enzymlogic

Designing selective 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 define selectivity.

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 design of selective covalent 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 inhibitor may improve the rate of inactivation (k_{inact}) at the expense of inhibitor binding (K_I) or vice versa.

In order to minimize potential off-target effects, the right balance between efficiency of inactivation and selectivity is needed.

A recent study demonstrated how IC_{50} inadequately reflects the selectivity profile of the BTK irreversible inhibitors ibrutinib and acalabrutinib. Although acalabrutinib seems to be more selective than ibrutinib when comparing IC_{50} alone[3], the assessment of selectivity using K_{inact}/K_I ratios corroborate clinical data, demonstrating similar safety profiles between the therapies.[1-3]

COVALfinder® helps you to modulate the therapeutic window and safety profile of irreversible drugs

Features and benefits

- Accurate.
- Robust and 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 k_{obs}, 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.

Selectivity profiling of irreversible EGFR inhibitors using COVALfinder®

EGFR is a receptor tyrosine kinase, member of the ErbB family together with HER2, HER3 and HER4. EGFR overexpression is frequent in many types of human malignancies, including non-small cell lung cancer (NSCLC). Afatinib, Dacomitinib and Neratinib are quinazoline based irreversible pan-ErbB drugs. Afatinib and Dacomitinib have been approved for the treatment of EGFR mutated NSCLC and Neratinib for the treatment of HER2-Positive breast cancer.

Here we study the selectivity profile of three irreversible EGFR inhibitors using COVALfinder®. The binding profiles for all the irreversible inhibitors showed a slow onset of EGFR, HER2 and HER4 inhibition (representative data for HER2 inactivation by Dacomitinib is shown in Fig.1, A). Each inhibitor with the three kinases tested also

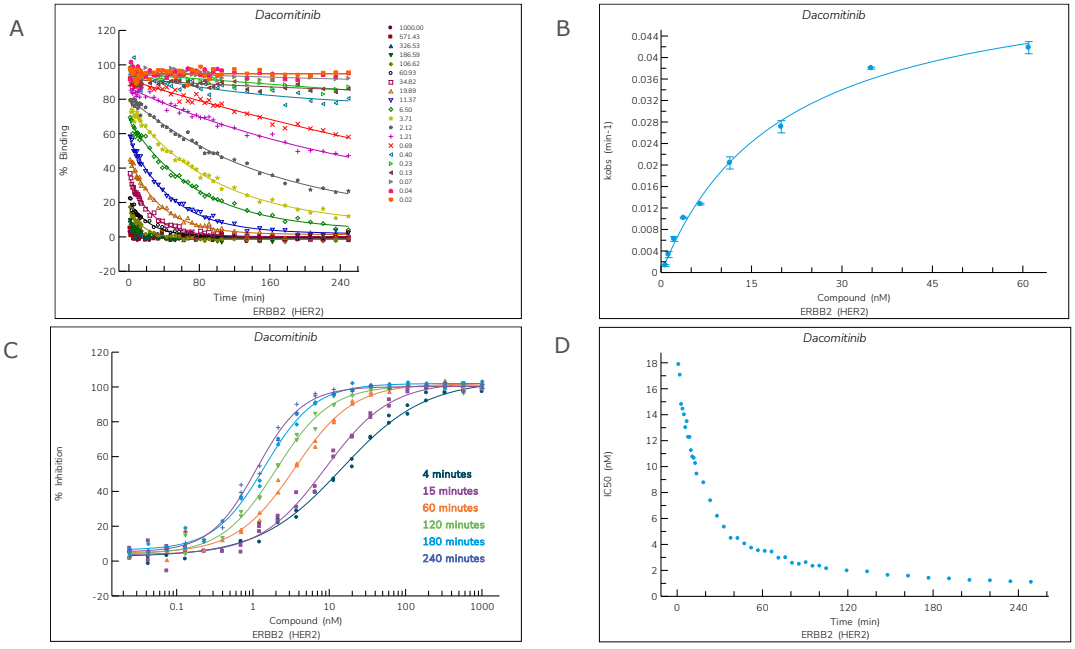

Figure 1. **Characterization of the kinetics of HER2 inactivation by the irreversible inhibitor Dacomitinib.** (A) Progress curve of HER2 incubated with increasing compound concentrations. (B) Dependence of k_{obs} on Dacomitinib concentration. (C) Dose-response curves over time. (D) IC_{50} values over time.

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, HER2 and HER4, the dependence of kobs 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.

Inactivation kinetics profiling is crucial for selectivity assessment

As can be seen in Table 1, if we take into account the IC_{50} values to calculate the selectivity ratio, all the inhibitors are pan-ErbB drugs. However, using k_{inact}/K_I the selectivity profile of Afatinib and Dacomitinib dramatically changes. Afatinib and Dacomitinib binds preferentially to EGFR and HER4 whereas Neratinib is a pan-ErbB inhibitor.

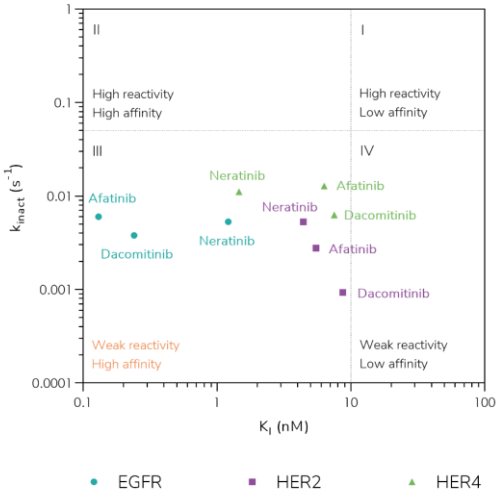

Figure 2. **Covalency quadrant scale.** Classification of covalent binders by target comparing the rate of covalent bond formation (k_{inact}) against affinity (K_I).

The covalency quadrant (Fig.2) clearly shows that the selectivity of Afatinib and Dacomitinib towards HER2 is due to a lower affinity (K_I) and reactivity (k_{inact}). These results may explain the efficiency of Afatinib and Dacomitinib in the treatment of EGFR mutated NSCLC and the efficiency of Neratinib in HER2-Positive breast tumors.

Compound	Target	KI (nM)	k_{inact}/KI ($M^{-1}s^{-1}$)	k_{inact} (s^{-1})	$T_{1/2}^{\infty}$ (min)	IC_{50} (nM)	IC_{50} selectivity	Efficiency selectivity
Dacomitinib	EGFR	0.2	$1.6x10^{7}$	$3.8x10^{-3}$	3.1	6.0	1	1
Afatinib		0.1	$4.6x10^{7}$	$6.0x10^{-3}$	1.9	0.5	1	1
Neratinib		1.2	$4.4x10^{6}$	$5.3x10^{-3}$	2.2	92	1	1
Dacomitinib	HER2	8.7	$1.1x10^{5}$	$9.3x10^{-4}$	12.4	46	8	**150**
Afatinib		5.5	$5.0x10^{5}$	$2.8x10^{-3}$	4.2	14	28	**91**
Neratinib		4.4	$1.2x10^{6}$	$5.3x10^{-3}$	2.2	59	1	4
Dacomitinib	HER4	7.5	$8.4x10^{5}$	$6.3x10^{-3}$	1.8	74	12	19
Afatinib		6.3	$2.0x10^{6}$	$1.3x10^{-2}$	0.9	1.0	2	23
Neratinib		1.5	$7.6x10^{6}$	$1.1x10^{-2}$	1.0	NA		0.6

Table 1. **Kinetic analysis of irreversible inhibition of ErbB family**. Calculation of the selectivity ratio is based on the IC_{50} values reported in the literature and k_{inact}/K_I values obtained with COVALfinder® for off-targets relative to EGFR. NA: no data available in the literature. Empty fields indicate that the selectivity ratio cannot be calculated.

References

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