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PRECLINICAL STUDIES

Dual targeting of bromodomain-containing 4 by AZD5153 and BCL2 by AZD4320 against B-cell lymphomas concomitantly overexpressing c-MYC and BCL2

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Summary
Despite the recent therapeutic progress, the prognoses of diffuse large B-cell lymphomas (DLBCLs) that concomitantly overexpress c-MYC and BCL2, i.e., double hit lymphoma (DHL) and double expressing lymphoma (DEL), remain poor. This study examined triple targeting of c-MYC, BCL2 and the B-cell receptor (BCR) signaling pathway for DHL and DEL. We first used AZD5153, a novel bivalent inhibitor for bromodomain-containing 4 (BRD4), in DHL- and DEL-derived cell lines, because BRD4 regulates disease type-oriented key molecules for oncogenesis. AZD5153 was more effective than conventional monovalent BRD4 inhibitors, JQ1 and I-BET151, in inhibiting cell proliferation of a DHL-derived cell line and two DEL-derived cell lines, with at least 10-fold lower half growth inhibitory concentrations. AZD5153 caused G1/S cell cycle blockade, while the apoptosis-inducing effect was relatively modest. At the molecular level, AZD5153 was potent in downregulating various molecules for oncogenesis, such as *c-MYC, AKT2* and *MAP3K*; those involved in the BCR signaling pathway, such as *CD19*, *BLNK* and *CD79B*; and those associated with B-cell development, such as *IKZF1*, *IKZF3, PAX5, POU2AF1* and *EBF1*. In contrast, AZD5153 did not decrease anti-apoptotic BCL2 proteins, and did not activate pro-apoptotic BH3-only proteins, except BAD. To augment cell death induction, we added a novel BH3-mimicking BCL2 inhibitor AZD4320 to AZD5153, and found that these two agents had a mostly synergistic antitumor effect by increasing cells undergoing apoptosis in all three cell lines. These results provide a rationale for dual targeting of BRD4 and BCL2 using AZD5153 and AZD4320 as a therapeutic strategy against DHL and DEL.

Keywords BRD4 inhibitor · AZD5153 · C-MYC · BCL2 · BH3-mimetic

Introduction

Diffuse large B-cell lymphoma (DLBCL), which is the most prevalent among non-Hodgkin lymphomas (NHLs), is a highly heterogenous disease subtype with diverse cytogenetic and genetic features that are closely associated with clinical manifestations, aggressiveness, treatment response and prognosis. For instance, DLBCL has been molecularly subcategorized into three gene expression profiling (GEP)-defined major cell of origin-based subtypes: germinal center B-cell-like (GCB), activated B-cell-like (ABC), and primary mediastinal DLBCL, and prognoses differ among subtypes [1, 2].

The treatment outcomes of most patients with DLBCL have been greatly improved by the advent of rituximab-containing immunochemotherapy, but those of approximately 20–30% of DLBCL cases harboring concomitant high expression of *a bona fide* oncogene c-MYC and an apoptosis-inhibitor BCL2 remain extremely poor, even in cases treated by intensive immunochemotherapy, including that supported by autologous or allogeneic hematopoietic stem cell transplantation [3–8]. Overexpression of c-MYC and BCL2 occurs *via* various

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mechanisms in DLBCL. Concomitant chromosomal translocations involving *c-MYC* and *BCL2* rearrangements account for approximately 5–10% of DLBCL cases, and these are currently designated as double hit lymphoma (DHL). On the basis of distinct cytogenetics and clinical outcomes from DLBCL, the 2016 revision of the World Health Organization (WHO) classification has separated DHL from DLBCL and generated a new category for DHL that is designated as high-grade B-cell lymphoma with translocations involving c-MYC and BCL2 or BCL6 [3, 6, 9]. Unregulated overexpression of c-MYC is also induced through insertional mutagenesis, gene amplification, and deregulation of *PVT-1* mRNA or microRNAs [10–14], while BCL2 overexpression is induced by gene amplification, transcriptional upregulation or deregulation of microRNA in approximately 20–40% of DLBCL patients [14–16]. As such, DLBCL overexpressing c-MYC and BCL2 through translocation-unrelated molecular mechanisms has been designated as double expressing lymphomas (DEL) [6]. DHL is more common in GCB DLBCL, and the impact of *BCL2* translocation on poor outcome is limited to the GCB subtype in the context of *c-MYC* translocation. In contrast, DEL occurs in both GCB and ABC subtypes, and confers a similar poor prognosis on the two subtypes. In DEL, increased c-MYC protein expression is the only predictor of a poor outcome in a case with increased BCL2 [4, 5, 15].

Regardless of the nature of the underlying mechanisms, c-MYC overexpression promotes indefinite cell proliferation, genomic instability and immune escape [17], while BCL2 overexpression provides resistance to cell death stimuli, including both mitochondria- and death receptor-mediated apoptosis, in neoplastic B cells [18, 19]. Accordingly, concomitant overexpression of c-MYC and BCL2 displays strong synergy for lymphomagenesis [20]. While our previous study indicated that cell survival or death depends more on BCL2 than on c-MYC in DEL, c-MYC is still an attractive therapeutic target, since c-MYC inhibition synergistically augments the antitumor effect of BCL2 inhibition in DEL-derived cell lines [11, 21, 22]. In addition to c-MYC and BCL2, B-cell receptor (BCR) signaling is also a central therapeutic target in DLCBL, since the activation of 'chronic' or 'tonic' BCR signaling is essential for proliferation and survival of neoplastic B cells in DLBCL. GCB DLBCL relies exclusively on tonic BCR signaling, which activates the AKT pathway, while chronic active BCR signaling, which interacts with the NF-kappa B signaling pathway, is required for cell survival in ABC DLBCL [23]. Indeed, development of various agents, such as ibrutinib against BTK or lenalidomide for IKZF, has underscored the importance of targeting of pathways for BCR signaling or B-cell development in DLBCL, while their efficacy has been mostly limited to ABC DLBCL.

Bromodomain-containing 4 (BRD4), a member of the bromodomain and extra-terminal domain (BET) protein family, promotes expression of pivotal molecules in disease development, maintenance and progression in various cancers, including lymphoma. c-MYC has been repeatedly reported as a BRD4 target regardless of cancer type, while BRD4 also targets diverse disease-specific "driver" and "suppressor" molecules for cancer development and progression in a disease context-dependent manner. Monovalent BRD4 inhibitors JQ1 and I-BET exert their anti-tumor effects *via* induction of apoptosis and cell senescence, which are accompanied by c-MYC downregulation in DLBCLs and multiple myeloma [21, 24–34]. Therefore, several BRD4/BET inhibitors are currently under clinical development as cancer treatment [35, 36]. AZD5153 is a novel potent, selective, and orally available BET/BRD4 bromodomain inhibitor that bivalently ligates two bromodomains in BRD4 simultaneously, and therefore, is chemically distinct from the commonly described benzodiazepine-based monovalent molecules, such as I-BET151 and JQ1. The bivalent binding mode allows for efficient displacement of BRD4 from chromatin at lower drug concentrations, and is expected to give increased cellular and antitumor activity compared with monovalent inhibitors [37, 38]. Given the MYC-mediated synthetic lethality concept that a MYC-driven tumor is under dual control of MYC and non-MYC oncogenes and that MYC-dependent tumor cells may be killed by targeting partner oncogenes of MYC [39], in this study we investigated the cellular and molecular effects of AZD5153 on DEL- and DHL-derived cell lines, and further explored a strategy for triple targeting of c-MYC, BCL2 and the BCR signaling pathway against DEL and DHL.

Materials and methods

Cell lines and reagents

Two DEL-derived cell lines, KPUM-MS3 and KPUM-UH1 [11], and a DHL-derived cell line, STR-428 (JCRB Cat# JCRB 1384) were used in the study. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin in a highly humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Two conventional BRD4 inhibitors, JQ1 (Cat# 1268524–69-1) and I-BET151 (GSK1210151A), were purchased from Sigma Aldrich (St. Louis, MO), and a BH3-mimetic, ABT-263 (Cat# 923564–51-6), was obtained from Cayman Chemical (Ann Arbor, MI). A novel BRD4 inhibitor, AZD5153, ((3R)-4-[2-[4-[1-(3-Methoxy-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-4-piperidyl]phenoxy]ethyl]-1,3-dimethyl-piperazin-2-one), a novel BH3-mimicking dual BCL2/BCLxL inhibitor, AZD4320, and a novel MCL1 specific inhibitor, AZD5991, were provided by Oncology, IMED Biotech Unit, AstraZeneca R&D (Boston, MA). The study was conducted with the approval of the institutional ethical review board of Kyoto Prefectural University of Medicine.

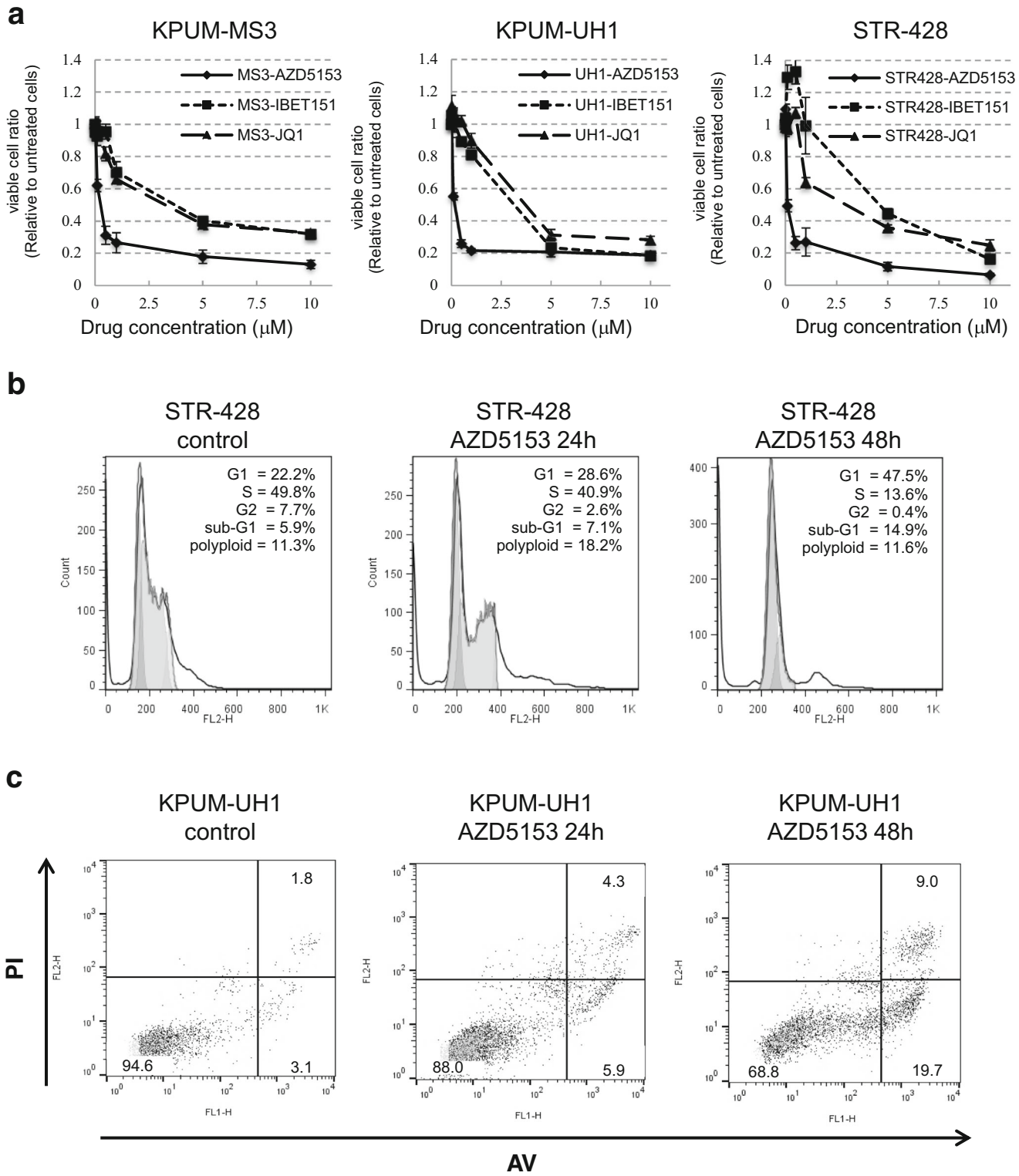

Fig. 1 Antitumor effects of AZD5153 on DEL- and DHL-derived cell lines. **a** Growth inhibitory effects of AZD5153 on KPUM-MS3, KPUM-UH1, and STR-428 cells. Cells were treated by the indicated concentrations of AZD5153, I-BET151, or JQ1 for 72 h, and subjected to a modified MTT assay. **b** Cells were treated at the IC_{50} of AZD5153 for 24 and 48 h, and subjected to DNA content analysis using flow cytometry. Cell cycle distribution was calculated using FLOWJO software. AZD5153 induced G1/S blockade in all three cell lines. Data in STR-428 cells are shown as representative. **c** Cells were treated at the IC_{50} of AZD5153 for 24 and 48 h, subjected to Annexin-V (AV)/propidium iodide (PI)-counterstaining, and analyzed using flow cytometry. AZD5153 caused a substantial increase in cells undergoing apoptosis in all three cell lines. Data in KPUM-UH1 cells are shown as representative

Assay for growth inhibition

Cells seeded at 2.0×10^5 cells/ml were treated with various concentrations of AZD5153, JQ1, I-BET151, AZD4320, ABT263 and AZD5991 for 72 h and the growth inhibitory effect was evaluated by a modified MTT assay using a Cell Counting Kit-8 (Dohjindo Molecular Technologies, Japan). Values are expressed as the mean ± standard error (S.E.) of three independent experiments in cell proliferation assays.

Assays for apoptosis and cell cycle

For examination of apoptosis, cells were counterstained with Annexin V-FITC (AV) and propidium iodide (PI), and subjected to flow cytometric analysis. For DNA content (cell cycle) analysis, cells were permeabilized and fixed with ice-cold 70% ethanol overnight, stained with PI, and then analyzed by flow cytometry using a FACS Calibur (BD Biosciences, NJ). Data obtained were analyzed using FLOWJO software Ver. X (Tomy Digital Biology, Tokyo, Japan).

In vitro drug combination assays

To examine the combinatory growth inhibitory effects of AZD5153 and AZD4320, cells were treated with five concentrations (0.25, 0.5, 1.0, 2.0, 4.0 x IC_{50}) for 72 h, and subjected to a modified MTT assay. Fractional effect concentrations (i.e., a fractional effect of 0.25 equals a 25% growth inhibitory effect) and the combination index (CI) were calculated with CalcuSyn (Biosoft, Cambridge, UK). This method facilitates

Table 1 Top 30 genes commonly upregulated by AZD5153 in KPUM-UH1, STR-428 and KPUM-MS3 cells

Gene symbol		Gene description	KPUM-UH1 (−fold)	STR-428 (−fold)	KPUM-MS3 (−fold)	Mean (−fold)
1	*SERPINI1*	serpin peptidase inhibitor, clade I (neuroserpin), member 1	11.41	3.89	3.87	6.39
2	*HEXIM1*	hexamethylene bis-acetamide inducible 1	4.52	8.00	2.84	5.12
3	*SAT1*	spermidine/spermine N1-acetyltransferase 1	3.25	2.07	9.22	4.85
4	*TMEM2*	transmembrane protein 2	4.13	2.11	4.92	3.72
5	*JUN*	jun proto-oncogene	1.80	5.16	3.70	3.55
6	*ELL2*	elongation factor, RNA polymerase II, 2	4.77	1.63	3.85	3.42
7	*SLFN5*	schlafen family member 5	3.14	3.07	4.00	3.40
8	*ZSWIM6*	zinc finger, SWIM-type containing 6	3.12	2.85	4.03	3.33
9	*NEU1*	sialidase 1 (lysosomal sialidase)	3.44	2.92	3.47	3.28
10	*NMT2*	N-myristoyltransferase 2	3.56	4.01	2.12	3.23
11	*CSRNP2*	cysteine-serine-rich nuclear protein 2	3.97	2.79	2.73	3.16
12	*CCDC92*	coiled-coil domain containing 92	3.36	4.05	2.06	3.16
13	*HIST2H4A*	histone cluster 2, H4a	3.61	2.35	2.98	2.98
14	*SESN3*	sestrin 3	2.52	2.48	3.93	2.98
15	*TXNIP*	thioredoxin interacting protein	2.63	4.23	2.02	2.96
16	*TM7SF2*	transmembrane 7 superfamily member 2	1.98	3.22	3.59	2.93
17	*PEG10*	paternally expressed 10	2.24	1.62	4.88	2.92
18	*DCXR*	dicarbonyl/L-xylulose reductase	2.35	3.84	2.31	2.83
19	*PLXNA3*	plexin A3	2.75	2.60	2.84	2.73
20	*RNF19B*	ring finger protein 19B	3.65	1.97	2.49	2.70
21	*ARHGAP26*	Rho GTPase activating protein 26	2.28	2.49	3.33	2.70
22	*CNNM4*	cyclin M4	2.35	2.40	3.22	2.66
23	*CAPRIN2*	caprin family member 2	3.32	2.04	2.55	2.64
24	*LPAR3*	lysophosphatidic acid receptor 3	2.85	2.24	2.78	2.62
25	*SLC6A8*	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	2.04	1.99	3.77	2.60
26	*TAX1BP3*	Tax1 (human T-cell leukemia virus type I) binding protein 3	3.23	2.03	2.52	2.60
27	*C1orf63*	chromosome 1 open reading frame 63	2.12	3.11	2.55	2.59
28	*TESK2*	testis-specific kinase 2	3.06	2.05	2.65	2.59
29	*ARRDC3*	arrestin domain containing 3	2.58	2.56	2.57	2.57
30	*WDR47*	WD repeat domain 47	3.22	2.12	2.36	2.57

quantification of synergism (CI < 1) and antagonism (CI > 1) at different doses and effect levels. CI calculations were conducted on the assumption that drug mechanisms were not mutually exclusive. To examine combinatory effects on induction of apoptosis, cells were treated at IC_{50s} of AZD5153, AZD4320 or their combination for 24 h, and were subjected to an assay for apoptosis using flow cytometric analysis.

Microarray analysis and signal pathway analysis

KPUM-MS3, KPUM-UH1 and STR-428 cells were treated without or with AZD5153 at IC_{80} for each cell line for 12 h. Total RNA was isolated using a mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA), and the gene expression prolife (GEP) was analyzed with Affymetrix Gene Chip arrays and GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Array data analysis was carried out with Affymetrix GeneChip operating software ver. 1.0., and genes showing at least a 1.5- or 0.67-fold difference in expression levels from those of untreated cells were considered to be positive. Signal pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (RQ-PCR)

Total RNA was extracted as described, and complementary DNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK). The mRNA levels were analyzed with an ABI Prism 7300 system (Applied Biosystems, Branchburg, NJ). RQ-PCR was performed using Power Up SYBR® Green Master Mix with UNG with a 7300 Real Time PCR System (Applied Biosystems) and the following primers: MYC-S: 5'-TCG GAT TCT CTG CTC TCC TC-3′ and MYC-A: 5'-TCC TCA TCT TCT TGT TCC TCC TC-3′ for detection of *c-MYC*, BLNK-S: 5′-AAA GCA CCT CCA AGT GTT CC-3′ and BLNK-A: 5′-TGA GTC CGA GTG CTC ATC TG-3′ for *BLNK*, MAPK3-S: 5'-CCT GGA AGC CAT GAG AGA TG-3′ and MAPK3-A: 5′-TGT TGA TGA GCA GGT TGG AG-3′ for *MAPK3*, CD19-S: 5'-TTC TTC CTC CTC TTC CTC ACC-3′ and CD19-A: 5'-CCC AGG CTG AGT TTT AAG AAG G-3′ for *CD19*, AKT2-S: 5′-TGG TAT ACC GCG ACA TCA AG-3′ and AKT2-A: 5'-CCC ACA GAA GGT TTT CAT GG-3′ for *AKT2*, BCL2L1-S: 5′-ATG CAG GTA TTG GTG AGT CG-3′ and BCL2L1-A: 5'-CTG CTG CAT TGT TCC CAT AG-3′ for *BCL2L1*, CD79B-S: 5'-CCA AAG GAT

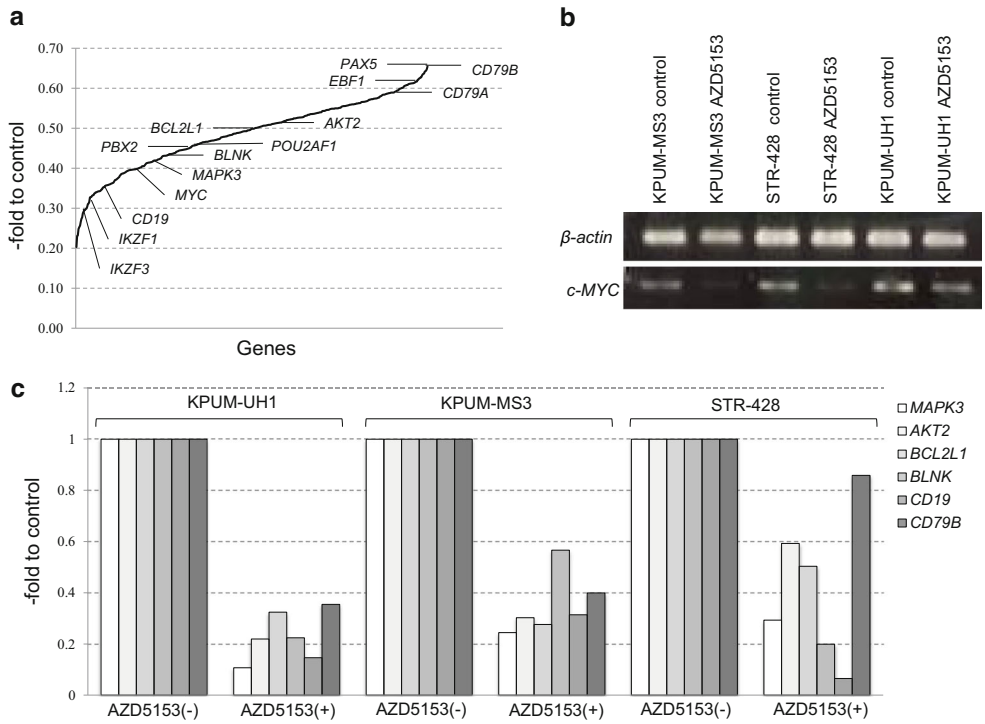

Fig. 2 Effect of AZD5153 on gene expression in DEL- and DHL-derived cell lines. **a** Expression levels of 494 genes downregulated by 1.5-fold in KPUM-MS3, KPUM-UH1 and STR-428 cells treated by AZD5153. Expression levels were 1.0 in untreated cells. Several genes involved in lymphomagenesis, B-cell receptor signaling and B cell development are listed. **b** Reverse transcriptase-polymerase chain reaction (RT-PCR) for *c-MYC* in the three cell lines with or without treatment with AZD5153. **c** Expression levels of *MAP3K, AKT2, BCL2L1, BLNK, CD19* and *CD79B* determined by quantitative RT-PCR in the three cell lines treated by AZD5153. Expression levels were 1.0 in untreated cells. Cells were exposed to the IC_{80} of AZD5153 for 12 h throughout these experiments

TCA GCA CCT TG-3′ and CD79B-A: 5’-TCA TAG GTG GCT GTC TGG TC-3′ for *CD79B*, BCL2-S: 5′-TGG ATG ACT GAG TAC CTG AAC C-3′ and BCL2-A: 5′-AAT CAA ACA GAG GCC GCA TG-3′ for *BCL2*, ACTB-S: 5’-GTC TTC CCC TCC ATC GTG-3′ and ACTB-A: 5′-AGG TGT GGT GCC AGA TTT TC-3′ for *ACTB* as an internal control.

Western blotting

Western blot analysis was performed as described previously [11, 40], using primary antibodies against β-actin (Sigma-Aldrich Cat# A2228), BCL2 (clone Bcl-2-100; Millipore Cat# 05–729, Upstate, Lake Placid, NY), BCL_XL (Santa Cruz Biotechnology Cat# sc-8392), MCL1(Santa Cruz Biotechnology Cat# sc-819), c-MYC (Santa Cruz Biotechnology Cat# sc-788), BID (Cell Signaling Technology Cat# 2002), PARP (Cell Signaling Technology Cat# 9542), AKT (Cell Signaling Technology Cat# 9272), Phospho-BAD (Ser112) (Cell Signaling Technology Cat# 9291S, Beverly, MA), BIM (Enzo Life Sciences Cat# ALX-804-527), BAD (Enzo Life Sciences Cat# ADI-AAP-020) and NOXA (Enzo Life Sciences Cat# ALX-804-408).

Table 2 Top 30 genes commonly downregulated by AZD5153 in KPUM-UH1, STR-428 and KPUM-MS3 cells

Gene symbol		Gene description	KPUM-UH1 (−fold)	STR-428 (−fold)	KPUM-MS3 (−fold)	Mean (−fold)
1	*ACSL5*	acyl-CoA synthetase long-chain family member 5	0.21	0.17	0.23	0.20
2	*MTMR2*	myotubularin related protein 2	0.38	0.12	0.19	0.23
3	*SIT1*	signaling threshold regulating transmembrane adaptor 1	0.29	0.27	0.14	0.23
4	*SASH3*	SAM and SH3 domain containing 3	0.38	0.20	0.16	0.25
5	*ZNF532*	zinc finger protein 532	0.32	0.24	0.20	0.25
6	*GTF3C6*	general transcription factor IIIC, polypeptide 6, alpha 35 kDa	0.35	0.20	0.22	0.26
7	*LRRFIP1*	leucine rich repeat (in FLII) interacting protein 1	0.34	0.18	0.27	0.27
8	*ALKBH8*	alkB, alkylation repair homolog 8 (*E. coli*)	0.31	0.30	0.21	0.27
9	*NSMAF*	neutral sphingomyelinase (N-SMase) activation associated factor	0.40	0.20	0.23	0.28
10	*IKZF3*	IKAROS family zinc finger 3 (Aiolos)	0.39	0.21	0.26	0.29
11	*PRDM10*	PR domain containing 10	0.20	0.20	0.48	0.29
12	*ZHX2*	zinc fingers and homeoboxes 2	0.34	0.29	0.25	0.29
13	*RNF214*	ring finger protein 214	0.37	0.35	0.17	0.30
14	*TSGA14*	testis specific, 14	0.38	0.30	0.21	0.30
15	*TCERG1*	transcription elongation regulator 1	0.33	0.37	0.20	0.30
16	*TMBIM4*	transmembrane BAX inhibitor motif containing 4	0.31	0.34	0.26	0.30
17	*TH1L*	TH1-like (Drosophila)	0.30	0.39	0.24	0.31
18	*MED24*	mediator complex subunit 24	0.38	0.30	0.25	0.31
19	*C1orf163*	chromosome 1 open reading frame 163	0.36	0.33	0.26	0.32
20	*ZMYND8*	zinc finger, MYND-type containing 8	0.48	0.29	0.21	0.33
21	*DCPS*	decapping enzyme, scavenger	0.43	0.34	0.21	0.33
22	*ITGA4*	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	0.44	0.19	0.35	0.33
23	*MEST*	mesoderm specific transcript homolog (mouse)	0.39	0.27	0.33	0.33
24	*IKZF1*	IKAROS family zinc finger 1 (Ikaros)	0.53	0.31	0.16	0.33
25	*HNRNPD*	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa)	0.33	0.31	0.36	0.33
26	*APBB1IP*	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	0.33	0.40	0.28	0.34
27	*IPO8*	importin 8	0.52	0.30	0.20	0.34
28	*MME*	membrane metallo-endopeptidase	0.50	0.29	0.22	0.34
29	*SENP1*	SUMO1/sentrin specific peptidase 1	0.42	0.36	0.24	0.34
30	*DOK3*	docking protein 3	0.40	0.26	0.36	0.34

Results

Effect of AZD5153 on DEL- and DHL-derived cell lines

We first examined the growth inhibitory effects of three BRD4 inhibitors (AZD5153, I-BET151 and JQ1) on KPUM-MS3, KPUM-UH1, and STR-428 cells by modified MTT assay. All three agents inhibited the growth of the three cell lines in a dose-dependent manner, and AZD5153 was more potent compared with I-BET151 and JQ1. IC_{50s} for AZD5153 were at least 10-fold lower compared with those for I-BET151 and JQ1 in all three cell lines; $IC50_s$ for AZD5153, I-BET151 and JQ1 were 0.263 μM, 2.821 μM and 3.385 μM, respectively, in KPUM-MS3 cells; 0.285 μM, 2.925 μM and 4.355 μM, respectively, in KPUM-UH1 cells; and 0.202 μM, 5.196 μM and 2.039 μM, respectively, in STR-428 cells (Fig. 1a). Regarding the mechanism of action, AZD5153 at its IC_{50} increased the G1 phase population and decreased cells in S to G2/M phases in a time-dependent manner in all three cell lines, indicating G1/S cell cycle blockade (Fig. 1b). In addition, a substantial increase of cells undergoing apoptosis during 48-h treatment with AZD5153 was detected by DNA content analyses: for example, STR-428 cells in subG1 phase increased from 5.9 to 14.9% (Fig. 1b). We also

Table 3 Canonical pathway regulated by AZD5153

	KPUM-MS3		KPUM-UH1		STR-428	
Rank	Ingenuity canonical pathways	-log(*p*-value)	Ingenuity canonical pathways	-log(p-value)	Ingenuity canonical pathways	-log(p-value)
1	Role of BRCA1 in DNA Damage Response	7.35	B Cell Receptor Signaling	7.16	B Cell Receptor Signaling	8.28
2	tRNA Charging	6.88	autophagy	3.60	Antigen Presentation Pathway	5.83
3	Protein Ubiquitination Pathway	6.56	CD27 Signaling in Lymphocytes	3.45	PI3K Signaling in B Lymphocytes	5.63
4	B Cell Receptor Signaling	6.07	SAPK/JNK Signaling	3.42	ATM Signaling	4.88
5	DNA Double-Strand Break Repair by Non-Homologous End Joining	5.92	Molecular Mechanisms of Cancer	3.40	OX40 Signaling Pathway	4.85
6	Pancreatic Adenocarcinoma Signaling	4.43	NF-κB Activation by Viruses	3.35	p53 Signaling	4.52
7	Hereditary Breast Cancer Signaling	4.38	Integrin Signaling	3.34	3-phosphoinositide Degradation	4.43
8	NRF2-mediated Oxidative Stress Response	4.35	PTEN Signaling	3.27	CD40 Signaling	4.11
9	PI3K Signaling in B Lymphocytes	4.01	PDGF Signaling	3.27	IL-4 Signaling	4.07
10	Endoplasmic Reticulum Stress Pathway	3.83	RANK Signaling in Osteoclasts	3.22	Superpathway of Inositol Phosphate Compounds	3.94
11	Chronic Myeloid Leukemia Signaling	3.45	Insulin Receptor Signaling	3.20	iNOS Signaling	3.89
12	Glioma Signaling	3.27	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.13	CD28 Signaling in T Helper Cells	3.83
13	Amyloid Processing	3.25	Pancreatic Adenocarcinoma Signaling	3.02	Cdc42 Signaling	3.76
14	CMP-N-acetylneuraminate Biosynthesis I	3.20	TNFR1 Signaling	2.98	TNFR1 Signaling	3.75
15	Cell Cycle: G1/S Checkpoint Regulation	3.05	JAK/Stat Signaling	2.94	phagosome maturation	3.70
16	CD27 Signaling in Lymphocytes	2.97	CD40 Signaling	2.90	TWEAK Signaling	3.67
17	RAR Activation	2.89	STAT3 Pathway	2.85	autophagy	3.67
18	Molecular Mechanisms of Cancer	2.89	Reelin Signaling in Neurons	2.79	CD27 Signaling in Lymphocytes	3.63
19	ATM Signaling	2.76	PI3K Signaling in B Lymphocytes	2.79	Endoplasmic Reticulum Stress Pathway	3.57
20	EGF Signaling	2.73	UVA-Induced MAPK Signaling	2.77	3-phosphoinositide Biosynthesis	3.57

investigated the potency of AZD5153 for apoptotic induction by AV/PI counterstaining. AZD5153 induced apoptosis by increasing AV(+)/PI(−) cells in the early apoptosis phase and AV(+)/PI(+) cells in the late apoptosis phase in all three cell lines (Fig. 1c). These results indicate that blockade of BRD4 by AZD5153 exerted antitumor activity against DEL- and DHL-derived cell lines via both G1/S cell cycle blockade and induction of apoptosis.

BRD4 targeting inhibits genes for oncogenesis, BCR signaling, and B-cell development

We next investigated the impact of AZD5153 on gene expression in DEL- and DHL-derived cell lines. Comparative GEP analyses between untreated and AZD5153-treated cells identified 270 genes that were commonly upregulated by more than 1.5-fold (Table 1, Supplementary Table S1) and 494 genes, including those involved in oncogenesis (*c-MYC, PBX2, AKT2, MAPK3,* etc.), the BCR signaling pathway (*CD19*, *CD79B, BLNK*, *EBF1,* etc.), and B-cell development (*IKZF1*, *IKZF3*, *PAX5, POU2AF1, EBF1,* etc.), were commonly downregulated by less than 0.67-fold (Fig. 2a, Table 2, Supplementary Table S2) in the three cell lines. RQ-PCR showed that *c-MYC* expression was downregulated to 11.3%, 21.7 and 14.4% by AZD5153 in KPUM-MS3, KPUM-UH1 and STR-428 cells, respectively (Fig. 2b). GEP results were also validated by RQ-PCR, which showed that AZD5153 downregulated expression of *MAPK3*, *BLNK*, *CD19*, *AKT2*, *BCL2L1* and *CD79B* in the three cell lines (Fig. 2c). Given these results, we further analyzed the pathways modulated by AZD5153. Ingenuity Canonical Pathway analysis revealed that BRD4 inhibition by AZD5153 modulated various pathways in a cell context-dependent manner, and pathways designated as "BCR signaling", "PI3K signaling in B-lymphocytes" and "CD27 signaling in lymphocytes" were commonly regulated by AZD5153 treatment in the three cell lines (Table 3).

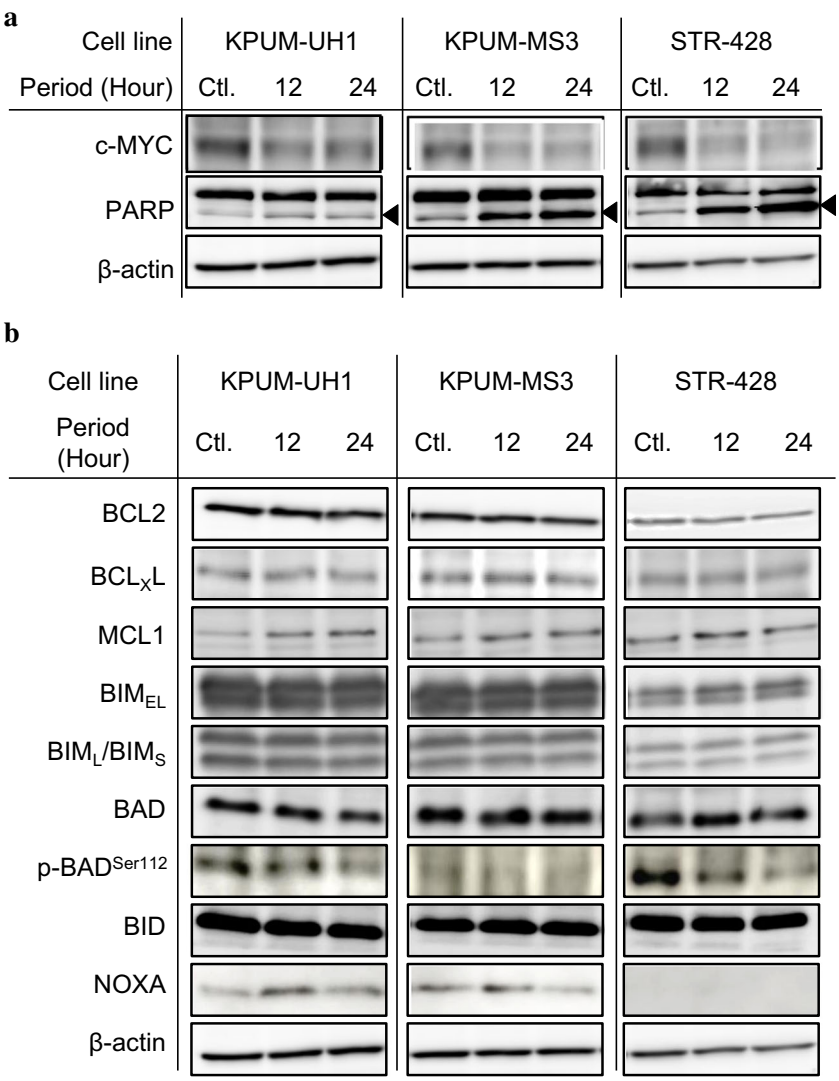

Fig. 3 Protein expression changes induced by AZD5153 treatment in DEL- and DHL-derived cell lines. KPUM-UH1, KPUM-MS3 and STR-428 cells were treated at IC_{80} for AZD5153 for the indicated periods. **a** c-MYC and PARP. Arrows indicate the cleaved form of PARP, indicating proteolytic cleavage during the apoptosis process. **b** Effects of AZD5153 on anti-apoptotic BCL2 family proteins and BH3-only proteins. NOXA was not detectable in STR-428 cells. β-actin was used as an internal control

Effect of AZD5153 on expression of pro- and anti-apoptotic BCL2 family proteins

Apoptosis is regulated by the interaction between pro-apoptotic BH3-only proteins and anti-apoptotic BCL2 family proteins, and its modulation is a pivotal determinant in chemotherapeutic killing of cancer cells [18, 19, 41]. AZD5153 induced apoptosis in substantial proportions of cells in both DEL- and DHL-derived cell lines; however, transcriptional changes of major members of the pro- and anti-apoptotic BCL2 family, including *BCL2*, by AZD5153 were not significant in comprehensive GEP analyses, except for *BCL2L1* (Tables 1, 2, Supplementary Tables S1, S2). At the protein level, despite c-MYC downregulation and PARP cleavage (Fig. 3a), BCL2 was not decreased by treatment with AZD5153. Although AZD5153 repressed BCL2L1 expression at the transcription level (Fig. 2a, Supplementary Table S2), its protein expression was extremely low at the basal level and was not apparently reduced by AZD5153. In addition, an unexpected slight increase of anti-apoptotic MCL1 occurred during treatment with AZD5153 (Fig. 3b). BAD, an apoptosis-promoting BH3-only protein, was activated through dephosphorylation, but there was no increase or activation of other BH3-only proteins, such as BIM, BID or NOXA, by AZD5153 (Fig. 3b). In addition, PUMA was not detectable regardless of AZD5153 treatment (data not shown).

AZD4320 potently augments the antitumor effect of AZD5153 in DEL- and DHL-derived cell lines

In the absence of prominent activation of endogenous BH3-only proteins, except BAD, we next investigated if addition of a synthetic BH3-mimetic could augment the cytotoxic effect of AZD5153 in the DEL- and DHL-derived cell lines. We first examined the antitumor-effects of two dual BH3-mimetics for BCL2 and BCL_XL, a conventional agent ABT-263 and novel AZD4320, on the three cell lines. As shown in Fig. 4a, AZD4320 and ABT-263 both inhibited proliferation of DEL- and DHL-derived cell lines in a dose-dependent manner, and AZD4320 was more active against all three cell lines compared with ABT-263. IC_{50s} for AZD4320 and ABT-263 were 0.026 μM and 1.958 μM in KPUM-MS3 cells; 0.017 μM, and 0.304 μM in KPUM-UH1 cells; and 0.17 μM and 0.344 μM in STR-428 cells. We also examined the effect of a MCL1-specific inhibitor, AZD5991, on the three cell lines, and found that AZD5991 was only active in STR-428 cells, with an IC_{50} of 0.12 μM, while up to 10 μM AZD5991 showed only a modest growth inhibitory effect on KPUM-MS3 and KPUM-UH1 cells (Fig. 4b). Based on these results, we selected AZD4320 as a candidate partner of AZD5153, and examined the combinatory effects of AZD5153 and AZD4320 in the three cell lines. This combination showed mostly synergistic, and at least additive, growth inhibitory effects on DEL- and DHL-derived cell lines (Fig. 5a), and profoundly increased cells undergoing apoptosis in all three cell lines (Fig. 5b and c).

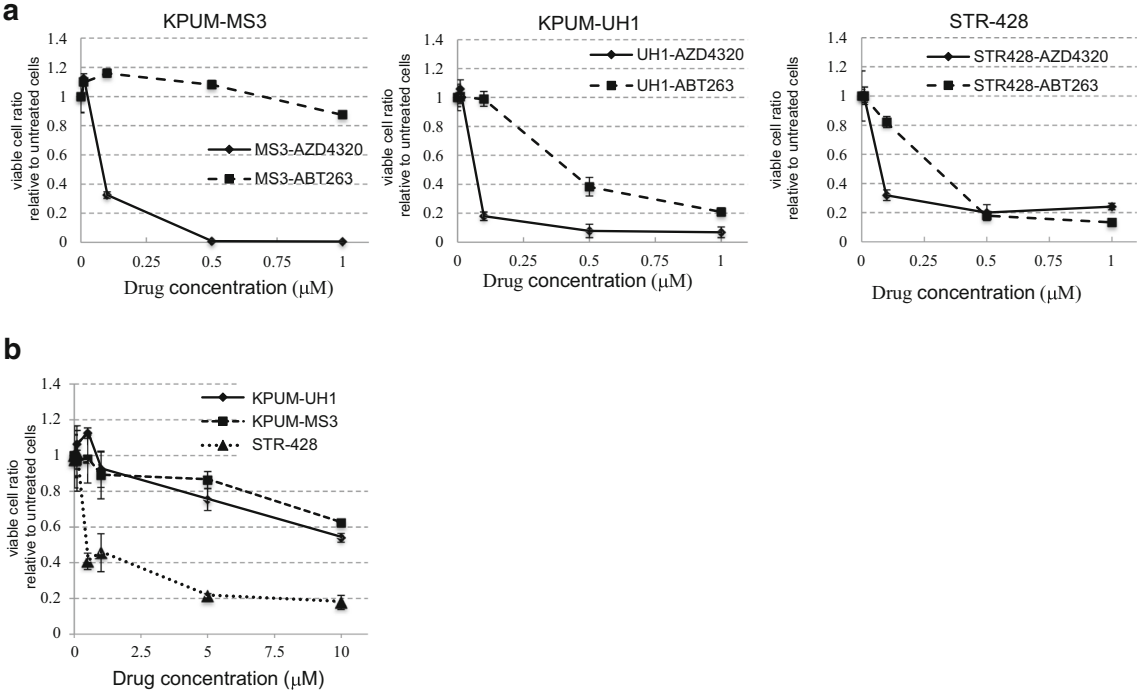

Fig. 4 Growth inhibitory effects of inhibitors for anti-apoptotic BCL2 family proteins. a KPUM-MS3, KPUM-UH1, and STR-428 cells were treated with the indicated concentrations of AZD4320 or ABT263 for 72 h, and subjected to a modified MTT assay. **b** The three cell lines were treated with the indicated concentrations of AZD5991 for 72 h, and subjected to a modified MTT assay

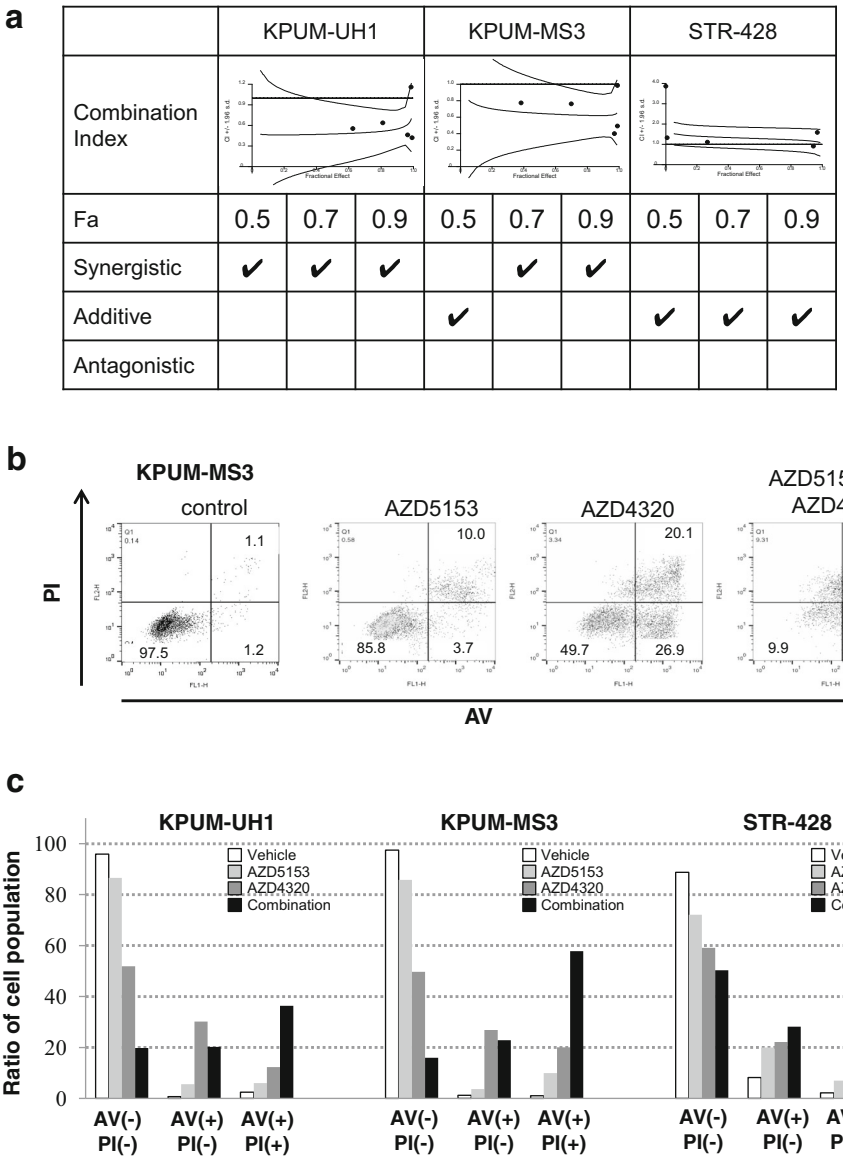

Fig. 5 Combinatory effects of AZD5153 and AZD4320 on DEL- and DHL-derived cell lines. **a** Growth inhibitory effects of a combination of AZD5153 and AZD4320 were examined in KPUM-MS3, KPUM-UH1 and STR-428 cells. Cells were treated with various concentrations of AZD5153 and AZD4320 in combination for 72 h, and subjected to a modified MTT assay. In the combination index (CI) figures, the X- and Y-axes represent fractional effects and the CI, respectively. The combinatory growth inhibitory effect was determined by CI ± 1.96 S.D. from three independent experiments. Fa: fraction affected. **b** Cells were treated with AZD5153 and AZD4320 at their IC_{50s} for 24 h, counter-stained by AV and PI, and subjected to flow cytometric analyses. AV(−)/PI(−) cells, AV(+)/PI(−) cells and AV(+)/PI(+) cells were considered to be viable cells, cells in the early apoptosis phase, and cells in the late apoptosis phase, respectively. Data in KPUM-MS3 cells are shown as representative. **c** Ratios of AV(−)/PI(−), AV(+)/PI(−) and AV(+)/PI(+) cells without treatment and after treatment with AZD5153 and/or AZD4320 at their IC_{50s} for 24 h are shown for KPUM-UH1, KPUM-MS3 and STR-428 cells

Discussion

In this study, we first investigated whether therapeutic targeting of BRD4 enables triple targeting of c-MYC, BCL2 and BCR signaling in DEL and DHL, as BRD4 frequently regulates expression of c-MYC and other disease-specific "tumor driver" oncogenes in solid cancers [25–34]. For instance, dual inhibition of c-MYC and BCL2 by BRD4 inhibition has been reported in neuroblastoma, acute leukemia, and renal cell carcinoma, but not in mantle cell lymphoma [42–46]. To investigate the role of BRD4 in DEL and DHL, we used a novel BRD4 inhibitor, AZD5153, in this study. AZD5153 can ligate two bromodomains in BRD4 simultaneously, and this property translates into enhanced pharmacologic activity. As expected, AZD5153 showed higher growth inhibitory potency against DEL- and DHL-derived cell lines, compared with JQ1 and I-BET151. The effects of AZD5153 on G1/S cell cycle blockade and induction of apoptosis in DEL- and DHL-derived cell lines were the same as those observed in DLBCL-derived cells treated by other BRD4 inhibitors like JQ1 [29, 32]. Also as expected, treatment with AZD5153 suppressed expression of various oncogenic molecules, including c-MYC, as well as a series of genes involved in BCR signaling and B cell development in DEL- and DHL-derived cell lines. Previous studies have revealed that the expressions of *c-MYC* and the major B cell regulatory master genes, *IKZF1* and *PAX5*, are under the direct transcriptional control of BRD4 in B cell lineage cells [28, 47]. In addition, *POU2AF1* which is essential for germinal center development and *CD79B* which is a crucial for BCR signaling have been also shown to be directly regulated by BRD4 at transcriptional level [28]. Thus, AZD5153 might downregulate *c-MYC* as well as several regulatory molecules those are critical for BCR signaling and B cell development by direct inhibition

of BRD4 binding, while it also caused repression of other B-cell physiology-associated molecules as downstream effectors of direct BRD4 targets.

In contrast to its promising effect on cell cycle blockade, the apoptosis-inducing effect of AZD5153 was relatively modest. This cytostatic effect of AZD5153 may be due to its modest impact on expressions of pro- and anti-apoptotic BCL2 family proteins. In contrast to effects in other cancers [42–46], AZD5153 did not cause BCL2 reduction in DEL- or DHL-derived cell lines. Thus, the role of BRD4 on BCL2 expression differs among disease types and even among patients with the same cancer [34]. In addition, among pro-apoptotic BH3-only proteins, only BAD was activated by AZD5153. BAD lacks direct apoptosis-inducing ability, but helps cells undergo apoptosis by displacing activator BH3-only proteins or activated BAX or BAK monomers from BCL2 and BCL_XL [18, 19, 41]. In contrast to an observation in mantle cell lymphoma [44], AZD5153 did not induce BIM, an activator BH3-only protein that directly promotes apoptosis by binding to and activating BAX and BAK, in DEL- and DHL-derived cell lines. To resolve this problem, we combined AZD5153 with a novel BH3 mimetic against BCL2 and BCL_XL, which was more effective than ABT-263. As expected, the combination of AZD5153 and AZD4320 showed enhancement of cell death of lymphoma cells carrying both BCL2 and c-MYC. Several BH3-mimetics, such as ABT-199 and ABT-263, have been utilized for hematologic malignancies, including NHLs, chronic lymphocytic leukemia, and acute leukemias [19, 48]. BH3-mimics enhance apoptosis-inducing effects of conventional anticancer agents, and synergistic antitumor effects have been reported with combinatory use of ABT-199 and JQ1 against DEL-derived cell lines [21], and with a combination of ABT-199 and the BTK inhibitor ibrutinib in ABC DLBCL, GCB DLBCL and follicular lymphoma [49]. Collectively, the current and previous studies provide a rationale for dual targeting of BRD4 and antiapoptotic BCL2 proteins as a therapeutic strategy against BCLs with concomitant overexpression of c-MYC and BCL2. Although resistance to BET inhibitors has been documented in other cancers [50, 51], our study also suggests that selection of an optimal combinatory partner that can overcome the molecular mechanism of resistance may not only restore, but also enhance the efficacy of a BRD4 inhibitor.

In conclusion, this study showed that a novel BRD4 inhibitor, AZD5153, is more effective in inhibiting cell proliferation of DHL- and DEL-derived cell lines in comparison with conventional BRD4 inhibitors, JQ1 and I-BET151. AZD5153 potently downregulates several common oncogenes, including *c-MYC*, as well as a series of genes specific for B-cell biology. Thus, the combination of AZD5153 and AZD4320 enables triple targeting of c-MYC, BCL2 and BCR signaling molecules, and may constitute an attractive therapeutic strategy against DEL and DHL.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in association with this study.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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