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ATP COMPETITION OF PI3K INHIBITORS USING PHOSPHOCELLULOSE PAPER APPROACH

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Abstract: Profiling small molecule inhibitors against lipid kinases are challenging owing to fact that lipid substrates/products are difficult to capture on membrane filters unlike protein kinase substrates. We have recently reported the development of an inexpensive & robust methodology for profiling lipid kinase inhibitors in a phosphocellulose plate format. This is a medium throughput 96 well format assay & validated with major PI3K isoforms using specific inhibitors. This unique assay methodology was further extended to inhibitor characterization and evaluated the mechanism of action (MOA) of PI3K inhibitors (LY294002 and TGX-221) with respect to ATP competitiveness against PI3 kinase β. This robust method can be easily adapted in any lipid kinase drug discovery program for compound profiling & in-depth mechanistic applications without having any potential artifacts.

Key Words: Phosphatidyl Inositol 3-kinase, Phosphocellulose, ATP Competition, PtdIns, PtdSer, IC_{50}

INTRODUCTION

The phosphoinositide 3-kinases (PI3K) plays a key role in cell surface receptor triggered signal transduction pathways which controls multitude complex functions like cellular responses controlling cell survival and cellular proliferation [1]. PI3 kinase phosphorylates D3 Hydroxyl position of membrane bound phospholipid phosphatidyl 4, 5 biphosphate (PIP2) to yield phospholipid phosphatidyl 3, 4, 5 triphosphate (PIP3). PI3K lipid kinase comprises Class I, Class-II and Class-III which differ in substrate specificity and regulation. Class-I is subdivided into Class-IA and Class-IB, which were extensively studied due to deregulation in majority of diseases like cancer, inflammatory diseases [9]. Class-IA comprises of p110α p110β p110δ as catalytic subunits with p85 and p55 as regulatory subunit, while Class-IB has p110γ as catalytic unit with p101 as regulatory subunit. Class-I generates PIP3 as second messenger in their respective signal transduction pathway. Class-IA PI3 kinase enzymes are primarily activated by receptor tyrosine kinases (RTKs), while Class-IB PI3 kinase enzymes by G-protein coupled receptor (GPCRs) [10]. Besides its function in cancer [2], numerous diseases have exhibited abnormal increase in the PI3K activity thereby emerging as a potential target in many disease areas like cardiovascular, autoimmune diseases etc [3] [6] [7] [8].

Screening for lipid kinase inhibitors has been typically challenging owing to fact that lipid substrates/products are difficult to capture on membrane filters unlike protein kinase substrates. In the quest for a cheap & robust lipid kinase assay platform, we developed a novel phosphocellulose plate based lipid kinase assay [5]. In this assay format, transferred phosphate (γ phosphate) can be directly measured thereby quantification of enzyme kinase activity is direct without the involvement of any additional chemicals or steps. Radioactive assay method eliminates the cross reactivity or interference of color reagents, fluorometric or quenching agents etc generated from the compounds/ buffers or other assay reagents. Other formats like fluorescence assay, luminescent assays or colorimetric assays use different antibodies and conjugated fluorophores thereby carrying the baggage of false positive or false negative results.

In our present study we have extended this novel method to study the mechanism of action of these inhibitors with respect to ATP competition. We have chosen PI3Kβ as target enzyme and two inhibitors (TGX-221 and LY294002) which have different potencies (nano molar to micro molar range) against PI3Kβ

MATERIALS AND METHODS

ATP competition assays were executed with varying concentrations of ATP. Concentrations selected spanned the Km of the respective enzyme. Based on the apparent Km of PI3Kβ as 25μM, five concentrations of ATP used were 1μM, 10μM, 25μM, 50μM and 100μM respectively [5]. TGX-221 and LY294002 served as reference compounds for the assay. TGX-221 is a specific PI3Kβ inhibitor whereas LY294002 is a pan PI3K (PI3Kα, β, γ and δ) inhibitor. Both the compounds were dissolved in DMSO and titrated in buffer maintaining uniformity in final optimized concentration of DMSO.

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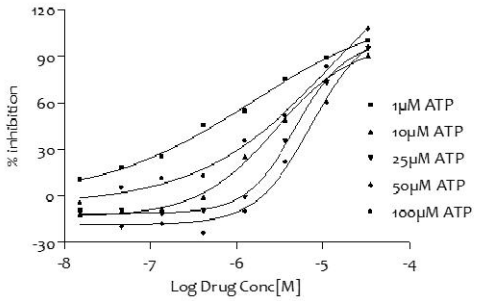
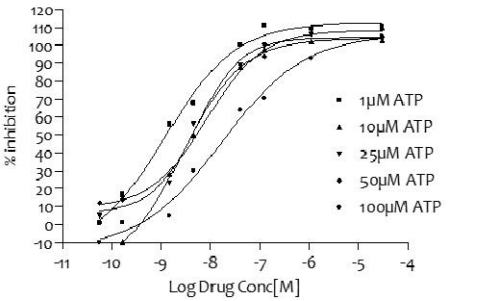
Starting concentrations of both inhibitors were selected as per the IC_{50} generated at apparent ATP Km and fixed for ATP competition studies.

Experiments were carried out as described earlier [5]. Briefly, 1µg per well pre-sonicated lipid mix (PIP + PS) at 1:1 ratio was used as substrate for the enzyme assay. Reactions were carried out using 50ng of enzyme and incubated for 2 hours at 30 degrees with gentle shaking in regular incubator. Reaction was terminated using stop solution (1:1 methanol: 1 N HCl) incubating 10 min at room temperature. The total reaction volume was transferred to pre-equilibrated phosphocellulose plates. Plates were washed for three times on vacuum manifold to remove unbound radioactivity. Microscint was added to the dried plates and read in a Top Count Radioactivity plate reader. Percentage inhibitions were calculated and IC_{50}s were generated for all the ATP concentrations deduced from Graph pad Prism software [Figure 1A and 1B].



	1µM ATP	10µM ATP	25µM ATP	50µM ATP	100µM ATP
EC50	1.473e-006	2.838e-006	4.823e-006	7.419e-006	1.286e-005

Figure 1A: ATP competition of LY94002 against PI3Kβ



	1µM ATP	10µM ATP	25µM ATP	50µM ATP	100µM ATP
EC50	1.253e-009	2.670e-009	5.431e-009	8.396e-009	1.727e-008

Figure 1B: ATP competition of TGX-221 against PI3Kβ

IC_{50} curves data for LY294002 and TGX-221 at different ATP concentrations:

Different concentrations of ATP (1µM, 10µM, 25µM, 50µM and 100µM): 5µCi [γP^{32}] ATP per reaction, PIP2: PI = 1µg/well, Temperature: 30 degrees, Enzyme concentration: 50ng/well/reaction in 50µl volume, Time for the reaction: 2 hours, Buffer: MOPS: Reaction was carried in 96 well plates: DRC were generated in Graph Pad Prism software.

RESULTS AND DISCUSSION

Majority of the kinase inhibitors discovered till date are known to be ATP competitive in nature [4]. At a certain concentration of ATP, stoichiometric amount of inhibitor is required to displace ATP from the enzyme's ATP binding pocket. This requirement increases proportionally with increase in ATP concentration and displays an increase in IC50 of the test compound. We successfully demonstrated the ATP competition in phosphocellulose plate format.

Although existing lipid kinase assay platforms are used for automated routine screening; nevertheless these methodologies have limitation in inhibitor characterization because of multiple interfering agents such as antibodies, tracers, enzymes etc. in the supplied reagents, which might alter the true inhibitor property & thereby make the inhibitor characterization virtually impossible. In our previous report, we studied the enzyme assay development and kinetics in detail with known inhibitors & validated against all PI3K isoforms which is amicable for routine screening campaign. In our present study the ATP competition property of the two PI3 kinase inhibitors (TGX-221 and LY294002) having IC50s ranging from nano molar to micro molar range were examined using the phosphocellulose paper binding technology [5].

Our present findings demonstrated robust IC_{50}s for the known competitive PI3K inhibitors (TGX-221 and LY294002 both tested against PI3Kβ) at different concentrations of ATP. IC_{50}s for both inhibitors at different ATP concentrations were tabulated [Table 1A and 1B]. The detection mode being radioactive and direct, an excellent slope is maintained even within a limited range of ATP variation. This sensitivity is unique to this methodology and hasn't been demonstrated in other assay methods to our knowledge [Figure 1C and 1D]. Additionally, it's very difficult to study the substrate competition with potent compounds which are in nano molar range. Phosphocellulose paper radioactive based assay does not have this limitation and nano molar compounds can be studied for mechanism of action (MOA) and distinct IC_{50}s generated at different ATP concentrations. In the present study, PI3K beta specific nano molar compound TGX-221 was investigated with the present phosphocellulose format & provided insights on ATP competition [FIGURE: 1B and 1D]. Additionally, LY294002 which is in micro molar range also demonstrated ATP competition [FIGURE: 1A and 1C]. In the process of drug discovery, when compounds are designed to study SAR, it's very essential to know mechanism of compound inhibition. Very fine SAR

changes can be easily identified as this methodology is radioactivity based & inherently very sensitive. This in turn helps driving the medicinal chemistry efforts involved in a programme into the right direction.

Table 1A: IC_{50} values of LY294002 against PI3Kβ at different ATP concentrations

ATP Concentrations	IC_{50} in µM
1 µM ATP	1.47
10 µM ATP	2.83
25 µM ATP	4.8
50 µM ATP	7.4
100 µM ATP	12.86

Table 1B: IC_{50} values of TGX-221 against PI3Kβ at different ATP concentrations

ATP Concentrations	IC_{50} in nM
1 µM ATP	1.25
10 µM ATP	2.6
25 µM ATP	5.44
50 µM ATP	8.4
100 µM ATP	17

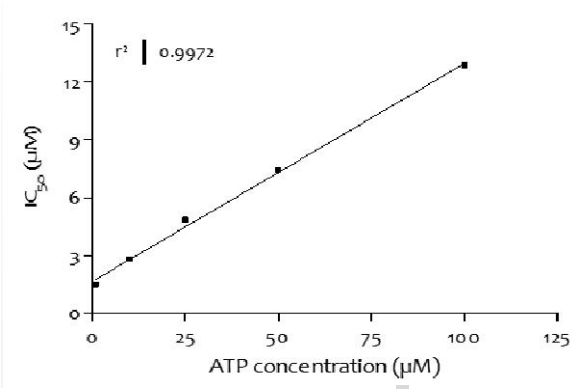


Figure 1C: ATP competition PI3Kβ inhibition with LY94002

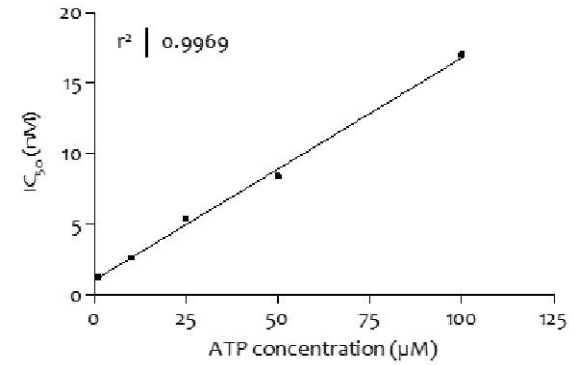


Figure 1D: ATP competition of PI3Kβ inhibition with TGX-221

Linearity graphs showing IC_{50}s of LY294002 and TGX-221 against PI3Kβ

Linear regression graphs were plotted taking ATP concentrations in X-axis and IC_{50} values in Y-axis in Graph pad Prism software.

In luminescence, fluorescence, HTRF & other platforms, investigating compound properties require multiple rounds of optimizations as there are a lot of interfering factors. The assay needs to be re-standardized for appropriate concentrations of the interfering agents (antibodies, fluorescent tracers etc.). Present phosphocellulose methodology does not require further optimization steps as it is known to be direct method (PIP3 detection). Like any other screening assay, good signal/ background (S/B) is essential to get robust results. For radioactive assay, S/B is dependent on [γP^{32}] ATP and this should be used fresh.

CONCLUSION

Radioactive assays are direct and gold standard. The phosphocellulose paper assay method proved a highly sensitive, cost effective, medium throughput profiling platform & fared well in inhibitor property characterization experiments providing appreciable information in dissecting the possible ATP competition. This unique phosphocellulose paper based assay methodology has the potential to provide insights into the compound mechanism of action and can be effectively included in the screening cascade of any lipid kinase drug discovery programme, particularly the ones dealing with PI3Ks.

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