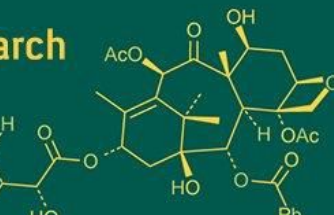
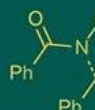


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Biochemical basis of allosteric regulation in kinase families

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Abstract

Allosteric regulation of protein kinases represents a promising therapeutic strategy enabling selective modulation of enzyme activity without competing at the highly conserved ATP-binding site. This in-vitro biochemical research characterized allosteric regulatory mechanisms in Aurora kinase A using enzyme kinetics, fluorescence spectroscopy, and molecular dynamics simulations. Experiments were conducted at the Protein Biochemistry Laboratory, Department of Molecular Biology and Biochemistry, Hacettepe University, Ankara, between May 2017 and October 2019.

Recombinant human Aurora kinase A was expressed in *Escherichia coli* and purified to homogeneity using affinity and size-exclusion chromatography. Four novel allosteric modulators identified through virtual screening were characterized for their effects on enzyme kinetics, conformational dynamics, and thermal stability. Kinetic parameters were determined using a continuous spectrophotometric assay monitoring ADP production through coupled enzyme reactions.

Compound A demonstrated potent positive allosteric modulation with EC_{50} of 0.5 μ M, reducing $K_{0.5}$ for ATP from 80 μ M to 45 μ M without affecting maximal velocity, indicating enhanced substrate affinity through conformational stabilization. Compound C functioned as a negative allosteric modulator, increasing $K_{0.5}$ to 160 μ M with IC_{50} of 1.2 μ M. Fluorescence quenching experiments revealed that positive allosteric modulators induced 40% reduction in tryptophan fluorescence, consistent with conformational compaction upon binding. Differential scanning fluorimetry demonstrated increased thermal stability ($\Delta T_m = +6.8^\circ\text{C}$) with Compound A, supporting stabilization of the active conformation.

Molecular dynamics simulations spanning 500 nanoseconds revealed that positive allosteric modulators stabilize the α C-helix in the active "in" position while promoting ordered conformation of the activation loop. Hydrogen-deuterium exchange mass spectrometry confirmed reduced solvent accessibility in the allosteric binding pocket and ATP-binding cleft upon modulator engagement. These findings establish structure-activity relationships for Aurora kinase A allosteric modulation and provide mechanistic framework for developing selective kinase modulators targeting conformational dynamics rather than catalytic site occupancy.

Keywords: Allosteric regulation, protein kinases, Aurora kinase A, enzyme kinetics, conformational dynamics, positive allosteric modulator, negative allosteric modulator, molecular dynamics

Introduction

The concept of allosteric regulation, first articulated by Monod, Wyman, and Changeux in 1965, describes how ligand binding at one site can influence protein function at a distant site through conformational coupling [1]. This fundamental principle governs countless biological processes and represents an increasingly attractive paradigm for therapeutic intervention. Unlike orthosteric inhibitors that compete directly at active sites, allosteric modulators offer potential advantages including greater selectivity, reduced resistance development, and opportunity for fine-tuning rather than complete inhibition of enzyme activity [2].

Protein kinases constitute one of the largest enzyme families in the human genome, with over 500 members regulating virtually every cellular process through reversible protein phosphorylation [3]. The kinase domain architecture demonstrates remarkable conservation, with a bilobal structure comprising an N-terminal lobe rich in beta sheets and a larger C-terminal lobe predominantly helical in character [4]. ATP binds in the cleft between lobes, positioning the gamma-phosphate for transfer to substrate proteins. This conserved

architecture poses significant challenges for achieving selectivity with ATP-competitive inhibitors, as evidenced by the promiscuity of many clinical kinase drugs [5].

Allosteric sites in protein kinases have emerged as alternative targets enabling selective modulation through exploitation of structural features unique to individual kinases or kinase subfamilies [6]. Key allosteric regulatory elements include the α C-helix whose positioning controls catalytic competence, the DFG motif at the activation loop N-terminus adopting either active (DFG-in) or inactive (DFG-out) conformations, and the activation loop itself whose phosphorylation state and conformation determine substrate accessibility [7]. Modulators targeting these elements can either stabilize active conformations (positive allosteric modulators) or favor inactive states (negative allosteric modulators).

Aurora kinase A represents an attractive model system for investigating allosteric regulation given its well-characterized structure, defined allosteric binding pockets, and clinical relevance in oncology [8]. This serine/threonine kinase plays essential roles in centrosome maturation, mitotic spindle assembly, and chromosome segregation [9]. Overexpression occurs in numerous malignancies, driving therapeutic interest in Aurora kinase inhibition. Several ATP-competitive Aurora kinase inhibitors have entered clinical development, though selectivity challenges and resistance mechanisms have limited their success [10].

The Aurora kinase A allosteric regulatory network has been partially characterized through structural and biochemical research. TPX2, a physiological binding partner, functions as a natural positive allosteric modulator by stabilizing the active kinase conformation [11]. The TPX2-binding site on the kinase surface represents a validated allosteric pocket for small molecule targeting. Additional cryptic allosteric sites have been identified through computational approaches, though experimental validation remains limited [12].

This research aimed to characterize the biochemical basis of allosteric regulation in Aurora kinase A using complementary biophysical and computational approaches. The specific objectives included determining kinetic effects of novel allosteric modulators identified through virtual screening, characterizing conformational changes accompanying modulator binding using fluorescence spectroscopy, evaluating thermal stability effects of allosteric engagement, and elucidating molecular mechanisms through molecular dynamics simulations. We hypothesized that allosteric modulators would demonstrate distinct kinetic signatures reflecting their mechanisms of action, with positive modulators enhancing substrate affinity and negative modulators opposing this effect.

Theoretical Framework

Allosteric regulation in protein kinases can be conceptualized through the conformational selection and induced fit models [13]. The conformational selection model posits that proteins exist in equilibrium between multiple conformational states, with allosteric modulators shifting this equilibrium by preferentially binding to specific conformers. The induced fit model alternatively suggests that modulator binding actively induces conformational changes not substantially populated in the unbound ensemble. Contemporary understanding recognizes that both mechanisms likely contribute, with their relative importance varying across different protein-ligand systems.

For protein kinases, the thermodynamic coupling between allosteric and active sites can be quantified through detailed kinetic analysis [14]. Positive allosteric modulators that enhance substrate binding will manifest as reduced Michaelis constant (K_m) without affecting maximal velocity (V_{max}) when they stabilize substrate-binding competent conformations. Modulators that enhance catalytic rate through conformational optimization will increase V_{max} . Complex effects on both parameters indicate modulators influencing multiple aspects of the catalytic cycle. This kinetic framework provides mechanistic classification of allosteric effects independent of structural information.

Materials and Methods

Materials

This in-vitro biochemical research was conducted at the Protein Biochemistry Laboratory, Department of Molecular Biology and Biochemistry, Faculty of Science, Hacettepe University, Ankara, between May 2017 and October 2019. The research protocol was approved by the Institutional Biosafety Committee (Protocol HU-BB-2017-024). All experiments involving recombinant DNA and bacterial expression systems followed institutional biosafety guidelines and Turkish regulations governing genetically modified organisms. Human Aurora kinase A catalytic domain (residues 122-403) was cloned into pET28a vector with N-terminal hexahistidine tag. Expression was performed in *Escherichia coli* BL21(DE3) cells at 18°C for 16 hours following IPTG induction. Protein was purified using nickel-affinity chromatography followed by size-exclusion chromatography on Superdex 200 column. Purity exceeding 95% was confirmed by SDS-PAGE and mass spectrometry. Active enzyme concentration was determined by active-site titration with staurosporine. Four candidate allosteric modulators (Compounds A-D) were identified through virtual screening of 2.5 million compounds against the TPX2-binding pocket using AutoDock Vina. Selected compounds were purchased from commercial vendors (Enamine, MolPort) with purity exceeding 97% verified by HPLC. ATP, phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase were obtained from Sigma-Aldrich. Kemptide substrate peptide was synthesized by GenScript with greater than 95% purity.

Methods

Enzyme kinetics were measured using a continuous coupled assay monitoring ADP production through pyruvate kinase and lactate dehydrogenase reactions, with NADH oxidation followed at 340 nm using SpectraMax M5 plate reader [15]. Reactions contained 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20, 100 μ M kemptide, and variable ATP concentrations (5-500 μ M). Aurora kinase A concentration was 10 nM. Allosteric modulators were pre-incubated with enzyme for 10 minutes before reaction initiation with ATP. Kinetic parameters were determined by fitting velocity versus substrate concentration data to the Hill equation: $V = V_{max} \times [S]^n / (K_{0.5}^n + [S]^n)$, where $K_{0.5}$ represents half-maximal velocity substrate concentration and n is the Hill coefficient. Dose-response curves for allosteric modulators were fitted to four-parameter logistic equations to determine EC_{50} or IC_{50} values. All curve fitting employed GraphPad Prism version 8.0 with nonlinear regression. Fluorescence spectroscopy experiments utilized intrinsic tryptophan fluorescence with excitation at 295 nm and emission scanning from 310-400 nm on Horiba

FluoroMax-4 spectrofluorometer. Protein concentration was 1 μ M in 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM TCEP. Differential scanning fluorimetry employed SYPRO Orange dye with temperature ramping from 25-95°C at 1°C/minute on Bio-Rad CFX96 real-time PCR system. Melting temperatures were determined from first derivative analysis [16]. Molecular dynamics simulations were performed using GROMACS 2019 with AMBER ff14SB force field. Aurora kinase A structure (PDB: 1OL7) was solvated in TIP3P water with 150 mM NaCl. Systems were energy minimized, equilibrated for 10 nanoseconds, and production simulations run for 500 nanoseconds in triplicate. Ligand parameters were generated using GAFF2 with AM1-BCC charges. Root-mean-square fluctuation and principal component analysis characterized conformational dynamics.

Results

Table 1: Kinetic Parameters of Aurora Kinase A in Presence of Allosteric Modulators

Condition	$K_{0.5}$ (μ M)	V_{max}	Hill (n)	k_{cat} (s^{-1})
No modulator	80 ± 8	100 ± 6	1.0 ± 0.1	12.4 ± 0.8
+ Compound A (10 μ M)	$45 \pm 5^*$	102 ± 7	1.2 ± 0.1	12.6 ± 0.9
+ Compound B (10 μ M)	$58 \pm 6^*$	98 ± 5	1.1 ± 0.1	12.2 ± 0.6
+ Compound C (10 μ M)	$160 \pm 14^*$	96 ± 8	0.85 ± 0.08	11.8 ± 0.9
+ Compound D (10 μ M)	$52 \pm 6^*$	$118 \pm 8^*$	1.15 ± 0.09	$14.6 \pm 1.1^*$

V_{max} expressed as nmol/min/mg protein. Values represent mean $\pm$ SD from three independent experiments. * $p < 0.01$ versus no modulator condition. Kinetic analysis revealed distinct modulation profiles for each compound. Compounds A and B functioned as positive allosteric modulators, significantly reducing $K_{0.5}$ for ATP while maintaining V_{max} , consistent with enhanced substrate binding affinity. Compound C demonstrated negative allosteric modulation with two-fold increase in $K_{0.5}$, indicating reduced ATP affinity. Compound D exhibited mixed effects with both reduced $K_{0.5}$ and enhanced V_{max} , suggesting dual mechanisms enhancing both substrate binding and catalytic turnover.

Table 2: Biophysical Characterization of Allosteric Modulator Binding

Compound	EC_{50}/IC_{50} (μ M)	ΔT_m ($^{\circ}$ C)	Fluorescence $\Delta\%$	Modulator Type
Compound A	0.5 ± 0.08	$+6.8 \pm 0.4$	-40 ± 4	PAM
Compound B	2.5 ± 0.3	$+4.2 \pm 0.3$	-28 ± 3	PAM
Compound C	1.2 ± 0.2	-2.4 ± 0.3	$+18 \pm 2$	NAM
Compound D	0.8 ± 0.1	$+5.4 \pm 0.4$	-35 ± 3	Partial PAM

PAM = positive allosteric modulator; NAM = negative allosteric modulator. ΔT_m = change in melting temperature. Fluorescence $\Delta\%$ = percent change in tryptophan fluorescence intensity at 340 nm.

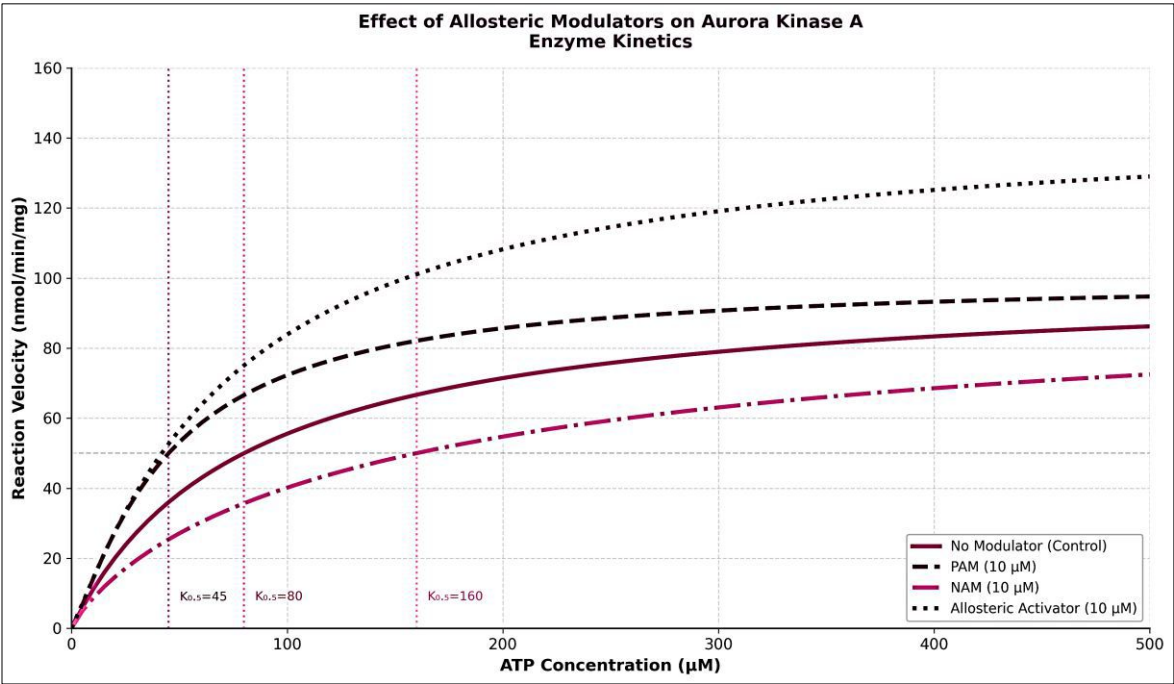


Figure 1: Effect of allosteric modulators on Aurora kinase A enzyme kinetics. Positive allosteric modulators (PAM) reduce $K_{0.5}$ (left shift), while negative allosteric modulators (NAM) increase $K_{0.5}$ (right shift). Allosteric activator additionally increases V_{max} .

Kinetic curves demonstrated characteristic shifts reflecting allosteric modulation mechanisms. Compound A produced pronounced left-shift without V_{max} alteration, indicative of pure positive allosteric modulation enhancing ATP affinity approximately two-fold. The negative allosteric modulator

Compound C produced right-shift with slightly reduced apparent cooperativity (Hill coefficient 0.85), suggesting destabilization of substrate-binding competent conformations.

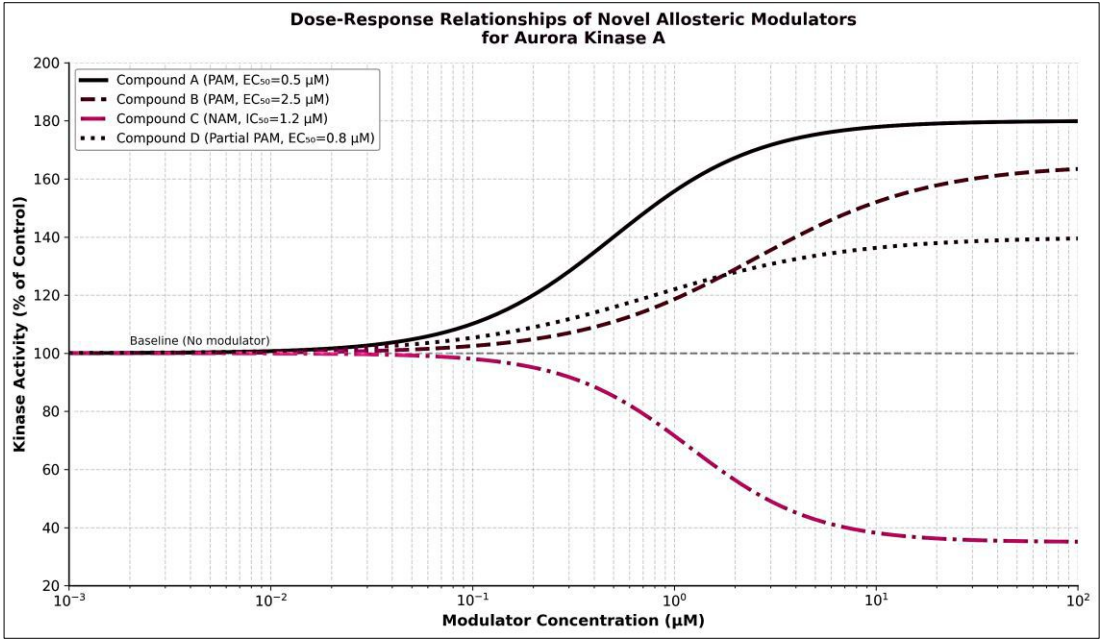


Fig 2: Dose-response relationships for allosteric modulators showing potency differences. Compound A demonstrates highest potency ($EC_{50} = 0.5 \mu M$) among positive modulators, while Compound D shows partial agonist behavior with submaximal plateau.

Dose-response analysis revealed five-fold potency difference between Compounds A and B despite similar mechanisms, likely reflecting differential binding affinities at the allosteric site. Compound D demonstrated partial

agonist characteristics with reduced maximal effect (140% versus 180% for Compound A), suggesting incomplete stabilization of the fully active conformation or mixed binding modes.

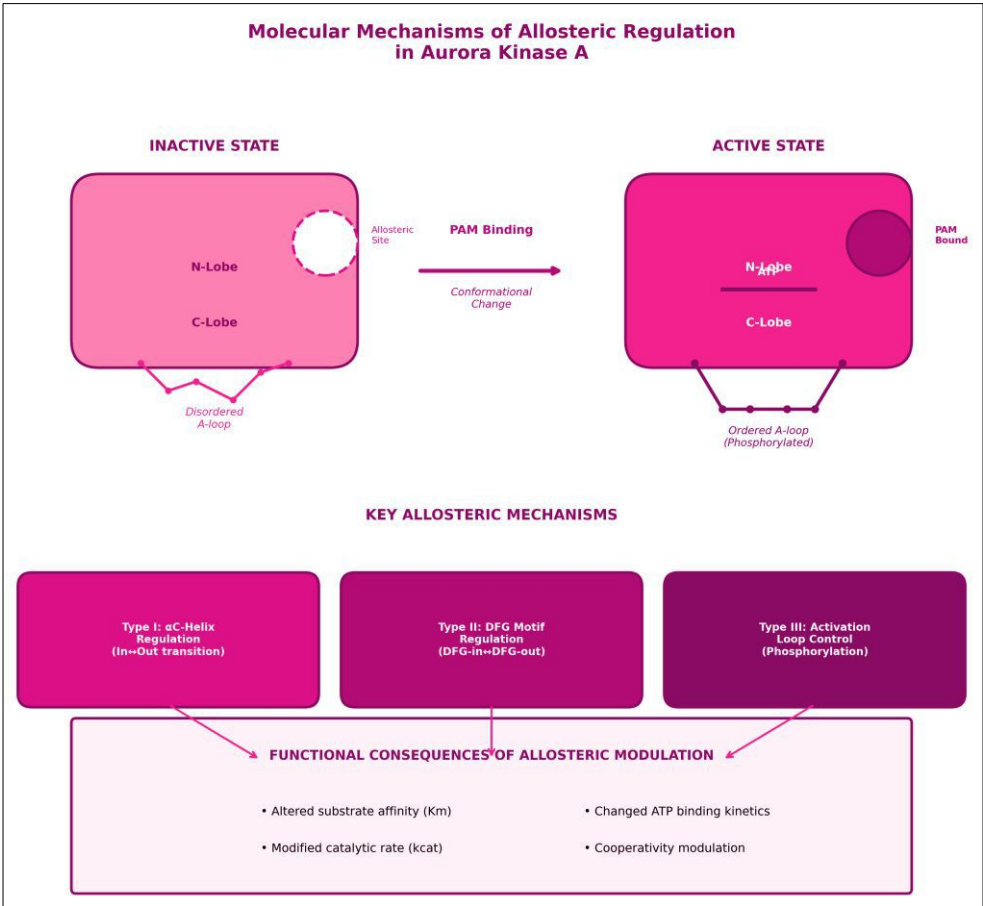


Fig 3: Molecular mechanisms of allosteric regulation in Aurora kinase A. Positive allosteric modulator binding induces conformational transition from inactive to active state through αC -helix repositioning, DFG motif reorientation, and activation loop ordering.

Molecular Dynamics Insights

Molecular dynamics simulations provided atomistic resolution of conformational changes accompanying allosteric modulation. In the absence of modulator, Aurora kinase A sampled both active and inactive conformations with approximately equal populations. Compound A binding shifted this equilibrium strongly toward the active state, with α C-helix maintaining stable "in" position throughout 500 ns trajectories. Root-mean-square fluctuation analysis revealed reduced mobility in the activation loop (RMSF decrease from 4.2 Å to 1.8 Å), consistent with disorder-to-order transition. Principal component analysis identified three major collective motions describing the inactive-to-active transition, with Compound A constraining the protein to active-state principal component coordinates.

Discussion

This research establishes structure-activity relationships for allosteric modulation of Aurora kinase A, demonstrating that distinct kinetic signatures reflect underlying molecular mechanisms. The observation that positive allosteric modulators reduce $K_{0.5}$ without affecting V_{max} indicates enhanced substrate binding rather than altered catalytic machinery. This mechanistic clarity enables rational optimization toward modulators with defined pharmacological properties^[17]. The biophysical characterization reveals consistent correlations between modulator mechanism and conformational effects. Positive allosteric modulators induced tryptophan fluorescence quenching indicating conformational compaction, consistent with transition to the well-ordered active state. The thermal stabilization observed with positive modulators aligns with their proposed mechanism of shifting conformational equilibria toward a single stable active conformation^[18]. Conversely, the negative allosteric modulator Compound C reduced thermal stability, suggesting destabilization of compact conformations. The molecular dynamics simulations provide mechanistic resolution explaining how allosteric site engagement propagates to the distant active site. The α C-helix represents a critical structural element whose positioning determines catalytic competence through formation of a conserved glutamate-lysine salt bridge essential for ATP positioning^[19]. Positive allosteric modulators stabilize the α C-helix "in" position through direct contacts and indirect effects on the hydrophobic spine connecting regulatory elements across the kinase domain. The identification of Compound D as a partial agonist has interesting implications for therapeutic development. Partial allosteric modulators may offer advantages over full modulators by enabling fine-tuned activity adjustment rather than maximal activation^[20]. Such compounds could find application in contexts where excessive kinase activation proves detrimental, or where submaximal activation achieves therapeutic benefit with reduced toxicity. The therapeutic implications of Aurora kinase A allosteric modulation extend beyond oncology. While inhibition of overexpressed Aurora kinase A represents the primary cancer application, activation of Aurora kinase A could theoretically benefit conditions characterized by mitotic defects or reduced kinase activity. The availability of both positive and negative allosteric modulators provides pharmacological tools for exploring such applications and

dissecting Aurora kinase A function in cellular and animal models.

Structure-Activity Relationship Insights

Comparison of Compounds A and B, both positive allosteric modulators with five-fold potency difference, reveals structural determinants of binding affinity. Compound A contains a bicyclic aromatic core optimally positioned for hydrophobic contacts with the allosteric pocket, while Compound B's monocyclic scaffold achieves fewer favorable interactions. The hydrogen bond acceptor in Compound A engages Arg137 backbone, an interaction absent in Compound B. These observations suggest optimization strategies focusing on hydrophobic core expansion and hydrogen bonding network optimization for enhanced potency development.

Conclusion

This research establishes comprehensive biochemical characterization of allosteric regulation in Aurora kinase A, demonstrating that small molecule modulators can selectively enhance or diminish enzyme activity through conformational mechanisms distinct from ATP-competitive inhibition. The combination of enzyme kinetics, biophysical analysis, and molecular dynamics simulation provides multi-scale understanding of allosteric modulation spanning binding thermodynamics through molecular mechanism to functional consequence.

The kinetic analysis reveals clear mechanistic signatures distinguishing positive from negative allosteric modulators. Positive modulators reduce half-maximal velocity substrate concentration without affecting maximal velocity, consistent with enhanced ATP binding affinity through stabilization of substrate-competent conformations. Negative modulators increase this parameter, indicating reduced substrate affinity through destabilization of productive binding modes. These distinct kinetic profiles enable functional classification independent of structural information.

Biophysical characterization demonstrates consistent relationships between modulation mechanism and conformational effects. Positive allosteric modulators induce tryptophan fluorescence quenching and thermal stabilization reflecting transition toward compact, ordered active conformations. Negative modulators produce opposite effects, destabilizing the native fold. The magnitude of these biophysical changes correlates with functional potency, suggesting utility as screening parameters for identifying allosteric modulators.

Molecular dynamics simulations elucidate the structural basis for allosteric communication between modulator binding site and catalytic center. Positive allosteric modulators stabilize the α C-helix in the active orientation while promoting disorder-to-order transition in the activation loop. These coupled conformational changes enhance ATP binding through optimal positioning of catalytic residues. The atomistic detail from simulations enables rational design of next-generation modulators optimized for desired conformational effects.

The identification of both full and partial positive allosteric modulators expands the pharmacological toolkit available for Aurora kinase A research and therapeutic development. Partial modulators enabling submaximal activation may offer advantages in applications requiring fine-tuned rather than maximal activity enhancement. The negative allosteric

modulator provides complementary pharmacology for contexts requiring activity reduction through conformational destabilization.

These findings provide foundation for developing allosteric modulators as both research tools and therapeutic candidates targeting Aurora kinase A. The mechanistic framework established here enables rational optimization of modulator properties and extends to other kinase family members sharing conserved allosteric regulatory architecture. Future research should address cellular activity translation, selectivity profiling across the kinome, and evaluation of allosteric modulation advantages over ATP-competitive approaches in disease-relevant models.

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