# A Practical and Efficient Method for Determining $k_{inact}/K_I$ of Covalent Fragments

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## Introduction

- The clinical success of covalent drugs such as ibrutinib and osimertinib has renewed interest in covalency for rational drug design, and the discovery of $KRAS^{G12C}$ covalent inhibitors reveals the potential of covalency for targeting traditionally "undruggable" proteins.
- Mass spectrometry (MS) enables direct detection of covalent protein-ligand adducts and can be applied to proteins not amenable to an enzymatic assay. High-throughput intact protein MS platforms are frequently employed to screen electrophilic fragment libraries and characterize covalent binders in dose-response (DR) and time-course (TC) format.
- The ratio $k_{inact}/K_I$ offers the most rigorous assessment of irreversible binders as $IC_{50}$ values do not fully capture the time-dependent mechanism of irreversible binders. $k_{inact}/K_I$ is also the preferred means of predicting *in vitro* and *in vivo* target occupancy and therapeutic effect.[1]
- However, the gold-standard full DRTC approach to obtain $k_{inact}/K_I$ values is resource-intensive and requires specialized data analysis, limiting the routine use of $k_{inact}/K_I$ for SAR.
- We present a practical alternative: the intact protein MS diagonal DRTC (dDRTC) method, which demonstrates accurate measurement of $k_{inact}/K_I$ over more than three orders of magnitude, and sufficiently high throughput (8x increase) and rank-ordering to accelerate SAR interpretation.

## Methods

- Compounds dispensed via ECHO 650 into 384-well plate
- 0.8 µM $KRAS^{G12C}$ (inactive GDP state) protein incubated with desired compounds at selected doses and time points (see table)
- Reactions were quenched with 4% formic acid
- Samples ran on RF-MS (Models: RapidFire 365, 6230 TOF, Agilent)
- Charge-state envelopes were deconvoluted with MassHunter BioConfirm

| Series 1 | | Series 2 | |
|---|---|---|---|
| Time, s | Concentration, µM | Time, s | Concentration, µM |
| 15 | 1.65 | 120 | 1.56 |
| 60 | 1.65 | 360 | 3.13 |
| 120 | 3.8 | 900 | 6.25 |
| 360 | 5.8 | 1800 | 12.5 |
| 900 | 10.9 | 3600 | 25 |
| 1800 | 25 | 10800 | 50 |
| 3600 | 57 | 21600 | 100 |
| 10800 | 85 | 72000 | 200 |


Incubate protein with small molecule

Data acquired using RapidFire Time-of-Flight (RF-TOF)

Deconvoluted spectrum showing $KRAS^{G12C}$ 30% bound by ARS-853

## Overview of diagonal DRTC (dDRTC)

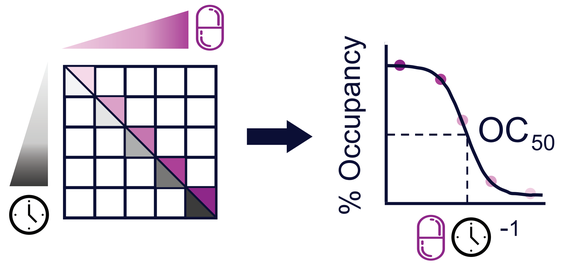


**Figure 1: A schematic representation of the diagonal DRTC (dDRTC) method to determine $k_{inact}/K_I$.** For an *n x n* checkerboard with varying time on one axis and varying dose on another, only the diagonal sampling (in color) is measured. After running samples through intact protein MS, an occupancy curve can be fit to determine $OC_{50}$, the dose and time which yields 50% occupancy, which is converted to $k_{inact}/K_I$. Y = % Occupancy, and X = the inverse product of dose and time in units of $k_{inact}/K_I$ ($M^{-1}s^{-1}$).

## dDRTC reflects full DRTC for ARS-853

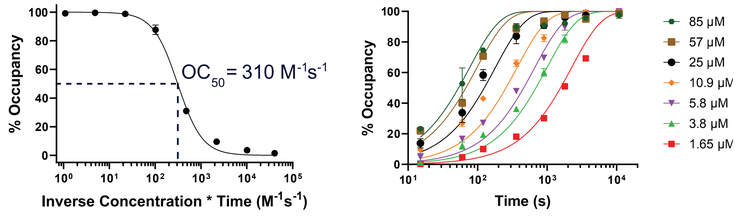


**Figure 2: Comparison of dDRTC vs. gold-standard full DRTC checkerboard approach using $KRAS^{G12C}$ occupancy by ARS-853 quantified via intact protein MS.** Assay dose and time conditions prepared according to Series 1 scheme. Error bars indicate standard error of the mean (SEM). A. $OC_{50}$ curve from the diagonal slice of the 8x8 checkerboard (n = 2, 8 data points per experiment). Data was fit using logistic equations commonly applied to DR curves. Equation (2) was used to convert the $OC_{50}$ to a $k_{inact}/K_I$ value of 215±7.1 $M^{-1}s^{-1}$, closely matching the reported literature value of 250±20 $M^{-1}s^{-1}$.[3] B. Full DRTC curves with global fitting (n = 2, 64 data points per experiment), yielding a $k_{inact}/K_I$ of 274.2 ± 0.8 $M^{-1}s^{-1}$.

## Simulations position dDRTC for early stage of programs when (I) << $K_I$

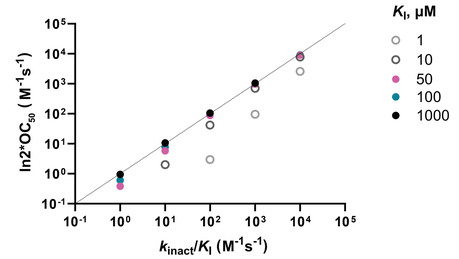


**Figure 4: Simulated experiments provide guidance for applying dDRTC.** 60 datasets were simulated across a broad range of $k_{inact}$ (0.0001 - 1 $s^{-1}$) and $K_I$ (1 - 1000 µM) values in KinTek Global Kinetic Explorer.[4] Simulations predicted no deviation between dDRTC $OC_{50}$-derived $k_{inact}/K_I$ values ($ln2*OC_{50}$) plotted on the Y-axis and true $k_{inact}/K_I$ (X-axis) for covalent modifiers with reversible affinities $K_I \geq 50$ µM (filled circles). The dotted line represents a perfect positive correlation where $ln2*OC_{50} = k_{inact}/K_I$. Deviation from true $k_{inact}/K_I$ increases as $K_I$ approaches (I).

## Practical considerations

**Table 1. Comparison of the decision factors for choosing an assay to quantify the covalent modifier potency $k_{inact}/K_I$.**

| Factors influencing assay decision | Biochemical Assays* | Intact Protein MS dDRTC | Intact Protein MS DRTC | SPR |
|---|---|---|---|---|
| Specialized equipment / expertise required | No | Yes | Yes | Yes |
| Sophisticated software for data fitting required | No | No | Yes | Yes |
| Throughput | High | High | Low | Low |
| Protein consumption | Low | Low | High | Low |
| $k_{inact}/K_I$ upper limit | $10^3$-$10^4$ | $10^5$ | $10^5$ | $10^6$ |
| Unambiguous covalent detection | No | Yes | Yes | Yes** |
| Protein tag / probe / functional assay required | Yes | No | No | No*** |

"High"-throughput as noted here indicates an assay that is amenable to weekly SAR studies, whereas "Low"-throughput assays are not practical. *Examples include continuous enzymatic activity and probe competition assays; **Unambiguous covalent detection may be affected by the surface stability (drift in the signal); ***Affinity tag (e.g., Avi-tag) is often required for protein capture on the surface.

## Relating half-maximal occupancy ($OC_{50}$) and $k_{inact}/K_I$

Building on derivations by Copeland et. al,[2] we established a relationship between $OC_{50}$ and $k_{inact}/K_I$. We began with the following pseudo first-order rate equation under the assumption (I) » (P),

$$[PI_t] = [PI_{eq}](1 - e^{-k_{obs}t}) \qquad (1)$$

We use P, I, and PI notation for protein, ligand, and protein-ligand complex, respectively. $[PI_t]$ is the %PI (i.e. covalent occupancy) at time $t$ and $[PI_{eq}]$ is 100% maximum occupancy. We converted equation (1) as follows:

$$\%PI_t = 100(1 - e^{-k_{obs}t})$$

With $k_{obs} = \frac{k_{inact}[I]}{K_I+[I]}$,

$$\%PI_t = 100\left(1 - e^{-\frac{k_{inact}[I]}{K_I+[I]}t}\right)$$

When $[I] \ll K_I$,

$$e^{-\frac{k_{inact}}{K_I}[I]t} = 1 - \frac{\%PI_t}{100}$$

$$-\frac{k_{inact}}{K_I}[I]t = \ln\frac{100 - \%PI_t}{100}$$

$$\frac{k_{inact}}{K_I} = -\frac{\ln\frac{100 - \%PI_t}{100}}{[I]t}$$

We defined the $OC_{50}$ as an estimate of the dose and time value ($M^{-1}s^{-1}$) that yields 50% occupancy: $OC_{50} = \left(\frac{1}{[I]\,t}\right)_{\%PI=50\%}$

Thus, $\frac{k_{inact}}{K_I} = \frac{\ln 2}{[I]\,t} = \ln 2\, OC_{50}$ (2)

## dDRTC is accurate across three orders of magnitude

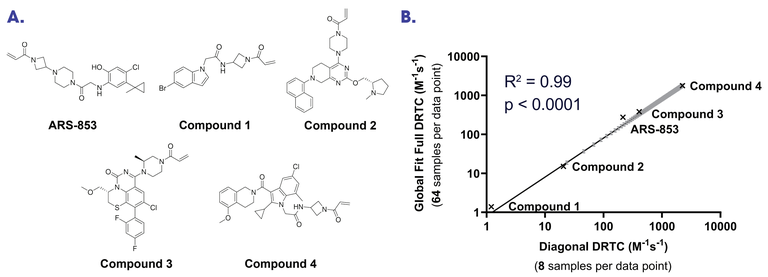


**Figure 3: Five $KRAS^{G12C}$ covalent inhibitors spanning over 3 orders of potency magnitude selected to compare $k_{inact}/K_I$ obtained via dDRTC (8 samples per data point) vs. full DRTC (64 samples per data point).** A. Compound 1 (early fragment) was run with Series 2, all others were run with Series 1. B. $k_{inact}/K_I$ determined from full DRTC vs. diagonal DRTC (n = 2). The linear regression analysis demonstrated a strong correlation and a consistent rank ordering between methods. Average % difference between the gold-standard approach and dDRTC was 20%, showing the dDRTC approach can accurately reflect $k_{inact}/K_I$ while sparing resources (reagents and time) at a sufficiently high throughput to enable weekly SAR.

## Conclusions

- We present a streamlined approach to measuring inactivation kinetics of covalent fragments.
- The dDRTC method increased throughput **8x** while maintaining accuracy within a 20% difference on average of the gold-standard approach.
- Our simulations extend the characterization of the dDRTC method and provide guidelines on how to best implement it depending on the stage of the program.
- The efficiency gains with dDRTC allowed $k_{inact}/K_I$ to be determined for every compound during routine SAR, and this approach is used to drive SAR in early programs at Frontier.

## References

1. Hopper, M. *et al.* Relative Selectivity of Covalent Inhibitors Requires Assessment of Inactivation Kinetics and Cellular Occupancy: A Case Study of Ibrutinib and Acalabrutinib. *J Pharmacol Exp Ther* **372**, (2020).
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3. Hansen, R., Peters, U., Babbar, A., Chen, Y., Feng, J., Janes, M. R., Li, L. S., Ren, P., Liu, Y., & Zarrinkar, P. P. (2018). The reactivity-driven biochemical mechanism of covalent KRASG12C inhibitors. *Nature Structural and Molecular Biology*, 25(6), 454–462. https://doi.org/10.1038/s41594-018-0061-5
4. Johnson, K. A., Simpson, Z. B. & Blom, T. Global Kinetic Explorer: A new computer program for dynamic simulation and fitting of kinetic data. *Anal Biochem* **387**, (2009).