Binding kinetics in drug discovery – A current perspective

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Structure-kinetic relationships
   3.1. Conformational changes and flexibility
      3.1.1. Nexavar (Sorafenib) induces a DFG out movement in Raf1
      3.1.2. Compound and target rigidity affects residence time on Pseudomonas aeruginosa LpxC
      3.1.3. Conformational adaptation upon inhibitor binding in DOT1L results in longer residence time
   3.2. Hydrogen bonds and hydrophobicity
      3.2.1. Long residence time of CDK8/CycC inhibitors depend on hydrophobic stacking
      3.2.2. Aromatic stacking could drive kinetics in ERK1/2
      3.2.3. Hydrophobic interactions between Melagatran and Thrombin increase residence time
   3.3. Water molecules
   3.4. Covalent and reversible-covalent binders
4. Methods for investigation of binding kinetics in drug discovery
   4.1. Binding assay formats
      4.1.1. Direct measurement of binding kinetics
      4.1.2. Indirect measurement of binding kinetics
   4.2. Functional assay formats
      4.2.1. Enzymatic activity assays
      4.2.2. Cellular activity assays
      4.2.3. Ex vivo and in vivo assays
   4.3. Considerations for the choice of assay formats
   4.4. Assay configurations and readout technologies
      4.4.1. Assays for investigation of target-compound interactions in solution
         4.4.1.1. Radioligand binding kinetic assays
         4.4.1.2. Fluorescent-labeled ligand binding kinetic assays
            4.4.1.2.1. Non-homogenous fluorescent assays
            4.4.1.2.2. Homogenous fluorescent assays
         4.4.1.3. Bioluminescent label binding kinetic assays
      4.4.2. Biosensor-based assays
         4.4.2.1. Electro-optical biosensors
         4.4.2.2. Electromechanical biosensors
   4.5. Structure-based analysis
   4.6. Current use and future trends for binding kinetics methods
5. Integration of binding kinetics in the lead discovery process
6. Acknowledgement
7. References

1. ABSTRACT

The impact of target binding kinetics (BK) on the clinical performance of therapeutic agents is presently a topic of intense debate in drug discovery. While retrospective studies suggest that BK is a differentiating parameter in marketed medicines, it is yet unclear how this information could be used to prioritize drug candidates during lead optimization. Motivated by the question whether BK can be understood and rationally optimized, we review the most relevant literature in the field, with special focus on selected examples from our organization.
First we discuss structure-kinetic relationships (SKR), and how they can be influenced by factors such as conformational changes, molecular flexibility, hydrogen bonds, hydrophobicity, water molecules and (reversible-) covalent bonds. We then introduce the methodologies currently used for the investigation of BK parameters, briefly commenting on their strengths, weaknesses and future trends. Finally, we present our current perspective on the integration of BK in the drug discovery process, aiming to stimulate further thoughts on this important subject.

2. INTRODUCTION

Despite technological progress, the attrition rate is high in modern target-based drug discovery. The reasons for failure are diverse. In the clinic, a large proportion of drug candidates between phase II and submission fail due to lack of efficacy (56% between 2011 and 2012) (1). A recent analysis by Bunnage concluded that ~24 development candidates were required to achieve a single NME (new molecular entity) approval. In his dataset, comprising 14 large pharmaceutical enterprises within the period from 2005 to 2009, the greatest attrition (75%) occurs in phase II, in which the efficacy of a project’s novel molecule is directly tested in a clinical proof-of-concept study (2).

These failure rates call for a continued reevaluation and adaption of the selection criteria of candidate molecules. Swinney et al. have noted that among FDA-approved small molecule therapeutics there appears to be an enrichment of “non-equilibrium” compounds, i.e. compounds that are displaying a molecular mode of action (MMoA) other than a direct equilibrium competition with an endogenous ligand (3). Therefore, it has been suggested that it may be a generally beneficial strategy to aim for such “non-equilibrium” MMoAs in order to improve discovery effectiveness and reduce attrition (4, 5). Slow dissociation, i.e. increased target residence time, is one facet of such a “non-equilibrium” MMoA.

The use of binding kinetics as a parameter in the drug discovery process is influenced by three factors. First, we need a detailed molecular understanding of binding kinetics. Therefore, we will review important structural parameters that influence drug binding kinetics to soluble targets. The modulation of binding kinetics on membrane protein targets, e.g. GPCRs has recently been reviewed elsewhere (6, 7). Secondly, robust assays are needed during drug discovery. We will highlight current techniques employed for monitoring drug binding kinetics and describe their quality and throughput. Finally, the integration of binding kinetics measurements needs to be considered in the drug discovery process. In our last chapter, we will focus on the implementation of binding kinetics in current workflows in the pharmaceutical industry.

3. STRUCTURE-KINETIC RELATIONSHIPS

The equilibrium binding ($K_d$) or functional affinities ($IC_{50}$) in vitro are considered to be key parameters to optimize drug molecules during rational lead optimization for in vivo efficacy. During this process, protein structures in complex with the lead candidate help to understand structural features that influence the binding affinity. There is a lack of structural information to rationalize binding kinetics, especially because kinetics are a dynamic process which is challenging to be resolved with normally used structural methods (8). In this part of the review we aim to describe structural features that are discussed to influence binding kinetics. Also, we will correlate residence times to equilibrium constants for each case to understand if the observed effects can be attributed to binding kinetics. We will divide this chapter into four parts, each of which will cover examples to an observed structural feature.

3.1. Conformational changes and flexibility

Both target and ligand can undergo conformational changes during binding. This can either be a conformational change in the protein to accommodate a compound in its binding site or the protein can also induce a different conformation in the ligand upon binding (9, 10). Likewise, during drug-target binding, the target could select a ligand with a distinct conformation from a large variety of others. In a free energy diagram, binding kinetics are dependent on the free energy barrier of the transition state between the different binding states (Figure 1). These mostly short lived transition states cannot be directly observed. One commonly used option to gain a better understanding of the binding process are molecular dynamics simulations (11). From Figure 1 it becomes obvious that a modification of binding kinetics can be achieved in multiple ways. For example if the TD complex is destabilized it results in an increased free energy barrier when everything else is kept constant. As a consequence, the $k_{on}$ will be lower and residence time of the drug on its target longer. Conformational changes that lead to a prolonged residence time are reported in the literature. Resulting from the energy diagram, the residence time of a drug on its target depends on the time scale of the conformational change. Therefore, especially slow structural changes can affect drug-target residence time.

We present here an example from our development, a kinase that undergoes a DFG out movement which is an example representative of various kinases. We also present a bacterial protein where rigidification of the compound leads to an extended residence time. And finally we will have a close look at a compound that induces a new allosteric binding pocket on the protein surface.

3.1.1. Nexavar (Sorafenib) induces a DFG out movement in Raf1

There are four known classes in kinase inhibition: Type I inhibitors bind ATP competitively at the ATP-binding
Sorafenib is a multi-kinase inhibitor that mainly targets Raf, VEGFR, PDGFR, KIT and RET kinases (14). Consistent with its multiple targets it shows a broad antitumor activity. As one of its main targets, Sorafenib inhibits Raf kinase isof orm 1 (15). Sorafenib is a type II inhibitor and binds to the inactive conformation of the DFG motif. For this, it interacts and replaces the phenyl ring of the DFG motif with its trifluoromethyl moiety at the position that the phenyl ring would occupy in the active Raf-1 (16). Sorafenib has a long residence time of 489 min on Raf-1 as measured in a reporter displacement assay and an IC_{50} of 6 nM in \textit{in vitro} Raf1 kinase inhibition experiments (15, 17). On other kinases in its activity spectrum, Sorafenib has similar K_{D}s but different residence times (17). Sorafenib binds to CDK8/CycC with an affinity of 30 nM and has a long residence time of 576 min. In contrast, the kinase DDR1 has a similar affinity of 72 nM but a shorter residence time of 24 minutes. This may theoretically result in a shorter target inhibition time course. A situation where selectivity is achieved by similar binding affinities but different binding kinetics is called kinetic selectivity. Sorafenib was also shown to induce a DFG out movement on VEGFR2 where its residence time is a magnitude lower around 55 min (18). In this case, the IC\textsubscript{50} values in a biochemical assay correlate with an increase in residence time, 6nM for Raf1 and 90nM for VEGFR2.

Other kinases that undergo a DFG out movement during drug binding also show an extended residence time. GSK1070916 induces a DFG out movement in Aurora B and Aurora C and has long residence times of \textgreater 480 min and 270 min respectively (19). CDK2 was also shown to be able to undergo a conformational change to accommodate type II binders (20). In this example, compounds that induce the DFG out conformation show an extended residence time. It has to be noted that a possible bias towards type II binders and prolonged residence times exist because a lot of effort was put into the development of new entities that show these qualities (21). It should be noted that type I binder that only induce minor structural rearrangements can also show very long residence times (22).

### 3.1.2. Compound and target rigidity affects residence time on \textit{Pseudomonas aeruginosa} LpxC

LpxC deacylates a precursor of bacterial lipidA during biosynthesis (23). It is an interesting antibacterial target because it is essential in many bacteria. It has a catalytic zinc ion at its active site and a hydrophobic cleft that binds lipidA’s myristoyl chains during synthesis (24). CHIR-090 is an inhibitor of LpxC with a two-step binding mechanism. First, an encounter enzyme-inhibitor complex is formed, which is then followed by a slow isomerization to the more stable non-covalent LpxC-inhibitor complex (23). In a NMR guided structure analysis, binding of CHIR-090 does not induce large conformational
Binding kinetics in drug discovery: Current perspective

Figure 2. Kinases have a bilobal structure: At the N-lobe, the helix alpha C is located which undergoes a structural rearrangement upon ATP binding. At the C-lobe, the peptide substrates bind. Between the two lobes is the hinge region that together with the N-lobe is responsible for ATP binding. The activation loop is a regulatory structure to modulate kinase activity at the C-lobe. It contains a conserved DFG motif that changes its conformation between inactive (DFG out) and active (DFG in) upon ATP binding. At the same time, the activation loop and helix alpha C move and result in an adaptation in the hinge region. Type II binders like Sorafenib stabilize the inactive DFG out conformation (12).

3.2. Hydrogen bonds and hydrophobicity

Hydrogen bonds are crucial in the design for higher affinity and specificity for drug molecules. Especially the formation of water shielded hydrogen bonds in energetically unfavorable transition states determine long residence times (32). On the other hand, hydrophobic stacking interactions can contribute significantly to the affinity of drugs to their target and likewise their kinetics (33). We will review three examples that depend on hydrophobic stacking interactions to increase their residence time.

3.2.1. Long residence time of CDK8/CycC inhibitors depend on hydrophobic stacking

Amongst other targets, Cyclin-dependent kinase 8/cyclin C (CDK8/CycC) is targeted by sorafenib and imatinib that both bind in the DFG out mode (type II binders) (34). They induce a movement of the DFG motif close to the ATP binding site that makes their deep binding site accessible and inactivates the kinase (see section 3.1.1.). The structure-kinetic relationship between CDK8/CycC and various compounds was analyzed (35). First, in a new experimental approach, lead like compounds were soaked into a co-crystal structure that was stabilized by a compound in the DFG in conformation (Type I binder) (see Figure 2). After soaking of their various compounds, the co-crystallized compound got replaced in all cases while a DFG out conformation in the crystal structure was induced. The ligands investigated in this study differed tremendously in their residence time measured in a time resolved fluorescence energy transfer (TR-FRET, see section 4.1.2.) displacement assay (see section 4.1.2.) from less than 1.4 min up to 1944 min which seems to be in contrast to the connotation that conformational changes...
upon binding could be crucial for prolonging residence time (see section 3.1.). Long residence compounds also showed very high affinity in the nM range. Crystal structures revealed different interactions in various regions. For shorter residence time binders (below 100 min) additive hydrogen bonding between the respective compounds and CDK8 hinge region seems to correlate with increasing residence time. For long residence time compounds (longer than 1000 min) crucial interactions at the hinge region are not observable, but large hydrophobic π-stacking in the front pocket around an Arg seem to expand their residence time. They argue that in fact the “breathing motion” responsible for nucleotide binding and release in CDK8 is blocked by binding in the front pocket and thereby results in a longer residence time (36).

3.2.2. Aromatic stacking could drive kinetics in ERK1/2

In the case of ERK1/2 protein, that is part of the cell proliferation signaling, diverse drugs were approved that target multiple cancer types. In most cases, ERK1/2 co-crystal structures with compounds revealed type I binders that stabilize the DFG in state (37). Compound SCH772984 was shown to be highly selective and potent especially against PLX4032 and GSK1120212 resistant cancer cells (38). The structure-kinetic analysis for this compound to ERK1/2 and other off-target kinases was approached by Chaikuad et al (39). In the co-crystal structure, SCH772984 induced a new, allosteric binding pocket between the αC-helix and the phosphate binding loop (P loop pocket) adjacent to the ATP binding pocket. Tight binding of the inhibitor was explained from the structure by three key interactions between protein and compound. First, two hydrogen bonds were formed with the hinge region, second, the DFG in motif was stabilized and third, a π stacking interaction and water mediated hydrogen bonding in the new allosteric P loop pocket. Binding was accompanied with a large binding enthalpy but strongly compensated by entropy and resulted in a K_D of 200 nM for SCH772984 on ERK1/2. Other kinases that are inhibited by SCH772984 were identified with KinomeScan (40). Amongst them are the atypical kinase Haspin and the MAPK C-JUN kinase 1 (JNK1). In co-crystal structures with both off target kinases, SCH772984 bind to the active site, as a type I inhibitor. In correlation with other type II or allosteric binders, SCH772984 shows a slow off rate when interacting with ERK1/2 but not with Haspin. A bio-layer interferometry (BLI, see section 4.4.2.1.) assay resulted in a dissociation half-life of about 25-80 min for ERK1/2. Another type I ERK1/2 inhibitor VTX-11 that stabilizes the DFG in conformation had a similar long residence time in BLI measurements and affinity in ITC measurements as SCH772984. Its dissociation half-life was in the same range as for the latter, about 35-46 min. Therefore, residence time cannot solely be attributed to a conformational change. In a mutagenesis approach in the P loop pocket the authors found two tyrosines, located in the αC helix and P-loop that mediated or induced the binding of both inhibitors, VTX-11 and SCH772984. In the case of the allosteric pocket binder, SCH772984, the binding was explained by π stacking interaction between compound and a tyrosine that establishes the new P-loop pocket by stabilizing a distorted P-loop conformation. In the case of VTX-11, its chlorobenzene group is able to distort the P-loop by replacing a tyrosine that in turn is then able to stack to the tyrosine in the αC-helix. Therefore on ERK1/2 aromatic stacking interactions either induced or mediated by the compound seem to be crucial to increase the residence time. In metastatic adenocarcinoma breast cancer cells, both slow dissociating compounds showed prolonged phosphorylation inhibition after washout in agreement with their measured residence time in vitro.

3.2.3. Hydrophobic interactions between Melagatran and Thrombin increase residence time

Thrombin is a serine protease in the coagulation cascade and cleaves fibrineogen to fibrin. Thrombin’s active site is in a deep pocket with an aspartic acid at the bottom and it is quite flexible and allows hydrophobic compounds to bind (41). Melagatran is a zwitterionic active site thrombin inhibitor. It forms an electrostatic interaction with its positively charged amidinium to the aspartate which is responsible for a fast association kinetic and an extended hydrophobic interaction with its other end to stabilize the complex (42). Winquist et al thought to take advantage of their internal lead optimization program to understand the relationship between thermodynamics and kinetics by exchanging a carboxyl residue on Melagatran with amines with increasing chain length and hydrophobicity (43). By using SPR measurements, they found that k_on reached diffusion limit for all of their compounds. From all compounds tested, Melagatran had the slowest k_on in an SPR assay but was average in its nM affinity and 100 s residence time at 25°C. This was explained with a crystal structure where its carboxylate moiety is repelled by a glutamate residue in the active site. The extended hydrophobic interactions by increasing the aliphatic chain length and substitution results in an increased residence time on Thrombin, but gets saturated after a certain length. The authors argue that this could be due to shielding the amide hydrogen from water in the binding site and maintaining a favorable hydrogen bond to a glutamate, which is dependent on size of the compound. They also point out how unpredictable slight conformational changes in the protein resulted in different enthalpic and kinetic contributions. Additionally, different substituents could change the water network that also can have an influence on binding kinetics (44).

3.3. Water molecules

Solvation effects are recognized as important factors to understand binding affinities (45). Water molecules in the vicinity of hydrophobic patches drive the association of molecules that replace them from
binding kinetics in drug discovery: Current perspective

the protein surface (46). These movements of water molecules on a hydrophobic surface can influence binding kinetics because they represent a kinetic barrier during dissociation. Likewise, desolvation phenomena modulate binding kinetics as well (47).

In a recent review on G protein coupled receptors (GPCR), the importance of binding kinetics on the efficacy of compounds was highlighted (6). Adenosine receptor A2A is a GPCR that is important for inflammatory and motor activity disorders (48). In a study with ten different agonists binding to A2A receptor the kinetics measured in a radioligand displacement or competition association assay yielded residence times between 3 min to 250 min (49). Their respective efficacy in whole cell assays using impedance monitored change in cell morphology or in a biochemical cAMP assay correlate well with their respective residence times but not with their Kd constants. Long residence time was observed for agonists with a ribose substituent that is able to bind deeper in the main pocket after displacement of structural water molecules that is entropically favored (50). There, the ribose is able to interact via hydrogen bonds and non-polar interactions and the agonists long residence times are likely a result from an entropic stabilized interaction in the binding pocket.

3.4. Covalent and reversible-covalent binders

Covalent bond formation between drugs and their targets sometimes raises concerns because an exposed reactive group could potentially react with a variety of off-targets causing adverse effects. There are however a number of safe and efficacious covalently binding drugs (51, 52). A famous example from Bayer is Aspirin which is the most employed drug to regulate homeostasis and to reduce inflammation (53). It irreversibly acetylates a serine residue in COX-1 and 2 in a hydrophobic channel that avoids passage of a substrate (54, 55). Another example is VX-950 (Telaprevir) a reversible-covalent protease inhibitor that was developed for hepatitis C virus treatment. It is a ketoamide and forms covalent but reversible bonds with a serine residue in the N3S-4A protease (56). Since a covalent bond represents the ultimately longest residence time on a target, we here would like to present one example were different residence times on a kinase was achieved with a reversible covalent inhibition approach.

Bruton’s tyrosine kinase (BTK) is a cytosolic protein important for B cell survival, cell cycle progression, and proliferation in response to B cell stimulation (57). It possesses a non-catalytic cysteine that is targeted by a marketed drug, ibrutinib, by forming an irreversible covalent bond with its acrylamide warhead (58). Bradshaw et al target the same non-catalytic cysteine outside of the ATP binding site but with a reversible covalent cyanoacrylamide by inverting its orientation relative to the kinase active site (59). Thereby, they have new possibilities for variation in the molecule to modulate binding kinetics. They coupled multiple amine containing linkers to the cyanoacrylamide carboxyl group and influenced the steric and electronic environment with branched alkyl capping groups in the drug molecule. A covalent bond between the cysteine and the electrophilic beta-carbon in the cyanoacrylamide was formed as shown by crystallography but which is not present in BTK unfolding experiments. This indicates its reversible character. By using a fluorescent irreversible binder in a competition experiment they found biochemical residence times between five minutes to seven days without a strong correlation in their respective enzymatic IC50s. If only IC50s were considered for optimization, a long residence time compound would likely have been overlooked. Residence time was modulated by subtle structural changes in the linker and polar branched alkyl capping group but not predictable. The long occupancy of BTK with the inverted acrylamide was shown in Ramos B cell washout experiments by using AlphaScreen. Here, the biochemical residence time directly translated into occupancy of BTK in cells, and a measurable cellular effect. A translation of the long residence time effect in in vivo efficacy in rats was possible, with a fast plasma clearance but still high occupancy of 40% BTK in their cells after 24h for the slow dissociating compound. The approach has been shown to be transferable to kinase FGFR1, which also harbors an addressable cysteine.

4. METHODS FOR INVESTIGATION OF BINDING KINETICS IN DRUG DISCOVERY

First efforts to investigate the binding dynamics of drugs date back to the 1960’s (60-62). Initial quantitative studies were performed using direct radioisotope labeling (63, 64) or stopped-flow fluorescence (65-67). In 1984, the equations describing the kinetics of competitive radioligand binding predicted by the law of mass action were introduced, and soon first kinetic data for unlabeled compounds competing with a radioactive tracer appeared in the literature (68, 69). In the early 1990’s, the first SPR biosensor that was able to measure real time kinetic data was brought into the market (70-72). Nowadays various types of biosensor-based instruments are capable of measuring binding kinetics with high precision and throughput. In addition, laboratory automation facilities enable the application of radioactivity or fluorescence based technologies for high-throughput determination of kinetic parameters in competitive set ups (8).

In this chapter, we give a brief overview about several techniques that can be used for the determination of kinetic on- and off rates for small molecules. We will categorize them according to their formats and readout technologies, discussing their practicability, throughput and information content. Moreover, we are going to comment on their complementarity and positioning within the drug discovery process. In the end we will mention
Binding kinetics in drug discovery: Current perspective

several novel technologies whose development should be closely followed in the future by drug discovery scientists interested in the topic.

Binding kinetics can be studied in a variety of biological systems ranging from isolated recombinant soluble or membrane proteins, to endogenous targets expressed in cultured cells, or even in animal models. Experiments can be designed to have either binding or functional (e.g. enzyme activity or second-messenger assays) readouts. In general, binding assays are most convenient for precise determinations of binding kinetics parameters. On the other hand, functional cell and in vivo assays are expected to be closer to the physiological and patho-physiological situation. Cellular background can affect binding, and certain compounds show different on- or off-rates depending on whether they are tested in membrane preparations or whole cells (73-75).

4.1. Binding assay formats

Binding assays can be performed in both direct and indirect (competitive) formats, and are often performed with purified recombinant target proteins under non-physiological conditions. In consequence, these assays rather serve to rank compounds according to their binding kinetics. A few examples from the literature show that binding studies can also be performed under more physiologic conditions (e.g. 37°C) and in whole cells (e.g. (76-78)) or animals (e.g. (76, 79)).

4.1.1. Direct measurement of binding kinetics

There are two main approaches for direct measurements of compound binding kinetics: The first consists of labeling the compound of interest and measuring the binding to its target protein in vitro (80) or in vivo (81) (see below). The second type of approach is the so-called “label-free”, which is most frequently enabled by biosensor technologies (section 4.4.2 and 4.6). For the labeling strategy, two variants are possible: 1) The incorporation of a radioisotope to the ligand of interest, which is expensive, time-consuming, and has very low throughput, e.g. in vitro (80) and in vivo (81). 2) Alternatively, one can make use of the proteins intrinsic fluorescence changes upon binding or the compound’s intrinsic fluorescence properties to monitor its binding kinetics, an approach for which only limited examples are known, since auto-fluorescent drug-like molecules are often unwanted in drug discovery programs (82).

In the simplest direct binding procedure, concentration dependent compound binding to recombinant proteins is quantified over time, allowing calculation of the pseudo first order parameter \( k_{\text{obs}} \), from which the rate constants \( k_{\text{on}} \) and \( k_{\text{off}} \) can be derived by plotting \( k_{\text{obs}} \) vs. compound concentration. Another way to determine the dissociation rate constant \( k_{\text{off}} \) is by monitoring the dissociation of pre-saturated compound-protein complexes once the unbound compound has been removed or hindered from binding either by addition of an excess of unlabeled (or non-signaling) competitive ligands or by rapid (“jump”) dilution with a large amount of a given medium (83). A combination of both, jump dilution and excess of unlabeled competitive ligand is recommendable, since an excess of unlabeled compound added to the diluent may limit rebinding effects.

A special way to follow compound binding kinetics in a direct fashion is by monitoring target conformational changes induced by compound binding. One striking example is the – so called – FliK (fluorescent labels in kinases) assay in which special dyes covalently attached in the DFG loop of kinases will change their fluorescent properties with movement of this loop from the “DFG-in” to the “DFG-out” conformation (84, 85).

4.1.2. Indirect measurement of binding kinetics

Indirect binding kinetics assays monitor the association and dissociation of labeled ligands in the presence or absence of unlabeled test compounds. To this end, mainly two approaches have been described: either I) simultaneous competitive target binding of an unlabeled compound and a labelled tracer (77, 86, 88) or II) displacement of pre-bound tracer or unlabeled compound by the test compound or the labeled tracer, respectively (59, 87, 89, 90). By using the binding kinetics parameters of the tracer (previously determined in direct binding experiments), the kinetic rate constants of the compound can be calculated based on appropriate mathematical models: Competitive binding allows for simultaneous determination of both, \( k_{\text{on}} \) and \( k_{\text{off}} \) using the competitive binding kinetics equations (91), whereas displacement assays solely enable \( k_{\text{on}} \) and \( k_{\text{off}} \) determinations via \( k_{\text{obs}} \) or exponential decay models.

The advantages of indirect measurements are evident: If a competing (labeled) ligand is available, various compounds of interest can be investigated using the same assay protocol, and there is no need for immobilization of targets or labeling of each compound of interest. Therefore, indirect methods enable a wider range of application and a higher throughput mode as compared to the direct binding assays. A further advantage is, that a high affinity and highly selective tracer can be applied leading to little non-specific binding. For biosensors, a competitive ligand can enhance the respective transducer signal (by e.g. mass, size, dye effect). Therefore, the indirect formats allow for improved sensitivity and specificity (92). More information about biosensor transducers is provided in section 4.4.2.2

A number of variations of indirect assay formats aim to increase throughput (for more details refer to (93)). In brief, most of them simplify methods for analysis of binding kinetics and solely allow for estimation and ranking of compounds according to their dissociation rate without quantification of kinetic rate constants:
1) Heise et al (94) identified slow dissociating ligands by comparison of (apparent) affinities determined after 30 min versus 10 h. For slow-dissociating compounds equilibrium is not reached after 30 min leading to a significant shift in the dose response curve after 10 h.

2) Vanderpool et al (95) performed a displacement assay and measured the signal output at two points in time: I) shortly after mixing of all assay components (labeled tracer and pre-equilibrated compound-target complex) and II) after 20 min of incubation. Since tracer binding increases the signal, the difference between the second and first point in time ($\Delta F$) is large for slowly dissociating compounds. In contrast, $\Delta F$ is small for rapidly dissociating compounds that dissociate prior to the first measurement. This method allows for identification and ranking of slow dissociating compounds, while fast dissociating compounds and non-binders cannot be discriminated.

3) Guo et al (96) simplified the competitive binding assay in a similar way: They developed a dual-point competition association assay measuring the signal output at two points in time: I) at the time when the target binding of the labeled tracer without compound reaches equilibrium, II) at a time, when equilibrium should be reached for simultaneous competitive target binding of compound and labeled tracer. They calculated the ratio of the first and the second point in time ("kinetic rate index" KRI) to categorize for compounds with long and short residence times: When the compound dissociates faster from the target than the tracer, the tracer signal will increase until it reaches equilibrium and the KRI will be smaller than 1. If the compound dissociates slower from the target than the tracer, the signal will overshoot and then decrease until equilibrium is reached, so that the KRI is bigger than 1. If no binding of the compound occurs, KRI will be equal to 1. This method allows for classification according to the off-rates without quantification of kinetic rate constants. Since, also the on-rate influences the time course of the signal (e.g. for simple 1-step binding, $k_{obs} = k_{off} (1 + [\text{compound}] / K_i) = k_{off} [\text{compound}] + k_{on}$), the described behavior is solely true as long as the competition strength for test compounds, i.e. $(\text{compound}) / K_i$, is maintained comparably (91). Therefore compounds should initially be ranked according to their competition strength followed by ranking according to the KRI, keeping in mind that one must pay attention to BK to reach equilibrium and reliably determine $K_i$.

Only a few assay formats have been described which allow for high-throughput quantification of binding kinetics. They all rely on displacement or competition of fluorescent labeled probes and are performed in microtiter plates and with multimode plate readers:

1) Neumann et al (97) described a fluorescent probe displacement assay suited for the determination of binding kinetics. Precise experimental procedures, as well as the readout technology of the assay are undisclosed, but follow up publications show that at least kinase inhibitors can be successfully evaluated with this technology (34).

2) Uitdehaag et al. (98) presented a high throughput competitive TR-FRET assay for profiling of CSF1R inhibitors with regard to their target residence time. To this end the authors simplified the mathematical procedures described by Motulsky and Mahan in order to overcome technical and computing challenges and increase the throughput.

3) Based on the same principle, a novel homogenous kinetic probe competition assay (kPCA) format was recently developed and presented by us (77). The method makes use of the pump-based injection systems recently integrated in several TR-FRET plate readers to enable real time measurement of binding kinetics with unprecedented sampling frequency. The absence of lag times between sample mixing and reading and the high kinetic resolution of the method allow more reliable quantification and determination of fast on- and off-rates. This feature makes possible the use of the original Motulsky and Mahan equations to fit the kinetic traces without any simplification or shortcuts. Compared to displacement TR-FRET assays (59), a further advantage of the method is that high quality results can be obtained with much lower protein and tracer concentrations – since "jump dilution" is not necessary.

4.2. Functional assay formats

Functional assays only allow for indirect investigation of binding kinetics. They are more complex than binding assays and therefore often more difficult to interpret, but they are expected to be better predictors for the in vivo situation. While the methods for the characterization of slow binding enzyme inhibitors with functional assays have become a standard in drug discovery laboratories (99), the use of cell-based or in vivo functional readouts remains largely confined to isolated examples for individual targets and compounds.

4.2.1. Enzymatic activity assays

The time course analysis of enzymatic activity (enzyme reaction progress curve analysis) is the method most frequently used for the determination of the kinetic dissociation rate constants. It can either be performed I) after addition of different compound concentrations to the enzyme (e.g. (100)), or II) after inducing dissociation of a pre-saturated enzyme-compound complex by jump dilution (e.g. (83, 101)) or removal of unbound compounds.
The enzymatic activity is often followed by using pseudo-substrates, such as chromogenic (100) or fluorescent substrates (101, 102). The enzymatic conversion of the substrate leads to a changing readout signal, which can either be monitored directly over time or the reaction can be stopped at different time intervals. To allow for the calculation of binding kinetic parameters, the enzymatic mechanism of action has to be known and the mathematical models should be adapted for slow and tight binding inhibitors (100, 103, 104). Zhang et al. present various experimental design and data analysis guidelines for kinetic profiling by enzyme progress curve analysis (105).

Re-equilibration kinetics experiments can be applied to estimate the duration of receptor occupancy without prior knowledge about the mechanism of action (106, 107) and for ranking of compounds according to their dissociation rate: Dose-response curves are studied for agonists added to pre-equilibrated antagonist-receptor complexes. With increasing antagonist concentrations, fast dissociating antagonists provoke a rightward shift of the dose-response curve, whereas slow dissociating compounds additionally cause a reduced maximal response when detection takes place before equilibrium is reached (106). For non-covalent reversible binders, this response reduction is time dependent and the signal will recover. A control experiment is recommended since the described response reduction is also observable for all other effects leading to a reduced number of receptors available for agonist binding – such as receptor internalization (see also (93)).

A method commonly used for rapid determination of slow binding enzyme inhibitors is the determination of biochemical IC_{50} values with and without target-compound pre-incubation. Typically the IC_{50}s for slow binding compounds shifts to lower values after pre-incubation (108).

4.2.2. Cellular activity assays

Cellular activity assays can be used to study the kinetics of a cellular response upon compound exposure. This assay format captures phenomena such as the internalization of receptors upon drug binding, cell content dependence of binding (e.g. microenvironment, compartmentation, different redox potentials, and ion concentrations) and cellular amplification of the response. The complexity of the parameters influencing the response can cause difficulties in interpretation of the data, and erroneous estimation of the occupancy of the target over time can have a negative influence on the conclusions about off-rates of the compounds. On the other hand, such cellular activity assays can lead to a better understanding of the compound’s mode of action: e.g. comparison of the cellular activity results with previously otherwise quantified residence times, might reveal if the pathway activation upon drug binding is irreversible (e.g. often observed for apoptosis pathway) or reversible. Only the latter variant would require long residence times.

The most frequently used cellular activity assay is a washout experiment: Living cells – pre-equilibrated with the compounds of interest – are washed to remove unbound compounds, further incubated and lysed at different time intervals to analyze the activity of the target by e.g. ELISA or western blotting. In 2004 Wood et al. evaluated the duration of the effects of the EGFR inhibitors Lapatinib, Tarceva and Iressa after washing away free compound and found that the rate of recovery of receptor phosphorylation in the tumor cells reflected the inhibitor off-rate from the purified intracellular domain (22). Later, Qingsong Liu et al. (102) monitored the recovery of mTORC1- and PI3K-dependent phosphorylation over time (quantified by immunoblotting) after treatment with specific inhibitors. Alternatively, monitoring of changing cellular activity upon compound association and dissociation can be performed by kinetic measurement of other parameters, such as e.g. secondary messengers, proliferation or mass redistribution. An interesting example was contributed by Borovska et al. (109), which performed electrophysiological recording using the whole-cell voltage-clamp technique to follow compound induced current changes as a measure for the on- and offset of receptor inhibition.

4.2.3. Ex vivo and in vivo assays

Functional ex vivo and in vivo assays directly reflect the in vivo situation, but probably due to their complexity and the need for animal testing, they are rarely conducted. The in vivo part is often restricted to the administration and distribution of the drug, followed by sampling and ex vivo functional analysis. E.g. Wang A et al. (101) administered monkeys with different doses of compounds and collected blood samples at different time intervals, isolated plasma and measured activity of the target DPP (plasma dipeptidyl peptidase) to determine the occupancy of the target over time. Tillotson et al. developed a novel a pharmacodynamics activity assay to directly measure Hsp90 occupancy with inhibitors in cells and tumor tissue (110).

4.3. Considerations for the choice of assay formats

The choice for the appropriate assay format is a balancing act to extract as much kinetic information as possible using the simplest assay available which also provides the highest throughput and lowest costs. These features are often not compatible (Figure 3), and therefore scientists have to make a decision to choose the most appropriate method, based on the current state of knowledge, the number of compounds and targets of interest as well as the anticipated strength of the output information. For example in the scope of drug discovery, early stage high throughput screening
applications demand for universally applicable, fast and cost-effective assays – such as competition binding or displacement assays. Whereas in later stages of the drug discovery value chain, when the number of drug candidates decreases, more complex and fairly labor-intensive functional assay formats might be more appropriate to get a better predictability of \textit{in vivo} effects. Nevertheless, simple quantification of kinetic parameters using binding assays can also be convenient for lead optimization based on kinetics. Here we would recommend a combination of both, binding and functional assays.

\section*{4.4. Assay configurations and readout technologies}

The assay formats described above can be performed in various configurations, and make use of different principles to measure optical, electrical or mechanical changes caused by compound-target interactions over time. The readout technology chosen has an impact on whether labeling and immobilization of compounds and targets are needed. It also influences the sensitivity, kinetic resolution, protein demands and throughput of the assays. For example, if the readout technology can directly discriminate between bound and unbound molecules, there is no need for separation. This feature allows one-step mix-and-read assays, where binding signals are directly measured upon mixing of the reagents with the compound and target. These so called homogenous assays provide advantages in terms of simplicity, higher kinetic resolution and throughput.

In the following sections, we will present some of the technologies currently used for the kinetic characterization of small molecule ligands. We will first discuss various assay configurations for radioactive, fluorescent and luminescent labels, and later focus on label-free binding kinetic assays that are based on biosensors with electrooptical, electromechanical or electrical readout.

\subsection*{4.4.1. Assays for investigation of target-compound interactions in solution}

\subsubsection*{4.4.1.1. Radioligand binding kinetic assays}

Radioactive labeling of compounds implies the replacement of specific atoms by their radioactive isotope, with the result that binding properties are rather unaffected. In conventional radioligand binding assays bound and unbound radioactive molecules have to be separated either by filtration (soluble / solubilized targets, suspended cells) or washout (adherent cells). These non-homogenous assays have typically a fixed endpoint and multiple-handling-steps and the experimenter often has to deal with non-specific binding to the filters, accumulation of radioactivity on the filter unit and large volumes of liquid waste (111).

Radiolabeling is often the label of choice for lower throughput applications such as direct binding assays. However, – as stated above – there are also publications performing competition binding (86, 88) and displacement (86-88) assay formats with radioligands to quantitatively determine binding kinetic parameters. Jump dilution displacement filtration assays limit but do not eliminate rebinding. Vauquelin et al (112) compared previously different multi-step procedures to determine kinetic off-rates using radioligand binding assays. Guo et al (93) describe a number of different assay formats addressing the problem of the relatively labor intensive and time consuming work required for the radioligand binding method: These include re-equilibration kinetics experiments, comparison of apparent affinities determined after 30 min and 10 h and the determination of the KRI of a competition binding experiment (see section 4.1.2).

An alternative to the standard filtration assay is the scintillation proximity based assay SPA (113), a homogenous assay format in which light is emitted upon binding of the radioligand to the target immobilized on beads (containing the scintillant). In this way no scintillation cocktail and no separation step is required leading to reduced amounts of radioactive waste, higher throughput and improved kinetic resolution. Major limitations of the SPA (as compared to the filtration method) are: lower counting efficiency, more expensive assay components, non-proximity effects at high radioligand or bead concentrations, quenching by colored compounds, bead settling effects and higher signal variation (111). Moreover, the required immobilization of the target on beads can influence target binding properties.

\subsubsection*{4.4.1.2. Fluorescent-labeled ligand binding kinetic assays}

There is a variety of excitation and detection modes for fluorescence readout (see below). In general fluorescent labels provide advantages in
terms of sensitivity, safety and multiplexing (since several fluorescent molecules with different emission wavelength are simultaneously measurable). Drawbacks as compared to radioligand labeling can result from background fluorescence, photobleaching and steric hindrance by the more bulky label. However, in the time-resolved fluorescence setups the background fluorescence is limited (see section 4.4.1.2.2. TR-FRET) and photostable fluorophores can decrease bleaching problems. The non-homogenous fluorescent methods are analogous to radioligand binding studies, whereas the homogenous fluorescent methods opened up new possibilities for higher-throughput applications.

4.4.1.2.1. Non-homogenous fluorescent assays

Fluorescence intensity (FI) / fluorescence spectroscopy was used to study binding kinetics decades ago (65-67, 114) and is still used (115, 116). The main fields of application are functional assays (101) or stopped flow analysis (115). In the stopped-flow spectrometers, solutions with target and compound (or target-compound complex and competitor) are combined in a mixing chamber and also fast reaction kinetics can be studied by immediately starting to observe e.g. circular dichroism or fluorescence intensity changes (in the emission spectrum) upon binding or unbinding. Stopped flow spectroscopy is still the fastest method for the determination of binding kinetics with resolutions in the millisecond levels. A problem of FI measurements is that both bound and unbound molecules fluoresce, so that their respective contributions to the fluorescence intensity have to be calculated (66) which is prone to errors. Alternatively unbound molecules have to be separated from the complex (117). Further drawbacks result from high background signals due to autofluorescence of cells and cell components as well as the relatively high level of non-specific signals.

An interesting application of fluorescent readouts for the determination of binding kinetics, is the use of confocal microscopy to monitor the association and dissociation rates of labeled ligands while binding to receptors expressed in the surface of living cells (118, 119).

4.4.1.2.2. Homogenous fluorescent assays

Fluorescence polarization (FP) can be applied to study binding kinetics in a homogenous format (120). Fluorophores excited by linear polarized light also emit polarized light. If a fluorescent ligand binds to its target (in solution), the rotation of the large ligand-target complex is significantly decelerated as compared to the small unbound ligand. This can be monitored within the lifetime of fluorescence activation, since the rotation influences the axes of polarization: Fluorescence anisotropy decreases with rotation speed. This difference in anisotropy of fully bound, partially-bound and unbound ligand can be monitored over time to measure binding kinetics.

To detect large differences in rotation, the receptor and ligand concentration should be in a similar range and a relatively large amount of protein is required. This assay is homogenous and can distinguish between the bound and unbound state in solution, but the high background signals due to autofluorescence remains a problem so that the technique is rather used for purified targets.

Fluorescence correlation spectroscopy (FCS) is a similar technique: Large ligand-target complexes diffuse significantly slower as compared to the small unbound ligands, allowing for distinguishing bound and unbound state in a small volume by investigation of the fluctuation of fluorescence intensity caused by Brownian motion. In addition to the average diffusion time also the average number of fluorescent particles (molecules or cells) can be measured. The amount of required protein is smaller as compared to FP assays.

Fluorescence resonance energy transfer (FRET) is the non-radiative process of energy transfer from an excited donor chromophore to an acceptor chromophore. The efficiency of this energy transfer is inversely related to the sixth power of distance between donor and acceptor, which can be used to study ligand-target inactions in a homogenous, mix-and-read assay format. Both the ligand and the target have to be labeled with either the donor or the acceptor molecule, which will work as a FRET pair only at close proximity (≈ 9 nm or less).

To increase sensitivity by reducing the background signals, FRET can be combined with time-resolved fluorometry to the so called time resolved-fluorescence resonance energy transfer (TR-FRET) technique. TR-FRET reagents are available from various sources (59, 77, 95). Typically, long-lived emitting fluorophores (lanthanides such as Terbium or Europium) are used as FRET donors, while acceptor dyes can go from green to far red regions of the spectrum. TR-FRET unites the simplicity and speed of a homogenous assay format with excellent signal-to-noise ratios, low protein demands and high sensitivity and is a powerful tool for high-throughput applications. The kinetic resolution of TR-FRET readout is higher as compared to radioligand binding but lower as compared to SPR and the data obtained are comparable to other technologies (77, 121, 122). Many publications using TR-FRET for quantification of binding kinetic rates are based on the displacement assay format (59, 89, 90, 95, 122, 124) but – as commented above – the method has been recently used for true competitive binding kinetics experiments (77). Among the advantages of this application (compared to displacement assays) are the absence of pre-incubation step and delay times between sample mixing and reading, which allows association and dissociation rates to be measured in the same experiment and a more reliable analysis of compound with fast association and
dissociation kinetics (see end of section 4.1.2 and (77)).

In addition to application for binding assays (TR-)FRET technology is also widely applied for functional assays (e.g. (101, 102)), including a homogenous setup of the re-equilibration experiments.

4.4.1.3. Bioluminescent label binding kinetic assays

2012 Luker et al (76) used a Gaussia luciferase protein fragment complementation assay to study binding of small molecule inhibitors to their targets in cells and living mice. As an alternative to straight luminescent applications, bioluminescence resonance energy transfer (BRET) can be exploited to study ligand-target interactions: BRET is similar to the FRET approach but uses a bioluminescent donor. The BRET signal increases with proximity of a fluorescently labeled compound and an (e.g.) luciferase-tagged target. L. A. Stoddart et al recently demonstrated the applicability of BRET for monitoring of kinetics of ligand binding to GPCRs in living cells (78). A favorable feature of BRET is the avoidance of problems caused by photobleaching of the donor, autofluorescence and dual excitation of both, acceptor and donor. Most of these FRET limitations are also reduced by using TR-FRET (125, 126). Drawbacks of BRET are the necessity of a bioluminescent fusion protein for target labeling and the relatively low sensitivity, but for light-sensitive tissues BRET can be superior to TR-FRET (125, 126). Moreover BRET assays can be performed in living cells (158).

4.4.2. Biosensor-based assays

In the most concise definition, a biosensor is an analytical device combining bio-recognition of analytes by a biological sensitive element with a transducer element for translation of the docking event into an electrically measurable signal. The emphasis of this chapter concerns application for binding kinetics analysis and the whole biosensor system composed of the following subunits: 1) A sample delivery system, 2) a bio-recognition entity (e.g. target, compound or microorganism), 3) a transducer based on a physical (electrical, electromechanical) or (electro-) optical principle, 4) an electronic system for signal processing (e.g. amplification) and 5) a signal output unit. Biosensor assays produce and evaluate signals arising directly from the binding of biomolecules on a sensor-surface: The interaction causes physical or chemical changes, so that a label is not required for detection but immobilization and special instrumentation. Immobilization can cause protein activity problems and mass transport contribution to binding data. The most beneficial property of biosensors is the label-free, highly sensitive and real-time detection of binding events. Microfluidic delivery systems are preferred over batch operation, as it is essential to separate bound from free compounds to limit rebinding and determine binding kinetics accurately.

In this section we describe the biosensors which have been – to our knowledge – adopted in drug discovery laboratories, and for which peer-reviewed literature is available. Other promising technologies and instruments will be briefly touched in the last section of this section (see 4.6).

4.4.2.1. Electro-optical biosensors

The first optical biosensor available to drug discovery scientists for the determination of kinetic rate constants was based on the transducer principle surface plasmon resonance (SPR). To measure the binding of compounds to the target protein, the protein is immobilized onto the sensor surface – a thin metal film (typically a gold layer) coated on a glass block (typically a prism) – and the compounds are injected in a continuous flow in solution. The phenomenon of SPR can be exploited to follow the binding interactions in real-time: While total internal reflection of a light beam the electromagnetic field component propagates along the metal film and the non-illuminated side of the sensor surface, creating an exponentially attenuating evanescent wave. This evanescent wave can excite the plasmons on the metal surface in a resonant manner. Due to the resonance energy transfer to the surface plasmons, the intensity of the reflected light is reduced at a specific angle of incidence. The surface plasmon resonance angle is influenced by the refractive index close to the surface. If a compound binds to the immobilized target, the local refractive index and therefore the angle of SPR changes, so that the SPR signal can be used as a measure for mass concentration on the surface and for real-time monitoring and determination of association and dissociation. Today, most publications about research on binding kinetics using biosensors are based on SPR transducer. SPR is well suited for many soluble proteins whereas membrane proteins cause difficulties in terms of reproducibility and robustness arising from stability and orientation issues during immobilization into lipid. However, there are also many publications demonstrating the usability of SPR to study binding kinetics for membrane proteins (113, 127, 128). To face the stabilization problem, several techniques evolved in recent years: 1) Stabilization by a small number of point mutations (Heptares StaR technology (129)) for binding kinetics SPR screening of membrane proteins (127); 2) Robust immobilization strategies based on detergent-free insertion of membrane proteins into lipid bilayer nanodiscs (113) and 3) Advances in standard solubilization strategies (128). SPR devices provide nearly real-time kinetic resolution and typically analyze 3 ligands at a time or with a more complex microfluidic setup up to 12 ligands at a time. This rather lower throughput might be increased in future devices for SPR imaging with a biosensor array. However, for optical based biosensors multiplexing remains a challenge, since the optical components in the readout and disposable are costly in terms of price and energy.
Binding kinetics in drug discovery: Current perspective

Bio-layer interferometry (BLI) is a further optical sensing principle for the analysis of biomolecular interactions (130). Binding of compounds to the target proteins immobilized on the sensor surface produces an increase in the optical thickness and induces a wavelength shift and therefore a shift in the interference pattern of the reflected light. Association and dissociation can be followed in real-time. A beneficial property compared to SPR is that changes in the refractive index of the surrounding medium and changes in the flow rate cannot be followed in real-time. A beneficial property compared to SPR is that changes in the refractive index of the surrounding medium and changes in the flow rate cannot affect the signal output.

4.4.2.2. Electromechanical biosensors

Surface acoustic wave biosensor (SAW) sensors are a class of microelectromechanical systems (MEMS) able to measure mass differences originated by binding and unbinding of molecules to the sensor surface, along with changes in the viscoelasticity as induced by conformational rearrangements. Recently Heyn et al demonstrated the applicability of SAW for the characterization of small-molecule binding to the GPCR CXCR4 in its native conformation in the membrane environment (131) and showed complementarity to the results generated by SPR analyses. They claim that their approach obviates the need for often laborious and time-consuming solubilization or stabilization of membrane proteins as required for SPR.

The patch clamp technique has been used to assess the binding kinetics of drugs. In this method, a special pipette suction a membrane surface area (a patch) of a cell including a few ion channel proteins creating a gigaohm seal to measures the current across the membrane patch (132). This electrophysiological approach can be used to study compound induced current changes as a measure for the on- and offset of receptor inhibition (109) and for measuring binding kinetics – also of fast dissociating ligands (133).

4.5. Structure-based analysis

The methods and technologies described above are used to investigate binding kinetics rate constants. Additional structural information gained by biophysical methods (x-ray crystallography; nuclear magnetic resonance (NMR) and molecular dynamics simulation) can help to expand our knowledge about structure-kinetic relationships. Most frequently, the drug bound target structure is compared to the apo target structure, while taking the kinetic rate constants into account (134). As an alternative, relaxation dispersion NMR spectroscopy allows for tracking of protein-folding at a millisecond time resolution. The high-resolution structural information can be exploited to study local transition states upon ligand binding and unbinding (135, 136). Additionally, molecular dynamics simulation enables the computational calculation of transition states at a femtosecond time resolution. Since dissociation can last from seconds to hours or days, the simulation of dissociation (137) is more complex and more difficult than the simulation of usually fast association (138). Site-directed mutagenesis can confirm theories about the importance of distinct structural groups for regulation of binding kinetics (87). The better understanding of structural features influencing kinetic rates may help us to alter the stability of ground and transition states and to adjust residence times accordingly (8).

4.6. Current use and future trends for binding kinetics methods

The portfolio of assay options and readout technologies which can be accessed by drug discovery scientists has expanded in the past decades, and is expected to grow further by optimization of currently existing methods and by development of novel readouts such as new biosensors. None of the existing technologies (a summary is presented in Table 1 and Figure 4) satisfies the criteria required for application at every step of the value generation chain (see below). Understanding their advantages and disadvantages with regard to the targets they can access (e.g. membrane protein or soluble target), sensitivity, associated costs, throughput, information content (duration of action, IC\textsubscript{50}, K\textsubscript{D}, K\textsubscript{obs}, k\textsubscript{on}, k\textsubscript{off}) and kinetic resolution is key to make the best use of them. Among the most significant limitations of the technologies discussed above rebinding, ligand depletion and mass transport effects stand out. The phenomena of re-binding – when a drug repeatedly binds to its target rather than diffusing far away – cannot be avoided in some assay setups. On the one hand, re-binding hampers the determination of the real dissociation rate of a drug on its target. Seen from another perspective, re-binding can be handled as improved predictor of \textit{in vivo} reality: Interaction with membranes or limited diffusion due to compartmentation leads to increased re-binding \textit{in vitro} and \textit{in vivo} and can thereby further enhance the duration of action. Therefore it can be advantageous to study both – compound-target binding without or with limited re-binding.
for quantification of kinetic rate constants and the same setup with rebinding. But it should be mentioned that especially batch assays often lead to increased rebinding compared to the open \textit{in vivo} situation, where the drug is eliminated over time. In this context, microfluidic setups allow for complete elimination of unbound compounds, when the compound-target complex is immobilized. Biosensor-based methods are often heavily affected by the background signals of standard co-solvents such as DMSO. Although correction methods have been developed to deal with this issue, there is a need for biosensors whose readouts are solvent-insensitive.

Luckily, new technologies are emerging which can overcome the limitations of the existing ones. Although concrete application examples of these methods in the small molecule drug discovery set up are missing, they may become important in the near future: I) Among the electro-optical biosensors, resonant waveguide-grating (RWG) (123, 139) and optical waveguide grating (OWG) sensors (140) are quite promising technologies. Besides being applicable to isolated targets, they allow for whole cell dynamic mass redistribution (DMR) assays. Initially developed for batch microtiter plate-based formats, recent literature shows that microfluidic setups can regulate and eliminate rebinding by removal of unbound compounds to study both, the effect of a drug on its target with and without rebinding to estimate residence times (141). II) The newest electro-optical sensor which can been applied to quantify small molecule binding kinetics, is based on grating coupled interferometry (GCI) (122, 142, 143). This technique promises to increase the kinetic resolution and sensitivity of biosensors while decreasing the costs of materials.

<table>
<thead>
<tr>
<th>Table 1. Technologies for investigation of binding kinetics</th>
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<tr>
<td>Assay configuration and readout technology</td>
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<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Radioactivity filtration</td>
</tr>
<tr>
<td>scintillation proximity</td>
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<tr>
<td>Fluorescence fluorescence intensity</td>
</tr>
<tr>
<td>fluorescence polarization</td>
</tr>
<tr>
<td>RET (FRET, TR-FRET, BRET)</td>
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<tr>
<td>Biosensor technologies surface plasmon resonance</td>
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<tr>
<td>bio-layer interferometry</td>
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<tr>
<td>Stopped flow fluorescence based or circular dichroism depending on readout</td>
</tr>
</tbody>
</table>

The different technologies have their advantages and disadvantages with regard to the assessable targets (e.g. membrane protein or soluble target), ease-of-use, throughput and kinetic resolution. Some methods need a dedicated instrumentation (stopped flow spectrometers and biosensors) while others utilize cross-functional devises (such as plate readers). \(^{(1)}\) indirect = based on binding competition with labeled ligand \(^{(2)}\) combination of the time required to separate bound from unbound molecules (where required) and the cycle times of current instrumentation: +: > 100 s; ++: > 10 s; +++: > 1 s; ++++: > 0.1 s; ++++++: < 0.1 s \(^{(3)}\) based on literature reports, current instrument specification, and indirect detection mode: low: > 5 compounds per day, moderate: > 50 compounds per day; high: > 500 compounds per day \(^{(4)}\) soluble targets (ST), membrane preparations (MP), cells/tissue slices (C/TS)
Along these lines, back scattering interferometry (144), is a promising technology which is currently used only for steady state affinity measurements, but can potentially be applied for the determination of binding kinetics if the target is immobilized in the flow cell system. III) In the category of the electromechanical biosensors, quartz crystal microbalances offer a new biophysical principle for the study of small molecules binding to their receptor in real time: namely binding related mass changes on a sensor surface leading to a shift in the mechanical resonance frequency (127). Improvement of the attenuation caused by fluids (127), one is the major drawbacks of the method not present in others such as SAW may increase the acceptance of the technology in drug discovery laboratories. IV) Electrical biosensors are another emerging field bearing plenty of promise. Here compound-target interactions on the sensor surface are directly translated into an electrical signal. As there is no need for the integration of expensive optical components, electrical sensors have a higher potential for reasonable-priced multiplexing and high throughput applications as compared to optical sensors. Among them, silicon nanowire field effect transistors (FET) have been shown to be applicable for binding kinetics studies (145). V) As an alternative to DMR, label-free sensing of integrated cell responses can be performed by using impedance-based biosensors (146). It has been shown that the technology is capable of capturing smallest changes (directed by cytoskeleton and) induced by compound binding and the appropriate biomolecular response, and thereby can be applied to get functional efficacy and duration of action information (49). As for the electro-optical DMR sensor, here the question will be how to engineer microfluidics in the current instrumentation in order to eliminate rebinding effects. VI) Finally, other established methods such as isothermal calorimetry (ITC) and capillary electrophoresis are being repurposed for binding kinetics measurements (147, 148). Another example is the kinetic exclusion assay (KinExA) technology (149), which combines spectrofluorimetry and chromatography principles, and has been successfully applied to characterize antibody antigen interactions (150, 151).

Most technologies presented in this chapter have their rightful place at different stages of the drug discovery process. While microtiter plate based methods are applicable for screening and hit to lead campaigns, lower throughput and higher precision techniques are more suited to characterize leads and clinical candidates. In general, we recommend an overall interpretation of binding kinetics data based on the results from various assay formats and readout technologies.

5. INTEGRATION OF BINDING KINETICS IN THE LEAD DISCOVERY PROCESS

A typical screening cascade for a target-based lead discovery program is constituted of in vitro assays, cell-based assays and in vitro PK, (15, 152, 153) followed by in vivo characterization for few selected compounds. In most cases, in vitro assay formats are run in equilibrium mode as these formats permit an adaption to high-throughput screening technology. Consequently, in vivo assays are typically the first assays truly performed as “open systems” (4). Thus, it appears prudent to consider binding kinetics at an earlier stage, i.e. before entering animal trials.

As described earlier, novel assay systems such as the kPCA assay allow an early integration of binding kinetics data in the lead discovery process. At the same time, in our view, the investigation of binding kinetics for a typical small molecule lead discovery project that aims at substrate-competitive inhibition, for most cases has an increased chance to pay off if the compounds of interest have reached at least a double-digit nanomolar IC₅₀ given that the biochemical assay is performed under balanced conditions (154). Assuming that the kₚₒ of a typical lead-like small molecule is rarely below 10⁵ mol⁻¹ s⁻¹, it is obvious that the resulting target residence time is insignificant: (kₚₒ; 10⁵ mol⁻¹ s⁻¹, Kₛₒ; 100 nM, kₒᵣ; 10⁻² s⁻¹, thus RT: 1.67 min). In our view, the investment to establish a binding kinetics assay for a small molecule lead discovery project has an increased chance to pay off if the compounds of interest display a potency of at least 100 nM or lower, more rigorously 10 nM or lower, in a balanced biochemical assay. It should be noted however that this rule-of-thumb only applies in the case of a competitive agonism or antagonism that is elicited by one-to-one stoichiometry. It does not apply for multiple binding of inhibitor molecules or any form of cooperativity, or other complex mechanisms of inhibition such as partial or mixed types or two-state binding reactions, which may include reversible covalent binding (see section 3.4) as well as conformational adaption phenomena (4).

In our experience, IC₅₀₆ below 100 nM are usually found in the lead evaluation and lead optimization phase. Here, a decision to follow up selected chemical clusters can be leveraged with binding kinetic data (98). At this stage, it is not uncommon to encounter a set of chemical matter that differs with regards to its binding kinetic properties despite displaying similar functional inhibition in equilibrium assays. An informed selection of chemical matter that displays desired kinetic properties can provide valuable guidance for medicinal chemistry. Thus, we consider the lead evaluation and lead optimization phase the phase during which binding kinetics data can most likely generate a positive impact for project success (155).

As described earlier, Swinney, Copeland and others have correctly pointed out that a number of successful drugs do not display substrate-competitive binding, but instead display uncompetitive binding, which has to be considered in the binding kinetic
assay (4-5, 9). To conclude, it appears mandatory that an informed lead discovery project team pays close attention to signs of deviations from one-state reactions or non-substrate competitive mode-of-actions. Another important facet to consider in the context of lead optimization projects is the target protein turnover rate, i.e., the rate that describes the kinetics of a target protein’s degradation and de novo synthesis in the target tissue or, as surrogate, in cultured cells of a relevant cell line. A recent study concluded that in NIH3T3 fibroblasts, the median half-life of 5028 analyzed protein is 46 h. Only 2.6% of the analyzed proteins have a half-life less than 6 h (130 out of 5028) (156). Nevertheless, it should not be taken for granted that a target protein of interest displays a sufficiently slow protein turnover that permits optimization of target residence time (while de-prioritizing plasma exposure levels over time). Cell-based washout assays that are based on so-called mechanistic readouts are important contributions since these type of assays provide a first evidence of the relevance of a long-lasting inhibition.

It is thus important to allow flexibility in a project’s screening cascade, since the screening cascade ultimately determines the project’s ability to capitalize on serendipitous discoveries (157). Binding kinetic assays are an important contribution that can assist in characterization of MMoAs. In our view, technological advances in surface plasmon resonance as well as optical readers as well as an increasing availability of chemical probes will increasingly facilitate the early and routine determination of BK parameters in lead discovery projects. Yet, it is crucial to consider all known contributing factors of target biology and physiology to allow an informed decision whether a BK parameter such as the target residence time is a sensible parameter to optimize leads for.

In conclusion, the implementation of binding kinetic assays in small molecule lead discovery projects should focus on the critical path of the project and should allow a rapid adaption of the project’s screening cascade based on the individual project’s requirement. These requirements are in many cases primarily outlined by the target’s biology. At the same time, we recommend balancing effort against information content when opting for binding kinetics and when selecting an assay system for the latter.

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