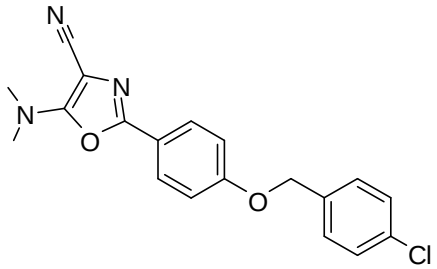
Project: Inhibitors of *Bacillus stearothermophilus* Pyruvate Kinase

Probe: 2-(4-(4-chlorobenzyloxy)phenyl)-5-(dimethylamino)oxazole-4-carbonitrile



SID: 862236
MLS000042013-01
Internal IDs: Class: Pyruvinb; Type: Bacruvinb; Shown: Bacruvinb-A

PubChem Primary Bioassay Identifier (AID): 361

SID	IC50 (μM)	Antitarget	Selectivity*
862236	0.25	Human PK M2	≥240 (Inactive @60 μM)

*Selectivity = antitarget IC50/target IC50

Assigned Assay Grant #: ML0005

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Douglas Auld; NIH Chemical Genomics Center
Human pyruvate kinase (M2 isoform) was provided by Wael M. Rabeh, Lyudmila Nedyalkova and Hee-Wan Park; Structural Genomics Consortium, 100 College St. Toronto, Ontario, Canada.

Assay or Pathway Target: *Bacillus stearothermophilus* Pyruvate Kinase

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Assay provider information

Specific Aim: We performed proof-of-principle research aimed at discovering and optimizing selective inhibitors of pyruvate kinase (PK). The research has two specific aims: 1) to engineer inhibitors for bacterial PKs as leads for anti-infective agents. 2) To use structure-based, analogue synthesis and medicinal chemical principles to identify inhibitors of therapeutically useful PKs from infectious pathogens and other species including human where inhibitors have been considerably more difficult to obtain.

Significance: PK is a critical metabolic enzyme operating in the ultimate step in glycolysis where it catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP (see Figure 1). PK from *bacillus stearothermophilus (*bPK*)* is an allosteric enzyme activated by monophosphate sugars such as AMP or ribose 5-phosphate and therefore shows different allosteric

regulation than human isozymes that are regulated by the bis-phosphorylated sugar, fructose1,6-bisphosphate. Inhibitors of bPK have been identified through a proof-of-principle qHTS assay. This scaffold is currently being examined as means to understand and design chemical series that are capable of inhibiting the PKs from *bacillus* and other bacterial species. As well, specific inhibition of bPK may be useful in the metabolic engineering of folic acid[1]. No small molecule inhibitors (other than metabolites or PEP substrate analogs) have been previously described for PK.

Rationale: The implementation of bPK assay was used as a proof-of principle study of quantitative HTS (qHTS)[2], an approach where multiple concentrations are tested for every compound in large chemical libraries to allow determination of concentration-response curves (CRCs) for every compound in a high-throughput mode (~1 CRC/sec was achieved in the present assay). The data generated for bPK has also been used by other laboratories to test hierarchical classification of chemical scaffolds alogorithms[3] and to test the effect of data-processing procedures on the sensitivity and specificity of hit detection[4].

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Pyruvate Kinase

List of PubChem bioassay identifiers generated for this screening project (AIDS):

AID	Target	Concentration	Bioassay type
361	bPK- luciferase	57μM – 3.7 nM	Primary qHTS
411	luciferase	57μM – 3.7 nM	qHTS profile database

Primary Assay Description as defined in PubChem:

Overview:

Pyruvate kinase (partially purified from *Bacillus stearothermophilus*) was assayed for its ability to generate ATP from ADP using phosphoenolpyruvate (PEP) as a substrate. ATP generation was detected in a coupled reaction by luciferase-mediated luminescence, an ATP-dependent process. Pyruvate kinase substrates, PEP and ADP, were present in the assay at Km and 10-fold below Km respectively. The enzyme was assayed at an intermediate level of activity to screen for inhibitors and activators. In addition, ribose 5 phosphate, a positive modulator of pyruvate kinase, was included in the assay at AC10 in order to identify possible potentiators.

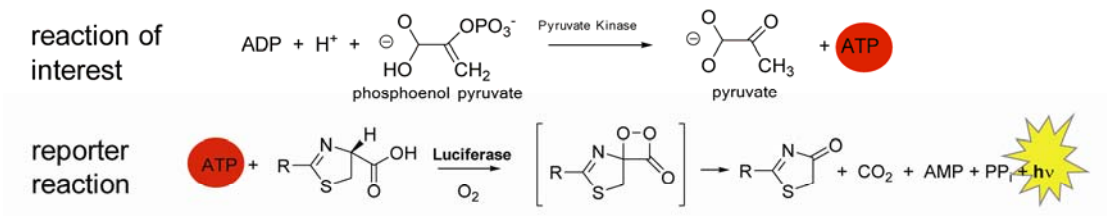
Protocol:

The qHTS 1536-well protocol is shown in Figure 1. Briefly, three μL of substrate mix (final concentration, 50 mM imidazole pH 7.2, 50 mM KCl, 7 mM MgCl2, 2 mM PEP, 0.1 mM ADP, 0.7 nM ribose 5 phosphate, 0.01% Tween 20, 0.05% BSA) was dispensed into 1536 well plates. Twenty three nL of compound were delivered by a pin tool and 1 uL of enzyme mix (final concentration, 10 nM pyruvate kinase, 50 mM imidazole pH 7.2, 0.05% BSA) was added. After centrifugation at 1000 rpm for less than a minute, plates were incubated at room temperature for 2 hours. Three μL of detection and kinase stop mix (PKLight, Cambrex) was added, the plates centrifuged, and luminescence read by a ViewLux (Perkin

Elmer) at 5 second exposure/plate. Data were normalized to the controls for AC_{100} stimulation (5 μM ribose 5 phosphate) and to AC_{100} inhibition (110 μM luteolin).

Center Summary of the Primary Screen:

Assay principle and protocol:



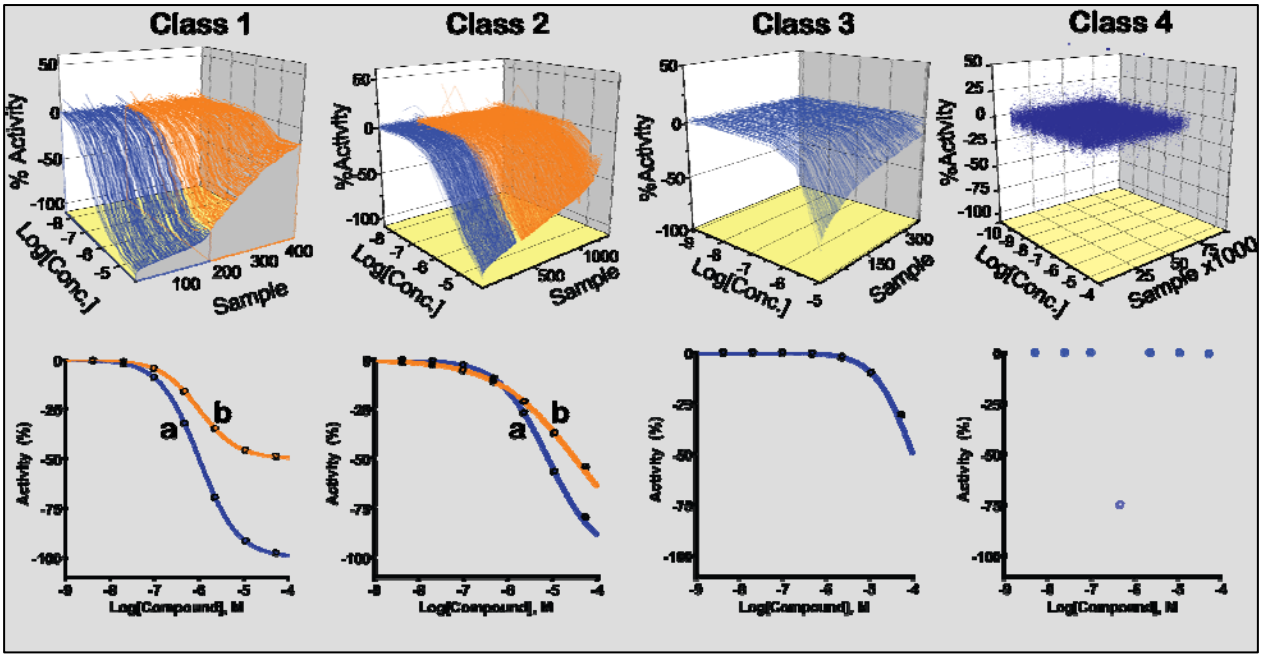
Bacillus PK Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3uL	Substrate mix (PEP =0.5 mM, ADP = 0.1 mM)
2	Compound	23 nL	Control plate
3	Control	23 nL	Compound library
4	Reagent	1uL	2 nM final PK; activator R5P; Apigenin Inhibitor
5	Time	75 min	Room temp
6	Reagent	2uL	Cambrex PK-Light (plus kinase stop)
7	Detector	luminescence	Viewlux

Step	Notes
1	Dispenser 1 (tip 1-4) Head B substrate
2	Control plate
3	Compound library 57 μM-0.7 nM; 1:5 dilutions
4	Dispenser 2 (tips 1-4)= 1X; col 4: no enzyme
5	Room temp
6	Cambrex PK-Light (plus kinase stop) Head C Dispenser 1 8-tips
7	Viewlux (2 sec, 2X binning)

Figure 1: Pyruvate kinase-firefly luciferase coupled assay. *Top*, the assay used to detect the formation of ATP catalyzed by PK by coupling to firefly luciferase. *Bottom*, the protocol used to perform qHTS against PK on the Kalypsys robotic system. Compounds were screened at seven concentrations (57μM – 3.7 nM).

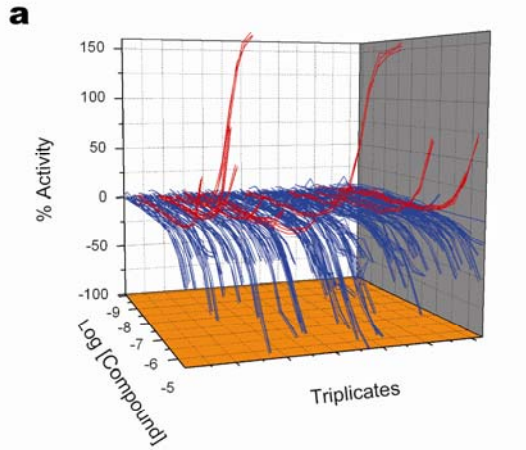
Identification of Pyruvinbs: Following the qHTS the CRC data was subjected to a classification scheme to rank the quality of the CRCs as described by Inglese and co-workers[2] (see scheme 1). Briefly, CRCs are placed into four classes. Class 1 contains complete CRCs showing both upper and lower asymptotes and r^2 values > 0.9. Class 2 contains incomplete CRCs lacking the lower asymptote and shows r^2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. Once an active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed

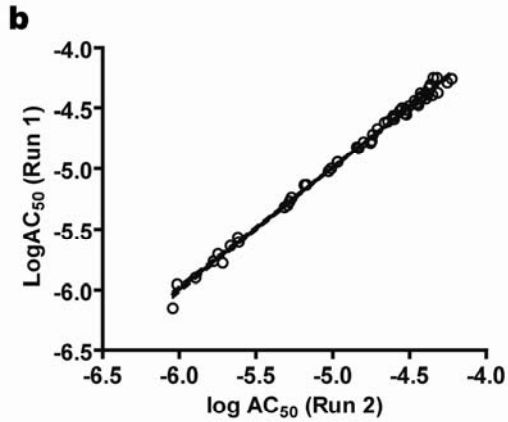
by using Leadscope (Leadscope Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data. The data was also compared to a complete qHTS profile database against firefly luciferase (PubChem AID: 411)[5] to eliminate firefly luciferase inhibitors.



Scheme 1: Example qHTS data and classification scheme for assignment of resulting curve-fit data into classes. **Top**, qHTS curve-fit data from AID 361 binned into curve classifications 1-4 based classification criteria. **Below**, Examples of curves fitting the following classification criteria: Class 1 curves display two asymptotes, an inflection point, and r2 ≥0.9; subclasses 1a (blue) vs. 1b (orange) are differentiated by full (>80%) vs. partial (≤ 80%) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a (blue) and 2b (orange) are differentiated by a max response and r2, >80% and >0.9 or <80% and <0.9, respectively. Class 3 curves have a single left-hand asymptote, no inflection point, and a response >3SD the mean activity of the sample field. Class 4 defines those samples showing no activity across the concentration range.

qHTS summary of assay results:





c

qHTS Assays Performance Summary	
Parameter	***qHTS***
Compounds (total # tested)	107,610
Sample-concentration measurements (sample wells)	1,037,696
Total wells	1,187,328
Plates Screened	773
Plates Failed QC	3
Plates Re-run for QC	163
RZ'	0.68 +/- 0.08
Signal / Background	6.9 +/- 1.4
CV	12 +/- 5
Screening System	Kalypsys
Software for stat analysis	In-house software

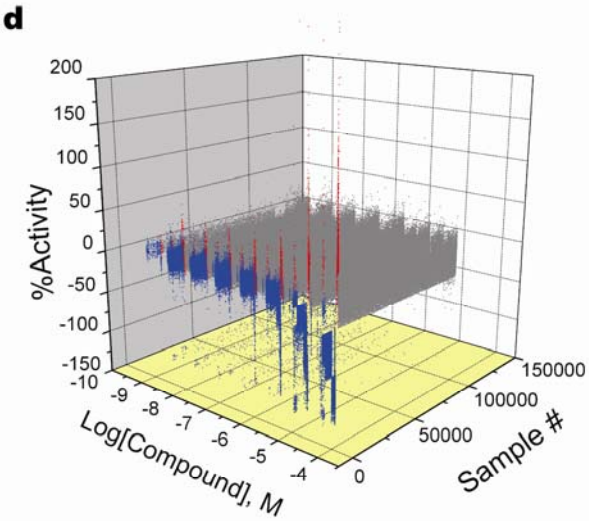


Figure 2: Validation and qHTS performance of the bPK-luciferase assay. **a**) Inter-screen data from triplicate qHTS runs of the Prestwick collection. Data for 104 compounds fitting to CRCs with inhibitory (blue) or stimulatory (red) activity are shown. Lines connect the data points for each compound titration and replicate. **b**) Representative correlation plot of compounds with AC_{50} <60 μM identified from runs1 and 2 (r^2 = 0.98; n =58; median MSR = 1.1). For runs 1 vs. 3 and 2 vs. 3, r^2 = 0.99 and 0.98, respectively **c**) Summary of the performance of the bPK qHTS. **d**) Data for the entire qHTS for compounds that fit to inhibitor CRCs (blue) or activating CRCs (red) as well as inactive compounds that did not show a CRC (grey).

SAR of Bacruvinb series:

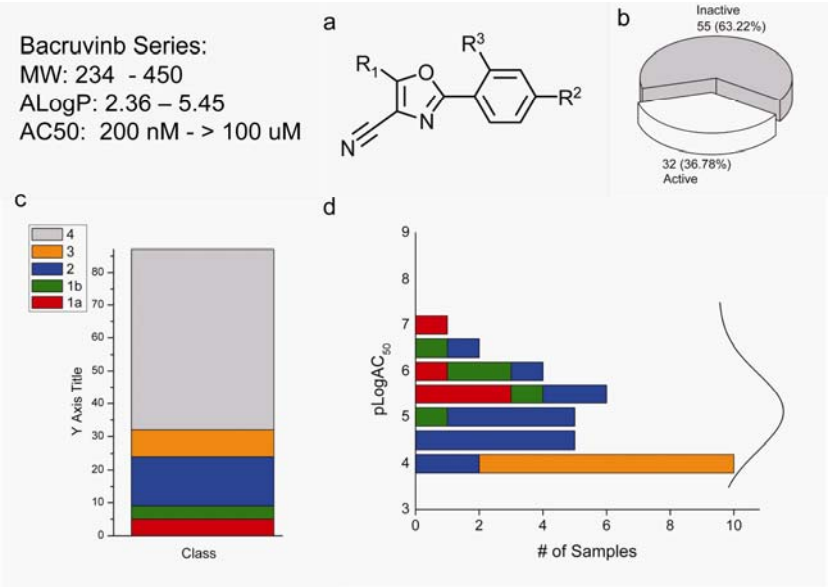
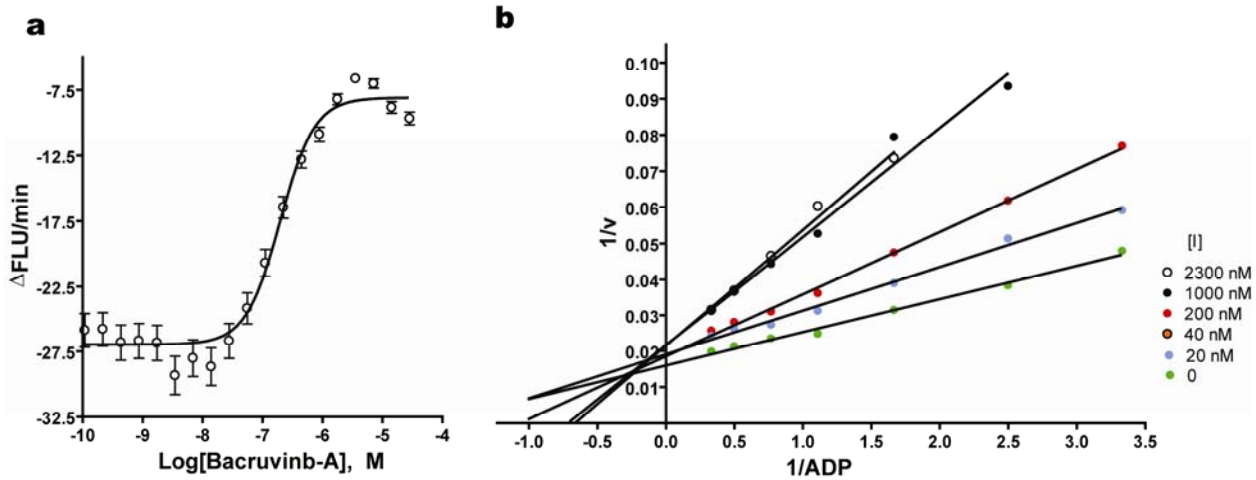


Figure 3: Pharmacological profile of the probe inhibitor series. **a**) MCS **b**) The number of active and inactive analogs identified. Curve Classes 1–3 (white) and Class 4 (light gray) is indicated in the pie chart. **c**) CRC class distribution **d**) Distribution of active CRC class vs potency values. A normal distribution is shown. CRCs corresponding to Class 1a (red, high efficacy), Class 1b (green, low efficacy), Class 2 (blue, incomplete CRCs), Class 3(orange, single point activity), and inactive (gray) are depicted in the bar charts.

Probe Characterization



Mode of action for Bacruvinb-A:

Figure 4: Bacruvinb-A acts as a mixed-type inhibitor with respect to ADP and shows a K_i = 200 nM. **a**) IC_{50} of Bacruvinb-A determined in the LDH coupled assay using a kinetic mode of detection. Concentration of ADP was 0.1 mM and the concentration of PEP was 4 mM. b) Variation of ADP at 4 mM PEP in the presence of the indicated concentrations of Bacruvinb-A [I].

Has this compound been provided to the MLSMR?

Yes, MLS000042013-01

Canonical SMILES: CN(C)C1=C(N=C(O1)C2=CC=C(C=C2)OCC3=CC=C(C=C3)Cl)C#N

InChI: InChI=1/C19H16ClN3O2/c1-23(2)19-17(11-21)22-18(25-19)14-5-9-16(10-6-14)24-12-13-3-7-15(20)8-4-13/h3-10H,12H2,1-2H3

Description of secondary assays used to optimize/characterize probe structure:

The luciferase profile database (PubChem AID: 411) was used to determine if the compound was active at the coupling enzyme used in the bPK-luciferase luminescent primary assay. See Auld et al.[5] for details of the luciferase qHTS.

Bacillus PK-LDH Coupled Assay Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	3uL	Substrate mix (PEP =0.5 mM, ADP = 4 mM; 200 µM NADH)
2	Compounds	23 nL	Test Compound
3	Reagent	1uL	Enzyme mix: 15 nM bPK/1 µM LDH final conc.
4	Detector	NADH fluorescence	Viewlux

Step	Notes
1	FRD dispense; Buffer 50 mM Tris-Cl pH 8.0, 200 mM KCL,15 mM $MgCl_2$
2	24-point titration starting at 57 µM; 1:2 dilutions
3	Source of LDH (rabbit muscle Sigma)
4	Viewlux kinetic read 30 sec intervals

A PK-LDH coupled assay was used to measure the depletion of NADH following reduction of pyruvate by LDH[6]. The protocol is given below:

Known probe properties:

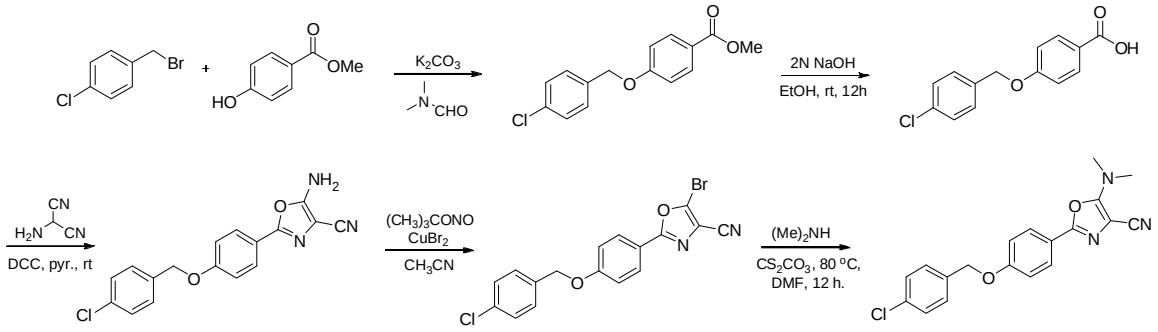
Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

Molecular Weight	353.80224 [g/mol]
Molecular Formula	$C_{19}H_{16}ClN_3O_2$
XLogP	4.8
H-Bond Donor	0
H-Bond Acceptor	5
Rotatable Bond Count	5
Exact Mass	353.093104
MonoIsotopic Mass	353.093104
Topological Polar Surface Area	62.3
Heavy Atom Count	25

Compound preparation:

Compound is prepared in DMSO at 10 mM stock concentration. Assays described above have 0.6% DMSO final concentration in buffer.

The general synthetic strategy for the preparation of the probe is as follows:



Probe availability:

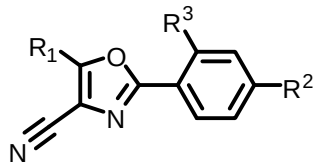
Aliquots of MLS000042013-01 are available from the NCGC upon request.

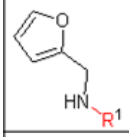
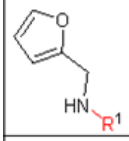
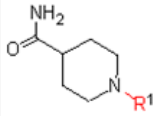
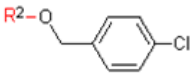
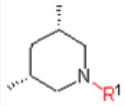
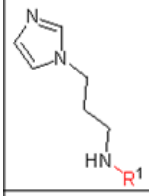
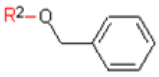
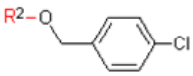
Appendices

Comparative data on probe, similar compound structures and prior probes:

Other than phosphomycin, no prior probes for bPK have been described.

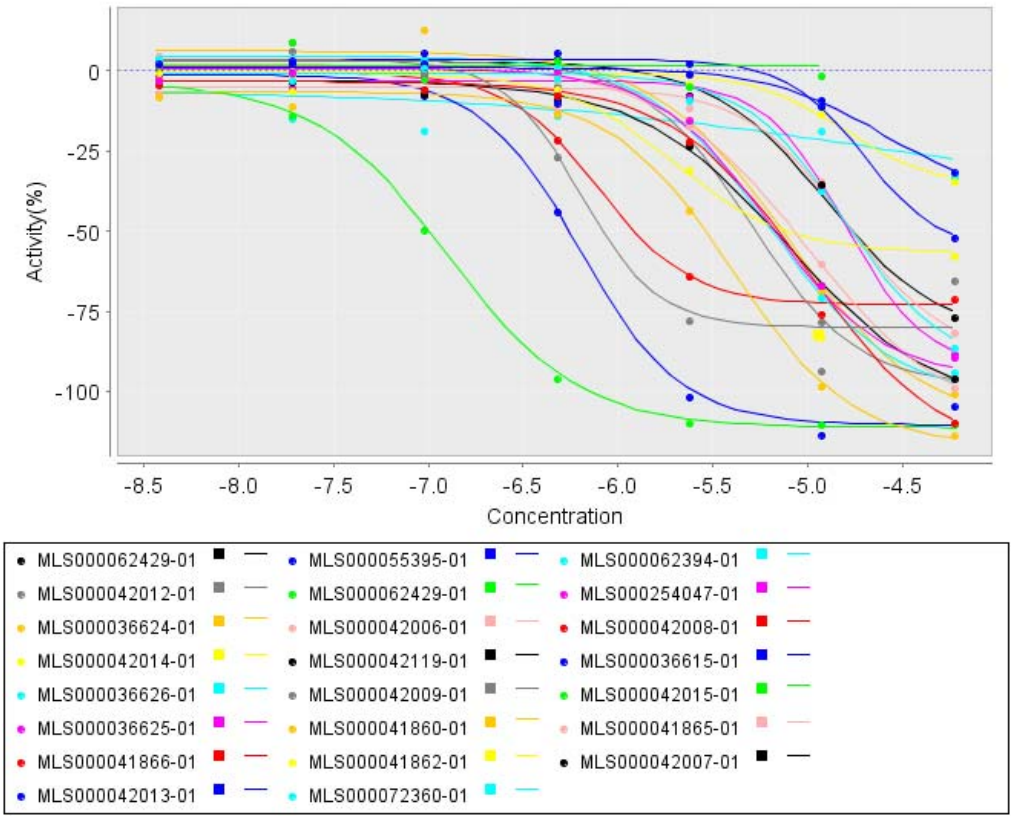
Representative active and inactive analogs from the qHTS



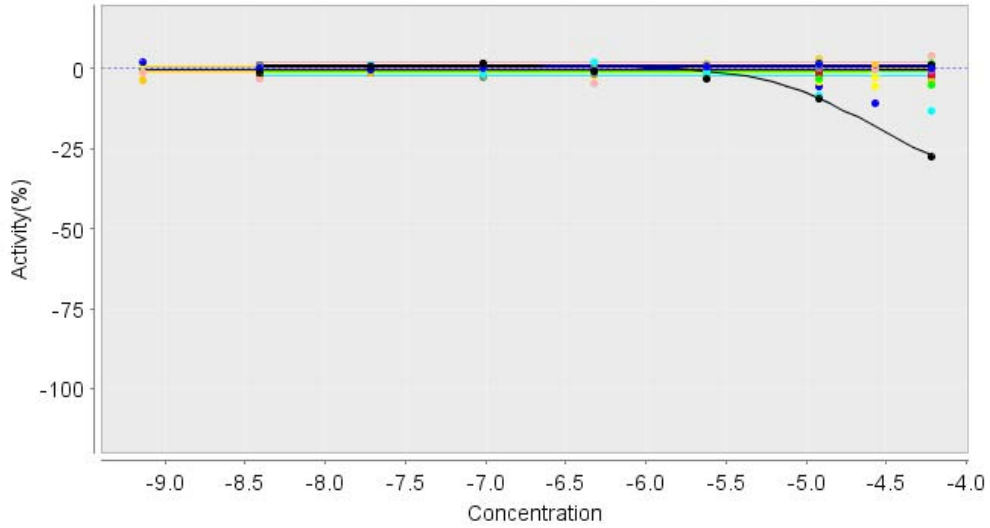
R1	R2	R3	NCGC ID	pub chem sid	Curve Class	Rank	AC50 (uM)	Act Max Conc	Hill Coeff	MW	ALogP
	Cl	H	NCGC00072191-01	4257445	4		inactive	-8		300	4.07
	H	Cl	NCGC00053176-01	4255774	4		inactive	-3		300	4.07
		H	NCGC00067413-01	862867	-1.1	1/20	0.08	-92	1.1	437	4.15
	H	Cl	NCGC00052762-01	4255152	4		inactive	1		316	4.35
		H	NCGC00066999-01	863475	-1.1	10/20	4.3	-93	1.1	400	4.49
		H	NCGC00067332-01	862236	-1.1	2/20	0.18	-79	1.4	354	4.51

Comparative data showing probe specificity for target

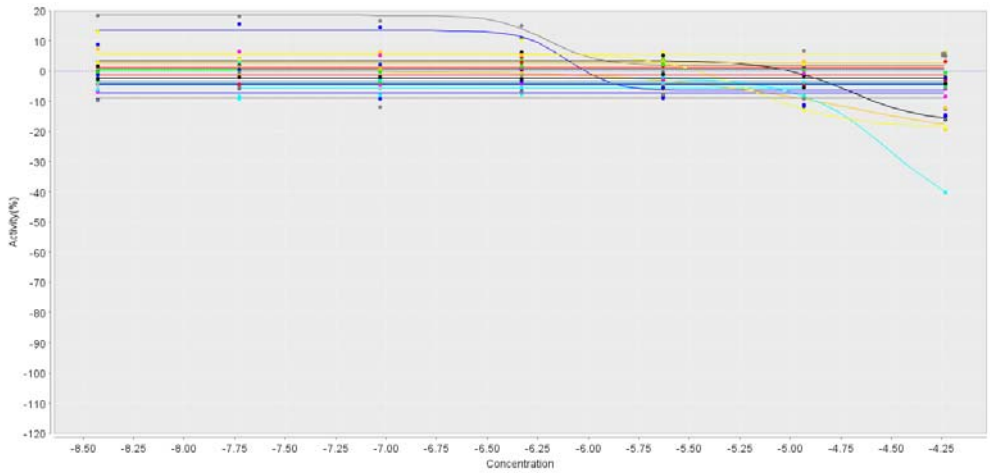
Activity in bPK-luciferase coupled assay



Activity in luciferase assay:



Activity in human PK M2-luciferase assay:



Comparison with PubChem assays:

SID: 862236 (Bacruvinb-A) has been tested in 220 assays found within PubChem and was designated as active in only seven of these that are listed below.

AID	Target	Tested Concentrations	Bioassay type
361	bPK- luciferase	57 µM – 3.7 nM	Primary qHTS (luminescent)
893	HSD17B4, Hydroxysteroid Dehydrogenase	57 µM – 3.7 nM	Primary qHTS (fluorescent)
886	Hydroxyacyl-Coenzyme A, Dehydrogenase Type II	57 µM – 3.7 nM	Primary qHTS (fluorescent)
431	Cytotoxicity HPDE-C7K cells	10 µM	HTS (fluorescent)
709	Autofluorescence at 339/460 nm	57 µM – 3.7 nM	Profile qHTS (fluorescent)
590	Autofluorescence at Alexa 350	57 µM – 3.7 nM	Profile qHTS (fluorescent)
589	Autofluorescence at 4-MU	57 µM – 3.7 nM	Profile qHTS (fluorescent)

Interpretation:

Bacruvinb-A may show blue fluorescence that would explain the activity in spectroscopic profiling assays and the two dehydrogenase assays. The inactivity in 213 PubChem assays would suggest a highly selective compound.

Bibliography

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2. Inglese, J. *et al.* Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* **103**, 11473-11478 (2006).
3. Schuffenhauer, A. *et al.* The scaffold tree--visualization of the scaffold universe by hierarchical scaffold classification. *J Chem Inf Model* **47**, 47-58 (2007).
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5. Auld, D.S. *et al.* Characterization of chemical libraries for luciferase inhibitory activity. *J Med Chem* **51**, 2372-2386 (2008).
6. Hannaert, V. *et al.* The putative effector-binding site of *Leishmania mexicana* pyruvate kinase studied by site-directed mutagenesis. *FEBS Lett* **514**, 255-259 (2002).