The Bruton tyrosine kinase inhibitor CC-292 shows activity in mantle cell lymphoma and synergizes with lenalidomide and NIK inhibitors depending on nuclear factor-κB mutational status

Ibrutinib is a first-in-class covalent inhibitor of Bruton tyrosine kinase (BTK) approved in the European Union and USA for the treatment of patients with relapsed/refractory mantle cell lymphoma (MCL).[1] Despite ibrutinib's promising activity, recent data suggest that primary and secondary resistance is common in MCL patients treated with this agent. Mutations within the BTK active site (Cys481Ser) have been described in MCL as a secondary mechanism of ibrutinib resistance.[2] For primary resistance unrelated to BTK mutations, recent studies suggest the presence of alternative mechanisms. One investigation indicate that MCL cell lines exhibiting ibrutinib sensitivity depend on chronic activation of the B-cell receptor (BCR) leading to the activation of the classical nuclear factor-κB (NF-κB) pathway, whereas ibrutinib-resistant MCL cell lines rely on the alternative NF-κB pathway and anti-BTK therapy would be unlikely to be of benefit.[3]

CC-292 is a highly selective, oral, small molecule inhibitor that shows greater selectivity than ibrutinib against BTK.[4-5] Both single-agent and combination trials with CC-292 are ongoing in patients with a wide variety of B-cell lymphoproliferative disorders. The aim of this study was to evaluate the antitumor profile of CC-292 in MCL, together with its impact on cellular activation, migration and tumor-stroma crosstalk. We also explored possible combination strategies to enhance CC-292 activity.

We first investigated the antitumor effects of CC-292 in five MCL cell lines (REC-1, MINO, UPN-1, MAVER-1 and Z138) after 72 h of treatment. CC-292 (10-1000 nM) had a cytostatic effect in a subset of cell lines, with REC-1, MINO and UPN-1 appearing to be the most sensitive, while MAVER-1 and Z138 were the most resistant to

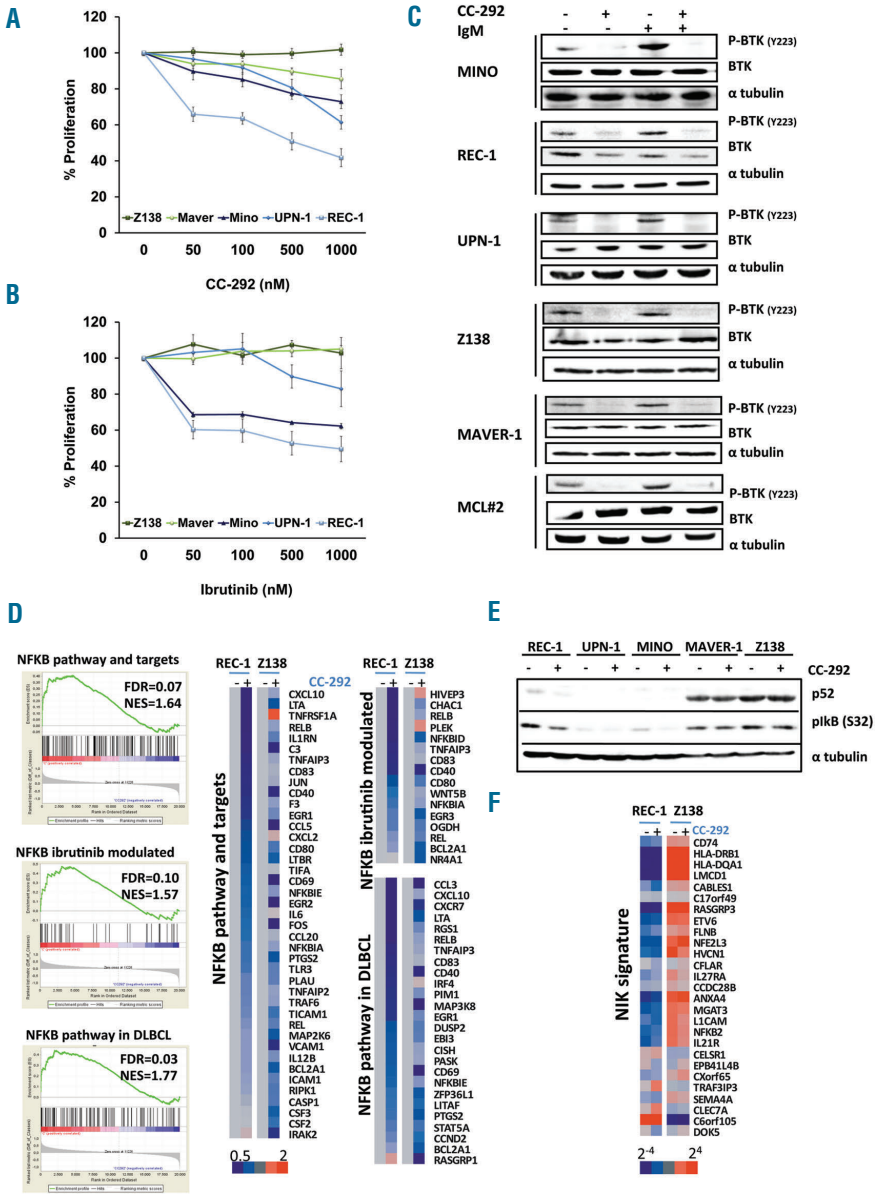

Figure 1. Mantle cell lymphoma cell lines show differential response to CC-292-mediated BTK inhibition, depending on NF-κB activation status. (A and B) Viability (MTT assay) of MCL cell lines treated with CC-292 or ibrutinib at the indicated doses for 72 h. Values are referred to the untreated control, and results are the mean ± SEM of three to nine independent experiments. (C) MCL cell lines and primary cells were pre-incubated with vehicle or 1 μM CC-292 for 1 h at 37°C. Samples were then stimulated with 25 μg/mL anti-IgM or treated with medium alone (control) for 30 min and pBTK and BTK expression were assessed by western blot, using α-tubulin as a loading control. (D) REC-1 and Z138 cell lines were treated with 1 μM CC-292 for 48h. RNA isolated and subjected to gene expression profiling using the Affymetrix platform (HG-U219). Gene set enrichment analysis was performed using custom genes related to the NFκB pathway.[3,15] Enrichment plots and heatmaps of the leading edges are shown. NES indicates normalized enriched score; FDR, false discovery rate. Threshold FDR<0.10 and NES>1.5 (E) MCL cell lines were treated for 6 h with 1 μM CC-292, and pIkB and p52/100 expression were assessed by western blot, using α-tubulin as a loading control. (F) NIK gene signature expression[7] of REC-1 and Z138 cell lines treated as in (D).

CC-292, following a trend similar to that for ibrutinib (Figure 1A,B). CC-292 induced marginal apoptosis (10-15%) in the most sensitive cell lines (UPN-1 and REC-1) (*Online Supplementary Figure S1*). Identification of Tyr223 pBTK is considered a surrogate marker for kinase activity.[6] MCL cell lines pre-incubated with CC-292 were IgM-stimulated to mimic BCR activation. As displayed in Figure 1C, CC-292 significantly reduced both constitutive and IgM-induced BTK phosphorylation at the Y223 residue in MCL cell lines and primary cells, independently of their sensitivity to the inhibitor.

To examine the molecular basis of CC-292 sensitivity and resistance, we then used gene expression profiling to compare the molecular changes induced by this inhibitor in the sensitive cell line REC-1 and the resistant cell line Z138. Gene set enrichment analysis (GSEA) showed that the NF-κB pathway was one of the pathways most significantly regulated by CC-292, and downmodulation of NF-κB target genes was much more prominent in the sensitive cell line REC-1 than in the resistant cell line Z138 (Figure 1D). In accordance, CC-292 downregulated the expression of the surrogate marker for NF-κB activation, pIkB (Ser32), only in MCL cell lines sensitive to CC-292 (Figure 1E). These results are in agreement with those reported by Rahal *et al.*[3] with the first-in-class BTK inhibitor ibrutinib, showing that MCL cell lines resistant to BCR inhibitors display activation of the alternative NF-κB pathway, associated with genetic lesions in this pathway. The two cell lines resistant to CC-292, MAVER-1 and Z138, harbor a *TRAF3* biallelic deletion or a nonsense mutation in *TRAF2* genes, respectively.[3] We confirmed constitutive activation of the alternative NF-κB pathway in these cell lines, as shown by cleavage of the p100 subunit into p52 by western blotting (Figure 1E). Likewise, we analyzed the expression of genes related to NF-κB-inducing kinase (NIK) activity, a central kinase of the NF-κB alternative pathway which allows this p100 processing, in the REC-1 and Z138 cell lines, together with their regulation by CC-292. As shown in Figure 1F, genes belonging to the NIK signature[7] were prominently expressed in Z138 compared to REC-1, and CC-292 did not significantly downmodulate their expression in any of these cell lines.

We then sought to determine the effect of CC-292 on cellular activation after BCR stimulation. MCL cell lines, both sensitive (UPN-1) and resistant (MAVER-1) to CC-292 and primary cells bearing wt*BIRC3* (MCL#3, MCL#6) or mut*BIRC3* (MCL#1, MCL#7) (*Online Supplementary Table S1 and Figure S2*) were stimulated with IgM for 24 h in the presence or absence of 1 μM CC-292. The *BIRC3*

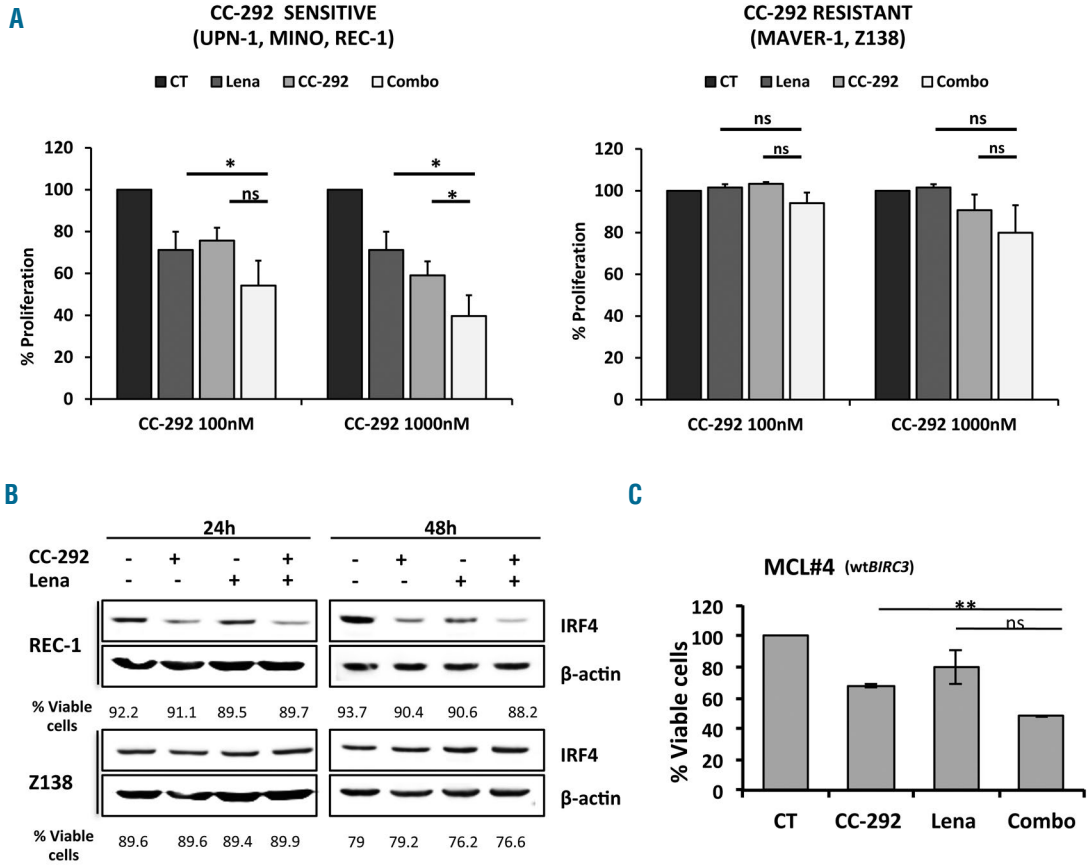

Figure 2. CC-292 cooperates with lenalidomide in CC-292-sensitive mantle cell lymphoma cells. A) CC-292-sensitive (MINO, REC-1 and UPN-1) and CC-292-resistant (MAVER-1 and Z138) cell lines were exposed to either CC-292 (100 nM or 1000 nM) or lenalidomide (5 μM) or their combination for 72 h. Viability was assessed by an MTT assay and results are displayed referred to the untreated control. (B) REC-1 and Z138 cell lines were treated with 100 nM CC-292 and/or 5 μM lenalidomide for 24 and 48 h. IRF4 was assessed by western blot using β-actin as a loading control. (C) Primary cells from a representative MCL patient with wt*BIRC3* (MCL#4) were cultured with stromaNKtert feeder cells and treated with 1 μM CC-292 with or without 5 μM lenalidomide for 72 h. The number of viable MCL cells was quantified by annexin-V and CD19 labeling followed by flow cytometry analysis. Results are shown referred to the untreated control.

gene encodes for cIAP2, a key component of the alternative NF-κB pathway that regulates NIK protein degradation; its inactivation leads to NIK protein stabilization.[8] As shown in *Online Supplementary Figure S3A,B,* CC-292 significantly decreased the IgM-induced expression of CD69 and CD86 in both MCL cell lines and primary samples, irrespective of NF-κB mutational status. As CCL3 and CCL4 are reported to be induced after BCR engage-

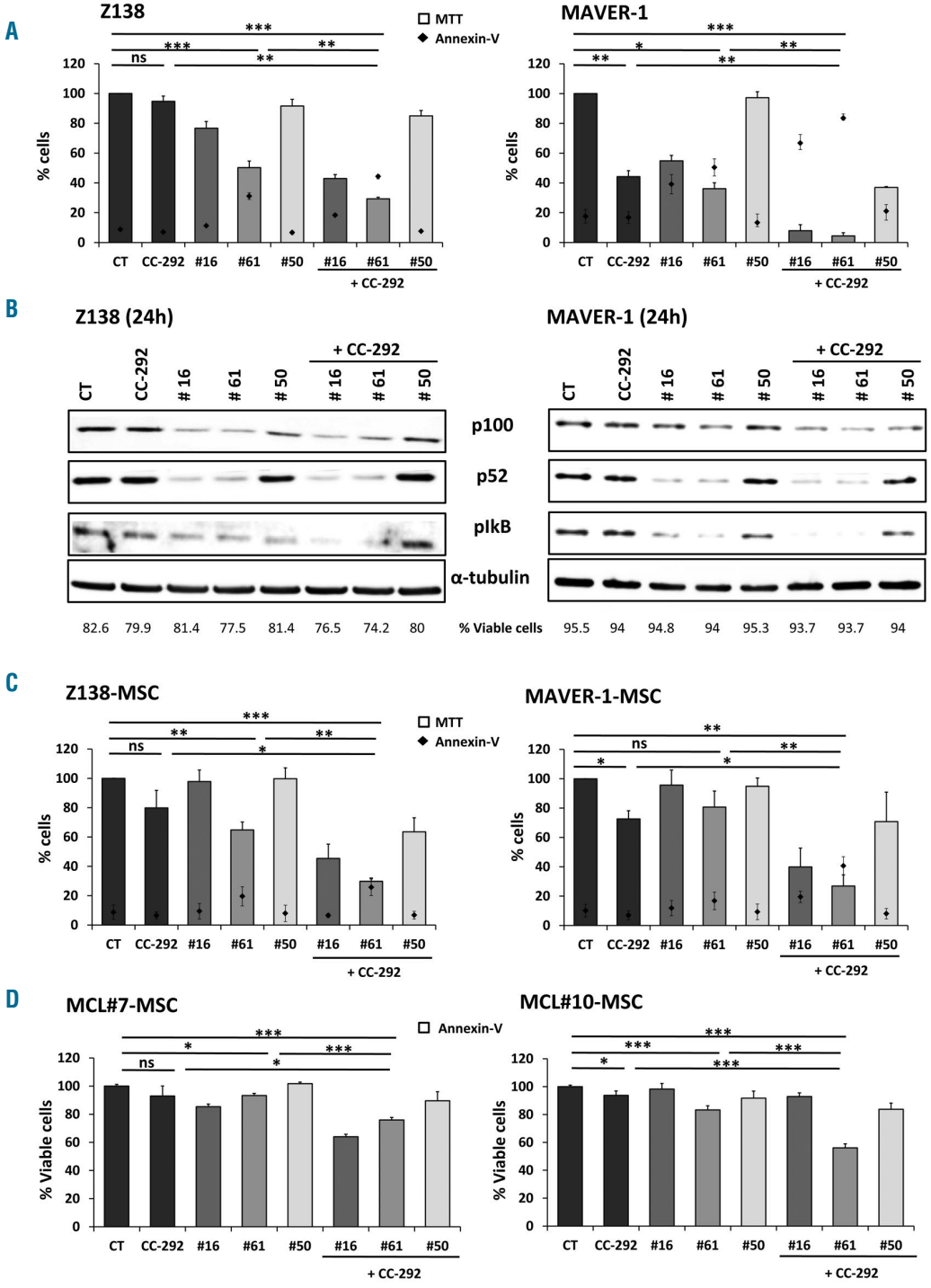

Figure 3. CC-292 cooperates with NIK inhibitors in CC-292-resistant mantle cell lymphoma cells. (A) Z138 and MAVER-1 were exposed to either 1 μM CC-292 or 1 μM of NIK inhibitors AM-0216 (#16) and AM-0561 (#61), the inactive isomer AM-0650 (#50) or their combination for 6 days. The total number of viable cells was analyzed by the MTT proliferation assay (bars) and viability by annexin-V labeling (dots). Values are referred to an untreated control and results are expressed as the mean ± SD of three independent experiments. (B) Z138 and MAVER-1 were treated with either 1 μM CC-292 or 1 μM NIK inhibitors or the combination for 24 h at 37°C, and the expression of NF-κB proteins was assessed by western blot. (C) MAVER-1 and Z138 were co-cultured with stromaNKtert in the presence or absence of 1 μM CC-292 and 1 μM NIK inhibitors for 6 days. The total number of viable cells was analyzed by the MTT proliferation assay (bars) and viability by annexin-V labeling and CD19 labeling(dots). Values are referred to an untreated control and results are expressed as the mean ± SD of three independent experiments.. (D) Primary cells from MCL patients with mut*BIRC3* were cultured with stromaNKtert feeder cells and treated with 1 μM CC-292 with or without 1 μM NIK inhibitors for 6 days. The number of viable MCL cells was quantified as before. Results are shown referred to the untreated control.

ment in MCL cells,[9] we investigated the effect of CC-292 on the secretion of these chemokines. CC-292 efficiently decreased the IgM-induced secretion of CCL3 and CCL4 in both CC-292-sensitive and -resistant cell lines and in supernatants from primary MCL cells with *BIRC3* inactivated by deletion of one allele and mutation of the other (*Online Supplementary Figure S3C*).

BTK is involved in B-cell trafficking mediated by the chemokine receptors CXCR4/CXCR5.[10] We measured the direct effect of CC-292 on migration of two MCL cell lines (UPN-1, Z138) and four primary cells (MCL#1, MCL#2, MCL#10, MCL#11). CXCL12-induced migration of MCL cell lines was diminished after CC-292 treatment, although the extent of inhibition was greater in the CC-292-sensitive cell line UPN-1 (34.4%) than in the CC-292-resistant cell line Z138 (18.8%) (*Online Supplementary Figure S4A*). Regarding the primary cases, we found that CC-292 significantly inhibited CXCL12-induced migration of those cells with no mutations in the alternative NF-κB pathway (MCL#2, MCL#11) (22.2% and 55.1% of inhibition, respectively), whereas it was not able to interfere with CXCL12-induced migration of MCL#1 and MCL#10, bearing mut*BIRC3* (*Online Supplementary Figure S4A*).The basis of these different chemotactic responses may lie in the recently demonstrated requirement of both canonical and alternative NF-κB pathways for this biological process.[11] Moreover, CXCL12 ligation to CXCR4 induced immediate and prominent pERK1/2 activation (phosphorylation at Thr202/Tyr204 residues). Treatment with CC-292 efficiently reduced ERK activation in UPN-1 and to a lesser extent in Z138 while it had no effect in MCL#10, according to the results obtained in the CXCL12-migration assay (*Online Supplementary Figure S4B*).

Since the antitumor activity of CC-292 is limited, we investigated whether a greater therapeutic effect could be achieved by combining this inhibitor with the immunomodulatory agent lenalidomide. In the cell lines with primary sensitivity to CC-292 (MINO, REC-1, UPN-1), lenalidomide significantly increased the antitumor activity of the BTK inhibitor when used both at low and at high doses. In contrast, no cooperation was found between these two agents in the CC-292-resistant cell lines (MAVER-1 and Z138) (Figure 2A). It has been described that lenalidomide downregulates the expression of IRF4, a NF-κB target, in sensitive MCL cell lines.[12] In this line, the combination efficiently reduced IRF4 expression in CC-292-sensitive cases, while expression remained unaffected in CC-292-resistant cases (Figure 2B). This cooperative effect was likewise observed in the MCL-stromaNKtert co-culture system of primary cells (MCL#4) with no mutations in the alternative NF-κB pathway regulators (Figure 2C).

Finally, we determined the potential activity of specific NIK inhibitors[13,14] in those MCL cell lines resistant to CC-292 due to activation of the alternative NF-κB pathway. Z138 and MAVER-1 were treated for 6 days with two NIK inhibitors, AM-0216 (#16) and AM-0561 (#61), or with an isomeric control of AM-0216 [AM-0650 (#50)], in the presence or absence of 1 μM CC-292. AM-0216 and AM-0561 were active in both cell lines, with MAVER-1 being the more sensitive. It is worth noting that combining AM-0216 and AM-0561 with an inhibitor of the canonical NF-κB pathway, such as CC-292, resulted in a significant cooperative effect in terms of cell growth inhibition and apoptosis induction both in MAVER-1 and in Z138 (Figure 3A). Analysis of the p52 levels, as a surrogate marker of activation of the alternative NF-κB pathway, indicated that while CC-292 exerted no effect on p100 processing, AM-0216 and AM-0561 induced a remarkable decrease in p52 levels, while these remained unaffected in the presence of the isomeric control AM-0650 (Figure 3B). More importantly, pIκB completely disappeared in the cells treated with the combination, indicating that total inhibition of the NF-κB pathway was achieved (Figure 3B). When co-cultured with stromaNKtert cells, Z138 and MAVER-1 became less sensitive to NIK inhibitors, although the cooperation with the CC-292 and NIK inhibitors was maintained (Figure 3C). We validated these results in primary MCL cases bearing inactivation of *BIRC3* in the MCL-stromaNKtert co-culture system. As shown in Figure 3D, the combination of CC-292 and NIK inhibitors was significantly active in primary MCL cases with *BIRC3* inactivation (MCL#7 and MCL#10), confirming the results obtained with MCL cell lines.

In summary, CC-292 shows an anti-proliferative profile conditioned by the existence of activating mutations in alternative NF-κB pathway genes. It is worth noting that CC-292 blocks BCR activation, independently of the presence of mutations on *TRAF2/3* or *BIRC3* genes. However, CC-292 interferes with CXCL12-induced migration mostly in MCL cells without activation of the alternative NF-κB pathway. In sensitive cell lines, CC-292 activity was significantly enhanced by co-treatment with lenalidomide. For MCL cases bearing mutations in the alternative NF-κB pathway, inhibitors of NIK produced cytostatic and cytotoxic responses in MCL cell lines and primary cultures. These substantial effects of CC-292 on MCL, especially in combination regimens, warrant further investigation in the clinical setting.

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