Evaluation of covalent BTK inhibitors using COVALfinder®

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De-risking covalent BTK drug discovery

BTK is a prominent therapeutic target for hematologic cancers and an attractive target for treating autoimmune diseases. BTK inhibitors can be divided into 2 types: covalent irreversible and non-covalent reversible inhibitors.

Currently, 6 approved BTK inhibitors (Ibrutinib, Acalabrutinib, Zanubrutinib, Tirabrutinib and Orelabrutinib) target the kinase domain of BTK, forming a covalent bond with Cys481. Remibrutinib is another irreversible inhibitor that exhibits a good kinase selectivity due to binding to the non-phosphorylated form of BTK and is being evaluated in the clinic for urticaria and asthma. Recently, a hybrid inhibitor with the ability to establish a reversible covalent bond with Cys481 and temporarily inactivate BTK (Rilzabrutinib) has entered phase 3 clinical trials for the treatment of pemphigus and immune thrombocytopenic purpura[1,2].

Here, we present the kinetic characterization of covalent BTK inhibitors using COVALfinder® as a successful approach to identify novel covalent drugs, interpret SAR and modulate the safety profile of irreversible drugs.

How COVALfinder® assays work

COVALfinder® is a robust kinetic platform that delivers all key inactivation (k_{inact}, k_{inact}/K_I, $T_{1/2}\infty$) and affinity (K_I) parameters for irreversible binders.

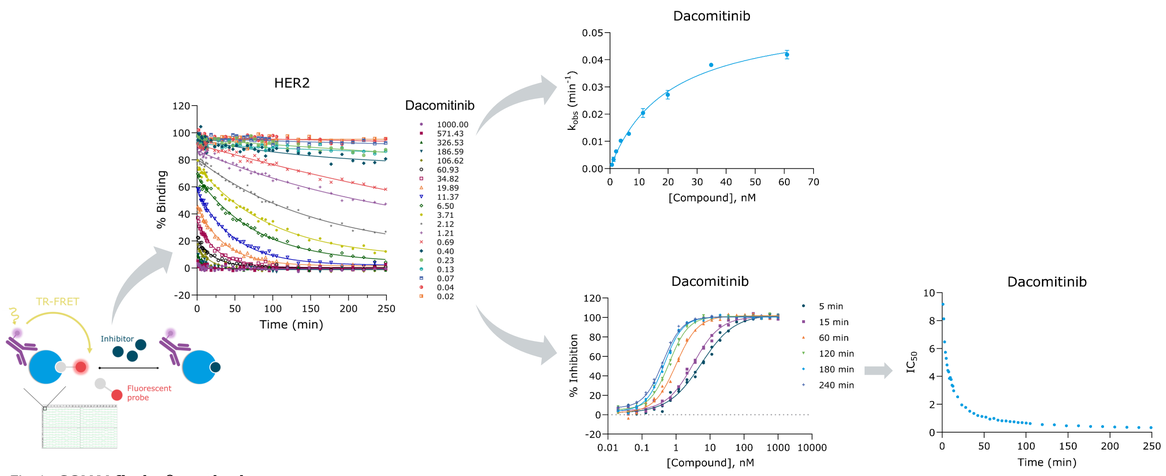


Fig 1. **COVALfinder® method.**

FEATURES

- k_{inact}, K_I, k_{inact}/K_I and $T_{1/2}^{\infty}$.
- Robust, reproducible and precise.
- Broad dynamic range.
- Activated and non-activated targets.
- Rapid turnaround time.

APPLICATIONS

- Discrimination between irreversible and reversible drugs.
- Selectivity profiling for irreversible drugs.
- Understanding PK/PD disconnects.

Differentiation of conformation-specific BTK irreversible drugs by inactivation kinetics

The crystal structure of Ibrutinib, Acalabrutinib, Zanubrutinib and Remibrutinib shows the stabilization of different inactive states of BTK[3,4]. Ibrutinib, Acalabrutinib and Zanubrutinib occupy the back pocket of BTK kinase domain when the C-helix is rotated outwards (C-helix out) and the activation loop adopts the DFG-in conformation. In contrast, Remibrutinib occupy the H3 pocket, which is present when the activation loop Tyr551 is unphosphorylated. Consequently, Remibrutinib blocks the activation loop phosphorylation and stabilize it in the DFG-out conformation[5].

- Ibrutinib, Acalabrutinib and Zanubrutinib inactivate similarly the phosphorylated and non-phosphorylated forms of BTK, indicating that the inhibitors may occupy the back pocket present in both forms.
- Whereas the inactivation efficiency (k_{inact}/K_I) of Remibrutinib is 140-fold higher with the non-phosphorylated form. This may explain the exquisite selectivity profile of this inhibitor[5].

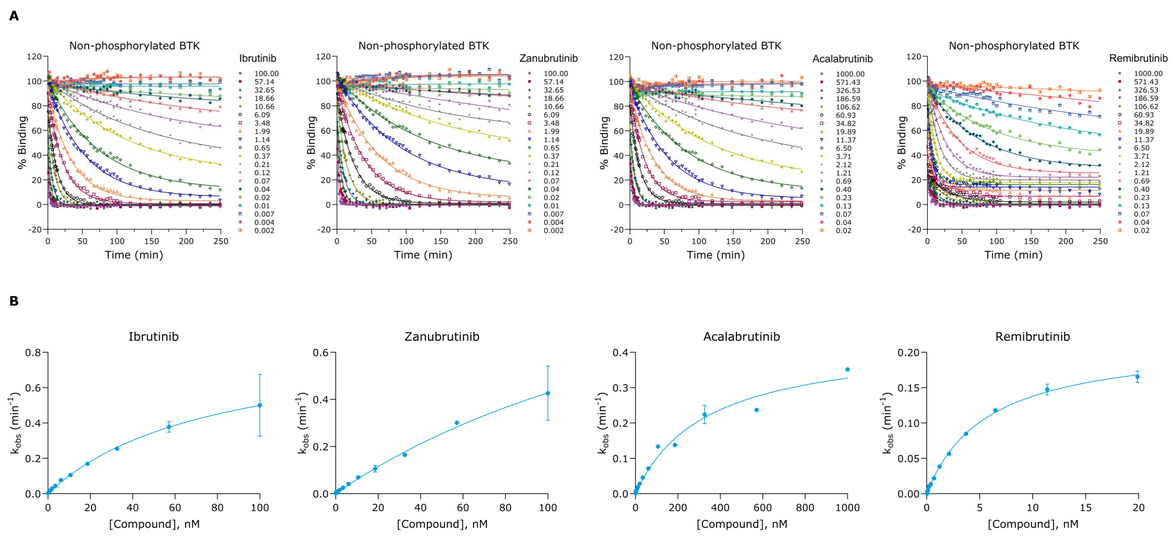


Figure 2. **Characterization of the kinetics of non-phosphorylated BTK inactivation by the covalent-irreversible inhibitors Ibrutinib, Zanubrutinib, Acalabrutinib and Remibrutinib using COVALfinder®.** (A) Progress curve of BTK incubated with increasing compound concentrations. (B) Dependence of k_{obs} on inhibitor concentration.

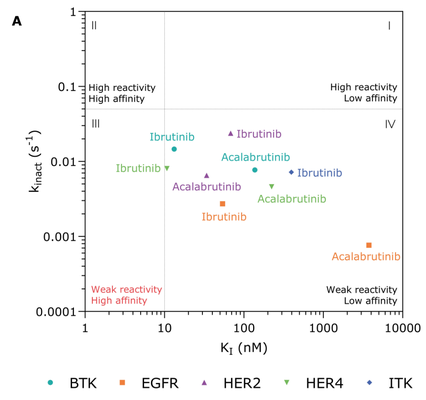
	Phosphorilated BTK				Non-phosphorylated BTK			
Compound	**K_I (nM)**	**k_{inact}/K_I ($M^{-1}s^{-1}$)**	**k_{inact} (s^{-1})**	**$T_{1/2}\infty$ (min)**	**K_I (nM)**	**k_{inact}/K_I ($M^{-1}s^{-1}$)**	**k_{inact} (s^{-1})**	**$T_{1/2}\infty$ (min)**
Ibrutinib	13	$1.1x10^6$	$1.5x10^{-2}$	0.8	14	$7.2x10^5$	$1.0x10^{-2}$	1.1
Zanubrutinib	37	$4.6x10^5$	$1.7x10^{-2}$	0.7	55	$2.7x10^5$	$1.5x10^{-2}$	0.8
Acalabrutinib	138	$5.6x10^4$	$7.7x10^{-3}$	1.5	118	$5.8x10^4$	$6.8x10^{-3}$	1.7
Remibrutinib		$1.6x10^4$			1.6	$2.3x10^6$	$3.6x10^{-3}$	3.2

Table 1. **Kinetic analysis of the irreversible inhibition of BTK forms**. K_I, k_{inact}, k_{inact}/K_I and $T_{1/2}\infty$ of Ibrutinib, Zanubrutinib, Acalabrutinib and Remibrutinib for the phosphorylated and non-phosphorylated form of BTK are shown.

Ibrutinib and Acalabrutinib selectivity profile

Irreversible inhibition involves an initial binding step driven by affinity and a time-dependent inactivation step driven by covalent bond formation. As IC_{50} does not reflect both steps, its use may fail to define selectivity. In order to minimize potential off-target effects, the right balance between efficiency of inactivation and selectivity is needed.

- Ibrutinib irreversibly inhibits kinases which carry a Cys at the same position of BTK: ITK, EGFR, HER2, HER4.
- Acalabrutinib preferentially inactivates HER2 and HER4. Selectivity towards EGFR is due to a lower affinity (K_I) and reactivity (k_{inact}).



B

Compound	Target	k_{inact}/K_I ($M^{-1}s^{-1}$)	k_{inact} (s^{-1})	$T_{1/2}\infty$ (min)	K_I (nM)
Ibrutinib	BTK	$1.1x10^6$	$1.5x10^{-2}$	0.8	13
	HER4	$7.5x10^5$	$8.0x10^{-3}$	1	11
	HER2	$3.5x10^5$	$2.4x10^{-2}$	0.5	68
	EGFR	$5.1x10^4$	$2.7x10^{-3}$	4	54
	ITK	$1.8x10^4$	$7.2x10^{-3}$	2	397

Compound	Target	k_{inact}/K_I ($M^{-1}s^{-1}$)	k_{inact} (s^{-1})	$T_{1/2}\infty$ (min)	K_I (nM)
Acalabrutinib	BTK	$5.6x10^4$	$7.7x10^{-3}$	2	138
	HER4	$2.1x10^4$	$4.6x10^{-3}$	3	224
	HER2	$1.9x10^5$	$6.5x10^{-3}$	2	34
	EGFR	$2.0x10^2$	$7.6x10^{-4}$	15	3757
	ITK	No inhibition			

Figure 3. A) **Covalency quadrant scale.** Classification of Ibrutinib and Acalabrutinib by target comparing the rate of covalent bond formation (k_{inact}) against affinity (K_I). B) **Kinetic analysis of irreversible inhibition of BTK, HER4, HER2, EGFR and ITK by Ibrutinib and Acalabrutinib.** Calculation of the selectivity ratio is based on each parameter for off-targets relative to BTK. Ratios >10 are highlighted in yellow, while those >100 are highlighted in green. Red indicates a similar profile.

In addition, Ibrutinib but not Acalabrutinib, inhibits reversibly several other kinases.

- In all cases, off-target occupancy and dissociation is rapid (seconds to few minutes).
- Ibrutinib maintain activity against BTK owing to prolonged covalent engagement, while the transient reversible inhibition of off-targets is minimized.

A

Compound	Target	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	Residence time (min)	K_d (nM)
Ibrutinib	LCK	7.58E+06	7.19E-03	2.3	0.9
	FYN B	1.53E+06	3.81E-03	4.4	2.5
	LYNA	3.44E+06	1.30E-02	1.3	3.8
	SRC	3.02E+06	2.27E-02	0.7	7.5
	RIPK2	7.93E+05	1.42E-02	1.2	18
	LATS1	3.20E+06	6.37E-02	0.3	20
	ARAF	1.58E+06	3.50E-02	0.5	22
	FLT4 (VEGFR3)	1.72E+06	7.23E-02	0.2	42
	RET	4.57E+05	4.07E-02	0.4	89
	ACK (TNK2)	1.48E+05	1.38E-02	1.2	93

B

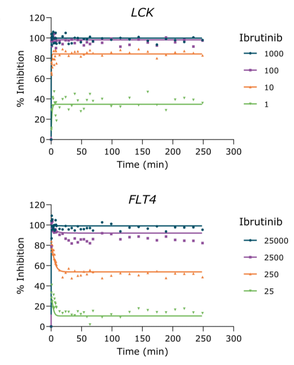
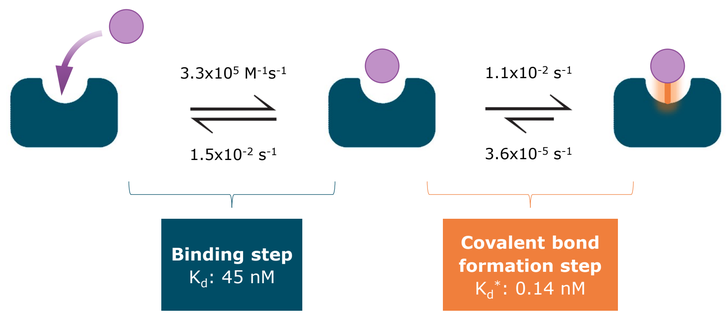


Figure 4. A) **Kinetic analysis of the reversible inhibition of Ibrutinib with all kinases that have shown a K_d value lower than 100 nM using the KINETICfinder® platform.** Residence time values within the range of seconds are highlighted in green, while those within the range of minutes are highlighted in yellow. B) **Kinetic curves of Ibrutinib** for two representative kinases obtained with KINETICfinder®.

Analysis of BTK reversible-covalent drugs

COVALfinder® and KINETICfinder® platforms has been used to provide an in-depth understanding of the binding mechanism of the covalent-reversible inhibitor Rilzabrutinib.

- Our results confirm that BTK-Rilzabrutinib complex formation takes place in reversible sequential events.
- Rilzabrutinib binding is the initial event and the covalent bond formation with Cys481 of BTK the second.
- The high affinity of Rilzabrutinib (K_d*: 0.14 nM) is a result of the extraordinary stability of the BTK-Rilzabrutinib complex (k_{off}: 2.0×10^{-5} s^{-1}, residence time: 812 min).



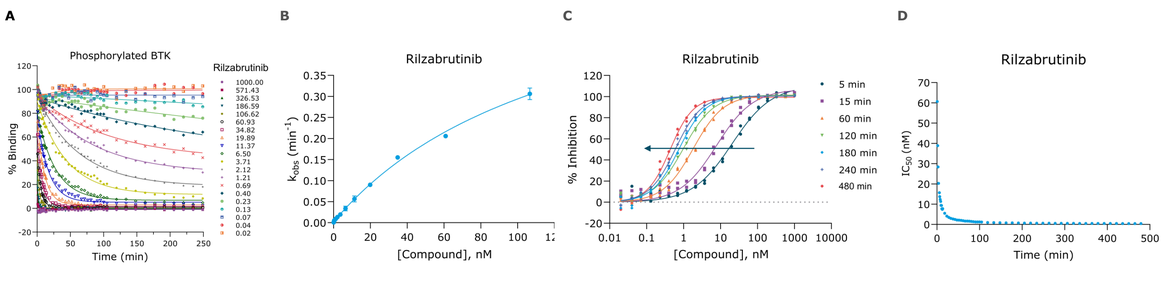


Figure 5. **Characterization of the kinetics of BTK inactivation by the covalent-reversible inhibitor Rilzabrutinib using COVALfinder®.** (A) Progress curve of BTK incubated with increasing compound concentrations. (B) Dependence of k_{obs} on Rilzabrutinib concentration. (C) Dose-response curves over time. (D) IC_{50} values over time.

Discussion & Significance

- The analysis of K_I, k_{inact} and k_{inact}/K_I of irreversible BTK inhibitors using COVALfinder® reveals that Ibrutinib, Acalabrutinib and Zanubrutinib inactivate similarly the phosphorylated and non-phosphorylated forms of BTK. These results show that the inhibitors may occupy the back pocket present in both forms.
- Our results confirm that Remibrutinib occupy the H3 pocket, which is only present in BTK when the activation loop Tyr551 is unphosphorylated: the k_{inact}/K_I obtained with the COVALfinder® assay of non-phosphorylated BTK is 140-fold higher than the phosphorylated BTK.
- The evaluation of k_{inact}/K_I of Ibrutinib and Acalabrutinib towards several kinases which carry a Cys at the same position of BTK displays that Acalabrutinib is more selective.
- Our kinetic platform for reversible binders KINETICfinder® shows that Ibrutinib transiently inhibits several other kinases, suggesting that the risk of undesired side effects would be minimized.
- COVALfinder® enables a detailed characterization of both covalent-reversible and irreversible drugs.
- Rilzabrutinib is a covalent-reversible inhibitor that bind to BTK in two-reversible steps with a fast association rate ($3.3x10^5$ $M^{-1}s^{-1}$) and very long residence time (812 min).

1.-Zhang D. et al (2021) Molecules 26(16): 4907. 2.-De Sousa M.E. et al (2021) Molecules. 26(23): 7411. 3.- Lin D.Y. (2023) PLoS One. 18(8): e0290872. 4.- Li W. et al (2023) Mol Cancer Ther. 5.- Angst D. et al (2020) J Med Chem 63(10):5102-5118.