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Biochemical characterization of novel inhibitors targeting Abl and Src tyrosine kinases

Caratterizzazione biochimica di nuovi inibitori diretti contro le tirosin-chinasi Abl e Src

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Abstract

Tyrosine kinases are central to cellular signaling pathways, and their dysregulation is closely associated with the development of several malignancies. In this study, the biochemical properties, inhibitory potency, and selectivity of two newly synthesized small molecule candidates, GC21 and GC22, were evaluated against the Abl and Src tyrosine kinases. A series of enzyme-based inhibition assays, dose–response analyses, and kinetic measurements were performed to characterize the interaction of these compounds with their molecular targets. GC21 inhibited Src potently, with an IC_{50} of approximately 65.71 nM, and produced consistently high inhibition across all tested concentrations. In contrast, GC21 inhibited Abl weaklier, with an IC_{50} of approximately 218.12 nM, indicating selectivity for Src over Abl. GC22 displayed a different profile, showing moderate inhibitory activity toward both kinases. IC_{50} values of roughly 246 nM for Src and 311 nM for Abl indicated its non-selective nature and considerably lower potency compared with GC21. These results reveal that structural variations within this compound series can lead to substantial differences in kinase affinity and selectivity. Overall, the findings identify GC21 as a promising lead candidate for the development of selective Src inhibitors, while GC22 serves as a reference compound for understanding structure–activity relationships within this molecular class. These biochemical results provide a basis for future optimization and the rational design of next-generation kinase inhibitors.

Keywords: Tyrosine kinases, Abl, Src, GC21, GC22, Kinase inhibition, IC_{50}, Selectivity profiling, Small-molecule inhibitors, Biochemical characterization

Riassunto

Le tirosin chinasi svolgono un ruolo fondamentale nei processi di segnalazione cellulare e la loro deregolazione è strettamente associata all'insorgenza e alla progressione di diverse neoplasie. In questo studio sono state investigate le proprietà biochimiche, la potenza inibitoria e la selettività di due nuove piccole molecole sintetizzate, GC21 e GC22, nei confronti delle tirosin chinasi Abl e Src. A tal fine, sono stati eseguiti saggi enzimatici di inibizione, analisi dose-risposta e studi cinetici per caratterizzare l'interazione di questi composti con i rispettivi bersagli molecolari.

GC21 ha mostrato una marcata attività inibitoria nei confronti di Src, con un valore di IC_{50} pari a circa 65,71 nM, indicativo di un'elevata potenza nel range nanomolare e di un'efficiente soppressione dell'attività chinasica. Le elevate percentuali di inibizione osservate a tutte le concentrazioni testate hanno ulteriormente confermato la robustezza della sua attività. Al contrario, GC21 ha evidenziato un'inibizione significativamente inferiore nei confronti di Abl, con un valore di IC_{50} pari a circa 218,12 nM, indicando una spiccata selettività per Src rispetto ad Abl.

GC22 ha mostrato un profilo differente, caratterizzato da un'attività inibitoria moderata nei confronti di entrambe le chinasi. I valori di IC_{50}, pari a circa 246,8 nM per Src e 311 nM per Abl, suggeriscono una limitata selettività e una potenza complessivamente inferiore rispetto a GC21.

Questi risultati dimostrano come variazioni strutturali all'interno di questa serie di composti possano determinare differenze sostanziali nell'affinità e nella selettività verso le chinasi bersaglio. Nel complesso, i dati ottenuti identificano GC21 come un promettente lead compound per lo sviluppo di inibitori selettivi di Src, mentre GC22 rappresenta un utile termine di confronto per comprendere le relazioni struttura-attività all'interno di questa classe molecolare. Le evidenze biochimiche emerse da questo studio forniscono una solida base per future attività di ottimizzazione e per la progettazione razionale di inibitori delle chinasi di nuova generazione.

Parole chiave: Tirosin-chinasi, Abl, Src, GC21, GC22, Inibizione chinasica, IC_{50}, Profilo di selettività, Piccole molecole inibitrici, Caratterizzazione biochimica.

1. Introduction

1.1 Protein kinases: structure, function and biological relevance

1.1.1 Protein phosphorylation and kinase activity

Protein kinases constitute one of the largest and most extensively studied families of enzymes in eukaryotic organisms. These enzymes catalyze the transfer of the γ-phosphate group from high-energy phosphate donors, primarily adenosine triphosphate (ATP), to specific substrates through a process known as phosphorylation (Theivendren et al., 2021). Depending on the nature of their substrates, kinases can be classified into protein kinases, lipid kinases, and carbohydrate kinases. Among these groups, protein kinases are the most abundant, and their extensive involvement in cellular regulation has made them a major focus of biological research (Karunanidhi et al., 2025).

Protein phosphorylation is one of the most important post-translational modifications in living organisms. By covalently attaching a phosphate group to target proteins, kinases induce changes in protein conformation, enzymatic activity, intracellular localization, stability, and interactions with other macromolecules (Seok, 2021). Phosphorylation can activate or inhibit signaling pathways depending on the extracellular and intracellular stimuli a cell receives (Zhang et al., 2023).

Figure 1 illustrates the general mechanism through which kinases regulate cellular processes by mediating reversible phosphorylation events. The attachment of a phosphate group introduces a bulky, negatively charged moiety that changes how the target protein folds and which partners it can bind, thereby switching its function on or off. This regulation is effective because it is reversible: the modification persists only while signaling demands it and is removed once phosphatase activity prevails. A protein can therefore cycle repeatedly between its two states, with the time spent in each set by the relative activity of the kinases that add the phosphate and the phosphatases that remove it. This balance, not a single irreversible event, lets phosphorylation encode graded and rapidly adjustable cellular responses.

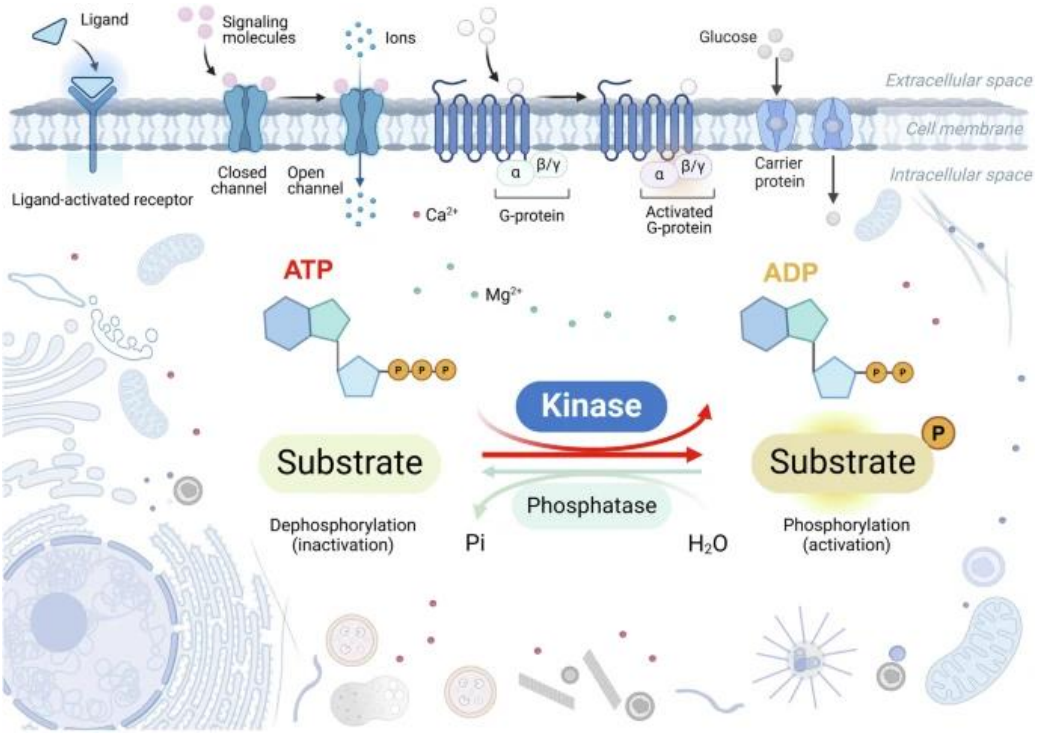


Fig. 1. Schematic diagram of the general working mechanism of kinases (Wu et al., 2025).

In eukaryotic cells, phosphorylation most commonly occurs on serine, threonine, and tyrosine residues (Reményi, 2022). The general reaction catalyzed by protein kinases is represented in Figure 2.

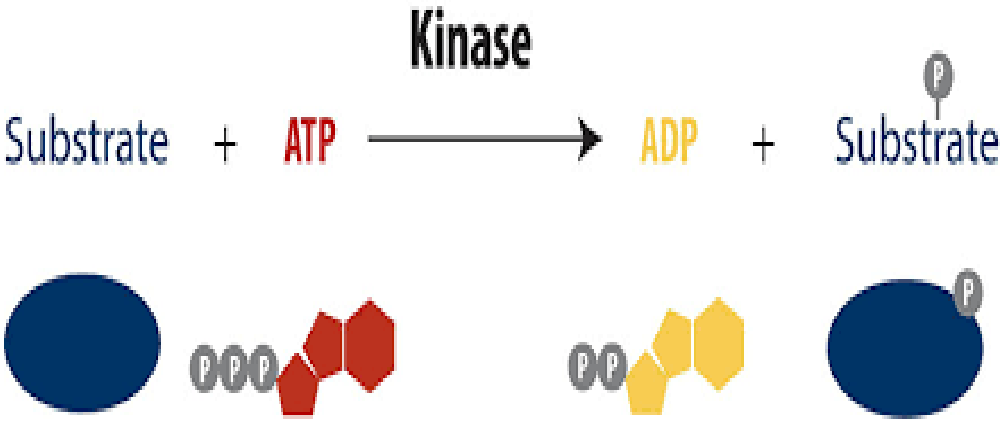


Fig. 2. Reaction catalyzed by protein kinases (Panneerselvam et al., 2021).

At the biochemical level, phosphorylation is energetically favorable due to the high-energy phosphoanhydride bonds present in ATP (Müller et al., 2019). However, efficient phosphate transfer

requires precise molecular positioning of both ATP and substrate within the kinase active site. Protein kinases facilitate this process by stabilizing the transition state through conserved amino acid residues and, in some cases, coordinated metal ions such as Mg^{2+}. These interactions reduce the activation energy of the reaction and ensure efficient phosphotransfer (Kung & Jura, 2019).

Protein kinases can be either catalytically active enzymes or kinase-like proteins that have partially lost catalytic activity during evolution. Despite differences in enzymatic function, both classes regulate cellular signaling networks and contribute significantly to physiological and pathological processes. Because phosphorylation controls numerous cellular functions, alterations in kinase activity can profoundly affect cell behavior and contribute to disease development (Anderson et al., 2023).

Approximately 30% of human proteins are estimated to undergo phosphorylation at some stage of their life cycle (Foroughi et al., 2019). Although serine phosphorylation accounts for the majority of phosphorylation events, tyrosine phosphorylation exerts particularly important regulatory effects because of its involvement in growth factor signaling, immune responses, and oncogenic transformation (Steinberg, 2018).

In addition to regulating individual protein function, phosphorylation coordinates complex signaling networks that govern cellular homeostasis. A single phosphorylation event can trigger the assembly of multiprotein complexes by creating docking sites for phospho-recognition domains such as SH2, PTB, and 14-3-3 domains (Getz et al., 2019). Through these interactions, phosphorylation enables the rapid transmission and amplification of intracellular signals, allowing cells to integrate information from multiple extracellular and intracellular stimuli. In this way, a limited set of signaling molecules can produce many distinct biological responses (Ullo & Case, 2023).

Phosphoproteomic technologies have shown how extensive protein phosphorylation is within eukaryotic cells. Large-scale mass spectrometry studies have identified hundreds of thousands of phosphorylation sites distributed across proteins involved in virtually every cellular process. These analyses have demonstrated that phosphorylation is not restricted to signaling proteins but also affects transcription factors, metabolic enzymes, structural proteins, transporters, and components of the protein degradation machinery (Stopfer et al., 2021).

The reversibility of phosphorylation distinguishes it from many other post-translational modifications and contributes significantly to its regulatory potential. Protein phosphatases continuously oppose kinase activity by removing phosphate groups from target proteins. The balance between kinase-mediated phosphorylation and phosphatase-mediated dephosphorylation determines the phosphorylation status of signaling proteins and ultimately influences cellular responses. Disruption of this balance can lead to aberrant signaling and has been associated with numerous pathological

conditions, including cancer, diabetes, neurodegenerative disorders, and inflammatory diseases (Hitrec et al., 2021).

Phosphorylation also contributes to the spatial and temporal organization of signaling pathways. By regulating protein localization, phosphorylation can control the recruitment of signaling molecules to specific cellular compartments, including the plasma membrane, endosomes, mitochondria, and nucleus (Martinez-Val et al., 2021). Furthermore, transient phosphorylation events allow signaling pathways to be activated and terminated rapidly, ensuring precise control of cellular responses. This temporal regulation is particularly important in processes requiring rapid adaptation, such as immune activation, stress responses, and cell-cycle progression (Qi & Zhang, 2020).

1.1.2 Classification of protein kinases

The human kinome comprises more than 500 protein kinase genes and represents approximately 2% of the entire human genome (Wilson et al., 2018). Advances in genomic sequencing and structural biology have enabled the identification of 478 conventional protein kinases containing the canonical eukaryotic protein kinase domain, together with approximately 40 atypical kinases that exhibit kinase activity despite lacking the classical catalytic architecture (Patow et al., 2024).

Protein kinases are commonly classified according to the amino acid residues they phosphorylate. This classification reflects substrate specificity and functional specialization within cellular signaling pathways. The three major categories include:

- Serine/threonine kinases, which phosphorylate serine and threonine residues;
- Tyrosine kinases, which specifically phosphorylate tyrosine residues;
- Dual-specificity kinases, which are capable of phosphorylating both serine/threonine and tyrosine residues (Ahiri & Aboulmouhajir, 2026).

From an evolutionary and structural perspective, protein kinases are further organized into several major groups, including AGC kinases, CAMK kinases, CMGC kinases, CK1 kinases, STE kinases, tyrosine kinases (TKs), tyrosine kinase-like kinases (TKLs), and atypical protein kinases (Ahsan et al., 2023). Each group contains multiple kinase families that participate in distinct biological processes and signaling cascades. The diversity and evolutionary relationships of human kinases are illustrated in Figure 3.

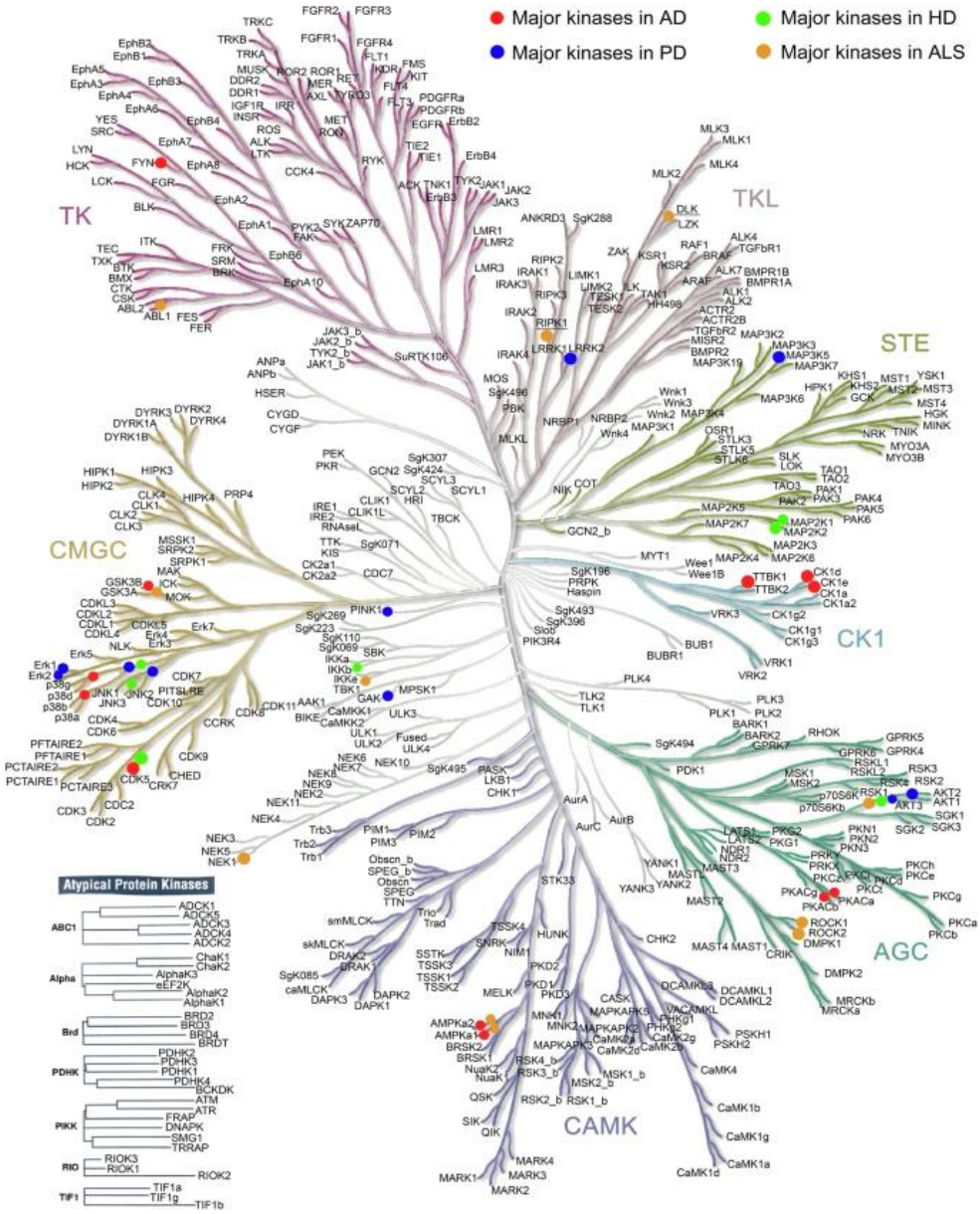


Fig. 3. Kinase classification map (Modi & Dunbrack Jr, 2019).

Despite considerable sequence variability among kinase families, most eukaryotic protein kinases share a highly conserved catalytic domain. More than 280 human kinase structures have been experimentally determined, revealing common structural features involved in ATP binding, substrate recognition, and catalytic activity (Tinker et al., 2018). The N-terminal region of the catalytic domain contains a glycine-rich loop that participates in ATP binding, whereas a conserved aspartate residue located within the catalytic loop is essential for phosphotransfer activity. This catalytic core is conserved across eukaryotes, consistent with the central regulatory role of phosphorylation (Attwood et al., 2021).

The conserved catalytic domain adopts a characteristic bilobal fold in Figure 4. The smaller N-terminal lobe is composed mainly of β-sheets and contains the glycine-rich loop (the G-loop, with its conserved Gly-x-Gly-x-x-Gly motif) that positions the phosphates of ATP, together with the αC helix, whose orientation is central to regulation. The larger, predominantly α-helical C-terminal lobe harbors the catalytic and activation loops and provides the docking surface for protein substrates. ATP binds in the cleft between the two lobes, where the adenine ring is anchored by hydrogen bonds to the hinge region connecting them. This same cleft is the site at which the great majority of clinical kinase inhibitors bind, which is why its architecture is so relevant to drug discovery (Arter et al., 2022).

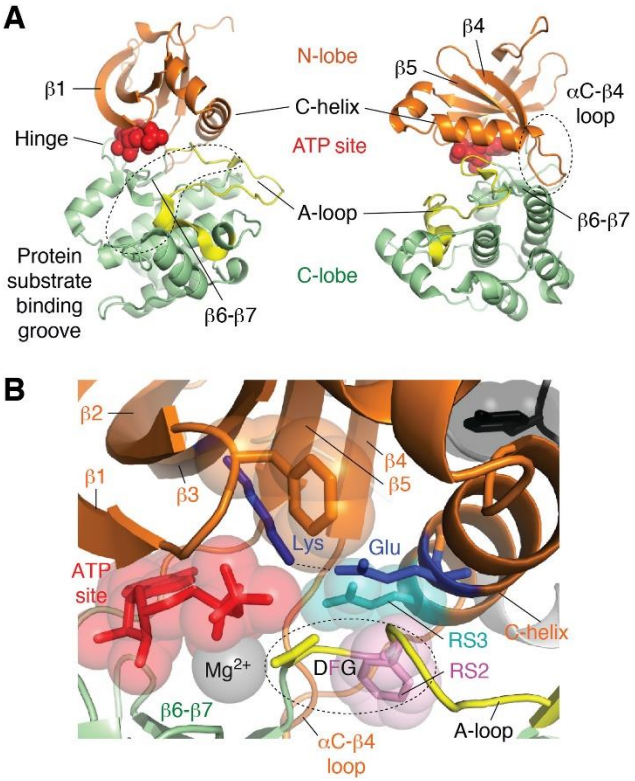


Fig. 4. Architecture of the protein kinase catalytic domain, showing the N- and C-terminal lobes, the hinge region, the ATP-binding cleft, the αC-helix, and the activation loop (Arter et al., 2022).

Catalysis depends on a small set of strictly conserved residues. A lysine in the N-lobe and a glutamate in the αC helix form a salt bridge that correctly positions ATP; the aspartate of the His-Arg-Asp (HRD) motif in the catalytic loop acts as the catalytic base, while the aspartate of the Asp-Phe-Gly (DFG) motif at the start of the activation loop coordinates the magnesium ions required for phosphotransfer. The integrity of the active site is further organized by two stacked sets of hydrophobic residues, the regulatory and catalytic spines, which assemble only in the active enzyme

and align the structural elements needed for catalysis. Because these residues and motifs are conserved across essentially the entire kinome, they constrain how selectively the active site can be targeted by inhibitors (Endicott et al., 2012).

A defining feature of protein kinases is their ability to switch between active and inactive conformations, which provides a structural basis for their regulation in Figure 5. In the active state, the αC helix rotates inward ("αC-in") to complete the lysine–glutamate salt bridge, and the DFG motif points its aspartate into the active site ("DFG-in"), aligning the catalytic machinery. Inactive states are structurally more varied: the αC helix may swing outward ("αC-out"), breaking the salt bridge, or the DFG motif may flip ("DFG-out"), withdrawing the aspartate and opening an additional hydrophobic pocket adjacent to the ATP site. These inactive conformations are functionally important for drug design, because inhibitors that recognize them — rather than the highly conserved active state — can achieve greater selectivity. This conformational plasticity recurs throughout the present work, since both Src and Abl are regulated through transitions between such states (Vijayan et al., 2015).

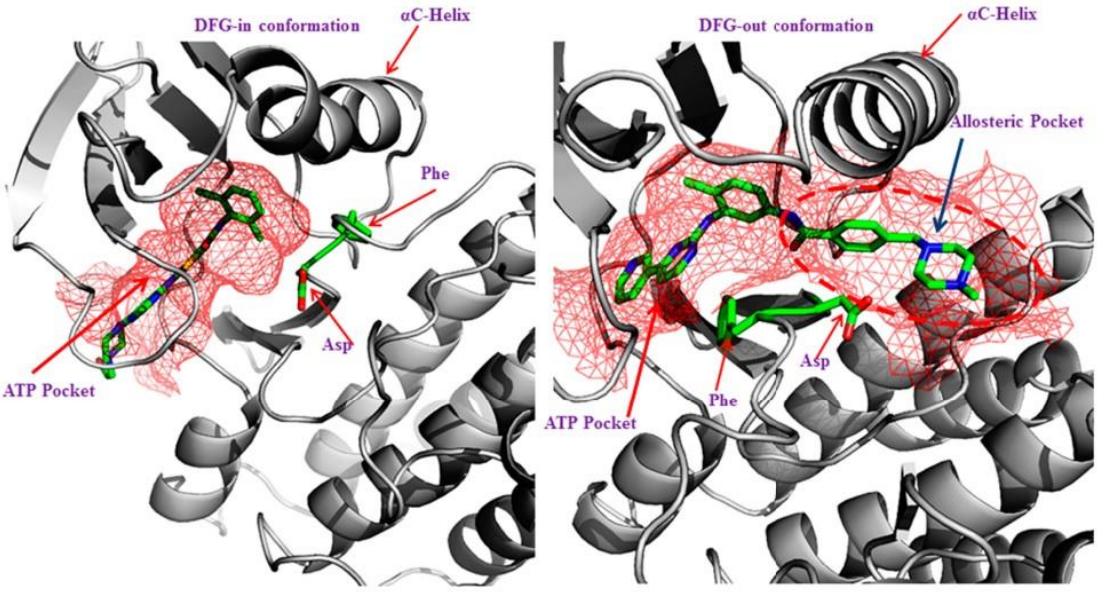


Fig. 5. Active (DFG-in) and inactive (DFG-out) conformations of the ABL kinase domain, bound to dasatinib and imatinib respectively (Vijayan et al., 2015).

Beyond their classification based on substrate specificity, protein kinases can also be categorized according to their evolutionary origin and structural organization. Comparative genomic analyses have demonstrated that kinase families arose through multiple gene duplication and diversification

events during evolution, resulting in the extensive kinase repertoire observed in modern eukaryotes (Bradley & Beltrao, 2019).

The AGC kinase group includes protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC), which are primarily regulated by second messengers such as cyclic nucleotides and diacylglycerol (Jiang et al., 2022). The CAMK family comprises calcium/calmodulin-dependent kinases that function as key mediators of calcium signaling and are essential for synaptic plasticity, muscle contraction, and immune responses (Tokumitsu & Sakagami, 2022). Members of the CMGC group, including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3 (GSK3), and CDC-like kinases, are particularly important regulators of cell-cycle progression, transcription, and cellular stress responses (Chowdhury et al., 2023; Whitaker & Cook, 2021).

Dysregulation of CMGC kinases has been implicated in cancer, neurodegenerative disorders, and developmental abnormalities. Similarly, STE kinases function upstream of MAPK signaling cascades and contribute to the transmission of extracellular signals toward intracellular transcriptional programs (Amusan & Guo, 2026; Kim & Lee, 2023).

The human kinome represents one of the best-characterized enzyme superfamilies and has become an important framework for understanding cellular signaling. Mapping of kinase interactions has revealed highly interconnected signaling networks in which individual kinases may participate in multiple pathways simultaneously (Ross et al., 2023).

1.1.3 Protein kinases in signal transduction and disease

Protein kinases occupy a central position in intracellular signaling networks and regulate virtually every aspect of cellular physiology, including proliferation, differentiation, metabolism, migration, survival, apoptosis, and cellular stress responses (Jia et al., 2020). Through the reversible phosphorylation of target proteins, they act as molecular switches that allow cells to perceive, process, and respond to extracellular and intracellular stimuli.

Signal transduction is typically initiated when extracellular signaling molecules, such as growth factors, cytokines, hormones, or extracellular matrix components, interact with cell-surface receptors. Receptor activation triggers intracellular cascades involving protein kinases and second messengers, leading to the sequential propagation of signals toward specific cellular effectors and transcription factors. Through these cascades, extracellular cues are translated into coordinated biological responses, including changes in gene expression, metabolism, and cell behavior (Kamepalli et al., 2025).

One of the defining properties of kinase-mediated signaling is signal amplification. Activation of a relatively small number of receptors can generate robust cellular responses through multiple rounds of phosphorylation, enabling cells to react efficiently even to weak stimuli. Equally important is the reversibility of phosphorylation. Protein phosphatases continuously counterbalance kinase activity by removing phosphate groups from target proteins, thereby maintaining signaling homeostasis and preventing inappropriate pathway activation. The dynamic equilibrium between kinases and phosphatases determines the intensity and duration of signaling events and is essential for normal cellular function (Hitrec et al., 2021).

To ensure accurate regulation, kinase activity is controlled through multiple mechanisms, including phosphorylation, protein–protein interactions, allosteric regulation, conformational changes, and subcellular localization (Lim & Mayer, 2024). Disruption of these regulatory processes can result in persistent signaling and pathological cellular behavior.

Dysregulation of kinase activity may arise through activating mutations, gene amplification, chromosomal translocations, aberrant protein expression, or loss of negative regulatory mechanisms. These alterations frequently lead to constitutive activation of signaling pathways, promoting uncontrolled proliferation, resistance to apoptosis, genomic instability, metabolic reprogramming, and malignant transformation (Okabe & Kaneda, 2021; Yang et al., 2022). Beyond cancer, abnormal kinase signaling has also been implicated in inflammatory diseases, autoimmune disorders, cardiovascular pathologies, metabolic syndromes, and neurodegenerative conditions (Canovas & Nebreda, 2021; Yan et al., 2024).

Several major signaling pathways exemplify the biological importance of protein kinases. Among the best characterized is the RAS–RAF–MEK–ERK (MAPK) pathway, which transmits signals from activated receptor tyrosine kinases to the nucleus and regulates cell proliferation, differentiation, and survival. Aberrant activation of this pathway, often resulting from mutations in RAS or RAF proteins, is a common feature of numerous human malignancies and has led to the development of clinically successful targeted therapies (Bahar et al., 2023).

Another key signaling network is the phosphoinositide 3-kinase (PI3K)–AKT–mTOR pathway, which integrates signals derived from growth factors, nutrients, and cellular energy status. This pathway regulates protein synthesis, metabolism, cell growth, and resistance to apoptosis. Hyperactivation of PI3K–AKT–mTOR signaling, frequently caused by activating mutations in PI3K or loss of the tumor suppressor PTEN, contributes to tumor progression, metastatic potential, and therapeutic resistance in a wide range of cancers (Miricescu et al., 2020).

Protein kinases are also essential regulators of immune and inflammatory responses. The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway mediates signaling downstream of numerous cytokine receptors and provides a direct link between extracellular immune stimuli and transcriptional responses. Dysregulated JAK–STAT signaling has been associated with autoimmune diseases, chronic inflammatory disorders, and hematological malignancies, highlighting the broad pathological relevance of kinase-dependent signaling beyond oncology (Hu et al., 2021; Rizwi et al., 2023). Importantly, signaling pathways do not function as isolated linear cascades. Instead, they operate within highly interconnected regulatory networks characterized by extensive cross-talk and feedback mechanisms. Positive and negative feedback loops modulate signal intensity and duration, while interactions between distinct pathways allow cells to integrate multiple environmental cues and coordinate complex biological responses. Although this interconnectedness increases signaling flexibility and robustness, it can also promote disease progression and therapeutic resistance by enabling compensatory activation of alternative pathways when a single signaling node is inhibited (Lovly & Shaw, 2014).

Because abnormal kinase activity underlies many human diseases, protein kinases have become some of the most important therapeutic targets in modern medicine. The successful development of kinase inhibitors has demonstrated the clinical value of targeting dysregulated signaling pathways, particularly in oncology, where kinase-directed therapies have transformed the treatment of several malignancies. Continued efforts to characterize kinase signaling networks, identify novel druggable targets, and understand the molecular basis of therapeutic response and resistance remain central objectives in both academic and pharmaceutical research (Li et al., 2024).

The importance of kinase signaling is particularly evident in tyrosine kinases, whose deregulation frequently drives oncogenic transformation and disease progression. Consequently, tyrosine kinases have become a major focus of targeted drug development and represent the primary subject of the following sections.

1.2 Tyrosine kinases as therapeutic targets in cancer

1.2.1 Receptor and non-receptor tyrosine kinases

Tyrosine kinases constitute a specialized subgroup of protein kinases that catalyze the phosphorylation of tyrosine residues on target proteins. Tyrosine phosphorylation accounts for only a small fraction of total cellular phosphorylation events, yet exerts disproportionate regulatory influence (Salokas et al., 2022).

Based on their structural organization and cellular localization, tyrosine kinases are generally divided

into two major classes: receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs) (Eshaq et al., 2024).

Receptor tyrosine kinases (RTKs) are transmembrane proteins comprising an extracellular ligand-binding domain, a single transmembrane helix, and an intracellular kinase domain. Binding of specific extracellular ligands, such as growth factors, cytokines, or hormones, induces receptor dimerization and activation, initiating intracellular signaling cascades that regulate diverse cellular processes (Antenucci, 2019). In the absence of ligand, most RTKs exist at the cell surface as inactive monomers, with the intracellular kinase domain maintained in a low-activity conformation. Ligand binding promotes the formation or stabilization of receptor dimers, bringing the two intracellular kinase domains into proximity and enabling trans-autophosphorylation. Phosphorylation of residues within the activation loop stabilizes the kinase domain in its active conformation, while phosphorylation of additional tyrosines outside the catalytic core generates docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (Ebrahimi et al., 2023). Several RTK families have been identified in humans, including the epidermal growth factor receptor (EGFR) family, platelet-derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs), fibroblast growth factor receptors (FGFRs), and hepatocyte growth factor receptor (MET). Although these families differ in extracellular domain architecture and ligand specificity, they share a common mechanism of ligand-induced dimerization and activation, with structural variation largely determining differences in ligand selectivity and downstream signaling output (Nandi et al., 2022). This diversity lets cells respond to many different extracellular signals. Growth factors, cytokines, hormones, and extracellular matrix components can all activate specific receptor families, enabling precise regulation of proliferation, differentiation, metabolism, survival, and tissue homeostasis. In multicellular organisms, RTKs are particularly important during embryonic development, where they regulate cell fate determination, organogenesis, and morphogenetic processes (Prenzel et al., 2000).

In contrast, non-receptor tyrosine kinases (NRTKs) lack transmembrane domains and function entirely within the intracellular environment. Rather than being activated directly by extracellular ligands, these enzymes are recruited and activated through interactions with membrane receptors, adaptor proteins, or intracellular signaling complexes.

Major NRTK families include the Src family kinases (SFKs), Abl kinases, Janus kinases (JAKs), focal adhesion kinase (FAK), and Tec family kinases (Tsygankov, 2003).

NRTKs integrate signals arising from multiple upstream pathways, functioning as signaling hubs that connect membrane receptors to downstream effectors. Individual NRTKs can therefore be engaged by several distinct receptor systems; SFKs, for instance, are recruited downstream of RTKs, integrins,

and immune receptors alike, while FAK is activated primarily through integrin-mediated adhesion but can also respond to growth factor signaling. Such convergence allows structurally unrelated upstream signals to be channeled into shared downstream outputs (Tsytlonok et al., 2019; Wang et al., 2026).

SFKs and FAK act together at focal adhesions to regulate actin dynamics underlying cell migration, JAKs phosphorylate STAT proteins to drive transcriptional programs involved in immune cell differentiation, and Tec family kinases couple antigen receptor engagement to downstream transcriptional activation in lymphocytes, reflecting the functional specialization of individual NRTK families despite their shared catalytic mechanism (Gocek et al., 2014).

Because they are not activated directly by extracellular ligands, NRTKs act as intracellular intermediates that coordinate inputs from several receptor systems (de Pins et al., 2021).

An important feature of many non-receptor tyrosine kinases is their ability to shuttle between different cellular compartments. Depending on activation status and interacting partners, these proteins may localize to the plasma membrane, cytoplasm, cytoskeleton, endosomal structures, or nucleus. Such dynamic localization enables them to regulate distinct cellular functions and respond rapidly to environmental changes (Jones et al., 2014).

The physiological functions of both RTKs and NRTKs require precise regulation of kinase activity. Under normal conditions, activation is transient and tightly controlled through phosphorylation, dephosphorylation, protein-protein interactions, intracellular localization, and receptor internalization. Disruption of these regulatory mechanisms can result in persistent kinase activation and pathological signaling (Metibemu et al., 2019) (Fox et al., 2019).

The interplay between receptor and non-receptor tyrosine kinases contributes significantly to signaling complexity. In many cases, activation of RTKs results in recruitment and activation of NRTKs such as Src or Abl, which subsequently phosphorylate additional substrates and expand the signaling response. This hierarchy produces signaling networks capable of highly coordinated biological outcomes while retaining regulatory flexibility.

1.2.2 Oncogenic activation of tyrosine kinases

The oncogenic potential of tyrosine kinases was first recognized through studies of retroviral transforming genes, several of which were later found to encode constitutively active versions of cellular tyrosine kinases, providing some of the earliest direct evidence linking kinase activity to malignant transformation (Bache et al., 2004).

Several mechanisms can lead to constitutive activation of tyrosine kinases. One of the most common

is gene amplification, resulting in overexpression of kinase receptors at the cell surface. Increased receptor density enhances signaling output and may promote ligand-independent activation. This phenomenon has been extensively documented for members of the EGFR family in various solid tumors (Rocha-Lima et al., 2007). At sufficiently high receptor concentrations, dimerization can occur stochastically even in the absence of ligand, since the probability of two receptor molecules encountering one another at the membrane increases with their local density. This effect is compounded in tumors that simultaneously overexpress both a receptor and its cognate ligand, establishing autocrine signaling loops in which tumor cells continuously stimulate their own growth without dependence on external growth factor sources. Amplification of MET, for example, has been observed in subsets of gastric and lung cancers, where it drives signaling independent of hepatocyte growth factor availability and has been specifically associated with resistance to therapies targeting other receptor pathways (Du & Lovly, 2018).

Activating point mutations represent another important mechanism of oncogenic kinase activation. Such mutations may stabilize the active conformation of the kinase domain, increase catalytic efficiency, or impair negative regulatory mechanisms. Constitutively active kinase signaling promotes uncontrolled cell proliferation while simultaneously suppressing apoptotic pathways (Shortt & Johnstone, 2012). The precise location of a mutation within the kinase domain often determines its functional consequence: mutations affecting the activation loop tend to lock the kinase in its active conformation, whereas mutations near the ATP-binding pocket can alter substrate affinity or, in some cases, confer resistance to small-molecule inhibitors that compete for the same binding site. Exon 19 deletions and the L858R substitution in EGFR, recurrent in non-small cell lung cancer, illustrate this principle directly, as both alterations destabilize the receptor's autoinhibited conformation and permit kinase activation independent of ligand engagement (Ke et al., 2017). A comparable pattern is observed with activating mutations in KIT in gastrointestinal stromal tumors, where mutations clustering within the juxtamembrane domain disrupt the autoinhibitory function normally provided by this region (Tarn et al., 2005).

Chromosomal translocations constitute a particularly important mechanism in hematological malignancies. The best-known example is the Philadelphia chromosome, generated by a reciprocal translocation between chromosomes 9 and 22 (Kurzrock et al., 2003). This rearrangement creates the BCR-ABL fusion gene, which encodes a constitutively active tyrosine kinase responsible for the development of chronic myeloid leukemia (CML). The discovery of BCR-ABL provided one of the earliest demonstrations that a specific kinase abnormality could act as a primary oncogenic driver. The oncogenic fusion protein achieves constitutive activity through loss of the regulatory sequences normally present in native ABL, while the BCR-derived portion of the fusion promotes

oligomerization, further enhancing kinase activity independent of any upstream activating signal. This same principle, gene fusion eliminating autoinhibitory elements while introducing a domain that drives constitutive dimerization, recurs across numerous other kinase fusions identified in human cancer, including ALK rearrangements in non-small cell lung cancer and anaplastic large cell lymphoma, ROS1 fusions in lung adenocarcinoma, and recurrent NTRK gene fusions identified across a wide range of tumor types regardless of tissue origin (Di Marco & Voena, 2022). The recognition that such fusions can occur across diverse cancers irrespective of their site of origin has contributed to a broader shift in oncology toward classifying certain tumors according to their underlying molecular drivers rather than solely by anatomical location.

Similarly, abnormal activation of Src family kinases has been observed in numerous solid tumors, including breast, colorectal, lung, pancreatic, and ovarian cancers (Martellucci et al., 2020). Although activating mutations in Src are relatively uncommon, increased kinase expression, altered regulation, and enhanced signaling through upstream receptors frequently result in elevated Src activity. Aberrant Src signaling promotes tumor growth, invasion, angiogenesis, and metastatic dissemination (Summy & Gallick, 2006). Because Src activity is governed primarily by upstream regulatory inputs rather than by intrinsic mutation, its dysregulation in cancer often reflects broader alterations elsewhere in the signaling network, such as overexpression of upstream RTKs or loss of the regulatory kinase Csk, which normally phosphorylates an inhibitory tyrosine residue near the Src C-terminus to maintain the kinase in an inactive state. This indirect mode of activation illustrates a broader principle in oncogenic kinase signaling: a kinase need not itself be genetically altered to become a significant driver of malignant behavior, provided that the regulatory network constraining its activity is sufficiently disrupted elsewhere (Thomas & Brugge, 1997).

Persistent tyrosine kinase signaling also contributes to genomic instability, a hallmark of cancer characterized by the accumulation of genetic alterations over time (Mahajan & Mahajan, 2015). Enhanced proliferative signaling may overwhelm DNA repair mechanisms and increase replication stress, thereby facilitating the acquisition of additional mutations that further promote malignant transformation. As tumors evolve, selective pressures favor clones capable of exploiting kinase-dependent signaling networks to gain growth and survival advantages (Hanahan & Weinberg, 2011).

The tumor microenvironment also modulates tyrosine kinase activity. Cancer-associated fibroblasts, immune cells, endothelial cells, and extracellular matrix components produce growth factors and cytokines capable of activating tyrosine kinase receptors in both tumor and stromal cells. This reciprocal communication establishes a dynamic signaling network that promotes angiogenesis, immune evasion, invasion, and metastatic dissemination (Shiga et al., 2015).

Kinase signaling heterogeneity within tumors has emerged as an important factor shaping treatment

outcomes. Individual cancer cells may exhibit distinct patterns of kinase activation, contributing to intratumoral diversity and therapeutic resistance. This heterogeneity represents a major challenge for targeted therapy, as inhibition of a single kinase pathway may leave alternative signaling routes available for tumor survival. A clearer understanding of these interactions is needed to develop more effective therapies (Zhu et al., 2009).

1.2.3 Development of tyrosine kinase inhibitors

The recognition that oncogenic tyrosine kinases drive malignant transformation has motivated the development of small-molecule tyrosine kinase inhibitors (TKIs), which act primarily by binding to the ATP-binding pocket of the kinase catalytic domain and have become one of the most successful classes of anticancer agents (Ayala-Aguilera et al., 2021).

Tyrosine kinase inhibitors are commonly classified by how and where they engage the kinase in Figure 6. Type I inhibitors bind the ATP pocket of the active conformation, with the αC helix and DFG motif in their catalytically aligned positions; because they recognize a state common to many kinases, they tend to be potent but less selective. Type II inhibitors bind the inactive "DFG-out" conformation, extending beyond the ATP site into an adjacent hydrophobic pocket exposed when the activation loop is displaced; this pocket is less conserved, so type II compounds can achieve greater selectivity, and imatinib is the prototypical example. Type III inhibitors are truly allosteric, binding sites entirely outside the ATP cleft and modulating activity without competing with ATP. This framework underlies much of the development described below and is revisited when interpreting the behavior of the compounds studied in this work (Łukasik et al., 2021).

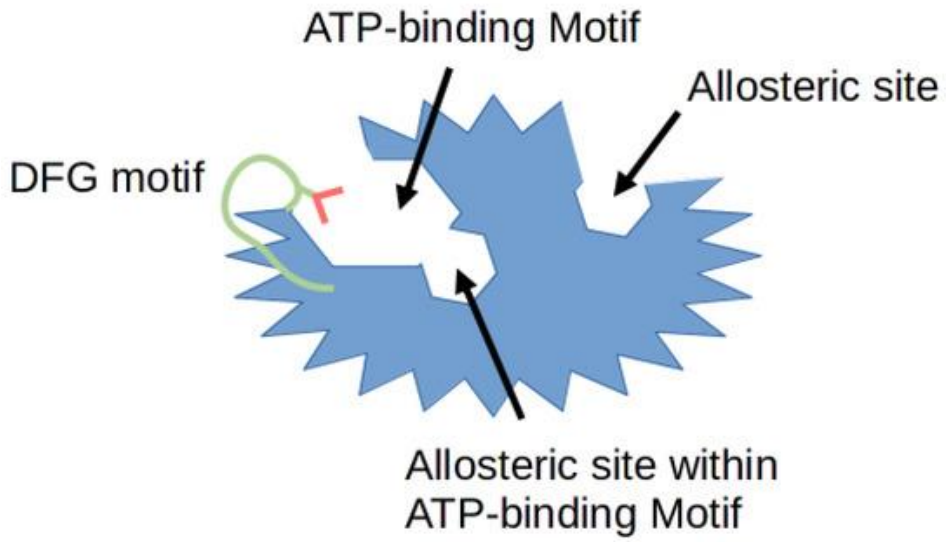


Fig. 6. Binding modes of kinase inhibitors, showing type I (active, DFG-in conformation), type II (inactive, DFG-out conformation), and type III (allosteric) sites relative to the ATP pocket (Łukasik et al., 2021)

The evolution of tyrosine kinase inhibitors has generally been divided into successive generations characterized by improvements in potency, selectivity, and resistance management. First-generation inhibitors were primarily designed to target the ATP-binding site of specific oncogenic kinases and demonstrated that selective kinase inhibition could achieve substantial clinical efficacy. Imatinib, a selective inhibitor of BCR-ABL kinase, is the archetypal example: clinical studies demonstrated remarkable efficacy in chronic myeloid leukemia, transforming a previously fatal disease into a manageable chronic condition and establishing the paradigm for molecularly targeted therapy (Druker, 2004; Weisberg et al., 2006; Weisberg et al., 2005).

Following the introduction of imatinib, numerous kinase inhibitors were developed against a broad range of therapeutic targets. Several agents targeting EGFR, VEGFR, FGFR, RET, ALK, MET, and other kinases have subsequently entered clinical practice and significantly improved outcomes for patients with diverse malignancies (Lai et al., 2018).

Second-generation inhibitors were subsequently developed to overcome resistance mechanisms that emerged during prolonged treatment. These compounds typically display increased binding affinity and broader activity against mutant kinase variants. Many of these agents were specifically engineered to retain activity against kinase domain mutations that compromised the efficacy of first-generation inhibitors, either by adopting a binding mode less sensitive to structural changes in the ATP-binding pocket or by exploiting alternative contacts within the kinase domain not utilized by the original compound. In chronic myeloid leukemia, drugs such as dasatinib and nilotinib demonstrated improved efficacy in patients who developed resistance or intolerance to imatinib (Martinelli et al., 2007). Dasatinib was found to inhibit a broader range of BCR-ABL kinase domain mutants than imatinib, while also exhibiting activity against Src family kinases, reflecting a less restrictive binding mode within the ATP pocket. Nilotinib, developed as a structural analogue of imatinib with optimized binding interactions, achieved substantially higher potency against BCR-ABL while retaining a comparatively similar selectivity profile (Rix et al., 2007).

Third-generation inhibitors were specifically engineered to target resistance-associated mutations while preserving activity against the primary oncogenic kinase. Rather than relying on broad-spectrum activity across multiple kinase domain mutants, as was largely the case with second-generation compounds, this generation of inhibitors emerged from a more deliberate, structure-guided design process aimed at directly addressing the specific mutations most frequently responsible for

treatment failure. Ponatinib, for example, was developed to inhibit the T315I gatekeeper mutation of BCR-ABL, one of the most clinically challenging resistance mutations identified in leukemia patients (Zhou et al., 2011). The gatekeeper residue occupies a position at the entrance to the ATP-binding pocket, and its substitution to isoleucine in the T315I mutant introduces a bulky side chain that sterically obstructs the binding of most existing inhibitors while leaving the kinase's catalytic function intact. Ponatinib's design specifically accounted for this structural change, incorporating a binding configuration capable of accommodating the altered pocket geometry and thereby restoring inhibitory activity against a mutation that had rendered both first- and second-generation BCR-ABL inhibitors ineffective (Rix et al., 2007).

A further design strategy involves covalent inhibitors, which form an irreversible bond with a nucleophilic residue, typically a cysteine, situated near the ATP pocket. By covalently modifying their target, these compounds achieve prolonged inhibition that persists until new kinase protein is synthesized, and they can reach residues unique to a particular kinase, improving selectivity. This approach has been most successfully applied to mutant EGFR in non-small cell lung cancer, where later-generation covalent inhibitors were developed specifically to target activating and resistance mutations while sparing the wild-type receptor. The trade-off is that permanent target modification raises the potential for off-target reactivity, which must be carefully controlled during design (Abdeldayem et al., 2020).

Despite these achievements, important challenges remain. One major limitation is the emergence of acquired resistance, frequently caused by mutations within the kinase domain that reduce inhibitor binding affinity (Barouch-Bentov & Sauer, 2011). Such mutations rarely emerge uniformly throughout a tumor; rather, they typically arise within small, pre-existing subpopulations of cells that are subsequently selected for and expanded under the selective pressure imposed by continuous drug exposure, allowing resistant clones to eventually dominate the tumor population despite their initial rarity.

Another significant challenge concerns selectivity. Because the ATP-binding site is highly conserved among kinases, many inhibitors interact with multiple targets. While multi-target inhibition may sometimes be therapeutically advantageous, particularly when a tumor depends on more than one kinase pathway for survival, it can also increase toxicity and complicate interpretation of pharmacological effects, since adverse outcomes observed during treatment may stem from inhibition of unintended targets rather than from the primary mechanism of action under investigation. This lack of selectivity also complicates efforts to attribute clinical efficacy to a specific molecular target, making it more difficult to establish clear structure-activity relationships that could guide the design of more refined inhibitors. Off-target activity can additionally influence the toxicity profile of a given

compound in ways that are not always predictable from its primary target alone, as inhibition of structurally related kinases involved in normal physiological processes, such as cardiac or vascular homeostasis, can produce clinically significant adverse effects unrelated to the drug's intended anticancer activity. These limitations have driven the development of next-generation inhibitors with better selectivity, higher potency, and alternative mechanisms of action (Morphy, 2010).

In addition to ATP-competitive compounds, increasing attention is directed toward allosteric inhibitors and molecules targeting the regulatory domains involved in protein–protein interactions. Because allosteric sites are generally less conserved than the ATP pocket, such compounds often display greater target selectivity and may remain effective where resistance mutations reduce the affinity of ATP-competitive inhibitors (Hantschel et al., 2012).

The clinical success of asciminib, an allosteric inhibitor of ABL1, has established the therapeutic potential of this strategy; its mechanism is described in Section 1.4.4 (Choi, 2023).

Structural studies and computational drug-design methods have further improved the identification and optimization of new kinase inhibitors, particularly those active against resistant disease.

1.2.4 Measuring kinase activity and inhibition

The biochemical characterization of a kinase inhibitor depends on a reliable, quantitative measure of catalytic activity. Because protein kinases transfer the γ-phosphate of ATP to a substrate and release ADP, an assay can in principle follow any of three quantities: consumption of ATP, appearance of phosphorylated substrate, or production of ADP. The available assay formats differ mainly in which of these they detect and in how the signal is generated, and the choice of format shapes throughput, cost, and the kinds of artefact to which the measurement is vulnerable (Ma et al., 2008).

Radiometric assays, historically the reference method, follow the transfer of ^{32}P or ^{33}P from labelled ATP directly onto the substrate. They report catalysis without the need for antibodies or coupling enzymes and tolerate a wide range of substrates, but the handling of radioisotopes, the associated waste, and the limited throughput have reduced their routine use (Hastie et al., 2006). Antibody-dependent fluorescence methods instead detect the phosphorylated product with a phospho-specific antibody; time-resolved fluorescence resonance energy transfer (TR-FRET) and fluorescence-polarization formats are widely used for this purpose and are well suited to high-throughput screening, although they require a validated antibody for each phosphorite and are sensitive to optical interference from colored or fluorescent compounds (Jia et al., 2008). Mobility-shift assays take a different approach, physically separating the phosphorylated and unphosphorylated forms of a labelled peptide by microfluidic capillary electrophoresis and quantifying the ratio of the two; the

readout is direct and largely insensitive to optical artefacts, but the instrumentation is specialized and the substrate must carry a fluorescent tag (Ma et al., 2008).

Luminescent ADP-detection assays occupy a complementary niche. Rather than detecting the phosphorylated product, they quantify the ADP generated by the reaction: the remaining ATP is depleted, the accumulated ADP is converted back to ATP, and the regenerated ATP drives a luciferase reaction whose light output is proportional to kinase activity. The format is homogeneous, antibody-free, and independent of the identity of the substrate, which makes it broadly applicable across kinases and convenient for inhibitor profiling (Zegzouti et al., 2009). These properties motivated its use in the present work, and the specific protocol is described in Section 3.

Several considerations apply regardless of format. The substrate may be a full-length protein or a short peptide bearing the target residue; peptides are reproducible and inexpensive but may lack the docking interactions that influence catalysis on the physiological substrate (Ma et al., 2008). The ATP concentration is equally important: because most small-molecule inhibitors compete with ATP, the measured half-maximal inhibitory concentration (IC_{50}) depends on the ATP concentration used and on the affinity of the enzyme for ATP, so potencies are strictly comparable only when these are held constant or explicitly corrected (Section 3.5) (Knight & Shokat, 2005). Finally, assay quality is commonly judged from the separation between positive and negative controls, expressed as the Z′-factor, and from the behavior of a reference inhibitor included in every run; here dasatinib served this role, confirming that the assay responded as expected before the test compounds were evaluated (Zhang et al., 1999).

Table 1. Comparison of common biochemical formats for measuring kinase activity and inhibition.

Assay format	**Quantity detected**	**Main advantages**	**Main limitations**
Radiometric ($^{32}P/^{33}P$)	Phosphate transferred to substrate	Direct; substrate-flexible; few optical artefacts	Radioactive waste; low throughput
TR-FRET / fluorescence polarisation	Phosphorylated product (antibody)	High throughput; homogeneous	Needs validated phospho-antibody; optical interference
Mobility-shift (capillary electrophoresis)	Ratio of phospho/non-phospho peptide	Direct; resistant to optical artefacts	Specialised instrument; tagged substrate
Luminescent ADP detection (ADP-Glo)	ADP produced by the reaction	Antibody-free; substrate-independent; suited to profiling	Indirect; sensitive to background ATP/ADP

1.2.5 Structure–activity relationships and selectivity

The optimization of a kinase inhibitor is guided by its structure–activity relationship (SAR): the correspondence between defined chemical features of a compound series and the resulting biological activity. By comparing analogues that differ at a single position, it becomes possible to attribute changes in potency or selectivity to substituents and to identify which parts of a scaffold should be retained or modified (Zhang et al., 2009). In the kinase field, SAR is interpreted against a small number of recurring contact regions: the hinge, where most ATP-competitive inhibitors form the hydrogen bonds that anchor them in the pocket; the region behind the gatekeeper residue, access to which varies between kinases and often governs selectivity; and the solvent-exposed front of the pocket, which tolerates substituents that tune physicochemical properties without contacting the conserved core. The conformation an inhibitor recognizes—active or inactive, in the binding-mode classification introduced in Section 1.2.3—further constrains which structural features are productive. Selectivity is the second axis along which inhibitors are evaluated, and it carries direct therapeutic weight: activity against unintended kinases can produce toxicity and confound the interpretation of pharmacological effects, whereas a defined selectivity profile allows a phenotype to be attributed to inhibition of a particular target. Selectivity can be assessed at several levels of resolution. The simplest is a pairwise selectivity ratio—the quotient of the potencies measured against two kinases, as reported for Src and Abl in Section 4.4. Broader characterization relies on profiling a compound across many kinases and summarizing the result with metrics such as the selectivity score, defined as the fraction of a kinase panel inhibited beyond a chosen threshold, the partition index, or the Gini coefficient of the activity distribution (Cheng et al., 2010; Graczyk, 2007; Karaman et al., 2008). These measures complement one another: a pairwise ratio answers a focused question about two related enzymes, while a panel-wide score situates a compound within the kinome as a whole.

A point specific to ATP-competitive inhibitors deserves emphasis, because it bears directly on how the present results are interpreted. The IC_{50} measured for such a compound reflects not only its intrinsic affinity but also the affinity of the enzyme for ATP, since the two molecules compete for the same site. Two kinases with different Michaelis constants for ATP can therefore yield different IC_{50} values for the same inhibitor even when its true affinities are similar. Converting IC_{50} to an inhibition constant (K_i) through the Cheng–Prusoff relationship removes this dependence and provides a more faithful basis for comparing affinities across kinases (Section 3.5). A selectivity ratio expressed at the level of K_i may consequently differ from the same ratio expressed at the level of IC_{50}, a distinction that becomes important when the two target kinases differ appreciably in their ATP affinity (Cheng & Prusoff, 1973; Knight & Shokat, 2005).

1.2.6 Resistance to kinase inhibitors

The clinical durability of kinase inhibitors is limited by acquired resistance, a theme recurring in several of the cases already discussed (Sections 1.2.3 and 1.4.4). Resistance develops through several distinct routes and separating them is useful because each calls for a different response in inhibitor design (Lovly & Shaw, 2014). The mechanisms are conventionally grouped into those that alter the drug target itself and those that allow the cell to survive despite continued inhibition of that target.

Target-site mutations are the most direct route. The clearest example is mutation of the gatekeeper residue, which controls access to the pocket behind the ATP site: substitution with a bulkier amino acid sterically blocks inhibitor binding while leaving catalysis largely intact, as in the T315I mutation of BCR-ABL introduced in Section 1.4.4. Other substitutions act more subtly by shifting the conformational equilibrium of the kinase. Mutations in the activation loop or surrounding elements that stabilize the active state reduce the affinity of inhibitors which require the inactive, DFG-out conformation, and therefore selectively erode the efficacy of type II compounds (Section 1.2.3). In each case the kinase retains function while its affinity for the inhibitor falls, so the enzyme continues to drive proliferative signaling in the presence of the drug (Daub et al., 2004).

A second group of mechanisms leaves the target unchanged. Amplification or overexpression of the kinase can raise the amount of active enzyme beyond what a given concentration of inhibitor can suppress. Alternatively, the cell may engage parallel or downstream pathways that render the inhibited kinase dispensable, a phenomenon described as bypass signaling, in which activation of an alternative receptor or effector restores flux through the same proliferative programs the inhibitor was intended to interrupt. Pharmacokinetic factors form a third category: increased drug efflux through membrane transporters, altered metabolism, or limited tissue penetration can lower the effective intracellular concentration so that inhibition is incomplete even when the target remains fully sensitive in vitro (Lovly & Shaw, 2014).

These mechanisms have shaped successive generations of inhibitor design. Where resistance is driven by a specific mutation, later ATP-competitive compounds have been engineered to accommodate the altered residue, as with the activity of ponatinib against the T315I gatekeeper mutant (Section 1.4.4). Where the ATP site itself is compromised, allosteric inhibitors that bind less conserved and less mutation-prone regions offer an alternative, illustrated by the myristoyl-pocket inhibitor asciminib (Sections 1.2.3 and 1.4.4). A defined selectivity profile contributes as well, because limiting activity against unintended kinases reduces the toxicity that constrains dosing and so widens the margin within which a resistant target can still be suppressed. The continued search for new chemical scaffolds combining favorable potency with selectivity, of which the compounds characterized in this work are examples, belongs to this same effort to stay ahead of resistance (Section 2).

1.3 Src kinase

1.3.1 Structure and regulation of Src

Src is the prototypical member of the Src family kinases (SFKs), a group of non-receptor tyrosine kinases that regulate intracellular signaling pathways (Tatosyan & Mizenina, 2000). The family comprises eight members in vertebrates — Src, LCK, LYN, BLK, HCK, FYN, FGR, and YES — which share a highly conserved structural organization and catalytic mechanism. They differ mainly in their tissue distribution: Src, FYN, and YES are expressed broadly across most cell types, whereas LCK, BLK, HCK, FGR, and LYN are largely restricted to hematopoietic and immune cells, where individual members serve specialized signaling roles. A common catalytic core combined with distinct expression patterns lets the family carry out a shared biochemical function across many cellular contexts (Brian 4th & Freedman, 2021).

The modular architecture of Src is essential for its regulatory function. As illustrated in Figure 7, Src family kinases contain an N-terminal SH4 domain involved in membrane localization through myristoylation, followed by a Unique Domain (UD), the SH3 and SH2 regulatory domains, and the catalytic kinase domain (SH1). A short C-terminal regulatory tail contains a critical tyrosine residue whose phosphorylation status controls kinase activity (Ortiz et al., 2021).

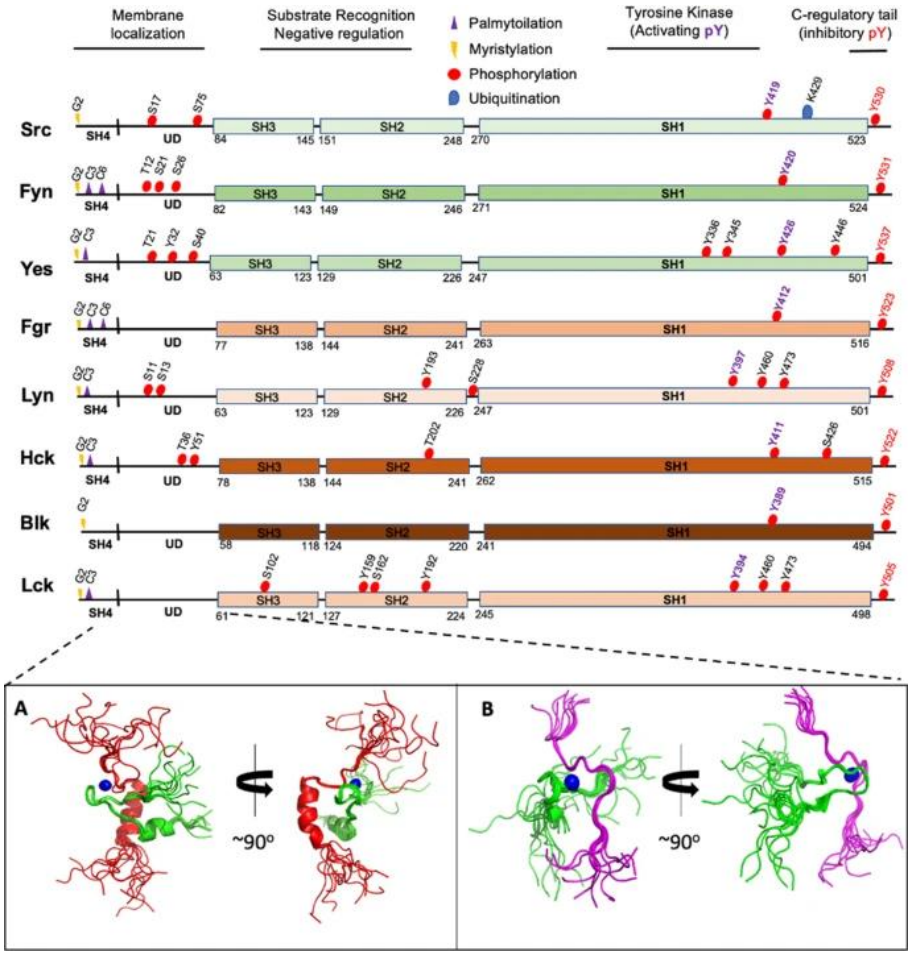


Fig. 7. Primary structure of Src family kinases (Ortiz et al., 2021).

The activity of Src is tightly regulated through intramolecular interactions that hold the enzyme in an autoinhibited, "assembled" conformation in the absence of activating signals. The central regulatory element is a tyrosine residue within the short C-terminal tail (Tyr527 in chicken c-Src; Tyr530 in the human enzyme). When this residue is phosphorylated, it engages the SH2 domain of the same molecule, folding the C-terminal tail back against the regulatory apparatus. Simultaneously, the SH3 domain docks onto the linker connecting the SH2 and catalytic (SH1) domains, which adopts a left-handed polyproline type II helix conformation despite lacking a canonical proline-rich motif. Together these two latches — the phospho-Tyr527/530–SH2 interaction and the SH3–linker interaction — clamp the regulatory domains against the back of the kinase domain and lock Src in a closed, catalytically repressed state (Boggon & Eck, 2004).

This clamped architecture suppresses catalysis not by occluding the active site directly but by imposing an unproductive geometry on the kinase domain. In the inactive conformation the αC helix of the N-lobe is rotated outward ("αC-out"), disrupting the conserved Glu310–Lys295 salt bridge required to coordinate ATP, while the activation loop collapses into a conformation that blocks substrate access. The enzyme therefore remains essentially silent as long as the intramolecular

network is intact (Tong et al., 2017).

Activation requires disruption of these stabilizing interactions, which can be achieved through two principal routes. First, dephosphorylation of the C-terminal regulatory tyrosine abolishes its affinity for the SH2 domain and releases the tail. Second, the SH2 and SH3 domains can be competitively displaced by higher-affinity external ligands — such as phosphotyrosine motifs on activated receptors or proline-rich sequences in focal adhesion proteins — which draw the regulatory domains away from their intramolecular partners. In either case, release of the clamp allows the αC helix to rotate inward ("αC-in"), restoring the catalytic salt bridge, and frees the activation loop, which becomes phosphorylated at its own tyrosine (Tyr416 in chicken; Tyr419 in human) through trans-autophosphorylation. This modification stabilizes the open, active conformation and the substrate-binding platform, producing a fully catalytically competent kinase (Boggon & Eck, 2004).

The phosphorylation status of the C-terminal tyrosine — and therefore the equilibrium between the closed and open states — is governed by opposing enzymes. The C-terminal Src kinase (Csk), together with its homolog Chk, phosphorylates this residue to enforce the inactive state, whereas protein tyrosine phosphatases including PTPα, PTP1B, SHP-1, and SHP-2 remove the phosphate to license activation. Src activity in the cell thus reflects the net output of this kinase–phosphatase balance acting on a single critical residue (Ingley, 2008).

One important mechanism of Src activation involves β-arrestin-mediated recruitment of Src family kinases to activated seven-transmembrane receptors (7TMRs). Classically, β-arrestins were identified as negative regulators of GPCR signaling: following agonist binding, the receptor is phosphorylated by G-protein-coupled receptor kinases (GRKs), generating high-affinity docking sites for β-arrestins. Their recruitment uncouples the receptor from heterotrimeric G proteins (desensitization) and targets it for clathrin-mediated internalization. However, β-arrestins are now recognized as multifunctional scaffolds that, beyond terminating G-protein signaling, actively nucleate the assembly of new signaling complexes — including those containing Src family kinases — thereby initiating a second, G-protein-independent wave of signal transduction (Perry & Lefkowitz, 2002).

The interaction between β-arrestins and SFKs is mediated by binding of proline-rich and other motifs within β-arrestin to the SH3 and catalytic domains of the kinase, recruiting the otherwise cytosolic SFK to the activated receptor at the plasma membrane. This spatial re-localization positions the kinase in proximity to its substrates and physically couples receptor activation to downstream effector pathways. As illustrated in Figure 8, this mechanism is not restricted to a single receptor or kinase but represents a general signaling paradigm employed by multiple 7TMRs, each recruiting distinct SFK members to elicit cell-type-specific responses (Perry & Lefkowitz, 2002).

Three representative examples highlight the functional versatility of this module. At the β2-

adrenergic receptor (β2AR) and the neurokinin-1 receptor (NK1R), β-arrestin recruits c-Src, leading to activation of the ERK1/2 mitogen-activated protein kinase cascade and the propagation of proliferative and survival signals. In neutrophils, the interleukin-8 receptor CXCR1 uses β-arrestin to recruit the SFK members Hck and c-Fgr, triggering granule release and contributing to the inflammatory effector functions of these cells. At the endothelin A receptor (ETAR), β-arrestin recruits the SFK Yes, which promotes translocation of the glucose transporter GLUT4 to the plasma membrane and stimulates glucose uptake. Together, these examples show how a conserved β-arrestin–SFK scaffold is adapted to markedly different outputs (MAPK signaling, immune cell activation, and metabolic regulation), depending on the receptor engaged and the family member recruited (Perry & Lefkowitz, 2002).

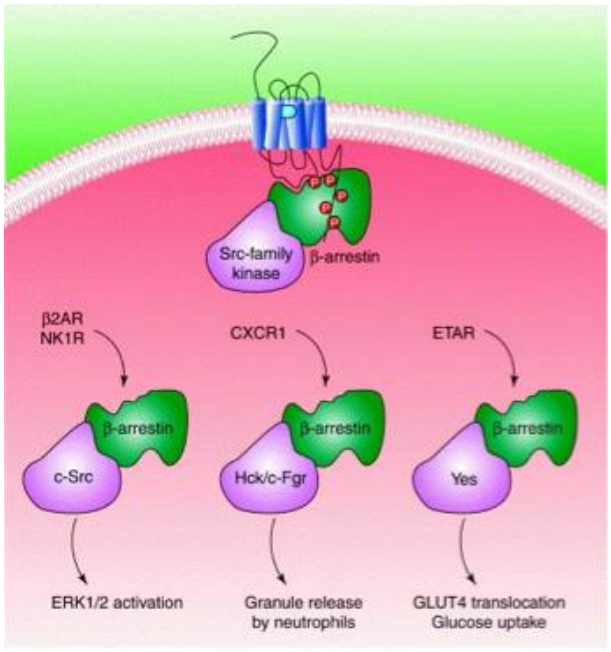


Fig. 8. Seven-transmembrane-span receptors (7TMRs) signal to members of the Src family of tyrosine kinases through β-arrestin-mediated recruitment mechanisms (Perry & Lefkowitz, 2002).

This reversible transition between the closed, autoinhibited conformation and the open, active state is summarized in Figure 9 (Dongre & Weinberg, 2019).

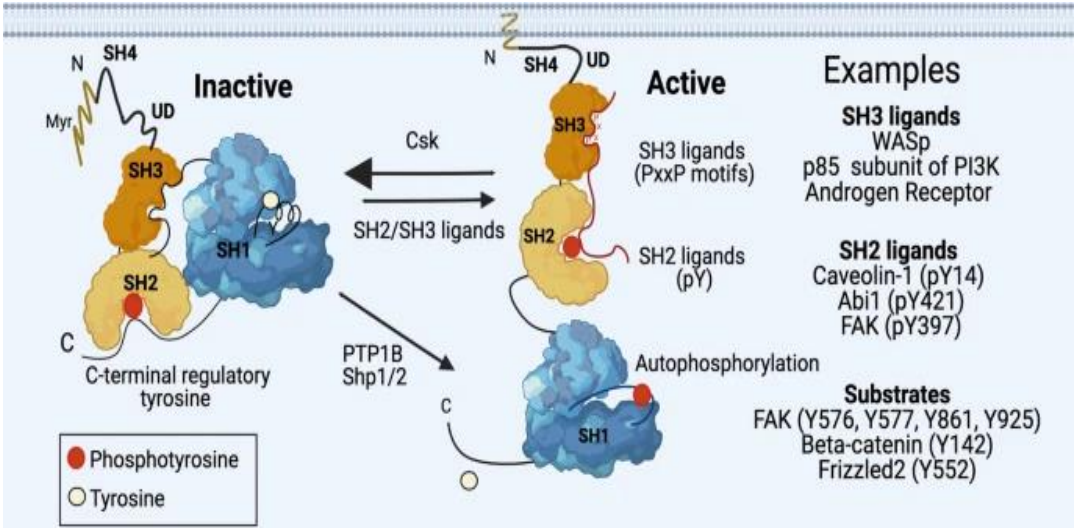


Fig. 9. Regulation of Src family kinase activity, showing the transition between the inactive (assembled) and active (open) conformations. The closed state is stabilized by phosphorylation of the C-terminal regulatory tyrosine and by SH2/SH3 intramolecular interactions; Csk phosphorylates this tyrosine to maintain the inactive state, whereas the phosphatases PTP1B and Shp1/2 promote activation. Representative SH3 ligands, SH2 ligands, and substrates are indicated (Dongre & Weinberg, 2019).

1.3.2 Biological functions of Src

Src regulates many physiological processes through signaling pathways. It exerts this influence through the phosphorylation of a large and diverse substrate repertoire distributed across the cell — at the plasma membrane, throughout the cytoplasm, on the cytoskeleton, and within the nucleus. Because these substrates feed into distinct effector pathways, the biological outcome of Src activity is highly context-dependent, determined by the cell type, the activating stimulus, and the subcellular location at which the kinase is engaged (Guarino, 2010).

One of the best-characterized functions of Src is its involvement in integrin signaling and focal adhesion dynamics. Upon integrin-mediated adhesion to the extracellular matrix, focal adhesion kinase (FAK) autophosphorylates at Tyr397, creating a high-affinity docking site for the Src SH2 domain. The resulting FAK–Src complex is mutually activating: Src phosphorylates additional tyrosines within FAK to maximize its catalytic output, and the assembled complex then phosphorylates a set of downstream structural and adaptor proteins — including p130Cas, paxillin, and cortactin — that govern the assembly, maturation, and turnover of focal adhesions. Through cortactin and related substrates, Src also regulates branched actin polymerization and cytoskeletal remodeling at the cell periphery. By controlling the dynamic disassembly of adhesions at the leading

edge, this FAK–Src axis underlies directional cell migration and is essential during embryonic development, tissue remodeling, wound healing, and immune cell trafficking (Katoh, 2024).

Src additionally acts as a positive modulator of receptor tyrosine kinase signaling. Beyond being recruited and activated by receptors such as EGFR, PDGFR, VEGFR, and MET, Src reciprocally phosphorylates these receptors at regulatory sites distinct from their own autophosphorylation residues (for example EGFR at Tyr845), thereby enhancing and prolonging their output. The downstream cascades engaged through this crosstalk (the RAS and PI3K pathways) are described in Section 1.1.3 (Cao, 2013).

In addition, Src family kinases are central to the immune system, where tissue-restricted expression of individual members supports highly specialized functions. In T lymphocytes, the family member LCK initiates antigen-receptor signaling by phosphorylating the immunoreceptor tyrosine-based activation motifs (ITAMs) of the T-cell receptor complex and recruiting the downstream kinase ZAP-70, whereas LYN, FYN, and BLK perform analogous proximal roles in B-cell receptor signaling. In myeloid cells, members such as HCK, FGR, and LYN regulate the effector responses of macrophages and neutrophils. This division of labor — different family members serving different lineages while sharing a conserved activation mechanism — allows the Src family to govern activation, differentiation, and effector output across the immune system (Okutani et al., 2006).

1.3.3 Src in cancer progression

The association between Src and cancer has historical significance because Src was the first proto-oncogene ever identified. Studies performed on the Rous sarcoma virus demonstrated that a viral gene, v-Src, was capable of inducing malignant transformation in infected cells. Subsequent investigations revealed that v-Src originated from a normal cellular gene, c-Src, establishing the concept that alterations of endogenous cellular genes can drive tumorigenesis (Simatou et al., 2020). This discovery fundamentally changed our understanding of cancer biology and contributed to the emergence of modern molecular oncology.

The extent of Src deregulation varies across tumor types, and in several cancers elevated Src activity is associated with disease stage and outcome. Increased Src expression or activity has been reported in colorectal, breast, pancreatic, ovarian, lung, and head-and-neck carcinomas, often in association with advanced stage and reduced differentiation. In hormone-receptor-positive breast cancer, Src has been linked both to proliferative signaling and to the emergence of endocrine resistance. This pattern — modest deregulation across many epithelial tumors rather than dramatic activation in a few — fits Src acting as a signal amplifier that potentiates other oncogenic inputs, and explains why it is seldom

the sole driver yet frequently contributes to the aggressive behavior of established tumors (Summy & Gallick, 2003).

In addition to promoting proliferation and survival, Src drives tumor cell invasion and metastatic dissemination. Metastasis is a complex multistep process involving detachment from the primary tumor, degradation of the extracellular matrix, intravasation into the bloodstream, survival in circulation, extravasation into distant tissues, and colonization of secondary organs. Src signaling contributes to several of these steps by regulating cytoskeletal dynamics, focal adhesion turnover, and cellular motility (Wang et al., 2015). At the cellular level, much of Src's pro-invasive activity converges on actin-rich membrane protrusions known as invadopodia. These concentrate Src together with cortactin, the adaptor Tks5, and matrix metalloproteinases, focusing proteolysis onto discrete points of contact with the extracellular matrix. By phosphorylating cortactin and Tks5, Src promotes invadopodia assembly and localized matrix degradation, allowing tumor cells to breach basement membranes. This links the focal-adhesion and cytoskeletal substrates Src regulates directly to invasive capacity (Leong et al., 2014).

Src also has a defined role in epithelial-to-mesenchymal transition (EMT). During EMT, epithelial cells lose their characteristic polarity and cell-cell adhesion properties while acquiring mesenchymal features associated with increased migratory and invasive capacity. Src activation promotes the phosphorylation of proteins involved in adherens junctions and focal adhesions, leading to disruption of epithelial architecture and enhanced metastatic potential (Noshita et al., 2023).

Src also contributes to tumor angiogenesis through regulation of vascular endothelial growth factor (VEGF) signaling. By modulating endothelial cell migration, proliferation, and vascular permeability, Src facilitates the formation of new blood vessels that supply oxygen and nutrients to growing tumors. Increased angiogenesis not only supports tumor expansion but also creates additional routes for metastatic dissemination (Sivaraman Siveen et al., 2017).

Recent evidence further suggests that Src participates in the development of resistance to anticancer therapies (Yu et al., 2020). Activation of Src-dependent signaling pathways can compensate for inhibition of other oncogenic drivers, allowing cancer cells to maintain survival and proliferative signaling despite therapeutic intervention (Higuchi et al., 2021). This phenomenon has been observed in several malignancies treated with EGFR inhibitors, HER2-targeted therapies, and hormonal treatments (de Melo Gagliato et al., 2016; Peng & Tan, 2023).

Because Src acts on proliferation, invasion, angiogenesis, and resistance at once, selective Src inhibition is an attractive strategy, particularly in combination with other targeted agents.

1.3.4 Current Src inhibitors and limitations

Given its importance in tumor biology, Src has been extensively investigated as a pharmacological target. Several clinically approved tyrosine kinase inhibitors exhibit activity against Src family kinases, including dasatinib, bosutinib, ponatinib, and vandetanib. However, these compounds were primarily developed for other kinase targets and generally display broad kinase inhibition profiles (Roskoski Jr, 2015).

Dasatinib, bosutinib, and ponatinib are approved mainly for the treatment of chronic myeloid leukemia through inhibition of BCR-ABL, while vandetanib is primarily used in medullary thyroid carcinoma through inhibition of RET signaling (Lee et al., 2021; Okafor et al., 2021). Although all these agents inhibit Src family kinases, Src inhibition is typically only one component of their pharmacological activity.

Clinical studies have demonstrated that Src inhibition alone often produces limited efficacy in solid tumors. Src does not act in isolation but within highly interconnected networks marked by redundancy and feedback. As a result, inhibiting Src alone can be bypassed through compensatory pathways, and durable responses are unlikely in advanced cancers carrying multiple oncogenic alterations (Pelaz & Tabernero, 2022; Shah et al., 2018).

Because the ATP pocket is highly conserved (Section 1.2.3), selectivity remains a central challenge in Src inhibitor development. An alternative strategy targets the non-catalytic regulatory domains SH2 and SH3, which mediate the protein–protein interactions required for assembly of Src signaling complexes. Because these molecules disrupt complex assembly rather than competing for the conserved ATP pocket, they may achieve greater specificity and reduce adaptive resistance (Dionne et al., 2022; Kasembeli et al., 2025).

The binding-mode classification described in Section 1.2.3 applies directly to Src. Most clinically used SFK inhibitors, including dasatinib, are type I compounds that bind the active conformation, whereas type II inhibitors engaging the less-conserved DFG-out pocket can offer greater selectivity. Beyond the ATP site, the success of myristoyl-pocket inhibition in the related ABL kinase has prompted interest in comparable allosteric vulnerabilities in Src, although Src lacks an equivalent well-defined site and such approaches remain investigational (Roskoski Jr, 2015).

A conceptually distinct strategy is targeted protein degradation. Proteolysis-targeting chimeras (PROTACs) recruit an E3 ubiquitin ligase to the kinase, marking it for proteasomal destruction rather than merely blocking catalysis. For Src this offers two theoretical advantages: it removes the kinase's scaffolding and protein-interaction functions, not only its catalytic output, and it may be less vulnerable to the compensatory activation that limits ATP-competitive inhibitors. Early Src-directed degraders have been reported, though none is yet in clinical use and selectivity among family

members remains unresolved. Alongside SH2/SH3-directed and allosteric approaches, degraders illustrate the wider move beyond the conserved ATP pocket (Yu et al., 2021).

Because of the extensive structural similarity shared among members of the Src family, and between Src and numerous other tyrosine kinases, many Src inhibitors also target unrelated kinases, increasing the risk of off-target effects and dose-limiting toxicities. Furthermore, compensation among different SFK members may reduce the effectiveness of selective Src blockade (Teli et al., 2023).

The limited selectivity of many ATP-competitive inhibitors may contribute to adverse effects resulting from inhibition of unintended targets. Hematological toxicity, gastrointestinal disturbances, cardiovascular complications, and immunological alterations have all been reported for multitarget kinase inhibitors exhibiting Src activity (Negi et al., 2021).

Fragment-based discovery, computational modeling, and structure-guided design have helped identify new scaffolds that target previously unexplored regulatory regions of Src (Mishra et al., 2024), with the goal of achieving greater specificity and efficacy.

Developing Src inhibitors with better potency, selectivity, and alternative mechanisms therefore remains an active area of drug discovery and provides the rationale for investigating new chemical entities such as those studied here.

1.4 Abl kinase

1.4.1 Structure and regulation of Abl

The Abl family of non-receptor tyrosine kinases consists of two members, ABL1 and ABL2 (also known as Arg) (Siveen et al., 2018). These proteins are essential for the regulation of cellular architecture, migration, proliferation, adhesion, and survival. ABL1 was originally identified as the cellular homolog of the transforming gene carried by the Abelson murine leukemia virus, whereas ABL2 was subsequently discovered through sequence homology analyses (Luttman et al., 2021).

ABL kinases are characterized by a multidomain architecture that integrates regulatory and catalytic functions. As illustrated in Figure 10, both ABL1 and ABL2 contain an N-terminal cap region followed by SH3, SH2, and kinase domains, which collectively regulate kinase activity through intramolecular interactions. The C-terminal region contains additional motifs involved in DNA binding, actin binding, microtubule association, and cytoskeletal regulation (Tse & Verkhivker, 2015).

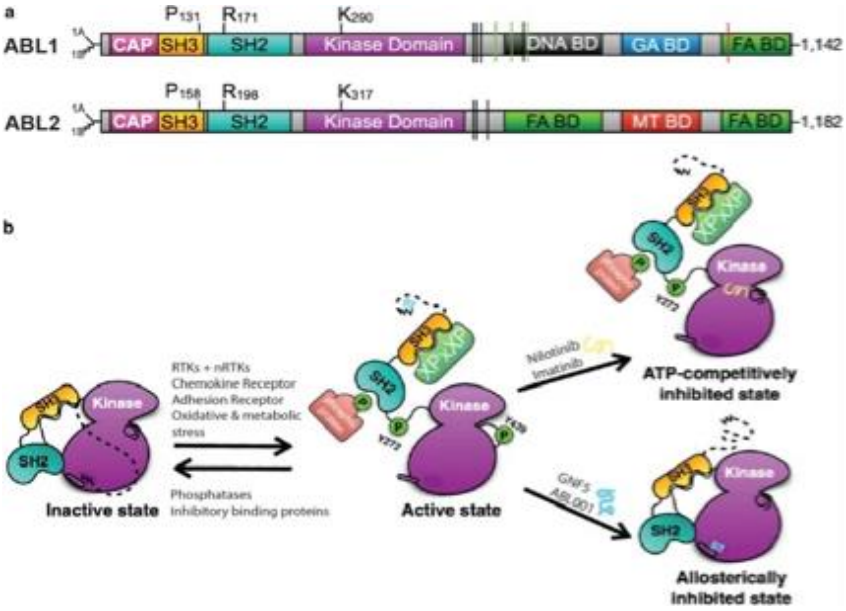


Fig. 10. Representation of the Abl structural domains and regulation of Abl kinase (Luttman et al., 2021).

Under physiological conditions, Abl kinases cycle between inactive and active conformations. The inactive state is maintained through intramolecular interactions involving the SH3 and SH2 domains, which constrain the catalytic domain. Activation occurs in response to diverse extracellular and intracellular signals, including growth factor stimulation, integrin engagement, oxidative stress, chemokines, and DNA damage. These stimuli induce conformational changes that relieve autoinhibition and promote kinase activation (Xie et al., 2020).

Tight regulation of Abl activity matters because aberrant activation can profoundly alter signaling pathways controlling cell growth, migration, and survival (Khatri et al., 2016). Consequently, multiple mechanisms have evolved to ensure tight control of Abl kinase signaling under normal physiological conditions.

One of the most distinctive characteristics of ABL1 is its ability to shuttle between the nucleus and cytoplasm. Under physiological conditions, nuclear-cytoplasmic trafficking is tightly regulated and allows the kinase to respond to specific cellular stimuli (Ge et al., 2023). Nuclear Abl participates in DNA damage responses, regulation of transcription factors, and control of apoptosis, whereas cytoplasmic Abl primarily regulates cytoskeletal organization, cell adhesion, and migration (Low et al., 2024).

Autoinhibition of Abl kinase activity is mediated through a sophisticated network of intramolecular interactions involving the SH3 domain, SH2 domain, kinase domain, and N-terminal myristoylated cap. These interactions stabilize an inactive conformation that prevents uncontrolled kinase activity (Wu et al., 2024). Structural studies have demonstrated that disruption of this autoinhibitory

arrangement represents a critical step in kinase activation and can occur through multiple physiological and pathological mechanisms (Wu et al., 2024).

The structural basis of this autoinhibition is now well defined. The N-terminal myristoyl group, attached to the cap region, inserts into a hydrophobic pocket in the C-lobe of the kinase domain, bending the αI helix and creating a docking surface against which the SH2 and SH3 domains can assemble. The SH3 domain engages the proline-containing linker connecting the SH2 and kinase domains, while the SH2 domain clamps onto the back of the kinase C-lobe. Together these contacts lock the catalytic domain in a closed, assembled conformation in which the αC helix is rotated outward, and the active site is catalytically unproductive. This arrangement is conceptually analogous to the SH2/SH3 clamp that maintains Src in its inactive state, but it is reinforced in Abl by the additional myristoyl latch, giving Abl a second, lipid-dependent layer of control that Src lacks (Hantschel et al., 2012; Wu et al., 2024).

Disruption of any of these interactions shifts the equilibrium toward the active state. Engagement of the SH3 or SH2 domains by external ligands, dephosphorylation events, or removal of the myristoyl contact releases the clamp, allowing the αC helix to rotate inward and the activation-loop tyrosine to be phosphorylated, producing the fully active kinase. A key regulatory difference from Src lies in the C-terminus: whereas Src is held inactive by an intramolecular phosphotyrosine–SH2 interaction involving its C-terminal tail, Abl lacks this C-terminal regulatory tyrosine and instead depends primarily on the cap and myristoyl-based mechanism. This distinction explains why Csk, the kinase that switches Src off through C-terminal phosphorylation, does not regulate Abl in the same way (Chong et al., 2005).

The clinical importance of this regulatory architecture becomes evident in the BCR-ABL fusion protein. Fusion of the BCR sequence to the N-terminus of ABL1 displaces the myristoylated cap, abolishing the myristoyl-dependent autoinhibitory latch, while the BCR-derived coiled-coil domain promotes oligomerization and trans-autophosphorylation. The combined loss of autoinhibition and forced clustering renders the kinase constitutively active. This structural understanding directly informs inhibitor strategy: it explains both why ATP-competitive inhibitors can suppress the active kinase and why restoring the vacant myristoyl pocket, as achieved by allosteric agents, represents a rational means of re-imposing the regulation the fusion has lost (Hantschel et al., 2012).

These conformational states have direct pharmacological consequences. Like other tyrosine kinases, Abl populates a spectrum of conformations defined by the position of the αC helix and the orientation of the conserved Asp-Phe-Gly (DFG) motif at the start of the activation loop. In the active "DFG-in" state the catalytic machinery is aligned, whereas in the inactive "DFG-out" state the motif flips, opening an adjacent hydrophobic pocket. Type I inhibitors such as dasatinib recognize the active

DFG-in conformation, while type II inhibitors such as imatinib and nilotinib exploit the DFG-out state, which contributes to their selectivity. The ability of Abl to adopt these distinct states is therefore not only central to its physiological regulation but also a key determinant of how, and how selectively, it can be drugged (Roskoski Jr, 2015).

Structural studies based on X-ray crystallography and cryo-EM have improved our understanding of Abl conformational changes and facilitated the design of new inhibitors. These studies continue to improve our understanding of kinase regulation and facilitate the rational design of inhibitors capable of targeting specific structural states associated with disease (Amatya et al., 2019)

1.4.2 Physiological functions of Abl

Abl kinases participate in numerous cellular processes essential for normal tissue homeostasis. Through interactions with actin filaments, microtubules, adhesion complexes, and signaling proteins, they regulate cytoskeletal organization, cell polarity, migration, and morphogenesis (Zhang et al., 2026).

Activation of Abl kinases by growth factors and adhesion receptors allows cells to coordinate changes in cytoskeletal dynamics during migration and tissue remodeling. In addition, Abl proteins contribute to the cellular response to DNA damage by regulating checkpoint activation, DNA repair pathways, and apoptosis (Kuwano et al., 2016). The DNA-damage response illustrates the distinct role of nuclear ABL1. Following genotoxic stress, nuclear ABL1 is activated downstream of the ataxia-telangiectasia mutated (ATM) kinase and contributes to the cellular decision between repair and death. It phosphorylates substrates involved in the damage response and can promote pro-apoptotic signalling, for example through effects on p53 and the related p73, so that severely damaged cells are eliminated rather than propagated. This places ABL1 at a checkpoint linking genome integrity to cell survival, a role quite separate from its cytoplasmic functions in cytoskeletal regulation and one that depends on the nuclear–cytoplasmic shuttling described above (Khatri et al., 2016; H. Zhang et al., 2021).

Another important physiological function of Abl kinases involves regulation of cell-cell and cell-matrix interactions (Rizzo et al., 2015). Through modulation of adhesion structures and signaling complexes, Abl contributes to tissue architecture and coordinated cellular movement. At the molecular level, the cytoskeletal functions of Abl are mediated through a defined set of effectors. Abl kinases phosphorylate and regulate actin-regulatory proteins, including members of the Ena/VASP family, the actin nucleator cortactin, and the WAVE regulatory complex, thereby controlling actin polymerization at the cell periphery. Through these substrates, Abl influences the formation of

lamellipodia, filopodia, and membrane ruffles that drive cell migration, and it couples signals from growth factor receptors and integrins to localized re-modelling of the actin network. This effector set links Abl directly to the same migratory machinery that becomes relevant when Abl signaling is deregulated in invasive tumors (Khatri et al., 2016; Wang & Pendergast, 2015).

The physiological functions of Abl kinases extend beyond the regulation of cytoskeletal dynamics and cellular migration. Increasing evidence indicates that ABL1 and ABL2 contribute to the coordination of cellular responses to environmental stress, including oxidative stress, nutrient deprivation, hypoxia, and genotoxic damage. Through interactions with multiple signaling pathways, Abl proteins help maintain cellular homeostasis under conditions that threaten cell survival (Hussein, 2021).

Abl kinases also contribute to embryonic development. Studies performed in genetically modified animal models have demonstrated that disruption of Abl signaling affects organ development, tissue architecture, and morphogenesis. These observations underscore the importance of precise Abl regulation throughout developmental processes and highlight the broad biological significance of this kinase family (Yildirim et al., 2025).

In the nervous system, Abl kinases have been implicated in neuronal differentiation, axonal guidance, synapse formation, and maintenance of neuronal connectivity. Although the precise molecular mechanisms remain incompletely understood, accumulating evidence suggests that Abl-dependent signaling contributes to the establishment and maintenance of functional neural networks (Franco-Villanueva et al., 2015).

Through these varied roles, Abl kinases act as signaling integrators that link extracellular cues to structural, metabolic, and transcriptional responses — which is also why their dysregulation has such broad consequences in disease.

1.4.3 Abl dysregulation in cancer

Deregulation of Abl signaling is strongly associated with cancer development and progression. The best-known example is the BCR-ABL fusion protein generated by the t(9;22) translocation (the Philadelphia chromosome; see Section 1.2.2), a constitutively active kinase that is the molecular hallmark of chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemias (Zhu & Gao, 2019)

Beyond hematological malignancies, increasing evidence indicates that ABL1 and ABL2 also contribute to the progression of solid tumors. Elevated expression or activation of Abl kinases has been observed in several cancers, where they promote migration, invasion, epithelial-to-

mesenchymal transition (EMT), and metastatic dissemination (Li et al., 2015).

The contribution of Abl kinases to solid tumors has been documented across several cancer types. In breast cancer, ABL1 and ABL2 are activated downstream of receptor tyrosine kinases and have been linked to invasion and metastatic behavior, while in lung and gastric cancers Abl activity has been associated with enhanced migration and disease progression. Importantly, in solid tumors the Abl kinases are generally not activated by the BCR-ABL fusion that characterizes CML, but rather through upstream signals — growth factor receptors, oxidative stress, and chemokine pathways — that relieve their normal autoinhibition. This distinction matters therapeutically, because it implies that Abl-directed inhibitors developed for leukemia might be repurposed against solid tumors in which Abl is activated by non-genetic mechanisms (Li et al., 2015; Wang & Pendergast, 2015).

At the mechanistic level, Abl kinases promote invasion through the same cytoskeletal and adhesion effectors that underlie their physiological functions. By regulating actin dynamics, focal-adhesion turnover, and the activity of Rho-family GTPases, activated Abl enhances cell motility and the capacity to remodel and penetrate the extracellular matrix. Abl signaling has also been implicated in epithelial-to-mesenchymal transition, the same dedifferentiation program described earlier for Src, contributing to loss of epithelial adhesion and acquisition of a migratory phenotype. The convergence of Src and Abl on overlapping invasive machinery provides part of the biological rationale for considering them together as therapeutic targets (Wang & Pendergast, 2015).

As shown in Figure 11, Abl kinases integrate signals originating from receptor tyrosine kinases, integrins, oxidative stress pathways, and metabolic stress responses. These signaling inputs converge on ABL1/2, which subsequently regulate multiple processes involved in tumor progression, including invasion, dissemination, extravasation, colonization of distant organs, and metastatic outgrowth (AlAbdi et al., 2024)

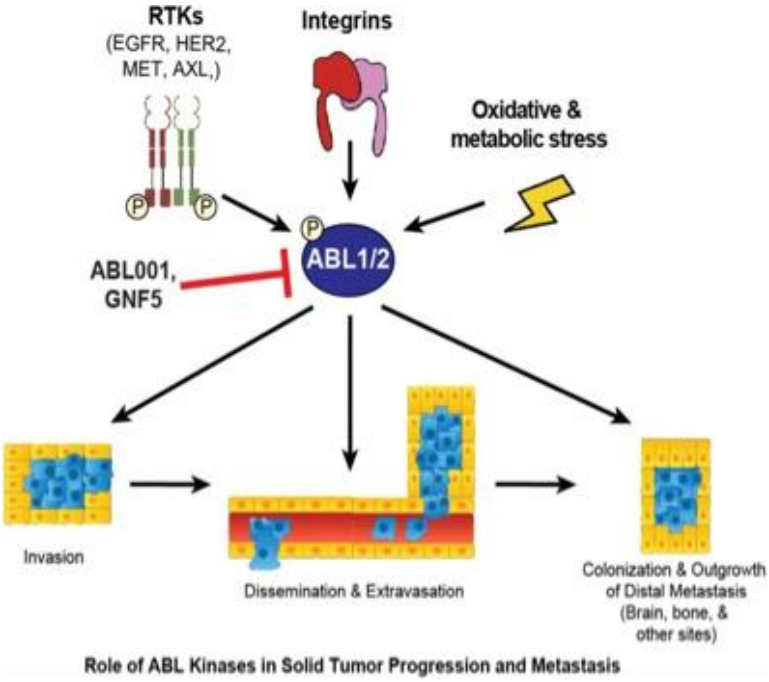


Fig. 11. The ABL kinases, ABL1 and ABL2 promote tumor progression and metastasis (AlAbdi et al., 2024).

Recent studies have also highlighted the importance of Abl signaling in metabolic adaptation and stress responses within tumor cells. In hereditary leiomyomatosis and renal cell carcinoma (HLRCC), ABL1 promotes tumor growth through signaling pathways involving mTOR, HIF1α, and NRF2, thereby supporting aerobic glycolysis and protection from proteotoxic stress (C. Zhang et al., 2021).

1.4.4 Abl inhibitors and clinical relevance

The discovery that constitutive Abl activation drives chronic myeloid leukemia led to one of the most significant breakthroughs in targeted cancer therapy. Prior to targeted therapy, treatment options for CML were limited and long-term prognosis was poor; the ATP-competitive inhibitor imatinib transformed this picture, demonstrating that selective inhibition of a single oncogenic kinase could produce durable responses and dramatically improve survival(Cohen et al., 2021; Zitvogel et al., 2016).

Since the introduction of imatinib, several second- and third-generation Abl inhibitors — including dasatinib, nilotinib, bosutinib, and ponatinib — have been developed to overcome resistance and improve efficacy, and are now used in the treatment of Philadelphia chromosome-positive leukemias (Soverini et al., 2019). Many of these compounds also inhibit Src family kinases to varying degrees, reflecting the structural similarity between the two families.

Resistance nevertheless remains an important clinical challenge. More than one hundred mutations have been identified within the BCR-ABL kinase domain, many of which reduce inhibitor binding, while activation of compensatory signaling pathways can further limit efficacy. Among these mutations, the T315I gatekeeper substitution has attracted particular attention because it confers resistance to several first- and second-generation inhibitors (Liu et al., 2021).

The development of ponatinib represented a major advance in overcoming T315I-mediated resistance, with structural modifications enabling efficient inhibition of the mutant while retaining activity against the native kinase. However, its increased potency has been associated with cardiovascular adverse events, highlighting the balance between efficacy and safety in kinase inhibitor development (Liu et al., 2021).

More recently, approaches distinct from ATP competition have emerged. The allosteric inhibitor asciminib binds the myristoyl pocket of ABL1 and restores an autoinhibited conformation resembling that of the native kinase (Ureshino & Kimura, 2026). This pocket is normally occupied by a lipid modification on native ABL1 that folds back onto the kinase domain to keep it inactive; because the BCR-ABL fusion lacks this myristoylation it loses access to that regulatory mechanism, yet the pocket itself remains structurally intact and pharmacologically accessible. Asciminib exploits this by occupying the vacant pocket directly, effectively restoring the autoinhibitory conformation the fusion would otherwise lack — a mode of action described as STAMP inhibition, in reference to its specific targeting of the ABL myristoyl pocket (Rea et al., 2021). Because this mechanism differs fundamentally from ATP-competitive inhibition, asciminib may retain activity against resistant variants and can be combined with conventional inhibitors to achieve more durable responses.

Beyond leukemia, growing evidence indicates that Abl signaling contributes to tumor invasion, metastatic dissemination, and adaptation to microenvironmental stress in several solid tumors, and experimental suppression of Abl can reduce invasion, metastasis, and survival signaling (Wang & Pendergast, 2015). The therapeutic potential of Abl inhibition therefore extends beyond hematological malignancies.

Despite these limitations, Abl remains one of the most clinically validated kinase targets in oncology (Røsland & Engelsen, 2015). Continued efforts to develop more selective inhibitors, overcome resistance, and define the context-dependent functions of Abl signaling are expected to expand the clinical utility of this kinase family.

The structural kinship between Src and Abl provides a coherent rationale for seeking compounds active against both. The two share a closely related SH3–SH2–kinase regulatory architecture and a highly similar ATP cleft, which is why agents such as dasatinib and bosutinib inhibit both families.

This shared architecture is also the central obstacle to selective inhibition. The ATP-binding pocket,

the site targeted by most inhibitors, is among the most conserved features of the kinase fold, so a molecule complementary to the Src pocket is often complementary to Abl, and frequently to other kinases as well. Selectivity therefore depends on exploiting the small differences that do exist — in the residues lining the pocket, in the size and accessibility of the region behind the gatekeeper, and in the conformational states each kinase preferentially adopts. The capacity of a scaffold to read these subtle differences, rather than the conserved core, is what ultimately determines whether it can discriminate between two related kinases (Roskoski Jr, 2015; Teli et al., 2023).

Against this background, dual Src/Abl activity can be either a liability or a designed feature. Indiscriminate inhibition across many kinases is undesirable, but controlled activity against a defined pair such as Src and Abl may be advantageous, since both kinases contribute to overlapping processes in tumor invasion and progression, and co-inhibition could limit the compensatory signaling that undermines single-target agents. The practical question for medicinal chemistry is how to tune a single scaffold along the Src–Abl axis: how small structural changes shift the balance of potency from one kinase toward the other (Tse & Verkhivker, 2015).

It is in this context that GC21 and GC22 were evaluated against both kinases, relating their structural differences to measurable differences in potency and selectivity

2. Aim of the work

Based on the therapeutic relevance of tyrosine kinases and the continuing need for improved kinase-directed therapies, the present study aimed to evaluate the biochemical properties of two newly synthesized small-molecule inhibitors, designated GC21 and GC22, as potential modulators of Abl and Src kinase activity.

To achieve this objective, recombinant human Src and Abl kinases were investigated using in vitro enzymatic assays based on the ADP-Glo™ Kinase Assay platform. This luminescence-based method enables quantitative measurement of ADP production during kinase-catalyzed phosphorylation reactions and therefore provides a reliable assessment of kinase activity under controlled experimental conditions.

The inhibitory activity of GC21 and GC22 was evaluated through dose–response experiments designed to determine the half-maximal inhibitory concentration (IC_{50}) for each compound against both target kinases. In addition, kinetic analyses were performed to estimate inhibition constants and to characterize the interaction between the inhibitors and their respective molecular targets. Comparison of the inhibitory profiles of the two compounds allowed assessment of differences in

potency and selectivity toward Src and Abl kinases.

Particular attention was devoted to identifying potential structure–activity relationships that could explain the distinct biological behavior of the two molecules. Understanding how relatively small structural modifications influence kinase inhibition may provide important information for future optimization efforts and facilitate the rational development of more selective and potent inhibitors.

The results obtained may serve as a foundation for future medicinal chemistry optimization and for the design of next-generation inhibitors directed against therapeutically relevant tyrosine kinases.

3. Material and Methods

3.1. Enzymes, substrates and reagents

Recombinant human Src and Abl kinases were obtained from commercial sources and used for in vitro enzymatic assays. Kinase activity was measured using the ADP-Glo™ Kinase Assay kit (Promega) according to the manufacturer's instructions with minor modifications. ATP served as the phosphate donor in the kinase reactions. Magnesium acetate and NP-40 were included to support enzymatic activity and maintain homogeneous reaction conditions. Dithiothreitol (DTT) was included in the buffer system as a reducing agent. Peptide substrates specific for Src and Abl kinases were used at concentrations at least twice their apparent K_m values to ensure near saturating substrate conditions during the reactions. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to the required working concentrations. The final concentration of DMSO was kept constant at 10% in all reactions. Dasatinib was included in each experiment as a reference inhibitor. To minimize adsorption of proteins and peptides to plastic surfaces, all reaction mixtures were prepared in protein low-binding tubes and subsequently transferred to white 384 well plates for luminescence measurements.

3.2 Kinase reaction conditions

All kinase reactions were carried out in a total volume of 10 µL. Separate reaction systems were prepared for Src and Abl kinases, each consisting of three components: Mix A, Mix B, and the ADP mix. The detailed compositions of these mixtures for the Src and Abl assays are summarized in Table 2 and Table 3, respectively. Mix A contained the recombinant kinase, the corresponding substrate peptide, NP-40 detergent, and the reaction buffer. Mix B consisted of ATP and reaction

buffer, and magnesium acetate (MgAc). The ADP mix served as the ADP control for the ADP-Glo™ luminescent detection system. All reactions were prepared using a 5× reaction buffer, containing 40 mM MOPS/NaOH (pH 7.0) and 1 mM EDTA, used for both the Src and Abl assays. Following reaction assembly, Src kinase mixtures were incubated at 30 °C for 10 minutes, whereas Abl kinase mixtures were incubated at 30 °C for 20 minutes prior to the addition of the ADP-Glo™ reagents.

Table 2. Src kinase assay reaction mixes:

Component	Mix A (1×)	Mix B (1×)	ADP mix (1×)
Reaction buffer (5×)	**1 µL**	**1 µL**	**1 µL**
NP-40 (0.0026%)	**1 µL**	**–**	**–**
Src peptide (500 µM)	**1 µL**	**–**	**–**
Src WT enzyme	**3.5 ng (0.035 µL)**	**–**	**–**
MgAc (200 mM stock)	**–**	**0.5 µL**	**0.5 µL**
ATP (10 mM stock)	**–**	**0.1 µL**	**–**
ADP (1 mM stock)	**–**	**–**	**1 µL**
H_2O (free)	**0.965 µL**	**3.4 µL**	**2.5 µL**
Total volume	**4 µL (Mix A)**	**5 µL (Mix B)**	**5 µL (ADP mix)**

Table 3. Abl kinase assay reaction mixes:

Component	Mix A (1×)	Mix B (1×)	ADP mix (1×)
Reaction buffer (5×)	**1 µL**	**1 µL**	**1 µL**
NP-40 (0.0026%)	**1 µL**	**–**	**–**
Abl TIDE peptide	**50 µM (0.67 µL)**	**–**	**–**
Abl WT enzyme	**0.1 µL**	**–**	**–**

MgAc (200 mM stock)	**–**	**0.5 µL**	**0.5 µL**
ATP (10 mM stock)	**–**	**0.1 µL**	**–**
ADP (1 mM stock)	**–**	**–**	**1 µL**
H_2O (free)	**1.23 µL**	**3.9 µL**	**2.5 µL**
Total volume	**4 µL (Mix A)**	**5 µL (Mix B)**	**5 µL (ADP mix)**

3.3 Inhibition assay procedure

Inhibition experiments were performed by pre-incubating the enzyme with test compounds before initiating the phosphorylation reaction. For each reaction, 4 µL of Mix A were combined with 1 µL of inhibitor solution. For the uninhibited control, 1 µL of pure DMSO was added instead of inhibitor. The mixtures were pre-incubated for 5 minutes at room temperature to allow interaction between enzyme and inhibitor. The kinase reaction was initiated by adding 5 µL of Mix B, resulting in a final reaction volume of 10 µL. Reactions were then incubated at 30 °C for 10 minutes for Src and 20 minutes for Abl. Control reactions included positive control (enzyme control) Mix A + DMSO + Mix B, representing 100 % enzyme activity; negative control (no-enzyme control) buffer, substrate, and ATP without enzyme to determine background luminescence. ADP control (ADP standard tube): a defined amount of ADP in buffer processed with ADP-Glo reagents to verify assay performance and maximal signal. Dasatinib was tested under the same conditions and served as a reference inhibitor to validate assay sensitivity and assay performance. All measurements were performed at least in duplicate.

3.4 ADP-Glo™ Detection Procedure

After completion of the kinase reaction, ADP production was quantified using the ADP-Glo™ Kinase Assay detection system. First, 10 µL of ADP-Glo™ reagent were added to each 10 µL kinase reaction to terminate enzyme activity and deplete the remaining ATP. Samples were incubated at room temperature for approximately 40-50 minutes. Subsequently, 20 µL of Kinase Detection Reagent were added to each reaction. This reagent converts ADP generated during the kinase reaction into ATP and simultaneously initiates the luciferase dependent luminescent reaction. Samples were incubated again at room temperature for 30-60 minutes while protected from light.

For luminescence measurement, reactions were transferred into white 384 well plates, and signals were recorded as relative light units (RLU) using a GloMax Discover microplate reader (Promega).

3.5 Data analysis

Luminescence readings were normalized against negative (no-enzyme) and positive (uninhibited) controls. Inhibition curves were plotted using GraphPad Prism 7.0, and IC_{50} values were calculated by nonlinear regression fitting to the following equation:

$v = V / (1 + (I / IC_{50}))$

where v is the observed activity, V the activity in the absence of inhibitor, and I the inhibitor concentration. The apparent inhibition constant K_i was estimated assuming ATP-competitive inhibition:

$K_i = IC_{50} / (1 + [ATP] / K_m)$

Kinetic parameters K_m and V_{max} were derived from Michaelis–Menten analysis:

$v = (V_{max} [S]) / (K_m + [S])$

All experiments were performed in at least two independent biological replicates, each containing two technical replicates. For every plate, raw luminescence (RLU) values were corrected against the no-enzyme negative control to subtract background signal, and the corrected values were normalized to the uninhibited (DMSO-only) positive control, defined as 100% kinase activity. Percentage inhibition was then calculated for each inhibitor concentration, and dose–response curves were fitted by nonlinear regression in GraphPad Prism 7.0 to obtain IC_{50} values, reported as mean ± standard deviation.

Apparent inhibition constants (K_i) were derived from the IC_{50} values using the Cheng–Prusoff equation for ATP-competitive inhibition, taking the assay ATP concentration as 100 µM and the apparent K_m(ATP) values determined in our laboratory as 95.95 µM for Src and 26.64 µM for Abl (Di Maria et al., 2022). Because the K_i depends on the ratio of [ATP] to K_m, the markedly lower K_m of Abl relative to Src was taken into account when comparing the two kinases. Dasatinib was included in every assay as a reference inhibitor to confirm assay performance.

4. Results and Discussion

4.1 Initial screening of GC21 and GC22

The biological activity of the synthesized compounds GC21 and GC22 was evaluated through in vitro

kinase inhibition assays against Abl and Src kinases. The inhibitory potency of the compounds was determined by calculating the half-maximal inhibitory concentration (IC_{50}) and the inhibition constant (K_i). These parameters provide quantitative information about the potency of the compounds and their apparent interaction with the enzyme. The IC_{50} values were obtained from dose–response experiments based on the percentage of enzyme inhibition at different inhibitor concentrations. All experiments were performed independently, and the resulting data were analyzed to determine the potency of each compound. The calculated IC_{50} and K_i values for the tested compounds are presented in Table 4. The obtained results indicate that both compounds exhibit inhibitory activity in the nanomolar range against the investigated kinases.

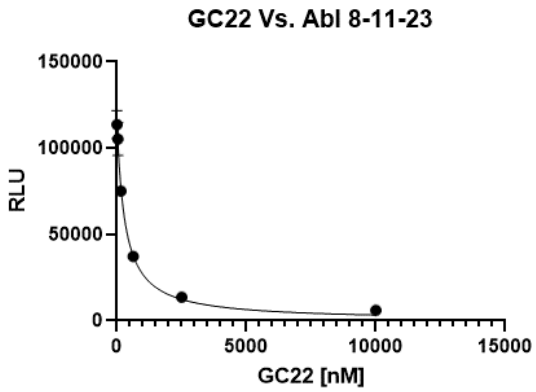
Table 4. Inhibitory activities of compounds GC21 and GC22 against Abl and Src kinases.

Compound	Target kinase	IC_{50} (nM)	K_i (nM)
GC21	Abl	218.12 ± 30.39	45.9
GC21	Src	65.71 ± 14.71	32.2
GC22	Abl	311.6 ± 36.25	65.5
GC22	Src	246.8 ± 29.53	120.8

4.2 Inhibitory activity against Abl kinase

Detailed dose–response experiments were performed to evaluate the inhibitory activity of GC21 and GC22 against Abl kinase. Enzymatic activity was measured using the ADP-Glo™ Kinase Assay following incubation with increasing concentrations of each compound. The resulting inhibition curves were analyzed by nonlinear regression to determine IC_{50} and K_i values**.**

The inhibitory profile obtained for GC22 is presented in Figure 12. Increasing concentrations of the compound produced a progressive decrease in Abl kinase activity, demonstrating a clear concentration-dependent inhibitory effect. At the highest concentration tested (10 μM), inhibition exceeded 94%, indicating that GC22 is capable of nearly complete suppression of Abl catalytic activity under saturating conditions. Intermediate concentrations generated proportional reductions in enzyme activity, confirming specific interaction with the kinase target. Nonlinear regression analysis yielded an IC_{50} value of 311.6 ± 36.3 nM and a corresponding K_i value of 65.5 nM. These values indicate that GC22 acts as a moderate inhibitor of Abl kinase, displaying activity in the submicromolar range but relatively limited potency compared with clinically optimized tyrosine kinase inhibitors.

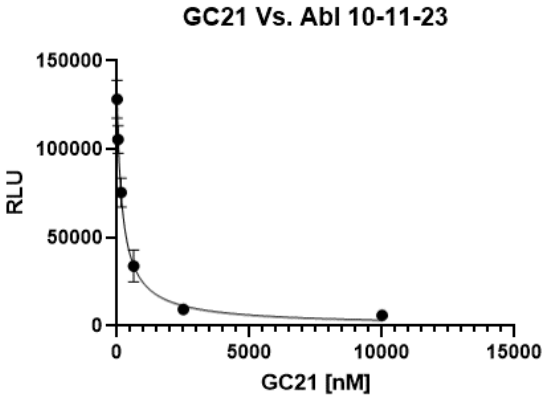


ID50 con exp	
Best-fit values	
V	114845
ID50	311.6
n	1.013
Std. Error	
V	2911
ID50	36.25
n	0.09612

Fig. 12. Dose–response curve of GC22 against Abl kinase.

The inhibitory behavior of GC21 toward Abl kinase was subsequently evaluated. As shown in Figure 13, GC21 produced a steeper dose–response curve and a more pronounced reduction in enzymatic activity throughout the concentration range tested. Analysis of the fitted curve generated an IC_{50} value of 218.1 ± 30.4 nM and a K_i value of 45.9 nM. Both parameters indicate stronger inhibition of Abl kinase compared with GC22. The approximately 1.4-fold reduction in IC_{50} suggests that structural modifications present in GC21 enhance productive interactions within the Abl ATP-binding site.

The smooth sigmoidal shape of both inhibition curves is consistent with a classical concentration-dependent inhibition mechanism. Overall, both compounds demonstrated biologically relevant inhibition of Abl kinase; however, GC21 emerged as the more potent inhibitor, exhibiting lower IC_{50} and K_i values. These results indicate that GC21 represents the most promising scaffold for further optimization within this compound series.



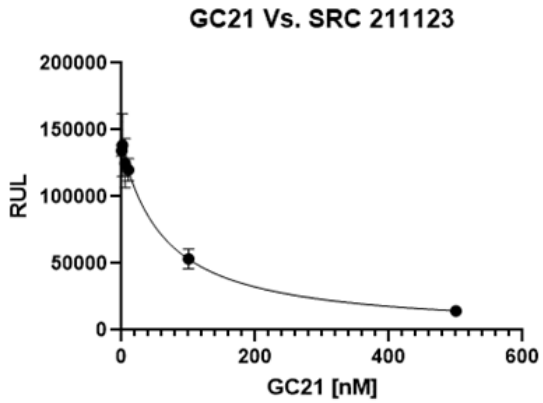
ID50 con exp	
Best-fit values	
V	128013
ID50	218.1
n	0.9532
Std. Error	
V	3841
ID50	30.39
n	0.1033

Fig. 13. Dose–response curve of GC21 against Abl kinase.

The inhibitory activity observed for both compounds against Abl kinase is particularly relevant considering the importance of Abl signaling in oncogenesis. Although the measured IC_{50} values are higher than those reported for clinically optimized inhibitors such as imatinib, nilotinib, and ponatinib, the submicromolar activity of these early-stage compounds is encouraging and suggests that both molecules possess structural features compatible with productive interaction within the Abl catalytic domain.

4.3 Inhibitory activity against Src kinase

The inhibitory activity of GC21 and GC22 was evaluated against Src kinase using the ADP-Glo™ Kinase Assay. Since Src is a key non-receptor tyrosine kinase involved in signaling pathways regulating cell proliferation, migration, invasion, and metastatic progression, the identification of potent Src inhibitors represents an important objective in targeted anticancer drug discovery. Dose–response analyses were therefore performed to determine the potency and selectivity of the synthesized compounds toward this therapeutically relevant target.



ID50 con exp	
Best-fit values	
V	136017
ID50	65.71
n	1.045
Std. Error	
V	4857
ID50	14.71
n	0.1959

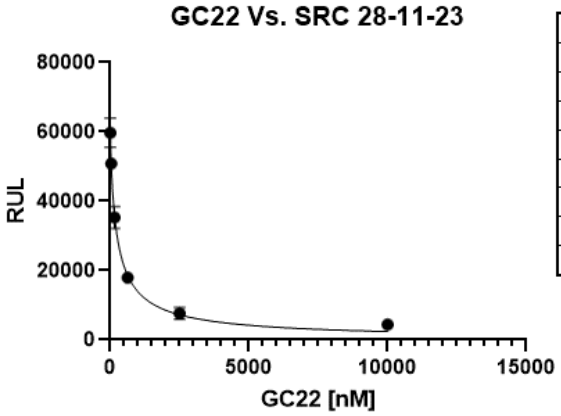
Fig. 14. Dose–response curve of GC21 against Src kinase.

The inhibition profile obtained for GC21 against Src kinase revealed a strong concentration-dependent reduction in enzymatic activity. The dose–response curve exhibited the typical sigmoidal shape expected for a reversible kinase inhibitor and showed a marked decrease in kinase activity even at relatively low concentrations. Near-complete inhibition was achieved at the highest concentrations tested, indicating highly efficient suppression of Src catalytic activity. Nonlinear regression analysis generated an IC_{50} value of 65.71 ± 14.71 nM and a corresponding K_i value of 32.2 nM. This is the strongest inhibitory activity observed among all compound–kinase combinations investigated in this study. The low-nanomolar IC_{50} indicates good potency, and the K_i — obtained after correcting for the assay ATP concentration relative to the Src K_m — confirms an effective interaction with the Src ATP-binding pocket.

The high potency of GC21 toward Src kinase is clearest when compared with its activity against Abl. Whereas inhibition of Abl required concentrations exceeding 200 nM to achieve 50% suppression of enzyme activity, only approximately 66 nM GC21 was sufficient to inhibit Src kinase by 50%. This approximately 3.3-fold difference between Src and Abl strongly suggests a preferential interaction with structural features specific to the Src catalytic domain. Such selectivity is desirable because it may allow more precise modulation of Src-dependent signaling pathways while reducing potential off-target effects associated with broader kinase inhibition.

The pronounced difference between the activities of GC21 and GC22 suggests that specific structural elements present in GC21 contribute markedly to target recognition. Although the precise molecular interactions remain to be elucidated, GC21 likely establishes more favorable contacts within the Src ATP-binding site. Structural studies such as molecular docking or crystallography could identify the determinants responsible for the observed selectivity.

The inhibitory potency of GC21 toward Src kinase, with an IC_{50} value of approximately 65.71 nM, warrants comparison with clinically established Src inhibitors. Dasatinib, one of the most potent approved inhibitors of Src family kinases, displays IC_{50} values in the low nanomolar range (0.5–1 nM against Src), while bosutinib exhibits IC_{50} values of approximately 1.2 nM. Although GC21 does not reach the sub-nanomolar potency of these optimized clinical agents, its activity in the low nanomolar range is encouraging for an early-stage, non-optimized compound. Moreover, unlike dasatinib and bosutinib, which were primarily developed as BCR-ABL inhibitors and display broad multi-kinase inhibition profiles, GC21 demonstrates a measurable degree of selectivity toward Src over Abl. This selectivity profile, even if preliminary, may represent a pharmacologically relevant starting point for the development of more selective Src-directed agents with potentially improved tolerability.



ID50 exp	
Best-fit values	
V	60061
ID50	246.8
n	0.8568
Std. Error	
V	1444
ID50	29.53
n	0.07195

Fig. 15. Dose–response curve of GC22 against Src kinase.

The inhibitory activity of GC22 toward Src kinase was also evaluated under identical experimental conditions. As shown in Figure 15, GC22 produced a concentration-dependent reduction in kinase activity; however, substantially higher concentrations were required to achieve inhibition levels comparable to those observed for GC21.

Analysis of the dose–response data yielded an IC_{50} value of 246.8 ± 29.53 nM and a K_i value of 120.8 nM. Although GC22 remained active within the submicromolar range, its inhibitory potency was considerably lower than that of GC21. The approximately fourfold increase in IC_{50}, and the correspondingly higher K_i, indicate a reduced ability to inhibit Src under these conditions.

Comparison of the two Src inhibition profiles clearly demonstrates the influence of structural modifications on biological activity. While both compounds inhibit Src kinase, GC21 exhibits notably

greater potency. The differences are particularly evident at lower inhibitor concentrations, where GC21 maintains strong inhibitory activity whereas GC22 displays a more gradual reduction in enzyme function. These observations suggest that specific structural features present in GC21 establish additional favorable interactions within the ATP-binding pocket, resulting in enhanced target recognition and improved inhibitory efficiency.

Taken together, the results obtained for Src kinase establish a clear ranking of inhibitory activity within the investigated compound series. GC21 emerged as the most potent inhibitor, displaying nanomolar activity and the lowest IC_{50} of the series, whereas GC22 exhibited a weaker but still measurable inhibitory effect. These findings highlight Src kinase as the preferred molecular target of GC21 and provide a strong rationale for further development of this compound as a potential Src-directed therapeutic lead.

4.4 Comparative analysis of Abl and Src inhibition

A comparative analysis of the inhibitory profiles obtained for Abl and Src kinases revealed substantial differences in both potency and selectivity between the two investigated compounds. GC21 exhibited markedly stronger inhibition of Src kinase than of Abl kinase, with IC_{50} values of 65.71 ± 14.71 nM and 218.12 ± 30.39 nM, respectively. This approximately 3.3-fold difference indicates a clear preference of GC21 for Src. The corresponding K_i values for GC21 are 32.2 nM against Src and 45.9 nM against Abl.

In contrast, GC22 displayed a considerably less selective inhibitory profile. The IC_{50} values obtained for Src and Abl were 246.8 ± 29.53 nM and 311.6 ± 36.25 nM, respectively, indicating only a modest difference in potency between the two targets. Similarly, the calculated K_i values remained within the same order of magnitude, suggesting weaker discrimination between the catalytic domains of Src and Abl. To quantify the relative preference of each compound between the two kinases, selectivity ratios were calculated as the ratio of Abl to Src values for both IC_{50} and K_i in Table 5.

Table 5. Src/Abl selectivity of GC21 and GC22, expressed as the ratio of Abl to Src values.

Compound	**Src IC_{50} (nM)**	**Abl IC_{50} (nM)**	**IC_{50} selectivity (Abl/Src)**	**Src K_i (nM)**	**Abl K_i (nM)**	**K_i selectivity (Abl/Src)**
GC21	65.71	218.12	3.3-fold	32.2	45.9	1.4-fold
GC22	246.8	311.6	1.3-fold	120.8	65.5	0.5-fold

These ratios confirm that GC21's preference for Src is clear at the IC_{50} level but modest once corrected

to K_i, while GC22 shows little consistent selectivity between the two kinases.

When the two compounds are compared directly, GC21 emerged as both the most potent and the most selective inhibitor identified in this study. In particular, its activity against Src was approximately fourfold greater than that of GC22 (246.8 vs 65.71 nM) and was associated with a correspondingly lower K_i. Conversely, GC22 behaved as a moderate inhibitor of both kinases, displaying lower potency and limited target selectivity.

GC21 also achieves a favorable balance between potency and selectivity — strong inhibitory activity together with measurable target preference — which is desirable in lead development, since very high potency with poor selectivity can cause off-target effects, while high selectivity with weak activity limits efficacy.

4.5 General discussion

The two compounds examined here behave consistently with ATP-competitive inhibition of the kinase catalytic domain, the assumption on which the K_i values were derived (Section 3.5). Within this framework, the divergent behaviour of GC21 and GC22 is most readily explained by differences in how each scaffold engages the regions of the pocket that distinguish Src from Abl. Because the catalytic core common to the two kinases is highly conserved (Section 1.4), a compound that contacts only this core is expected to inhibit both enzymes comparably—the pattern observed for GC22, whose similar potencies against Src and Abl point to recognition of conserved features. The preference of GC21 for Src, by contrast, implies that part of its scaffold reaches features less uniformly shared between the two kinases, such as the environment behind the gatekeeper or the residues lining the entrance to the pocket (Section 1.2.5). The structural basis of this preference cannot be assigned from biochemical data alone and would require the docking or crystallographic studies outlined in Section 5.3.

The relationship between the IC_{50}- and K_i-based selectivity merits closer attention, because the two give a different impression of the same compound. At the level of IC_{50}, GC21 prefers Src over Abl by roughly threefold; once corrected to K_i, that preference narrows to less than half as much. As set out in Section 1.2.5, this is the expected consequence of the lower ATP affinity of Abl under the assay conditions: a portion of the apparent selectivity reflects the different ATP-binding characteristics of the two enzymes rather than a difference in the affinity of the inhibitor itself. The practical implication is that selectivity claims for ATP-competitive compounds should be framed in terms of K_i, and that the modest intrinsic preference of GC21 is more realistic than the IC_{50} ratio alone would suggest. This distinction also has a bearing on the cellular setting, where ATP is present at concentrations far above

those used in vitro; for an ATP-competitive inhibitor, higher ambient ATP raises the effective concentration required for inhibition and can compress differences between targets, so the selectivity measured biochemically should be regarded as an upper estimate of what would be seen in cells.

Viewed against established agents, GC21 is best understood as an early, unoptimised starting point rather than a finished inhibitor. Its low-nanomolar potency toward Src is short of the sub-nanomolar activity of clinically optimised compounds (Section 4.3), but it is accompanied by a measurable preference for Src over Abl, whereas the approved dual Src/Abl agents were optimised for broad activity rather than discrimination between the two families. A scaffold that already reads, however modestly, the small differences between two closely related kinases is a more promising basis for selective optimisation than one that does not, since selectivity is generally easier to build upon than to introduce. On this reasoning GC21, rather than GC22, is the appropriate focus for the medicinal-chemistry steps proposed in Section 5.3.

These interpretations are bounded by the limitations set out in Section 5.2. The mode of inhibition was assumed rather than established, the compounds were tested against only two kinases and at a single ATP concentration, and no cellular or structural data are yet available. The conclusions drawn here therefore concern the biochemical behaviour of the compounds and the rationale for prioritising GC21, and are intended to frame, rather than pre-empt, the further work required to test them.

5. Conclusions

5.1 Summary of findings

The present study aimed to evaluate the inhibitory activity, potency, and selectivity of two newly synthesized small molecules, GC21 and GC22, against the non-receptor tyrosine kinases Abl and Src. Through a combination of enzymatic inhibition assays, dose–response analyses, and kinetic parameter determination, the biochemical properties of both compounds were successfully characterized.

The results demonstrated that the two molecules exhibit markedly different inhibitory profiles despite belonging to the same chemical series. GC21 consistently showed superior inhibitory activity compared with GC22 and emerged as the most potent compound investigated in this work. GC21's preference for Src over Abl indicates a measurable degree of target selectivity. In contrast, GC22 exhibited weaker inhibition of both kinases and only limited selectivity, suggesting a broader but less efficient binding profile.

The comparative analysis highlighted the importance of structural features in determining kinase recognition and inhibition. The substantial differences observed between GC21 and GC22 indicate that relatively small chemical modifications can markedly affect inhibitory potency and target selectivity. These findings provide valuable information regarding the structure–activity relationships governing this class of compounds and support the concept that selective kinase inhibition can be achieved through rational molecular optimization.

From a drug discovery perspective, GC21 represents the most promising lead compound identified in this study. Its nanomolar potency toward Src kinase, combined with its preferential activity relative to Abl, suggests that this scaffold may serve as a suitable starting point for the development of more selective Src-directed inhibitors. Such compounds could prove valuable both as research tools for studying Src-dependent signaling pathways and as potential candidates for future therapeutic development.

Although the present work was limited to biochemical characterization using purified enzymes, the data generated provide a solid foundation for further investigations. Overall, the study successfully achieved its objective of identifying and characterizing novel tyrosine kinase inhibitors and contributes to the ongoing effort to develop more potent and selective kinase-targeted agents. The results obtained establish GC21 as a promising scaffold for future optimization and support continued research aimed at the development of next-generation tyrosine kinase inhibitors.

5.2 Limitations of the study

Several limitations should be considered when interpreting the results of the present study. First, the inhibitory activity of GC21 and GC22 was evaluated exclusively through in vitro enzymatic assays. Although these experiments provide reliable information regarding kinase inhibition, selectivity and inhibitor potency, they do not fully reproduce the complexity of cellular systems, where multiple signaling pathways interact simultaneously and may influence the biological response to kinase inhibition.

A second limitation concerns the restricted number of molecular targets investigated. The study focused solely on Abl and Src kinases, whereas protein kinases belong to a large superfamily characterized by significant structural conservation, particularly within the ATP-binding pocket. Consequently, it remains possible that GC21 and GC22 may interact with additional kinases not examined in the present work.

Furthermore, no cell-based experiments were included in this study. As a result, the effects of GC21

and GC22 on cellular proliferation, apoptosis, migration, and intracellular signaling pathways were not evaluated. Similarly, potential cytotoxic effects and cellular uptake properties remain unknown. The inhibitory activity observed in biochemical assays may not necessarily translate into equivalent activity in living cells, where factors such as membrane permeability, protein binding, intracellular ATP concentration, and metabolic stability can significantly affect compound performance.

Another limitation relates to the absence of structural and mechanistic investigations. Techniques such as molecular docking, molecular dynamics simulations, or X-ray crystallography could provide valuable information regarding the molecular interactions responsible for the observed differences in potency and selectivity.

A further limitation concerns the determination of the inhibition constants. The K_i values reported here were derived from IC_{50} values using the Cheng–Prusoff relationship, which assumes competitive inhibition with respect to ATP. The actual mode of inhibition of GC21 and GC22 — competitive, non-competitive, or mixed — was not established experimentally, for example by measuring activity across a range of ATP concentrations. The K_i values should therefore be regarded as apparent estimates valid under the conditions used rather than as definitive constants.

In addition, the assays were performed in duplicate at a single ATP concentration and with a single peptide substrate. Because IC_{50} values in ATP-competitive systems depend on the ATP concentration used, the potencies reported are specific to these conditions, and a larger number of independent replicates would allow more robust estimation of potency and its associated error.

Finally, the compounds were not counter-screened for nonspecific inhibition mechanisms such as colloidal aggregation, which can produce apparent dose-dependent inhibition in biochemical assays. Although the well-behaved sigmoidal curves and nanomolar potencies are consistent with specific, reversible inhibition, orthogonal experiments would be needed to formally exclude such artefacts.

Despite these limitations, the biochemical characterization performed in this work provides a sound basis for identifying promising lead compounds and represents an important first step toward the development of more potent and selective tyrosine kinase inhibitors.

5.3 Future perspectives

The findings obtained in this study open several avenues for future research. An important next step will be the evaluation of GC21 and GC22 against a broader kinase panel in order to determine their selectivity profiles more comprehensively. Such analyses would clarify whether the compounds act as highly selective inhibitors or exhibit activity toward additional members of the human kinome.

Given the close structural similarity among Src-family kinases, it would also be valuable to test GC21 against other family members such as Lyn, Fyn, and Yes. This would establish whether the selectivity seen between Src and Abl extends to discrimination *within* the Src family, which is one of the central challenges in developing SFK-directed inhibitors.

Future investigations should also include cell-based assays to assess the biological activity of these compounds in a more physiologically relevant context. Evaluating their effects on cancer cell proliferation, apoptosis, migration, and intracellular signaling pathways would provide important information regarding their potential therapeutic relevance and help establish whether the biochemical inhibition observed in vitro translates into cellular efficacy.

Structural studies represent another valuable direction for future work. Computational approaches such as molecular docking and molecular dynamics simulations, together with experimental techniques including X-ray crystallography, could help identify the molecular determinants responsible for the different inhibitory profiles of GC21 and GC22. A detailed understanding of inhibitor–kinase interactions would facilitate rational optimization of the most promising compounds.

Particular attention should be devoted to the further development of the GC21 scaffold. Given its superior potency and selectivity toward Src kinase, systematic medicinal chemistry optimization could be undertaken to improve binding affinity, selectivity, metabolic stability, and overall pharmacological properties. Such efforts may lead to the generation of more effective kinase inhibitors with enhanced therapeutic potential.

A logical next step would be to determine the mechanism of inhibition directly, by measuring enzyme kinetics across a range of ATP concentrations. Establishing whether GC21 and GC22 are ATP-competitive would confirm the basis of the K_i values reported here and clarify how the compounds engage the catalytic domain.

Finally, future studies should address the pharmacokinetic and in vivo behavior of these compounds. Investigations into absorption, distribution, metabolism, excretion, and toxicity, together with efficacy studies in suitable animal models, will be essential to determine whether these inhibitors possess characteristics compatible with further preclinical development.

Overall, the results presented in this thesis identify GC21 as a promising lead compound and provide a strong rationale for continued research aimed at the development of next-generation Src-directed tyrosine kinase inhibitors.

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