Original Article Chk1 inhibitor-induced DNA damage increases BFL1 and decreases BIM but does not protect human cancer cell lines from Chk1 inhibitor-induced apoptosis

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Abstract: V158411 is a potent, selective Chk1 inhibitor currently in pre-clinical development. We utilised RNAsequencing to evaluate the gene responses to V158411 treatment. BCL2A1 was highly upregulated in U2OS cells in response to V158411 treatment with BCL2A1 mRNA increased > 400-fold in U2OS but not HT29 cells. Inhibitors of Chk1, Wee1 and topoisomerases but not other DNA damaging agents or inhibitors of ATR, ATM or DNA-PKcs increased BFL1 and decreased BIM protein. Increased BFL1 appeared limited to a subset of approximately 35% of U2OS cells. Out of 24 cell lines studied, U2OS cells were unique in being the only cell line with low basal BFL1 levels to be increased in response to DNA damage. Induction of BFL1 in U2OS cells appeared dependent on PI3K/ AKT/mTOR/MEK pathway signalling but independent of NF-κB transcription factors. Inhibitors of MEK, mTOR and PI3K effectively blocked the increase in BFL1 following V158411 treatment. Increased BFL1 expression did not block apoptosis in U2OS cells in response to V158411 treatment and cells with high basal expression of BFL1 readily underwent caspase-dependent apoptosis following Chk1 inhibitor therapy. BFL1 induction in response to Chk1 inhibition appeared to be a rare event that was dependent on MEK/PI3K/AKT/mTOR signalling.

Keywords: Chk1, BFL1, MEK, DNA damage, kinase inhibitor

Introduction

DNA repair pathways have long been postulated as potential therapeutic targets for oncology therapeutics. The approval of numerous inhibitors of poly (ADP-ribose) polymerase (PARP; Olaparib, Rucaparib and Niraparib) for ovarian, fallopian tube and peritoneal cancer has validated this approach [1, 2]. One such potential area of interest is the DNA damage response (DDR) pathway with inhibitors of two of the central signalling kinases (Chk1 and ATR) currently in Phase 1 and 2 clinical trials [3].

DNA breaks arising from endogenous or exogenous DNA insults (including from cytotoxic chemotherapeutics) activate the DDR signalling pathway. DDR activation results in a range of cellular responses including DNA damage checkpoint activation and cell cycle arrest, initiation of DNA repair, regulation of transcription, and apoptosis. ATR and Chk1 kinases are key signalling components of the DDR response and are activated in response to DNA damage and replication stress. Chk1 is activated by ATR following the generation of ssDNA-dsDNA transitions whilst a second checkpoint kinase, Chk2, is activated by ATM in response to DNA breaks [4, 5].

Numerous Chk1 inhibitors have entered Phase 1 and 2 clinical trials in combination with cytotoxic chemotherapy, ionising radiation or as single agents (recently reviewed in [6, 7]). As monotherapy, Chk1 inhibitors generate S-phase DNA damage and mitotic catastrophe in human cancer cell lines. This leads to increased ATR activation (as measured by increased phosphorylation on serine 317 and 345) and pan-nuclear expression of yH2AX (a marker of DNA double strand breaks [8] or replication stress [9] with pan-nuclear yH2AX an indicator of lethal replication stress [10]). Tumour cells harbouring high levels of replicative stress or underlying DNA repair defects appear particularly hypersensitive to Chk1 inhibition. Whilst demonstrating some initial promise, no Chk1 inhibitors have so far managed to progress into Phase 3 registration trials with the majority terminating after Phase 1 trials. The reasons behind this are numerous and complex including (but not limited to) lack of efficacy, dose limiting toxicities (especially in combination), poor pharmaceutical properties (oral bioavailability, half-life), target patient population selection and drug combination selection.

V158411 is a potent, selective Chk1 inhibitor discovered using structure guided drug design [11] that exhibits preclinical activity as a single agent [12] and in combination with traditional cytotoxic chemotherapy. As a monotherapy, V158411 induces predominantly S-phase DNA damage and apoptotic as well as non-apoptotic cell death in a range of human cancer cell lines [12]. As part of an ongoing program evaluating the pharmacology of V158411 to guide clinical studies, we utilised RNA-sequencing (RNAseq) to evaluate the gene responses in a cell model that undergoes caspase-dependent apoptosis and a second model that did not following V158411 treatment. BFL1 was identified as one of the most upregulated genes following V158411 treatment and its role in cell death responses to Chk1 inhibition further profiled.

Materials and methods

Cell lines and cell culture

Cell lines were purchased from the American Type Culture Collection (ATCC, LGC Standards, Teddington, UK) or the European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Salisbury, UK), established as a low passage cell bank and then routinely passaged in our laboratory for less than 3 months after resuscitation. These were routinely cultured in media containing 10% FCS and 1% penicillin/streptomycin (complete media) at 37°C in a normal humidified atmosphere supplemented with 5% CO_2 . Cells were authenticated by short tandem repeat (STR) profiling (LGC Standards) and routinely checked for mycoplasma contamination.

Compounds

V158411 was from Vernalis (R&D) Ltd (Cambridge, UK). The other kinase inhibitors were purchased from Selleckchem (Houston, USA).

All were prepared as 20 mM DMSO stocks. Cytotoxics were purchased from the indicated suppliers and prepared as described: gemcitabine (Apin Chemicals Ltd, Oxford, UK), 20 mM in H₂O; camptothecin (LC Laboratories, Woburn, MA), 5 mM in DMSO; etoposide (LC Laboratories), 50 mM in DMSO; cisplatin (Selleckchem), 3.33 mM in 1% NaCl; and hydroxy-urea (Sigma Aldrich, Poole, UK), 0.5 M in H₂O. TNF- α , IL-1 β and IFN- γ were purchased from R&D Systems (Abingdon, UK), and Phorbol 12-myristate 13-acetate (PMA) and Lipopoly-saccharide (LPS) from Invivogen (Toulouse, France).

Antibodies

The antibodies used in this study and the dilutions used are listed in <u>Table S1</u>.

Immunoblotting

Cells were washed once with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). Protein concentration was determined using a BCA kit (Thermo Fisher Scientific, Hemel Hempstead, UK). Equal amounts of lysate were separated by SDS-PAGE and western blot analysis conducted using the antibodies indicated above. ImageJ software (NIH) was used for densitometric analysis.

Single cell immunofluorescent imaging

This was conducted as previously described [12] using the antibodies listed in <u>Table S1</u>.

Single cell mRNA expression imaging

Single cell mRNA expression was determined using a ViewRNA Cell Plus assay kit (Thermo Fisher Scientific) with probes against BCL2A1 (BFL1, VA6-20602-VCP, AF647) and B2M (Beta-2-Microglobulin, VA4-13460-VCP, AF488) according to the manufacturer's instructions. Cells were imaged with an Operetta high content imager (Perkin Elmer, Sear Green, UK) using a 40x high NA objective. Fluorescent spots were quantified using Harmony software (Perkin Elmer).

NF-ĸB reporter cell line generation

U2OS cells were transfected with a NF-KB-RE-NanoLuc reporter plasmid (N1111, Promega) and a stable cell line generated by Hygromycin B selection (Invitrogen). The reporter contains a PEST destabilized version of the NanoLuc gene (NlucP). NlucP reporter activity was determined using a Nano-Glo luciferase assay system (N1110, Promega) and a Victor Nivo plate reader (Perkin Elmer).

RNAseq expression analysis

RNAseq analysis was conducted by Lexogen (Vienna, Austria). RNA was extracted from treated cells using a SPLIT kit, an RNA library prepared using a QuantSeq 3' mRNA-Seq Library Prep Kit FWD from Illumina and then sequenced using NextSeq 75 cycle high output sequencing with v2 chemistry on a HiSeq 2500 platform (Illumina, San Diego, CA). Data was analysed using a QuantSeq proprietary data analysis pipeline by Lexogen. In brief, reads were aligned using STAR aligner, RSEOC was utilised for quality control, and DESeg2 for differential expression analysis. Gene Ontology enrichment analysis was conducted using ShinyGO v0.75 (http://bioinformatics.sdstate. edu/go/) [13].

Cell proliferation, cytotoxicity and apoptosis assay

Cell proliferation was determined using either sulphorhodamine B staining or CellTiter Glo (Promega). Live cell imaging experiments were conducted on an Operetta high content imager essentially as previously described [12].

Statistical analysis

Data was analyzed using either a one-way ANOVA with Dunnett's post-hoc analysis or a two-tailed t test using GraphPad Prism software (version 9.3.1, GraphPad Software, La Jolla, CA). *P < 0.05; **P < 0.01; ***P < 0.001.

Results

Chk1 inhibitors increase BFL1 expression in U2OS osteosarcoma cells

To further understand the cellular response to Chk1 inhibition, the mRNA changes following Chk1 inhibition by V158411 in the human colon cancer cell line HT29 and osteosarcoma cell line U2OS were determined using RNAseq. Volcano plots of differentially expressed genes

are illustrated in Figure S1. In U2OS cells, but not HT29 cells, the anti-apoptotic Bcl-2 family member BCL2A1 (BFL1) (Figures S1, 1A) was highly upregulated (> 400-fold). A small increase in Bcl-b (approximately 4-fold) but not in any of the other Bcl-2 family members was observed. Of the BH3-only genes, there was a small increase in NOXA and PUMA, and a small decrease in BIM. This increase in BCL2A1 mRNA appeared limited to a subset of U2OS cells with around 33% demonstrating increased BCL2A1 mRNA by imaging (Figures 1B, S2). These results were confirmed by western blotting (Figure 1C) with the Wee1 inhibitor AZD1775 (Table 1) but not the ATR inhibitor VX-970 also increasing BFL1 protein levels. Increases in BFL1 protein by V158411 and AZD1775 were dose dependent (Figure 1D) correlating closely with DNA damage induction and growth inhibition, and time dependent (Figure 1E) with 24-hour treatment necessary to observe increases in BFL1 expression. Inhibition of Chk1 or Wee1 induced a time dependent decrease in BIM protein levels that mirrored the increase in BFL1 with decreased BIM observed after 24 hours of treatment (Figure 1D). Chk1 and Wee1 inhibition increased NOXA slightly whilst Wee1i also increased PUMA expression. BID, BAD and BAX expression remained relatively unchanged.

BFL1 induction following Chk1 inhibition was not limited to V158411. Structurally distinct Chk1 inhibitors (LY2603618, MK8776 and PF-477736; Tables 1, S2) increased BFL1 in U2OS cells (Figure 2A) with BCL2A1 mRNA increases again limited to a subset of U2OS cells (Figure 2B). Inhibitors of ATM (KU-60019) or DNA-PKcs (NU7746), two additional kinases in the DDR, did not activate BFL1 expression (Figure 2A). This induction of BFL1 was not just limited to Chk1 inhibitors. Treatment of U2OS cells with topoisomerase inhibitors (camptothecin and etoposide) readily increased BFL1 expression (Figure 2A, 2B) whilst other DNA damaging cytotoxic drugs (gemcitabine, hydroxyurea and cisplatin) did not.

BFL1 is often co-induced with BcI-XL [14, 15]. In U2OS cells treated with V158411, little increase (< 2-fold) in BcI-XL mRNA was observed (**Figure 1A**). 48-hour treatment of U2OS cells with V158411, VX-970 or AZD1775 appeared to increase BcI-XL protein levels (**Figure 1C**). This increase in BcI-XL protein following inhibi-



Figure 1. Inhibition of Chk1 increases BFL1 and decreases BIM expression in U2OS cells. (A) HT29 or U2OS cells were treated with 1x γ H2AX EC₅₀ of V158411 for 24 hours (γ H2AX EC₅₀ values from [12]) and gene expression determined by RNAseq analysis. Values are the mean of 2 independent replicates. (B) U2OS cells were treated with 3x GI₅₀ of V158411 for 24 hours (GI₅₀ values from [12]) and BCL2A1 (BFL1) mRNA expression determined using ViewRNA technology. Each point represents an individual cell. Data is derived from 3 independent wells. Significance was determined by Student's t-test (***P < 0.001). U2OS cells were treated with (C) 3x GI₅₀ for the indicated times, (D) 3x GI₅₀ for 1-48 hours or (E) 0.1-3x GI₅₀ for 48 hours and protein expression determined by western blotting.

tor treatment did not occur consistently with increased BcI-XL following V158411 not occurring in other western blots (**Figures 1D**, **1E** and **2C**). None of the other Chk1 inhibitors or cytotoxic agents induced a strong induction of BcI-XL (**Figure 2C**).

TNF- α , IL-1 β or phorbol ester signalling increased BFL1 expression in U2OS cells

Previous studies have demonstrated that TNF- α [14, 16], IL-1 β [17] and PMA [18] can all induce BFL1 expression via an NF- κ B depen-

dent mechanism. We therefore evaluated the effect of these three on BFL1 expression in U2OS cells. TNF- α , IL-1 β and PMA all induced BFL1 expression with robust increases in BFL1 protein observed after 4-hour treatment with IL-1 β and PMA and 24 hours with TNF- α (Figure S3A). Bcl-XL remained unchanged following any of the treatments. Previous work has demonstrated that both PMA and etoposide induce BFL1 in the LNCaP prostate cancer cell line [18]. Treatment of LNCaP cells with PMA for 4 hours induced BFL1 but by 24 hours the levels of BFL1 had returned to base line (Figure S3B).

Cmpd	Machanism of Action	U20S 0	Pof	
Cilipu	Mechanism of Action	Mean	SD	. Nei
V158411	Chk1 Inhibitor	0.82	0.1	[11]
VX-970	ATR Inhibitor	3.5	0.77	[50]
AZD1775	Wee1 Inhibitor	0.27	0.036	[51]
Gemcitabine	Chain terminator & RNR inhibitor	0.012	0.001	
Camptothecin	Topo I inhibitor	0.25	0.003	
Etoposide	Topo II inhibitor	4.2	0.32	
LY2603618	Chk1 inhibitor	0.67	0.05	[52]
MK8776	Chk1 inhibitor	0.82	0.05	[53]
PF-477736	Chk1 inhibitor	0.71	0.06	[54]
Cisplatin	DNA alkylator	7.5	0.77	
Hydroxyurea	RNR inhibitor	2800	1100	
KU-60019	ATM inhibitor	24	9.2	[55]
NU7746	DNA-PKcs inhibitor	8.6	0.48	[56]

Table 1. Mechanism of action and GI_{50} values of agents used

In comparison, V158411 treatment of LNCaP cells for 6 to 72 hours had no effect on BFL1 protein levels (Figure 3A). Likewise, treatment of LNCaP cells with AZD1775, LY2603618, gemcitabine, etoposide, camptothecin or cisplatin did not induce BFL1 protein levels (Figure 3A). Similarly, PMA treatment of HeLa cells induced BFL1 and decreased BIM protein expression (Figure 3B) but V158411, LY26-03618, etoposide or camptothecin had no effect on BFL1 or BIM protein levels.

BFL1 induction is a rare response in cancer cell lines to Chk1 inhibitor therapy

A range of additional cancer cell lines (Table S3) were screened for BFL1 induction following treatment with V158411 (Figure 3C-E). Of these, three cell lines (A2058, HL60 and PANC-1) exhibited higher basal expression of BFL1 and, of these, two (HL60 and PANC-1) had increased BFL1 expression following V158411 treatment (Figure 3E). No discernible changes in Bcl-2, Bcl-XL or Mcl-1 were observed in cells following V158411 treatment. As observed with Chk1 inhibitors, no increase in BFL1 was observed in MES-SA or SJSA-1 cells treated with gemcitabine, camptothecin, etoposide or cisplatin (Figure S3C) or HT29, MDA-MB-231 or SKOV3 cells treated with gemcitabine or camptothecin (Figure S3D). No induction of BcI-XL was observed. However, in the PANC-1 cells. V158411 and LY2603618 strongly induced BFL1, and all of the DNA damaging agents moderately induced BFL1 (Figure S3E).

Chk1i-induced BFL1 expression appeared independent of NF-кВ transcription factor

To further understand the mechanism by which DDR or topoisomerase inhibitors increase BFL1 expression, we evaluated the effects of these molecules on NF-kB transcription factor signalling. NF-kB is the transcription factor most associated with BFL1 induction [14, 16, 19]. TNF- α , IL-1 β and PMA all induced BFL1 expression with robust increases in BFL1 protein observed after 4-hour treatment with IL-1 β and PMA and 24 hours with TNF- α (Figure S2). This increase in BFL1 appeared

NF- κ B dependent as PMA, TNF- α or IL-1 β all induced an NF-kB reporter stably expressed in U2OS cells (Figure 4A; Figure S4) and treatment of U2OS cells with TNF-a resulted in clear nuclear translocation of p65 (Figure 4B; Figure S4) and, to a lesser extent, phospho-p65, c-Rel and RelB (Figure S4). Chk1, Wee1 or topoisomerase inhibitors did not induce an NF-KB reporter stably expressed in U2OS cells (Figure 4A). V158411 treatment appeared to increase phosphorylation of NF-kB (p65) on serine 536 (Figure 4C, 4D). Using single-cell immunofluorescence, there appeared to be apparent nuclear translocation of p65, phosphor-p65 and p50 forms of NF-kB following V158411 treatment. However, on closer inspection of the single cell data, there appeared to be noticeable decreases in nuclear NF-KB in a fraction of the treated cells (Figure 4C) with the increased nuclear NF-kB correlating with DNA condensation and therefore cell death. Western blotting (Figure 4D) confirmed no distinct changes in other components of the NF-kB signalling pathway in U2OS cells treated with V158411.

We utilised the ChEA3 tool [20] to understand which transcription factors may be responsible for the observed changes in V158411 treated U2OS cells. In U2OS cells treated with V158411, ChEA3 predicted a strong association between the observed gene expression changes between AP-1 family transcription factors (FOSB, FOSL1, JUN, FOS and JUNB) and stress-induced transcription factor (ATF4)



Figure 2. Structurally diverse Chk1 inhibitors or topoisomerase inhibitors induce BFL1 mRNA and protein expression in U2OS cells. A. U2OS cells were treated with the indicated compounds (see <u>Table S2</u> for GI₅₀ values) for 48 hours. B. U2OS cells were treated with 3x GI₅₀ for 24 hours and BCL2A1 (BFL1) mRNA expression determined using ViewRNA technology. Each point represents an individual cell. Data is derived from 3 independent wells. Significance was determined by one-way ANOVA (***P < 0.001). C. U2OS cells were treated with 1x GI₅₀ of the indicated compounds for 48 hours.

(<u>Table S4</u>). Interestingly, there was no overlap in the transcription factors identified by ChEA3 in U2OS and HT29 cells treated with an equitoxic dose of V158411. Further work is required to understand the relevance of these transcription factors to BFL1 protein induction.

Gene Ontology enrichment analysis was conducted using ShinyGO on the differentially expressed genes with a log2 fold change > 3 and a false discovery rate < 0.01 (<u>Table S5</u>). In keeping with the above results, NF- κ B signalling was not identified as a pathway in which gene expression was enriched.

MEK, mTOR/PI3K or pan-TK kinase inhibitors abrogate V158411 induced BFL1 expression and restore BIM expression

PMA, a potent activator of PKC, increased BFL1 and decreased BIM expression in U2OS cells. Inhibition of PKC with sotrastaurin reversed these effects returning BFL1 and BIM protein to basal levels (Figure S5A). In comparison, sotrastaurin had little effect on BFL1 induction or BIM reduction by V158411. At the concentration used, sotrastaurin had no effect of U2OS cell viability (Figure S5B).

A range of kinase inhibitors (Table S6) were therefore screened for potential to inhibit the induction of BFL1 by V158411. From this, the MEK inhibitor trametinib, mTOR inhibitor AZD-8055, PI3K/mTOR inhibitor dactolisib, the PI3K inhibitor pictilisib, and the pan-tyrosine kinase inhibitors ponatinib, crizotinib and dovitinib all blocked BFL1 induction by V158411 (Figure 5A). IC₅₀ values for inhibition of BFL1-induction ranged from 41 nM to 1.6 µM (Figure 5B; Table S6). None of the kinase inhibitors dramatically affected the induction of DNA damage (as measured by increase in nuclear yH2AX) by V158411 (data not shown). Their effects on U2OS cell proliferation are highlighted in Table S5. In addition to reducing BFL1 induction by V158411, dactilosib, AZD8055, ponatinib and trametinib blocked the reduction in BIM1 (Figure 5C). As would be predicted from their mechanisms of action, trametinib inhibited the phosphorylation of ERK, and dactilosib and AZD8055 reduced AKT phosphorylation.

Inhibition of Chk1 increases AKT and ERK phosphorylation in U2OS cells

Inhibition of Chk1 in U2OS cells increased AKT and ERK phosphorylation (**Figure 6A**). These



Figure 3. Increased BFL1 expression following Chk1i was observed only in a subset of human cancer cell lines. A. LNCaP cells were treated with 1 or 3x GI_{50} of the indicated compounds for 48 hours. B. HeLa cells were treated with 100 ng/mL PMA for 4 hours, or 1 or 3x GI_{50} of the indicated compounds for 48 hours. C. Cells were treated with 0-3x GI_{50} of V158411 for 48 hours. D. Cells were treated with 3x GI_{50} of V158411 or AZD1775 for 48 hours. E. Cells were treated with 0-3x GI_{50} of V158411 for 48 hours. D. Cells were treated with 3x GI_{50} of V158411 or AZD1775 for 48 hours. E. Cells were treated with 0-3x GI_{50} of V158411 or AZD1775 for 48 hours. E. Cells were treated with 0-3x GI_{50} of V158411 for 48 hours.

changes appeared time (Figure 6B) and dose dependent (Figure 6C) with kinetics that appeared similar to the induction of BFL1 with 24-hour treatment necessary to increase pAKT. These results were confirmed by single cell immunofluorescent imaging with V158411 treatment increasing the fraction of cells staining positive for pAKT and pERK (Figure 6D; Figure S6A). This increase in pAKT occurred dose dependently following V158411 and AZD1775 treatment (Figure 6E) and correlated with increased V158411 DNA damage (as measured by increased nuclear vH2AX, Figure S7A). There was some variability in the fraction of cells that exhibited increased AKT phosphorylation between experiments. V158411 induces DNA damage in S-phase cells. As increased

pAKT correlated with increased γ H2AX, these differences in pAKT may reflect differences in the number of actively dividing U2OS cells at the point of V158411 addition.

Increased pAKT was only observed in U2OS cells treated with Chk1 inhibitors and not the topoisomerase inhibitors camptothecin and etoposide (Figures 6F, 6G, <u>S6B</u>, <u>S7B</u>). pERK was strongly induced by DNA damaging agents including gemcitabine, which did not induce BFL1 (Figure 6F) and to a greater extent than that induced by V158411. Increased AKT and ERK phosphorylation in response to Chk1 inhibitor treatment was observed in other cell lines. Increased pAKT was observed in SKOV3 cells treated with V158411 (Figure S7C) whilst



Figure 4. Chk1i-induced BFL1 expression appeared independent of NF-κB transcription factors. A. U2OS-NF-κB-RE reporter cells were treated with 3x Gl₅₀ V158411, AZD1775, LY2603618, camptothecin or etoposide, or 100 ng/mL PMA, 30 ng/mL TNF-α or 30 ng/mL IL-1β for 4-48 hours. Values are the mean \pm SD of 3 replicates. B. U2OS cells were treated with 10 ng/mL TNF-α for 30 minutes and the NF-κB nuclear intensity determined by single cell immunofluorescence (IF). C. U2OS cells were treated with 1 or 3x Gl₅₀ V158411 for 24 hours and the nuclear intensity of the indicated proteins determined by single cell IF. Each point represents an individual cell. Data is derived from 3 independent wells. Significance was determined by one-way ANOVA (*P < 0.05; **P < 0.01; ***P < 0.001). D. U2OS cells were treated with 3x Gl₅₀ V158411 for 24 hours.



Figure 5. MEK, mTOR/PI3K or pan-TK kinase inhibitors abrogate V158411 induced BFL1 expression and restore BIM expression. A. U2OS cells were treated with the indicated kinase inhibitors (see <u>Table S6</u> for concentrations) in combination with 3x GI_{50} V158411 for 24 hours. B. U2OS cells were treated with the indicated concentrations of kinase inhibitors in combination with 3x GI_{50} V158411 for 24 hours. C. U2OS cells were treated with 0.3 μ M kinase inhibitor plus 3x GI_{50} V158411 for 24 hours.

increased pERK was observed in SJSA-1 cells (Figure S7D). Gene Ontology enrichment analysis of the genes with upregulated expression identified significant fold enrichment in the MAPK cascade with 3.4-fold enrichment and a

false discovery rate of < 0.001 (<u>Table S6</u>). No differential gene expression of any of the main signalling components of the PI3K/AKT/mTOR/ MEK pathway after V158411 treatment was observed.



Figure 6. Chk1i and Wee1i increase AKT and ERK phosphorylation in U2OS cells. U2OS cells were treated with (A) 1x or 3x Gl₅₀ for 48 hours, (B) 3x Gl₅₀ for 1-48 hours, or (C) 0.1-3x Gl₅₀ for 48 hours. Protein expression was determined by western blotting. U2OS cells were treated with (D) 0-3x Gl₅₀ V158411 for 24 hours, (E) 0.1-3x Gl₅₀ V158411 or AZD1775 for 24 hours. Protein expression determined by single cell IF. Values are the mean of 3 independent wells \pm SD. (F and G) U2OS cells were treated with 1 or 3x Gl₅₀ of compound for 24 hours. Expression of indicated proteins was determined by single cell immunofluorescent imaging. Values are the mean of 3 independent wells \pm SD. Significance was determined by one-way ANOVA (*P < 0.05; **P < 0.01; ***P < 0.001).

Inhibition of BFL1 induction by trametinib was not limited to V158411

Trametinib appeared the most consistent kinase inhibitor in preventing BFL1 induction in U2OS cells by Chk1, Wee1 and topoisomerase inhibitors. BFL1 induction by V158411, LY260-3618, AZD1775, camptothecin and etoposide were all inhibited by trametinib and dactilosib (Figure 7A). In comparison, AZD8055 failed to block BFL1 induction by AZD1775 and, ponatinib, BFL1 induction by camptothecin. As well as blocking BFL1 induction, trametinib reversed the downregulation of BIM in U2OS cells treated with V158411, LY2603618, AZD1775, camptothecin or etoposide (Figure 7B). These effects were not limited to U2OS cells. In PANC-1 cells, trametinib inhibited BFL1 induction and BIM reduction following V158411, LY2603618, AZD1775, camptothecin or etoposide treatment (Figure 7C). Interestingly, trametinib reduced the high basal expression levels of BFL1 in the DMSO treated control PANC-1 cells.

BFL1 induction does not decrease Chk1 inhibitor induced apoptosis

BFL1 is a member of the Bcl-2 family of antiapoptotic proteins. In the A2058 cell line, which exhibits high basal levels of BFL1, V158411 induced high levels of apoptosis with around 70% staining positive for cleaved capsase-3/7 after 48 hours (**Figure 8A**). We have previously observed significant levels of apoptosis in HL60 cells, another cell line with high basal BFL1, after V158411 treatment [21]. Likewise, U20S readily underwent caspase-3/7 dependent apoptosis after V158411 treatment with around 63 and 67% of cells cleaved caspase-3/7 positive after 48 and 72 hours (**Figure 8A**). In the cell lines where little apoptosis was

Chk1 inhibition induces BFL1



Figure 7. MEK, mTOR/PI3K or pan-TK kinase inhibitors abrogate Chk1i, Wee1i or Topoisomerase inhibitor induced BFL1 expression and restore BIM protein levels. A. U2OS cells were treated with 0.3 μ M kinase inhibitor in combination with 3x GI₅₀ (Table S2) of the other indicated agents for 24 hours. B. U2OS cells were treated with 0.3 μ M Trametinib in combination with 3x GI₅₀ of the other indicated agents for 24 hours. C. PANC-1 cells were treated with 0.3 μ M Trametinib in combination with 3x GI₅₀ of the other indicated agents for 24 hours. C. PANC-1 cells were treated with 0.3 μ M Trametinib in combination with 3x GI₅₀ of the other indicated agents for 24 hours.

observed (MES-SA and SJSA-1), V158411 still inhibited the proliferation of these cells (Figure 8B). Given that BFL1 expression was upregulated in a subset of around 30% of U2OS cells, we examined BFL1 expression in attached (live) versus detached (dead) cells after V158411 treatment. At the earliest time point (48 hours), BFL1 was only detected in the adherent cells suggestive of some protective effect from V158411-induced apoptosis. However, after 72-hour treatment with V158411, significant amounts of BFL1 was detected in the detached as well as the attached cell populations (Figure 8C). These detached cells were apoptotic as determined by the detection of cleaved lamin A and cleaved PARP suggesting that these cells were still able to undergo caspase-dependent apoptosis despite the increased expression of BFL1. In the A2058 cells a similar pattern of BFL1 expression was observed with the detached, cleaved lamin A/ cleaved PARP positive population containing high levels of BFL1 (Figure 8D).

The effects of AZD8055 and trametinib, two kinase inhibitors that blocked BFL1 induction by V158411, on V158411 induced apoptosis and cell death were evaluated. In U2OS cells,

AZD8055 inhibited the induction of apoptosis by V158411 by 2-3 fold (Figure 8E). This actually translated into a greater reduction in cell proliferation compared to V158411 but not cell death. In comparison, trametinib increased the amount of V158411-induced apoptosis especially at the early time point of 24 hours and the lower concentration of 1x Growth Inhibitory 50 (GI₅₀) (Figure 8F). This translated into increased cell death at this concentration. However, at 3x GI₅₀ trametinib did not dramatically increase the amount of apoptosis or cell death above that observed with V158411 alone. The results with trametinib were somewhat complicated by the fact that trametinib as a single agent appeared to induce caspase-3/7 cleavage without any significant effects on cell proliferation or death. Finally, trametinib failed to potentiate cell killing by V158411 in PANC-1 cells. In the absence of trametinib, the V158411 GI 50 was 4.2 \pm 0.8 μ M compared to 4.1 \pm 1.5 μ M in combination with 1 µM trametinib.

Discussion

Numerous Chk1 inhibitors have been described to date and demonstrate exciting anti-tumour efficacy as single agents, in combination



Figure 8. Chk1 inhibition induces apoptosis despite increased BFL1 expression. (A) Cleaved Caspase 3/7 (CC3/7) and (B) total cells were determined using high content live cell imaging following treatment with 3x GI_{50} V518411. Values are the mean of 3 wells ± SD. (C) U2OS cells were treated with 3x GI_{50} V158411. At the indicated time points, attached (A) and detached (D) cells were separately lysed. Experiment was performed in triplicate and a representative experiment shown. (D) Cells were treated with 3x GI_{50} V518411 for 72 hours and analysed as in (C) (C, untreated control cells). U2OS cells were treated with 1 or 3x GI_{50} V518411 (V411) in combination with (E) 0.3 µM AZD8055 (AZD) or (F) 0.3 µM Trametinib (Tram). Apoptotic nuclei and relative cell number (determined as the cell number at time 0) were determined using live cell high content imaging. Values are the mean of 3 independent wells ± SD. Significance was determined by Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

with cytotoxic chemotherapy, and in combination with novel targeted therapies (such as PARP inhibitors). Despite these initially exciting pre-clinical results, no inhibitors have yet to progress beyond Phase 2 into Phase 3 registration trials with the majority terminating after

(••••••)				
Cell Line		BFL1 Prote	Apoptotic Cells (%)	
Example	Basal	+Chk1i	+Chk1i+MEKi	+Chk1i
U20S	Low	Increased	To basal	67
PANC1	High	Increased	Decreased cf basal	ND
A2058	High	No change	ND	71
SJSA-1	Low	No change	ND	25

 Table 2. Summary of cellular BFL1 response to Chk1 inhibition (Chk1i)

ND, not determined; MEKi, MEK inhibitor.

Phase 1 [6, 22, 23]. The reasons behind this are numerous and complex including (but not limited to) lack of efficacy, dose limiting toxicities (especially in combination), poor pharmaceutical properties (oral bioavailability, halflife), target patient population selection and drug combination selection.

To further evaluate the clinical potential of our own Chk1 inhibitor, V158411 [11], we utilised RNAseq analysis of V158411 treated cancer cells to evaluate the genome wide changes to inhibitor treatment. One of the most upregulated genes in response to V158411 treatment in the U2OS osteosarcoma cell line was BCL2A1 (BFL1). BFL1 is a member of the Bcl-2 family of anti-apoptotic proteins that also includes the much wider studied Bcl-2, Mcl-1 and Bcl-XL members [24, 25]. BFL1 has been implicated in the development of lymphomas and leukaemia's [26-28], and increased BCL2A1 is associated with resistance to the clinically approved Bcl-2 inhibitor Venetoclax [29, 30]. Upregulated BCL2A1/BFL1 has been identified in melanoma [31, 32] where it confers resistance to Braf inhibitors. Like the other members of the Bcl-2 family of proteins, BFL1 binds to pro-apoptotic Bcl-2 proteins (such as BAK and BAX) and proapoptotic BH3-only proteins (such as tBID, BIM, PUMA and NOXA) thereby preventing mitochondrial membrane permeability, cytochrome c release and caspase activation in response to apoptotic stimuli.

Chk1 inhibition by V158411 induces replication stress and DNA damage in a wide range of human cancer cell lines of differing tumour types [12, 21, 33]. Out of the 24 cell lines studied, U2OS cells were the only cell line with low basal BFL1 protein levels that were increased by V158411. The increase in BCL2A1 mRNA expression in response to Chk1 inhibitor induced DNA damage appeared restricted to a subset of around 30-40% of the U2OS cells. Three additional osteosarcoma cell lines were evaluated (HOSTE85, SAOS-2 and SJSA-1) but did not induce BFL1 protein in response to V158411-induced DNA damage. Increases were also observed in the HL60 myeloid leukaemia, and PANC-1 pancreatic cancer cell line but these two cell lines had high basal BFL1 expres-

sion levels. **Table 2** summarises the different BFL1 induction responses of example cell lines to Chk1 inhibition. The reason why U2OS, HL60 and PANC-1 cells were sensitive to BFL1 induction by V158411 was not immediately apparent. The cells all come from diverse tissue types (bone, blood and pancreas respectively), have no obvious common driver mutations, and vary widely in their sensitivity to growth inhibition by V158411 (0.82, 0.21 and 8.0 μ M respectively). Further work is ongoing to understand these differences in BFL1 induction response between different cell lines.

The increase in BCL2A1/BFL1 in U2OS cells was not limited to V158411 but was also induced by other Chk1 inhibitors, the Wee1 inhibitor AZD1775 and topoisomerase inhibitors. The topoisomerase inhibitors etoposide [18] and camptothecin [34] have both previously been demonstrated to increase BFL1. However, other inducers of DNA damage such as the cytotoxic chemotherapy drugs gemcitabine, hydroxyurea and cisplatin, and inhibitors of the DNA damage response proteins ATR, ATM and DNA-PKcs did not increase BFL1 expression. The fact that not all DNA damage resulted in BFL1 induction suggests that the cells are responding to either a specific kind of DNA damage and/or DNA damage induced at a specific point in the cell cycle.

In addition to increasing BCL2A1, V158411 increased BCL2L10 (Bcl-b) mRNA though to a much lesser extent (5.8-fold) than BCL2A1 (444-fold). Coupled to this was a decrease in BIM mRNA and protein levels. Likewise, there was a small increase in NOXA mRNA and protein levels. NOXA has been demonstrated to potently bind to BFL1 [35] and therefore increased NOXA expression may neutralise the anti-apoptotic functions of BFL1, or vice versa. Further evaluation of the effects of V158411

on NOXA expression may therefore be warranted. The mechanism by which BCL2A1 mRNA was increased in U2OS cells by V158411 so far appears unclear. Previous studies have demonstrated NF-kB as a significant transcriptional inducer of BCL2A1 [14, 16, 19]. NF-KB also upregulates Bcl-XL [14, 15, 36] with the two proteins often co-induced by NF-kB activating stimuli. Here we observed only an increase in BFL1 and not Bcl-XL and found no evidence for NF-kB activation in response to Chk1i, Wee1i or topoisomerasei treatment in U2OS cells. However, the U2OS cells still induced BFL1 in response to TNF- α , IL-1 β or PMA in an NF-kB dependent manner suggesting that this pathway is intact and functioning in U2OS cells. In melanoma cells, BCL2A1 is upregulated via MITF [31, 32] but as U2OS are osteosarcoma cells, it would appear unlikely that MITF is responsible in this cell line.

In prostate cancer, BCL2A1 was induced by both PMA and etoposide via a PKCδ-dependent mechanism [18]. V158411 induced BCL-2A1 via a different mechanism as the PKC inhibitor sotrastaurin inhibited BFL1 induction by PMA but not that by V158411. AKT and ERK phosphorylation were increased in V158411 treated U2OS cells and the induction of BFL1 by V158411 appeared dependent on signalling through the MEK/PI3K/AKT/mTOR dependent pathways. Inhibitors targeting these pathways effectively blocked BFL1 induction not only by Chk1 inhibitors but also by topoisomerase inhibitors as well. The MEK inhibitor Trametinib appeared the most active inhibitor of BFL1 induction by Chk1 or topoisomerase inhibitors. There is limited literature to date linking BCL2A1 with either the MEK or PI3K/AKT pathways. BCL2A1 has been demonstrated to be regulated by PI3K/AKT to control neutrophil survival and homeostasis [37] and eosinophil adhesion to IMR-32 cells increased BCL2A1 in a PI3K-MEK/ERK-NF-KB dependent mechanism to protect against neuronal induced apoptosis [38]. Clear synergy between Chk1 inhibitors and mTOR inhibitors [39-41] and MEK inhibitors [42-44] has been demonstrated with potentially the control of anti-apoptotic protein expression contributing to this.

A very recent study has demonstrated that BFL1 (BCL2A1) and Bcl-b (BCL2L10) are stabilised by Ubiquilin 4 (UBQLN4). UBQLN4 is a substrate of ATM and is phosphorylated (and thereby activated) in response to ATM-activating DNA damage including that induced by the topoisomerase inhibitor camptothecin [45]. BCL2A1 and BCL2L10 stabilisation by UBOLN4 reduced apoptosis in response to DNA damage in a mesothelioma cell model. Chk1 and Wee1 inhibitors activate ATM [12, 46, 47]. In this study, the induction of BFL1 protein by Chk1i, Wee1i and topoisomerasei-induced DNA damage appears to be via an increase in BCL2A1 mRNA with BCL2A1 mRNA increased over 400fold following V158411 treatment. BCL2L10 was also increased by V158411 but to a much lesser extent (around 5.8-fold). Likewise, inhibition of ATM with KU-60019 did not block increased BFL1 expression following V158411 treatment. However, increased protein stability through UBOLN4-induced stabilisation may contribute to increased BFL1 protein levels through reduced protein turnover.

Whilst BFL1 was induced following V158411induced DNA damage in U2OS cells, it did not appear to block apoptosis in these cells with high levels of BFL1 protein observed in apoptotic cells. Likewise cells with high basal levels of BFL1 (namely A2058 and HL60 [21]) appeared to readily undergo caspase dependent apoptosis in response to Chk1 inhibition. Blocking the V158411-induced induction of BFL1 with Trametinib did not increase the fraction of U2OS cells undergoing caspase-dependent apoptosis. There was still a subset of around 30-40% of the cells that were resistant to V158411-induced apoptosis. This is in direct contrast to studies with inhibitors of other Bcl-2 family proteins. The Bcl-2 inhibitor venetoclax (ABT-199) increased the fraction of apoptotic AML cells when combined with a Chk1 inhibitor [48] whilst the Bcl-2/Bcl-XL inhibitor navitoclax sensitised pancreatic cancer cells to apoptosis induced by the Chk1 inhibitor prexasertib [49].

This study relied on the transcriptional profiling of cancer cells treated with V158411. Whilst transcriptional profiling is relatively easy to perform, it does not give the absolute levels of protein compared to, for example, Reverse Phase Protein Arrays (RPPA) or quantitative proteomics. Another limitation of this study was that we analysed bulk gene expression changes. From the follow up studies, it was obvious that BFL1 induction (and therefore potentially other observed gene changes) occurred in a subset of treated cells. Single cell RNAseq analysis to identify changes co-associating with BFL1 induction would provide additional useful information. Chk1 inhibitors induce DNA damage predominantly in S-phase cells through increased replication stress. Therefore, the gene expression changes in response to V158411 treatment may be influenced by the fraction of cells in S phase at the time of treatment and the doubling rate of the cells.

In conclusion, DNA damage induced by Chk1, Wee1 or topoisomerase inhibitors increased BFL1 expression and downregulated BIM via an PI3K/AKT/MEK dependent (and NF-KB independent) pathway in a subset of U2OS cells. This increase in BFL1 appeared to be an attempt by the cells to block DNA damage induced apoptosis. This attempt to inhibit apoptosis was ultimately futile as the cells still underwent apoptosis. This strongly suggests that BFL1 induction following Chk1 inhibitor treatment is not a useful biomarker for stratifying patient responses to Chk1 inhibitor therapy. Further work is ongoing to further understand the mechanism of BFL1 induction in this cell model.

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Disclosure of conflict of interest

None.

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Target	MWT (kDa)	Supplier	Prod. Code	Spp.	Appl.	Dil.
BFL1	18	Abcam	ab246319	R	WB	1:1000
McI-1	40	SantaCruz	sc-819	R	WB	1:2500
Bcl-2	26	SantaCruz	sc-492	R	WB	1:2000
Bcl-XL	30	CST	2762	R	WB	1:5000
γH2AX	15	CST	9718	R	WB	1:5000
pChk1 (S345)	56	CST	2348	R	WB	1:2500
HSP90	90	Enzo	SPA-830	М	WB	1:5000
Chk1	56	CST	2360	М	WB	1:2500
β-actin	45	CST	4970	R	WB	1:5000
BIM	12, 15, 23	CST	2933	R	WB	1:1000
PUMA	23	CST	12450	Μ	WB	1:5000
NOXA	10	CST	14766	R	WB	1:1000
BID	15, 22	CST	2002	М	WB	1:1000
BAD	23	CST	9239	R	WB	1:1000
BAX	20	CST	5023	R	WB	1:1000
pAKT (S473)	60	CST	4060	М	WB	1:5000
					IF	1:400
AKT	60	CST	9272	R	WB	1:2500
pERK (T202/Y204)	42, 44	CST	9101	R	WB	1:5000
					IF	1:250
ERK	42, 44	CST	9102	М	WB	1:2500
Cleaved Lamin A	28	CST	2035	R	WB	1:2500
PARP	89, 116	CST	9542	R	WB	1:2500
NF-κB (p65)	65	CST	8242	R	WB	1:5000
					IF	1:400
рNF-кВ (р65) (S536)	65	CST	3033	R	WB	1:2500
NF-κB (p50)	50	CST	13586	R	WB	1:2500
					IF	1:200
NF-κB (p52)	52	CST	3017	R	WB	1:2500
RelB	70	Abcam	ab180127	R	WB	1:2500
					IF	1:500
c-Rel	78	CST	67489	R	WB	1:2500
					IF	1:800
ρΙκκα/β (S176/180)	85, 87	CST	2697	R	WB	1:1000
Ικκα	85	CST	11930	М	WB	1:2000
Ικκβ	87	CST	8943	R	WB	1:2000
plκBα (S32)	40	CST	2859	R	WB	1:1000
ΙκΒα	40	CST	4814	Μ	WB	1:2000
pSTAT3 (Y705)	79, 86	CST	9145	R	WB	1:2500
STAT3	79, 86	CST	9132	R	WB	1:2000
GAPDH	37	CST	2118	R	WB	1:10000

Table S1. Antibodies used in the study



Figure S1. Volcano plots of differential gene expression. Red dots, -log (false discovery rate) > 2.



Figure S2. Example images of BCL2A1 (red) or B2M (green) mRNA using ViewRNA technology in U2OS cells treated for 24 hours with DMSO or 3x GI_{50} V158411 for 24 hours. Arrows indicate example cells with increased BCL2A1 mRNA.

Coll Lino	Gl ₅₀ (μM)						
Cell Line	AZD1775	LY2603618	Camptothecin	Etoposide	Gemcitabine	Cisplatin	
PANC-1	0.29	7.3	0.01	1.6	0.023	6.8	
MES-SA			0.003	0.21	0.016	1.1	
SJSA-1			0.013	1.7	0.012	1.7	
U20S	0.27	0.67	0.25	4.2	0.012	7.5	
HeLa	0.31	2.6	0.30	27.7	0.018	15.6	
LNCaP	0.55	1.3	0.031	14.4	0.021	16.1	

Table S2. GI_{50} values of compounds used



Figure S3. A. U2OS cells were treated with 100 ng/mL PMA, 30 ng/mL TNF- α or 30 ng/mL IL-1 β for 4 or 24 hours, or 1 or 3x GI₅₀ V158411 for 48 hours. B. LNCaP cells were treated with 100 ng/mL PMA for 4 or 24 hours or 3x GI₅₀ V158411 for 0-72 hours. C and D. Cells were treated with 3x GI₅₀ of the cytotoxic chemotherapeutic agents for 48 hours. E. PANC-1 cells were treated with 3x GI₅₀ of the indicated compounds for 48 hours. Protein expression was determined by western blotting.

Cell Line	Tumour Type	Key Mutations	V158411 Gl ₅₀ (µM)	BFL1 Exp (CCLE LOG2)
U20S	Osteosarcoma	CCND2	0.82	-5.73
A2058	Metastatic melanoma	BRAF, TP53	0.26	3.48
AsPC-1	Pancreatic adenocarcinoma	BRAF, KRAS, TP53	1.2	-5.18
BxPC3	Pancreatic adenocarcinoma	BRAF, TP53	0.13	0.10
CAPAN-1	Pancreatic adenocarcinoma	KRAS	0.35	-2.64
HCT116	Colorectal carcinoma	KRAS, PIK3CA	2.0	-13.00
HeLa	Cervical adenocarcinoma	HPV+ve	0.97	-6.03
HL60	Acute promyelocytic leukaemia	NRAS, TP53	0.21	2.91
HOSTE85	Osteosarcoma		0.18	ND
HT29	Colorectal adenocarcinoma	BRAF, PIK3CA, TP53	0.65	-4.26
LNCaP	Prostate carcinoma	PTEN	0.27	-4.71
LoVo	Colorectal adenocarcinoma	KRAS, BRAF, CRAF	0.16	-6.11
MCF-7	Breast adenocarcinoma	PIK3CA	2.0	-4.20
MDA-MB-231	Breast adenocarcinoma	KRAS, BRAF, TP53	0.40	-4.40
MES-SA	Uterine sarcoma	CRAF, PTEN	0.29	-13.00
MiaPaCa-2	Pancreatic carcinoma	KRAS, TP53	0.16	-6.81
MV4;11	Biphenotypic B myelomonocytic leukaemia	FLT3	0.063	-1.67
NCI-H1975	Non-small cell lung adenocarcinoma	EGFR, PIK3CA, TP53	12.0	-2.12
PANC-1	Pancreatic adenocarcinoma	KRAS, TP53	8.0	2.73

	Table S3.	V158411 G	and	l mutation	status	of	cancer	cell	lines
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SAOS-2	Osteosarcoma	RB1	0.37	-13.00
SJSA-1	Osteosarcoma	KRAS, NRAS, TP53	0.61	-2.85
SKBr-3	Breast adenocarcinoma	TP53	0.022	-6.07
SKOV3	Ovarian adenocarcinoma	PIK3CA, TP53	0.28	-5.06
THP-1	Acute monocytic leukaemia	NRAS	0.22	-3.14

Chk1 inhibition induces BFL1

ND, not determined.



Figure S4. U2OS cells were treated with 1 or $3x \text{ Gl}_{50}$ V158411 for 24 hours and the nuclear intensity of the indicated proteins determined by single cell IF.

		, 0		LIT20	
	U20S			H129	
Rank	TF	Score	Rank	TF	Score
1	CSRNP1	2.5	1	MEIS3	9
2	FOSB	7.333	2	KCNIP3	14.5
3	NR4A3	7.667	3	HEYL	20.67
4	ATF3	11.5	4	CSRNP3	22
5	SNAI1	12.33	5	DPF1	38
6	FOSL1	12.6	6	FOXS1	47.33
7	JUN	14.5	7	CENPA	52
8	FOS	16.4	8	MYT1	54.67
9	EGR2	17	9	SP7	60
10	JUNB	18.75	10	FOXG1	64

Table S4	Transcription	factor	analysis	using	ChE43
Table 34.	паньсприон	ιασιοι	anaiyəiə	using	CHLAS

Pathway	Fold Enrichment	nGenes	Pathway Genes	Enrichment FDR
Cell surface receptor signalling pathway	2.29	57	3363	1.75E-06
Cell differentiation	1.89	65	4637	5.24E-05
Animal organ development	2.01	58	3893	5.24E-05
Cellular developmental process	1.85	65	4731	8.65E-05
MAPK cascade	3.43	24	944	9.88E-05
Response to lipopolysaccharide	5.31	15	381	9.88E-05
Regulation of response to stimulus	1.83	64	4707	9.88E-05
Anatomical structure morphogenesis	2.16	46	2871	1.06E-04
Cell activation	2.60	32	1658	1.45E-04
Response to molecule of bacterial origin	5.01	15	404	1.45E-04
Regulation of localization	2.10	46	2951	1.45E-04
Leukocyte activation	2.74	30	1475	1.45E-04
Cellular response to molecule of bacterial origin	6.35	12	255	1.45E-04
Response to bacterium	3.64	20	741	1.70E-04
Positive regulation of signal transduction	2.54	31	1644	2.93E-04
Cellular response to biotic stimulus	5.78	12	280	2.93E-04
Tissue development	2.28	37	2190	3.06E-04
Cellular response to chemical stimulus	1.91	52	3680	3.08E-04
Anatomical structure formation involved in morphogenesis	2.85	25	1183	4.15E-04
Cellular response to lipopolysaccharide	6.06	11	245	4.15E-04
Response to external stimulus	1.93	48	3352	5.22E-04
Circulatory system development	2.87	24	1127	5.22E-04
Positive regulation of cell communication	2.35	32	1835	6.74E-04
Response to oxygen-containing compound	2.36	32	1832	6.74E-04
Positive regulation of signalling	2.34	32	1842	7.00E-04
Cell migration	2.46	29	1590	7.76E-04
Regulation of molecular function	1.91	47	3321	7.76E-04
Myeloid leukocyte activation	3.26	19	785	8.03E-04
Response to lipid	2.92	22	1017	8.28E-04
Cellular response to organic substance	1.95	44	3045	9.11E-04

Table S5. Gene Ontology enrichment analysis



Figure S5. A. U2OS cells were treated with 100 ng/mL PMA for 4 or 24 hours, or 3x GI_{50} V158411 (V411) in the absence or presence of 1 μ M sotrastaurin. B. Effects of sotrastaurin on U2OS cell viability following 72-hour incubation.

Inhibitor	Kinases Inhibited	Conc Tested (µM)	Inhibition of BFL1 Induction (%)	Inhibition of BFL1 IC ₅₀ (µM)	72 h GI ₅₀ (µM)
KU-60019	ATM	3	9.5		
NU7441	DNA-PKcs	3	2.2		
Dactolisib	ΡΙ3Κα/β/δ/γ, mTOR, ATR	1	91.9	0.041	0.017
AZD8055	mTOR	1	83.4	0.15	0.055
Gefitinib	EGFR	1	4.0		
Dasatinib	Abl, Src, c-Kit	1	45.6		
Sunitinib	VEGFR2, PDGFRβ, c-Kit	1	19.7		
Imatinib	Abl, c-Kit, PDGFR	1	-24.9		
Ponatinib	Abl, PDGFRα, VEGFR2, FGFR1, Src	1	88.9	0.070	0.58
Selumetinib	MEK1/2	1	33.0	1.6	13.8
Trametinib	MEK1/2	1	96.6	0.066	0.23
Sorafenib	c-Raf, B-raf, VEGFR2	1	-4.0		
PF 3644022	MAPKAPK2	3	-21.8		
SB 202190	ρ38 ΜΑΡΚα/β	3	-17.9		
Selicilib	CDK1, CDK2, CDK5	3	15.1		
Neratinib	HER2, EGFR	1	30.8		
Crizotinib	c-Met, ALK, Ros	3	66.3	1.0	2.6
Buparlisib	ΡΙ3Κα/β/δ/γ	3	61.6		
Dovitinib	FLT3, c-Kit, FGFR1-3, VEGFR1-4 (InsR, EGFR, c-Met, EphA2, Tie2, IGFR1, HER2)	3	93.0	0.56	1.6
Linsitinib	IGF-1R, InsR	3	-4.6		
Pictilisib	ΡΙ3Κα/δ	3	73.3	> 3	0.59
ND-2158	IRAK4	3	38.4		
Tofacitinib	JAK1/2/3	3	17.0		

Table S6. Kinase Inhibitor screen-list of kinase inhibitors used



Figure S6. A. Example images of U2OS cells treated with 0 or $3x \text{ GI}_{50}$ V158411 for 24 hours and protein expression determined by single cell immunofluorescent imaging. B. U2OS cells were treated with $3x \text{ GI}_{50}$ of compound for 24 hours. Example IF images of U2OS cells are shown.



Figure S7. A. Dot plot of mean nuclear γ H2AX versus mean nuclear pAKT (S473) following 24-hour treatment of U2OS cells with 3x GI₅₀ V158411. Each dot represents a single cell. B. U2OS cells were treated with 1x GI₅₀ of the indicated compounds for 48 hours. C. Cells were treated with 3x GI₅₀ of the indicated compounds for 48 hours. D. Cells were treated with 0-3x GI₅₀ V158411 for 48 hours.