Use of the LanthaScreen® Eu Kinase Binding Assay for Type III kinase inhibitors

Type III kinase inhibitors are the most selective class of protein kinase inhibitors. In this publication we present the unexpected finding that almost all Type III inhibitors can displace an ATP-site probe, making their study using the TR-FRET LanthaScreen® Eu Kinase Binding Assay relatively simple.

Type III: The most selective class of protein kinase inhibitors

There are great clinical research and basic research needs to develop selective, high-affinity, small-molecule protein kinase inhibitors. Such compounds are useful for pathway elucidation and target validation in addition to drug discovery. To date the vast majority of kinase inhibitors discovered bind solely to the ATP-binding site and are referred to as Type I kinase inhibitors. However, ATP pockets are highly conserved among kinases, which makes the development of highly selective ATP-competitive inhibitors a challenge. Moreover, Type I inhibitors are effective only if they have sufficient potency to compete with high concentrations of intracellular ATP (typically in the low-millimolar range). In contrast, Type III kinase inhibitors (sometimes referred to as allosteric inhibitors) are compounds that bind exclusively to sites other than the ATP-binding site. A selection of commercially available Type III inhibitors is shown in Figure 1. Because they don't bind at the ATP-binding site, these compounds don't have to compete with intracellular ATP—in fact, some Type III compounds bind with higher affinity in the presence of ATP. Because Type III compounds bind to less-conserved sites on kinases, they are highly selective and are of increasing interest to the research and drug discovery communities. Furthermore, in contrast to the crowded patent landscape surrounding Type I compounds, Type III inhibitors occupy a largely open intellectual property arena. In terms of lead discovery, the possibility of identifying Type III compounds during primary screening and early-stage drug discovery is very attractive. We discuss the basic assay types used (activity and binding) to detect such compounds and highlight differences in their ability to detect various Type III inhibitors.

Current methods to detect Type III inhibitors

Both kinase activity assays and cell-based assays have been utilized to discover and develop allosteric inhibitors. For example, the MEK inhibitor U0126 was identified in a cell-based high-throughput screening campaign as a functional antagonist of AP-1 transcriptional activity [1]. Though cellular assays provide a more physiologically relevant system, target deconvolution and defining clear structure–activity relationships in these systems can be difficult.

Cellular assays as well as direct and cascade kinase activity assays have led to the discovery of Type III inhibitors. A cascade assay was used to discover the MEK inhibitor PD98059 [2], while a direct activity assay resulted in the discovery of the GSK3beta inhibitors VI and VII [3]. Direct kinase activity assays are simpler and easier to interpret

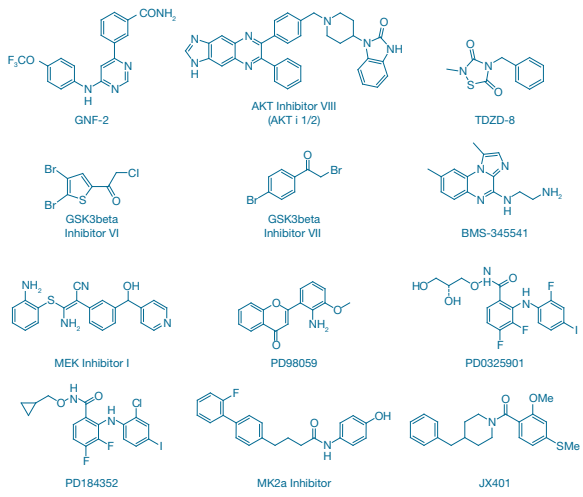


Figure 1. Commercially available Type III kinase inhibitors.

than cell-based and cascade assays. Some compounds, however, are not detected in a direct activity assay (or are only detected under specific conditions). For example, MK2a, a Type III inhibitor, blocks p38alpha activity only when MK2a is used as a substrate, but not when ATF2 or MBP is the substrate [4,5]. In addition, the MEK allosteric inhibitors PD98059 and U0126 are detected in cascade assays but are not detected in a direct activity assay with wild-type MEK. The ability to detect these compounds in cascade assays, which utilize inactive MEK, but not in direct activity assays is likely due to their preference for binding the non-phosphorylated form of MEK over the phosphorylated, wild-type MEK.

LanthaScreen® Eu Kinase Binding Assay

The LanthaScreen® Eu Kinase Binding Assay [6] (Figure 2) provides a simple means of assessing the ability of a compound to bind to specific kinases, which is particularly useful when investigating a target with no available activity assay, or when examining a compound binding to non-activated (e.g., non-phosphorylated) kinase states, which is known to occur for some Type III inhibitors. Even in cases where an activity assay is available, the simplicity of the LanthaScreen® Eu Kinase Binding Assay and its ability to generate real-time binding data provide advantages over many kinase assays. The basis of this technology is an ATP-competitive tracer, which binds to the kinase of interest and is detected by a europium-labeled anti-tag antibody also bound to the kinase of interest. When both tracer and antibody are bound, there is a high TR-FRET signal. When the tracer is displaced by a kinase inhibitor, there is a loss of the TR-FRET signal. It is clear that this format is useful for compounds that directly compete with the tracer for the ATP-binding sites. However, given the importance of Type III inhibitors, the applicability of this assay for detecting these compounds was investigated.

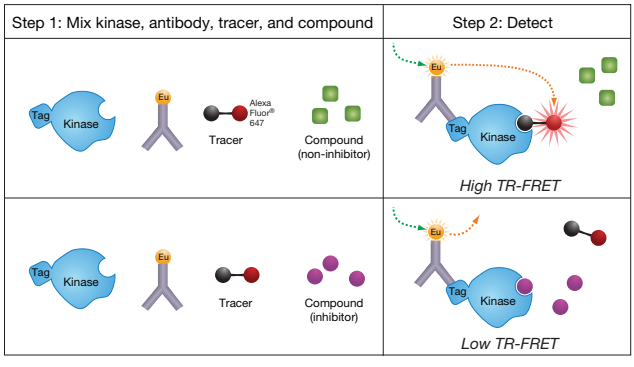


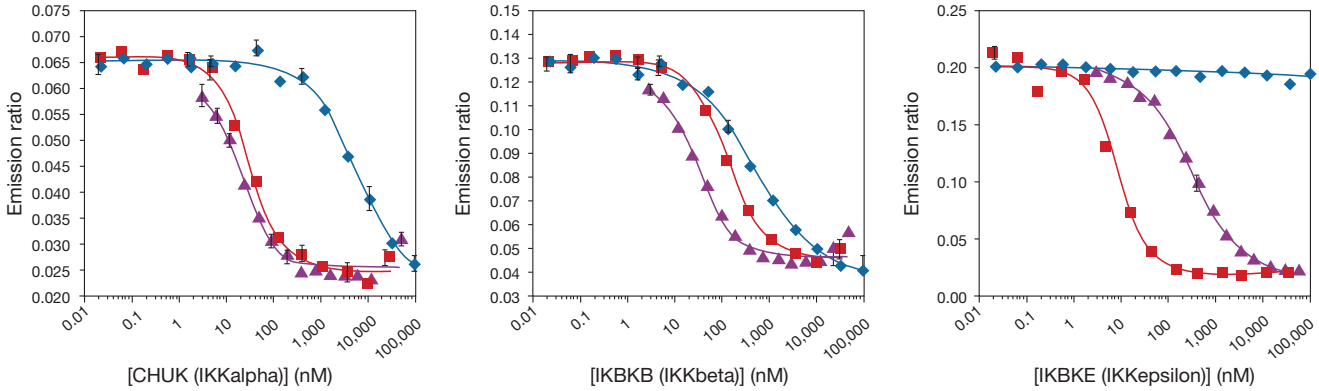
Figure 2. LanthaScreen® Eu Kinase Binding Assay schematic.

Type III inhibitors displace ATP-site probes

To test the ability of the LanthaScreen® Eu Kinase Binding Assay to detect Type III inhibitors, commercially available Type III compounds were tested in standard titration experiments with kinases they have been shown to inhibit (Figure 1). In almost all cases, the potency levels obtained in the LanthaScreen® assay matched reported activity assay values for these Type III inhibitors (Figures 3 and 4, Table 1). Even though these compounds do not bind directly to the ATP site, the majority must either alter the active site in a way that displaces the tracers or bind close to the active site. For example, the Type III inhibitors BMS-345541 (Figure 3, Table 1) and AKT Inhibitor VIII (Table 1), which target IKKbeta and AKT, respectively, exhibit binding assay potencies that are very similar to those observed with activity assays. The equivalence between the binding assay and activity assay also exists for most Type III molecules targeting GSK3beta, p38alpha, Abl1, and MEK (Table 1). Three MEK inhibitors that act in an uncompetitive manner with respect to ATP (i.e., they bind with higher affinity in the presence of ATP) demonstrate increased potency when binding assays are performed in the presence of a subsaturating level of ATP (Figure 4, Table 1). The sole example of a compound not detected in the binding assay but detected in activity assays is a substrate-specific inhibitor of p38alpha. However, this compound is only detected in activity assays when a specific substrate (MK2a) is used. Alternatively, Type III inhibitors that bind preferentially to non-active states (e.g., the MEK inhibitor PD98059) are detected only in binding assays and not in a direct activity assay utilizing wild-type MEK.

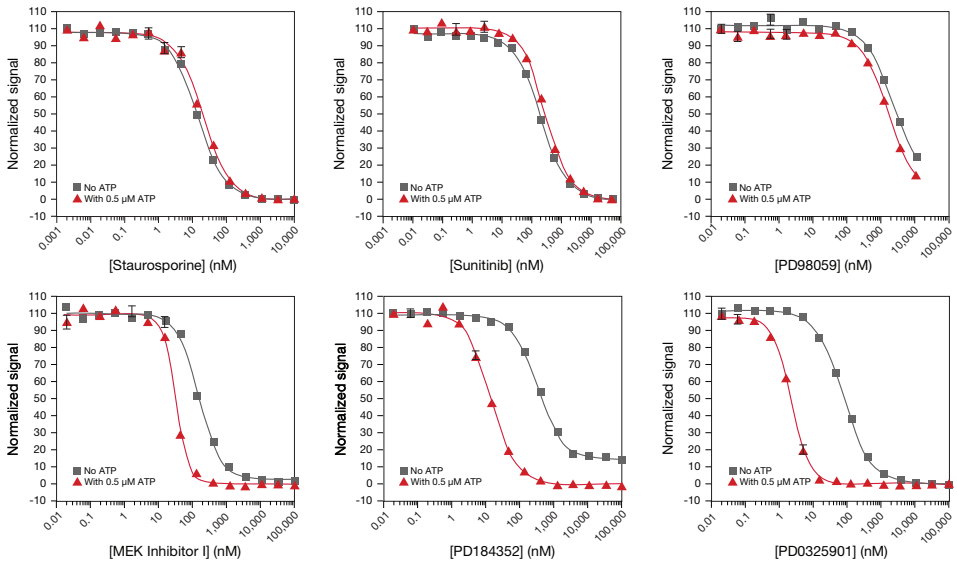
Considerations in selecting an assay format for high-throughput screening

When selecting a biochemical assay for primary screening, it is useful to consider the types of compounds that can be detected with each assay. In particular, it may be desirable to detect novel chemical entities that act allosterically or that bind to novel sites on a kinase. Testing of these commercially available Type III inhibitors showed that activity assays and the LanthaScreen® Eu Kinase Binding Assay are comparable in terms of the percentage of Type III inhibitors that are detected. However, there are two notable differences. First, activity assays (but not binding assays) can detect compounds that act to block phosphorylation of specific substrates. Second, the binding assay enables detection of compounds that bind preferentially to a non-activated state. In conclusion, the LanthaScreen® Eu Kinase Binding Assay provides a suitable option for detection and characterization of almost all known Type III kinase inhibitors.



		IC_{50} (nM)					
		CHUK (IKKalpha)		IKBKB (IKKbeta)		IKBKE (IKKepsilon)	
Compound	Inhibitor type	Binding assay	Literature	Binding assay	Literature	Binding assay	Literature
Staurosporine	Type I	25	NA	120	250	4.4	5.7
IKK Inhibitor VII	Type I	21	40	30.7	1.6	270	604
BMS-345541	Type III	7,500	4,000	620	~300	>100,000	>100,000

Figure 3. Detection of BMS-345541. Although the tracers developed for the binding assay utilize scaffolds of ATP-competitive Type I inhibitors, the assays can detect binding of many non–ATP-competitive compounds such as BMS-345541. Binding assays for IKKalpha (CHUK), IKKbeta (IKBKB), and IKKepsilon (IKBKE) were optimized and run with known Type I inhibitors (i.e., ATP-competitive) and BMS-345541. Data correlated well to literature values [7] based on activity measurements.



		IC_{50} values (nM)	
	Inhibitor type	No ATP	+ 0.5 µM ATP
Staurosporine	ATP-competitive	18	24
Sunitinib	ATP-competitive	160	310
PD98059	ATP-noncompetitive	1,920	1,600

		IC_{50} values (nM)	
	Inhibitor type	No ATP	+ 0.5 µM ATP
MEK Inhibitor I	ATP-uncompetitive	140	30
PD184352	ATP-uncompetitive	530	18
PD0325901	ATP-uncompetitive	70	2.5

Figure 4. Detection of Type III MEK1 inhibitors. Type III inhibitors of MEK1 have been identified that are either noncompetitive or uncompetitive with respect to ATP. ATP titrations were first performed under standard binding assay conditions to determine ATP K_m apparent (data not shown) and the assay tolerance in the presence of 0.5 µM ATP. Data are shown using non-activated MEK. ATP-uncompetitive compounds demonstrate a 5–28-fold increase in potency in the presence of 0.5 µM ATP. Data in the presence of ATP are consistent with reported literature values for activity-based assays using cascade assays or an intrinsically active truncation mutant [8,9,10].

Table 1. Summary of Type III inhibitor testing.

		IC_{50} values (nM)		
Kinase	Compound	Binding assay	Activity assay	Notes
AKT1 AKT2 AKT3	AKT Inhibitor VIII (AKT i 1/2)	70 240 1,590	58 210 2,120	pH domain-dependent [11]
ABL1 (non-activated)	GNF-2	100	240	Binds to myristate-binding pocket in the C-lobe of the kinase domain [12,13]
GSK3beta	TDZD-8	9,000	2,000	TDZD analog, noncompetitive with respect to ATP [14]
	GSK3beta Inhibitor VI	450	100	Irreversible, noncompetitive with respect to ATP [3]
	GSK3beta Inhibitor VII	230	500	Irreversible, noncompetitive with respect to ATP [3]
IKBKB CHUK(IKKalpha)	BMS-345541	620 7,500	300 4,000	Noncompetitive with respect to ATP [7]
MEK1 (non-activated)	PD98059	1,900	300–7,000* >10,000 in a direct activity assay	Blocks activation of MEK, noncompetitive with respect to ATP [15]
	U0126	60 (partial)	65–540	
MEK1 (non-activated)	PD0325901 PD184352 MEK Inhibitor I	2.5† 18† 30†	<1 to 20* 67–300* 12*	Uncompetitive with respect to ATP [8,9,10]
p38alpha	JX401	43	32	Noncompetitive with respect to ATP or substrate [16]
	MK2a Inhibitor	>10,000	330 for MK2a >3,000 MBP or ATF-2	MK2a inhibitor is not expected to compete off tracer as kinase is still active for MBP and ATF-2 substrates [4,5]

***** Coupled assays or direct assays using intrinsically active transaction mutant. † IC_{50} values determined when binding assay was performed in the presence of 0.5 µM ATP.

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Ordering information

Product	Quantity	Cat. No.
5X Kinase Buffer A	4 mL	PV3189
Kinase Tracer 178	25 µL	PV5593
Kinase Tracer 236	25 µL	PV5592
Kinase Tracer 199	25 µL	PV5830
LanthaScreen® Eu-anti-GST Antibody	25 µg	PV5594
LanthaScreen® Eu-anti-His Antibody	25 µg	PV5596
LanthaScreen® Eu-anti-DYKDDDDK	25 µg	PV6026
LanthaScreen® Eu-Streptavidin	25 µg	PV5899

Learn more about the LanthaScreen® Eu Kinase Binding Assay at www.invitrogen.com/bindingassay.

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