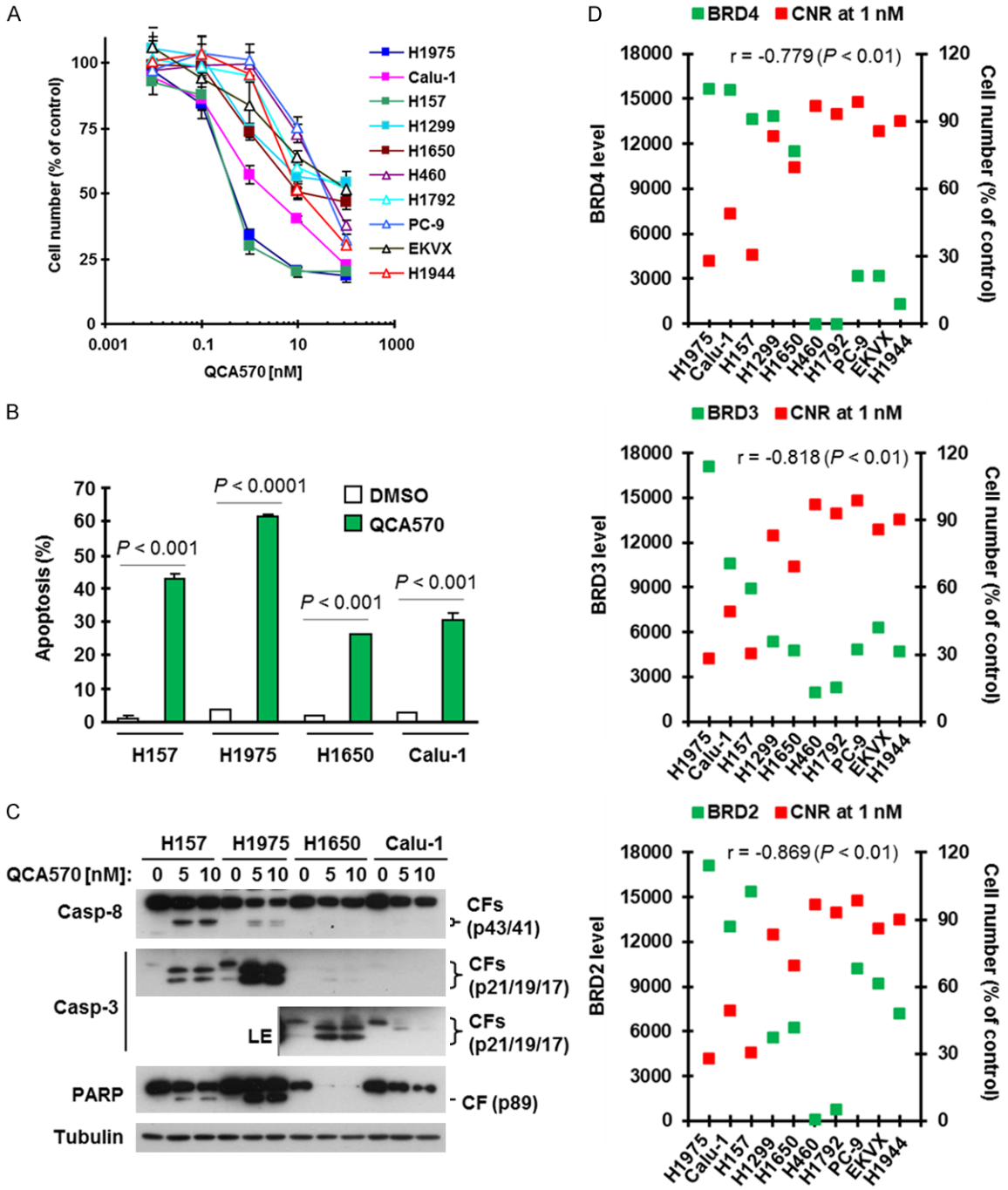

Figure 1. QCA570 decreases cell survival (A) and induces apoptosis (B and C) in human NSCLC cells and its potencies are positively correlated with high levels of BET proteins (D). (A) The indicated cell lines seeded in 96-well plates were treated with different concentrations of QCA570 for 3 days. Cell numbers were estimated with the SRB assay. The data are means ± SDs of four replicate determinations. (B and C) The given cell lines were exposed to DMSO and 5 nM (H157 and H1975) or 10 nM (H1650 and Calu-1) for 24 h (B) or different concentrations of QCA570 as indicated for 22 h (C). Apoptosis was determined by measuring annexin V-positive cells (B) with flow cytometry or detecting protein cleavage with Western blotting (C). CF, cleaved form; LE, longer exposure. (D) Basal levels of BRD4, BRD3 and BRD2 generated in our previous study [10] were correlated with the decrease in cell numbers caused by 1 nM QCA570. CNR, cell number reduction.

only transiently decreased c-Myc levels (e.g., before 6 h) in H1975 cells (**Figure 2D**), while reducing c-Myc levels quickly and sustainably in H157 cells (**Figure 2B**).

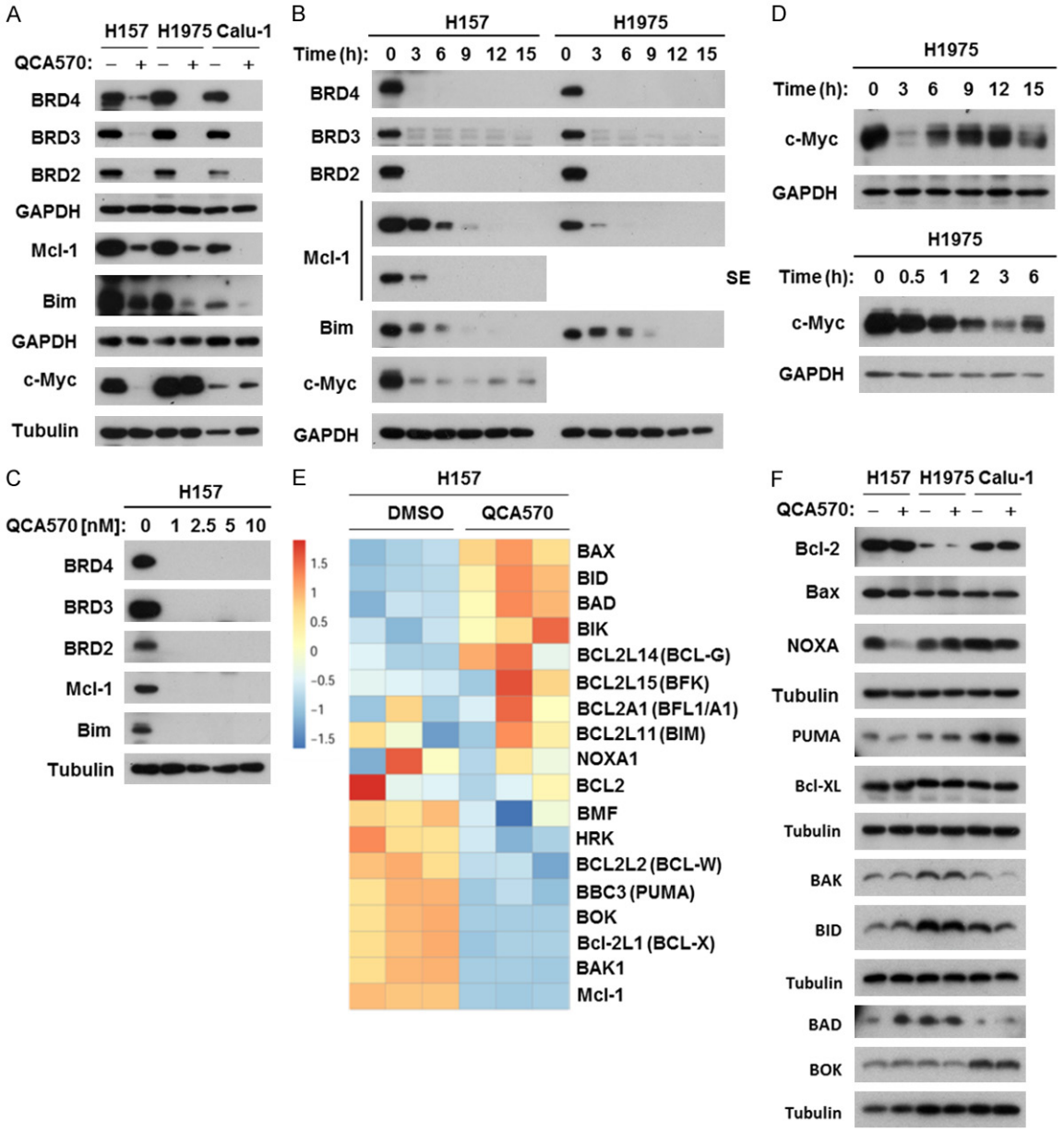

Figure 2. QCA570 primarily modulates the levels of BRDs, Mcl-1, Bim and c-Myc in human NSCLC cells. (A-D and F) The indicated cell lines were treated with DMSO or 10 nM QCA570 for 7 h (A and F), 10 nM QCA570 for different time as indicted (B and D) or varied concentrations of QCA570 for 7 h (C). The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blotting to detect the given proteins. SE, shorter exposure; LE, longer exposure. (E) H157 cells were treated with 5 nM QCA for 12 h and then harvested for preparation of total cellular RNA and subsequent RNA-seq analysis.

Considering that QCA570 induces apoptosis in NSCLC cells, we also examined its effects on modulation of the levels of Mcl-1 and Bim, two important apoptosis-regulating proteins regulated by another BET degrader across different NSCLC cell lines as reported in our previous study [10]. QCA570 indeed decreased the levels of both Mcl-1 and Bim across the three tested NSCLC cell lines (**Figure 2A**) in a fashion similar to what it did to BET protein in terms of potency, rapidness and durability (**Figure 2B** and **2C**).

By analyzing RNA-seq data generated from H157 cells treated with QCA570, we found that a few of the other Bcl-2 family genes were mod-

ulated: the expression of Bax, BID, BAD and BIK was elevated, whereas the expression of PUMA, Bcl-X_L, BOK and BAK was suppressed. Here we found that Mcl-1 mRNA expression was downregulated while Bim mRNA levels were not significantly altered (**Figure 2E**). However, we failed to validate the clear alterations of Bax, BAD, Bcl-X_L, BAK and BOK at protein levels in either of three tested NSCLC cell lines (**Figure 2F**). Slight increase of BID, apparent elevation of BAD and slight reduction of PUMA were detected in H157 cells, but not in H1975 and Calu-1 cells (**Figure 2F**). Bcl-2 expression was neither altered at mRNA (**Figure 2E**) nor at proteins level (**Figure 2F**) in H157 cells. NOXA was not altered at mRNA level (**Figure 2E**), but clearly decreased at protein level in H157 cells (**Figure 2F**). However, both proteins were not altered in H1975 and Calu-1 cells (**Figure 2F**). Hence, it appears that these Bcl-2 proteins play a less critical role in mediating QCA570-induced apoptosis.

QCA570 induces proteasomal degradation of BET proteins, Mcl-1, Bim and c-Myc in human NSCLC cells

We then determined whether QCA570 indeed induces degradation of BET proteins in human NSCLC cells. As presented in **Figure 3A**, the presence of MG132, a widely used proteasome inhibitor, rescued the reduction of not only BRD proteins, but also Mcl-1 and Bim, indicating that QCA570 does induce proteasomal degradation of BET proteins. In our RNA-seq analysis, QCA570 clearly decreased Mcl-1, but not Bim mRNA levels (less than 2 fold change; **Figure 3B**). The downregulation of Mcl-1 mRNA expression by QCA570 was further confirmed by RT-qPCR in both H157 and H1975 cells (Figure S1). To determine whether QCA570 exerts any effects on the degradation of Mcl-1 and Bim proteins since MG132 prevented these proteins from degradation in cells exposed to QCA570 as we just demonstrated, we conducted a CHX assay to compare the degradation rates of these proteins between DMSO- and QCA570-treated cells and found that the degradation rates of these proteins were shorter in QCA570-treated cells than those in DMSO-treated cells (**Figure 3C**). Therefore, the data suggest that QCA570 facilitates the degradation of these proteins as well.

c-Myc is also an unstable protein undergoing proteasomal degradation [21]. Our RNA-seq data in H1570 cells did not show c-Myc mRNA reduction (**Figure 3B**). Thus, we further conducted an experiment by checking the effects of MG132 on c-Myc reduction induced by H1975 and H157 cells and found that the presence of MG132 clearly rescued c-Myc reduction induced by QCA570 (**Figure 3D**). The CHX chase assay also showed that c-Myc was degraded faster in QCA570-treated cells than in DMSO-treated control cells (**Figure 3E**). These data together suggest that QCA570 facilitates c-Myc degradation.

Mcl-1 reduction contributes to QCA570-induced apoptosis in human NSCLC cells, which is not affected by Bim reduction

To determine whether Mcl-1 reduction contributes to QCA570-induced apoptosis, we compared the effects of QCA570 on induction of apoptosis between cells carrying a control vector and isogenic cells expressing ectopic Mcl-1. We found that QCA570 effectively induced cleavage of PARP and caspase-3 and increased annexin V-positive cells in both H157 and H1975 control cells, but minimally did so in these cell lines expressing ectopic Mcl-1 (**Figure 4A** and **4B**). Hence, enforced ectopic expression of Mcl-1 protected cells from undergoing QCA570-induced apoptosis. Interestingly, QCA570 effectively induced apoptosis in both H157 and H1975 deficient with Bim as it did in their corresponding parental cells (**Figure 4C** and **4D**), suggesting a Bim-independent apoptosis-inducing activity. In contrast, the BET inhibitor JQ-1 increased Bim levels in H157 and H1975 cells (**Figure 4E**) and had significantly reduced effects on induction of apoptosis in both H157/Bim-KO and H1975/Bim-KO cells (**Figure 4F**), indicating Bim-dependent induction of apoptosis.

QCA570 synergizes with osimertinib in decreasing the survival and inducing apoptosis of human EGFR-mutant (EGFRm) NSCLC cells with acquired resistance to osimertinib

Losing modulation of Mcl-1 is associated with emergence of acquired resistance to osimertinib [15]. Since QCA570 potently decreases Mcl-1 levels as demonstrated above, we checked whether QCA570 sensitizes osimertinib-resistant cells to osimertinib. We tested the combinational effects of QCA570 plus osimertinib in 3 different osimertinib-resistant cell lines and found that the QCA570 and

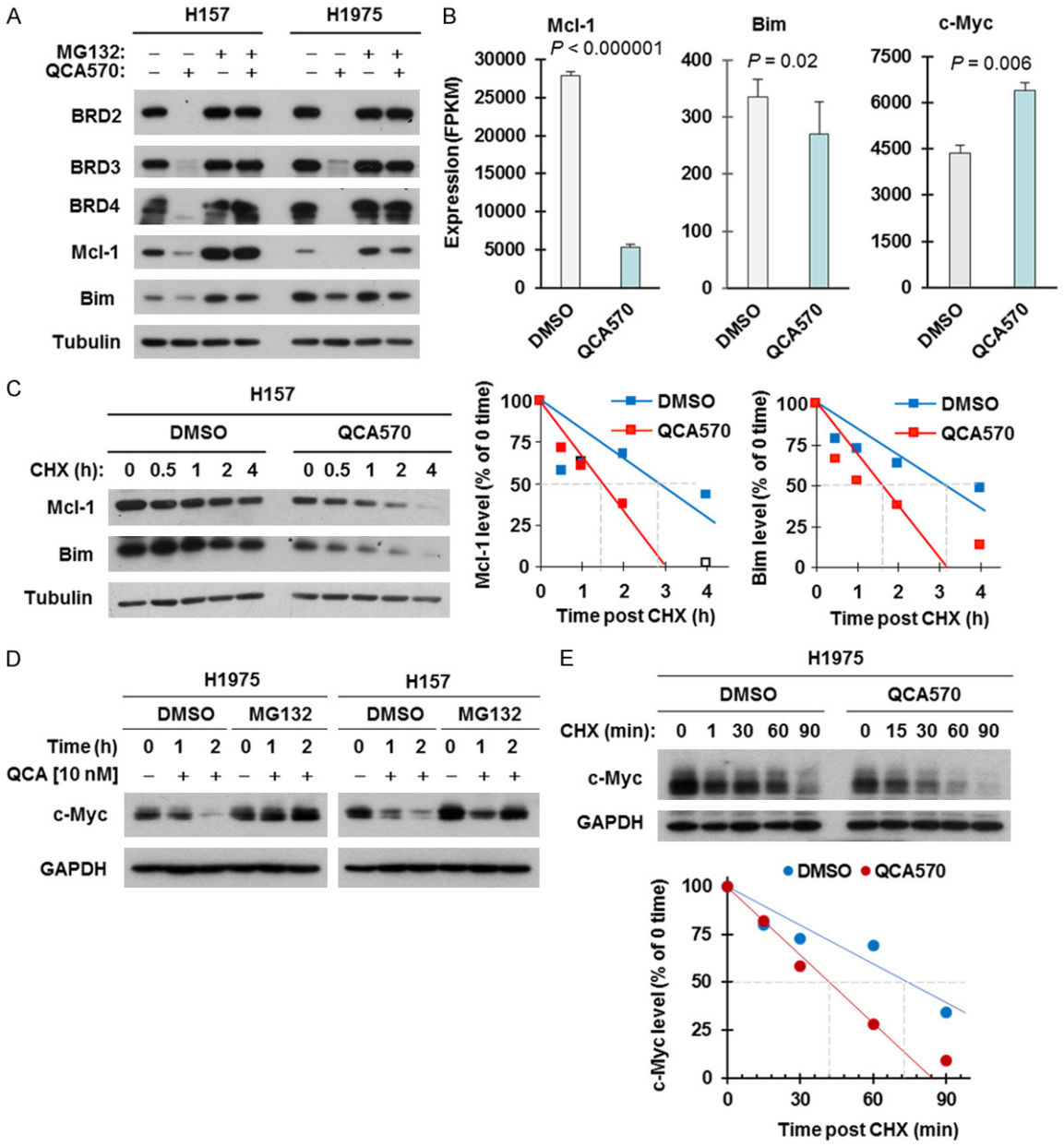

Figure 3. QCA570 induces proteasomal degradation of BET proteins, Mcl-1, Bim and c-Myc (A and C-E) with varied effects on modulation of Mcl-1, Bim and c-Myc transcription (B). (A and C-E) Both H157 and H1975 cells were pre-treated with 5 μM MG132 for 30 min before 10 nM QCA570 was added. After an additional 3 h (A) or the indicated times (D), the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Moreover, H157 (C) or H1975 (E) cells were treated with DMSO or 10 nM QCA570 for 4 h (C) or 1 h (E) and then exposed to 10 μg/ml CHX. Cells were then harvested at different times post CHX as indicated. The indicated protein alterations were detected with Western blotting. Protein densities were estimated with NIH Image J software and plotted as % of 0 time. (B) RNA-seq data from H157 cells exposed to 5 nM QCA570 for 12 h.

osimertinib combination was more effective than either agent alone in decreasing the survival of these cell lines (**Figure 5A**). The CIs were <1, indicating synergistic effects. Moreover, the combination was also significantly more active than either agent alone in increasing annexin V-positive cells (**Figure 5B**) and cleavage of PARP and caspase-3 (**Figure 5C**). Hence, the combination of QCA570 and osimertinib enhances induction of apoptosis in these osimertinib-resistant cells. In the colony formation assay that allows multiple cycles of the treatments, the combination of QCA570 and osimertinib was also more potent than

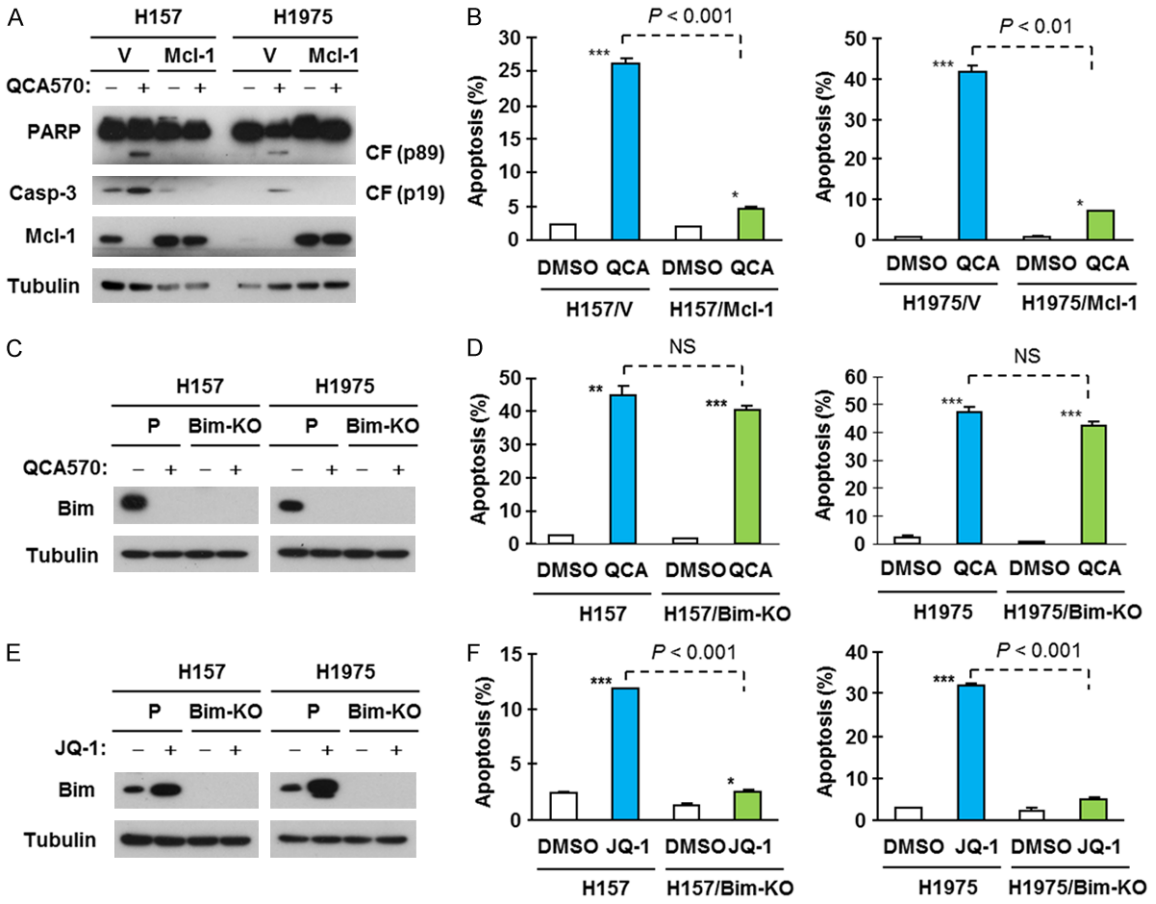

Figure 4. Enforced expression of ectopic Mcl-1 (A and B) compromises cell response to induction of apoptosis by QCA570, whereas Bim knockout does not affect QCA570-induced apoptosis (C and D) although it abrogates induction of apoptosis by JQ-1 (E and F). The indicated cell lines with ectopic Mcl-1 expression (A) or Bim knockout (C) as confirmed with Western blotting were exposed to DMSO or 10 nM QCA570 for 24 (A and C) or 48 h (B and D). Bim-KO cells were also treated with DMSO and 10 μM JQ-1, respectively, for 24 (E) or 48 h (F). Apoptosis was evaluated with annexin V/flow cytometry (48 h; B, D and F) and Western blotting (24 h; A). The data are means ± SDs of duplicate determinations. CF, cleaved form. $^{*}P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$ compared with DMSO control. NS, not significant.

either agent alone in inhibiting the formation and growth of PC-9/AR and HCC827/AR colonies (**Figure 5D**), further demonstrating the enhanced therapeutic efficacy of QCA570 and osimertinib combination.

QCA570 combined with osimertinib effectively inhibits the growth of human EGFRm NSCLC xenografts with acquired resistance to osimertinib in vivo

Given the promising *in vitro* effects of the QCA570 and osimertinib combination against the growth of osimertinib-resistant EGFRm NSCLC cell lines, we further examined the effect of this combination on the growth of osimertinib-resistant EGFRm NSCLC xenografts in nude mice. While each single agent alone exerted limited effects on suppressing the growth of HCC827/AR xenografts, the combination of QCA570 and osimertinib was significantly more active than either single agent in inhibiting the tumor growth (**Figure 5E**) with limited effect on enhancing reduction of body weight (**Figure 5F**), indicating that the combination is well tolerated and effective in inhibiting the growth of EGFRm NSCLC tumors with acquired resistance to osimertinib *in vivo*.

Enhanced Mcl-1 reduction critically contributes to augmented induction of apoptosis by the combination of QCA570 and osimertinib in osimertinib-resistant NSCLC cells

To get insight into the mechanisms by which QCA570 and osimertinib combination enhanc-

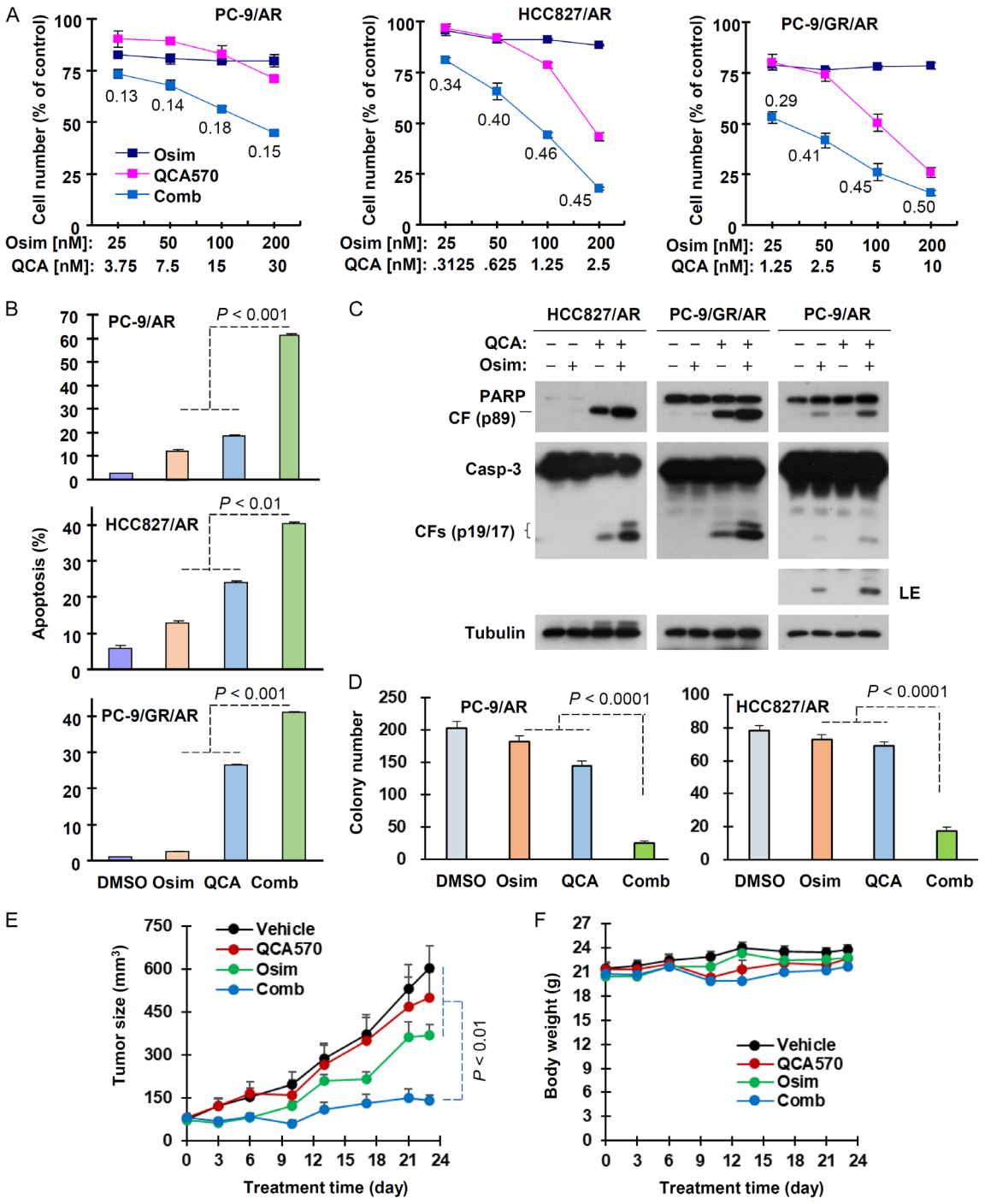

Figure 5. QCA570 combined with osimertinib synergistically decreases the survival of osimertinib-resistant cell lines (A) with augmented induction of apoptosis (B and C), enhances suppression of colony formation and growth (D) and augments suppression of the growth of osimertinib-resistant tumors in vivo (E and F). (A) The given cell lines were exposed to varied concentrations of agents alone or their combinations for 3 days. Cell numbers were estimated with the SRB assay. Data are means ± SDs of four replicate determinations. Numbers inside the graphs are CIs. (B) The given cell lines were exposed to DMSO, 200 nM osimertinib, 30 nM (PC-9/AR), 2 nM (PC-9/GR/AR) or 1 nM (HCC827/AR) QCA570 or QCA570 and osimertinib combination for 36 h (PC-9/GR/AR), 48 h (HCC827/AR) or 72 h (PC-9/AR). Apoptosis was determined with annexin V/flow cytometry. The data are means ± SDs of duplicate determinations. (C) The given cell lines were treated as described in *B* for 36 h. Proteins were detected with Western blotting. CF, cleaved form. (D) Both PC-9/AR and HCC827/AR cells seeded in 6-well plates were exposed to DMSO, 100 nM osimertinib, 15 nM (PC-9/AR) or 0.5 nM (HCC827/AR) QCA570, or osimertnib plus QCA570. The same treatments were applied every 3 days. After 12 days, the cell colonies were stained and photographed. The data are means ± SDs of triplicate determinations. (E and F) HCC827 xenografts were treated as described in "Materials and Methods". The data are means ± SEs of 6 tumors from 6 mice in each group.

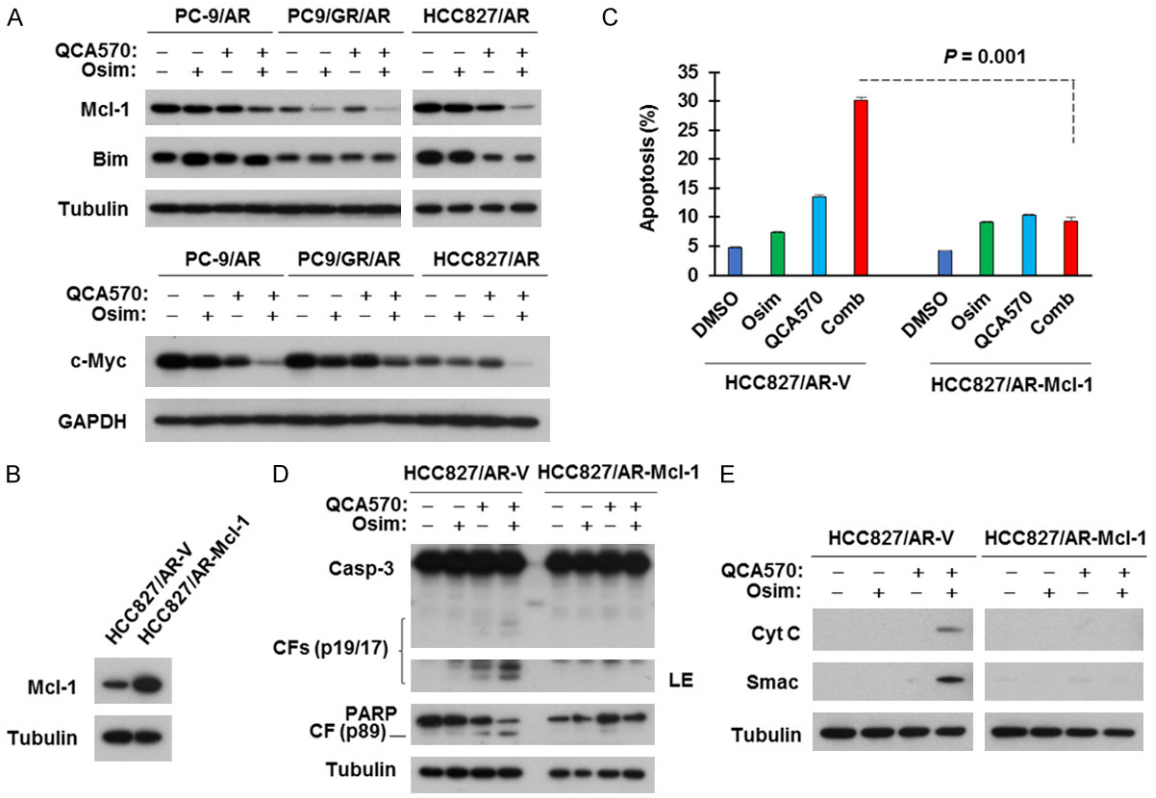

Figure 6. The combination of QCA570 and osimertinib enhances downregulation of Mcl-1 and c-Myc in osimertinib-resistant cells (A) and is ineffective in enhancing apoptosis including enhancement of Cyt C and Smac release from mitochondria in those expressing ectopic Mcl-1 (B-E). (A) The indicated cell lines were exposed to DMSO, 200 nM osimertinib, 30 nM (PC-9/AR), 2 nM (PC-9/GR/AR) or 1 nM (HCC827/AR) QCA570 or QCA570 and osimertinib combination for 12 h. The different proteins were detected by Western blotting. (B) Confirmation of ectopic Mcl-1 expression by Western blotting. (C and D) The indicated cell lines were treated with DMSO, 200 nM osimertinib, 1 nM QCA570 or QCA570 plus osimertinib for 48 h. The cells were then harvested for annexin V/flow cytometry (C) and Western blotting to detect protein cleavage (D). LE, long exposure. (E) The indicated cell lines were treated with DMSO, 200 nM osimertinib, 1 nM QCA570 or QCA570 plus osimertinib for 16 h. Cell were then harvested for preparation of cytosolic fraction and subsequent Western blotting.

es induction of apoptosis in osimertinib-resistant cells, we checked the impact of the combination on modulation of Mcl-1 and Bim, two critical proteins in mediating osimertinib-induced apoptosis in EGFRm NSCLC cells as we previously demonstrated [15]. Under the tested conditions, the combination of QCA570 and osimertinib was more effective than either single agent in decreasing Mcl-1 levels in the 3 tested osimertinib-resistant cell lines. The combination did not enhance Bim elevation in either of the tested cell lines. As observed in other NSCLC cell lines describe above, QCA570 at the tested condition clearly decreased Bim levels in HCC827/AR cells (**Figure 6A**). c-Myc is another targeted protein downregulated by QCA570 as demonstrated above. We found that the combination was also more potent than either agent alone in decreasing c-Myc levels in these three osimertinib-resistant cell lines (**Figure 6A**).

To demonstrate the involvement of Mcl-1 reduction in mediating enhanced induction of apoptosis by QCA570 and osimertinib combination, we enforced the expression of ectopic Mcl-1 in HCC827/AR cells (**Figure 6B**) and examined its impact on induction of apoptosis by the combination. While the combination of QCA570 and osimertinib enhanced induction of apoptosis in HCC827/AR-V control cells as evidenced by increasing annexin-V cells and cleavage of caspase-3 and PARP, these effects were lost in HCC827/AR-Mcl-1 cells (**Figure 6C** and **6D**), indicating that enforced ectopic Mcl-1 expression abrogates enhanced induction of

apoptosis by the QCA570 and osimertinib combination. Moreover, we also detected enhanced release of Cyt C and Smac from mitochondria, a critical event in the mitochondria-mediated apoptosis regulated by Mcl-1 [22, 23], in HCC827/AR-V cells, but not in HCC827/AR-Mcl-1 cells (**Figure 6E**), indicating that the QCA570 and osimertinib combination indeed enhances mitochondria-mediated apoptosis.

QCA570 downregulates the expression of multiple genes involved in regulation of several critical cancer-related signaling pathways

To have a general picture of QCA570 on suppressing the expression of other cancer-related genes, we analyzed the RNA-seq data generated from H157 and H1975 cell lines exposed to QCA570. Among the 3045 genes significantly and commonly regulated by QCA570 in both H157 and H1975 cell lines, commonly downregulated genes are largely associated with several signaling pathways that are critical for supporting cancer cell survival and proliferation including TGFβ, HIPPO, FoxO, hedgehog, Wnt, MAPK and DNA repair signaling pathways (Figure S2).

Discussion

Our recent study has demonstrated that the novel BET degrader, ZBC260, effectively decreases the survival of human NSCLC cells, particularly those with high levels of BRD4 protein. However, ranges of its IC_{50}s are varied widely from <10 nM to around 700 nM [10]. The current study reveals that the other novel BET degrader, QCA570, exerts much more potent effects than ZBC260 in decreasing the survival of human NSCLC cells with IC_{50}s ranging from 0.3 nM to 100 nM. QCA570 mechanistically shares with ZBC260 in inducing BET protein degradation and decreasing the levels of Mcl-1 and Bim and thus functionally exerts similar activity in decreasing cell survival with induction of apoptosis. Consistently, high levels of BET proteins in cells are significantly correlated with high sensitivity of NSCLC cell lines to QCA570. Nonetheless, our previous and current findings have provided strong preclinical support for further evaluation of BET degraders against NSCLC, particularly those with increased levels of BET proteins, in the clinic.

We noted that QCA570 facilitated the degradation of Mcl-1 in addition to suppressing its transcription; these effects are in agreement with what ZBC260 did on modulation of Mcl-1 [10]. These findings further support the notion that BET degraders affect not only gene transcription, but also protein degradation. Moreover, QCA570 functions similarly as ZBC260 does to induce Mcl-1 reduction-mediated apoptosis irrespective of Bim reduction since enforced expression of ectopic Mcl-1 protected cells from undergoing QCA570-induced apoptosis, whereas Bim knockout did not compromise the ability of QCA570 to induce apoptosis. Hence, the current findings with QCA570 further support the dominant role of Mcl-1 suppression in mediating apoptosis induced by BET degraders.

c-Myc is a putative target gene that mediates the cancer therapeutic activity of JQ-1 and other BET inhibitors [5, 24, 25]. However, an increased number of studies have suggested that these inhibitors exert c-Myc-independent activity [5, 26, 27]. JQ-1 even increased c-Myc levels in some lung cancer cell lines [12, 26]. Similar to ZBC260, QCA570 also generated mixed effects on modulation of c-Myc levels: it clearly reduced c-Myc levels in some NSCLC cell lines (e.g., H157), but not in others (e.g., Calu-1). Interestingly, QCA570 exerted a rapid, but transient effect on decreasing c-Myc levels in H1975; this effect is due to enhancement of c-Myc proteasomal degradation because QCA570 facilitated c-Myc degradation rate and MG132 were able to rescue c-Myc reduction by QCA570 in this cell line. It was recently reported that BRD4 negatively regulates c-Myc proteins stability by enhancing c-Myc ubiquitination and degradation through phosphorylating c-Myc at Thr58 [28]. Induction of BRD4 protein, but not inhibition, resulted in increased levels of c-Myc protein [28]. QCA570 strongly induced BRD4 degradation, but enhanced c-Myc degradation in H157 and H1975 cells, suggesting a different mechanism. Hence the regulation of c-Myc stability by BET proteins needs further investigation.

The intriguing finding in this study is the synergistic effects between QCA570 and osimertinib against different EGFRm NSCLC cell lines with acquired resistance to osimertinib as demonstrated *in vitro* and *in vivo*, suggesting its poten-

tial in overcoming acquired resistance to targeted therapy of lung cancer such as EGFR-targeted therapy. Mechanistically, we believe that enhanced induction of apoptosis is a critical event accounting for the enhanced effects of the combination on suppressing the growth of osimertinib-resistant cells. In addition to c-Myc suppression, Mcl-1 reduction should play an essential role in mediating the enhanced induction of apoptosis by the combination of QCA570 and osimertinib in different osimertinib-resistant EGFRm NSCLC cell lines given that this combination enhanced Mcl-1 reduction in these resistant cell lines and lost activity on induction of apoptosis in the resistant cell lines harboring overexpressed ectopic Mcl-1.

Given that QCA570 primarily affects gene expression through inhibiting BET-mediated gene transcription, it is reasonable to believe that induction of BET degradation by QCA570 should affect a broad range of gene expression subjected to this regulatory mechanism. Indeed, our RNA-seq data showed that there are over 3000 genes whose expression was modulated in both H157 and H1975 cells. We noted that many commonly downregulated genes were involved in regulation of several cancer-related signaling pathways including TGFβ, HIPPO, FoxO, hedgehog, Wnt, MAPK and DNA repair signaling pathways. We assume that suppression of these signaling pathways should contribute to the potent effect of QCA570 against the growth of NSCLC cells, which warrants further investigation in the future.

TGFβ [29-32], Hippo [33-35], Hedgehog [36-39], Wnt [40-42] and MAPK [15, 43-46] signaling pathways are all associated with emergence of acquired resistance to different EGFR-TKIs. Targeting either of these pathways exerts effect in overcoming acquired resistance to EGFR-TKIs. Therefore, we speculated that suppression of these signaling pathways by QCA57 likely contributes to its effect on overcoming osimertinib-acquired resistance. Further studies in this direction are also warranted.

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Disclosure of conflict of interest

The University of Michigan has filed a patent on QCA570 and its analogues for which Shaomeng Wang is a co-inventor. The patent has been licensed by Roivant Sciences Inc/Proteovant Therapeutics Inc, for which Shaomeng Wang is a paid consultant. The University of Michigan has received a research contract from Proteovant Therapeutics Inc for which Shaomeng Wang is the principal investigator on the contract. Suresh Y. Ramalingam is on consulting/advisory board for AstraZeneca, BMS, Merck, Roche, Tesaro and Amgen. No potential conflicts of interest were disclosed for other authors.

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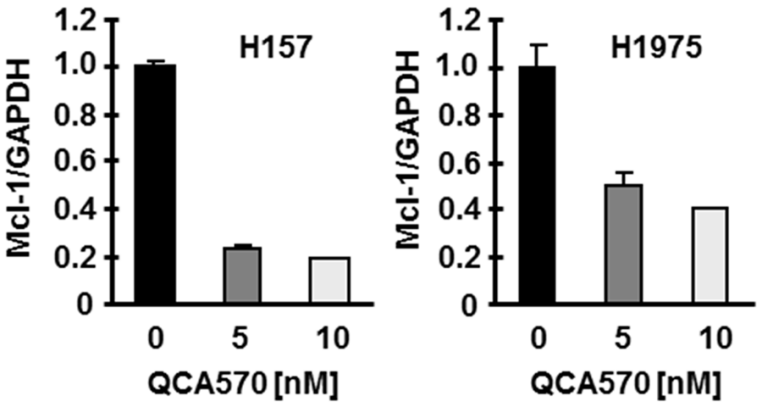

Figure S1. Validation of Mcl-1 downregulation by QCA570 at mRNA level in human NSCLC cells. The given cell lines were treated with the indicated concentrations of QCA570 for 4 h. RT-qPCR was used to detect mRNA of the tested genes. Data are means ± SDs of triplicate determinations.

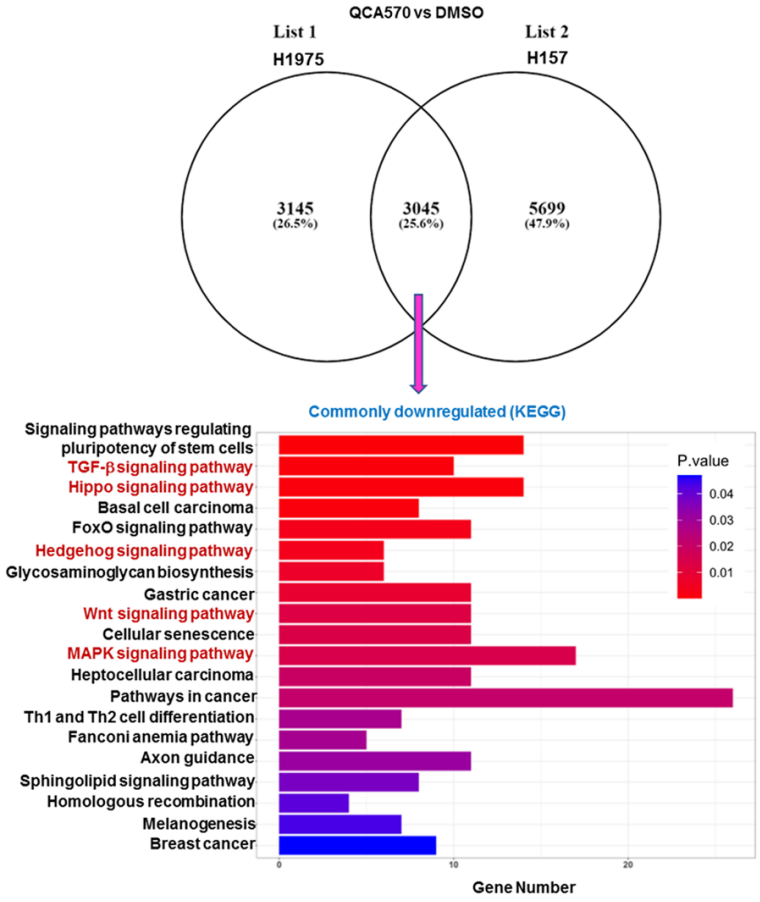

Figure S2. Some cancer-associated signaling pathways are commonly downregulated by QCA570 in human NSCLC cells. KEGG pathway analysis of RNA-seq data generated from H157 and H1975 cells treated with 5 nM for 12 h were conducted. Pathways in red are known to be connected to emergence of acquired resistance to different EGFR-TKIs.