Validation of an Automated Process for the Determination of Covalent Modifier Potency: K_{inact}/K_I of the Btk/Ibrutinib Model System

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Abstract

Targeting the covalent modification of proteins with compounds containing reactive functional groups has led to the development of several new potent and selective drugs, as demonstrated by the epidermal growth factor receptor (EGFR) and Bruton's tyrosine Kinase (BtK) active inhibitors used to treat various cancers. Recent publications[1] highlight an "evolving toolbox" of techniques and technologies implemented in covalent inhibitor discovery and design. Though historically covalent compound discovery has been conducted via structure guided design, direct covalent ligand screening of electrophilic compound libraries has been gaining popularity as a primary approach. The generation of "hit" lists has increased demands on secondary assays defining and rank ordering compound potency. The rate constant, K_{inact}/K_I, is the potency metric of irreversible inhibitors (akin to an IC_{50} value for reversible inhibitors). We have validated the use of simple and commercially available hardware and software products in an automated workflow for the determination of the rate constant K_{inact}/K_I using the BtK protein and Ibrutinib inhibitor model system. The throughput afforded by this workflow can enable the determination of full K_{inact}/K_I values for dozens of compounds per day. Additionally, minor modifications to plate prep protocols can enable the primary screening of electrophilic libraries of up to 10,000 compounds in 2-3 days.

Introduction

Covalently active molecules have long been a "nuisance" in practical high-throughput screening strategies due to their propensity to cause interference and confounding results in variety of optically and non-optically based screening methodologies, often leading to their removal from corporate compound collections. Having said this, the ability of these, typically though not always electrophilic, reactive groups to form covalent bonds with protein targets provides an alternate affinity above traditional non-covalent binding events. The prolonged target engagement afforded by these covalent interactions often results in increased potency and extended pharmacokinetic profiles.

Renewed interest in the development of targeted covalent modifying compounds, brought on in part by the commercial success of marketed drugs such as Imbruvica and Tagrisso, have fueled the expansion and advancement of covalent screening technologies and strategies. An assortment of mass spec based and non-mass spec based systems have emerged for the determination and characterization of covalent interactions, including activity based protein profiling via proteomic or gel based platforms and even phenotypic screening techniques.

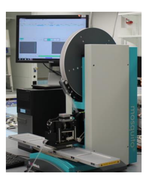
Of the many screening approaches available, one of the highest throughput alternatives is the use of intact mass MS. Intact mass MS utilizes software tools to "deconvolute" m/z envelopes into a single "intact" mass of a large biomolecule. Utilizing intact MS software tools, one can extract semi-quantitative information related specific protein constructs and their post reaction "adducted" products. The rate limiting factor in this workflow is how fast samples can be generated and introduced to the mass spectrometer for analysis. Developing systems that utilize standardized plate maps, automated robotics and high-throughput MS platforms can maximize the productivity of this already powerful workflow.

In the following studies, plates were prepared using a SPT Labtech Mosquito HTS nanoliter liquid handler for compound titration dispensing and a Formulatrix Tempest reagent dispenser for assay assembly and precision timing. Data was captured with a combination of the Agilent RapidFire 365 platform and an Agilent 6545 Q-TOF MS. A combination of vendor supplied, commercially available and in-house software (MassHunter, Bioconfirm, Prism, etc…) were used to convert raw MS data into deconvoluted profiles which were filtered down to only the quantitative information of relevant biomolecules used to generate the K_{inact}/K_I data products.

Assay Assembly Robotics

ASSAY-READY PLATE PREP

Assay-ready plates for kinetic screening are prepared by dispensing 300nL of compound using a **MOSQUITO HTS** nanoliter Liquid Handler (*SPT Labtech*).



Time (sec)	5				10				20				40				600				1800			
Conc (uM)	0.3125	0.625	1.25	2.5	0.3125	0.625	1.25	2.5	0.3125	0.625	1.25	2.5	0.3125	0.625	1.25	2.5	0.3125	0.625	1.25	2.5	0.3125	0.625	1.25	2.5
384 Master Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1
B	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1	CPD-1
C	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2
D	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2	CPD-2
E	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3
F	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3	CPD-3
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H	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4	CPD-4
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J	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5	CPD-5
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L	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6	CPD-6
M	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7
N	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7	CPD-7
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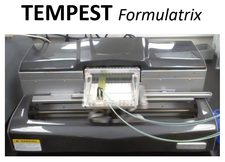
P	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8	CPD-8

PRECISE REAGENT DISPENSING FOR KINETIC ASSAY TIMING

Dispensation of protein (1.0 uM BtK, catalytic domain) and stop reagent (0.4% formic acid) were automated for precise timing using both the **ASSIST PLUS** with **VIAFLO** multichannel pipettes (*Integra Biosciences) and* **TEMPEST** reagent dispenser (*Formulatrix*). Intuitive programming of kinetic protocols on the **ASSIST PLUS** provides rapid development and adaptation of kinetic assays but is limited to intervals >30 seconds. Preliminary kinetics of BtK labelling by Ibrutinib using the Assist protocol revealed nearly complete adduction of BtK by Ibrutinib within 30 seconds necessitating a novel solution to be developed for more rapid timing, see Figure 1. Taking advantage of the TEMPEST's independent, contactless dispensing capability we developed a protocol with <5 sec timing by direct programming of the instrument via the command line Sequence Editor.

TEMPEST *Formulatrix*



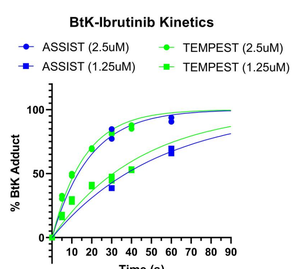
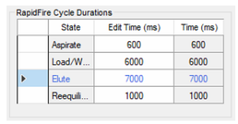


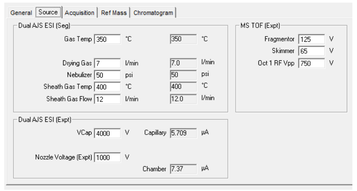
Figure 1: Rapid Kinetics of BtK labelling by Ibrutiinib.

High-throughput Mass Spectrometry

Stopped assays in 384-well plates were analyzed on an Agilent RapidFire 365 system by collecting a 10 uL sample on an Agilent C4 based packing material cartridge with a wash buffer of water/0.09% formic acid/0.01% trifluoracetic acid and an elution buffer of 80% acetonitrile/0.09% formic acid/0.01% trifluoracetic acid. The valve timings and flow rates are presented below.



The Agilent 6545 Q-TOF MS was set to collect a mass range of 100-3200 m/z at an acquisition rate of 3 spectra/second. The source parameters used are presented below.



Software Analysis

Raw data files were captured and split using the Agilent supplied MassHunter Workstation Software. Subsequently raw data files were deconvoluted to intact mass values utilizing the Agilent MassHunter Bioconfirm Software. This same software package was used to export "biomolecule" tables containing all relevant protein and protein:compound adduct information.

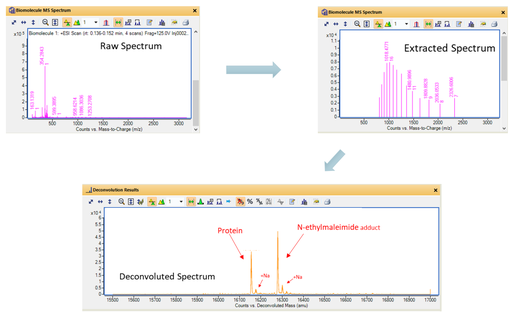


Figure 2: Raw data files are searched for patterns matching m/z envelopes consistent with the charge states of a single large biomolecule. These extracted chromatograms are then converted into intact mass values for individual biomolecules.

Exported biomolecule reports are then filtered to extract only relevant "parent protein" and "protein adduct" information.

Injection	PlateID	WellID	CompoundID	Parent/Adduct ID	Mass	Height	Area	Diff
Inj00024	BtK-8CPDS-Kinetic	A1	0.3125 uM Ibrutinib - 5 seconds	Parent Protein	32287.7783	629693	1795142	0.388016667
Inj00026	BtK-8CPDS-Kinetic	A2	0.625 uM Ibrutinib - 5 seconds	Parent Protein	32287.8049	477192	1462565	0.361416667
Inj00027	BtK-8CPDS-Kinetic	A3	1.25 uM Ibrutinib - 5 seconds	Parent Protein	32287.767	472028	1512448	0.399316667
Inj00027	BtK-8CPDS-Kinetic	A3	1.25 uM Ibrutinib - 5 seconds	Adduct	32727.866	38905	267648	0.800316667
Inj00028	BtK-8CPDS-Kinetic	A4	2.5 uM Ibrutinib - 5 seconds	Parent Protein	32287.8163	367717	1297583	0.350016667
Inj00028	BtK-8CPDS-Kinetic	A4	2.5 uM Ibrutinib - 5 seconds	Adduct	32728.0224	119017	618143	0.643916667

Kinetic Models for High-Throughput Characterization[2]

k_{obs}: %Total Occupancy = 100(1-exp(- **k_{obs}** * time))

Prism Model: *One Phase Association*

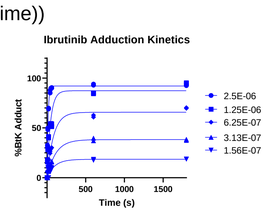
$Y=Y_0 + (Plateau-Y_0)*(1-exp(-K*x))$ *$Y_0 = 0$*, *Plateau = 100*

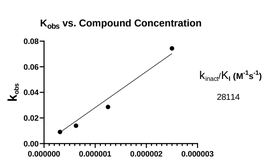
$Y=100*(1-exp(-K*x))$ K = **k_{obs}** , x = time

k_{inact}/K_I: **k_{obs}** = (kinact * [I])/(KI + [I])

Prism model: *linear fit, y = mx + b; b fixed to* 0

k_{obs} = **k_{inact}/K_I** * [I]





References

[1] Boike, L. *et al*, (2022) Nature Reviews Drug Discovery **21**, 881-898

[2] *Strelow J. M., (2017) A Perspective on the Kinetics of Covalent and Irreversible Inhibition SLAS Discovery 2017 (22) 3-20.*

Results

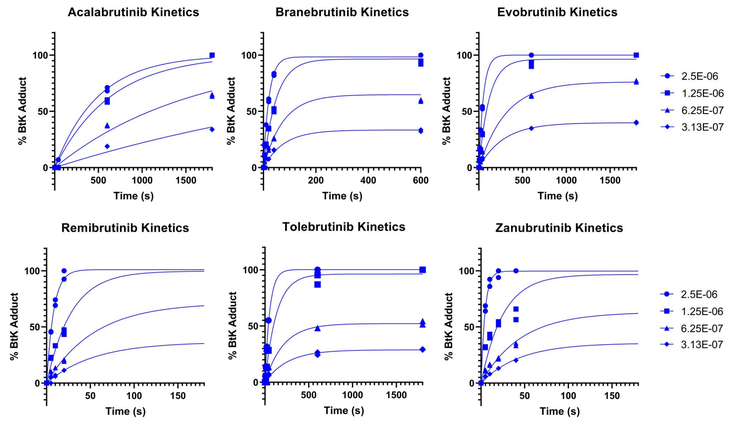


Figure 3: Rate plots of BtK Adduction for 6 approved/clinical candidates.

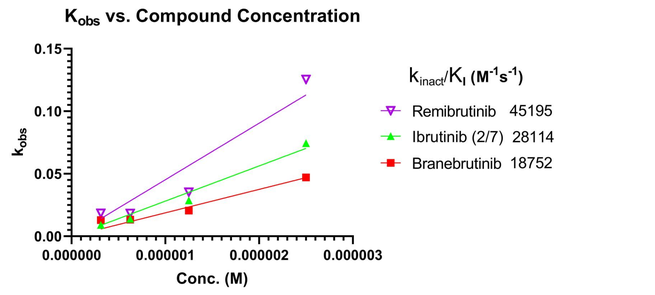


Figure 4: K_{inact}/K_I determination from rate data allows discrimination of covalent drug potency.

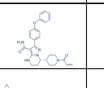
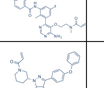
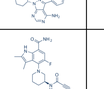
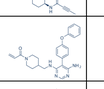
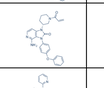
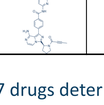
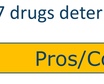
Inhibitor	Structure	Kinact/KI M-1 s-1	Development Phase
Zanubrutinib		76825	Approved
Remibrutinib		45195	``
Ibrutinib (2/7)		28114	Approved
Branebrutinib		18752	Ph II
Evobrutinib		7437	Ph II/III
Tolebrutinib		7199	Ph III
Acalabrutinib		900	Approved

Table 1: K_{inact}/K_I values for 7 drugs determined in a single HT kinetic run.

Pros/Cons

Pros

- Using this automated sample prep process, single 384-well plates representing eight compound each, can be prepared and assayed in 120 minutes.
- Using the RapidFire-365/Agilent 6545 Q-TOF platform, data can be taken from an entire 384-well plate in < 2 hours
- Automated sample preparation, sample processing and data analysis tolls provide for a largely handsfree workflow

Cons

- Intact mass MS can require large commitments in terms of protein consumption (as much as 60 picomoles per sample/23 nanomoles per 384-well plate)
- Kinetics can be underestimated for very fast acting covalent agents

Conclusions

- ✓ We have established an automated workflow utilizing commercially available hardware and software tools for the determination of K_{inact}/K_I values
- ✓ Using this workflow dozens of compounds can be interrogated, from initial sample prep to full data reporting, per 24-hour day
- ✓ Minor changes to the sample plate map can enable the primary screening of electrophilic libraries of up to 10,000 compounds in 2-3 days