LOXO-305, a highly selective and non-covalent next generation BTK inhibitor, inhibits diverse BTK C481 substitution mutations

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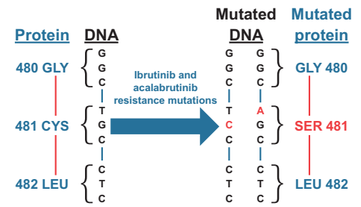
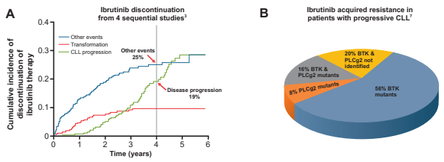
Introduction

- Bruton's tyrosine kinase (BTK) is an essential component of normal and malignant B-cell receptor signaling.
- Covalent BTK inhibitors have transformed the treatment of B-cell malignancies but are limited by off-target toxicity and acquired resistance, leading to eventual treatment discontinuation and disease progression. Emerging evidence suggests that acquired resistance is mediated predominantly by BTK C481 substitution mutations at the covalent BTK inhibitors' binding site. There is significant unmet clinical need for new treatment approaches that overcome acquired resistance and minimize toxicity.[1-5]
- LOXO-305 is a highly selective, non-covalent, next generation BTK inhibitor. We previously showed that LOXO-305 potently inhibited both wild-type (WT) BTK and BTK C481S-mediated kinase activity in enzyme and cell-based assays with nanomolar potency, and caused regression of BTK-dependent lymphoma tumors in mouse xenograft models. LOXO-305 was also more than 300-fold selective for BTK versus 370 other kinases tested and showed no significant inhibition of non-kinase off-targets at 1 μM.[6] In addition, preclinical ADME and pharmacokinetic experiments in two species predicted that LOXO-305 would likely have reasonable human exposure and sustained BTK C481S target coverage in patients at clinically achievable doses.



Mutation of BTK C481 prevents inhibitors from binding to BTK covalently.
Single nucleotide changes (substitution mutations) at C481 render BTK resistant to BTK inhibitors ibrutinib and acalabrutinib.



Resistance and intolerance limit covalent BTK inhibitors
- The appearance of BTK C481x mutations is the dominant reason for progressive CLL after covalent BTK inhibitors.[1-5,7]
- BTK C481x mutations prevent covalent BTK inhibitors from effective target inhibition.[1-5,7]

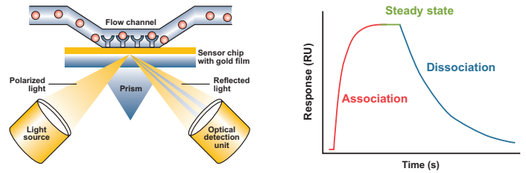
Objectives

- Here we describe the cellular activity of LOXO-305 against BTK C481 substitution mutations, including mutations identified in patients with acquired resistance to covalent BTK inhibitors. We further determine equilibrium-binding affinities for LOXO-305 for diverse mutant BTK enzymes and compare these activities to other clinically available BTK inhibitors.

Materials and Methods

- **Potency study in cells:** HEK293T cell lines transiently expressing WT BTK and BTK C481 substitution mutations were incubated with LOXO-305, ibrutinib or acalabrutinib for 30 min followed by the addition of orthovanadate. After 2 h, cells were lysed, and phosphorylated Y223 BTK and total BTK levels were detected by immunoblot (BTK WT, C481S, and C481T) or MesoScale (C481R). Bands and MSD signals were quantified, and the IC_{50} values calculated with GraphPad Prism.
- **Kinetic analysis by SPR:** WT biotinylated BTK 2-659 (BTN-BTK) and BTN-BTK C481S were purchased from Carna Biosciences. BTN-BTK C481R, and BTN-BTK C481T were expressed as N-terminal DYKDDDDK tagged, biotinylated proteins (~80 kDa) using a baculovirus expression system. The insect cells were broken by hypotonic lysis and the proteins were purified using DYKDDDDK tag antibody agarose. The equilibrium-binding affinities for targeted BTK inhibitors to BTK enzyme variants were determined by SPR using the Biacore T200. Purified BTN-BTK proteins were immobilized on a Series S Streptavidin sensor chip. Five increasing concentrations of each inhibitor plus blank controls were analyzed using a single cycle kinetics program with no regeneration step. Individual injection association and dissociation times were 120 s each with a final dissociation event of 1800 s. Association/dissociation rate constants were calculated by global fitting of the data to a 1:1 binding interaction model using T200 evaluation software v3.1.

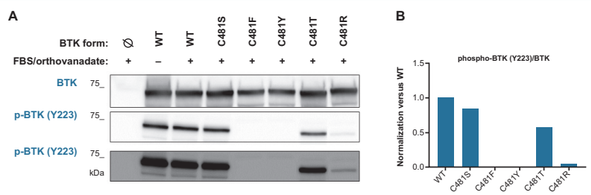
Surface Plasmon Resonance Binding Assay



Biacore T200 Handbook

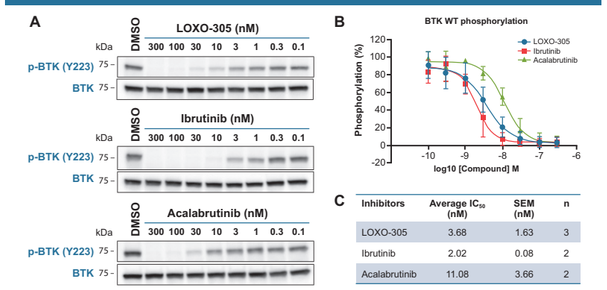
Results

Figure 1: Autophosphorylation of BTK Y223 in HEK293 cells expressing WT and C481X mutant forms of BTK



Western blot analysis of HEK293T cell lysates after transient transfection with WT and mutant forms of BTK. C481S mutant showed similar levels of phosphorylation compared with the WT form of BTK, C481T autophosphorylation was reduced by 50%, while C481R was weakly catalytically active. No Y223 phosphorylation was detected for C481F and Y substitutions. (A) Total BTK expression and phospho-BTK Y223 detection; two exposure times of the membrane are shown for Y223. (B) Bar graph showing the relative amount Y223 phosphorylation in BTK WT and mutant forms. Bands were quantified and the phospho-Y223 signal was normalized to total BTK.

Figure 2: LOXO-305, ibrutinib and acalabrutinib inhibit WT BTK Y223 autophosphorylation

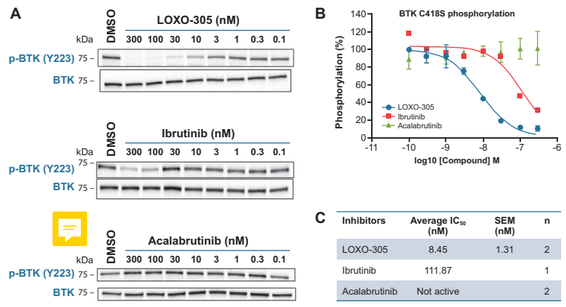


C

Inhibitors	Average IC_{50} (nM)	SEM (nM)	n
LOXO-305	3.68	1.63	3
Ibrutinib	2.02	0.08	2
Acalabrutinib	11.08	3.66	2

Evaluation of the potency of LOXO-305, ibrutinib and acalabrutinib on inhibiting BTK WT autophosphorylation. (A) Representative Western blot membranes for total and phospho-BTK. (B-C) BTK Y223 phosphorylation was normalized to total BTK, (B) dose-response curves expressed as percentage of the DMSO control, (C) table with average IC_{50} values and SEM.

Figure 3: LOXO-305 inhibits BTK C481S Y223 autophosphorylation with a >10 fold potency over ibrutinib and acalabrutinib

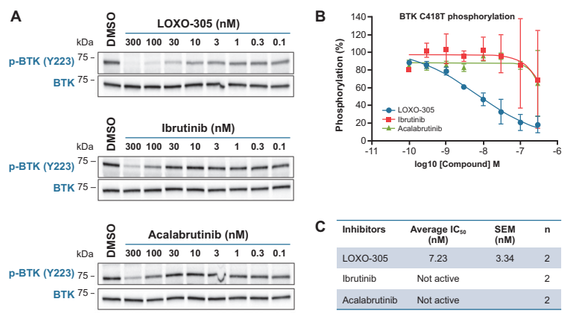


C

Inhibitors	Average IC_{50} (nM)	SEM (nM)	n
LOXO-305	8.45	1.31	2
Ibrutinib	111.87		1
Acalabrutinib	Not active		2

Evaluation of the potency of LOXO-305, ibrutinib and acalabrutinib on inhibiting BTK C481S autophosphorylation. (A) Representative Western blot membranes for total and phospho-BTK. (B-C) BTK Y223 phosphorylation was normalized to total BTK, (B) dose-response curves expressed as percentage of the DMSO control, (C) table with average IC_{50} values and SEM.

Figure 4: LOXO-305 inhibits BTK C481T Y223 autophosphorylation, while ibrutinib and acalabrutinib are inactive

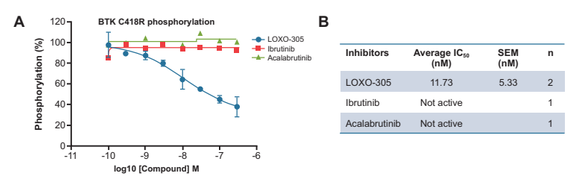


C

Inhibitors	Average IC_{50} (nM)	SEM (nM)	n
LOXO-305	7.23	3.34	2
Ibrutinib	Not active		2
Acalabrutinib	Not active		2

Evaluation of the potency of LOXO-305, ibrutinib and acalabrutinib on inhibiting BTK C481T autophosphorylation. (A) Representative Western blot membranes for total and phospho-BTK. (B-C) BTK Y223 phosphorylation was normalized to total BTK, (B) dose-response curves expressed as percentage of the DMSO control, (C) table with average IC_{50} values and SEM.

Figure 5: LOXO-305 inhibits BTK C481R Y223 autophosphorylation, while ibrutinib and acalabrutinib are inactive

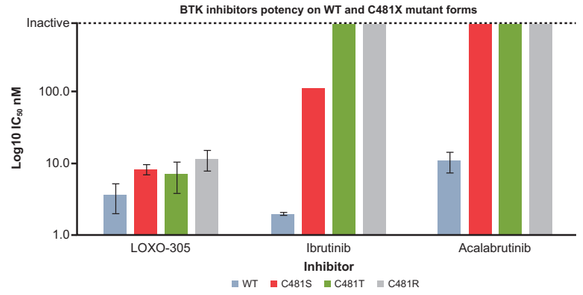


B

Inhibitors	Average IC_{50} (nM)	SEM (nM)	n
LOXO-305	11.73	5.33	2
Ibrutinib	Not active		1
Acalabrutinib	Not active		1

Evaluation of the potency of LOXO-305, ibrutinib and acalabrutinib in inhibiting BTK C481R autophosphorylation. Phosphorylation of BTK C481R and total BTK was analysed by MSD, and the phospho signal was normalized to total BTK. (A) Dose-response curves expressed as percentage of the DMSO control. (B) Table with average IC_{50} values and SEM.

Figure 6: LOXO-305 shows a superior potency in inhibiting BTK C481 substitutions versus commercially available BTK inhibitors



Data summary comparing the relative potencies of LOXO-305, ibrutinib and acalabrutinib in inhibiting BTK WT and C481 mutation substitutions.

Table 1: Biacore-generated kinetic data for BTK-inhibitor affinity interactions

LOXO-305	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	$t_{1/2}$ (min)	Acalabrutinib	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	$t_{1/2}$ (min)	Ibrutinib	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	$t_{1/2}$ (min)
BTK (WT)	6.0E+04	5.3E-05	216	BTK (WT)	N/A	N/A	N/A	BTK (WT)	N/A	N/A	N/A
C481S	2.7E+04	7.2E-05	156	C481S	2.4E+04	2.6E-03	4	C481S	6.7E+02	3.5E-04	33
C481R	5.9E+04	2.6E-05	450	C481R	8.8E+04	2.1E-03	5	C481R	4.6E+02	8.2E-04	14
C481T	3.1E+04	6.8E-06	1692	C481T	8.9E+04	1.2E-03	10	C481T	5.4E+02	2.3E-04	44

Biacore-generated data summary of the rate constants for WT BTK and 3 BTK C481 substitution mutations with LOXO-305 and 2 commercially available BTK inhibitors. The complex half-life time ($t_{1/2}$) is the time needed for dissociation of half of the complexes to their individual components and is calculated based on the dissociation rate constant ($\ln 2/k_d$).
k_a, association rate constant ($M^{-1}s^{-1}$); k_d, dissociation rate constant (s^{-1}); N/A, covalent inhibitor prohibits kinetic analysis.

Conclusions

- The next generation, non-covalent, highly selective BTK inhibitor LOXO-305 potently inhibited the cellular activity of BTK C481S, T and R mutations and displayed strong equilibrium binding to WT BTK and several BTK C481 substitution mutations. Together with high selectivity and significant BTK target coverage in vivo, these results indicate that LOXO-305 may overcome acquired resistance to covalent BTK inhibitors in patients without significant off-target toxicity. A phase 1 clinical trial for LOXO-305 is currently underway.

References

1. Woyach et al. *N Eng J Med*. 2014; 370:2286–2294.
2. Byrd et al. *N Eng J Med*. 2016; 374:323–332.
3. Woyach et al. *J Clin Oncol*. 2017; 35:1437–1443.
4. Xu et al. *Blood*. 2017; 129:2519–2525.
5. Hershkovitz-Rokah et al. *Br J Haematol*. 2018; 181:306–319.
6. Brandhuber et al. *Clin Lymphoma Myeloma Leuk*. 2019; 18, Suppl 1:S216.
7. Lampson et al. *Expert Rev Hematol*. 2018; 11:185–194.

Disclosures

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