ELSEVIER

Innovative strategies for measuring kinase activity to accelerate the next wave of novel kinase inhibitors

Tim S. Veth [1,2,§], Nynke M. Kannegieter [3,§], Erik L. de Graaf [3], Rob Ruijtenbeek [4], Jos Joore [3], Anna Ressa [3], Maarten Altelaar [1,2,*]

[1] Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Padualaan 8, Utrecht 3584 CH, The Netherlands
[2] Netherlands Proteomics Center, Padualaan 8, Utrecht 3584 CH, The Netherlands
[3] Pepscope, Nieuwe Kanaal 7, 6709 PA Wageningen, The Netherlands
[4] Genmab, Utrecht, The Netherlands

The development of protein kinase inhibitors (PKIs) has gained significance owing to their therapeutic potential for diseases like cancer. In addition, there has been a rise in refining kinase activity assays, each possessing unique biological and analytical characteristics crucial for PKI development. However, the PKI development pipeline experiences high attrition rates and approved PKIs exhibit unexploited potential because of variable patient responses. Enhancing PKI development efficiency involves addressing challenges related to understanding the PKI mechanism of action and employing biomarkers for precision medicine. Selecting appropriate kinase activity assays for these challenges can overcome these attrition rate issues. This review delves into the current obstacles in kinase inhibitor development and elucidates kinase activity assays that can provide solutions.

Keywords: Kinases; kinase activity assays; protein kinase inhibitors; phosphorylation; inhibitor development; mechanism of action; biomarkers

Introduction

Protein kinases have become a focal point for drug developers owing to their potential as drug targets.(p1) The human kinome comprises 557 curated protein kinases that regulate cellular signaling by phosphorylating proteins, leading to changes in function, structure, location and interactions.(p2),(p3) It is estimated that up to 75% of the human proteome can be phosphorylated, underlining the widespread role of kinases in cellular processes.(p4) Aberrant kinase signaling often results in diseases like cancer, autoimmune disorders and inflammation.(p5) Protein kinase inhibitors (PKIs) have shown promise in treating these diseases, such as targeting ALK in non-small-cell lung cancer (NSCLC), vascular endothelial growth factor (VEGF) in macular degeneration and JAK in rheumatoid arthritis.(p6),(p7),(p8),(p9)

The growing interest in PKI development has spurred advancements in kinase activity assays.(p10) Contemporary assays have shifted from relying on kinase abundance to directly measuring kinase activity in its endogenous environment.(p11) This demand for reliable assays has driven the development of numerous methods to assess kinase activity, resulting in a wide array of

Glossary: ALK, Anaplastic lymphoma kinase; BRAF, v-Raf murine sarcoma viral oncogene homolog B; CAMK, Calcium/calmodulin-dependent protein kinase; CDK6, Cyclin-dependent kinase 6; EGFR, Epidermal growth factor receptor; ERK4, Mitogen-activated protein kinase 4; FGFR, Fibroblast growth factor receptor 1; JAK, Janus kinase; MAP2K1, Mitogen-activated protein kinase kinase 1; MAP2K2, Mitogen-activated protein kinase kinase 2; PI3K, Phosphoinositide 3-kinase; PKC, Protein kinase C; ROCK, Rho-associated protein kinase; VEGF, Vascular endothelial growth factor.

* *Corresponding author. Altelaar, M. (m.altelaar@uu.nl)*
§ These authors contributed equally to this work.

assays employed in PKI development pipelines across academia and industry.[p10]

Although PKI development has seen much progress, there remains great potential for further improvement.[p1] Currently, >200 candidates are undergoing clinical trials ranging between Phase I and Phase IV (Figure 1a).[p12],[p13],[p14] The predicted attrition rate of these PKIs is 59%, indicating that most PKIs under evaluation will not receive market approval.[p1] PKI development has generated >70 approved PKIs (Figure 1b), targeting ~50 of the at least 200 kinases believed to be crucial in disease.[p1],[p6],[p15] Moreover, 80% of the approved PKIs are exclusive to oncology, whereas kinases are vital in nearly all major disease areas,[p16],[p17],[p18] leaving non-oncological diseases inadequately targeted.

Several challenges need to be addressed to exploit the opportunities for PKI development. Most challenges involve understanding a drug's mechanism of action (MOA), which encompasses the biochemical interactions through which a PKI exerts its effects.[p19,p20] Others involve the appropriate use of biomarkers to enable precision medicine. Biomarkers are objectively measured biological indicators of a patient's medical condition. They effectively stratify patient groups, predict clinical outcomes and ultimately maximize the clinical potential of a PKI.[p21],[p22]

Addressing these challenges necessitates the appropriate use of kinase activity assays, because each challenge demands specific biological and analytical assay properties. Each assay has strengths and limitations, making it more suitable for a subset of challenges. The unique requirements of the challenges and distinct properties of the kinase activity assays emphasize the importance of selecting the correct assay for the appropriate challenge. This selection is crucial for enhancing the PKI development pipeline. This review aims to clarify the complexity of kinase activity measurements to improve PKI development. We discuss kinase activity assays that excel in multiple biological and analytical properties required for PKI development challenges (excluding bioinformatic assays as they are reviewed elsewhere).[p23] We then elaborate on potential challenges encountered during PKI development. Lastly, we associate the most promising assays with the solutions to each challenge.

Protein kinase activity

Biochemically, protein kinase activity is gauged by the rate at which a kinase carries out its enzymatic reaction. Assessing this in a kinase cellular environment is complicated because various modulating factors tightly control kinase activity. These mechanisms are partly determined by genetics but are primarily influenced by the kinase environment. Cellular genetics can influence kinase activity through activating point mutations, frequently observed in cancer (Figure 2). PI3K and BRAF are among the most commonly mutated tumor-driving kinases, with point mutations leading to sustained activity.[p24],[p25],[p26] Mutated or not, kinase genes must be adequately expressed to impact cellular signaling. Although kinase expression determines the potential to initiate a kinase response, the correlation between expression levels and kinase activity is weak.

Studying the kinase in its endogenous environment is vital to understanding its activity.[p27] To begin, kinase–substrate proximity is essential to determine the available substrate pool. For instance, protein kinase C (PKC) family members can bind to distinct trafficking proteins, altering PKC cellular localization and exposing it to various substrates.[p28] Moreover, proteins that bind to kinases can act as scaffolding proteins, connecting kinases and substrates.[p29]

Post-translational modifications (PTMs) also finely tune kinase activation, with phosphorylation being the most common. PTMs can influence the biological behavior of proteins, such as intracellular trafficking, physically exposing or blocking binding sites, or achieve the same effect through allosteric refolding. For example, fibroblast growth factor receptor (FGFR) has multiple phosphorylation sites on its intracellular tail, enabling the binding and phosphorylation of different substrates.[p30] Kinases often possess multiple regulatory PTMs but one or two key phosphorylations primarily determine their activity.

Alternative substrates or chemical environments can also impact kinase activity. For instance, CaMK kinases are activated

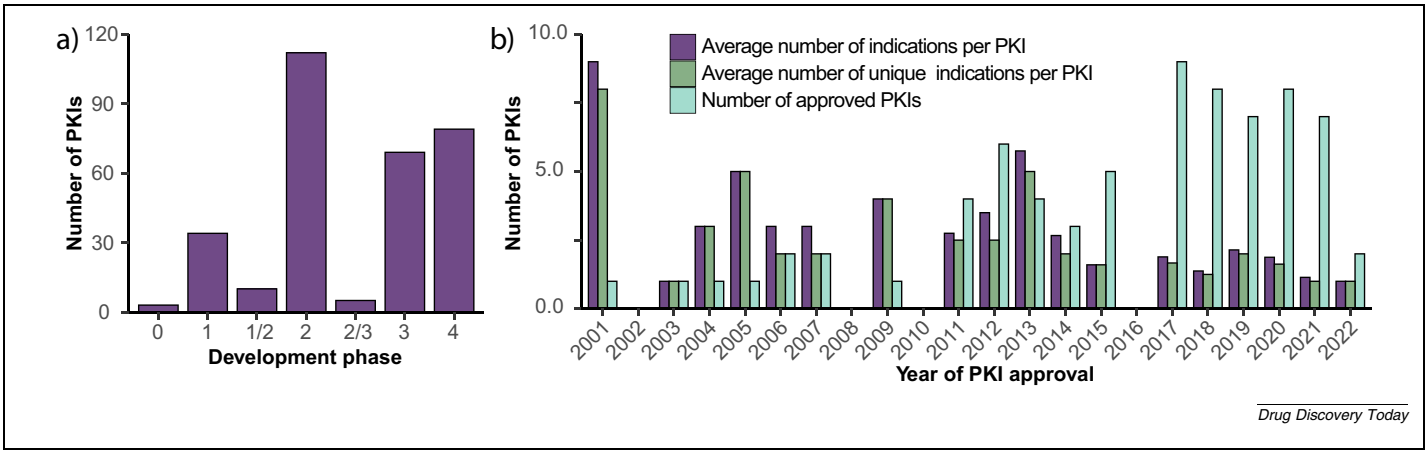

FIGURE 1

Overview of the protein kinase inhibitor (PKI) development pipeline. **(a)** Number of current PKIs in the different phases of drug development.[p86],[p123] The development Phases for I and II, and II and III are combined for some PKIs. **(b)** The average number of PKI indications, the average number of unique indications and the total number of approved PKIs per year. Unique indications are indications in different pathological fields or cancer types.

by autophosphorylation upon Calmodulin or Ca^{2+} binding, and pH alters the activity dynamics of cyclin-dependent kinase 6 (CDK6).[p31],[p32] Kinase folding, crucial for activity, is influenced by PTMs, which can refold kinases into active conformations, facilitating ATP and substrate binding. Activation loop phosphorylation is often considered the most important step for folding into an active conformation.[p33],[p34] Conserved domain analysis indicates that ~85% of kinases contain an activation loop, highlighting its importance in kinase activity. Once activated, nearby substrates can be phosphorylated, often leading to feedback loops and altered gene expression, producing activating or inactivating signals that further modulate cellular signaling.

The kinase activity assay toolkit

The intricate process of kinase activity regulation and protein phosphorylation indicates that various approaches can be employed to measure kinase activity (Figure 2). No single assay captures the full complexity of kinase activity, necessitating technological advancements to encompass as much of this complexity as possible. Each assay has unique strengths and weaknesses, requiring a thorough understanding to match these characteristics to the challenge at hand. This review focuses on assays that (i) quantify kinase activity and (ii) excel in multiple biological and analytical properties vital for PKI development challenges. The assays we excluded encompass bioinformatic platforms, assays that do not infer kinase activity (such as biochemical assays measuring target engagement rather than kinase activity) and assays that identify kinase activity incidentally. Unless otherwise specified, 'kinase activity assays' refers to this selection.

Biological and analytical assay parameters

Each assay yields different biological insights and biological precision. Improved biological precision can be ascribed to several assay properties (Table 1). First, assays should measure endogenous kinase activity without adding recombinant kinases, substrates or ATP,[p35] thereby ensuring a biologically relevant experimental system. Second, assays should perform measurements under relevant biological conditions, such as appropriate *in situ* or *in vivo* systems or disease models. Third, assays should directly measure kinase activity at the responsible kinase rather than predicting it based on substrate phosphorylation.[p36] Fourth, because cellular signaling pathways involve multiple kinase events, broader kinome coverage in the assay enhances the understanding of the kinome activity.[p37] Fifth, changes in kinase activity can impact the proteome, so assays providing additional protein expression information further increase biological relevance. Lastly, some assays offer information on PKI binding to its target (target binding), which is crucial for determining parameters such as dissociation constants or identifying on– or off-target effects.[p38] Additionally, some assays provide structural information to elucidate binding pockets and key residues required for PKI binding.[p39]

Apart from the differences in biological relevance, kinase activity assays possess unique analytical properties. Analytical properties include sensitivity, specificity, quantification accuracy, reproducibility, quantification, throughput, multiplexity and assay complexity (Box S1, see supplementary material online). These properties determine suitability of an assay for addressing specific scientific questions. The following section

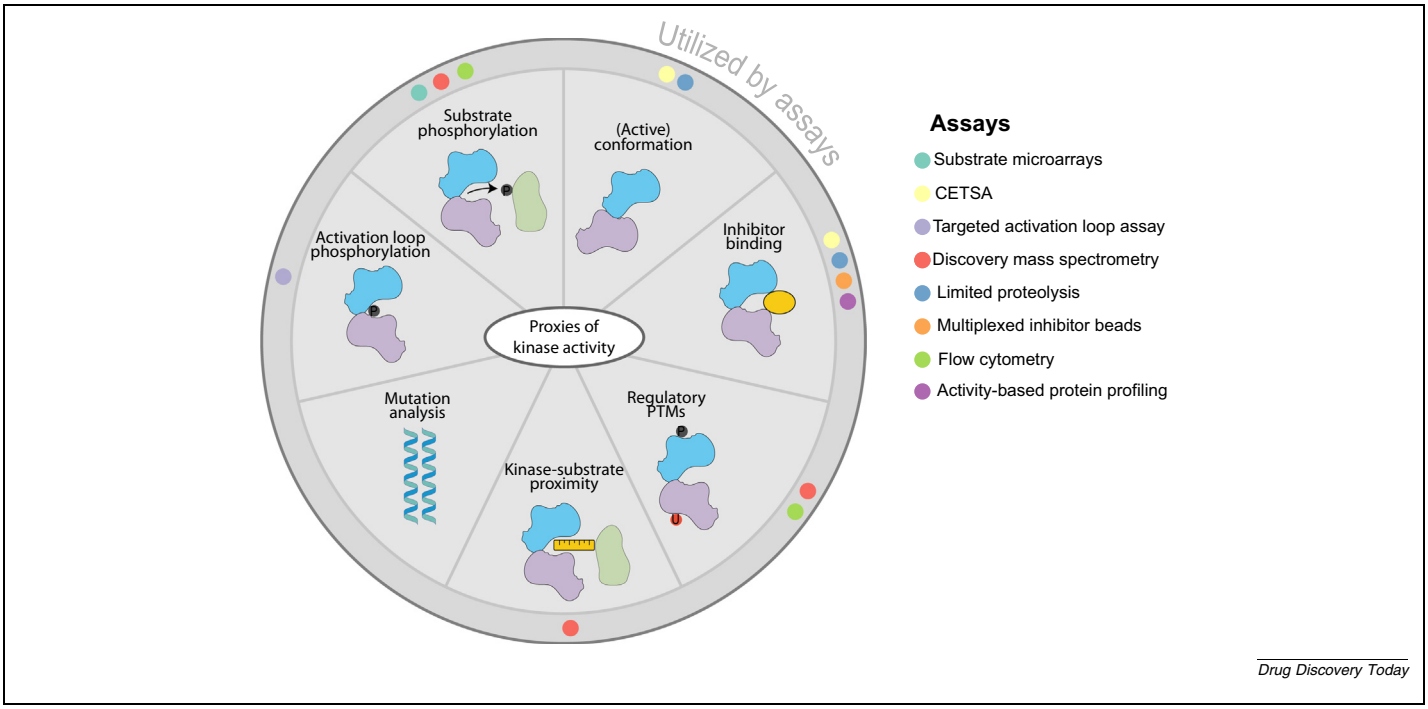

FIGURE 2

Kinase activity assays use diverse strategies to infer kinase activity. Illustration of how kinase activity assays employ various strategies to measure kinase activity. Each solid-filled circle within the figure symbolizes a specific kinase activity assay positioned according to its method of measuring kinase activity. Certain assays adopt diverse approaches for this purpose. The included assays excel in multiple biological and analytical properties crucial for protein kinase inhibitor (PKI) development challenges. Other proxies not covered here can be measured using alternative kinase activity assays.

TABLE 1

Biological and analytical properties of the assays described in this review.

Biological properties	Substrate microarrays	CETSA	Targeted activation loop assay	Discovery mass spectrometry	Limited proteolysis	Multiplexed inhibitor beads	Flow cytometry	Activity-based protein profiling
Detection of endogenous peptides		✓	✓	✓	✓	✓	✓	✓
Physiological relevant sample types	✓	✓	✓	✓	✓	✓	✓	✓
Direct			✓			✓	✓	✓
Kinome coverage			✓					
Measurement of additional substrates		✓		✓	✓			
Target binding		✓			✓	✓		✓
Analytical properties								
Sensitivity	✓		✓			✓	✓	✓
Specificity		✓	✓	✓	✓	✓		✓
Quantification accuracy							✓	
Reproducibility	✓	✓	✓	✓	?	✓	✓	✓
Quantification			✓				✓	
Throughput								
Multiplexity		✓	✓	✓	✓	✓		
Assay complexity	✓						✓	

Each assay excels in a subset of the biological and analytical properties, which are presented using a checkmark in the table. An assay is regarded to excel in a property when this is of the level needed to tackle challenges arising during protein kinase inhibitor (PKI) development (see Table S1 in supplementary material online). Unknowns due to insufficient available quantitative data are represented using a question mark (?).

will discuss how these assays aim to measure kinase activity. Understanding this is essential for identifying the most promising assays to address the challenges of PKI development, which will be explained subsequently.

Substrate microarrays

Substrate microarrays (Table 1) consist of synthetic non-phosphorylated peptides coated on plates or wells, which are known kinase targets for phosphorylation and are used to predict kinase activity.[p40] Kinase substrate surrogates are incubated with cell lysates or kinases. Subsequently, kinase activity is determined by quantifying substrate phosphorylation using phosphorylation-specific antibodies or heavy-labeled ATP. Substrate arrays are easy to use, providing quick predictions of kinase activity, making them a fast and simple tool. Several companies offer substrate arrays as a service or as ready-to-use products, such as Kinex™ and PamChip™. Some commercial substrate microarrays attempt to predict the activity of nearly the entire kinome. Importantly, these predictions are indirect and not endogenous measurements.

Cellular thermal shift assay

The cellular thermal shift assay (CETSA) (Table 1) is a label-free method that directly measures intracellular target engagement of PKIs by assessing protein thermal stability.[p41],[p42] Typically, cells are incubated with a PKI in a physiologically relevant environment. Post-treatment, samples are heated to denature and precipitate the proteins, and proteins in the remaining solution are quantified.[p43] PKI-bound kinases exhibit altered denaturation curves, remaining in solution longer.[p41] Detection and identification of stabilized kinases in the soluble fractions can be achieved through various approaches, including western blotting, proximity-based assays with specific probes or multiplexed quantitative mass spectrometry (MS). Several vendors provide materials such as probes or antibodies for CETSA experiments, whereas Pelago Bioscience™ offers CETSA as a service.

Targeted activation loop assay

Targeted activation loop assays (Table 1) use targeted MS methods to measure activation loop phosphorylation in kinases, indicative of kinase activation. Heavy labeled peptides are added as internal standards to provide high selectivity and confidence in identifying and quantifying kinase isoforms or phosphoproteoforms with different biological functions.[p44],[p45] Activation loop phosphorylations are quantified on a triple quadrupole mass spectrometer (SRM/MRM assays) or quadrupole-orbitrap/time-of-flight instrument (PRM assays). QuantaKinome™, a novel platform, monitors the activation loop phosphorylation status of >200 endogenous kinases and substrates in a sample.[p37] Commercial kits like SureQuant™ simplify PRM assays and increase sample throughput.[p46]

Discovery mass spectrometry

Discovery mass spectrometry in the context of kinase activity quantification (Table 1) uses liquid chromatography-mass spectrometry (LC-MS) to measure phosphorylated peptides from protein digests.[p47],[p48] Cells are lysed, proteins are digested and phosphorylated peptides are enriched before MS analysis. Label-free quantification methods are often used; however, alternative quantification methods using (non-)isobaric tags are also standardized. Ongoing developments include automated, sensitive and high-throughput sample preparation, faster and more-sensitive mass spectrometers, and better bioinformatics tools for predicting kinase activity.[p49],[p50],[p51] Discovery phosphoproteomics mass spectrometry is an established assay with different pipelines mainly established in specialized research laboratories but also commercially available (e.g., KScan™).

Limited proteolysis

Limited proteolysis coupled to MS (LiP-MS) (Table 1) predicts PKI binding and protein structural changes by assessing protease cleavage susceptibility alterations after perturbations.[p52],[p53] Machine-learning-based LiP-Quant assigns probability scores to peptides, indicating potential structural changes and EC_{50} scores.[p54] This method can predict binding pockets of PKIs, like the binding of selumetinib to MAP2K1 and MAP2K2.58 LiP-MS is commercially available from Biognosys™ and LiP-Quant software can be accessed online.

Multiplexed inhibitor beads

Multiplexed inhibitor beads (MIBs; Table 1) employ immobilized kinase inhibitors linked to beads or columns to purify interacting kinases, which are measured using techniques like MS.[p55] Columns containing multiple inhibitor layers capture as many kinases as possible. Kinase activity is solely quantified when inhibitors bind exclusively to the kinase active state.[p56],[p57],[p58] This strategy has identified off-target binding of numerous PKIs.[p19] In-house MIB protocols are available[p59] and KinomeScout by OmiScouts™ offers commercial kinome activity studies using MIBs.

Flow cytometry

Flow cytometry (Table 1) uses lasers to analyze labeled particles or single cells in fluid, such as labeled activating PTMs on kinases or resulting substrate phosphorylation with fluorescent markers.[p60] Flow cytometry is relatively simple, providing absolute quantification of the particles. However, challenges are the limited availability of suitable antibodies and probes and a limited probe multiplexity.[p61],[p62] Although no companies offer specific flow cytometry services for kinase activity, custom panels can be designed for kinase activity measurements.

Activity-based protein profiling

Activity-based protein profiling (ABPP; Table 1) uses chemical probes to bind targets, such as kinases, which are then isolated and quantified.[p63],[p64] Kinase-binding probes enable target enrichment before MS analysis.[p65] ATP analogs, like XO44, are commonly used probes, quantifying ~150 kinases per experiment.[p66] Custom probes can be made, yet steric hindrance must be considered. Probes can target active and inactive kinase conformations, limiting accurate quantification of kinase activity.[p67]

Connecting assays to the challenges

Selecting the appropriate kinase activity assay depends on the challenge being addressed and its biological and analytical properties requirements. It is crucial to compare the requirements with the properties of available kinase activity assays to make the right choice, because each assay excels in a specific subset of properties (Table 1; Table S1, see supplementary material online). The following section will discuss the challenges, their particular requirements and the most promising assays to tackle them.

PKI discovery and development challenges

Unlocking the underexplored potential of PKI development involves addressing various challenges that can arise across its different stages (Figure 3). Most of these challenges require an in-depth understanding of the PKI MOA. The PKI MOA refers to the biochemical interactions through which a PKI exerts its effects. The FDA does not require a complete understanding of MOA to approve initiating clinical studies.[p68],[p69] This has deterred pharmaceutical companies from implementing functional kinase activity assays alongside kinase binding assays, although such assays are crucial for the success of PKI candidates. Commonly used biochemical kinase activity assays, like the nano-BRET assay, only partially address the MOA challenge by determining target engagement without examining downstream pharmacodynamic effects. As a result, most PKI candidates lack a clear understanding of MOA and overall pharmacodynamic (PD) profile. Another way to unlock the potential of PKI development is to use biomarkers. Biomarkers, objectively measured biological markers indicative of a patient's medical state, have a crucial role throughout PKI discovery and development. The next sections will elaborate on five challenges to improve PKI development.

Challenge 1: illuminating the understudied dark kinome

The first challenge is illuminating the understudied dark kinome, comprising ~160 kinases whose function in human biology is poorly understood.[p70] Kinases belonging to the dark kinome are low in abundance, making them undetectable by most kinase activity assays. The inability to measure these kinases leads to a knowledge gap and a lack of proper antibodies, potent chemical probes and bioinformatics software that can predict their activity, role in disease and treatment targets.[p71],[p72] This vicious circle and importance of the dark kinome has resulted in numerous efforts to identify and prioritize the study of the dark kinome, such as the 'Illuminating the Druggable Genome' program which includes the study of the dark kinome and The Clinical Kinase Index.[p70],[p71],[p73] Nevertheless, the dark kinome remains understudied.

Most detected dark kinases have emerged through (genomic) screening studies.[p73],[p74],[p75] Although these screens can identify implicated kinases, they do not quantify kinase activity. The ideal assay should be highly sensitive to be compatible with measuring low-abundance kinases and quantifying kinase activity directly because algorithms and techniques for indirect quantification of dark kinases are still underdeveloped. For pharmaceutical target deconvolution, assays should be capable of measuring kinase activity in high throughput, because HTS is preferably used to screen for new targets. None of the discussed assays meet these requirements (Table 1) but the targeted activation loop assay fits best, although it currently lacks the required high throughput (Table 1, Figure 4). Nevertheless, using the targeted activation loop assay, dark kinases could reliably be quantified, such as ERK4.[p37] As a follow-up on the Dark Kinome Knowledgebase, efforts are being put into expanding the targeted repertoire to include the dark kinome.

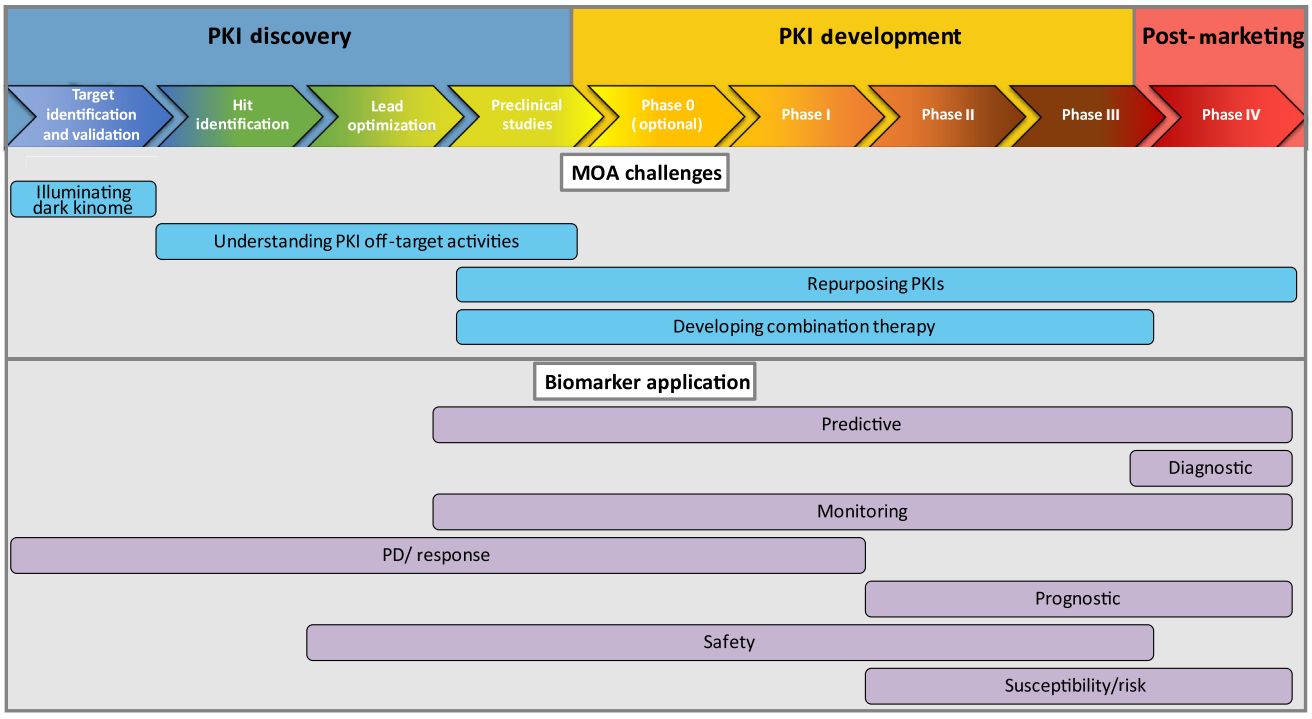

FIGURE 3

The relevance of MOA challenges and biomarker types in each phase of the PKI discovery and development pipeline. Abbreviations: MOA, mechanism of action; PKI, protein kinase inhibitor.

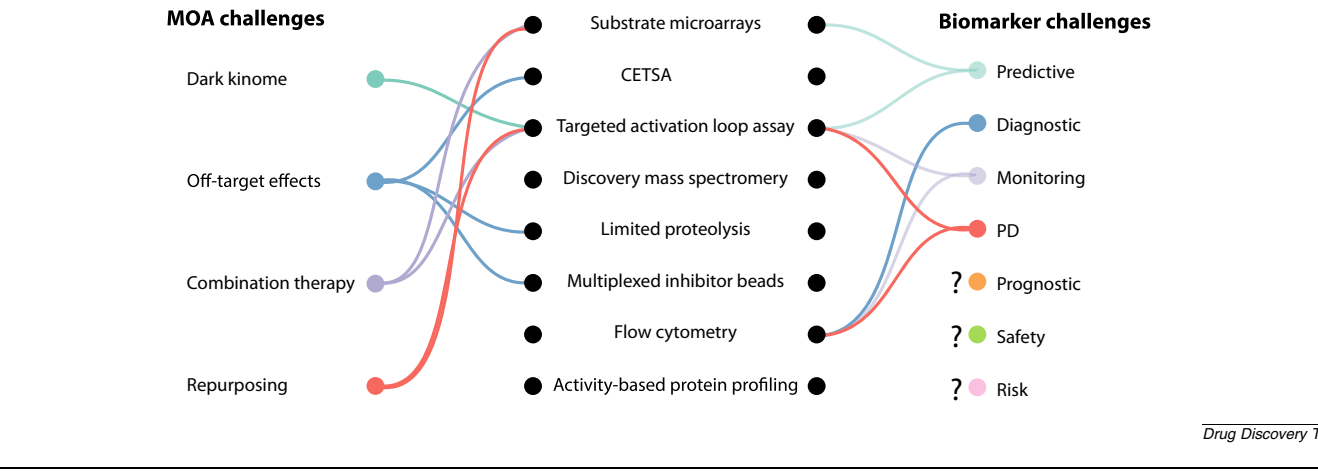

FIGURE 4

The best-fit kinase activity assay(s) for each mechanism of action (MOA) and biomarker challenge. The assays are shown in the middle and coupled with the MOA challenges (left) and biomarker challenges (right), for which they are the best choice. No assay meets the high standards needed for prognostic and risk biomarkers yet. Consequently, these are represented using a question mark (?).

Challenge 2: understanding PKI off-target activities

Despite researchers' efforts to develop highly selective PKI candidates, almost all PKIs are 'dirty' compounds, hitting (many) off-target proteins. This eventually leads to unexpected off-target toxicity emerging in later developmental stages owing to the knowledge deficit on the PKI candidate PD and off-target effects.[(p76),(p77),(p78)] Klaeger *et al.* demonstrate the lack of MOA knowledge by finding 20% additional and unknown off-target activities of their tested PKIs compared to the already known off-target activities.[(p19)] Notably, at least 25 PKIs show non-

kinase off-target effects, rarely studied in the hit identification and lead optimization phases.[p79] The unknown biological effects of the PKI on off-target kinases and non-kinases present a therapeutic potential that pharmaceutical companies have not yet exploited.

Numerous assays have been used to elucidate off-target PKI effects, for example ELISA, Alphascreen™ or TR-FRET.[p78],[p80] These assays have identified off-target activities because running large sample sets increases the likelihood of finding them. An optimal assay should maximize the chance of finding off-target effects through broad coverage of the kinome and proteome in a multiplexed fashion. The selected assays that fulfill most requirements are CETSA, limited proteolysis and multiplexed inhibitor beads (Table 1, Figure 4). However, none of these assays fit perfectly because they lack sensitivity or coverage. MIBs can be used to enrich for off-target effects. This approach has, for example, been used to find off-target effects of nearly 1200 PKIs.[p81] MIBs do not measure kinase activity directly; thus, obtaining reliable kinase activity measurements must be measured with alternative assays.

Challenge 3: repurposing abandoned PKI candidates and finding new opportunities for approved PKIs

Repurposing abandoned or approved PKIs is appealing because we already partly understand these PKIs. Exploiting this knowledge can lead to accelerated PKI development, lower development costs and reduced risk. Although approved PKIs are readily available for repurposing efforts, many abandoned PKI candidates are available through public repositories or companies that gather and/or provide these molecules.[p82],[p83] At least 62% of protein kinases that lack an approved PKI can be targeted using abandoned inhibitors with a preset minimum bioactivity.[p72]

Numerous repurposing strategies exist, including exploiting off-target activities of PKIs that were undesirable for one disease but could be beneficial in treating another disease.[p84] Another strategy is to use abandoned PKIs with high toxicity in one disease that can demonstrate efficacy in another disease at lower, less toxic doses. For instance, initially unsuccessful as an anticancer agent, saracatinib has completed Phase I clinical trials for Alzheimer's Disease treatment using lower, more-tolerable doses.[p85] Another strategy is repurposing PKIs for other disease areas, such as infectious diseases, immune disorders or even antibiotics.[p86],[p87]

An ideal kinase activity assay reveals mechanistic insights into PKI activity through broadly measuring kinase regulation, increasing the likelihood of identifying inhibited kinases and pathways. Ideally, PKI target binding is measured, providing a mechanistic insight into PKI effects.[p77],[p87],[p88] Analytically, employing a highly sensitive, reproducible and multiplexed assay is beneficial, because alternative targets for a abandoned) PKI can be unknown and scarce. The assay should be able to handle large compound libraries. The substrate microarray and targeted activation loop assay are the most suitable for this challenge, although none is capable of high-throughput measurements (Table 1, Figure 4). An example of such an approach is an effort that explored the possible repurposing of PKIs as antiplatelet drugs using a substrate assay, successfully identifying sunitinib.[p89],[p90]

Challenge 4: developing effective combination therapy

PKI attrition in clinical trials can be attributed to diseases not governed by a single kinase target or pathway. For instance, targeting a solitary kinase in cancer therapy can lead to intrinsic resistance, target mutations or acquired activation of alternative pathways.[p91] Employing a combination therapy involving another PKI or an alternative therapy represents a prominent strategy to overcome these challenges.[p92] Studies increasingly report the effectiveness of combination therapy, such as BRAF inhibitors combined with immune checkpoint inhibitors or EGFR inhibitors, and the combined inhibition of ROCK and EGFR in triple-negative breast cancer.[p93],[p94],[p95],[p96],[p97],[p98],[p99],[p100] Numerous challenges can be listed that limit the use of combination therapy.[p101] However, the most important factor is the absence of preclinical evidence that provides rationale for combination therapy, specifically the inadequate use of appropriate assays to provide this evidence. Combining drugs necessitates a deep understanding of biological interactions to maximize therapeutic response while mitigating the risk of adverse effects.

So, understanding the resistance mechanism and the MOA of individual drugs and their combinations in the specific disease model is crucial to developing successful PKI combination treatments.[p102],[p103] Current assays lack sensitivity and reproducibility in preclinical phases, hampering the ability to measure the selectivity and MOA of PKI combinations.[p102] Obtaining the information required to predict what drugs to use in combination therapy ideally includes multiplexed assays with high kinome and proteome coverage measuring direct kinase activity in a physiologically relevant context with preferably absolute quantification.[p99] Although imperfect, the most suitable assays meeting these requirements include substrate microarrays and targeted activation loop assays (Table 1, Figure 4). Especially in *in vitro* models, these assays have contributed to understanding combination therapy.[p104],[p105],[p106],[p107]

Challenge 5: identifying clinical biomarkers for precision medicine

The final challenge is identifying suitable activity-based clinical biomarkers.[p108],[p109] The Biomarkers, Endpoints and other Tools (BEST) resource categorizes biomarkers into seven types: diagnostic, monitoring, PD/response, prognostic, predictive, safety and susceptibility/risk.[p110] All seven can be used to improve PKI discovery and development.

Because biomarker quantification typically leads to clinical action, biomarkers must fulfill high standards regarding reliability, specificity, sensitivity and reproducibility. Numerous assays have aimed to meet these standards, including gene expression microarrays, discovery mass spectrometry, ELISA and PCR-based assays. Although these assays were successful, improved assays are desired because they provide indirect readouts, do not detect low abundant kinases or do not have the correct antibodies to measure kinase activity.[p11],[p109],[p111],[p112],[p113],[p114],[p115] Although different categories of biomarkers require slightly

different assay properties, kinase assays should be highly specific to measure endogenous kinase activity in relevant sample types, be low in complexity with interpretable results, highly specific, reproducible, accurate and that provide an absolute readout that can be compared to a preset threshold.(p116),(p117),(p118)

Four kinase activity assays are generally suitable for solving the biomarker challenge described above based on their properties (Table 1). Substrate microarrays are appropriate for developing predictive biomarkers because they are highly sensitive and reproducible and are not too complex to interpret. They show a strong association between the substrate biomarker and response rate, which is necessary to understand whether a patient will respond to a therapy.(p119),(p120) The targeted activation loop assay is a good fit for developing predictive biomarkers, monitoring biomarkers and PD biomarkers owing to its sensitivity and specificity in general and to the possibility for absolute quantification, reproducibility and kinome coverage, respectively. Flow cytometry is a suitable assay for developing diagnostic, monitoring or PD biomarkers. The assay can measure kinase activity in single cells, increasing the quality of these three biomarker types.(p121),(p122)

Concluding remarks and future perspectives

Opportunities remain to develop PKIs more effectively. Integrating kinase activity assays to address MOA and biomarker-related challenges could decrease attrition rates and enhance PKI efficiency. Kinase activity assays, with desirable biological and analytical properties, offer promising solutions. However, no one-size-fits-all assay exists, because each challenge has unique requirements and each assay possesses distinct properties. Careful selection of the appropriate assay for each challenge can require multiple assays. Enhancing the ability to address challenges with kinase activity assays necessitates further development. First, increasing throughput is vital, which can be achieved by investing in workflow automation, time reduction, robotics and artificial intelligence for data interpretation. Second, the complexity of kinase activity assays has made their application in hospital settings difficult. Barriers to clinical use include high costs, the need for specialized personnel, lack of governmental approval and limited throughput. Continued development of the kinase activity assays discussed in this review will help address these challenges and optimize their utility in clinical settings.

Selecting the optimal kinase activity assay to address PKI challenges in this review depends on their biological and technological properties. Further refinement of assay choice should consider the specific practical needs of each challenge. These include assay automation (e.g., using robotics) with some assays, such as mass spectrometry-based assays, requiring more time to automate than substrate microarrays. Another consideration is assay standardization, which is crucial for effective PKI development. For instance, daily clinical routine demands high validation, whereas early development stages might not require such stringent validation. Finally, cost implications should be considered, because HTS can rapidly escalate expenses owing to the large number of samples involved. In conclusion, kinase activity assays are evolving to meet the rigorous demands of effective PKI utilization and development. Although significant progress has been achieved, selecting the appropriate kinase activity assay(s) for each challenge remains crucial. Ongoing enhancements of these assays support the expansion and optimal use of the PKI toolkit, which is vital for addressing kinase-driven diseases.

Conflicts of interest

Maarten Altelaar is one of the patent holders of the described T-loop kinase activity assay QuantaKinome™. Nynke Kannegieter, Erik de Graaf, Jos Joore and Anna Ressa are former employees of Pepscope, which offered the kinase activity assay QuantaKinome™. All other authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Tim S. Veth: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Nynke M. Kannegieter:** Visualization, Writing – original draft, Writing – review & editing. **Erik L. de Graaf:** Conceptualization, Writing – original draft. **Rob Ruijtenbeek:** Writing – original draft. **Jos Joore:** Writing – original draft. **Anna Ressa:** Conceptualization, Writing – original draft. **Maarten Altelaar:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank Nick Veringmeier (JustPushPlay) for proofreading and improving the manuscript's readability. This work has been supported by EPIC-XS, project number 823839, funded by the Horizon 2020 programme of the European Union and the NWO funded Netherlands Proteomics Centre through the National Road Map for Large-scale Infrastructures program X-Omics, Project 184.034.019.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.drudis.2024.103907.

References

1. Attwood MM, Fabbro D, Sokolov AV, Knapp S, Schiöth HB. Trends in kinase drug discovery: targets, indications and inhibitor design. *Nat Rev Drug Discov*. 2021;20:839–861. https://doi.org/10.1038/s41573-021-00252-y.
2. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002;298:1912–1934. https://doi.org/10.1126/science.1075762.
3. Moret N et al. A resource for exploring the understudied human kinome for research and therapeutic opportunities. *Systems Biology*. 2020 http://biorxiv.org/lookup/doi/10.1101/2020.04.02.022277.
4. Sharma K et al. Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep*. 2014;8:1583–1594. https://doi.org/10.1016/j.celrep.2014.07.036.

5. Lahiry P, Torkamani A, Schork NJ, Hegele RA. Kinase mutations in human disease: interpreting genotype–phenotype relationships. *Nat Rev Genet*. 2010;11:60–74. https://doi.org/10.1038/nrg2707.
6. Ferguson FM, Gray NS. Kinase inhibitors: the road ahead. *Nat Rev Drug Discov*. 2018;17:353–377. https://doi.org/10.1038/nrd.2018.21.
7. Kremer JM et al. The safety and efficacy of a JAK inhibitor in patients with active rheumatoid arthritis: Results of a double-blind, placebo-controlled phase IIa trial of three dosage levels of CP-690,550 versus placebo. *Arthritis Rheum*. 2009;60:1895–1905. https://doi.org/10.1002/art.24567.
8. Reimand J, Wagih O, Bader GD. The mutational landscape of phosphorylation signaling in cancer. *Sci Rep*. 2013;3:2651. https://doi.org/10.1038/srep02651.
9. Vinores SA. Pegaptanib in the treatment of wet, age-related macular degeneration. *Int J Nanomed*. 2009. Published online.
10. Defert O, Boland S. Kinase profiling in early stage drug discovery: sorting things out. *Drug Discov Today Technol*. 2015;18:52–61. https://doi.org/10.1016/j.ddtec.2015.10.002.
11. Wang Y, Ma H. Protein kinase profiling assays: a technology review. *Drug Discov Today Technol*. 2015;18:1–8. https://doi.org/10.1016/j.ddtec.2015.10.007.
12. Cohen P, Cross D, Jänne PA. Kinase drug discovery 20 years after imatinib: progress and future directions. *Nat Rev Drug Discov*. 2021;20:551–569. https://doi.org/10.1038/s41573-021-00195-4.
13. Lightfoot HL, Goldberg FW, Sedelmeier J. Evolution of small molecule kinase drugs. *ACS Med Chem Lett*. 2018;10:153–160. https://doi.org/10.1021/acsmedchemlett.8b00445.
14. Roskoski R. Properties of FDA-approved small molecule protein kinase inhibitors. *Pharmacol Res*. 2019;144:19–50. https://doi.org/10.1016/j.phrs.2019.03.006.
15. Shapiro P. *Next generation kinase inhibitors: moving beyond the ATP binding/catalytic sites*. Springer International Publishing; 2020 https://link.springer.com/10.1007/978-3-030-48283-1.
16. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature*. 2001;411:355–365. https://doi.org/10.1038/35077225.
17. Sanchez-Vega F et al. Oncogenic signaling pathways in the cancer genome atlas. *Cell*. 2018;173:321–337.e10. https://doi.org/10.1016/j.cell.2018.03.035.
18. Xie Y et al. Glycan–protein cross-linking mass spectrometry reveals sialic acid-mediated protein networks on cell surfaces. *Chem Sci*. 2021;12:8767–8777. https://doi.org/10.1039/D1SC00814E.
19. Klaeger S et al. The target landscape of clinical kinase drugs. *Science*. 2017;358. https://doi.org/10.1126/science.aan4368 eaan4368.
20. Morgan S, Grootendorst P, Lexchin J, Cunningham C, Greyson D. The cost of drug development: a systematic review. *Health Policy*. 2011;100:4–17. https://doi.org/10.1016/j.healthpol.2010.12.002.
21. Friedman AA, Letai A, Fisher DE, Flaherty KT. Precision medicine for cancer with next-generation functional diagnostics. *Nat Rev Cancer*. 2015;15:747–756. https://doi.org/10.1038/nrc4015.
22. Schmidt KT, Chau CH, Price DK, Figg WD. Precision oncology medicine: the clinical relevance of patient-specific biomarkers used to optimize cancer treatment: precision oncology medicine. *J Clin Pharmacol*. 2016;56:1484–1499. https://doi.org/10.1002/jcph.765.
23. Franciosa G, Locard-Paulet M, Jensen LJ, Olsen JV. Recent advances in kinase signaling network profiling by mass spectrometry. *Curr Opin Chem Biol*. 2023;73. https://doi.org/10.1016/j.cbpa.2022.102260 102260.
24. Cicenas J, Zalyte E, Bairoch A, Gaudet P. Kinases and cancer. *Cancers*. 2018;10:63. https://doi.org/10.3390/cancers10030063.
25. Kang S, Bader AG, Zhao L, Vogt PK. Mutated PI 3-kinases: cancer targets on a silver platter. *Cell Cycle*. 2005;4:571–574. https://doi.org/10.4161/cc.4.4.1593.
26. Mendiratta G, Ke E, Aziz M, Liarakos D, Tong M, Stites EC. Cancer gene mutation frequencies for the U.S. population. *Nat Commun*. 2021;12:5961. https://doi.org/10.1038/s41467-021-26213-y.
27. Ochoa D et al. An atlas of human kinase regulation. *Mol Syst Biol*. 2016;12:888. https://doi.org/10.15252/msb.20167295.
28. Gould CM, Newton AC. The life and death of protein kinase C. *Curr Drug Targets*. 2008;9:614–625. https://doi.org/10.2174/138945008785132411.
29. Morrison DK. The 14–3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends Cell Biol*. 2009;19:16–23. https://doi.org/10.1016/j.tcb.2008.10.003.
30. Lew ED, Furdui CM, Anderson KS, Schlessinger J. The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. *Sci Signal*. 2009;2. https://doi.org/10.1126/scisignal.2000021.
31. Swulius MT, Waxham MN. Ca(2+)/calmodulin-dependent protein kinases. *Cell Mol Life Sci CMLS*. 2008;65:2637–2657. https://doi.org/10.1007/s00018-008-8086-2.
32. Yousuf M et al. Effect of pH on the structure and function of cyclin-dependent kinase 6. Singh S, ed.. *PLOS ONE*. 2022;17. https://doi.org/10.1371/journal.pone.0263693 e0263693.
33. Modi V, Dunbrack RL. Defining a new nomenclature for the structures of active and inactive kinases. *Proc Natl Acad Sci*. 2019;116:6818–6827. https://doi.org/10.1073/pnas.1814279116.
34. Nolen B, Taylor S, Ghosh G. Regulation of protein kinases: controlling activity through activation segment conformation. *Mol Cell*. 2004;15:661–675. https://doi.org/10.1016/j.molcel.2004.08.024.
35. Papachristou EK et al. A quantitative mass spectrometry-based approach to monitor the dynamics of endogenous chromatin-associated protein complexes. *Nat Commun*. 2018;9:2311. https://doi.org/10.1038/s41467-018-04619-5.
36. Y lmaz S, Ayati M, Schlatzer D, Çiçek AE, Chance MR, Koyutürk M. Robust inference of kinase activity using functional networks. *Nat Commun*. 2021;12:1177. https://doi.org/10.1038/s41467-021-21211-6.
37. Schmidlin T et al. High-throughput assessment of kinome-wide activation states. *Cell Syst*. 2019;9:366–374.e5. https://doi.org/10.1016/j.cels.2019.08.005.
38. Walkup GK et al. Translating slow-binding inhibition kinetics into cellular and in vivo effects. *Nat Chem Biol*. 2015;11:416–423. https://doi.org/10.1038/nchembio.1796.
39. De Souza N, Picotti P. Mass spectrometry analysis of the structural proteome. *Curr Opin Struct Biol*. 2020;60:57–65. https://doi.org/10.1016/j.sbi.2019.10.006.
40. Houseman BT, Huh JH, Kron SJ, Mrksich M. Peptide chips for the quantitative evaluation of protein kinase activity. *Nat Biotechnol*. 2002;20:270–274. https://doi.org/10.1038/nbt0302-270.
41. Ishii T et al. CETSA quantitatively verifies in vivo target engagement of novel RIPK1 inhibitors in various biospecimens. *Sci Rep*. 2017;7:13000. https://doi.org/10.1038/s41598-017-12513-1.
42. Shaw J et al. Positioning high-throughput CETSA in early drug discovery through screening against B-Raf and PARP1. *SLAS Discov*. 2019;24:121–132. https://doi.org/10.1177/2472555218813332.
43. Jafari R et al. The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat Protoc*. 2014;9:2100–2122. https://doi.org/10.1038/nprot.2014.138.
44. de Graaf EL, Altelaar AFM, van Breukelen B, Mohammed S, Heck AJR. Improving SRM assay development: a global comparison between triple quadrupole, ion trap, and higher energy CID peptide fragmentation spectra. *J Proteome Res*. 2011;10:4334–4341. https://doi.org/10.1021/pr200156b.
45. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics*. 2012;11:1475–1488. https://doi.org/10.1074/mcp.O112.020131.
46. Stopfer LE et al. High-density, targeted monitoring of tyrosine phosphorylation reveals activated signaling networks in human tumors. *Cancer Res*. 2021;81:2495–2509. https://doi.org/10.1158/0008-5472.CAN-20-3804.
47. Lemeer S, Heck AJ. The phosphoproteomics data explosion. *Curr Opin Chem Biol*. 2009;13:414–420. https://doi.org/10.1016/j.cbpa.2009.06.022.
48. Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: approaches, advances, and applications. *Annu Rev Biomed Eng*. 2009;11:49–79. https://doi.org/10.1146/annurev-bioeng-061008-124934.
49. Martinez-Val A et al. Spatial-proteomics reveals phospho-signaling dynamics at subcellular resolution. *Nat Commun*. 2021;12:7113. https://doi.org/10.1038/s41467-021-27398-y.
50. Post H et al. Robust, sensitive, and automated phosphopeptide enrichment optimized for low sample amounts applied to primary hippocampal neurons. *J Proteome Res*. 2017;16:728–737. https://doi.org/10.1021/acs.jproteome.6b00753.
51. Savage SR, Zhang B. Using phosphoproteomics data to understand cellular signaling: a comprehensive guide to bioinformatics resources. *Clin Proteomics*. 2020;17:27. https://doi.org/10.1186/s12014-020-09290-x.
52. Feng Y et al. Global analysis of protein structural changes in complex proteomes. *Nat Biotechnol*. 2014;32:1036–1044. https://doi.org/10.1038/nbt.2999.
53. Malinovska L et al. Proteome-wide structural changes measured with limited proteolysis-mass spectrometry: an advanced protocol for high-throughput applications. *Nat Protoc*. 2023;18:659–682. https://doi.org/10.1038/s41596-022-00771-x.
54. Piazza I et al. LiP-Quant, an automated chemoproteomic approach to identify drug targets in complex proteomes. *Published online*. 2019.
55. Duncan JS et al. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. *Cell*. 2012;149:307–321. https://doi.org/10.1016/j.cell.2012.02.053.

56. Godl K et al. An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc Natl Acad Sci*. 2003;100:15434–15439. https://doi.org/10.1073/pnas.2535024100.
57. Kurimchak AM et al. Kinome profiling of primary endometrial tumors using multiplexed inhibitor beads and mass spectrometry identifies SRPK1 as candidate therapeutic target. *Mol Cell Proteomics*. 2020;19:2068–2090. https://doi.org/10.1074/mcp.RA120.002012.
58. Stuhlmiller TJ, Earp HS, Johnson GL. Adaptive reprogramming of the breast cancer kinome. *Clin Pharmacol Ther*. 2014;95:413–415. https://doi.org/10.1038/clpt.2014.8.
59. Daub H et al. Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell*. 2008;31:438–448. https://doi.org/10.1016/j.molcel.2008.07.007.
60. McKinnon KM. Flow cytometry: an overview. *Curr Protoc Immunol*. 2018;120. https://doi.org/10.1002/cpim.40.
61. Kannegieter NM, Hesselink DA, Dieterich M, De Graav GN, Kraaijeveld R, Baan CC. Differential T cell signaling pathway activation by tacrolimus and belatacept after kidney transplantation: post hoc analysis of a randomised-controlled trial. *Sci Rep*. 2017;7:15135. https://doi.org/10.1038/s41598-017-15542-y.
62. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat Biotechnol*. 2002;20:155–162. https://doi.org/10.1038/nbt0202-155.
63. Miao W, Yin J, Porter DF, Jiang X, Khavari PA, Wang Y. Targeted proteomic approaches for proteome-wide characterizations of the AMP-binding capacities of kinases. *J Proteome Res*. 2022;21:2063–2070. https://doi.org/10.1021/acs.jproteome.2c00225.
64. Okerberg ES et al. High-resolution functional proteomics by active-site peptide profiling. *Proc Natl Acad Sci*. 2005;102:4996–5001. https://doi.org/10.1073/pnas.0501205102.
65. Van Bergen W, Heck AJR, Baggelaar MP. Recent advancements in mass spectrometry–based tools to investigate newly synthesized proteins. *Curr Opin Chem Biol*. 2022;66. https://doi.org/10.1016/j.cbpa.2021.07.001 102074.
66. Patricelli MP et al. In situ kinase profiling reveals functionally relevant properties of native kinases. *Chem Biol*. 2011;18:699–710. https://doi.org/10.1016/j.chembiol.2011.04.011.
67. Zhao Q et al. Broad-spectrum kinase profiling in live cells with lysine-targeted sulfonyl fluoride probes. *J Am Chem Soc*. 2017;139:680–685. https://doi.org/10.1021/jacs.6b08536.
68. Davis RL. Mechanism of action and target identification: a matter of timing in drug discovery. *iScience*. 2020;23. https://doi.org/10.1016/j.isci.2020.101487 101487.
69. Moffat JG, Vincent F, Lee JA, Eder J, Prunotto M. Opportunities and challenges in phenotypic drug discovery: an industry perspective. *Nat Rev Drug Discov*. 2017;16:531–543. https://doi.org/10.1038/nrd.2017.111.
70. Berginski ME, Moret N, Liu C, Goldfarb D, Sorger PK, Gomez SM. The dark kinase knowledgebase: an online compendium of knowledge and experimental results of understudied kinases. *Nucleic Acids Res*. 2021;49:D529–D535. https://doi.org/10.1093/nar/gkaa853.
71. Essegian D, Khurana R, Stathias V, Schürer SC. The clinical kinase index: a method to prioritize understudied kinases as drug targets for the treatment of cancer. *Cell Rep Med*. 2020;1. https://doi.org/10.1016/j.xcrm.2020.100128 100128.
72. Oprea TI et al. Unexplored therapeutic opportunities in the human genome. *Nat Rev Drug Discov*. 2018;17:317–332. https://doi.org/10.1038/nrd.2018.14.
73. Tamir TY, Drewry DH, Wells C, Major MB, Axtman AD. PKIS deep dive yields a chemical starting point for dark kinases and a cell active BRSK2 inhibitor. *Sci Rep*. 2020;10:15826. https://doi.org/10.1038/s41598-020-72869-9.
74. Giamas G et al. Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer. *Nat Med*. 2011;17:715–719. https://doi.org/10.1038/nm.2351.
75. Southekal S, Mishra NK, Guda C. Pan-Cancer Analysis of Human Kinome Gene Expression and Promoter DNA Methylation Identifies Dark Kinase Biomarkers in Multiple Cancers. *Cancers*. 2021;13:1189. https://doi.org/10.3390/cancers13061189.
76. Chaar M, Kamta J, Ait-Oudhia S. Mechanisms, monitoring, and management of tyrosine kinase inhibitors-associated cardiovascular toxicities. *OncoTargets Ther*. 2018;11:6227–6237. https://doi.org/10.2147/OTT.S170138.
77. Huang Y et al. A framework for identification of on- and off-target transcriptional responses to drug treatment. *Sci Rep*. 2019;9:17603. https://doi.org/10.1038/s41598-019-54180-4.
78. Lin A et al. Off-target toxicity is a common mechanism of action of cancer drugs undergoing clinical trials. *Sci Transl Med*. 2019;11. https://doi.org/10.1126/scitranslmed.aaw8412 eaaw8412.
79. Munoz L. Non-kinase targets of protein kinase inhibitors. *Nat Rev Drug Discov*. 2017;16:424–440. https://doi.org/10.1038/nrd.2016.266.
80. Hantschel O. Unexpected off-targets and paradoxical pathway activation by kinase inhibitors. *ACS Chem Biol*. 2015;10:234–245. https://doi.org/10.1021/cb500886n.
81. Reinecke M et al. Chemical proteomics reveals the target landscape of 1,000 kinase inhibitors. *Nat Chem Biol*. 2023. https://doi.org/10.1038/s41589-023-01459-3.
82. Drewry DH, Wells CI, Zuercher WJ, Willson TM. A perspective on extreme open science: companies sharing compounds without restriction. *SLAS Discov*. 2019;24:505–514. https://doi.org/10.1177/2472555219838210.
83. Wells CI et al. The Kinase Chemogenomic Set (KCGS): an open science resource for kinase vulnerability identification. *Int J Mol Sci*. 2021;22:566. https://doi.org/10.3390/ijms22020566.
84. Knapp S. New opportunities for kinase drug repurposing and target discovery. *Br J Cancer*. 2018;118:936–937. https://doi.org/10.1038/s41416-018-0045-6.
85. Gns HS, Gr S, Murahari M, Krishnamurthy M. An update on drug repurposing: re-written saga of the drug's fate. *Biomed Pharmacother*. 2019;110:700–716. https://doi.org/10.1016/j.biopha.2018.11.127.
86. Bournez C et al. Comparative assessment of protein kinase inhibitors in public databases and in PKIDB. *Molecules*. 2020;25:3226. https://doi.org/10.3390/molecules25143226.
87. Zhang Z et al. Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduct Target Ther*. 2020;5:113. https://doi.org/10.1038/s41392-020-00213-8.
88. Pushpakom S et al. Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discov*. 2019;18:41–58. https://doi.org/10.1038/nrd.2018.168.
89. Tullemans B et al. Multiparameter evaluation of the platelet-inhibitory effects of tyrosine kinase inhibitors used for cancer treatment. *Int J Mol Sci*. 2021;22:11199. https://doi.org/10.3390/ijms222011199.
90. Tullemans BME et al. Tyrosine kinase inhibitor sunitinib delays platelet-induced coagulation: additive effects of aspirin. *Thromb Haemost*. 2022;122:92–104. https://doi.org/10.1055/s-0041-1730312.
91. Knight ZA, Lin H, Shokat KM. Targeting the cancer kinome through polypharmacology. *Nat Rev Cancer*. 2010;10:130–137. https://doi.org/10.1038/nrc2787.
92. Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. *Cell*. 2017;171:1678–1691. https://doi.org/10.1016/j.cell.2017.11.009.
93. Cohen JB. Novel therapies for relapsed/refractory aggressive lymphomas. *Hematol Am Soc Hematol Educ Program*. 2018;2018:75–82. https://doi.org/10.1182/asheducation-2018.1.75.
94. Flaherty KT, Sosman JA, Atkins MB. New options and new questions: how to select and sequence therapies for patients with metastatic melanoma. *Am Soc Clin Oncol Educ Book*. 2012;32:524–530. https://doi.org/10.14694/EdBook_AM.2012.32.211.
95. Geyer CE et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*. 2006;355:2733–2743. https://doi.org/10.1056/NEJMoa064320.
96. Giaccone G et al. Combination therapy with gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, gemcitabine and cisplatin in patients with advanced solid tumors. *Ann Oncol*. 2004;15:831–838. https://doi.org/10.1093/annonc/mdh188.
97. Pancholi S et al. Tumour kinome re-wiring governs resistance to palbociclib in oestrogen receptor positive breast cancers, highlighting new therapeutic modalities. *Oncogene*. 2020;39:4781–4797. https://doi.org/10.1038/s41388-020-1284-6.
98. Ressa A et al. A System-wide approach to monitor responses to synergistic BRAF and EGFR inhibition in colorectal cancer cells. *Mol Cell Proteomics MCP*. 2018;17:1892–1908. https://doi.org/10.1074/mcp.RA117.000486.
99. Rontogianni S, Iskit S, van Doorn S, Peeper DS, Altelaar M. Combined EGFR and ROCK inhibition in triple-negative breast cancer leads to cell death via impaired autophagic flux. *Mol Cell Proteomics MCP*. 2020;19:261–277. https://doi.org/10.1074/mcp.RA119.001800.
100. Sundar R, Hong DS, Kopetz S, Yap TA. Targeting BRAF-mutant colorectal cancer: progress in combination strategies. *Cancer Discov*. 2017;7:558–560. https://doi.org/10.1158/2159-8290.CD-17-0087.

101. Boshuizen J, Peeper DS. Rational cancer treatment combinations: an urgent clinical need. *Mol Cell*. 2020;78:1002–1018. https://doi.org/10.1016/j.molcel.2020.05.031.
102. Jin H, Wang L, Bernards R. Rational combinations of targeted cancer therapies: background, advances and challenges. *Nat Rev Drug Discov*. 2023;22:213–234. https://doi.org/10.1038/s41573-022-00615-z.
103. Twarog NR, Connelly M, Shelat AA. A critical evaluation of methods to interpret drug combinations. *Sci Rep*. 2020;10:5144. https://doi.org/10.1038/s41598-020-61923-1.
104. Lukas M et al. Survey of ex vivo drug combination effects in chronic lymphocytic leukemia reveals synergistic drug effects and genetic dependencies. *Leukemia*. 2020;34:2934–2950. https://doi.org/10.1038/s41375-020-0846-5.
105. Song X, Leonhard WN, Kanhai AA, Steinberg GR, Pei Y, Peters DJM. Preclinical evaluation of tolvaptan and salsalate combination therapy in a Pkd1-mouse model. *Front Mol Biosci*. 2023;10:1058825. https://doi.org/10.3389/fmolb.2023.1058825.
106. Vallés-Martí A et al. Phosphoproteomics guides effective low-dose drug combinations against pancreatic ductal adenocarcinoma. *Cell Rep*. 2023;42. https://doi.org/10.1016/j.celrep.2023.112581 112581.
107. Wang WZ et al. Predicting ROR1/BCL2 combination targeted therapy of small cell carcinoma of the lung. *Cell Death Dis*. 2021;12:577. https://doi.org/10.1038/s41419-021-03855-w.
108. Colombo R, Moll J. Target Validation and Biomarker Identification in Oncology. *Mol Diagn Ther*. 2008;12:71–76. https://doi.org/10.1007/BF03256272.
109. Paweletz CP et al. Identification of direct target engagement biomarkers for kinase-targeted therapeutics. *PloS One*. 2011;6:e26459. https://doi.org/10.1371/journal.pone.0026459.
110. Group FNBW. BEST (Biomarkers, EndpointS, and Other Tools) Resource. *Food and Drug Administration (US)*. 2016 http://www.ncbi.nlm.nih.gov/books/NBK326791/.
111. Kelly K, West AB. Pharmacodynamic Biomarkers for Emerging LRRK2 Therapeutics. *Front Neurosci*. 2020;14:807. https://doi.org/10.3389/fnins.2020.00807.
112. Lohmann S et al. Gene expression analysis in biomarker research and early drug development using function tested reverse transcription quantitative real-time PCR assays. *Methods*. 2013;59:10–19. https://doi.org/10.1016/j.ymeth.2012.07.003.
113. Nakayasu ES et al. Tutorial: best practices and considerations for mass-spectrometry-based protein biomarker discovery and validation. *Nat Protoc*. 2021;16:3737–3760. https://doi.org/10.1038/s41596-021-00566-6.
114. Prassas I et al. False Biomarker Discovery due to Reactivity of a Commercial ELISA for CUZD1 with Cancer Antigen CA125. *Clin Chem*. 2014;60:381–388. https://doi.org/10.1373/clinchem.2013.215236.
115. Solé X et al. Discovery and validation of new potential biomarkers for early detection of colon cancer. *PloS One*. 2014;9:e106748. https://doi.org/10.1371/journal.pone.0106748.
116. Subtil F, Rabilloud M. Estimating the optimal threshold for a diagnostic biomarker in case of complex biomarker distributions. *BMC Med Inform Decis Mak*. 2014;14:53. https://doi.org/10.1186/1472-6947-14-53.
117. Wang X et al. Understanding LRRK2 kinase activity in preclinical models and human subjects through quantitative analysis of LRRK2 and pT73 Rab10. *Sci Rep*. 2021;11:12900. https://doi.org/10.1038/s41598-021-91943-4.
118. Wolf DM et al. Mechanism of action biomarkers predicting response to AKT inhibition in the I-SPY 2 breast cancer trial. *Npj Breast Cancer*. 2020;6:48. https://doi.org/10.1038/s41523-020-00189-2.
119. Labots M et al. Evaluation of a tyrosine kinase peptide microarray for tyrosine kinase inhibitor therapy selection in cancer. *Exp Mol Med*. 2016;48:e279. https://doi.org/10.1038/emm.2016.114.
120. Li S et al. Applications of Protein Microarrays in Biomarker Discovery for Autoimmune Diseases. *Front Immunol*. 2021;12. https://doi.org/10.3389/fimmu.2021.645632 645632.
121. Kannegieter NM et al. The effect of tacrolimus and mycophenolic acid on CD14 + monocyte activation and function. *PLOS ONE*. 2017;12:e0170806. https://doi.org/10.1371/journal.pone.0170806.
122. Skånland SS. Phospho flow cytometry with fluorescent cell barcoding for single cell signaling analysis and biomarker discovery. *J Vis Exp*. 2018;140:58386. https://doi.org/10.3791/58386.
123. Blue Ridge Institute for Medical Research. *Protein Kinase Inhibitors*. Accessed November 11, 2023. https://brimr.org/protein-kinase-inhibitors/