

Revealing intrinsic ATPase activity in kinases: Implications for drug discovery using ADP-Glo™ assay

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Abstract

Kinases are pivotal regulators of cellular signaling and are among the most targeted enzymes in drug discovery. Their downstream phosphorylation of substrates is a key step in many disease-associated signaling cascades. A kinase's biochemical activity, and the effect of its cognate inhibitors, is traditionally assessed through measurement of the phosphorylation of a relevant substrate. A kinase's intrinsic ATPase activity - ATP hydrolysis independent of substrate - remains an underexplored biochemical aspect of kinases which may require closer attention in assay development, to ensure that inhibitor activity and profiling are properly assessed. In this study, we employed the ADP-Glo™ assay to systematically evaluate ATPase activity across 300 of human recombinant kinases produced in house. In this system, about 10% of kinases exhibited substantial intrinsic ATPase activity in the absence of substrate, suggesting that under these conditions and for a number of kinases, significant ATP turnover may occur independently of canonical phosphorylation events.

Non-specific inhibitors (e.g., Staurosporine and K252a) and specific kinase inhibitors were used to examine and confirm the origin of the substrate-phosphorylation and ATPase activity. Select kinase mutants and their mutant-specific inhibitors were used to further examine the catalytic and ATPase activities.

Our observations provide a deeper insight into kinase biochemistry and underscore the importance of understanding kinase-substrate catalytic activity from background ATP hydrolysis, particularly in high-throughput screening and lead optimization, so as not to mischaracterize inhibitor potency.

Materials and Methods

All human recombinant kinases were produced by Carna Biosciences, Inc., and ATP consumption was measured with ADP-Glo™ (Promega). Mobility shift assay using BioPhase8800 (Scienc) is performed to check the inhibitory effect of JNK1 and EGFR inhibitors.

Detailed investigation of ATPase activity

The detailed investigation on each kinase is performed. Figure 4 shows the results of JNK2. As briefly mentioned above, JNK2 showed high ATPase activity. While its inactive form showed very low ATPase activity (please note that the unit of x-axis is $\mu\text{g/ml}$, not ng/ml), the activated JNK2 showed strong ATPase activity.

We next checked whether the ATPase activities are inhibited by nonspecific kinase inhibitors. Regardless of the strength of ATPase activity, both kinase and ATPase activities are suppressed by Staurosporine and K252a (Figure 5). We also compared the IC₅₀ of K252a against JNK1 and 2. Figure 6 shows both IC₅₀s of the ATPase and kinase activities are the same, within experimental variation. We compared IC₅₀s of different inhibitors against EGFR wild type and mutants (Table 1), including competitive/allosteric (non-competitive) and reversible/irreversible modes. We observed that both the non-specific and EGFR-specific inhibitors of different modalities showed the same IC₅₀ values within experimental variation. This suggests that the use of an ATP-competitive and even non-competitive/allosteric inhibitor which prevents or modifies ATP binding, produces IC₅₀s within experimental variation whether it is ATPase activity, or substrate phosphorylation that is used as the final readout. This suggests that for such inhibitors, ADP-Glo™ could be used as a readout across kinases which have a sufficiently high intrinsic ATPase value to provide an experimental window, even if no substrate exists.

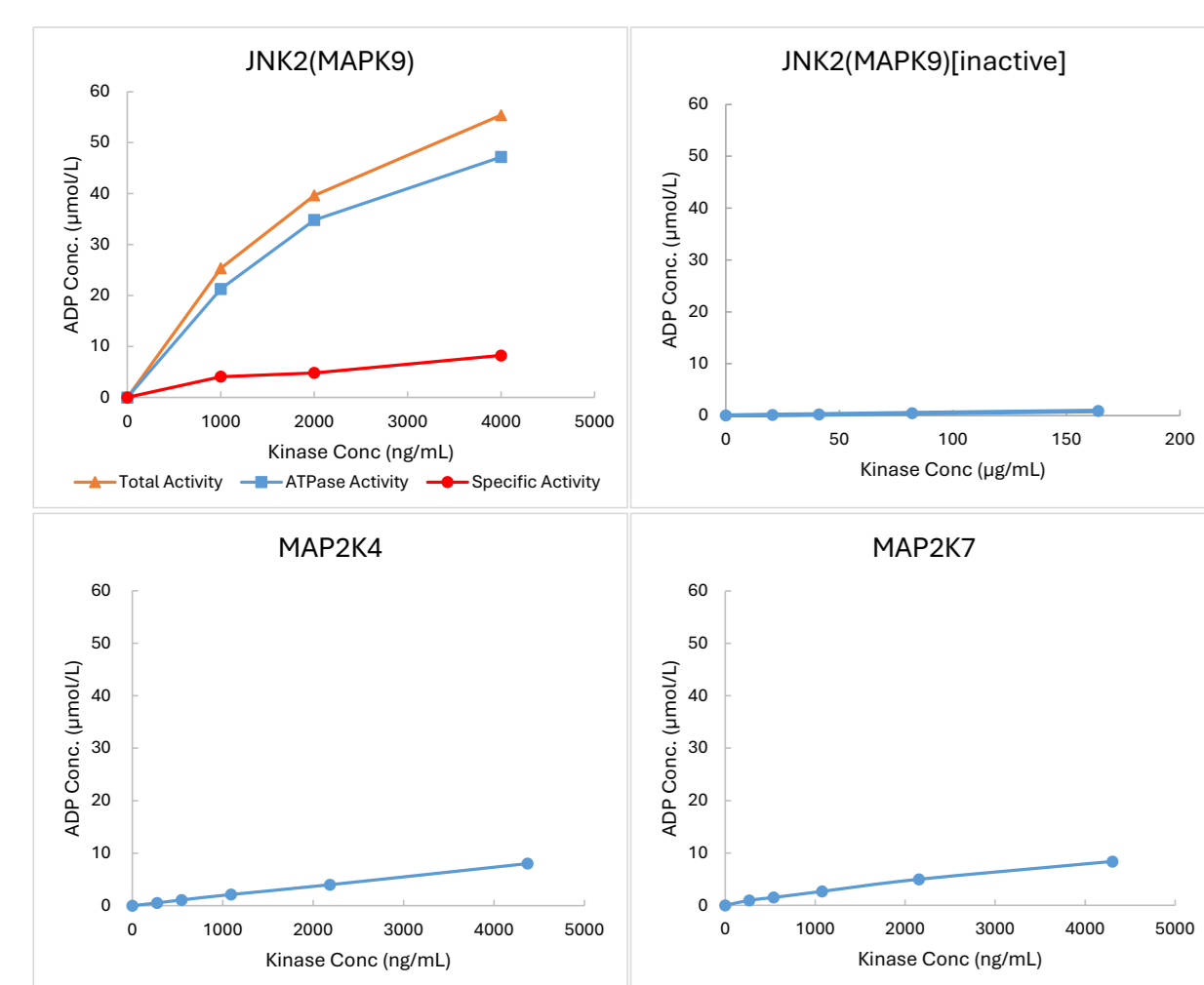


Figure 4. JNK2 ATPase activity was only observed in activated JNK2.

Inactive JNK2 is produced in E.coli and thereafter activated with the upstream kinases (MAP2K4 and MAP2K7). Neither inactive JNK2 (upper right) nor upstream kinases (lower left and right) showed strong ATPase activity indicates active JNK2 ATPase activity is purely derived from active enzyme.

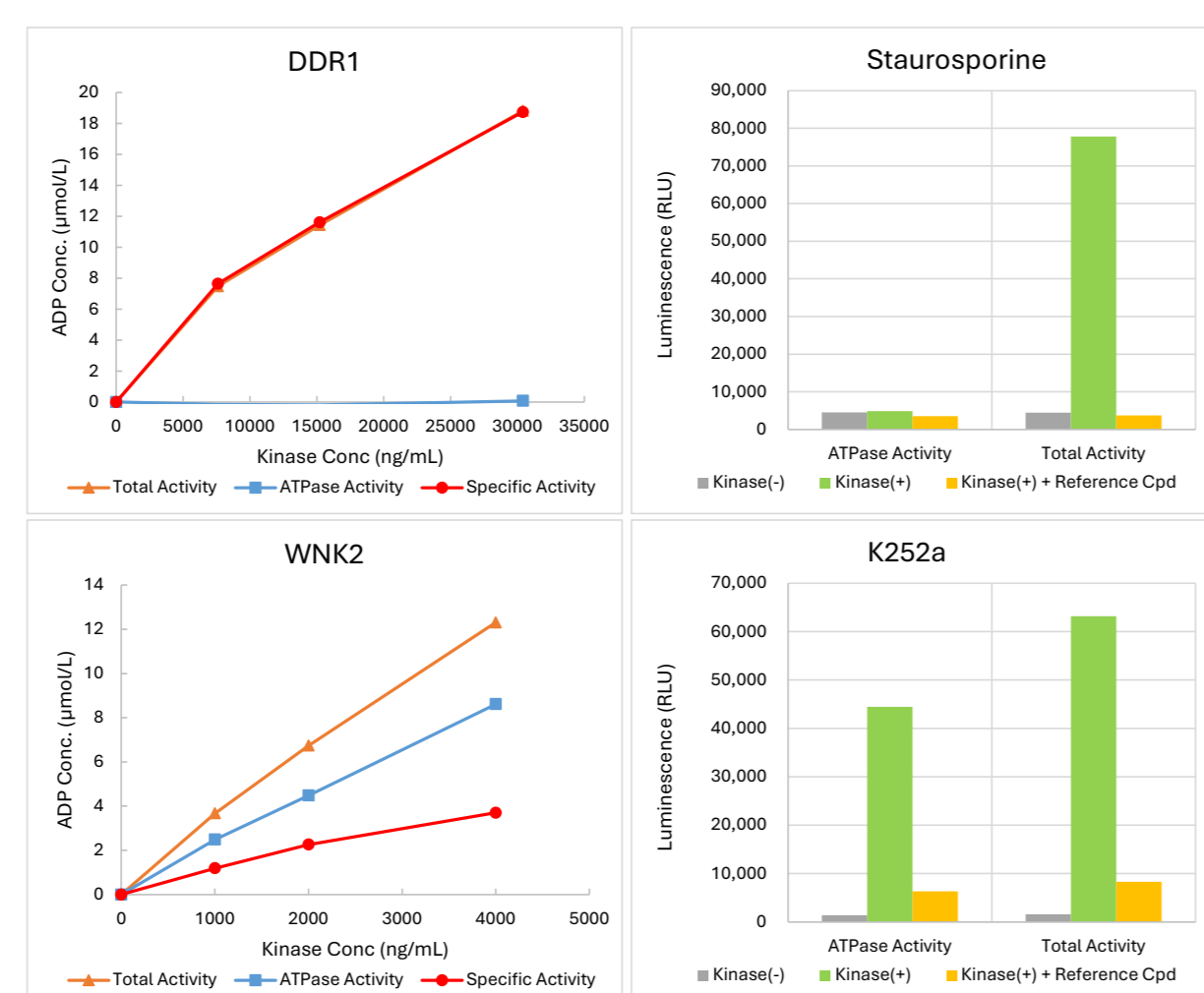


Figure 5. Inhibition of both kinase and ATPase activity by nonspecific kinase inhibitors.

Both kinase and ATPase activities are abrogated by non-specific kinase inhibitors such as Staurosporine and K252a.

Overview of ATPase activity

The ATPase activity reported here is defined as “catalytic activity in the absence of protein or peptide substrate, where water acts as the nucleophile for the hydrolysis of ATP to ADP and Pi”. To gain an overall understanding of the ATPase activity of the tested kinases, we analyzed the correlation between ATPase activity and other parameters. Most of the kinases have an ATPase activity lower than 30% (274), some (about 10%) showed relatively high ATPase activity (Figure 1). As shown in Figure 2, particularly strong activity is observed in certain kinase families such as MAPKs (Erk5, JNK1, 2, and 3), IKKs (IKK α , β and ϵ), and WNKs (WNK1, 2, 3, and 4). There was no significant correlation between enzyme concentration (Figure 3, left), purity (Figure 3, middle), and ATP Km (Figure 3, right). The observed rate of ATPase activity did not correlate with either the enzyme concentration or purity, we concluded the measured ATPase activity was not derived from impurity in the final preparation of our kinases. Km value reflects how easily ATP binds to the active site of the kinase. Therefore, a lower Km means higher affinity for ATP. The rate of catalysis of ATP to ADP + Pi by a kinase is controlled by the rate-limiting step of the overall process. This can be a chemical step, but is usually related to a product release step (ADP or Pi), as slow protein conformational changes are required for this to occur. Our observed ATPase activity represents the rate of ATP hydrolysis (to ADP and Pi), as measured by ADP release (the substrate for the ADP-Glo™ readout). Of note, we see no correlation with this rate and that of the ATP Km, which suggests that, as one would expect, the rate of ATP hydrolysis is not regulated by ATP affinity. Our result clearly showed ATP Km and ATPase activity reflect different aspects of enzyme function.

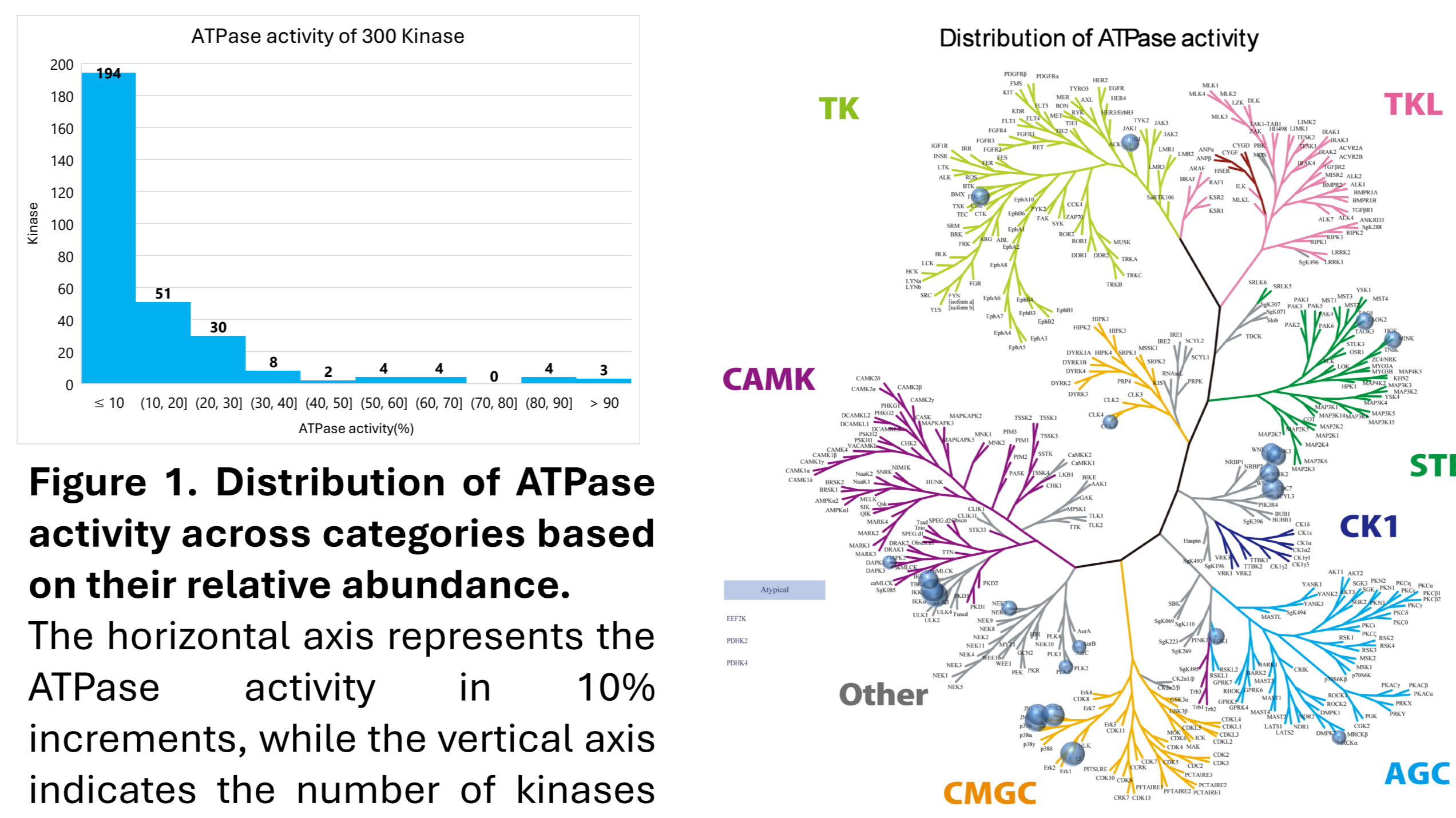


Figure 1. Distribution of ATPase activity across categories based on their relative abundance.

The horizontal axis represents the ATPase activity in 10% increments, while the vertical axis indicates the number of kinases belonging to each category. This bar graph illustrates the frequency of enzymes observed within each proportional range.

Figure 2. Kinases which show relatively high ATPase activity plotted on Kinome map.

Although the overall activity is dispersed, particularly strong activity is observed in certain kinase families. The bubble size shows the strength of ATPase activity (cut off; 30%).

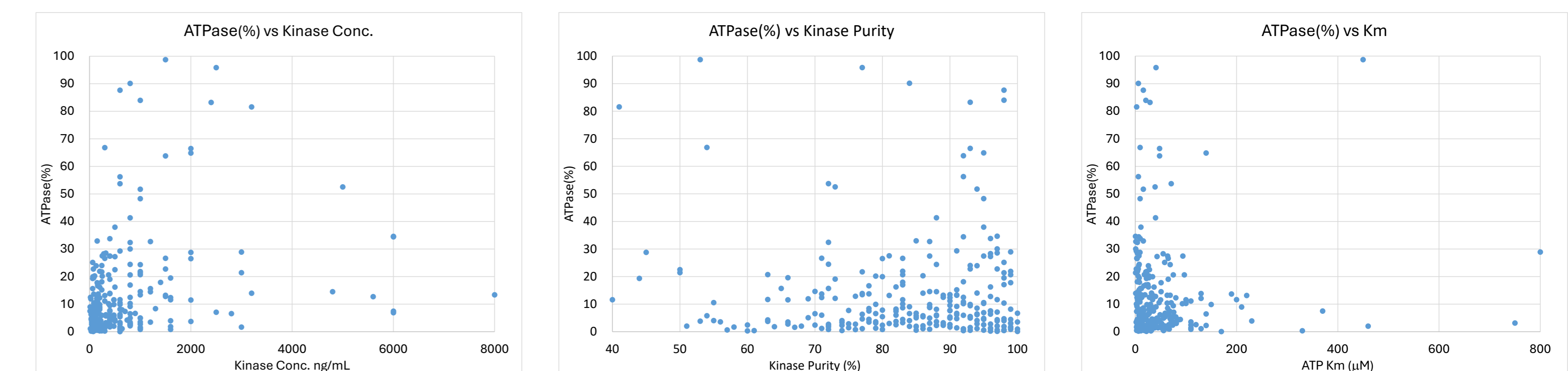


Figure 3. Correlation between ATPase activity and kinase concentrations, purities and ATP Km.

The X-axis shows kinase concentrations (left), purities (middle) and ATP Km (right), respectively and the correlation between these parameters and ATPase activities were plotted on the Y-axis. No obvious correlation was observed.

Conclusions

- The intrinsic ATPase activity is defined as “ATP hydrolysis in the absence of protein or peptide substrate”.
- The majority of kinases show an intrinsic ATPase activity lower than 30%, as compared to in the presence of substrate.
- Intrinsic ATPase activity was not derived from impurities in the final preparation of our kinases.
- The observed ATP Km and intrinsic ATPase activity show no correlation, suggesting that ATP Km and ATPase activity reflect different aspects of the catalytic process.
- Active JNK2 showed high intrinsic ATPase activity but neither inactive JNK2 nor upstream kinases showed this strong ATPase activity suggesting that activation of JNK2 increases its intrinsic ATPase (as well as its regular catalytic) activity.
- The EGFR mutants showed a range of intrinsic ATPase activities, which may reflect the role of the mutated residue in the catalytic process.
- Both nonspecific and specific kinase inhibitors of different competitive or allosteric modes, abrogated the ATPase and kinase activity. This suggests that ADP-Glo™ could act as a general inhibitor potency readout.

Table 1. IC₅₀ of various EGFR inhibitor against wild type and mutant EGFR kinases

Kinase	ATPase(%)	Staurosporine		Gefitinib		Osimertinib		JBI-09-063	
		ADP-Glo	MSA	ADP-Glo	MSA	ADP-Glo	MSA	ADP-Glo	MSA
EGFR	7.4	59	45	0.38	0.49	4.5	12	14	17
EGFR(C797S/L858R)	18	7.5	8.2	0.42	0.51	780	570	26	22
EGFR(D746-750)	6.0	7.8	17	0.46	0.30	2.4	2.4	>1000	>100
EGFR(D746-750/C797S)	0.54	11	11	0.26	0.35	300	300	>1000	>100
EGFR(D746-750/T790M)	7.0	1.1	0.70	>100	380	2.9	1.4	>1000	>100
EGFR(D746-750/T790M/C797S)	3.5	0.64	0.84	>100	550	200	120	>1000	>100
EGFR(D770_N771insNPG)	8.4	21	26	15	17	4.8	8.7	>1000	>1000
EGFR(L858R)	29	15	18	0.76	0.35	2.6	2.8	160	22
EGFR(L861Q)	7.7	53	75	0.38	0.70	2.5	4.0	9.3	10
EGFR(T790M)	16	3.2	2.3	>100	96	4.5	2.2	74	24
EGFR(T790M/C797S/L858R)	39	0.89	0.38	>100	>1000	360	43	34	34
EGFR(T790M/L858R)	29	1.1	1.2	>100	265	2.6	1.6	33	29

A comparison of the ATPase activity of EGFR wild and mutants. We observed different intrinsic ATPase activities across the EGFR wild type and mutant forms - which may reflect the role of the specific mutant residue in the process of ATP hydrolysis. We measured IC₅₀s of 3 EGFR-specific inhibitors with different modes of inhibition, Gefitinib (ATP-competitive, reversible), Osimertinib (ATP-competitive, covalent), and JBI-09-063 (allosteric/non-competitive, reversible). The measured IC₅₀s of the inhibitors in both ADP-Glo™ and MSA readouts were within experimental variation of each other.