enzymlogic

Discovering and de-risking irreversible inhibitors

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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 identify irreversible compounds, interpret structure-activity relationships (SAR), define selectivity and in vivo target occupancy.

Kinetic characterization allows for the determination of the rate (k_{inact}) and efficiency (k_{inact}/K_I) of inactivation as well as the potency (K_I).

This data is more useful for the optimization of irreversible inhibitors than IC_{50} values, since it allows for the improvement of both inhibitor binding and covalent bond formation. Modification of any part of an irreversible inhibitor may improve the rate of covalent bond formation (k_{inact}) at the expense of inhibitor binding to the target (K_I) or vice versa.

For example, in the optimization of kynurenine aminotransferase inhibitors, SAR showed changes in the kinact/KI with little change to the IC_{50}. In the case of EGFR, the k_{inact}/K_I values showed that improvements in the K_I were critical to achieving optimal cellular potency[1, 2].

COVALfinder® is the ideal hit-to-lead and lead optimization tool for irreversible drug discovery programs

Features and benefits

- Accurate.
- Robust.
- Reproducible.
- Sensitive.
- Broad dynamic range.
- Rapid turnaround.
- Standard known inhibitor in each plate.

Applications

- Modify compound binding (K_I) and reactivity (k_{inact}).
- Modulate the therapeutic index and safety profile of irreversible drugs.
- Distinguish between one- or two-step irreversible mechanisms.
- Understand PK/PD disconnects.

How COVALfinder® assays work

COVALfinder® is a highly tuned TR-FRET Kinetic Assay. Real-time binding of an active-site directed fluorescent probe is detected using a labeled anti-tag antibody, which binds to the target of interest. The binding of the probe to the target increases the TR-FET signal, whereas the displacement of the probe with a compound decreases the TR-FRET signal.

Assay process

1. **Performance:**
   - Pre-incubation of the target of interest, a fluorescent probe and labelled antibody.
   - 20-point 1.75-fold serial dilutions in duplicate of test compounds and a reference compound.
   - Up to 48 total binding and non-specific binding controls.
2. **Detection:** Reaction is monitored over time at room temperature.
3. **Analysis:**
   - Fitting of specific TR-FRET signals to a single-exponential equation to obtain kobs, the first-order rate constant for the interconversion between the initial and final degree of binding.
   - Production of dose-response curves at each time point in order to generate IC_{50} values, allowing easy inspection of time dependency.
   - Determination of K_I, k_{inact}, k_{inact}/K_I values and the mechanism of inhibition (one- or two-step) using a secondary plot of k_{obs} versus compound concentration and a statistical analysis.
4. **Quality control:** S/B, Z-value and MSR (within and between-run variability) for each assay.

Evaluation of irreversible EGFR inhibitors using COVALfinder®

Targeting EGFR with irreversible inhibitors like Afatinib and Dacomitinib has dramatically changed the therapeutic routine for lung adenocarcinoma patients. However, under therapeutic pressure resistant clones emerge. Osimertinib and Nazartinib have been designed to overcome acquired resistance induced by the T790M mutation with high specificity and reduced off-target WT EGFR-driven toxicities.

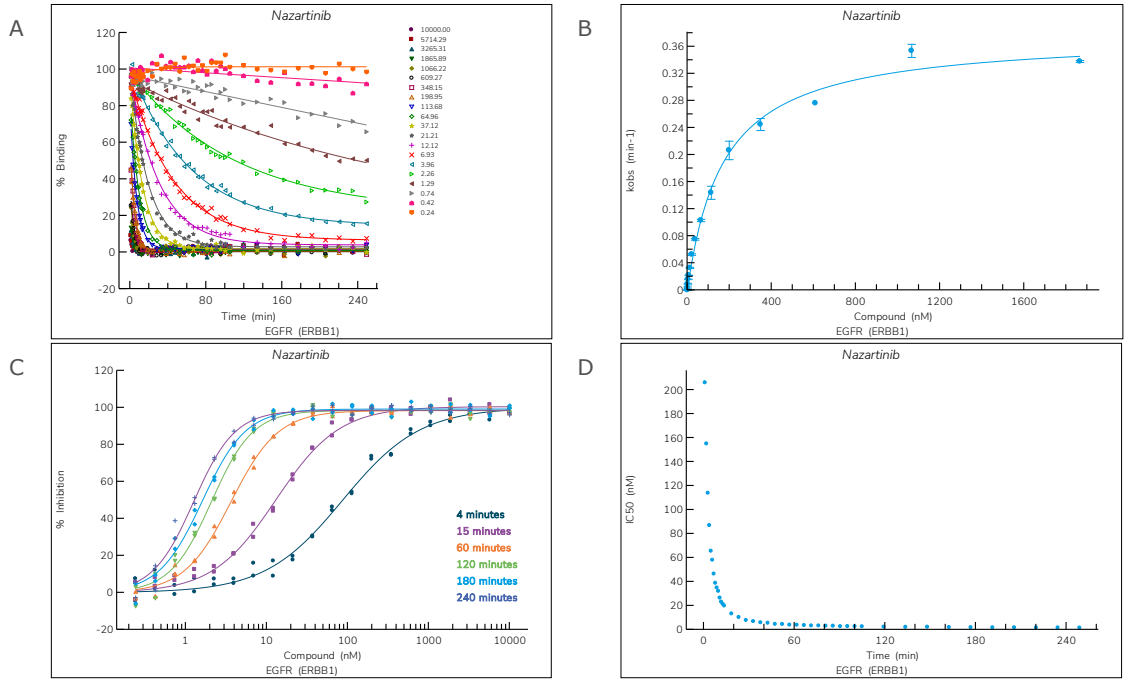

Figure 1. **Characterization of the kinetics of EGFR inactivation by the irreversible inhibitor Nazartinib.** (A) Progress curve of EGFR incubated with increasing compound concentrations. (B) Dependence of k_{obs} on Nazartinib concentration. (C) Dose-response curves over time. (D) IC_{50} values over time.

Identification of irreversible compounds

We have studied in detail the binding characteristics of seven irreversible EGFR inhibitors using COVALfinder®. The binding profiles for all the irreversible inhibitors showed a slow onset of inhibition (representative data for Nazartinib is shown in Fig.1, A). All of them also displayed the characteristic time-dependent reduction in IC_{50} due to the formation of the covalent complex over time (Fig.1, C and D). To confirm that these inhibitors were indeed binding irreversibly to EGFR, the dependence of k_{obs} on inhibitor concentration was evaluated (Fig.1, B).

A two-step, time-dependent inhibition was observed. This behavior is consistent with either slow onset reversible inhibition (the slower phase representing the rate of target isomerization) or irreversible inhibition (the slower phase representing target inactivation). However, in all cases the y-intercept was zero, which is only consistent with irreversible binding.

The k_{inact}, K_I, k_{inact}/K_I and $T_{1/2}^{\infty}$ (half-life for inactivation at infinite concentration of inhibitor) values obtained from this analysis are presented in Table. 1 and compared with those obtained from activity assays reported in the literature. Both, the mechanism of inhibition and the kinetic constants were successfully characterized for all the irreversible EGFR inhibitors[3-5].

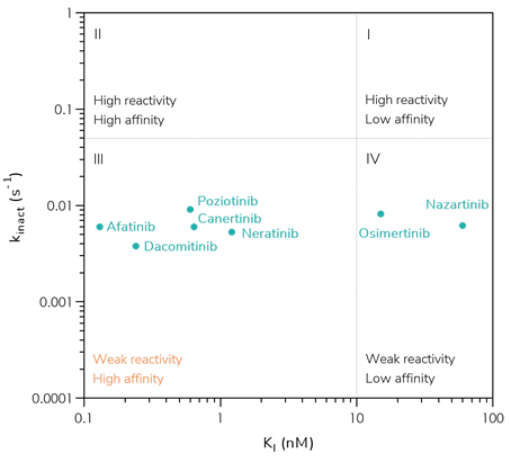

Figure 2. **Covalency quadrant scale.** Classification of EGFR covalent binders by comparing the rate of covalent bond formation (k_{inact}) against affinity (K_I).

Differentiation of drug candidates by inactivation kinetics

Overall, the quinazoline based irreversible EGFR drugs (Dacomitinib, Afatinib, Canertinib and Poziotinib) are extremely efficient (k_{inact}/K_I) with high affinity (K_I) and low specific reactivity (k_{inact}).

Neratinib has the same reactive substituent as Afatinib, but its affinity is 10-fold weaker and with 3-fold lower inactivation efficiency. As expected, pyrimidine based irreversible EGFR T790M inhibitors (Osimertinib and Nazartinib) show up to 500 times lower affinity and inactivation efficiency with similar weak reactivity.

	COVALfinder®				References			
Compound	KI (nM)	k_{inact} (s^{-1})	k_{inact}/KI ($M^{-1}s^{-1}$)	$T_{1/2}^{\infty}$ (min)	KI (nM)	k_{inact} (s^{-1})	k_{inact}/KI ($M^{-1}s^{-1}$)	$T_{1/2}^{\infty}$ (min)
Dacomitinib	0.24	$3.8x10^{-3}$	$1.6x10^{7}$	3	0.16	$1.5x10^{-3}$	$9.9x10^{6}$	11
Afatinib	0.13	$6.0x10^{-3}$	$4.6x10^{7}$	2	0.15	$9.0x10^{-4}$	$6.3x10^{6}$	19
Canertinib	0.64	$6.0x10^{-3}$	$9.5x10^{6}$	3	0.093	$2.9x10^{-3}$	$2.3x10^{7}$	6
Poziotinib	0.60	$9.1x10^{-3}$	$1.5x10^{7}$	1	<1	$8.3x10^{-4}$	>1.2E6	20
Neratinib	1.21	$5.3x10^{-3}$	$4.4x10^{6}$	2	7.1	$1.8x10^{-3}$	$2.5x10^{5}$	9
Nazartinib	60	$6.2x10^{-3}$	$1.0x10^{5}$	2	25	$5.2x10^{-3}$	$2.1x10^{5}$	3
Osimertinib	15	$8.2x10^{-3}$	$5.5x10^{5}$	1	14	$7.2x10^{-3}$	$5.3x10^{5}$	2

Table 1. **Kinetic analysis of irreversible inhibition of.EGFR** . A comparison of the kinetic values obtained with COVALfinder® and those reported in the literature is also shown.

References

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