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BET bromodomain inhibitor birabresib in mantle cell lymphoma: in vivo activity and identification of novel combinations to overcome adaptive resistance

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ABSTRACT

Background The outcome of patients affected by mantle cell lymphoma (MCL) has improved in recent years, but there is still a need for novel treatment strategies for these patients. Human cancers, including MCL, present recurrent alterations in genes that encode transcription machinery proteins and of proteins involved in regulating chromatin structure, providing the rationale to pharmacologically target epigenetic proteins. The Bromodomain and Extra Terminal domain (BET) family proteins act as transcriptional regulators of key signalling pathways including those sustaining cell viability. Birabresib (MK-8628/OTX015) has shown antitumour activity in different preclinical models and has been the first BET inhibitor to successfully undergo early clinical trials.

Materials and methods The activity of birabresib as a single agent and in combination, as well as its mechanism of action was studied in MCL cell lines.

Results Birabresib showed in vitro and in vivo activities, which appeared mediated via downregulation of MYC targets, cell cycle and NFKB pathway genes and were independent of direct downregulation of CCND1. Additionally, the combination of birabresib with other targeted agents (especially pomalidomide, or inhibitors of BTK, mTOR and ATR) was beneficial in MCL cell lines.

Conclusion Our data provide the rationale to evaluate birabresib in patients affected by MCL.

Key questions

What is already known about this subject?

- The Bromodomain and Extra Terminal domain (BET) proteins act as transcriptional regulators of key signalling pathways.
- The BET inhibitor birabresib (MK-8628/OTX015) has been the first in class to show early clinical activity.
- BET inhibitors have shown preclinical activity in different cancer models.

What does this study add?

- Mantle cell lymphoma cells exposed to birabresib undergo a downregulation of MYC targets, genes involved in cell-cycle regulation (but not *CCND1*) and NFKB and BCR signalling pathways.
- Benefit from birabresib combined with multiple targeted agents is seen in mantle cell lymphoma cells.
- Expression changes in mantle cell lymphoma cells after drug exposure suggest rational combinations.

How might this impact on clinical practice?

- Since patients with mantle cell lymphoma are still in need of therapeutic improvement, novel drug combinations based on a BET inhibitor can be designed.

INTRODUCTION

Mantle cell lymphoma (MCL) is characterised by the presence of the chromosomal translocation t(11;14)(q13;q32) with the juxtaposition of the *CCND1* gene to the *IGHV* locus and overexpression of cyclin D1.[1–3] Although the outcome of patients with MCL has improved,[1–3] there is still a considerable need for improvements in the treatment of these patients. MCL, like other cancers, present recurrent alterations in genes involved in maintaining chromatin structure and transcription machinery genes,[4 5] providing the rationale to pharmacologically target epigenetic proteins. The Bromodomain and Extra Terminal domain (BET) family proteins BRD2/3/4 are transcriptional regulators of pathways involved in cell viability and signalling pathways.[6] BET proteins bind to chromatin and facilitate histone H4-dependent transcription via RNA polymerase II.[6] BET inhibitors have shown promising preclinical[7–13] and early clinical activity[14] as antilymphoma agents. Exposure of cancer cells to

BET inhibitors results in a significant downregulation of key genes and pathways, such as MYC and its targets or genes involved in the NFKB or cell-cycle pathway.[7 8 10 11 13] Here, we report novel data obtained with the BET inhibitor birabresib (MK-8628/OTX015) as a single agent and in combination, including gene expression profiling (GEP) studies, to elucidate its mechanism of action and to identify combinations that might overcome adaptive resistance mechanisms.

MATERIALS AND METHODS

Cell lines and molecules

Used MCL cell lines (REC1, Jeko1, Maver1, Granta519, Mino, SP53 and UPN1)[15] were all validated for their cell identity by short tandem repeat DNA fingerprinting (Promega GenePrint 10 System kit) (online supplementary table 1). Birabresib was provided by OncoEthix (Lausanne, Switzerland), while the other compounds were bought from Selleckchem (Houston, Texas, USA).

In vivo experiment

NOD-Scid (NOD.CB17-*Prkdcscid*/NCrHsd) mice were subcutaneously inoculated with 15×10^6 REC1. Tumour volumes were calculated and analysed as previously described.[16] Mice maintenance and animal experiments were performed with study protocols approved by the local Swiss Cantonal Veterinary Authority (no. 10/2014). Pharmacokinetic analysis for birabresib plasma and tissue concentrations was performed as previously described.[17]

Cell proliferation, drug combinations and evaluation of synergism

The antiproliferative activity of birabresib as single agent and in combination was assessed as previously described.[7] Based on the Chou-Talalay Combination Index (CI),[18] the effect of the combinations was defined as beneficial if synergistic (CI <0.9) or additive (CI, 0.9–1.1).

Real-time PCR and western blotting

RNA was extracted using the RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). Real-time PCR and western blotting were performed as previously described.[15]

Gene expression profiling

GEP was done as previously described.[19] Transcript mapping was based on HG19 using the manufacturer's supplied annotation. Data were quantile normalised and batch corrected using ComBat.[20] Transcripts were ranked based on their differential expression between conditions using the empirical Bayes (paired) moderated t-test as implemented in the LIMMA R-package[21] and functional annotated using Gene Set Enrichment Analysis with gene sets from MSigDB 5.2[22] (hallmark, c2cpg, c2cp, c5), SignatureDB[23] and obtained in different experimental conditions,[7 13 15] applying a threshold based on false discovery rate (FDR) <0.1. Probes presenting a FDR, controlled by Benjamini-Hochberg algorithm, <0.05 and a log ratio >|0.3| were considered differentially expressed. Profiling data are available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database (GSE110134).

RESULTS

Birabresib shows in vivo activity in MCL

Since we previously reported in vitro activity of birabresib as single agent in MCL cell lines,[7] we first wanted to confirm its antitumour activity in an in vivo MCL model. For this purpose, REC1 cells were engrafted in NOD-Scid mice and treatments were carried out with birabresib (50 mg/kg once daily; Qd×7/w×5w) starting 3 days after the engraftment. After 14, 22, 26 and 30 days of treatment, tumour volumes were reduced by birabresib treatment with at least a twofold decrease when compared with control treated mice ($p<0.05$) (Figure S1online supplementary material 1), in agreement also with the recent data reported in another MCL xenograft model using the Z138 cell line.[24] Birabresib-treated mice did not show any weight loss compared with controls. Birabresib levels in plasma and tumour samples 4 hours after the last birabresib treatment showed a median value of 1951 ng/mL (95% CI 1021 to 5863) in plasma, which is equivalent to ~1.5 µM concentration, and 721 ng/g (95% CI 138.2 to 877.92) in tumour samples.

Birabresib does not downregulate CCND1 in MCL but affects MYC targets, NFKB signaling and other important biologic pathways

Birabresib has in vitro[7] and in vivo antitumour activity in MCL. Since BET inhibitors downregulate key cancer genes,[6 8] we assessed if birabresib downregulated cyclin D1, coded by the *CCND1* gene deregulated in MCL by the t(11;14) chromosomal translocation.[25] Four MCL cell lines were exposed to DMSO or birabresib (500 nM) for 4 and 24 hours. Only a slight decrease of *CCND1* mRNA expression levels was seen, while at protein level, cyclin D1 was not reduced (Figure S2online supplementary material 1).

Thus, to understand the mechanism of action of birabresib in MCL, we performed gene expression profiling analysis of the four MCL cell lines exposed to DMSO or birabresib (500 nM) for 2, 4, 8 or 12 hours. The gene expression profile changes were very similar across the four individual cell lines (Figure S3online supplementary material 1) and were merged for further analyses. Upregulated transcripts were enriched for genes related to p53 pathway, fatty acid metabolism, hypoxia, apoptosis, DNA repair, chromatin silencing, protein–DNA complex and RNA polymerase I promoter opening (figure 1A; Table S2Aonline supplementary table 2B). Downregulated transcripts were mainly enriched of MYC targets, interferon response, NFKB and MYD88 signalling, positive regulation of lymphocyte differentiation, HDAC targets silenced by methylation, and mitochondrial translation (figure 1B; Table S2Bonline supplementary table 2B). The upregulated transcripts comprised

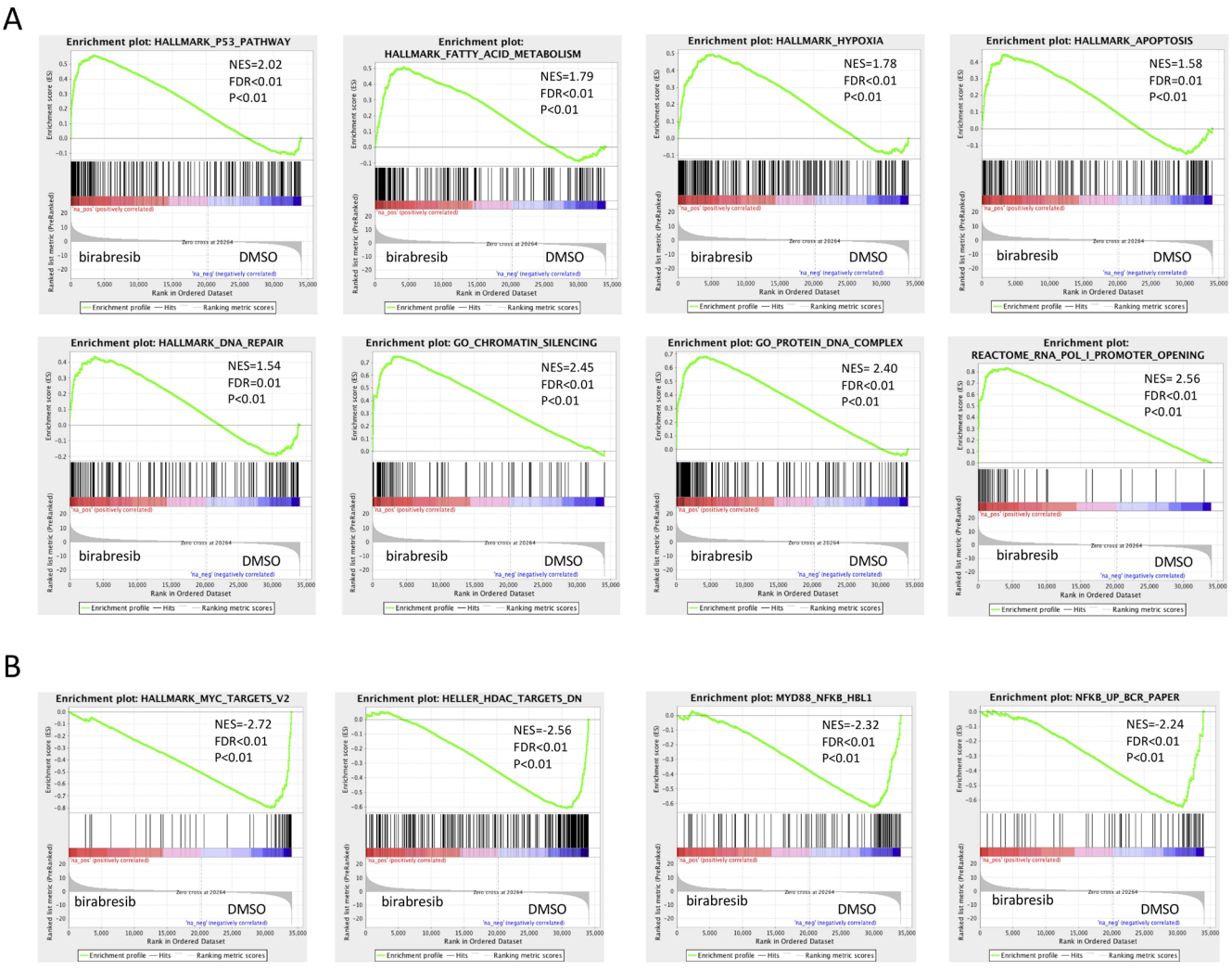

Figure 1 Birabresib regulates biologically relevant groups of genes in MCL. Representative Gene Set Enrichment Analysis plots illustrating the transcriptional expression signature enrichment in genes upregulated (A) and downregulated (B) after exposure for 4, 8, 12 and 24 hours of treatment with DMSO or 500 nM birabresib in the four MCL cell lines Jeko1, Maver1, REC1 and Granta519. Green line, enrichment score; bars in the middle portion of the plots show where the members of the gene set appear in the ranked list of genes. Positive or negative ranking metric indicate respectively correlation or inverse correlation with the profile. FDR, false discovery rate; NES, normalised enrichment score.

genes coding different histones (mostly from clusters 1–2), SESN3, *IRF7*, *FGFR3*, *TUBB3*, *HES6*, *DCXR*, *ULK1*, *PPP1R13B*, *CDKN2D*, *GADD45A*, *JUN* and *JUND*, while *NASPB*, *TNFRSF17*, *TLR10*, *FAIM3*, *CD19*, *CD72*, *MAP4K1*, *TLR7*, *NFKBIE*, *FCRL2*, *FCRLA*, *TNFRFSF13B*, *BLK* and *CD86* were among the downregulated transcripts (table 1, Table S2Conline supplementary table 2B). Validation of some of the observed changes, including analysis of MYC expression, are shown in Figure S4online supplementary material 1.

The birabresib signature was highly overlapping with the changes observed in diffuse large B-cell lymphoma (DLBCL) cells exposed to the same compound, in different tumour models treated with other BET inhibitors (including MCL cells exposed to JQ1) or with HDAC inhibitors (Figure S5online supplementary material 1, Table S3and table 3). Additionally, transcripts affected after birabresib treatment in MCL overlapped with the gene expression signatures obtained treating DLBCL cells with signalling inhibitors such as ibrutinib, idelalisib, duvelisib or bimiralisib[15] (Figure S6online supplementary material 1, Table S3and table 3), and were inversely associated with transcripts deregulated in the Jeko1 cell line bearing an acquired resistance to CHK1 inhibitor[26] (Table S3A-Bonline supplementary table 3).

Gene expression profiling identifies synergistic combinations

Since some of the transcripts and signalling pathways upregulated after birabresib exposure (signalling by FGFR, activation of MAPKK/ERK, DNA repair; Table S2online supplementary table 2) were suggestive of an adaptive resistance, and genes like FGFR3, c-JUN, STAT3 and MAP2K1 were upregulated after birabresib treatment (Figure S7online supplementary material 1), we

Table 1 Changes in gene expression levels in four mantle cell lymphoma (MCL) cell lines after exposure to birabresib

	Symbol	logFC
A		
ILMN_2109416	NAPSB	−1.71E+00
ILMN_1768016	TNFRSF17	−1.67E+00
ILMN_1719905	TLR10	−1.58E+00
ILMN_1671337	SLC2A5	−1.48E+00
ILMN_2414762	TLR10	−1.45E+00
ILMN_1700147	VPREB3	−1.33E+00
ILMN_1756595	SH3TC1	−1.26E+00
ILMN_1675191	GAPT	−1.26E+00
ILMN_2216582	LYL1	−1.26E+00
ILMN_1723043	NAPSB	−1.25E+00
ILMN_1691071	FCRLA	−1.22E+00
ILMN_1746148	LRRC33	−1.19E+00
ILMN_1738675	PTPN6	−1.19E+00
ILMN_1697554	SASH3	−1.18E+00
ILMN_1791329	FCRL2	−1.17E+00
ILMN_3242271	GAPT	−1.17E+00
ILMN_1775542	FAIM3	−1.16E+00
ILMN_2085862	SLC15A3	−1.16E+00
ILMN_1712431	FAM113B	−1.15E+00
ILMN_1780368	GPR18	−1.15E+00
ILMN_2264011	GRAP	−1.14E+00
ILMN_3207122	LOC644563	−1.14E+00
ILMN_2384056	GPER	−1.13E+00
ILMN_1782704	CD19	−1.13E+00
ILMN_1795298	GPER	−1.12E+00
B		
ILMN_1651496	HIST1H2BD	1.96E+00
ILMN_1732071	HIST2H2BE	1.55E+00
ILMN_1792689	HIST1H2AC	1.55E+00
ILMN_1659047	HIST2H2AA3	1.45E+00
ILMN_1758623	HIST1H2BD	1.45E+00
ILMN_1681437	DCXR	1.42E+00
ILMN_2144426	HIST2H2AA3	1.36E+00
ILMN_2115340	HIST2H4A	1.35E+00
ILMN_3242900	HIST2H2AA4	1.31E+00
ILMN_1813314	HIST1H2BK	1.27E+00
ILMN_1658702	HIST1H2BJ	1.27E+00
ILMN_1796179	HIST1H2BK	1.26E+00
ILMN_1794017	SERTAD1	1.25E+00
ILMN_1849494	EFR3B	1.21E+00
ILMN_1781374	TUFT1	1.19E+00
ILMN_1659490	LOC653158	1.18E+00
ILMN_1777061	ZSWIM6	1.18E+00
ILMN_3251587	LOC100008589	1.12E+00
ILMN_3238233	HIST2H4B	1.09E+00
ILMN_1694268	HES6	1.08E+00
ILMN_1768973	HIST2H2AC	1.07E+00
ILMN_1798181	IRF7	1.04E+00
ILMN_1708728	H2AFJ	1.01E+00
ILMN_1748831	PPP1R13B	1.01E+00
ILMN_3249110	CSRNP2	9.96E-01

Four MCL birabresib-treated cell lines were analysed for transcriptional changes and the resulting top 25 downregulated (A) and top 25 upregulated (B) transcripts are listed ranked by log2 ratios. logFC: log2 ratio. All p values and adjusted p values are <0.05.

pharmacologically blocked some of these combining OTX015 with inhibitors of FGFR (PD173074), MEK (pimasertib), SRC kinases (dasatinib), ATR (AZD6738), CHK1 (PF00477736) or WEE1 (AZD1775). We first assessed these three combinations in two cell lines, characterised by a different degree of sensitivity to the BET inhibitor.[7] All but one of the six novel combinations were synergistic in the very sensitive cell line Jeko1, but in the less sensitive Granta519, synergism was only observed when birabresib was combined with inhibitors of MEK, ATR, WEE1 and CHK1 (CI 0.18, CI 0.67, CI 0.4, CI 0.27, respectively), although they were not able to completely reverse its low sensitivity to the BET inhibitor (figure 2A). Based on these data, the combinations with the four compounds were expanded to five additional cell lines. The combination of birabresib with the ATR inhibitor was synergistic in 4/5 cell lines, and was associated with an increased DNA damage and induction of apoptosis, as shown by an increase in gamma H2AX and by cleaved PARP1, respectively, at immunoblotting (Figure S8online supplementary material 1). The other combinations showed benefit only in two or less cell lines (figure 2B). Finally, we studied a MCL cell line with secondary resistance to the CHK1 inhibitor,[26] observing that the combination with PF00477736 was synergistic also in this model (median CI 0.64; 95% CI 0.55 to 0.8).

Birabresib synergises with other targeted agents in MCL

We evaluated the combination of birabresib with other agents belonging to classes of drugs known to synergise with BET inhibitors[6 7 10–13 17] (figure 3). The combination with the second-generation immunomodulatory drug (IMID) pomalidomide was synergistic in all the seven cell lines. Both the addition of the mTOR inhibitor everolimus and of the BTK inhibitor ibrutinib were beneficial in 6/7 cell lines (synergistic in five and additive in one cell line). Since ibrutinib, like other signalling inhibitors, can induce an upregulation of transcripts encoding proteins belonging to the

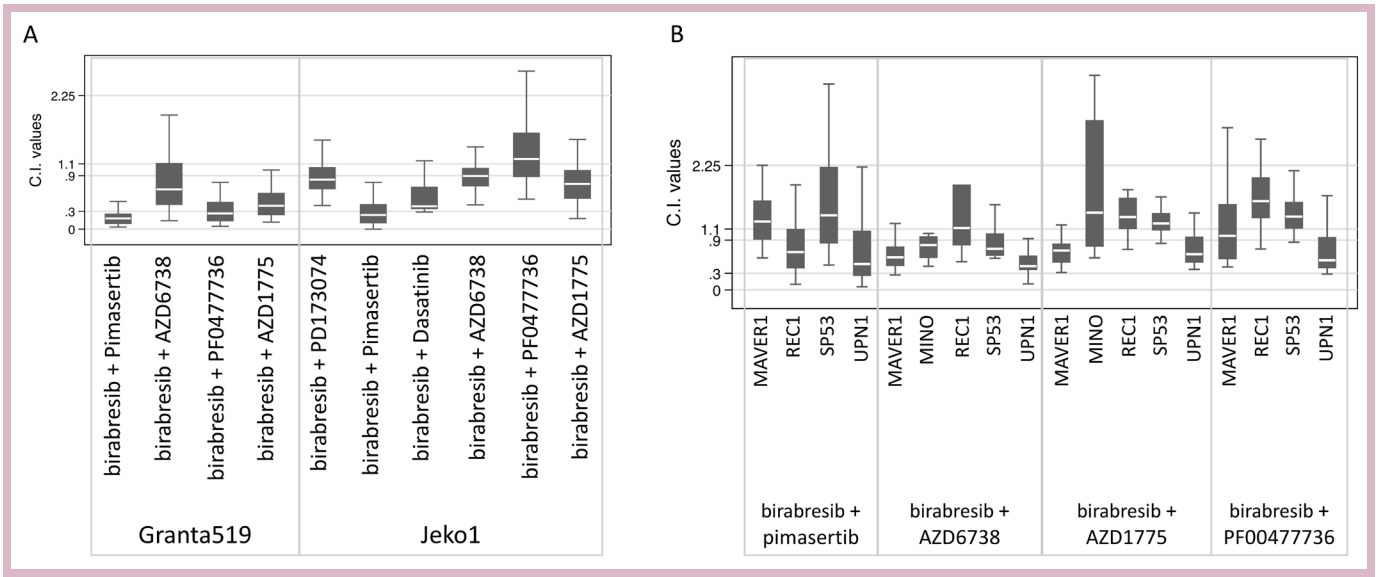

Figure 2 Birabresib is synergistic when combined with inhibitors targeting pathways that are upregulated after exposure to the BET inhibitor as a single agent. (A) Distribution of Chou-Talalay Combination Index (C.I.) values obtained in one birabresib-sensitive (Jeko1) and in one birabresib poor-sensitivity lymphoma cell line (Granta519), treated with different concentrations of the BET inhibitor birabresib in combination with the MEK inhibitor pimasertib, the SRC kinase inhibitor dasatinib, the FGRF inhibitor PD173074, the ATR inhibitor AZD6738, the CHK1 inhibitor PF00477736 or the WEE1 inhibitor AZD1775. C.I. values for birabresib/dasatinib and birabresib/PD173074 in Granta519 are not plotted due to a median value >3. (B) Distribution of Chou-Talalay Combination Index (C.I.) values obtained in Maver1, Mino, REC1, SP53 and UPN1 treated with different concentrations of the BET inhibitor birabresib in combination with the MEK inhibitor pimasertib, the ATR inhibitor AZD6738, the WEE1 inhibitor AZD1775 and the CHK1 inhibitor PF00477736. CI values for birabresib/pimasertib and birabresib/PF00477736 in Mino cell line are not plotted due to a median value >3. In each box plot, the line in the middle of the box represents the median and the box extends from the 25th to the 75th percentile (interquartile range, IQR); the whiskers extend to the upper and lower adjacent values (ie, ±1.5 IQR); outside values have been omitted from the figure. Y-axis, C.I. (C.I.<0.9, synergism; 0.9<C.I.<1.1, additive effect).

BCR pathway as a mechanism of adaptive resistance,[15] we compared the way birabresib and ibrutinib affected this class of genes. A few BCR signalling transcripts were differently affected after birabresib and ibrutinib treatment (Figure S9online supplementary material 1, Table S4and table 4), indicating that birabresib might counteract the ibrutinib-induced upregulation of BCR signalling genes.

Synergism was also observed when combining birabresib with pomalidomide, everolimus or ibrutinib in the cell line that was less sensitive to birabresib (Granta519)[7] for which the effect was more pronounced adding the mTOR inhibitor.

DISCUSSION

We have previously shown that birabresib has antiproliferative activity in MCL. We now extended this finding by demonstrating that birabresib also acts in vivo to delay tumour growth of the MCL REC1 cell line, in agreement with what recently reported using another xenograft model.[24] CCND1, the oncogenic driver in MCL, was neither downregulated at RNA nor at protein level by the BET inhibitor. Instead, birabresib preferentially affected the same biological pathways that are targeted by BET inhibitors (including birabresib) in other lymphoma types.[7 8 10 13] Birabresib determined a downregulation of MYC targets, genes involved in cell-cycle regulation (but not *CCND1*) and NFKB and BCR signalling pathways. While the cell cycle plays a central role in MCL pathogenesis,[27] NFKB and BCR signalling, despite being infrequently targeted by somatic mutations,[5 28] are also biologically relevant for MCL cells and represent valid therapeutic targets.[1 25 29 30]

As reported for BET inhibitors in different tumour models,[6] and particularly for birabresib in preclinical models of diffuse large B-cell lymphomas,[7 17] MCL cell lines also benefited from combination of the BET inhibitor with different targeted agents, namely the IMID pomalidomide, the BTK inhibitor ibrutinib and the mTOR inhibitor everolimus. Combination with the IMID compound appeared to be the most effective. This observation was supported by the mechanisms of action of the two classes of compounds, which are both able to affect the IRF4 pathway.[7 31] Indeed, we detected an upregulation of IRF7 following exposure to birabresib, which has also been observed in other studies combining birabresib or another BET inhibitor, CPI203 with the IMID lenalidomide in activated B-cell-like DLBCL[7] and in MCL, respectively.[11]

We also took advantage of the gene expression changes observed in MCL cells exposed to birabresib to identify novel combinations. The upregulation of genes such

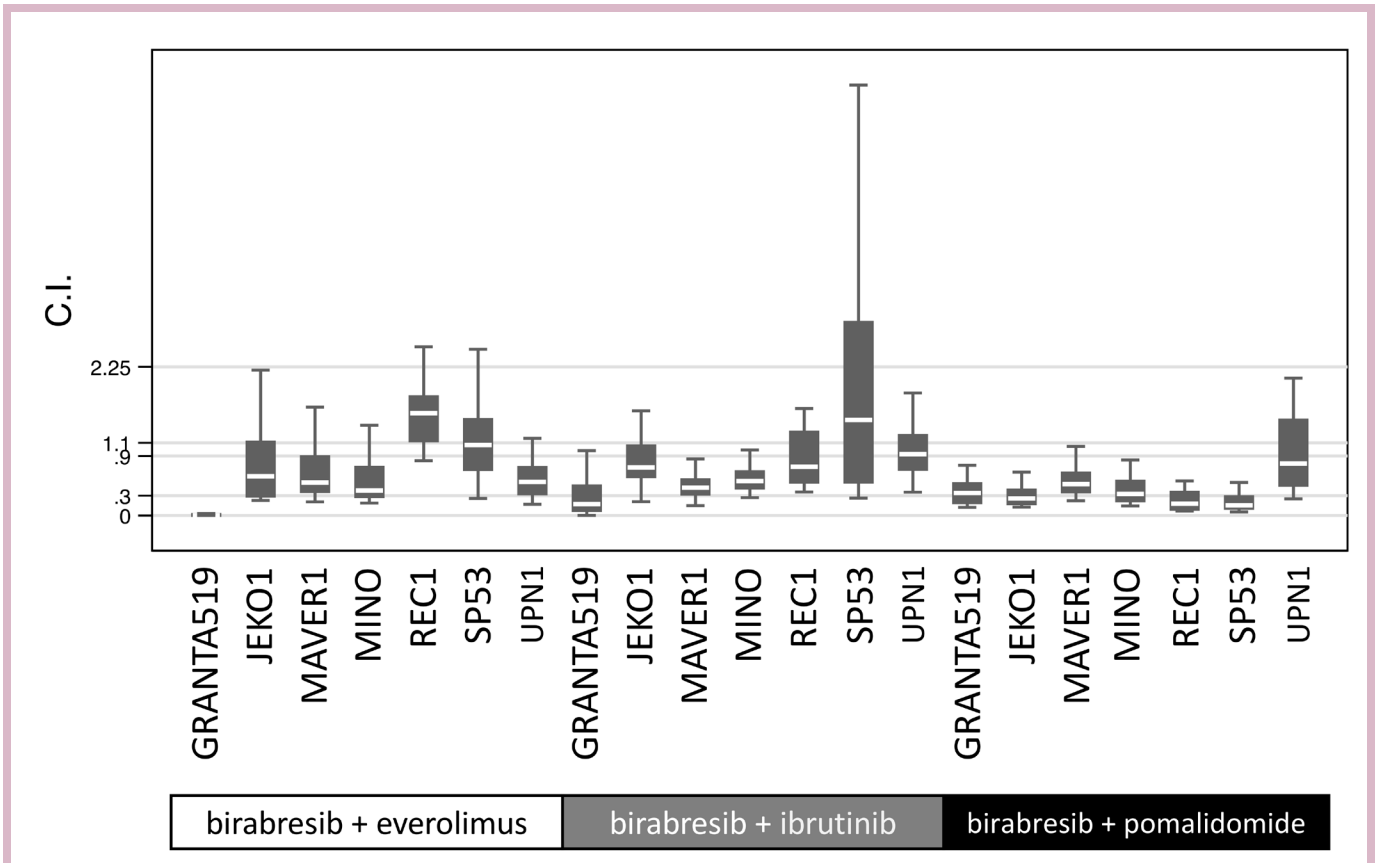

Figure 3 Birabresib shows synergistic efficacy in combination with other agents. Distribution of Chou-Talalay Combination Index (C.I.) values obtained in a panel of seven mantle cell lymphoma cell lines treated with different concentrations of birabresib in combination with everolimus, ibrutinib or pomalidomide. In each box plot, the line in the middle of the box represents the median and the box extends from the 25th to the 75th percentile (interquartile range, IQR); the whiskers extend to the upper and lower adjacent values (ie, ±1.5 IQR); outside values have been omitted from the figure. Y-axis, C.I. (C.I.<0.9, synergism; 0.9<C.I.<1.1, additive effect).

as MAP2K1, coding for MEK1, *FGFR3* and transcripts encoding DNA repair proteins suggested combination of birabresib with compounds targeting specific pathways. Treatment of cell lines with birabresib in combination an ATR inhibitor was the most active (beneficial in 6/7 cell lines), leading to increased DNA damage and induction of apoptosis. These data are further strengthened by similar data reported in solid tumours[32] and especially in murine MYC-driven lymphomas.[33] Improved activity was also seen when the BET inhibitor was combined with inhibitors of MEK, WEE1 or CHK1. The latter two classes of compounds are also in vitro and in vivo active in MCL as single agents and when combined.[34] Here, birabresib also inverted the gene expression signature associated with secondary resistance to the CHK1 inhibitor. Studies exploring these combinations in the in vivo setting will help to further define their efficacy.

Ibrutinib can induce increased transcription of BCR genes as an adaptive resistance mechanism.[15] Birabresib counterbalanced this effect, decreasing the expression of genes coding for positive regulators of BCR signalling (*STIM1*, *SHC1*, *SYK*, *CD79A*, *CD79B* and *BLK*) and that are upregulated by ibrutinib.[15] This observation provides further rationale for the combination of BET and BTK inhibitors and provided a possible mechanism for the synergism that is recurrently observed.[7 10 12 13 17 24 35]

In conclusion, the BET inhibitor birabresib showed antitumour activity in MCL as a single agent and in combination with other targeted agents, especially with IMIDs and inhibitors of mTOR, BTK and ATR. Birabresib mechanism of action appeared mediated via downregulation of genes involved the cell cycle, NFKB pathway and MYC targets'.

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Contributors CT cowrote the manuscript, designed and performed experiments, interpreted data; EB designed and performed experiments, interpreted data; EG wrote the manuscript, designed experiments and interpreted data; VR, AA, FS, AAM and MP performed experiments; AR performed gene expression profiling; IK and LuC performed data mining; EZ, EC and AS provided advice; LaC and MER provided advice and designed experiments; KR performed experiments; FB designed the study, interpreted data and cowrote the manuscript. CT and EB equally contributed. All authors have approved the final manuscript.

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