

## TO THE EDITOR:

## *BTK* mutations in patients with chronic lymphocytic leukemia receiving tirabrutinib

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In the clinic setting, there is now a plethora of Bruton tyrosine kinase inhibitors (BTKis), with either covalent or noncovalent binding to BTK, for malignant and autoimmune indications. Which BTKi is optimal for specific indications currently remains unclear; both relative efficacy and toxicities of an individual BTKi will depend on its precise mechanism of action. Chemical variables include the mechanism of binding of the BTKi within the adenosine triphosphate binding site of BTK and, in the case of covalent inhibitors, the mechanisms and kinetics of binding of the BTKi to the exposed cysteine-481 residue.

In the context of prolonged BTKi treatment required for chronic lymphocytic leukemia (CLL), variations in the binding of BTKi to BTK may be reflected in differences in patterns of *BTK* mutation. We read, with interest, the 2 recent publications in *Blood Advances* by Blombery et al<sup>1</sup> and Naeem et al,<sup>2</sup> and the recent publication in the *New England Journal of Medicine* by Wang et al,<sup>3</sup> describing patterns of *BTK* mutations associated with the covalent BTKi zanubrutinib and the noncovalent BTKi pirtobrutinib.

Patients with CLL treated with the first-in-class BTKi ibrutinib<sup>4-9</sup> or second-generation BTKi acalabrutinib<sup>10</sup> predominantly relapse with *BTK* C481S mutations, resulting in reduced binding affinity of the covalent BTKi to BTK. In comparison, zanubrutinib<sup>1,11</sup> and pirtobrutinib<sup>3</sup> demonstrated different *BTK* mutational patterns (supplemental Table 1). Blombery et al showed that patients with progressing disease while receiving zanubrutinib had enrichment for *BTK* L528W mutations.<sup>1,12</sup> Unlike C481S, which results in a kinase that retains activity, L528W prevents both the binding of covalent inhibitors and the binding of adenosine triphosphate, resulting in a “kinase-dead” protein. L528W and other non-C481 mutations within the kinase domain (including V416L, A428D, M437R, and T474I) have also been described at progression with pirtobrutinib.<sup>3</sup> In the case of 2 inactivating C481 mutations (C481F and C481Y), signaling to *PLCG2* may be maintained via a kinase-independent activation of hematopoietic cell kinase,<sup>13</sup> although it is unclear whether this bypass mechanism is initiated with L528W mutation.

In 9 patients with progressing disease while receiving pirtobrutinib in the BRUIN trial, multiple non-C481 *BTK* mutations were identified in 78% of the patients.<sup>14</sup> In contrast, non-C481 mutations were only rarely observed in patients with CLL treated with ibrutinib and acalabrutinib. Only 5% (6 out of a total of 115 previously reported patients with CLL) had disease progression with non-C481 mutations while receiving ibrutinib<sup>4-9</sup> (supplemental Table 2). In 67% of these patients, non-C481 *BTK* mutations occurred with concurrent C481 or *PLCG2* mutations, and all but 1 of these detected non-C481 mutations were at variant allele frequencies <8%.

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Data are available on request from the corresponding author, Martin J. S. Dyer ([mjds1@leicester.ac.uk](mailto:mjds1@leicester.ac.uk)).

The full-text version of this article contains a data supplement.

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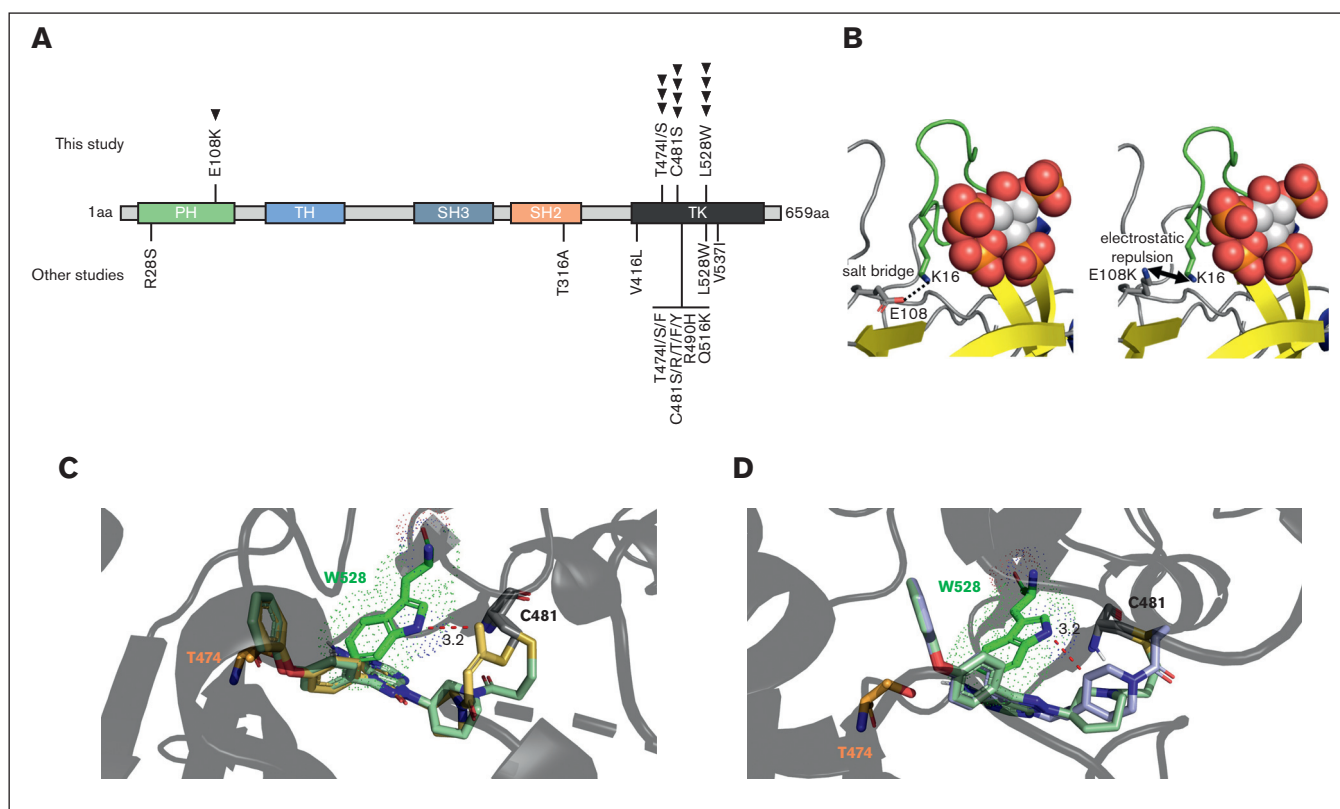
Here, we report a series of 9 patients with CLL whose condition progressed while receiving the covalent BTKi tirabrutinib<sup>15</sup> and who show a further distinct *BTK* mutational profile. *BTK* mutations were identified via both whole-exome sequencing and digital droplet polymerase chain reaction (ddPCR) and are summarized in Figure 1A and Table 1. All 9 patients with CLL who relapsed while receiving tirabrutinib had disease progression rather than Richter transformation; the duration of tirabrutinib administration before disease progression is shown in supplemental Figure 1, and clinical information is outlined in supplemental Table 3. There was a significant enrichment of non-C481 *BTK* mutations in patients treated with tirabrutinib compared with those treated with ibrutinib but not with those treated with zanubrutinib (supplemental Table 1). No *PLCG2* mutations were detected.

In total, 4 patients had a C481 mutation detected at relapse, of whom 1 had an additional T474I mutation, and 2 had both a T474I and L528W mutation. Only 1 patient exhibited a *BTK* C481 mutation as an isolated event. No patient had a T474 mutation in the absence of a C481 mutation. T474 mutations result in increased BTK kinase activity and decreased sensitivity to both covalent and noncovalent BTKi.<sup>16</sup> In 1 patient, T474I was acquired with an E108K mutation within the pleckstrin homology domain.

Modeling of E108K suggests an interruption of the salt bridge to BTK K16, with likely destabilization of the Ins(1,3,4,5)P4 binding site due to electrostatic repulsion via K16 of the  $\beta$ 1- $\beta$ 2 loop (Figure 1B). The previously described R28S has structurally similar consequences,<sup>6</sup> and such pleckstrin homology domain mutations will prevent the accumulation of activated BTK at the inner leaflet of the cell surface membrane, significantly impairing kinase activity.

In 2 male patients, L528W was observed without accompanying C481 and T474 mutations, with cancer cell fractions of 86.8% and 91%, suggesting a dominant resistance mechanism. Cancer cell fractions were calculated as variant allele frequencies via whole-exome sequencing, taking into account the copy number state of the mutation site and, thus, were unaffected by copy number variations of the X chromosome.

C481S and T474I mutations were detected in a sample from a patient with CLL (patient identified as CLL-6) 36 months before clinical relapse (supplemental Figure 1). Three patients treated with tirabrutinib lacked both *BTK* and *PLCG2* mutations, highlighting the role of alternative/nongenetic mechanisms of resistance. *PLCG2* mutational status of zanubrutinib-treated CLL has not been described but have been observed with pirtobrutinib (22% of the patients who relapsed).<sup>3</sup>



**Figure 1. BTK mutations in patients with CLL receiving tirabrutinib.** (A) Schematic domain structure of BTK with mutations identified in this study (top) (with each arrow denoting a single patient) and mutations identified in other studies of patients treated with ibrutinib, acalabrutinib, zanubrutinib, and pirtobrutinib (bottom). (B) Molecular modeling of the BTK pleckstrin homology (PH)-domain E108K mutation. Ins(1,3,4,5)P4 are shown as spheres, PH-domain residues of interest are shown as sticks. The  $\beta$ 1- $\beta$ 2 loop, which forms part of the Ins(1,3,4,5)P4 binding site is indicated in green. (Left) E108 and K16 form a salt bridge, which stabilizes the Ins(1,3,4,5)P4 binding site. (Right) the E108K mutation interrupts the salt bridge to K16 and is likely to destabilize the Ins(1,3,4,5)P4 binding site because of electrostatic repulsion via K16 of the  $\beta$ 1- $\beta$ 2 loop. (C-D) Molecular modeling of ibrutinib (pale green), tirabrutinib (yellow), and zanubrutinib (purple) bound to BTK. BTK W528 disrupts the binding of BTKi to BTK, with additional steric clash observed with tirabrutinib and zanubrutinib (denoted by red dotted lines). Mutated W528 is shown in bright green, and T474 shown in orange.

**Table 1. *BTK* mutations identified via whole-exome sequencing and digital droplet polymerase chain reaction in patients with CLL after relapse with tirabrutinib**

Patient	<i>BTK</i> amino acid change	WT codon	Mutant codon	CCF from WES (%)	VAF from ddPCR (%)
CLL-1	L528W	TTG	TGG	86.8	78.6
CLL-2	C481S	TGC	TCC	Not called	16.4
CLL-3			None detected		
CLL-4	C481S	TGC	TCC	22.4	34.5
	T474I	ACT	ATT	50.4	62.4
CLL-5	L528W	TTG	TGG	91.0	79.3
CLL-6	C481S	TGC	TCC	Not detected	1.9
	L528W	TTG	TGG	Not detected	0.71
	T474I	ACT	ATT	34.4	12
	E108K	GAA	AAA	56.9	Not tested
CLL-7			None detected		
CLL-8	C481S	TGC	TCC	Not called	17.9
	C481S	TGC	ACG	Not called	5.3
	L528W	TTG	TGG	Not detected	0.16
	T474I	ACT	ATT	5.0	5.8
CLL-9			None detected		

CCF calculated from WES and VAFs from ddPCR are included.

"Not detected" denotes mutations that were not called with our WES variant caller and were not visualized by eye in the raw sequencing read data.

"Not called" denotes mutations that were not detected with our WES variant caller but were visualized by eye on raw sequencing reads, likely because of low-confidence variant calling of low VAF mutations within low-coverage data.

CCF, cancer cell fraction; ddPCR, digital droplet polymerase chain reaction; VAF, variant allele frequency; WES, whole-exome sequencing; WT, wild-type.

Besides mutations to *BTK*, no other consistent genetic aberrations (including single-nucleotide variants or copy number aberrations) were detected that are considered to be important in the development of resistant disease (supplemental Figure 2), even for the 3 patients without detectable *BTK* and *PLCG2* mutations. Differential gene expression profiling was also carried out, with no consistent changes occurring at relapse in these cases (data not shown).

Because of the similarities between tirabrutinib and zanubrutinib with respect to the higher percentage of patients acquiring L528W mutations compared with ibrutinib, we compared the effect of *BTK* L528W on the binding of tirabrutinib, zanubrutinib, and ibrutinib to *BTK* in the context of mutated L528W using molecular modeling. We, and others, have shown that L528W caused steric hindrance, by which covalent BTKis are pushed away from the C481 covalent binding site, disrupting covalent interactions of all 3 inhibitors<sup>16</sup> (supplemental Figures 3 and 4). However, additional steric hindrance was caused by W528 when tirabrutinib and zanubrutinib, compared with ibrutinib, were bound to *BTK*. This is because of a predicted additional steric clash of W528 with the methyl group present in tirabrutinib (Figure 1C) and piperidine present in zanubrutinib (Figure 1D), both of which were 0.6Å closer to W528 compared with the groups present in ibrutinib.

Although individually based on small numbers of patients, these data collectively suggest that different BTKis may be associated with different mutational patterns of both *BTK* and *PLCG2*, which may be reflected in differential modes of the binding of the BTKi to *BTK*. We have demonstrated that tirabrutinib appears to induce C481, T474, and L528 mutations at approximately equal frequencies (44%, 33%, and 44% respectively). Of the patients who

developed a C481 mutation, 75% additionally developed a T474 mutation or both T474 and L528 mutations. This is different from patients treated with ibrutinib, in whom C481 mutations were predominant genetic drivers of resistance, and alternative *BTK* mutations were rare.

These data may have clinical implications because patients with disease progressing while receiving covalent BTKi with non-C481 mutations may have suboptimal responses with subsequent pirtobrutinib treatment, as demonstrated by Blombery et al,<sup>1</sup> presenting issues of cross-resistance. This highlights the need for the clinical development of high-sensitivity assays for the detection of non-C481 *BTK* mutations.

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**Contribution:** R.A.J. performed research, analyzed data, performed the bioinformatics analysis, and wrote the manuscript; C.S.T. analyzed data and performed bioinformatics analysis; S.J. performed research, analyzed data, and wrote the manuscript;

C.M.C. and S.L. performed research; R.G.B. and R.S. performed and analyzed molecular modeling-based analyses; V.M.S. analyzed data and wrote the manuscript; C.F. provided clinical samples and treated patients; H.S.W. and M.J.S.D. supervised the study, treated patients, and wrote the manuscript; and all authors approved the final version.

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