Dual inhibition of BCL-XL and MCL-1 is required to induce tumour regression in lung squamous cell carcinomas sensitive to FGFR inhibition

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Conflict of Interest

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Abstract

Genetic alterations in the fibroblast growth factor receptors (FGFRs) have been described in multiple solid tumours including bladder cancer, head and neck and lung squamous cell carcinoma (SqCC). However, recent clinical trials showed limited efficacy of FGFR-targeted therapy in lung SqCC, suggesting combination therapy may be necessary to improve patient outcomes. Here we demonstrate that FGFR therapy primes SqCC for cell death by increasing the expression of the pro-apoptotic protein BIM. We therefore hypothesized that combining BH3-mimetics, potent inhibitors of pro-survival proteins, with FGFR-targeted therapy may enhance the killing of SqCC cells. Using patient-derived xenografts and specific inhibitors of BCL-2, BCL-XL and MCL-1, we identified a greater reliance of lung SqCC cells on BCL-XL and MCL-1 compared to BCL-2 for survival. However, neither BCL-XL nor MCL-1 inhibitors alone provided a survival benefit in combination FGFR therapy *in vivo*. Only triple BCL-XL, MCL-1 and FGFR inhibition resulted in tumour volume regression and prolonged survival *in vivo*, demonstrating the ability of BCL-XL and MCL-1 proteins to compensate for each other in lung SqCC. Our work therefore provides a rationale for the inhibition of MCL-1, BCL-XL and FGFR1 to maximize therapeutic response in *FGFR1*-expressing lung SqCC.

Introduction

Lung cancer is the leading cause of cancer death worldwide with a five-year survival rate of 16%[1]. Non-small cell lung cancer (NSCLC) accounts for 85% cases and can be further divided into adenocarcinoma (ADC) and squamous cell carcinoma (SqCC). Cancer sequencing efforts have led to the successful development of targeted therapies for molecularly selected lung ADC, but no such targeted therapies are currently approved for SqCC. Fibroblast growth factor receptor 1 (*FGFR1*) gene amplification is detected in 20% of lung SqCC[2]. This observation generated a great interest in evaluating the role of *FGFR1* as a driver oncogene and a possible therapeutic target. Disappointingly, results from a recent clinical study show moderate efficacy[3], indicating that better biomarkers may be necessary to select drug-sensitive patients, and that combination therapy may be required to improve patients' survival outcomes[4]. We and others have recently shown that high levels of *FGFR1* RNA expression better predict response to FGFR tyrosine kinase inhibitors compared to *FGFR1* gene amplification[1,3,5]. However, we reported cytostatic activity occurred after single agent FGFR inhibition and limited cytotoxic effects were observed. Hence, we sought to evaluate whether combination therapy with potent inducers of apoptosis such as BH3-mimetics may increase tumour cell death.

Apoptosis can occur through two main pathways, the cell extrinsic pathway and the cell intrinsic pathway, which can be targeted by small molecule inhibitors to accelerate cellular apoptosis[6]. The intrinsic pathway involves BH3-only proteins BID, BAD, BIM, BMF, PUMA and NOXA, which have pro-apoptotic activity that can activate BAX and BAK. Once activated, BAX and BAK form pores in the mitochondria, resulting in cytochrome C release and apoptosis. Anti-apoptotic proteins include BCL-2, BCL-XL, BCL-W, A1/Bfl1 and MCL-1, which bind to BH3-only proteins as well as BAX/BAK and prevent cell death activation[6]. The fine balance of pro-survival and pro-apoptotic molecules determines if apoptosis can occur[7]. Interestingly, when the expression of both pro-survival and pro-apoptotic proteins are increased simultaneously, the cells are 'primed', or more susceptible, to cell death stimuli[4,8,9]. A number of BH3-mimetics have been designed to induce cell death, with the more recent synthesis of compounds that have selective activity against

each individual pro-survival protein of the BCL-2 family. ABT737 was one of the first BH3-mimetics described and neutralises BCL-2, BCL-XL and BCL-W[10], but the development of ABT199, also known as venetoclax, that targets BCL-2 only[11], A1331852, a selective inhibitor of BCL-XL[12] and S63845, an inhibitor of MCL-1[13] has enabled the specific targeting of molecules that drive cancer cell survival and induce cancer cell apoptosis. Recently, venetoclax has been granted approval for the treatment of certain hematological malignancies, highlighting the efficacy of this strategy for cancer control[14]. Given the cytostatic action of FGFR inhibitors in *FGFR1*-expressing lung SqCC, the combination of FGFR inhibitors and BH3 mimetics to effectively cause cancer cell death holds a great degree of promise[15].

Determining the integral pro-survival factors in *FGFR1*-altered lung cancer is necessary to design rational combination therapies. Both *MCL1* and *BCL2L1*, that encodes for BCL-XL, are amplified in lung cancer[6,16,17]. The efficacy of BCL-2/BCL-XL/BCL-W inhibition using ABT737 or ABT263 as a single agent or in combination with targeted therapies has been explored in preclinical and clinical trials in treating small cell lung cancer[7,18-20], yet studies in NSCLC remain limited. The combination of EGFR and ABT737 has shown efficacy in EGFR-mutant lung ADC[21], and also demonstrated efficacy in tumours with acquired EGFR inhibitor resistance[22]. A recent study identified a greater dependency on BCL-XL for survival compared to BCL-2 of both ADC and SqCC cell lines and demonstrated efficacious treatment of lung SqCC with single agent ABT737 therapy[23]. However, there is a need to assess the efficacy of BH3 mimetics in more clinically relevant models of lung SqCC, such as patient-derived organoids or xenografts (PDXs). In addition, further exploration of the downstream effects of FGFR inhibition on the intrinsic apoptotic pathway in such models will help to clarify which BH3 mimetic may be the most effective partner in FGFR combination therapy.

In this study, using our molecularly characterised PDX models of *FGFR1*-expressing lung SqCC[24] and cell lines, we identified upregulation of pro-apoptotic BIM after cytostatic FGFR inhibition. Using specific inhibitors of BCL-2 family proteins, we show that lung SqCC cells depended on BCL-XL or MCL-1 for survival *in vitro*, rather than BCL-2.

However, combination of BCL-XL and FGFR inhibition or MCL-1 and FGFR inhibition only resulted in modest survival outcomes *in vivo*, despite providing a short-term increase in tumour cell death. We hypothesized that MCL-1 may compensate for the blocking of BCL-XL during BCL-XL inhibition and vice versa. Consistently, triple BCL-XL, MCL-1 and FGFR therapy abrogated cell survival in every model tested and genetic knock-down of BCL-XL expression combined with FGFR and MCL-1 inhibition resulted in tumour regression and prolonged animal survival *in vivo*. Our work provides a rationale for further research into the delivery of BCL-XL and MCL-1 inhibitors to *FGFR1*-expressing lung SqCC.

Results

FGFR inhibition in lung SqCC does not induce apoptosis and alters the expression of BCL-2 family proteins

Using the *FGFR1*$^{+++}$ lung SqCC cell line H1581, previously shown to be sensitive to FGFR inhibition[2,25], we found that treatment with BGJ398[26], a pan-FGFR inhibitor (FGFRi), induced a significant delay in tumour growth and prolonged animal survival *in vivo* (Figure 1a). Although tumour cell proliferation was reduced with FGFR inhibition, no significant increase in apoptotic tumour cells was observed (Figure 1b), suggesting a cytostatic action of FGFR inhibition in lung SqCC as previously reported using PDX models[24]. Tyrosine kinase inhibitors have been shown to alter the expression of pro-survival and pro-apoptotic proteins of the BCL2 family in cancer cell lines, increasing their sensitivity to BH3 mimetics[21]. Consistently, treatment with BGJ398 increased the expression of the pro-apoptotic protein BIM in H1581, but not in H520, a FGFRi-resistant *FGFR1*^{amp} lung squamous cell carcinoma cell line (Figure 1c and d). In SqCC PDXs, long-term treatment with FGFRi revealed an upregulation of BCL-2, BCL-XL and BIM in the inhibitor-sensitive PDX 926 (Figure 1e), although another FGFRi-sensitive PDX (PDX 788) did not show such strong upregulation (Supplementary Figure 1a). No change in the expression of the BCL2-family proteins was observed in the FGFRi-resistant PDX 792 (Figure 1e). Overall, these data suggest treatment with FGFRi reduces cell proliferation and may prime lung SqCC cells for cell death, indicating that combining BCL-2, BCL-XL or MCL-1

inhibitors with FGFR targeted therapy may maximize tumour cell apoptosis, as well as abrogating cell proliferation.

Expression levels of BCL-2 family proteins in patient-derived xenografts are representative of lung SqCC

Using our SqCC PDXs, we aimed to determine whether BH3 mimetic and BGJ398 combination treatment could increase tumour cell death in addition to halting cell proliferation. We first assessed BCL-2 family protein expression levels in four PDXs with differing sensitivity to FGFRi. Western blot analysis showed that baseline expression levels of BCL-2 family members varied between tumours (Figure 2a). While BIM was expressed in all PDXs, PDX 926 expressed high levels of BCL-2 and PDXs 792 and 406 expressed greater amounts of BCL-XL. PDXs 788 and 406 expressed the highest levels of MCL-1 (Figure 2a). Protein expression levels did not coincide with gene amplifications, deletions or mutations in any PDXs (Supplementary Figure 2, Table S1). These results are consistent with data from The Cancer Genome Atlas[17,27] in which the pro-apoptotic protein BIM is found upregulated in both ADC and SqCC compared with normal tissue (Supplementary Figure 3a), whereas a range of expression levels of the pro-survival molecules is observed. In this large cohort of samples, only 5% of *FGFR1*-amplified SqCC also carried an amplification of *BCL2L1* or *MCL1* (Supplementary Figure 3b). We did not observe any association between anti-apoptotic *BCL-2*, *BCL-XL* or *MCL-1* RNA expression levels and lung SqCC patient survival (Supplementary Figure 3c). These results indicate that expression of BCL2 family proteins in our PDX panel is representative of that observed in the larger cohort of lung SqCC from TCGA data. Expression of pro-survival proteins in our PDXs therefore presents an opportunity for targeting them with BH3 mimetics.

Inhibition of BCL-XL or MCL-1 reduces cell viability in lung SqCC *in vitro*

We subjected all PDXs and cell lines to treatment with ABT737, a dual BCL-2/BCL-XL inhibitor[10]; ABT199, a BCL-2 specific compound[11]; A1331852, a BCL-XL specific inhibitor[12]; or S63845, a MCL-1 specific inhibitor[13]. In this short-term *in vitro* culture, PDX cells proliferate minimally, explaining the modest effect of BGJ398, an inhibitor of

cell proliferation, yet *in vivo* studies have further confirmed the sensitivity of these PDXs to FGFR inhibition[24]. We observed variation in the response of PDXs to the different inhibitors. PDX 406 and the H520 cell line showed limited or no sensitivity to the BH3 mimetics as single agents or in combination with BGJ398 (Figure 2b and c). Dual inhibition of BCL-2 and BCL-XL by ABT737 had a remarkable effect on PDXs 792 and 926 *in vitro*, possibly due to the low level of MCL-1 in these models (Figure 2b). Treatment with the BCL-2 specific inhibitor ABT199 had a reduced efficacy compared with the dual BCL-2/BCL-XL inhibitor in all PDXs, suggesting that BCL-XL was the molecular culprit behind the activity of the dual inhibitor. Indeed, treatment of PDXs with the BCL-XL specific compound A1331852 revealed the potency of the drug paralleled that of the dual inhibitor, where combination with BGJ398 decreased cell viability in FGFRi-sensitive PDXs 788 and 926 (Figure 2b). All PDXs tested were sensitive to MCL-1 inhibition and response was enhanced in the presence of BGJ398. Treatment of *FGFR1*-expressing cell lines with ABT199, A1331852 and S63845 revealed H1581 was only responsive to MCL-1 inhibition, and this was increased when combined with FGFR therapy (Figure 2c, Supplementary Figure 4a). MCL-1, BCL-2 or BCL-XL protein expression levels could not be correlated with sensitivity to the different BH3 mimetics in the PDXs and cell lines, similar to haematological cancers[14]. Expression levels of the pro-survival proteins of the BCL-2 family remained constant after short-term treatment with BH3 mimetics and BGJ398 (Supplementary Figure 4b and c). Collectively, these data establish that SqCC have a greater reliance on BCL-XL and MCL-1 than BCL-2 for survival, and dual inhibition of BCL-XL and FGFR, or MCL-1 and FGFR in FGFRi-sensitive tumours reduces cell viability *in vitro*.

Inhibiting BCL-XL or MCL-1 *in vivo* results in a modest survival benefit in lung SqCC

We then assessed the effect of A1331852 or S63845 therapy with BGJ398 in four-arm combination studies *in vivo*. Single agent MCL-1 inhibition or combination FGFR and MCL-1 targeted-therapy revealed no significant survival benefit of either treatment in PDX 788 *in vivo* (Figure 3a), the PDX that expresses high levels of MCL-1 and showed most sensitivity to the combination *in vitro* (Figure 2b). We then treated FGFRi-sensitive PDXs

788 and 926, which express similar levels of BCL-XL (Figure 2a), with A1331852 and BGJ398. A modest survival benefit of combination therapy was observed in PDX 788, due to a reduction in tumour cell proliferation driven by BGJ398 treatment, and an enhancement of apoptosis by A1331852 therapy (Figure 3b and c). There was no survival benefit of combination therapy in PDX 926, likely due to limited tumour cell apoptosis with combination treatment (Supplementary Figure 5a and b). We hypothesized that high levels of BCL-2 expression, and upregulation of BCL-2 with BGJ398 treatment, in this PDX (Figure 1d, 2a) may allow tumour cells to escape apoptosis driven by BCL-XL inhibition. However, treatment with the dual BCL-2/BCL-XL inhibitor ABT737 *in vivo* did not result in a survival benefit as single agent or in combination with BGJ398 (Supplementary Figure 5c). To assess whether BCL-XL inhibition could improve survival in lung SqCC that are not sensitive to FGFR inhibition, we evaluated the effect of A1331852 with standard chemotherapy in PDX 792, a PDX sensitive to its inhibition *in vitro* (Figure 2b). Combination of A1331852 with platinum-based chemotherapy did not result in increased efficacy *in vivo* compared with single agents (Supplementary Figure 5d). Overall, these results demonstrate that BCL-XL or MCL-1 inhibitors do not robustly prolong survival outcomes in SqCC as single agents or when combined with targeted or cytotoxic therapies.

Dual inhibition of MCL-1 and BCL-XL abrogates cell survival by inhibiting compensatory BIM association

Although we identified a dependency of lung SqCC on MCL-1 and BCL-XL for survival *in vitro*, *in vivo* treatment with BCL-XL or MCL-1 inhibitors as single agents or in combination with targeted or cytotoxic therapies revealed limited efficacy. We therefore hypothesized that a compensatory mechanism may exist between BCL-XL and MCL-1 during treatment of lung SqCC with BH3 mimetics. Accordingly, co-immunoprecipitation experiments showed a displacement of BIM to MCL-1 when BCL-XL was inhibited (Figure 4a), thus blocking efficacy of A1331852-driven apoptosis. To block the compensatory effect of each pro-survival protein, we assessed the effect of combining BH3 mimetics *in vitro*. A striking reduction in cell viability with dual BCL-XL and MCL-1 inhibition was observed in cell lines and PDXs ($p<0.001$, treated *vs* DMSO) compared with

combination BCL-2 and BCL-XL or BCL-2 and MCL-1 inhibition (Figure 4b, Supplementary Figure 6a). BLISS assays[28] further demonstrated a strong synergy between S63845 and A133852 in H520 and H1581 cell lines (Figure 4c), that was not observed with the other BH3 mimetic combinations (Supplementary figure 6b and c). We therefore concluded that inhibition of BCL-XL and MCL-1 is required to prevent compensation between the two molecules in order for SqCC cell death to occur.

BCL-XL and MCL-1 are synthetic lethal in lung SqCC

We next interrogated if the striking reduction in cell viability with dual A1331852 and S63845 treatment was due to *bona fide* synthetic lethality between BCL-XL and MCL-1 or off-target toxicities. Analysis of caspase activation showed cleavage of the intrinsic apoptosis-specific caspase-9 after treatment with combination A1331852 and S63845, demonstrating that the inhibitors exert their activity through activation of the intrinsic apoptotic pathway (Figure 5a). Furthermore, loss of BAX and BAK in mouse embryonic fibroblasts (MEFs) blocked the cell death induced by A1331852/S63845 combination therapy, indicating that the compounds exert on-target activity through induction of apoptosis (Figure 5b). Genetic depletion of BCL-XL with two different doxycycline-inducible CRISPR sgRNAs in H1581-Cas9-expressing cells (Figure 5c) and subsequent treatment of cells with doxycycline/S63845 showed strong synergy (Figure 5d), mimicking the effect of combining A1331852 and S63845 in lung SqCC. This result establishes the dramatic effect of dual BCL-XL/MCL-1 inhibition is not due to off-target toxicities. We therefore concluded that BCL-XL and MCL-1 are synthetic lethal in lung SqCC.

Triple inhibition of FGFR, BCL-XL and MCL-1 causes tumour regression

We then assessed if inhibition of FGFR, as well as BCL-XL and MCL-1 could further enhance cell death in lung SqCC. Addition of BGJ398 and A1331852 to H1581 and H520 treated with S63845 showed synergistic decreases in cell viability (Figure 6a). Interestingly, in both cell lines, treatment with S63845 induced stabilization of MCL-1 expression, as described previously[13] (Figure 6b). We noticed that this stabilization was reversed in the presence of A1331852 or BGJ398 and that, treatment with the three agents A1331852, S63845 and BGJ398 further reduced the levels of MCL-1 expression in FGFRi-

sensitive H1581 cell line (Figure 6b), prompting us to evaluate whether blocking BCL-XL, MCL-1 and FGFR would increase cell death and block tumour growth *in vivo*. However, combination treatment with BCL-XL and MCL-1 inhibitors at therapeutic doses *in vivo* resulted in acute liver toxicity. This observation is consistent with previous results showing that deletion of MCL-1 in the liver induced hepatotoxicity[29] and that loss of one allele of *BCL-XL* and one allele of *MCL-1* in the mouse liver was sufficient to induce hepatocyte apoptosis[30]. We therefore used our genetically engineered CRISPR/Cas9 H1581 cell line in which doxycycline induces knock-down of BCL-XL expression in the tumour cells *in vitro* (Figure 5c) and *in vivo* (Supplementary Figure 6d). Single agent BCL-XL inhibition by doxycycline or MCL-1 inhibition by S63845 showed limited survival benefit compared with vehicle, whilst the dual combination of BCL-XL and MCL-1 inhibition significantly slowed tumour growth (Figure 6c). BGJ398 treatment improved animal survival, and combination BGJ398 and BCL-XL inhibition or BGJ398 and MCL-1 inhibition provided a modest survival benefit compared with BGJ398 alone, as reported in our PDX models (Figure 6c, Figure 3a and b). Excitingly, triple combination inhibiting BCL-XL, MCL-1 and FGFRs resulted in tumour regression and complete response to treatment, as assessed by RECIST analysis[31] (Figure 6c and d). After the end of treatment (38 days), tumours grew rapidly in all mice except for those in the triple combination treatment group, resulting in a mean survival of 59 days compared with BGJ398 alone (42 days), or dual combination (47 days for both BGJ398/S63845 combination group and BGJ398/doxycycline group, Figure 6c). Altogether these data suggest dual BCL-XL and MCL-1 inhibition is necessary to prevent redundancy between the two molecules and induce tumour cell death, with FGFR inhibition further priming cells to respond to this dual combination and ultimately resulting in tumour regression.

Discussion

The development of efficient cancer treatment relies on the inhibition of multiple pathways intrinsic to tumour cells such as proliferation and avoidance of apoptosis, and micro-environmental factors that promote immune evasion and angiogenesis[32]. Here we show that the FGFR tyrosine kinase inhibitor BGJ398, a cytostatic compound[24], primes cells for apoptosis in patient-derived xenograft models of lung SqCC. We identified a greater

reliance of lung SqCC cells on BCL-XL and MCL-1 than BCL-2 for survival, yet compensatory feedback between BCL-XL and MCL-1 prevents efficacy of *in vivo* combination FGFR and BCL-XL or FGFR and MCL-1 targeted treatment. Our results indicate that combining FGFR tyrosine kinase inhibitors with inhibitors of both BCL-XL and MCL-1 is required to kill a significant proportion of tumour cells and prevent tumour growth in *FGFR1*-expressing lung SqCC.

Amplifications of *MCL1* and *BCL2L1* occur in 7% and 8% of lung SqCC[16,17], however gene amplification may not be a predictor of response to BH3 mimetics. Indeed, the lung SqCC cell line H520 harbours an amplification of *BCL2L1*[16] but did not respond to the BCL-XL inhibitor A-133852. In our molecularly annotated PDXs, we did not detect any amplification in the genes of the *BCL2* family and no correlation was found between protein expression levels of BCL-2 family members and response to therapy. SCLC is reported to have the highest expression of BCL-2 family members of all lung cancers and little efficacy is seen in using BH3 mimetic single agent therapy[33]. Similarly, in chronic lymphocytic leukemia and in breast cancer, genetic alteration in *BCL2* or protein expression did not predict response to BCL-2 inhibitors[14,34,35]. Further investigations in PDX models of *MCL1*- or *BCL2L1*-amplified SqCC will be necessary to evaluate whether correlations exist between molecular alterations and sensitivity to BH3 mimetics.

Targeted therapies to inhibit oncogenic kinases have been a paradigm shift in the age of personalized medicine, yet primary and acquired resistances frequently occur. There is therefore a challenge in cancer therapy to identify rational combination treatment strategies that enhance tumour cell death, as opposed to mere tumour cell growth arrest. We have recently identified *FGFR1*-overexpressing lung SqCC, and not all *FGFR1*-amplified lung SqCC, as sensitive to FGFR inhibition using patient-derived xenografts[24]. However, we demonstrated FGFR targeted therapy results in stabilized disease, rather than tumour regression. Pro-apoptotic molecules such as BIM and BAD have been identified as important for mediating the activity of other kinase inhibitors[21,36], yet the induction of BIM expression after FGFR therapy did not result in a significant increase in apoptosis. Higher levels of MCL-1 and BCL-2 have been reported in lung ADC after long-term therapy with

EGFR or ALK inhibitors[22,37,38], and co-treatment with EGFR inhibitors and ABT737 produced a more durable response in *EGFR*-mutant ADC. In addition, glioblastomas with acquired resistance to EGFR inhibitors can be re-sensitized to EGFR therapy by combining with ABT737[39]. However, the case in *FGFR1*-altered lung SqCC appears more complex, as BCL-2/BCL-XL, BCL-XL or MCL-1 inhibition combined with FGFR inhibition failed to produce prolonged responses compared to single agent FGFR tyrosine kinase inhibitor. Although BH3 mimetics are able to enact short-term increases in tumour cell apoptosis, the ability for each anti-apoptotic protein to compensate for each other prevents long-term survival benefits with single BH3 mimetic treatment.

We demonstrate that in order to combat compensatory mechanisms of intrinsic anti-apoptotic molecules, dual BCL-XL and MCL-1 inhibition is necessary to reduce SqCC cell viability. Such compensatory mechanisms have been previously reported, where genetic knock down of MCL-1 potentiates the response of hematological and solid cancer cells to BCL-2/BCL-XL inhibition by ABT737[40,41]. Indeed, MCL-1 upregulation is associated with acquired resistance to ABT737 in lymphoma[42]. However, tolerance issues prevent the delivery of therapeutic doses of newly developed BCL-XL and MCL-1 inhibitors *in vivo*. A better refinement of the therapeutic window of each agent or indirect reduction of MCL-1 levels may be a possible strategy. This approach has previously been achieved using CDK inhibitors or flavopiridol to reduce MCL-1 levels, where combination with ABT737 drastically improved cell death outcomes[41-43]. Similar experiments combining FGFR, CDK and BCL-XL inhibitors in lung SqCC may prove to be therapeutically viable. Our work therefore provides a framework for further investigation into the *in vivo* delivery of dual BCL-XL and MCL-1 inhibitors combined with FGFR-targeted therapy to produce durable treatment responses in *FGFR1*-overexpressing lung SqCC.

Methods

Human tumour collection and PDX generation

SqCC PDXs were generated as previously described[24]. Briefly, human lung tumors from surgical resections (SqCC) were implanted subcutaneously into the flanks of NOD.SCID^{prkdc}Il2rγ$^{-/-}$ (NSG) mice before twice weekly monitoring for tumor development, before subsequent expansion in further tumour passages. Mice were culled at ethical endpoint (tumor volume of 600 mm^3 as calculated by width*length^2/2). All PDXs were confirmed to recapitulate the primary patient tumour and characterised for their *FGFR1* RNA expression by RNA ISH. Genomic characterization of PDXs by RNAseq, SNP array and whole exome sequencing was carried out as previously described[24]. Written informed consent was obtained from all patients by the Victorian Cancer BioBank prior to inclusion in the study, according to protocols approved by the Human Research Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (WEHI) (approval #10/04). NSG mice (8-12 weeks old males) were bred at the WEHI breeding facility and maintained in our animal facilities according to institutional guidelines. All animal experiments were approved by the WEHI Animal Ethics Committee (approval #2013.028).

PDX tumor cell preparation for in vitro sphere assays

Single cell suspensions of PDX tumor samples were prepared and cultured as previously described[24]. Cells were left to recover for a minimum of 1hr, and then drugs added at the following concentrations: BGJ398 1 μM (Active Biochemicals), ABT-737 500 nM (Active Biochemicals), ABT-199 500 nM (Selleck), A-1331852 500 nM (synthesized by WEHI chemists according to published methods[12], (Patent WO 2013055897) or S63845 500 nM (Servier). Cells were grown for 72h at 37ºC in 5% CO_2 and 5% O_2 before cell viability was assessed by the MTS assay according to manufacturer's instructions (Promega).

PDX *in vivo* assays

PDXs at passage 4 were defrosted and washed with PBS before counting. 200,000 to 500,000 cells were then injected in a 50:50 PBS:matrigel mix into the flanks of NSG mice.

Tumors were measured twice weekly and treatment started when tumors were between 70 – 120mm^3. Mice were assigned to treatment groups based on average tumor volume per group. Mice were culled either 48h after treatment start for short term signaling experiments or when tumors reached a volume of 600mm^3 for long term survival studies. Tumors were collected and pieces were either fixed in 10% formalin for histological analyses or snap frozen for protein analyses. Mice were treated with either 30mg/kg BGJ398 (Active Biochemicals) by oral gavage 5 consecutive days a week for 5 weeks in vehicle (33% PEG300, 5% dextrose); alone or in combination with 25 mg/kg A1331852 by oral gavage 5 consecutive days a week for 5 weeks in vehicle (2.5:10:27.5:60 DMSO:ethanol:PEG400:Phospal); 50 mg/kg ABT737 by intraperitoneal injection every day for 10 days in vehicle (4.5:5:10:27 Tween80:5% dextrose at pH 1:DMSO:propylene glycol); 25 mg/kg S63845 by intravenous injection twice a week for 5 weeks in vehicle (40% 2-hydroxypropyl-B-cyclodextrin, 50nM HCl). Cisplatin (Hospira) was delivered by intraperitoneal injection once every 3 weeks at 4 mg/kg diluted in PBS in combination with A1331852. In experiments combining A1331852 with BGJ398, BGJ398 was dissolved in the vehicle for A1331852. Appropriate vehicle controls were included for each experiment.

Cell line *in vitro* and *in vivo* assays

Cell lines H520 and H1581 were obtained from the American Type Culture Collection and maintained according to the manufacturer's instructions. WT and Bak$^{-/-}$ Bax$^{-/-}$ mouse embryonic fibroblasts were generated from mouse embryos and then immortalized with SV40 large T antigen as previously described[40]. Mycoplasma testing was performed on passage 3 cells; passage 7-10 cells were used for all experiments. Cells were plated at a density of 2,000 (H1581) or 5,000 (H520) cells per well in 96-well plate (BD Falcon). Cells were left to recover 24 hrs, and then drugs added for combination studies (BGJ398 500 nM for H520, 15 nM for H1581; ABT-199 1 μM; A-1331852 1 μM and S63845 1 μM). Cells were grown for a further 72h at 37^{o}C in 5% CO_2 before cell viability was assessed by the Cell Titer Glo assay according to manufacturer's instructions (Promega). For *in vitro* cell assays to assess synergy between different drugs, combination effects were determined using the Bliss independence method[28]. Doxycycline was added for CRISPR/Cas9 assays

(1 μg/mL). For *in vivo* assays, 400,000 to 500,000 H1581 cells were injected in PBS into the flanks of NOD;SCID;IL-2Rγ$^{-/-}$ mice. When tumours reached 70 – 120 mm^3 in volume, mice began treatment as listed above for PDX assays, and tumours processed at ethical endpoint (tumour volume 600 mm^3 to 1000 mm^3).

Immunostaining

Samples were fixed in formalin for 24 hrs at room temperature then paraffin embedded. Slides were dewaxed using standard histology protocols. Antigen retrieval was performed using citrate buffer (10mM, pH 6). Sections were blocked in 10% goat serum, incubated with antibodies overnight at 4°C followed by HRP-conjugated secondary antibodies (Vector). Antibodies used were Ki67 (B56, BD Pharmingen) or cleaved caspase 3 Asp175 (polyclonal, Cell Signaling). For quantification of the Ki67 and cleaved caspase 3 (CC3) immunostaining, treatment groups were first blinded. Ki67 positive or CC3 positive cells were counted in 3 different fields of view.

Western blotting

Fresh tumors were snap-frozen and crushed in liquid nitrogen. Cell lines were centrifuged and the pellets snap frozen. Samples were then lysed in KalbC buffer (1% Triton X-100, 50nM Tris, pH 7.5, 1mM EDTA, 150 mM NaCl, 1mM NaV, 2mM NaF, Roche complete mini protease inhibitor cocktail, Roche PhosSTOP phosphatase inhibitor cocktail). Protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) and run on 10% Bis-Tris gels (Invitrogen) before transferring onto PVDF membranes (Immobilon). Membranes were blocked in 10% milk for 1hr at RT. Primary antibodies were incubated overnight at 4ºC: BCL-2 (C-2, Santa Cruz), BCL-XL (54H6, Cell Signaling), MCL-1 (D35A5, Cell Signaling), BIM (C34C5, Cell Signaling), B-actin (AC-15, Sigma) and caspase-9 (polyclonal, Cell Signaling). Secondary conjugated antibodies (Southern Biotech) were incubated for 1 hr at room temperature before developing (Amersham).

Co-immunoprecipitation

H1581 and H520 were incubated with 5 μM of S63845 or A1331852 for 5h. Cells were

then lysed in lysis buffer (20 mM Tris.HCL, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% digitonin, and 10% glycerol), and lysates were precleared by incubation with protein G sepharose for 1 hour at 4 ^{o}C and then immunoprecipitated with 5 μg of anti-MCL-1 (SC819, Santa-Cruz Biotechnology) antibody and protein G- sepharose. Beads were washed, and immunoprecipitate was eluted in SDS-sample buffer before western blot analysis for BIM (C34C5, Cell Signaling) and MCL-1 (D35A5, Cell Signaling).

Generation of inducible CRISPR/Cas9 sgRNA BCL-XL cell line

H1581 cells were infected with lentiviruses expressing Cas9 mCherry and doxycycline-inducible sgRNA GFP. Constitutive Cas9 and inducible guide RNA vectors targeting BCL-XL have previously been described[45]. mCherry+ GFP+ cells were sorted by flow cytometry using a FACS Aria (BD Biosciences). To induce expression of the sgRNA *in vitro*, doxycycline was added to a final concentration of 1 μg/mL. To induce expression of sgRNA *in vivo*, mice had access to doxycycline food (Specialty Feeds). In triple combination *in vivo* assay combining BGJ398, doxycycline feed and S63845 treatment, S63845 dose was reduced to 25 mg/kg by intravenous injection once a week.

Analysis of TCGA RNA-seq data

We obtained raw RNA-seq subread-aligned counts for lung adenocarcinoma, squamous cell carcinoma and normal lung from The Cancer Genome Atlas (TCGA) project, GEO accession number GSE62944[46]. We annotated and filtered TCGA data. To visualise the distribution of expression levels for genes of interest in adenocarcinoma, squamous cell carcinoma and normal lung, log2 CPM values for a given gene were plotted as a box and whiskers plot across all samples within each of the above groups. Kaplan Meier plots were derived from http://kmplot.com/analysis[47].

mRECIST analysis

mRECIST analysis was performed by adapting the method described by Gao *et al.*[31]. The response was determined by comparing tumor volume change at time t to its baseline: % tumor volume change = ΔVol_t = 100% × (($V_t - V_{initial}$) / $V_{initial}$). The BestResponse was the minimum value of ΔVol_t for $t \geq 10$ d. For each time t, the average of ΔVol_t from $t = 0$ to t

was also calculated. We defined the BestAvgResponse as the minimum value of this average for $t \geq 10$ d. This metric captures a combination of speed, strength and durability of response into a single value. The criteria for response (mRECIST) were adapted from RECIST criteria[48] and defined as follows (applied in this order): mCR, BestResponse < −95% and BestAvgResponse < −40%; mPR, BestResponse < −50% and BestAvgResponse < −20%; mSD, BestResponse < 50% and BestAvgResponse < 40%; mPD, not otherwise categorized.

Statistics

Data represent mean ± SEM (standard error of the mean). Student's *t* tests were performed using GraphPad Prism software and applied to each experiment as described in the figure legends. A p value less than 0.05 was considered significant. To determine significance in Kaplan-Meier survival analyses, the Mantel-Cox test was used. *p < 0.05, **p < 0.01, ***p < 0.001.

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References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA: A Cancer Journal for Clinicians* 2015; **65**: 5–29.

2 Weiss J, Sos ML, Seidel D, Peifer M, Zander T, Heuckmann JM *et al.* Frequent and Focal FGFR1 Amplification Associates with Therapeutically Tractable FGFR1 Dependency in Squamous Cell Lung Cancer. *Science Translational Medicine* 2010; **2**: 62ra93–62ra93.

3 Nogova L, Sequist LV, Perez Garcia JM, Andre F, Delord J-P, Hidalgo M *et al.* Evaluation of BGJ398, a Fibroblast Growth Factor Receptor 1-3 Kinase Inhibitor, in Patients With Advanced Solid Tumors Harboring Genetic Alterations in Fibroblast Growth Factor Receptors: Results of a Global Phase I, Dose-Escalation and Dose-Expansion Study. *J Clin Oncol* 2017; **35**: 157–165.

4 Weeden CE, Solomon B, Asselin-Labat M-L. FGFR1 inhibition in lung squamous cell carcinoma: questions and controversies. *Cell Death Dicsov* 2015; : 1–5.

5 Wynes MW, Hinz TK, Gao D, Martini M, Marek LA, Ware KE *et al.* FGFR1 mRNA and Protein Expression, not Gene Copy Number, Predict FGFR TKI Sensitivity across All Lung Cancer Histologies. *Clinical Cancer Research* 2014; **20**: 3299–3309.

6 Strasser A, Cory S, Adams JM. EMBO Member's ReviewDeciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *The EMBO Journal* 2011; **30**: 3667–3683.

7 Merino D, Bouillet P. The Bcl-2 family in autoimmune and degenerative disorders. *Apoptosis* 2009; **14**: 570–583.

8 Potter DS, Letai A. To Prime, or Not to Prime: That Is the Question. *Cold Spring Harb Symp Quant Biol* 2017; **81**: 131–140.

9 Merino D, Khaw SL, Glaser SP, Anderson DJ, Belmont LD, Wong C *et al.* Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* 2012; **119**: 5807–5816.

10 Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; **435**: 677–681.

11 Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, Dayton BD *et al.* ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* 2013; **19**: 202–208.

12 Leverson JD, Phillips DC, Mitten MJ, Boghaert ER, Diaz D, Tahir SK *et al.* Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and

define improved strategies for cancer therapy. *Science Translational Medicine* 2015; **7**: 279ra40.

13 Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G *et al.* The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature* 2016; : 1–20.

14 Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF *et al.* Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N Engl J Med* 2016; **374**: 311–322.

15 Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2protein family: implications forphysiology and therapy. *Nat Rev Mol Cell Biol* 2014; **15**: 49–63.

16 Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* 2011; **463**: 899–905.

17 Hammerman PS, Lawrence MS, Voet D, Jing R, Cibulskis K, Sivachenko A *et al.* Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 2012; **489**: 519–525.

18 Lam LT, Lin X, Faivre EJ, Yang Z, Huang X, Wilcox DM *et al.* Vulnerability of Small-Cell Lung Cancer to Apoptosis Induced by the Combination of BET Bromodomain Proteins and BCL2 Inhibitors. *Molecular Cancer Therapeutics* 2017; **16**: 1511–1520.

19 Gandhi L, Camidge DR, Ribeiro de Oliveira M, Bonomi P, Gandara D, Khaira D *et al.* Phase I Study of Navitoclax (ABT-263), a Novel Bcl-2 Family Inhibitor, in Patients With Small-Cell Lung Cancer and Other Solid Tumors. *J Clin Oncol* 2011; **29**: 909–916.

20 Gardner EE, Connis N, Poirier JT, Cope L, Dobromilskaya I, Gallia GL *et al.* Rapamycin Rescues ABT-737 Efficacy in Small Cell Lung Cancer. *Cancer Res* 2014; **74**: 2846–2856.

21 Cragg MS, Kuroda J, Puthalakath H, Huang DCS, Strasser A. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. *PLoS Med* 2007; **4**: 1681–89– discussion 1690.

22 Fan W, Tang Z, Yin L, Morrison B, Hafez-Khayyata S, Fu P *et al.* MET-Independent Lung Cancer Cells Evading EGFR Kinase Inhibitors Are Therapeutically Susceptible to BH3 Mimetic Agents. *Cancer Res* 2011; **71**: 4494–4505.

23 Zeuner A, Francescangeli F, Contavalli P, Zapparelli G, Apuzzo T, Eramo A *et al.* Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-X. 2014; **21**: 1877–1888.

24 Weeden CE, Holik AZ, Young RJ, Ma SB, Garnier J-M, Fox SB *et al.* Cisplatin Increases Sensitivity to FGFR Inhibition in Patient-Derived Xenograft Models of Lung Squamous Cell Carcinoma. *Molecular Cancer Therapeutics* 2017. doi:10.1158/1535-7163.MCT-17-0174.

25 Guagnano V, Kauffmann A, Wohrle S, Stamm C, Ito M, Barys L *et al.* FGFR Genetic Alterations Predict for Sensitivity to NVP-BGJ398, a Selective Pan-FGFR Inhibitor. *Cancer Discovery* 2012; **2**: 1118–1133.

26 Guagnano V, Furet P, Spanka C, Bordas V, Le Douget M, Stamm C *et al.* Discovery of 3-(2,6-Dichloro-3,5-dimethoxy-phenyl)-1-{6-[4-(4-ethyl-piperazin-1-yl)-phenylamino]-pyrimidin-4-yl}-1-methyl-urea (NVP-BGJ398), A Potent and Selective Inhibitor of the Fibroblast Growth Factor Receptor Family of Receptor Tyrosine Kinase. *J Med Chem* 2011; **54**: 7066–7083.

27 Network TCGAR. Comprehensive molecular profiling of lung adenocarcinoma. *Nature* 2014; : 1–9.

28 Prichard MN, Prichard LE, Baguley WA, Nassiri MR, Shipman C. Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. *Antimicrob Agents Chemother* 1991; **35**: 1060–1065.

29 Vick B, Weber A, Urbanik T, Maass T, Teufel A, Krammer PH *et al.* Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes. *Hepatology* 2008; **49**: 627–636.

30 Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T *et al.* Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. *Hepatology* 2009; **50**: 1217–1226.

31 Gao H, Korn JM, Ferretti SEP, Monahan JE, Wang Y, Singh M *et al.* High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat Med* 2015; : 1–11.

32 Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* 2011; **144**: 646–674.

33 Greenberg EF, McColl KS, Zhong F, Wildey G, Dowlati A, Distelhorst CW. Synergistic killing of human small cell lung cancer cells by the Bcl-2-inositol 1,4,5-trisphosphate receptor disruptor BIRD-2 and the BH3-mimetic ABT-263. *Cell Death and Dis;* 2015; : 1–10.

34 Vaillant F, Merino D, Lee L, Breslin K, Pal B, Ritchie ME *et al.* Targeting BCL-2 with the BH3 Mimetic ABT-199 in Estrogen Receptor-Positive Breast Cancer. *Cancer Cell* 2013; **24**: 120–129.

35 Oakes SR, Vaillant F, Lim E, Lee L, Breslin K, Feleppa F *et al.* Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. *Proc*

Natll Acad Sci USA 2012; **109**: 2766–2771.

36 Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, Huang DCS *et al.* Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci USA* 2006; **103**: 14907–14912.

37 Thiagarajan PS, Wu X, Zhang W, Shi I, Bagai R, Leahy P *et al.* Transcriptomic-metabolomic reprogramming in EGFR-mutant NSCLC early adaptive drug escape linking TGFβ2-bioenergetics-mitochondrial priming. *Oncotarget* 2016; **7**: 82013–82027.

38 Ye M, Zhang Y, Zhang X, Zhang J, Jing P, Cao L *et al.* Targeting FBW7 as a Strategy to Overcome Resistance to Targeted Therapy in Non-Small Cell Lung Cancer. *Cancer Res* 2017; **77**: 3527–3539.

39 Wykosky J, Hu J, Gomez GG, Taylor T, Villa GR, Pizzo D *et al.* A Urokinase Receptor-Bim Signaling Axis Emerges during EGFR Inhibitor Resistance in Mutant EGFR Glioblastoma. *Cancer Res* 2015; **75**: 394–404.

40 van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 2006; **10**: 389–399.

41 Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 Down-regulation Potentiates ABT-737 Lethality by Cooperatively Inducing Bak Activation and Bax Translocation. *Cancer Res* 2007; **67**: 782–791.

42 Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* 2010; **115**: 3304–3313.

43 Raje N. Seliciclib (CYC202 or R-roscovitine), a small-molecule cyclin-dependent kinase inhibitor, mediates activity via down-regulation of Mcl-1 in multiple myeloma. *Blood* 2005; **106**: 1042–1047.

44 Weeden CE, Chen Y, Ma SB, Hu Y, Ramm G, Sutherland KD *et al.* Lung Basal Stem Cells Rapidly Repair DNA Damage Using the Error-Prone Non homologous End-Joining Pathway. *PLoS Biol* 2017; **15**: e2000731.

45 Gong JN, Khong T, Segal D, Yao Y, Riffkin CD, Garnier JM *et al.* Hierarchy for targeting prosurvival BCL2 family proteins in multiple myeloma: pivotal role of MCL1. *Blood* 2016; **128**: 1834–1844.

46 Rahman M, Jackson LK, Johnson WE, Li DY, Bild AH, Piccolo SR. Alternative preprocessing of RNA-Sequencing data in The Cancer Genome Atlas leads to improved analysis results. *Bioinformatics* 2015; **31**: 3666–3672.

47 Szász AM, Lánczky A, Nagy Á, Förster S, Hark K, Green JE *et al.* Cross-validation

of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget* 2016; **7**: 49322–49333.

48 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl. Cancer Inst*. 2000; **92**: 205–216.

Figure Legends

Figure 1. Treatment with FGFR inhibitors in lung SqCC does not induce apoptosis and alters the levels of expression of proteins of the BCL-2 family

(**a**) *In vivo* drug response of mice harboring H1581 tumors (500,000 cells/mouse) to BGJ398 (30 mg/kg, 5 days a week by oral gavage). Left panel depicts tumor volume where data is mean ± SEM, right panel displays Kaplan Meier survival analysis. n = 10 mice vehicle group, n = 9 mice BGJ398 group, significance determined by Mantel-Cox test. **p<0.01. (**b**) Representative immunohistochemistry showing expression of Ki67 and cleaved-caspase 3 (CC3) in mice harboring H1581 tumors 48 h after treatment with BGJ398 (30 mg/kg every day by oral gavage). Right panel shows quantification of Ki67 and CC-3 immunostaining. Images were quantified by first blinding groups and counting Ki67 or CC-3 positive cells in 3 different fields of view. n = 7 mice per group, data is mean ± SEM. Significance determined by Student's t-test. Scale bar, 500 μm. (**c**) Cell viability curve for FGFR1^{+++} H520 and H1581 cell lines after 72h of treatment with increasing concentrations of BGJ398. Data represents mean ± SEM, n = 3 independent experiments. (**d**) Western blot showing expression levels of BCL-2 family members after 24 h treatment of lung SqCC cell lines with BGJ398 (2 μM). (**e**) Left panel depicts expression levels of BCL-2, BCL-XL, MCL-1 and BIM after BGJ398 treatment in FGFR inhibitor-sensitive and inhibitor-resistant PDXs. Tumors collected after 2 weeks of BGJ398 treatment *in vivo* (14 days at 30 mg/kg by oral gavage). Right panel shows the quantification of the Western blots by densitometry. Data represents mean ± SEM, n = 6 mice per treatment. Significance determined by student's t test compared to vehicle control, **p<0.01.

Figure 2. Treatment with BH3 mimetics reveals greater efficacy of BCL-XL or MCL-1 inhibition compared to BCL-2 inhibition in lung SqCC.

(**a**) Western blot showing expression levels of BCL-2, BCL-XL, MCL-1 and BIM in four human lung squamous cell carcinoma PDXs. (**b**) Cell viability of lung squamous cell carcinoma PDXs treated in vitro for 72 hours with ABT737 (500 nM), ABT199 (500 nM), A1331852 (500 nM), S63845 (500 nM) as single agent or in combination with BGJ398 (1 μM). Data represents mean ± SEM, n = 4 – 8 tumours per PDX, significance determined

by Student's t-test between DMSO control and single agent treatment groups; or between BGJ398 single agent and combination treatment groups. (**c**) Cell viability of H520 (left) and H1581 (right) 72 h after treatment with BGJ398 (500 nM for H520 and 15 nM for H1581), ABT199 (1 μM), A1331852 (1 μM) or S63845 (1 μM) as single agents or in combination. Data represents mean ± SEM, n = 3 independent experiments, significance determined by Student's t-test.

Figure 3. Inhibiting MCL-1 or BCL-XL *in vivo* as single agents or in combination with BGJ398 has a modest survival benefit in lung SqCC PDXs.

(**a**) *In vivo* drug response of PDX 788 to BGJ398 (30 mg/kg, 5 days a week by oral gavage) or S63845 (25 mg/kg, 2 times a week, i.v.) as single agents or in combination. Left panel depicts tumor volume where data is mean ± SEM, right panel displays Kaplan-Meier survival analysis. n = 8 mice per group, significance determined by Mantel-Cox test. ***p<0.001. (**b**) *In vivo* drug response of PDX 788 to BGJ398 (30 mg/kg, 5 days a week by oral gavage), A1331852 (25 mg/kg, 5 days a week by oral gavage) single agents or in combination. Left panel depicts tumor volume where data is mean ± SEM, right panel displays Kaplan Meier survival analysis. n = 8 mice per group for vehicle and BGJ398, n = 7 mice per group for A1331852 and combination, significance determined by Mantel-Cox test. **p<0.01. (**c**) Left panel depicts representative immunohistochemistry for Ki67 and cleaved caspase 3 (CC3) on PDX 788. Tumours collected 48 h after first BGJ398 (30 mg/kg, every day by oral gavage) and/or A1331852 (25 mg/kg, every day by oral gavage) treatment. Scale bar, 500 μm. Right panel shows quantification of immunohistochemical data. Images were quantified by first blinding groups and counting Ki67 or CC3 positive cells in three different fields of view. Data represents mean ± SEM, n = 6 mice per group, significance determined by Student's t-test.

Figure 4. Dual inhibition of BCL-XL and MCL-1 synergizes to induce tumour cell death *in vitro*.

(**a**) Immunoprecipitation-western blot showing the binding of BIM to immunoprecipitated MCL-1 in H1581 treated for 5 hours with A1331852 (5 μM) or S63845 (5 μM). (**b**) Cell viability of PDX 788 and 926 treated for 72 hours with S63845 (500 nM), ABT-199 (500

nM) and A1331852 (500 nM) with and without BGJ398 (1 μM). Data represents mean ± SEM, n = 3 – 4 tumours per PDX. (**c**) H520 and H1581 cells were treated with increasing concentrations of A1331852 and S63845 for 72 hours and then subjected to viability assays followed by BLISS score analysis. BLISS synergy values are > 0 on the vertical axis. Data represents averages of 3 independent experiments.

Figure 5. BCL-XL and MCL-1 are synthetic lethal in lung SqCC**.**

(**a**) Western blot showing cleavage of caspase 9 in H1581 and H520 treated for the indicated time with A1331852 (2 μM) and S63845 (2 μM) as single agents or in combination. (**b**) Wild-type mouse embryonic fibroblasts (MEFs) and $BAK^{-/-}BAX^{-/-}$ MEFs were treated with increasing concentrations of A1331852 and S63845 for 72 hours and then subjected to viability assays followed by BLISS score analysis. BLISS synergy values are > 0 on the vertical axis. Data represents averages of 3 independent experiments. (**c**) Western blot showing the reduced expression of BCL-XL after treatment with doxycycline in CRISPR/Cas9 H1581 cells expressing two different sg-RNA targeting BCL-XL. (**d**) Cell viability of BCL-XL sg-RNA H1581 cell lines (sgBCL-XL 1 and sgBCL-XL 2) treated with doxycycline and increasing concentrations of S63845 for 72h. Data represents mean ± SEM, n = 3 independent experiments.

Figure 6. Triple inhibition of BCL-XL, MCL-1 and FGFR results in tumour regression in FGFRi-sensitive lung squamous cell carcinoma.

(**a**) Cell viability of H520 and H1581 72 hours after treatment with increasing concentrations of S63845 and A1331852, with and without BGJ398 (250 nM for H520 and 3 nM for H1581). Data represents mean ± SEM, n = 3 independent experiments. (**b**) Western blot showing expression levels of BCL-2, BCL-XL, MCL-1 and BIM in H520 and H1581 24 hrs after treatment with BGJ398 (2 μM), A1131852 (2 μM) and S63845 (2 μM) as single agents or in combination. All samples were grown in the presence of a caspase inhibitor to prevent cell death (QVD, 10 μM). (**c**) *In vivo* drug response of doxycycline-inducible CRISPR/Cas9 BCL-XL sgRNA 1 H1581 cells to BGJ398 (30 mg/kg, 5 days a week by oral gavage), S63845 (25 mg/kg, once a week, i.v.) and doxycycline (administered in feed once tumour reached 70 – 120 mm^3) as single agents or in combination. Black bar indicates total duration of the treatment. Data depicts tumor

volume where data is mean ± SEM, n = 8 mice per group, significance determined by Mantel-Cox test. ***p<0.001, **p<0.01, *p<0.05. Right panel shows Kaplan-Meier survival curve. Black bar indicates total duration of the treatment. (**d**) Summary of compound sensitivity in CRISPR/Cas9 BCL-XL sgRNA 1 H1581 cells during continuous therapy. The best average response was used to make response calls (see mRECIST analysis in Methods), and each square represents a mouse. A total of 8 treatment groups were tested with 8 mice per group. Arrow (CR->PD, PR>PD, SD>PD) indicates disease progression; -> indicates progression seen after 24 or 38 days of treatment as indicated. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.