Chapter

Protein Kinase Inhibitors - Selectivity or Toxicity?

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Abstract

Protein kinases are attractive therapeutic targets for various indications including cancer, cardiovascular, neurodegenerative and autoimmune diseases. This is due to the fact that they play key roles in the regulation of cell cycle, metabolism, cell adhesion, angiogenesis, regeneration and degeneration. Protein kinase families share a common catalytic core and hence usually display clear sequence and structural similarity. These sequence and structural similarities can lead to a lack of selectivity and off-target toxicity of drug candidates. The lack of selectivity can be beneficial but can also cause adverse toxicities which result in the discontinuation of promising drug candidates. The chapter reviews the challenges and common toxicities of protein kinase inhibitors and the latest advances in in-vitro and in-silico assays to screen for selectivity. The various methods for quantifying selectivity of kinase inhibitors and future directions including emerging more selective and safer kinase inhibitors have also been discussed.

Keywords: Selectivity, Kinase inhibitors, Toxicity

1. Introduction

Protein kinases belong to a huge family of more than 500 enzymes that phosphorylate proteins in response to an external stimulus, via transfer of the γ-phosphate group from ATP to serine, threonine, or tyrosine residues on the target protein [1]. By doing so they regulate the function of many proteins, and mediate and influence a variety of cellular processes including proliferation, metabolism, adhesion, angiogenesis, regeneration and degeneration [2]. Since most protein kinases are involved in pivotal biological reactions, it is not surprising that dysregulation of the activities of kinases is the hallmark of many pathological conditions such as cancer [3], autoimmunity [4], inflammation [5], and neurological disorders [6]. In particular, genetic alteration in various protein kinases is associated with their over-expression and disease pathology, but also with drug response and resistance. Accordingly, more than 250 kinase inhibitors (KIs) are currently undergoing clinical trials and more than 50 have been approved for use by the Food and Drug Administration (FDA) [7]. Even though most therapeutic agents are mainly for oncologic indications such as imatinib (first small molecule KI approved for chemotherapy in 2001), gefitinib, sorafinib, erlotinib, dasatinib and crizotinib, there are also emerging KIs for other indications

such as rheumatoid arthritis (RA), inflammatory bowel disease, alopecia areata, psoriasis, idiopathic pulmonary fibrosis, organ rejection prophylaxis, glaucoma and neurodegenerative diseases such as Alzheimer disease [8]. In fact, KIs have become one of the most prevalent druggable targets with an estimated one-third of drug discovery programs aimed at developing KIs. Kinase inhibitors have transformed the treatment of many cancers and are showing the same promise for other indications. However, their development has been challenging due to severe toxicities observed in preclinical studies and clinical trials [9, 10].

Most of the approved KIs are small molecules, which may be classified into six main groups according to their binding site on the enzyme [11, 12]: KIs which bind to the ATP pocket in the active conformation of a kinase are classified as Type I; KIs that bind adjacent to the ATP pocket (adenine binding residues) of the unphosphorylated inactive conformation of kinases are classified as Type II; non-ATP competitive inhibitors that bind within the cleft between the small and large lobes close to the ATP binding pocket are classified as Type III; allosteric inhibitors that bind away from the ATP cleft are classified as Type IV; agents that span two distinct regions of the protein kinase domain are classified type V inhibitors; and agents that form covalent bonds with their target enzyme are classified type VI inhibitors [7].

These different binding modes not only influence the potency and mechanism of action of these inhibitors, but may also affect their selectivity and consequently their safety profile. Since type I inhibitors bind to the well-conserved ATP binding site shared by most protein kinases, these inhibitors are often less selective, and interact with multiple members of the protein kinase family. For example, most of the ATP-competitive inhibitors such as imatinib, nilotinib, dasatinib, bosutinib, and ponatinib that successfully target the oncoprotein kinase ABL1, exhibit notable off-target activities on kinases such as c-KIT, CSF1R, and PDGFRA/B [8] **Figure 1** shows an overlay of the crystal structures of five representative kinases from different families with their respective small molecule inhibitors bound to the ATP active site, demonstrating why type I inhibitors usually have low selectivity. This cross reactivity is associated with toxicities in the clinic and often leads to discontinuation of promising drug candidates during development as will be described in this chapter. Type II KIs demonstrate improved selectivity profiles compared with type I inhibitors because they bind adjacent to the ATP binding pocket in the inactive kinase conformational state, a site which is less conserved within the kinome [14]. Type III-VI inhibitors, which are non-ATP competitive inhibitors that often do not bind to conserved residues, offer greater selectivity and reduced toxicity compared to other types of KIs as they are likely to have less off-target effects [15–17]. In addition, the toxicity profiles of KIs depend on which kinase was targeted, the affinity of the inhibitor for the particular kinase and on the role that this kinase plays in intracellular signaling and overall cell function.

Off-target activity of KIs are not necessarily limited to kinases. Non-kinase off-targets of KIs often remain undiscovered, and may lead to misinterpretation of the cause of toxicity [18]. That is, KIs may also bind to other enzymes, ion channels and/or receptors and thus performing a full binding profile is important to understand all potential targets as well as subsequently assessing off-target activity. However, off-target activity is not always a cause for concern for therapeutic agents as the off-target activity may have positive therapeutic outcomes. Multi-targeted KIs may target a pathway at different points e.g. upstream and downstream and thus multi-targeted KIs may have improved efficacy compared to more selective inhibitors. Additionally, KIs with off-target activity in different pathways may be beneficial for targeting different indications [19, 20].

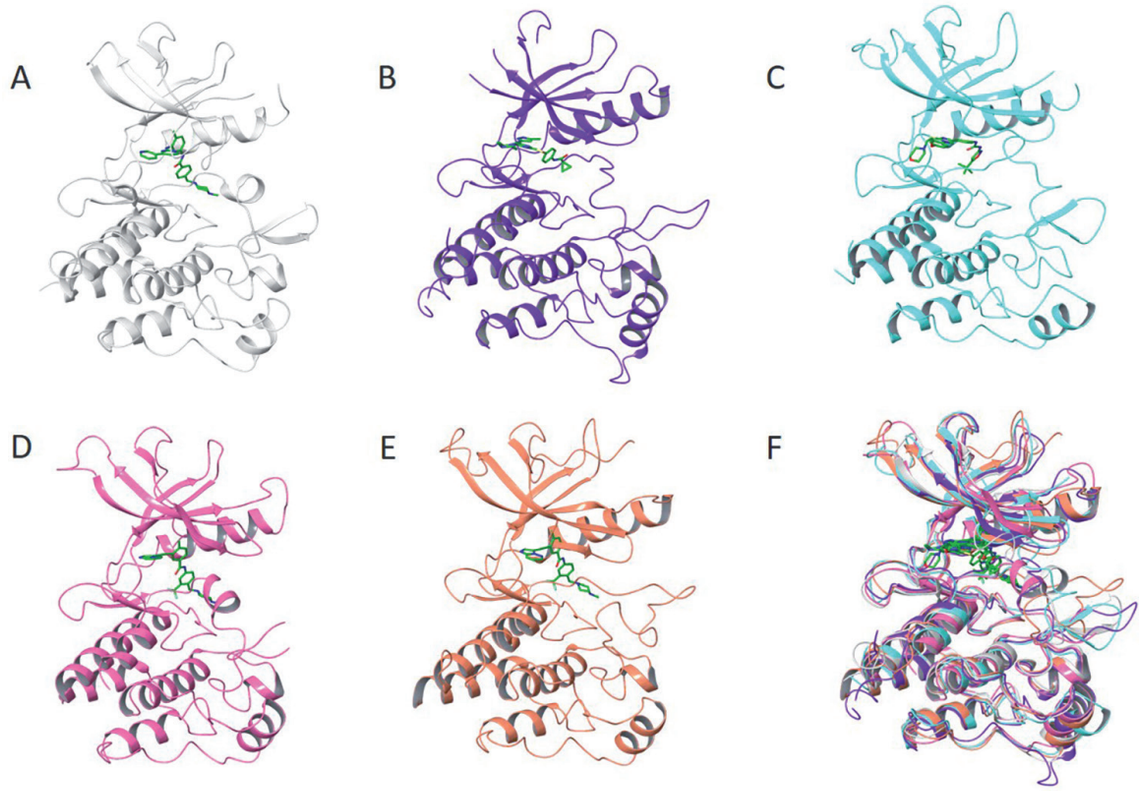

Figure 1.
Crystal structures of representative kinases complexed with small molecule inhibitors at the conserved ATP active site. A) Human Abl kinase domain in complex with imatinib. PDB ID: 2HYY. B) DDR1 bound to VX-680. PDB ID: 6BRJ. C) Crystal structure of the FLT3 kinase domain bound to the inhibitor quizartinib, PDB ID: 4XUF. D) The ROR1 Pseudokinase Domain Bound To Ponatinib. PDB ID: 6TU9. E) FGFR4 in complex with Ponatinib. PDB: 4UXQ. F) overlay of the five crystal structures (A-E) showing the high structural homology between the different kinases. The small molecule inhibitors are shown in green sticks. Images were drawn using the maestro software [13].

In addition to small molecule inhibitors, there are also several monoclonal antibodies (mAbs) that specifically and selectively target and block the extracellular domain of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR, HER1, ErbB-1), and human epidermal growth factor receptor 2 (HER2, ErbB-2) [21]. By targeting the extracellular part of the receptor tyrosine kinase, the mAb is able to block the binding of the natural ligand, avoid receptor conformational rearrangement essential to the activation of the kinase and thus the activation of the downstream signaling pathways. Even though mAbs therapies are often considered as very specific and safer therapeutic agents, anti-EGFR antibodies, such as cetuximab and panitumumab, have been associated with severe skin, renal, and gastrointestinal toxicities as they block essential cellular signaling pathways [22, 23]. In addition, the development of chemoresistance in a large portion of patients due to the ability of cells to re-activate pro-angiogenic factors via alternate pathways (i.e. increasing VEGF production), also hampered the success of these drugs in the clinic [24].

This chapter will review the challenges of developing safe protein KIs, the latest advances in assays to screen for selectivity and future directions.

2. Common toxicities with kinase inhibitors

Adverse effects from KIs may be classified into on- and off-target toxicities. "On-target" toxicity results when the inhibition of the targeted kinase is responsible

for both the intended pharmacodynamic effect and the unintended toxicity. On the contrary, "off-target" toxicity is the result of low selectivity and inhibition of kinases for which the drug was not intended.

KIs are associated with undesirable adverse reactions that impact on quality of life and compliance of patients, even though less than traditional chemotherapy. These effects include cardiovascular toxicity, hepatotoxicity, hematotoxicity, dermatological and ocular toxicities, gastro-intestinal symptoms, hyperphosphatemia and tissue mineralization [25]. For example, EGFR-Tyrosine KIs (TKIs) are typically associated skin rash, diarrhea, hepatotoxicity, stomatitis, interstitial lung disease and ocular toxicity, but these effects are usually mild in most cases [26]. In this chapter we decided to focus on the mechanism of three major toxicities: cardiovascular toxicity, hepatotoxicity and hematotoxicity. This decision was not based on the incidence of the side effects but rather the impact of these particular side effects on patients. These side effects have a great impact on patients due to the effects on their well-being and compliance to treatment. In addition, these particular side effects are a major cause for discontinuing the development of many molecules. Therefore, improving the selectivity of compounds in a manner that will not interfere with or avoid the mechanisms outlined below is important.

2.1 Cardiovascular toxicity

Many protein kinases are critical for normal function of cardiomyocytes and/or the vasculature, and thus their inhibition results in "on-target" cardiotoxicity due to the overlap in the targeted pathway for cancer progression and the pathway for regulation of cardiac function [27]. Protein kinase signaling plays a significant role in cardiac hypertrophy (increase in cell size) under physiological conditions in response to an increased workload, such as exercise or pregnancy, but the relative importance of individual kinases is not clear. Microarray analysis of protein kinase mRNA expression in dozens of non-failing human heart biopsies detected 402 protein kinase mRNAs that are constantly expressed under normal, non-pathologic conditions [28]. Therefore, it is not surprising that many KIs have a cardiovascular (CV) toxicity warning and precautions in their US prescribing information. The adverse cardiac events listed in the US prescribing information for cancer patients following treatment with KIs include: QT prolongation, hypertension, left ventricular dysfunction (LVD), congestive heart failure (CHF), acute coronary syndromes (ACS), and myocardial infarction (MI) [27]. For example, targeting VEGF is associated with hypertension, and targeting human epidermal growth factor receptor 2 (HER2), VEGFR, and/or Bcr-Abl is often accompanied with LVD and CHF [29, 30]. The consequence cardiac toxicity among new KIs due to "on-target" activities and lack of selectivity, limits development of new drugs even when targeting life-threatening therapeutic indications such as cancer.

Not all KIs, however, are prone to cardiotoxicity. More selective KIs targeting the Janus kinase (JAK) family, such as tofacitinib targeting Janus kinase (JAK) 1/3 for the treatment of RA, psoriatic arthritis, ulcerative colitis, and the selective JAK2 inhibitor, fedratinib, are not associated with cardiotoxicity (see **Table 1**). Similarly, inhibitors of mammalian target of rapamycin (mTOR) such as sirolimus, temsirolimus and everolimus developed for different malignancies are also not accompanied with cardiotoxicity (see **Table 1**, US prescribing information for sirolimus, temsirolimus and everolimus) despite the role of mTOR signaling in cardiac physiology [31]. These examples demonstrate that cardiotoxicity may be overcome by increasing selectivity.

Drug	Year of approval	primary target	Indication	Most common adverse reactions and warnings (as mentions in the US prescribing information)
BCR-Abl inhibitors				
Bosutinib	2012	BCR-Abl	Chronic myelogenous leukemias	Diarrhea, nausea, thrombocytopenia, rash, increased alanine aminotransferase, abdominal pain, increased aspartate aminotransferase, thrombocytopenia, vomiting, anemia, fatigue, pyrexia, cough, headache, alanine aminotransferase, and edema
Dasatinib	2006	BCR-Abl, SRC kinase	Chronic myelogenous leukemias	Myelosuppression (thrombocytopenia, neutropenia, and anemia may occur), fluid retention events, diarrhea, headache, skin rash, hemorrhage, dyspnea, fatigue, nausea, and musculoskeletal pain
Imatinib	2001	BCR-Abl	Philadelphia chromosome-positive CML or ALL, aggressive systemic mastocytosis, chronic eosinophilic leukemias, dermatofibrosarcoma protuberans, hypereosinophilic syndrome, gastrointestinal stromal tumors, myelodysplastic/ myeloproliferative disease	Cytopenias, particularly anemia, neutropenia, and thrombocytopenia, hepatotoxicity, heart failure and left ventricular dysfunction, edema, nausea, vomiting, muscle cramps, musculoskeletal pain, diarrhea, rash, fatigue and abdominal pain
EGFR and VEGFR inhibitors				
Cetuximab	2004	EGFR	Head and neck cancer and colorectal cancer	Fatal infusion reactions and Cardiopulmonary arrest. Cutaneous adverse reactions (including rash, pruritus, and nail changes), headache, diarrhea, and infection
Panitumumab	2006	EGFR	Colorectal Cancer	Skin toxicities (i.e., erythema, dermatitis acneiform, pruritus, exfoliation, rash, and fissures), paronychia, hypomagnesemia, fatigue, abdominal pain, nausea, diarrhea, and constipation
Erlotinib	2004	EGFR	NSCLC, pancreatic cancers	NSCLC: rash, diarrhea, anorexia, fatigue, dyspnea, cough, nausea, infection and vomiting. Pancreatic cancer: fatigue, rash, nausea, anorexia, diarrhea, abdominal pain, vomiting, weight decrease, infection, edema, pyrexia, constipation, bone pain, dyspnea, stomatitis and myalgia.
Gefitinib	2015	EGFR	NSCLC	Skin reactions and diarrhea, interstitial lung disease and Hepatotoxicity

Drug	Year of approval	primary target	Indication	Most common adverse reactions and warnings (as mentions in the US prescribing information)
Lapatinib	2007	HER2 and EGFR	HER2-positive breast cancers	Hepatotoxicity, diarrhea, palmar-plantar erythrodysesthesia, nausea, rash, vomiting, and fatigue. May prolong the QT interval in some patients or decreases in left ventricular ejection fraction,
Pazopanib	2009	VEGFR	Advanced renal cell carcinoma, advanced soft tissue sarcoma	Hepatotoxicity Advanced renal cell carcinoma: diarrhea, hypertension, hair color changes (depigmentation), nausea, anorexia, and vomiting. Advanced soft tissue sarcoma: fatigue, diarrhea, nausea, decreased weight, hypertension, decreased appetite, vomiting, tumor pain, hair color changes, musculoskeletal pain, headache, dysgeusia, dyspnea and skin hypopigmentation. Prolonged QT, Arterial thrombosis, cardiac dysfunction and hemorrhagic events were reported.
Sorafinib	2005	VEGFR	Hepatocellular carcinomas, renal cell carcinomas, thyroid cancers (differentiated)	Diarrhea, fatigue, infection, alopecia, hand-foot skin reaction, rash, weight loss, decreased appetite, nausea, gastrointestinal and abdominal pains, hypertension, and hemorrhage.
Sunitinib	2006	VEGFR	Gastrointestinal stromal tumors, pancreatic neuroendocrine tumors, renal cell carcinomas	Hepatotoxicity, fatigue, asthenia, fever, diarrhea, nausea, mucositis/stomatitis, vomiting, dyspepsia, abdominal pain, constipation, hypertension, peripheral edema, rash, hand-foot syndrome, skin discoloration, dry skin, hair color changes, altered taste, headache, back pain, arthralgia, extremity pain, cough, dyspnea, anorexia, and bleeding
JAK inhibitors				
Tofacitinib	2012	JAK1/ JAK3	RA, psoriatic arthritis, ulcerative colitis	Series infections, malignancy, upper respiratory tract infections, headache, diarrhea and nasopharyngitis
Baricitinib	2018	JAK1/ JAK2	RA	Series infections, malignancy and thrombosis, upper respiratory tract infections, nausea, herpes simplex, and herpes zoster
Ruxolitinib	2011	JAK1/ JAK2	Myelofibrosis	Thrombocytopenia, anemia, neutropenia bruising, dizziness and headache
Fedratinib	2019	JAK2	Myelofibrosis	Diarrhea, nausea, anemia, and vomiting

Drug	Year of approval	primary target	Indication	Most common adverse reactions and warnings (as mentions in the US prescribing information)
mTOR inhibitors				
Sirolimus	1999	mTOR	Kidney transplant, lymphangioleiomyomatosis	Kidney transplant: peripheral edema, hypertriglyceridemia, hypertension, hypercholesterolemia, creatinine increased, abdominal pain, diarrhea, headache, fever, urinary tract infection, anemia, nausea, arthralgia, pain, and thrombocytopenia Lymphangioleiomyomatosis: stomatitis, diarrhea, abdominal pain, nausea, nasopharyngitis, acne, chest pain, peripheral edema, upper respiratory tract infection, headache, dizziness, myalgia, and hypercholesterolemia
Temsirolimus	2007	mTOR	Advanced renal cell carcinomas	Rash, asthenia, mucositis, nausea, edema, and anorexia, anemia, hyperglycemia, hyperlipemia, hypertriglyceridemia, elevated alkaline phosphatase, elevated serum creatinine, lymphopenia, hypophosphatemia, thrombocytopenia, elevated AST, and leukopenia.
Everolimus	2009	mTOR	HER2-negative breast cancers, pancreatic neuroendocrine tumors, renal cell carcinomas	Advanced RCC: stomatitis, infections, asthenia, fatigue, cough, and diarrhea. SEGA: stomatitis, upper respiratory tract infection, sinusitis, otitis media, and pyrexia.
CDK inhibitors				
Abemaciclib	2017	CDK4/6	breast cancer	Diarrhea, neutropenia, nausea, abdominal pain, infections, fatigue, anemia, leukopenia, decreased appetite, vomiting, headache, alopecia, and thrombocytopenia
Palbociclib	2015	CDK4/6	Estrogen receptor- and HER2-positive breast cancers	Neutropenia, infections, leukopenia, fatigue, nausea, stomatitis, anemia, alopecia, diarrhea, thrombocytopenia, rash, vomiting, decreased appetite, asthenia, and pyrexia
Ribociclib	2017	CDK4/6	Combination therapy for breast cancers	Neutropenia, nausea, fatigue, diarrhea, leukopenia, alopecia, vomiting, constipation, headache and back pain

Table 1.
List of currently approved KIs, their primary targets, identified off-targets, indication and most common adverse reactions in humans as defined in the drug labels.

2.2 Hepatotoxicity

Hepatotoxicity has been reported for several TKIs and it is estimated to affect approximately 5% of patients [32]. The clinical effects have ranged from mild elevation in transaminases to progressive irreversible cirrhosis, which has resulted in death, and are dependent on the specific interaction between the drugs and the individual patients [33, 34]. As most of TKIs are metabolized by hepatic cytochrome P450 enzyme system, clinicians should be aware of potential hepatotoxicity with TKIs in patients with liver dysfunction. However, the mechanism for liver toxicity with KIs is not fully clear. Paech et al. [35] studied several approved oncologic KIs (erlotinib, imatinib, lapatinib, and sunitinib) associated with liver toxicity in human hepatocyte cell lines and in isolated mouse liver mitochondria focusing on ATP metabolism. The authors proposed that imatinib (Bcr-Abl TKI) and sunitinib (multiple receptor TKI) induce mitochondrial dysfunction and by inhibiting complex I and/or III of the electron transport chain of the mitochondria required for its function and glycolysis. Lapatinib (HER2 and EGFR TKI) affected mitochondria only weakly but inhibited glycolysis, and erlotinib (EGFR TKI) showed a slight cytotoxicity in both cell models investigated, although it did not affect the mitochondria ATP content in all cell types and did not impair oxidative metabolism. Similar mechanism of inhibition of mitochondrial complex I leading to impaired mitochondrial and myocyte proliferation was published for imatinib and dasatinib (Bcr-Abl and Src TKI) by Bouitbir et al. using C2C12 murine myoblasts and myotubes as well as human rhabdomyosarcoma (RD) cells [36].

In contrast to liver toxicity, it appeared in recent years that TKIs may also prevent or reverse hepatic disease. Accumulating evidence suggests that hepatic stellate cells (HSC) play a pivotal role in hepatic fibrogenesis [37], and that phosphorylation of transcription factors by kinases such as RSK [38] or focal adhesion kinase (FAK) [39] promotes stellate cell activation and survival. Therefore, inhibition of these kinases either as on-targets or off-targets may reduce hepatic disease.

2.3 Hematotoxicity

Hematotoxicity of KIs includes adverse effects on blood-forming organs such as bone marrow or on the constituents of blood, including platelets, leukocytes (white blood cells) and erythrocytes (red blood cells). One particular hematotoxicity caused by KIs is myelosuppression, also known as bone marrow suppression. Myelosuppression is a decrease in bone marrow activity that results in reduced production of blood cells manifested as anemia (decrease in erythrocytes), neutropenia (decrease in leukocytes), or thrombocytopenia (decrease in platelets). VEGF and its receptors are essential for production of mature blood cells [40, 41], as well as are principal regulators of blood vessel formation (angiogenesis) [42]. Accordingly, hematologic toxicities have been observed in clinical studies with several multi-kinase inhibitors such as VEGFRs KIs (i.e. pazopanib, sorafenib, and sunitinib) [43], although the frequency and severity varies among the different multi-kinase inhibitors, depending on their selectivity and affinity to other kinases [44].

Inhibitors of another two kinases which play roles in hematopoietic activities, FLT3 and mTOR, are also associated with hematotoxicities. A broad range of hematopoietic activities are mediated through interactions of c-Kit ligand (KL) and FLT3 ligand with their receptors: stem cell factor (SCF) and receptor-type tyrosine-protein kinase FLT3, respectively [45]. The signaling through SCF and FLT3 is essential for optimal

production of mature haematopoietic cells from stem cells [44]. FLT3 is more critical for the generation of lymphoid progenitors, whereas SCF regulates erythroid and myeloid platelet-derived growth factor (PDGF) progenitor cells. mTOR inhibitors, such as temsirolimus and everolimus, are also associated with a significant increased risk of developing anemia and thrombocytopenia [46], although they have great therapeutic potential in hematologic diseases such as leukemia, lymphoma, myeloma [47]. One possible explanation is that the effect of mTOR inhibition on erythropoiesis could be the antigrowth effect of these KIs on erythrocytes consequently leading to a lower production and decreased size when compared to normal growth [48]. Thus, low selectivity of various KIs towards the above kinases might explain the accompanying hematotoxicity (i.e. imatinib inhibits FLT3 as an off-target).

3. Management of kinase inhibitors associated toxicities

The overall risk for development of the discussed toxicities is different between inhibitors, indications, patients, and patient medical history, and usually the benefit to the patient exceeds the risk associated with development of these adverse events. That is, cardiotoxicity can be managed by routine monitoring via methods such as electroencephalogram, cardiac biomarkers, and blood pressure during the course of treatment, in addition to a comprehensive collection of past medical history and risk factors to identify those at increased risk. When there is a risk for hepatotoxicity, monitoring and management of serum liver chemistry such as elevations of Alanine transaminase (ALT), total bilirubin (TBL) and Alkaline phosphatase (ALP) may identify liver injury during treatment and may suggest reduction in dosage or replacement of KI. Likewise, anemia may be monitored by hematology testing and treatment of iron supplements or erythropoiesis stimulating agents. Regardless, avoiding these side effects via the development of more selective KIs is more advantageous for improving the quality of life of the patients and economy rather than management of the side effects post-marketing. The complex nature of kinase signaling and the challenges of developing selective KIs, suggest that early prediction of selectivity for new molecules and their potential for adverse events in the clinic at preclinical stages may assist and improve the development of future, safer KIs. Proper use of the tool box of preclinical assays as described below may accelerate this goal by identifying and comparing the selectivity of new KIs.

4. Development of selective Protein Kinase Inhibitors

4.1 Methodologies and assays to profile protein kinase inhibitors

Due to the evolving knowledge of the importance of kinases in cell biology and function and their problems of selectivity, multiple methodologies have been developed to profile KIs. Both radiometric and non-radiometric approaches are utilized today. Non-radiometric assays can assess ligand-kinase binding or kinase enzymatic activity. However, the radiometric approach assessing kinase enzymatic activity is still considered the "gold standard". Given the increasing demand for kinase profiling assays and evolution of technology, several companies have developed fee-for-service assays and assay kits [49]. The different methodologies and assays are discussed in the sections below and listed in **Table 2**.

Assay Type			Fee for service Assay	Reference
Enzymatic Activity Assays	Radioactive methods	Filtration binding assays	Reaction Biology Corporation HotSpot™	[50]
			Promega SignaTECT ™	[51]
		Scintillation proximity assay	Reaction Biology Corporation ^{33}PanQinase™	[50]
	Fluorescent-based assays	Fluorescent intensity assays	DiscoverX ADP Quest™	[52]
			DiscoverX ADP Hunter™	[52]
			BellBrook Labs Transcreener® ADP^2 FI Assay	[53]
		Fluorescent polarization assays	Molecular Device IMAP	[54]
			Bellbrook Labs Transcreener® ADP FP Assay	[53]
		Fluoresccent resonance energy transfer (FRET)	Invitrogen Z'-LYTE	[54]
			PerkinElmer Alpha Kinase assays	[55]
		Time resolved Fluorescence (TRF)	PerkinElmer DELIFA	[56]
		Time resolved Fluorescence resonance Energy transfer (TR-FRET)	Invitrogen LanthaScreen Activity assay	[57]
			Invitrogen Adapta Assay	[58]
			PerkinElmer Lance Ultra kinase Assay	[59]
			Bellbrook Labs Transcreener® ADP TR-FRET Assay	[53]
			Molecular Device IMAP	[54]
		Luciferase reporter Assays	Promega kinase-Glo	[60]
		Electrophoretic Mobility Shift assays	PerkinElmer (formerly Caliper Life Sciences) Nanosyn Assay	[61]
Ligand-kinase binding assays	Competitive binding assay	Immobilized ligand	DiscoverX (formerly Amit Biosciences) KINOMEscan® Assay	[62]
		TR-FRET	Invitrogen's LanthaScreen Eu Kinase binding assay	[63]
	Differential Scanning Fluorimetry			[64]

Table 2.
Kinase inhibitor profiling assays.

Identifying, quantifying and optimizing the selectivity of compounds became critical for both drug development and development of tool compounds for basic research [19]. Building our knowledge on the selectivity of compounds and creating databases aid in the development of structure-selectivity relationships that will improve rationale design of selective KIs, progress drug discovery and promote inhibitor optimization [65].

Selectivity of KIs is well acknowledged as a challenge in the development of safe drugs and tool compounds. 'Uni-specificity' refers to compounds that inhibit a single kinase more potently than any other kinases. Very few protein KIs demonstrate 'uni-specificity', emphasizing the unresolved issue of selectivity amongst protein KIs [20]. Investigating and comparing the selectivity of KIs early in their development is pivotal to developing more selective and consequently safer KIs. Multiple approaches were used to investigate the selectivity of KIs. One approach was to concentrate on the ability of a single KI to inhibit kinases within a specific subfamily due to the similarity in the ATP binding domain. However, importantly, inhibitor type does not guarantee selectivity. Additionally, Anastassiadis et al. [20] demonstrated that a substantial percentage of kinase off-target activity occurred outside the subfamily of interest. For example, 24% of off-target effects of TKIs occurred in the serine/threonine kinase subfamily [20]. Another approach was to focus on the ability of a single KI to inhibit kinases within particular pathways due to shared functionality. A third and most comprehensive approach is to profile KIs against a variety of kinases across the kinome both within and between subfamilies. As technology and assay methodology developed, use of broad screening panels became more common and revealed that compounds historically believed to be selective were in fact not. Today, the best-practice and recommended approach is to screen KIs in different kinase assay formats against multiple related and unrelated kinases. The broad screening panels should be quantitative and systematic with objective criteria to compare between studies. It is important that the assays used are optimized, robust, reliable and standardized for multiple, varied kinases. It is also preferable for practical use of the assays that they are suitable for high-throughput designs and economical [19, 20].

4.2 Enzymatic Activity Assays

4.2.1 Radiometric methods

4.2.1.1 Filtration binding assay

The use of [^{32}P]-or [^{33}P]-ATP in a kinase reaction allows phosphorylation of a substrate peptide or protein to be measured directly. The filtration binding assay approach is the most preferable and the benchmark against which other methodologies are compared. Following a kinase reaction utilizing the radiolabeled ATP molecules, the labelled substrates are bound to capture membranes such as P81 ion exchange filter paper and unchanged ATP/unbound phosphate is washed away. One such assay termed HotSpot™ was developed by Reaction Biology Corporation and Anastassiadis et al. [20] utilized Reaction Biology Corporation's HotSpot™ assay to demonstrate the selectivity of 178 KIs against 300 recombinant protein kinases. Promega's SignaTECT assay operates on a similar principle, however, the SignaTECT assay utilitizes biotinylated substrates and a biotin capture membrane to capture the biotinylated substrates with the radiolabeled phosphate for detection [51].

4.2.1.2 Scintillation proximity assay

In order to overcome the necessity for separation and washing steps of the filtration binding assay, the "mix and read" scintillation proximity assays were developed [49]. Reaction Biology Corporation's ^{33}PanQinase™ is an example of a scintillation proximity assay whereby the reaction with [^{33}P]-ATP is performed using microtiter plates coated with scintillant for detection [50]. A variety of radio isotypes can be utilized in a scintillation proximity assay [49].

Despite the advantages of radiometric methodologies such as universality across kinases and low error signal (low false-positive and false-negative rates), the major disadvantage of the radiometric methodology is the specialized waste disposal and safety precautions required for working with radioactive material. As a consequence, non-radioactive methodologies were developed.

4.2.2 Fluorescent based methods

4.2.2.1 Fluorescence Intensity assay

A number of fluorescence intensity assays are available whereby the readout is simply fluorescent intensity (FI). DiscoverX ADP Hunter™ and ADP Quest™FI assays use linked reactions that use ADP, pyruvate kinase, pyruvate oxidase and horseradish peroxidase to convert a fluorescent dye precursor (ADHP (10-Acetyl-3,7-dihydroxyphenoxazine) to fluorescent resorufin, the source of the fluorescent intensity signal [52]. Bellbrook Labs Transcreener® ADP FI Assay measures ADP levels. This is accomplished by utilizing an IRDye® QC-1 quencher conjugated to an anti-ADP antibody to quench the signal from an ADP Alexa Fluor® 594 tracer. Once ADP is produced, ADP displaces the fluorescent tracer allowing detection of the fluorescent signal from the fluorescent tracer [53].

4.2.2.2 Fluorescent polarization assays

Exciting molecules with polarized light promotes rotational movement. The speed of the rotational movement depends on the molecular weight of the compound. That is, high molecular weight molecules rotate slower than low molecular weight molecules. Molecular Device developed a "mix and read" FP assay whereby following kinase reaction a fluorescently labelled, phosphorylated substrate binds to a large nanoparticle increasing the molecular weight, decreasing rotational speed and increasing the polarization of the phosphorylated peptide or protein [54]. However, false-positive and false-negatives have been reported utilizing the FP methodology [49].

4.2.2.3 Fluorescent resonance energy transfer (FRET)

Fluorescent resonance energy transfer (FRET) relies on the transfer of energy between donor and acceptor molecules that occurs when the two molecules come into close proximity and following excitation of the donor molecule. Many companies have utilized this technology to develop assays for kinase profiling including Invitrogen's Z'-LYTE Kinase Assay utilized in their SelectScreen Kinase Profiling Service. In invitrogen's Z'-LYTE Kinase Assay, two fluorescent proteins that make up FRET donor and acceptor molecules are added to substrates, then following the

kinase reaction, the non-phosphorylated substrates are cleaved by a protease interrupting the energy transfer between the donor and acceptor fluorophores. Therefore, the emission wavelength differs depending on if the substrate was phosphorylated or not. The assay relies on identification of the amino acid sequence of the relevant substrate and has been validated for more than 200 kinases [49, 66].

4.2.2.4 Time-resolved fluorescence (TRF)

Time-resolved fluorescence (TRF) involves the use of fluorophores that decay over a longer period of time than traditionally used fluorophores and the decay following light excitation of these fluorophores can be monitored as a function of time. Lanthanide chelates such as Europium, Samarium and Terbium are examples of such non-traditional fluorophores with long decay times. PerkinElmer's DELFIA® assay is available as a TRF assay that utilizes lanthanide chelate tagged antibodies to detect phosphorylated substrates [49, 56].

4.2.2.5 Time-resolved fluorescence resonance energy transfer (TR-FRET)

A time-resolved fluorescence resonance energy transfer (TR-FRET) assay combines the principle of FRET with donor and acceptor molecules with TRF whereby one of the molecules is a fluorophore with an extended decay time [49]. For example, in addition to Invitrogen's Z'-LYTE FRET assay, Invitrogen also offer several TR-FRET assays (LanthaScreen Activity assay, LanthaScreen Eu Kinase binding assay [discussed below in Section 1.3.2] and Adapta Assay). The Adapta Assay utilizes a Europium-labeled anti-ADP antibody and an Alexa Fluor® 647 labeled ADP tracer to examine ADP levels following a kinase reaction. Without the presence of ADP, the anti-ADP antibody and ADP tracer bind to create the TR-FRET signal. ADP disrupts the binding of the antibody and tracer reducing the signal. Alternatively, the LanthaScreen® activity assay involves a fluorophore-labelled substrate that when phosphorylated is bound by Terbium-labeled antibody to generate the FRET signal [57, 58]. Similarly, PerkinElmer's Lance® Ultra kinase assay utilizes U*Light*™-labeled substrate that when phosphorylated is bound by Europium-labeled antibody to generate the FRET signal. Similar to the Z'-LYTE Kinase Assay, the LanthaScreen Activity assay and Lance® Ultra kinase assay both rely on identification of the amino acid sequence of the relevant substrate [59].

4.2.3 Luciferase reporter assay

Cell-based luciferase reporter assays that produce bioluminescence are common to monitor the activity of cellular processes. Cell-based luciferase reporter assays require luciferin, luciferase enzyme and sometimes ATP. The Firefly luciferase enzyme converts luciferin into oxiluciferin in the presence of ATP emitting a light photon. As the conversion of luciferin to oxiluciferin relies on ATP, the luciferase enzyme can be used to detect the amount of ATP following a kinase reaction and the amount of luminescence positively correlates with the amount of ATP [49, 67]. An example of a commercially available luciferase assay for kinase profiling is Promega's Kinase-Glo® platform [60].

It is also important to differentiate when utilizing a luciferase reporter assay between compounds that legitimately inhibit a kinase from compounds that interfere with the assay itself. That is, luciferase itself may be targeted by KIs [67].

Dranchak et al. [67] investigated the ability 367 compounds in the GSK published protein kinase inhibitor set to inhibit two commonly used types of luciferase enzymes (firefly luciferase [FLuc; ATP-dependent] and renilla renififormis luciferase [RLuc; ATP-independent]). Approximately 6% of the KIs inhibited FLuc activity whereas approximately 0.5% inhibited RLuc activity. Therefore, the specific luciferase utilized and the potential interactions of KIs with the luciferase should be taken into consideration when choosing an appropriate assay platform for kinase profiling using luciferase [67].

4.2.4 Electrophoretic Mobility Shift Assays

Phosphorylation of a substrate causes an increase in the negative charge of the substrate. As a consequence, phosphorylated substrates can be separated and detected using electrophoretic technologies. The Caliper Life Sciences' Nanosyn Assay takes advantage of these properties of phosphorylated substrates (usually fluorescently labelled peptides and proteins) and utilizes a microfluidic chip for the assay reaction and electrophoretic detection [49, 61, 68]. Elkins et al. [68] utilized this assay to examine the selectivity of the GSK published protein kinase inhibitor set against 224 recombinant kinases with a 50% inhibition threshold and identified that there are different kinases targeted by multiple compounds, kinases not inhibited by any of the compounds and kinases inhibited by only one compound.

4.3 Ligand-kinase binding assays

Measuring the effect of ligands on the enzymatic activity of kinases has traditionally been the "go to" approach. However, enzymatic activity assays require individual optimization in order to receive an acceptable signal-to-noise ratio as well as identification of upstream signaling partners and applicable substrates. The requirements for optimization of enzymatic activity assays cannot always be met and individual optimization of the assays for each kinase can be costly and time consuming. Therefore, binding assays were developed utilizing different technologies including competitive binding assays and differential scanning fluorimetry to facilitate high-throughput kinase profiling [64, 69]. Although suitable to enable high-throughput screening, ligand-kinase binding assays do not always predict enzymatic activity of a compound with the particular kinase [20, 69].

4.3.1 Competitive binding assays

One option for a competitive binding assay is Invitrogen's LanthaScreen Eu Kinase binding assay using TR-FRET technology as described in Section 4.2.2.5. That is, the assay operates whereby a Europium-labeled antibody is targeted against a tagged kinase and an Alexa Fluor® 647 labeled substrate are used to generate the FRET signal. Inhibitor binding to the kinase prevents substrate binding and disrupts the FRET signal [63].

An alternative approach is the DiscoverX's KINOMEscan assay utilizing immobilized ligands [62]. The KINOMEscan platform was originally published by Ambit Biosciences. The KINOMEscan platform involves phage or DNA tagged kinases and immobilized ligands. The ligands are biotinylated and then bound to streptavidin-coated magnetic beads attached to solid supports. The immobilized ligands compete with compounds for binding to the kinase. ATP-binding and allosteric KIs can

compete with the immobilized ligands. Kinase that is unbound to the immobilized ligand is removed via wash steps. The amount of kinase bound to the immobilized ligands is then quantified using qPCR identifying the phage or DNA tag attached to the kinase. The assay has been validated for more than 450 kinases (wild-type and mutant) [69–71]. Utilizing this technique, Davis et al. [69] screened 72 KIs and demonstrated that type II inhibitors are more selective than type I inhibitors with the majority of type II inhibitors demonstrating binding to 20% or less of the total kinases screened. However, a KI belonging to the type II class does not guarantee selectivity with a subset of type II inhibitors binding to 40–50% of kinases screened [69].

4.3.2 Differential Scanning Fluorimetry

Upon binding of a ligand to a protein, such as a kinase, the thermal properties of the protein stabilize and the melting temperature increases. The unfolded, unbound kinase that is not stabilized is detected by a dye that binds to unfolded proteins and fluoresces. This methodology is termed differential scanning fluorimetry (DSF). SYPRO orange is a suitable fluorescent dye for this application due to the high signal-to-noise ratio and comparably high excitation wavelength [64, 72]. Fedorov, Niesen and Knapp [64] demonstrated that the data generated using the KINOMEscan assay developed by Ambit Biosciences and described above (Section 4.3.1) highly correlated with their data generated using DSF with an r^2 value of 0.949. A major advantage of the thermal stability shift methodology is that knowledge of the amino acid sequence of the substrate or kinase is not required and specialized antibodies are also not required [64]. Additionally, Anastassiadis, et al. [20] compared the DSF method to Reaction Biology Corporation's radiometric method and found a significant correlation between the two methodologies. However, the DSF method did demonstrate false-positives and false-negatives and thus emphasizing that binding does not necessarily predict enzymatic activity [20].

4.4 Computational analysis

Computational kinase selectivity profiling methods can be used to predict the selectivity of KIs and rationally design KIs with desired profiles across a large number of kinases and avoid the limitations of activity and binding assays. However, computational approaches are dependent on the quality of the available structure–activity data or require extensive computational analysis [73]. Improved computational approaches take advantage of the conservational nature of the kinome and shared binding patterns of KIs as well as profiling data generated for multiple kinases across the classes and kinome and kinase 3D structures. Kinase inhibition profiling data including both positive and negative (little or no effect) data are invaluable to the evaluation of computational approaches for predicting KI selectivity [74]. The large volume of data generated to date paved the way for the development of machine learning and artificial intelligence approaches that allow for prediction of results for KIs and kinases not included in the dataset [19, 73–75]. With the accumulation of structure–activity data over the years, the literature has been minded to create both commercially and publically available databases with data from diverse sources such as ChEMBL, Kinase SARfari and GVK Biosciences kinase inhibitor database. The kinase-inhibitor profiling panels already discussed here such as those generated by Karaman [70], Anastassiadis [20] and Davis [69] as well as other databases such as the 3D structures available in PDB were also used to generate numerous computational approaches. The size of the

gatekeeper residue, hydrogen and covalent bonds, the flexibility of the hinge loop connecting kinase domains as well as kinase-inhibitor data were all used to generate computational approaches. The KI data can be represented either as binary yes-no or weighted by affinity or inhibitory activity and can be used to generate connectivity maps to predict either kinases, KIs or diseases [74]. Lo et al. [73] developed a computational approach based on searching for structural similarity of the ligand binding sites and determining a PocketFEATURE score (PFS). Specifically, a kinase database entitled 'KinomeFEATURE' of approximately 2850 kinase structures was constructed to predict selectivity of 15 known KIs with greater than 90% accuracy. Therefore, computational approaches are becoming more widely used and useful for the purpose of predicting selectivity and rationally design KIs [73].

4.5 Quantification of selectivity

In order to aid in the ability to compare data between studies and assay methodologies to identify and compare the selectivity of inhibitors, different quantitative measures of selectivity have been developed. These measures involve condensing large datasets into single values for each inhibitor. Such measures include the selectivity score, Gini coefficient score and selectivity entropy score described below. However, the selectivity score for a particular KI takes into consideration all kinases in a dataset and rely upon the size and diversity of the kinases in the dataset [76].

4.5.1 Selectivity score

In order to generate a selectivity score for a particular inhibitor, the number of kinases bound by this specific inhibitor with $K_d$or IC_{50} values that meet predefined threshold criteria should be divided by the total number of distinct kinases screened in a specific assay [20, 69, 70]. The predefined threshold can be a specific concentration e.g. 3 μM [S(3 μM)] or percentage e.g. 50% [S(50%)]. For example, Karaman et al. [70] from Amit Biosciences utilized the KINOMEscan to screen 38 KIs against 317 kinases and calculated selectivity scores S(3 μM) and S(100 nM) whereby K_d < 3 μM and K_d < 100 nM, respectively. Via the use of the selectivity score, Karaman et al. [70] showed that the compounds screened demonstrated a fairly even distribution of selectivity scores from 0.01 to 0.57 (1–57%).However, Karaman et al. [70] also demonstrated that the composition of the kinases in the screened database significantly affected the outcome. That is, the selectivity score varied greatly by adjusting the number of kinases screened and randomly selecting kinases preserved the results better than a systematic selection. Karaman et al. [70] also described a selectivity score to describe off-target affinities whereby a ratio of the K_d for the off-target to the K_d of the primary target is generated and then the number of ratios below 10 is then divided by the total number of distinct kinases assayed. This selectivity score was also termed S(10x). Later, Davis et al. [69] (Amit Biosciences) also used the KINOMEscan to screen 72 KIs against 442 kinases and demonstrated that the majority (64%; 46 out of 72) of inhibitors bound to <20% of the total distinct kinases screened by them (totaling 386) with a S(3 μM) score of <0.2.

The selectivity score is not limited to the assay type or assay technology facilitating comparisons between compounds, studies and assays [70]. The selectivity score is, however, dependent on the threshold set for defining the score such as 3 μM vs. 100 nM or 50% vs. 70% inhibition [20].

4.5.2 Gini Coefficient

The Gini coefficient has been described as a less arbitrary tool for evaluating selectivity compared to the selectivity score described above as the Gini coefficient does not rely on defining a threshold value. To calculate the Gini coefficient, the % inhibition of a compound at a single concentration is rank ordered, summed and normalized to generate a plot of rank order vs. fraction of cumulative effect of each target. Then, the deviation from the linear plot is calculated and a greater deviation from the linear plot indicates a less specific compound. A Gini score of 1 indicates an inhibitor that targets one kinase whereas a score of 0 indicates a compound that equally inhibits all kinases screened. Even though the Gini coefficient does not rely on a defined threshold, the coefficient relies on the single concentration tested and is thus inherently limited [20, 76].

4.5.3 Selectivity Entropy Value

Following the use of selectivity scores and the Gini coefficient to quantitatively describe inhibitor selectivity, Uitdehaag and Zaman [76] introduced the entropy value to overcome the limitations of the selectivity score and Gini coefficient score. The entropy value was previously utilized in a diverse range of fields such as thermodynamics and chemistry, and it is based on the concept that inhibitor binding to multiple kinases will assume a Boltzmann distribution [76]. The entropy equation utilized by Uitdehaag and Zaman [76] involves 5 steps based on the association constant (K_a, the inverse of the K_d or IC_{50} values).

A resulting selectivity entropy (S_{sel}) value of 0 indicates an inhibitor that targets one kinase and the higher the S_{sel} the less specific the compound is for the kinases profiled. Uitdehaag and Zaman [76] compared the selectivity entropy value to the selectivity score S(3 μM), K_a-Gini (Gini scores based on association constants), S(10x) and partition index (not outlined in this review) for data generated using the KINOMEscan compared to a radioactive filter binding enzymatic activity assay by Millipore. The two methodologies produced highly correlated data using the selectivity entropy score, S(3 μM) and K_a-Gini with r^2 correlation values of 0.93, 0.92 and 0.99, respectively, showing that these three scores are relatively robust. Uitdehaag and Zaman [76] also demonstrated that type II and III inhibitors are more selective than type I inhibitors consistent with the conclusions drawn by Davis et al. [69] using the selectivity score S(3 μM).

4.6 Graphical representation of selectivity

For qualitative rather than quantitative analysis of a compound selectivity, different graphical representations have been used to demonstrate the interaction of a compound with the whole kinome or kinase panel in the assay. Interaction maps and heat maps have been used to represent and compare compound selectivity. Interaction maps are based on the phylogenetic tree. In interaction maps, a circle is overlaid on a kinase in the kinase phylogenetic tree representing interaction of the compound with the kinase and the size of the circle represents potency. That is, the larger the circle the higher affinity the compound has for the kinase [68, 70, 76]. Graphical representations allow for visual rankings of selectivity and these visual observations should align with any quantitative measure chosen [76].

4.7 Cell-based vs. Cell-free selectivity assays

Regardless if binding or enzymatic activity assays are chosen, it is important to understand that cell-free in vitro assays may not reflect the activity in cellular systems. As such, it is recommended to follow-up cell-free in vitro selectivity assays with cell-based selectivity assays in vitro or in vivo animal model systems [65, 70, 77].

Cell-based assays are more complex than cell-free based assays and this complexity contributes to the discrepancies between the assays. One source of complexity in cell-based assays is the fact that the phosphorylation state in cells relies on the balance between phosphorylation of proteins by kinases and de-phosphorylation of proteins by phosphatases and this can lower the required concentration of KIs for kinase inhibition. Additionally, our understanding of phosphatase systems and their regulators is more limited. Moreover, sensitivities differ when assessing different cellular activities such as calcium release, IL-2 secretion or proliferation of T-cells [65] as measures for enzymatic reaction and selectivity.

An important difference between cell-based and cell-free selectivity assays is that in the cell-based assay the KI must penetrate the cell membrane and the cellular compartments in order for the inhibitor to reach its target. Different factors determine if a KI reaches its target within a cell. A KI can penetrate a cell either via a diffusion concentration gradient or active transport and a KI can also be actively pumped out of cells via efflux mechanisms. Size, lipophilic properties, aqueous solubility, plasma membrane partitioning and plasma protein binding, for in vivo assays, will impact the ability of a KI to reach its target within a cell. It is important to test the KI once the compound reaches steady state within the cell and therefore it is routine practice to pre-incubate cells with the KI prior to performing a cell-based kinase activity assay [65].

The metabolic activity and ATP concentration in cells will also affect inhibitor activity within cells. In order to compare IC_{50} values to K_d values, it is best practice to use ATP concentrations equal to the Michaelis constant for ATP ($K_{M,\ ATP}$) for the particular kinase in an enzymatic assay based on the Cheng-Prusoff equation: $IC_{50} = K_i(1 + [ATP]/K_{M,\ ATP})$, whereby K_i is the dissociation constant. However, ATP concentrations are generally higher in cellular systems compared to the $K_{M,\ ATP}$ and differences in ATP concentrations may be a source of discrepancy between cell-based and cell-free selectivity assays [65, 76].

An additional potential difference between cell-based and cell-free selectivity assays is that some cell-free screening panels use truncated forms of the kinases and interactions between a kinase and inhibitor may differ in the setting of the full-length kinase.

The conformational state of the kinase should also be taken into consideration when assaying the interaction with a KI as many kinases can adopt different conformational states. For example, inhibitors that bind to the inactive state of a kinase can stabilize the kinase in this state which may not occur in a cell-free assay with a truncated form of the kinase [20].

5. Overcoming toxicity with more selective kinase inhibitors

The first generation of KIs, including imatinib, which was the first KI approved by the FDA in 2001 for patients with chronic myeloid leukemia (CML), demonstrated acceptable toxicity profiles in oncology patients compared with traditional

chemotherapeutic agents, as well as comparable efficacy even though the first generation KIs were not highly selective. However, the acceptable toxicity and risk–benefit ratio are higher for oncology patients than for patients with conditions that are not severely debilitating or life-threatening such as chronic inflammatory disease [78]. Therefore, there is a growing need to overcome lack of selectivity and off-target toxicities with newly developed KIs for different indications.

An example of overcoming toxicity by improving selectivity and by lowering affinity for off-targets is coming from the JAK family of non-receptor tyrosine kinases that has gained great interest as therapeutic targets. The JAKs transduce signals from a multitude of cytokines and growth factors via the JAK–STAT (Signal Transducers and Activators of Transcription) pathway and are involved in various inflammatory and autoimmune diseases [79, 80]. Currently, there are four JAK inhibitors approved for clinical use: tofacitinib (for RA, psoriatic arthritis, ulcerative colitis), baricitinib (for RA), ruxolitinib and fedratinib (for myelofibrosis). These JAK inhibitors demonstrate that improved selectivity results in more favorable safety profile, and also emphasize that the existence of off-target binding does not automatically predict unfavorable safety profiles because the off-target activity plays a major role. That is, lower activity of these JAK inhibitors on their off-targets result in lower toxicity. Since JAK inhibitors block downstream signaling of a variety of cytokines relevant for normal physiology, various severe adverse effects were often predicted for these inhibitors. However, clinical trials of tofacitinib have shown an acceptable safety profile, with infection and cytopenias (due to the blockage of myelopoietic growth factor signaling through JAK2) being the major adverse events, and without any increased risk of developing malignancies [81]. Likewise, the selective JAK1/JAK2 inhibitor baricitinib, which offers an effective treatment for RA, demonstrates little effect on hematology parameters, with uncommon neutropenia (<1% patients) and no higher risk of infection [82]. A reduction in natural killer (NK) cell numbers was observed in some patients, but there was no evident association between the low NK cell count and the incidence of infections. Ruxolitinib is another potent JAK1/JAK2 inhibitor demonstrating dose-dependent inhibition of the JAK2/STAT signaling and inhibition of cell growth that is dependent on JAK2 activation, with a sixfold selectivity for JAK1/JAK2 over Tyk2 and approximately a 130-fold selectivity for JAK1/JAK2 over JAK3 [83]. Despite this high selectivity, thrombocytopenia and anemia are side effects of ruxolitinib which can be dose- or even treatment-limiting adverse events and patients who discontinue ruxolitinib have miserable outcomes, making this situation an area of significant unmet need [84, 85]. Recently, fedratinib was approved by the FDA as a different drug for myelofibrosis overcoming some of the toxicities and resistance observed in ruxolitinib-refractory patients [86]. Fedratinib is selective for JAK2 over other JAKs and the common side effects reported with this drug include anemia, gastrointestinal symptoms, and elevations in liver transaminases [87]. Fedratinib also has a black box warning for encephalopathy, although this occurred only in about 1% of the treated patients [87]. Overall, increasing selectivity of JAK inhibitors may decrease the side effect burden on the patient but this cannot be avoided completely due their mechanism of action on important signal transduction pathways.

In an attempt to develop more selective second-generation JAK inhibitors, there are more than 20 other JAK inhibitors undergoing clinical trials, with varying selectivity profiles, for a variety of autoimmune diseases, including psoriasis, alopecia areata, ankylosing spondylitis and lupus [87]. Within the JAK family, JAK3 inhibitors are emerging as potential targets for the design of more selective KIs. JAK3 is a potential target for inflammatory diseases and has a more defined function than

other JAKs, which participate in multiple cellular processes. JAK3 associates only with the common γ-chain receptor, and it is expressed selectively in lymphoid and myeloid cells. Consequently, selective JAK3 inhibition may be beneficial in suppressing inflammatory responses with less off-target effects and markedly reduced adverse effects [80]. The main concern that has arisen recently with JAK inhibitors from pharmacovigilance (real-world data analysis) is a higher risk of thromboembolism [88, 89]. Conflicting data indicates that higher thromboembolic risk may be related to the specificity of JAK inhibitor action, such that preferentially blocking one signaling pathway upsets the balance between pro and anti-thrombotic activities [90]. It could be that thromboembolic complications are not a general class effect of JAK inhibitors, but might be related to inhibition of a specific JAK, aging and mutations in JAKs and the patient clinical history.

Even though the threshold for acceptable toxicities is higher for oncology patients due to the higher risk–benefit ratio that is tolerated, effort continues to be invested into producing more selective anti-cancer KI treatments with fewer side effects because these drugs fail in clinical trials due to overt toxicity. Promising and selective anti-cancer agents are cyclin-dependent kinases (CDKs) inhibitors. CDKs are important players in the regulation of cell division and proliferation, and numerous drugs that target CDKs have been developed to treat cancers over the past 20 years. The clinical trials with the first CDK inhibitors were discontinued due to severe toxicities. These toxicities were related to the low selectivity of the CDK inhibitors, since CDKs such as CK1 and CDK2 that are essential for maintaining the growth and function of normal cells were also inhibited [91]. More recently approved CDK inhibitors for the treatment of patients with breast cancer, including palbociclib, ribociclib, and abemaciclib, which exhibit selectivity for CDK4/6 over other CDKs, are associated with a lower numbers of life-threatening side effects [92]. These inhibitors do not have cross reactivity with other CDKs nor with other kinases in general and therefore have an improved safety profile [93]. Other selective CDK inhibitors are currently under development [94].

6. Conclusion and future perspectives

Hundreds of diseases including various cancers, Alzheimer's disease and autoimmune are associated with kinase mediated phosphorylation of proteins, and therefore discovery of selective KIs is still an urgent need. Selectivity is a known challenge for the development of KIs as safe therapeutics and as reliable tool compounds to investigate biological activities. Therefore, it is important to investigate the selectivity of KIs against kinase panels as well as against other targets such as other enzymes, ion channels and receptors. As a consequence, the technologies for assessing the selectivity of KIs are constantly evolving and have become increasingly sophisticated. In particular, the development of techniques that measure inhibitor profiles in environments that mimic human physiology would provide more reliable and human relevant results on the KI selectivity. Optimization of KIs, either by structure based drug-design, identifying and targeting allosteric sites or enhancing affinity for the on-target kinase, improves selectivity, and in general more selective KIs were shown to have more favorable safety profiles. The recently approved CDK and JAK inhibitors provide a proof of concept that safer and more effective KIs can be developed for both oncology indications and non-oncology indications such as chronic inflammatory diseases. Recent progress in nanomedicine and targeted therapy offers improvement

of drug efficacy, and allow for specific delivery of TKIs to the diseased cells using special nanocarriers, thereby reducing the incidence of adverse events [95]. Yet, TKI-nanomedicines are in the initial stages of development and, although they have great potential, they still have a long way ahead of them. Overall, achieving improved target selectivity and reduced off-target-mediated toxicity using efficient compound screening and profiling technologies, providing targeted therapies and overcoming resistance will further pave the way for novel, selective and safe KIs as promising therapeutics in oncology and beyond.

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