A Novel Sox-based Continuous and Homogeneous Assay for the Discovery of Inhibitors of Inactive and Active AKT

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ABSTRACT

The activity of protein kinases can play a critical role in the aberrant activation of oncogenic signaling pathways which can drive tumorigenesis and malignant transformation in cancer. AKT has been shown to be both upregulated and mutated in cancer cells allowing it to serve as a driver of cancer cell growth and progression. Inhibition of AKT activation and activity are both attractive targets for effective drug discovery in cancer. We developed continuous, homogeneous assays for full length unactive AKT1, AKT2, and AKT3. A peptide substrate, modified with a sulfonamido-oxine fluorophore (Sox), utilizes chelation-enhanced fluorescence to enable a real-time readout of AKT activity as phosphorylation occurs. First, a subset of 30,000 existing Sox-containing sequences were evaluated to find ones that AKT1 can phosphorylate, selecting for natural substrates, assay robustness, and AKT specificity. A physiologically relevant peptide substrate was identified and used to develop a kinetic assay to monitor AKT1 activation and substrate phosphorylation. Unactive AKT1 was incubated with DOPS/DOPC and phosphatidylinositol 3,4,5-trisphosphate (PIP3) which mimics the plasma membrane, allowing the Pleckstrin Homology (PH) domain of AKT to bind, leading to a conformational change that enables subsequent phosphorylation of Thr-308, Ser-473, and Tyr-474 by PDK1 and MK2 for full activation. Upon initiating the assay, active AKT phosphorylates the sensor peptide, and the resulting signal is read in kinetic mode (enabling a progress curve in every well) using a fluorescence intensity readout (Ex/Em 360/485 nm). A novel assay was developed for AKT activation and substrate phosphorylation utilizing AQT0076, DOPS/DOPC, PIP3, PDK1, and MK2. With a mix of classical AKT inhibitors and allosteric inhibitors, we can demonstrate inhibition of both active AKT activity and inactive AKT activation via dose-response measurements. Conclusions: A robust, homogeneous assay was developed to simultaneously monitor AKT activation and substrate phosphorylation kinetically over time. Through a continuous assay format, one can capture both steady-state rates and rate acceleration as a function of inhibitor concentration, allowing for accurate quantitation of both classes of AKT inhibitors in a single experimental format. As such, this assay can be used in drug discovery to evaluate potential inhibitors of both AKT activation and subsequent substrate phosphorylation to prevent cancer cell growth and progression.

FIGURE 1. ASSAY DEVELOPMENT - PHYSIOLOGICAL SUBSTRATES

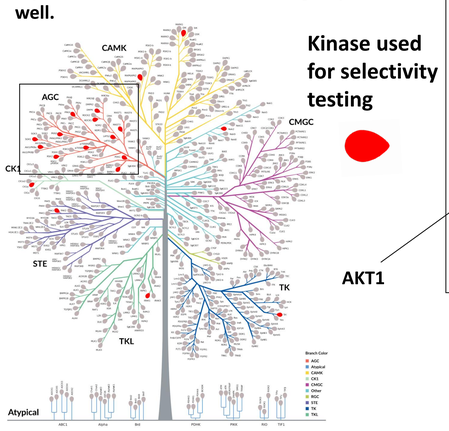
Table 1

Natural Substrate	Phosphorylation Site	Core Sequence
YAP	Ser127	AHSSPAS
BAD	Ser99, Ser134	RSRSAPP, RPKSAGT
Caspase 9	Ser196	RFSSLHF
FOXO1	Ser256, Ser319, Thr24	RAASMDN, RTSSNAS, RSCTWPL
FOXO3	Ser253, Thr32	MDNSNKY, RTNSNAS
FOXO4	Ser197, Ser262, Thr32	RAASMDS, RSSSNAS, RSCTWPL
ASK1	Ser83	RGSSVGG
IKKa	Thr23	RLGTGGF
CREB	Ser119	RRPSYRK
Mdm2	Ser166, Ser190	RAISETE, DSISLSF
mTOR	Thr2173	RKLTLMG
PRAS40	Thr246	LNTSDFQ

We tested a variety of substrates derived from natural/physiological AKT substrate sequences utilizing our extensive Cys-Sox Substrate Library (currently >30,000). Physiological substrate sequences often decrease the likelihood of off-target hits in biochemical and cell-based assays due to an increase in target specificity.
Some of the core sequences that were used are in Table 1, with the phosphorylation site highlighted in red. For the initial testing, we first selected sensor peptides that yielded robust signals (those with reaction rates >300 RFU/pmole/min) with active AKT kinases.

FIGURE 2. ASSAY DEVELOPMENT - SELECTIVITY TESTING

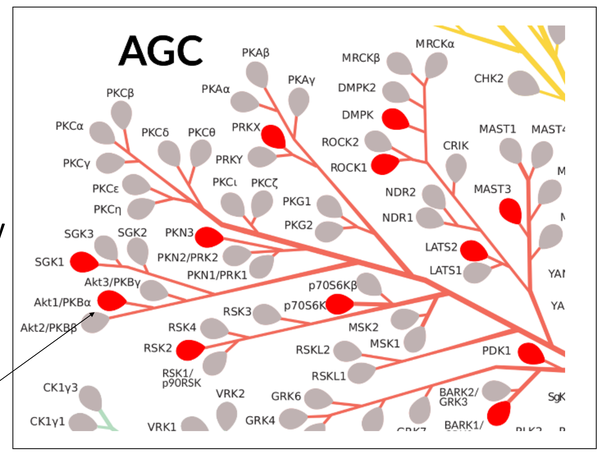
After creating a custom sensor peptide panel with strong AKT reaction rates, we proceeded to run selectivity experiments with an array of active kinases throughout the kinome. We focused on the AGC kinase group which includes AKT, although we tested representative kinases from other groups as well.

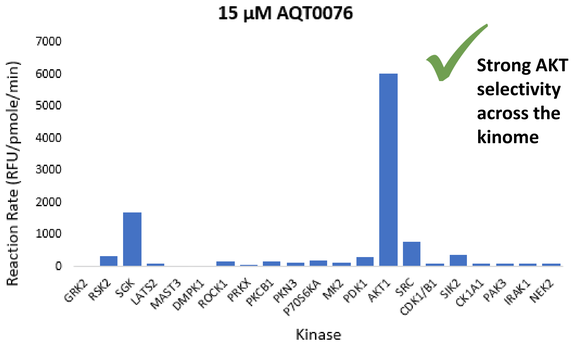


Full Kinome Tree

Kinase used for selectivity testing

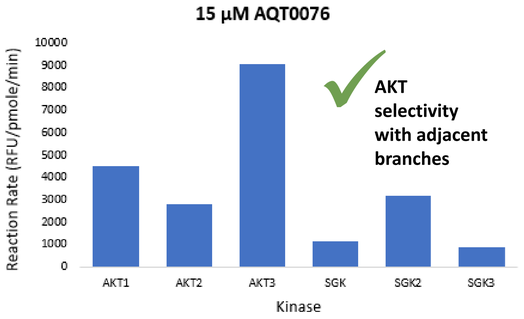
AKT1





Strong AKT selectivity across the kinome

We ran an initial assay for kinase selectivity, followed by an AKT/SGK panel to further narrow down the panel by assay strength and selectivity. From both rounds of testing, AQT0076 showed the best selectivity and activity for AKT1.



AKT selectivity with adjacent branches

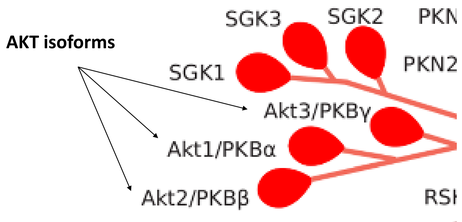
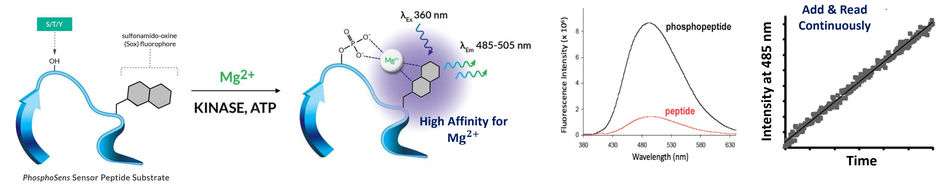


FIGURE 3. THE PHOSPHOSENS® ASSAY

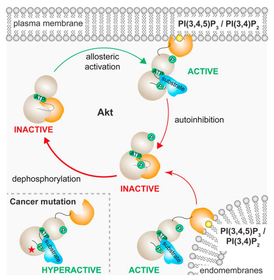


Peptide sequences are synthesized using solid-phase methods with the Sox fluorophore coupled through the sulfhydryl group of a cysteine residue proximal to a protein kinase phosphorylation site, such as a tyrosine, serine, or threonine. Upon addition of a kinase, the peptide is phosphorylated. In the presence of magnesium ion, a chelation complex is formed with the phosphate group, resulting in fluorescence enhancement of the Sox fluorophore **monitored continuously** as fluorescence intensity.

The standard PhosphoSens® AKT Assay used for active AKT enzymes is illustrated in Figure 3. This assay can be utilized to detect inhibition of inhibitors of active AKT by monitoring phosphorylation of peptides with sequences similar to physiologic AKT substrates. We also wanted to develop an assay that would detect inhibition of unactivated AKT preventing its activation. For this assay, we tried to mimic cellular conditions by using unactivated AKT kinase, lipids like those in the cellular lipid bilayer, and cofactors/kinases necessary for AKT activation. AKT Activation Assays were developed for AKT1, AKT2, and AKT3, as well as AKT1 (E17K), since this AKT enzyme is of research interest and endemic to many disease states (Chen et al. 2020).

FIGURE 4. ASSAY OPTIMIZATION

Figure 4A
AKT Activation in the Cell



Ebner et al. Mol Cell 65:416 (2017)

Workflow

1. **Compare Lipids (6:0 & 18:1)**
2. **Compare Unactive AKT from different sources**
3. **Enzyme Titration**
4. **Lipid/PIP3 Titration**
5. **PDK1 Titration**
6. **MK2 Titration**
7. **ATP Titration**
8. **Substrate Titration**
9. **DMSO Titration**
10. **Control Inhibitor Titrations**
11. **MK2 /PDK1 Counter Screens**

Figure 4B
Lipid/PIP3 6.0 vs 18.1

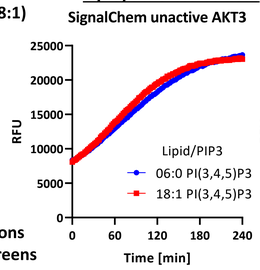


Figure 4C
Background Comparison

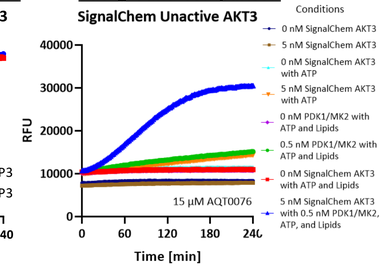


Figure 4D
Unactive AKT3 Enzyme Titration

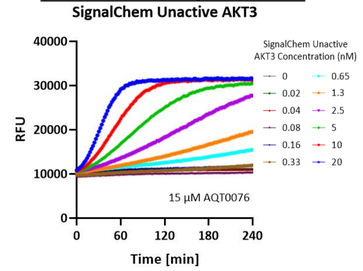


Figure 4E
Lipid/PIP3 18.1 Titration

Figure 4F
PDK1 Titration

Figure 4G
MK2 Titration

Figure 4H
ATP Titration

Figure 4I
AQT0076 Titration

Figure 4A illustrates the activation of AKT in the cell. We developed assays to mimic this activation *in vitro*. Figure 4B shows data for the comparison of DOPS/DOPC/PIP3 vesicles made with differing lengths of PIP3 with unactive AKT3. Both lipid vesicles increase activity from unactivated AKT3 . Figure 4C illustrates the background for unactive AKT3 alone and for assay components alone. There is a small amount of unactive AKT3 that can be activated by ATP alone as is indicated in orange. There is also a small increase in signal with PDK1/MK2/ATP/Lipid Vesicles. Figure 4D shows an enzyme titration for unactivated AKT3. The reaction is linear down to 0.65 nM unactive AKT3. Figure 4E shows a titration of DOPS/DOPC/PIP3 vesicles. The reaction rate continues to increase to the highest concentration tested (4.0 µM). Figure 4F and 4G show titrations of PDK1 and MK2, respectively. The rates for PDK1 increase to 63 pM, while MK2 rates increase to 250 pM. Figure 4H and 4I show titrations of ATP and the AQT0076 substrate, respectively. The ATP K_m is 680 µM, while the K_m for AQT0076 is 13 µM.

FIGURE 5. ASSAY VALIDATION WITH COMMERCIAL CONTROL COMPOUNDS

Final Assay Conditions:

Reaction Conditions:
54 mM HEPES, pH 7.5
1 mM ATP
1.2 mM DTT
0.012% Brij-35
0.52 mM EGTA
1% glycerol
0.2 mg/ml BSA
10 mM $MgCl_2$
15 µM AQT0076
Inhibitor, or 2% DMSO
1 µM 18:1 PI(3,4,5)P3 Avanti Polar Lipids
10 µM Upstate Lipid Blend (DOPC/DOPS (1:1, wt%))
2 nM SignalChem Active AKT1 Cat#/Lot#: A16-10G/A4630-10) amino acids 1-480 (end); N-terminal GST tag
OR
0.4 or 6 nM Unactivated SignalChem AKT1 (Cat#/Lot#: A16-14G/Q187-2) amino acids 1-480 (end); N-terminal GST tag
0.04 or 0.6 nM SignalChem PDK1 (Cat#/Lot#: P14-10H/I013-1) amino acids 1-556 (end); N-terminal HIS tag
0.04 or 0.6 nM Carna BS MK2 (Cat#/Lot#: 02-142/22CBS-0444B) amino acids 1-400 (end); N-terminal GST tag
coexpressed with HIS tagged p38B amino acids 1-364 (end) and HIS tagged MAP2K6 amino acids 1-334 (end)

Reaction Set Up:
0.4 µl 50x Compound Dilutions in DMSO
2 µL water OR 10x PDK1/MK2 Mix
4 µL activated AKT OR unactivated AKT (5x in EDB) or EDB
13.6 µL Reaction Mix with ATP, DTT, CSox Substrate, and Lipids
(variable 60 min preincubation times)
20 µL Final reaction volume
Reaction was run at 30˚C for 240 minutes, reading continuously

DMSO Titration

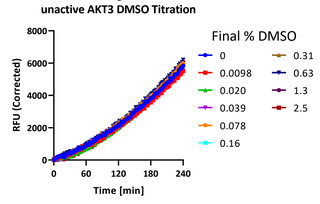


Figure 5A

Counter Screens: MK-2206 Titration with PDK1 and MK2

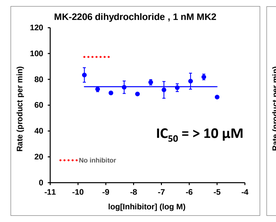


Figure 5F

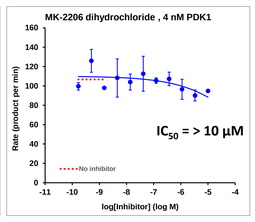


Figure 5G

Ipatasertib Titration with unactive and active AKT1

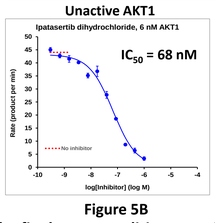


Figure 5B

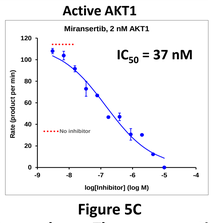


Figure 5C

MK-2206 Titration with unactive and active AKT1

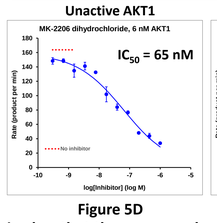


Figure 5D

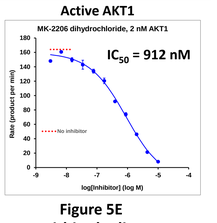


Figure 5E

Summary Table

Inhibitor	Enzyme	Enzyme Conc. (nM)	AKT1 Substrate Phosphorylation IC_{50} (nM)	MK2 Counter Screen IC_{50} (nM)	PDK1 Counter Screen IC_{50} (nM)
Borussertib	Active AKT1	2.0	106	> 10 µM	> 10 µM
Borussertib	Unactive AKT1	0.40	26	> 10 µM	> 10 µM
Ipatasertib dihydrochloride	Active AKT1	2.0	37	> 10 µM	> 10 µM
Ipatasertib dihydrochloride	Unactive AKT1	6.0	68	> 10 µM	> 10 µM
Miransertib	Active AKT1	2.0	143	> 10 µM	> 10 µM
Miransertib	Unactive AKT1	6.0	8.2	> 10 µM	> 10 µM
MK-2206 dihydrochloride	Active AKT1	2.0	912	> 10 µM	> 10 µM
MK-2206 dihydrochloride	Unactive AKT1	6.0	65	> 10 µM	> 10 µM

The final assay conditions are shown above. These were optimized so that the assay can be run within the linear range of the assay with a low unactive AKT concentration to avoid tight binding range, while still maintain a strong rate. As observed in Figure 5A, DMSO was tolerated up to at least 2.5% final. None of the compounds tested inhibited MK2 or PDK1 as shown in Figures 5F and 5G. Figures 5B, 5C, 5D, and 5E show examples of IC_{50} values for two control compounds tested with unactive and active AKT1. There is no significant difference between the IC_{50} values in the unactive vs active AKT1 assays with Ipatasertib which has been reported to be an ATP competitive inhibitor. The IC_{50} value for MK-2206 is significantly lower in the unactive AKT1 assay, which might be because MK-2206 has been reported to be an allosteric inhibitor which can bind in the PH domain of AKT and prevent conformational changes/enzyme activation.

SUMMARY AND NEXT STEPS

- **We developed continuous biochemical assays for both active and unactive AKT1, AKT2, AKT3, and AKT1 (E17K), and assayed four known AKT inhibitors: Borussertib, Ipatasertib dihydrochloride, Miransertib, and MK-2206 dihydrochloride.**
- **With these four unique inhibitors, we were able to show allosteric inhibitors that preferentially inhibited the inactive AKT conformation, and an ATP competitive inhibitor that inhibited the active and unactive conformations of AKT equally.**
- **Since we observed good AKT selectivity for AQT0076 with the kinome profiling mini-panel, we can follow-up with a Kinsight™ full kinome panel to further optimize this substrate for testing in a lysate assay.**
- **We plan to further analyze the initial rates in the unactive AKT assay by utilizing fits to acceleration kinetics and first derivative analysis to determine acceleration coefficients for AKT activity, and compound inhibition. A preliminary example is shown in Figure 6. Determining the potencies vs. AKT activation and substrate phosphorylation can be determined independently from the same set of dose-response progress curves.**

Figure 6
Fit to Acceleration Kinetics
$Y = P_0 + V_0t + \frac{1}{2}at^2$

Best-fit values
P_0 = 0 RFU
V_0 = 33 RFU/min
a = 2.6 RFU/min²