NX-5948 promotes selective, sub-nanomolar degradation of inhibitor-resistant BTK mutants

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Abstract

Small molecule kinase inhibitors have revolutionized the treatment of hematological malignancies by suppressing signaling pathways essential for tumor cell survival. Bruton's Tyrosine Kinase (BTK) inhibitors are widely used in the clinic for treatment of patients with B cell malignancies. Acquired resistance mutations, however, can reduce or eliminate their efficacy and represent a growing challenge. Mutations at C481 dramatically reduce the binding of covalent BTK inhibitors, whereas other clinically-observed mutations such as V416L, T474I, and L528W reduce or eliminate the activity of next-generation non-covalent inhibitors. Some of these mutations abolish BTK kinase activity while retaining intact BCR signaling and BTK-dependent growth, indicating that mutant BTK elicits scaffold-mediated signaling essential for malignant B cell survival [1].

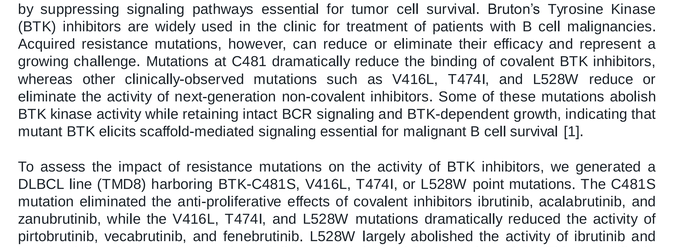
To assess the impact of resistance mutations on the activity of BTK inhibitors, we generated a DLBCL line (TMD8) harboring BTK-C481S, V416L, T474I, or L528W point mutations. The C481S mutation eliminated the anti-proliferative effects of covalent inhibitors ibrutinib, acalabrutinib, and zanubrutinib, while the V416L, T474I, and L528W mutations dramatically reduced the activity of pirtobrutinib, vecabrutinib, and fenebrutinib. L528W largely abolished the activity of ibrutinib and zanubrutinib, whereas V416L substantially reduced the activity of acalabrutinib. This variability in BTK inhibitor sensitivity to resistance mutations complicates treatment decisions for patients who relapse on BTK inhibitors, promoting the use of agents that can more broadly target resistance mutations.

Here we assessed the activity of NX-5948, a heterobifunctional degrader molecule that induces the targeted degradation of BTK. We used surface plasmon resonance to evaluate the binding of NX-5948 to WT and mutant (C481S, T474I, V416L and L528W) BTK proteins. NX-5948 binds potently to BTK WT, C481S and T474I with single-digit nanomolar affinities, but loses potency against the V416L (14-fold) and L528W (33-fold) mutants. Despite this reduction in binary binding affinity, NX-5948 induces sub-nanomolar degradation of all mutant forms of BTK and potently suppresses expression of activation markers and proliferation in TMD8 cells harboring these mutations. We propose that the positive cooperativity that NX-5948 induces between BTK and the E3 ligase cereblon contributes to its potent and sustained degradation activity against BTK resistance mutants.

We also assessed the selectivity of NX-5948 by global proteomics and observed exquisite selectivity across cell types and conditions. The exceptional potency, selectivity, and activity of NX-5948 against BTK mutants warrant its investigation in clinical settings that develop diverse inhibitor resistance. A phase 1a/b trial of NX-5948 for patients with relapsed or refractory B-cell malignancies is ongoing (NCT05131022).

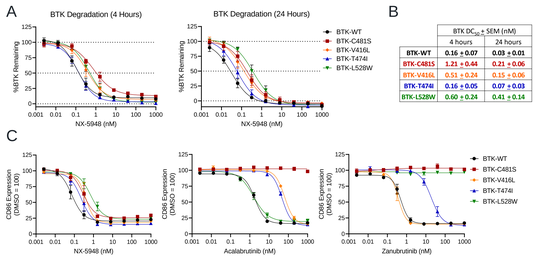
Results

Figure 1. Acquired resistance mutations reduce or eliminate the anti-proliferative effects of BTK inhibitors



TMD8 cells harboring WT BTK or knock-in BTK mutations (C481S, V416L, T474I, or L528W) were incubated with non-covalent (A) and covalent (B) BTK inhibitors for 72 hours, and viability was assessed using CellTiter-Glo 2.0 (Promega). The percentage of viable cells in compound-treated samples was normalized to DMSO-treated controls. (C) GI-50 values were calculated for compounds and lines in (A) and (B) and represent the concentration of compound that reduces cell growth by 50%. GI-50 values were capped at the highest assay concentration (5000 nM) for compounds that did not achieve 50% killing. Curves and GI-50 values are averaged from n ≥ 3 independent experiments. Mean ± SEM is displayed in (A) and (B).

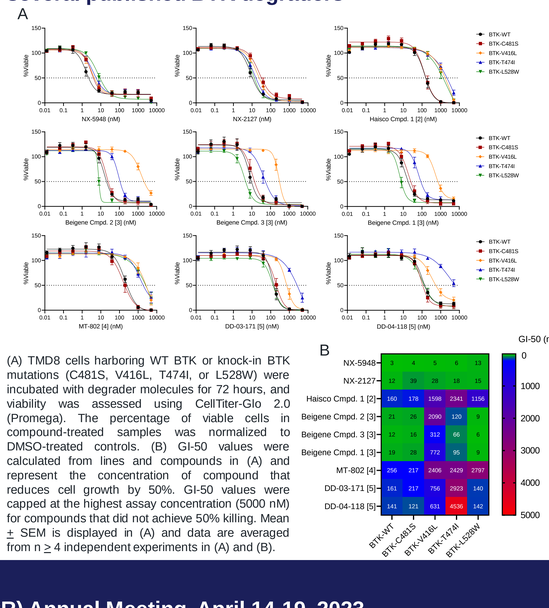
Figure 2. NX-5948 degrades mutant BTK and modulates activation markers in inhibitor-resistant TMD8 cells



	BTK DC_{50} ± SEM (nM)	
	4 hours	24 hours
BTK-WT	0.16 ± 0.07	0.03 ± 0.01
BTK-C481S	1.21 ± 0.44	0.21 ± 0.06
BTK-V416L	0.51 ± 0.24	0.15 ± 0.06
BTK-T474I	0.16 ± 0.05	0.07 ± 0.03
BTK-L528W	0.60 ± 0.24	0.41 ± 0.14

(A) TMD8 cells harboring WT BTK or knock-in BTK mutations (C481S, V416L, T474I, or L528W) were incubated with NX-5948 for 4 or 24 hours, and BTK degradation was assessed by flow cytometry. (B) BTK DC_{50} values were calculated for degradation curves in (A) and correspond to the concentration of compound that promotes 50% degradation of BTK. (C) TMD8 cells harboring WT or mutant BTK were treated with NX-5948 or inhibitors for 24 hours and surface CD86 expression was quantified by flow cytometry. Data were normalized to DMSO-treated controls and averaged from n = 3 independent experiments. Mean ± SEM is displayed.

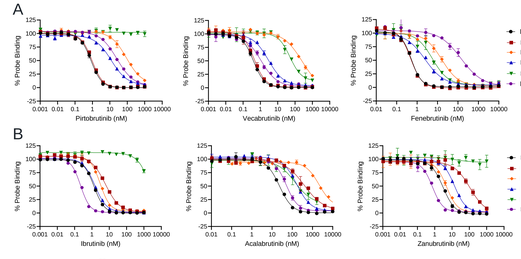
Figure 3. NX-5948 demonstrates broad coverage of BTK inhibitor resistance mutations, in contrast to several published BTK degraders



(A) TMD8 cells harboring WT BTK or knock-in BTK mutations (C481S, V416L, T474I, or L528W) were incubated with degrader molecules for 72 hours, and viability was assessed using CellTiter-Glo 2.0 (Promega). The percentage of viable cells in compound-treated samples was normalized to DMSO-treated controls. (B) GI-50 values were calculated from lines and compounds in (A) and represent the concentration of compound that reduces cell growth by 50%. GI-50 values were capped at the highest assay concentration (5000 nM) for compounds that did not achieve 50% killing. Mean ± SEM is displayed in (A) and data are averaged from n ≥ 4 independent experiments in (A) and (B).

Results (Continued)

Figure 4. Acquired resistance mutations reduce the binding of covalent and non-covalent BTK inhibitors to BTK

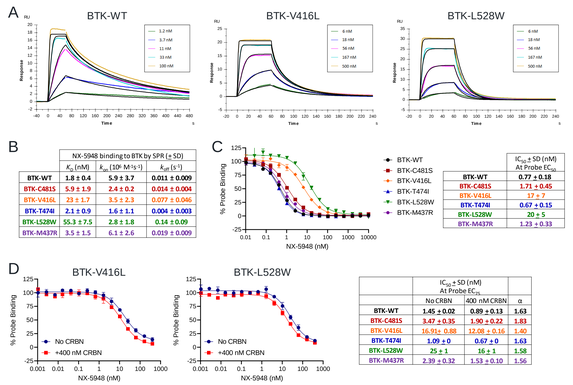


C

	IC_{50} ± SD (nM)					
	Pirtobrutinib	Vecabrutinib	Fenebrutinib	Ibrutinib	Acalabrutinib	Zanubrutinib
BTK-WT	0.76 ± 0.26	0.38 ± 0.06	0.68 ± 0.26	1.4 ± 0.1	26.6 ± 2.4	2.7 ± 0.3
BTK-C481S	0.83 ± 0.28	0.44 ± 0.06	0.69 ± 0.28	6.2 ± 0.3	467 ± 30	101 ± 6
BTK-V416L	130 ± 67	144 ± 45	21 ± 4	3.8 ± 0.7	4101 ± 1331	4.7 ± 0.1
BTK-T474I	13 ± 3	2.71 ± 0.32	2.74 ± 1	1.8 ± 0.3	189 ± 21	12.1 ± 1.5
BTK-L528W	>1000	84 ± 18	7.60 ± 1.94	>1000	240 ± 47	>1000
BTK-M437R	47 ± 22	1.01 ± 0.18	114 ± 36	0.28 ± 0.07	52.7 ± 5.9	0.61 ± 0.11

(A-B) Representative biochemical probe-based TR-FRET competition assay data for non-covalent (A) and covalent (B) BTK inhibitors for WT and mutant BTK. Inhibitors were tested using 2.5-fold dilutions starting from 1 μM (or 10 μM for acalabrutinib and fenebrutinib). (C) Average IC_{50} values for n ≥ 3 experiments are shown in table.

Figure 5. NX-5948 binds cooperatively with CRBN and BTK, either WT or mutant, overcoming the observed reduction in binary binding affinity to some mutants



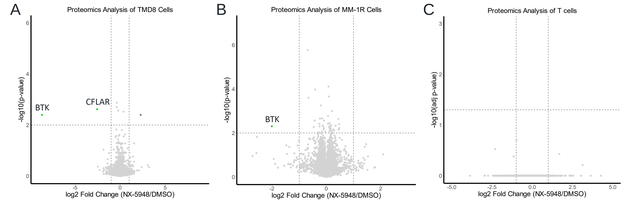
	NX-5948 binding to BTK by SPR (± SD)		
	K_D (nM)	k_{on} (10^4 $M^{-1}s^{-1}$)	k_{off} (s^{-1})
BTK-WT	1.8 ± 0.4	5.9 ± 3.7	0.011 ± 0.009
BTK-C481S	5.9 ± 1.9	2.4 ± 0.2	0.014 ± 0.004
BTK-V416L	23 ± 1.7	3.5 ± 2.3	0.077 ± 0.046
BTK-T474I	2.1 ± 0.9	1.6 ± 1.1	0.004 ± 0.003
BTK-L528W	55.3 ± 7.5	2.8 ± 1.8	0.14 ± 0.09
BTK-M437R	3.5 ± 1.5	6.1 ± 2.6	0.019 ± 0.009

	IC_{50} ± SD (nM) At Probe EC_{50}
BTK-WT	0.77 ± 0.18
BTK-C481S	1.71 ± 0.45
BTK-V416L	17 ± 7
BTK-T474I	0.67 ± 0.15
BTK-L528W	20 ± 5
BTK-M437R	1.23 ± 0.33

	IC_{50} ± SD (nM) At Probe EC_{50}		
	No CRBN	400 nM CRBN	α
BTK-WT	1.45 ± 0.02	0.89 ± 0.13	1.63
BTK-C481S	3.47 ± 0.35	1.90 ± 0.22	1.83
BTK-V416L	16.91± 0.88	12.08 ± 0.16	1.40
BTK-T474I	1.09 ± 0	0.67 ± 0	1.63
BTK-L528W	25 ± 1	16 ± 1	1.58
BTK-M437R	2.39 ± 0.32	1.53 ± 0.10	1.56

(A) Representative sensorgrams of NX-5948 binding to BTK WT, V416L and L528W. Measurements were taken using a Biacore S200 instrument at 25°C, pH 7.5. (B) SPR binding parameters of NX-5948 to WT and mutant BTK, reported as mean ± SD of n ≥ 3 independent measurements. (C) Representative biochemical probe-based TR-FRET competition assay data for NX-5948 with BTK WT and mutants. NX-5948 was tested using 2.5-fold dilutions starting from 10 μM at probe EC_{50}. (D) Representative biochemical probe-based TR-FRET cooperativity assay data for NX-5948 with BTK-V416L and BTK-L528W. NX-5948 was tested using 2.5-fold dilutions starting from 1 or 10 μM at probe EC_{75} in the absence and presence of saturating concentration of cereblon. Average IC_{50} values from n = 2 independent experiments are shown in probe displacement tables.

Figure 6. NX-5948 promotes selective degradation of BTK across cell types



(A) TMD8 cells were treated with 50 nM NX-5948 for 6 hours and analyzed by global proteomics. TMD8 cells are dependent on BTK for survival. CFLAR (c-FLIP) downregulation has been observed with BTK inhibition and likely represents a secondary effect of BTK degradation. (B) MM-1R cells were treated with 100 nM NX-5948 for 24 hours and analyzed by global proteomics. MM-1R cells express BTK but are not dependent on BTK expression for short term in vitro survival. No significant off-targets were identified under these conditions (C) Primary human T cells from 4 donors were treated with 100 nM for 4 hours and analyzed by global proteomics. T cells do not express BTK, and no significant off-targets were identified under these conditions. In (A) and (B), unadjusted p-value is displayed, and adjusted p-value is displayed in (C).

Conclusions

- NX-5948 promotes potent degradation of WT BTK at 4 (DC_{50}=0.16 nM) and 24 hours (DC_{50}=0.03 nM)
- Acquired resistance mutations reduce or abolish the anti-proliferative activity of BTK inhibitors since small molecule inhibitors need to occupy the BTK active site continuously in order to suppress pathway activation and prevent tumor cell proliferation. Single amino acid changes in the active site are often sufficient to significantly reduce target occupancy.
- In contrast to occupancy-driven pharmacology, targeted protein degraders employ event-driven pharmacology, inducing ternary complexes with an E3 ligase to promote target ubiquitylation. Furthermore, the additional interactions between the target protein and E3 ligase can increase the stability of ternary complexes relative to binary complexes.
- NX-5948 induces positive cooperativity between BTK and CRBN and retains the ability to degrade C481S, V416L, T474I, and L528W mutant BTK irrespective of the observed loss of binary binding affinity of NX-5948 to V416L and L528W.
- NX-5948 displays superior coverage of novel BTKi resistance mutations compared to published BTK degraders, particularly in the context of T474I and V416L mutations.
- Downregulation of CD86 expression on TMD8 cells by inhibitors and degraders closely correlates with the anti-proliferative effects of NX-5948.
- NX-5948 downregulates CD86 expression on mutant TMD8 cells and retains the ability to inhibit proliferation of cells harboring C481S, V416L, T474I, and L528W mutations
- BTK degradation by NX-5948 is highly selective in proteomics assessments; no significant direct off-targets were identified in primary T cells, TMD8 cells, nor MM-1R cells treated with NX-5948
- A phase 1a/b trial of NX-5948 for patients with relapsed or refractory B-cell malignancies is ongoing (NCT05131022).

References

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[4] Buhimschi et al. 2018. Biochemistry 57(26): 3564-3575.
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Disclosures

All authors are past or current employees of Nurix Therapeutics and hold company stock or stock options.

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