Binding kinetics in drug discovery – A current perspective

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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 K_i) 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_{off} 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
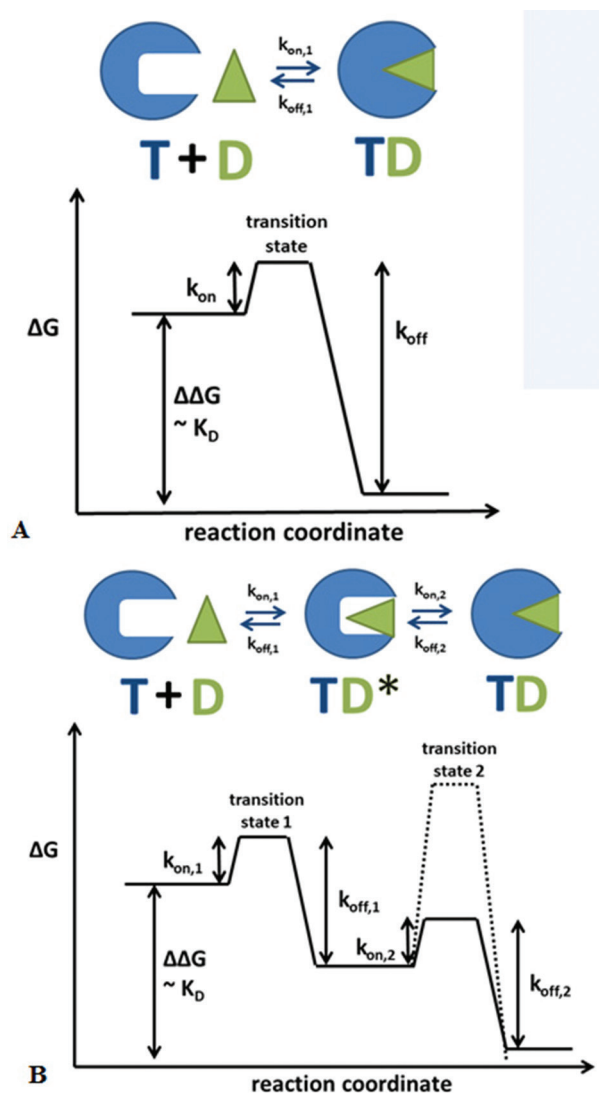
site and stabilize an open conformation of the conserved Asp-Phe-Gly (DFG) sequence in the activation loop, the so called DFG-in conformation (Figure 2). Type II inhibitors also bind to the active site but target the inactive DFG-out conformation of the activation loop in the kinase. Type III inhibitors induce a new allosteric binding site adjacent to the ATP binding pocket whereas type IV inhibitors are also allosteric binders but remote from the active site (13).

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 isoform 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 *in vitro* Raf1 kinase inhibition experiments (15, 17). On other kinases in its activity spectrum, Sorafenib has similar K_Ds 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_{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 >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 increased 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 *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
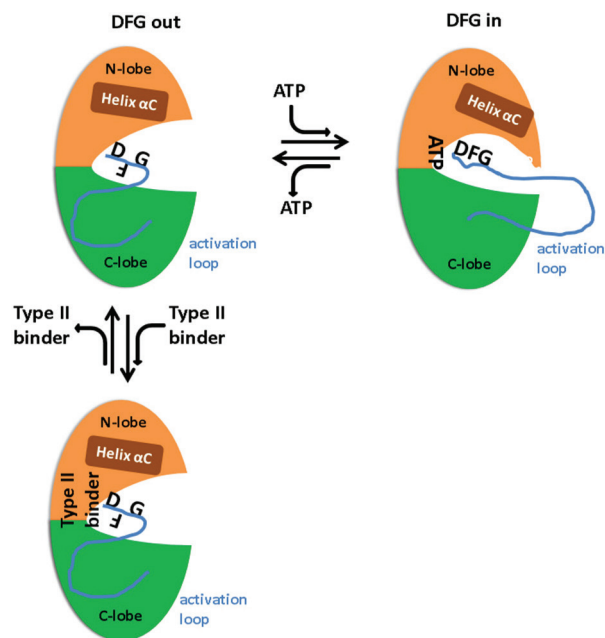
Figure 2. Kinases have a bilobal structure: At the N-lobe, the helix alpha C is located which undergoes a structural rearrangement upon ATP binding. At the C-lobe, the peptide substrates bind. Between the two lobes is the hinge region that together with the N-lobe is responsible for ATP binding. The 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).

changes in LpxC (25). However, flexibility in the acyl binding pocket of LpxC is required to accommodate and reorganize around rigid CHIR-090. Binding kinetics were measured in a biochemical assay. In a mutational study, protein rigidification reduces residence time and is resolved by using a more flexible analog of CHIR-090. In a recent study, Walkup *et al* analyzed a set of CHIR-090 derivatives kinetically (26). All their derivatives show similar slow binding kinetics as the lead compound CHIR-090 in a biochemical, rapid dilution assay at 37°C. The residence time of compounds span from 6 to 150 min and correlate with bacterial *Pseudomonas aeruginosa* growth inhibition IC_{50}. Therefore, the off-rates are likely dependent on a ground state stabilization of the final enzyme inhibitor complex (see Figure 1).

3.1.3. Conformational adaptation upon inhibitor binding in DOT1L results in longer residence time

DOT1L is a human methyltransferase of histone H3 which leads to remodeling of the chromatin structure and control of gene transcription (27). It plays an essential role during MLL-rearranged leukemia, when an inactive part of the Mll gene is fused amongst others to DOT1L that results in an upregulated activity of MLL leukemia relevant proteins (28). Inhibitors like EPZ003696 that

target the DOT1L activity were shown to selectively kill MLL cells (29). Basavapathruni *et al* solved crystal structures and measured surface plasmon resonance (SPR) of multiple analogs of EPZ003696 DOT1L inhibitor with low K_i constants that were designed during their lead discovery project (30). Within their aminonucleoside series, that covers K_i constants from 0.3-845 nM, all compounds have similar k_{on} rates limit but varying k_{off} rates, with residence times spanning from 5 s - 56 min mirroring the K_i constants. For the compounds with the longest residence time that carry an extended aminonucleoside, they found in the crystal structure that a new hydrophobic pocket is formed to accommodate them. Within the optimized series, the interaction with the new pocket was critical to expand efficacy in a cellular proliferation and methylation assay and correlated well with their respective residence times. Starting from this structure which had unfavorable pharmacokinetics, a new compound was developed that showed even longer residence time of 24 hrs (31). The compound EPZ-5676 opens an even larger hydrophobic pocket with additional contact surfaces. The increase in residence time correlates with a decrease in K_i.

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 4.4.1.2.2.) 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 Pi 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 Pi 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 fibrinogen 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

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 A_{2A} 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 K_i 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 IC_{50}s. If only IC_{50}s 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

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_{obs}, from which the rate constants k_{on} and k_{off} can be derived by plotting k_{obs} vs. compound concentration. Another way to determine the dissociation rate constant (k_{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_{on} and k_{off} using the competitive binding kinetics equations (91) whereas displacement assays solely enable k_{on} and k_{off} determinations via k_{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.

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 (ΔF) is large for slowly dissociating compounds. In contrast, ΔF 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, $k_{obs} = k_{off} (1 + [compound] / K_i) = k_{on} [compound] + k_{off})$, the described behavior is solely true as long as the competition strength for test compounds, i.e. (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
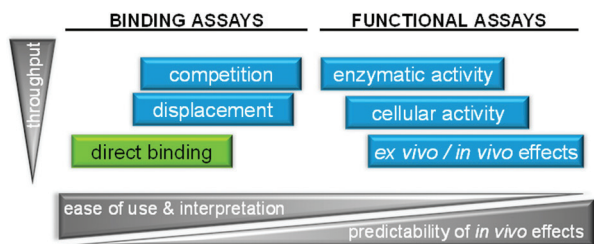
Figure 3. Comparison of assay formats for direct (green) and indirect (blue) investigation of binding kinetics: Binding assays are most convenient for (high throughput) determinations of binding kinetics parameters. Thereby, competition binding allows the highest throughput, as it allows for determination of kon, koff and KD in one experiment, whereas displacement assays only determine off-rates and additionally require a pre-incubation step. In contrast, the more complex and difficult to interpret functional assays better reflect the physiological reality, but quantification of binding kinetics is complicated or not feasible (especially for cellular and *in vivo* assays).

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 *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.

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.

4.4.1. Assays for investigation of target-compound interactions in solution
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.

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

Fluorecence 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 a (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.
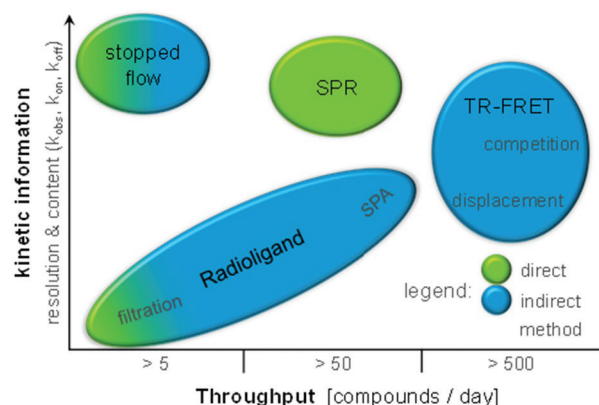
Figure 4. Comparison of readout technologies for investigation of binding kinetics in terms of throughput and information content.

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 withe 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 shift the interference pattern and will not 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 suctions 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_{50}, K_i, K_D, k_{obs}, k_{on}, k_{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 rebinding – when a drug repeatedly binds to its target rather than diffusing far away – cannot be avoided in some assay setups. On the one hand, rebinding hampers the determination of the real dissociation rate of a drug on its target. Seen from another perspective, rebinding can be handled as improved predictor of *in vivo* reality: Interaction with membranes or limited diffusion due to compartmentation leads to increased rebinding *in vitro* and *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 rebinding

Table 1. Technologies for investigation of binding kinetics

Assay configuration and readout technology		Label	Detection mode	Homogenous assay	Kinetic resolution[2] [s]	In solution *vs.* immobilized target	Throughput[3] [compounds per day]	Suitable for[4]	Possible assay formats
Radioactivity	filtration	ligand/probe labeling; no interference by label; radiation hazards & waste management	direct and indirect[1]	no	+	in solution	moderate	ST, MP, C/TS	binding; enzymatic activity
	scintillation proximity			yes	++	immobilization on particles		ST, MP, C	
Fluorescence	fluorescence intensity	ligand/probe labeling (and target labeling for RET); interference by label possible; multiplexing possible	direct and indirect[1]	no	+	in solution	moderate	ST, MP, C	binding; enzymatic activity; cellular activity
	fluorescence polarization			yes	+++				
	RET (FRET, TR-FRET, BRET)						high	ST, MP, C/TS	
Biosensor technologies	surface plasmon resonance	no	direct and indirect[1]	yes	++++	immobilization on surface	moderate	ST, (MP)	binding
	bio-layer interferometry								
Stopped flow	fluorescence based or circular dichroism	depending on readout	direct and indirect[1]	required	+++++	in solution	low	ST, MP, C	binding; enzymatic activity

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 utilized 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)

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 as compared to the open *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 und 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.

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 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 IC_{50} given that the biochemical assay is performed under balanced conditions (154). Assuming that the k_{on} of a typical lead-like small molecule is rarely below 10^5 mol^{-1} s^{-1}, it is obvious that the resulting target residence time is insignificant: (k_{on}: 10^5 mol^{-1} l^{-1}, K_D: 100 nM, k_{off}: 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, IC_{50}s 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 protein 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.

6. ACKNOWLEDGEMENT

7. REFERENCES

1. J. Arrowsmith and P. Miller: Trial watch: phase II and phase III attrition rates 2011-2012. *Nat Rev Drug Discov*, 12 (8), 569 (2013)
    DOI: 10.1038/nrd4090

2. M. E. Bunnage: Getting pharmaceutical R&D back on target. *Nat Chem Biol*, 7 (6), 335-9 (2011)
    DOI: 10.1038/nchembio.581

3. D. C. Swinney: Biochemical mechanisms of drug action: what does it take for success? *Nat Rev Drug Discov*, 3 (9), 801-8 (2004)
    DOI: 10.1038/nrd1500

4. P. J. Tummino and R. A. Copeland: Residence time of receptor-ligand complexes and its effect on biological function. *Biochemistry*, 47 (20), 5481-92 (2008)
    DOI: 10.1021/bi8002023

5. R. A. Copeland, D. L. Pompliano and T. D. Meek: Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov*, 5 (9), 730-739 (2006)
    DOI: 10.1038/nrd2082

6. J. D. Hothersall, A. J. Brown, I. Dale and P. Rawlins: Can residence time offer a useful strategy to target agonist drugs for sustained GPCR responses? *Drug Discovery Today*
    DOI: 10.1016/j.drudis.2015.07.015

7. C. Hoffmann, M. Castro, A. Rinken, R. Leurs, S. J. Hill and H. F. Vischer: Ligand Residence Time at G-protein-Coupled Receptors-Why We Should Take Our Time To Study It. *Mol Pharmacol*, 88 (3), 552-60 (2015)
    DOI: 10.1124/mol.115.099671

8. F. Schiele, P. Ayaz and A. Mueller-Fahrnow: The Use of Structural Information to Understand Binding Kinetics. In: *Thermodynamics and Kinetics of Drug Binding*. Wiley-VCH Verlag GmbH & Co. KGaA, (2015)
    DOI: 10.1002/9783527673025.ch12

9. R. A. Copeland: Conformational adaptation in drug-target interactions and residence time. *Future Med Chem*, 3 (12), 1491-501 (2011)
    DOI: 10.4155/fmc.11.112

10. D. D. Boehr, R. Nussinov and P. E. Wright: The role of dynamic conformational ensembles in biomolecular recognition. *Nat Chem Biol*, 5

(11), 789-96 (2009)
DOI: 10.1038/nchembio.232

11. V. L. Schramm: Enzymatic transition states, transition-state analogs, dynamics, thermodynamics, and lifetimes. *Annu Rev Biochem*, 80, 703-32 (2011)
DOI: 10.1146/annurev -biochem -061809 -100742

12. N. Jura, X. Zhang, N. F. Endres, M. A. Seeliger, T. Schindler and J. Kuriyan: Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. *Mol Cell*, 42 (1), 9-22 (2011)
DOI: 10.1016/j.molcel.2011.03.004

13. M. Tong and M. A. Seeliger: Targeting conformational plasticity of protein kinases. *ACS Chem Biol*, 10 (1), 190-200 (2015)
DOI: 10.1021/cb500870a

14. S. Wilhelm, C. Carter, M. Lynch, T. Lowinger, J. Dumas, R. A. Smith, B. Schwartz, R. Simantov and S. Kelley: Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov*, 5 (10), 835-44 (2006)
DOI: 10.1038/nrd2130

15. S. M. Wilhelm, C. Carter, L. Tang, D. Wilkie, A. McNabola, H. Rong, C. Chen, X. Zhang, P. Vincent, M. McHugh, Y. Cao, J. Shujath, S. Gawlak, D. Eveleigh, B. Rowley, L. Liu, L. Adnane, M. Lynch, D. Auclair, I. Taylor, R. Gedrich, A. Voznesensky, B. Riedl, L. E. Post, G. Bollag and P. A. Trail: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*, 64 (19), 7099-109 (2004)
DOI: 10.1158/0008-5472.CAN-04-1443

16. P. T. C. Wan, M. J. Garnett, S. M. Roe, S. Lee, D. Niculescu-Duvaz, V. M. Good, C. G. Project, C. M. Jones, C. J. Marshall, C. J. Springer, D. Barford and R. Marais: Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF. *Cell*, 116 (6), 855-867 (2004)
[doi not found]

17. L. Neumann, K. von König and D. Ullmann: Chapter Twelve - HTS Reporter Displacement Assay for Fragment Screening and Fragment Evolution Toward Leads with Optimized Binding Kinetics, Binding Selectivity, and Thermodynamic Signature. In: *Methods in Enzymology*. Ed C. K. Lawrence. Academic Press, (2011)
DOI: 10.1016/B978-0-12-381274-2.00012-1

18. K. Okamoto, M. Ikemori-Kawada, A. Jestel, K. von König, Y. Funahashi, T. Matsushima, A. Tsuruoka, A. Inoue and J. Matsui: Distinct Binding Mode of Multikinase Inhibitor Lenvatinib Revealed by Biochemical Characterization. *ACS Medicinal Chemistry Letters*, 6 (1), 89-94 (2015)
DOI: 10.1021/ml500394m

19. K. Anderson, Z. Lai, Octerloney B. Mcdonald, J. D. Stuart, Eldridge N. Nartey, Mary A. Hardwicke, K. Newlander, D. Dhanak, J. Adams, D. Patrick, Robert A. Copeland, Peter J. Tummino and J. Yang: Biochemical characterization of GSK1070916, a potent and selective inhibitor of Aurora B and Aurora C kinases with an extremely long residence time1. (2009)
DOI: 10.1042/BJ20090121

20. L. T. Alexander, H. Möbitz, P. Drueckes, P. Savitsky, O. Fedorov, J. M. Elkins, C. M. Deane, S. W. Cowan-Jacob and S. Knapp: Type II Inhibitors Targeting CDK2. *ACS Chemical Biology* (2015)
DOI: 10.1021/acschembio.5b00398

21. D. C. Miller, G. Lunn, P. Jones, Y. Sabnis, N. L. Davies and P. Driscoll: Investigation of the effect of molecular properties on the binding kinetics of a ligand to its biological target. *MedChemComm*, 3 (4), 449-452 (2012)
DOI: 10.1039/c2md00270a

22. E. R. Wood, A. T. Truesdale, O. B. McDonald, D. Yuan, A. Hassell, S. H. Dickerson, B. Ellis, C. Pennisi, E. Horne, K. Lackey, K. J. Alligood, D. W. Rusnak, T. M. Gilmer and L. Shewchuk: A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res*, 64 (18), 6652-9 (2004)
DOI: 10.1158/0008-5472.CAN-04-1168

23. A. W. Barb and P. Zhou: Mechanism and inhibition of LpxC: an essential zinc-dependent deacetylase of bacterial lipid A synthesis. *Curr Pharm Biotechnol*, 9 (1), 9-15 (2008)
DOI: 10.2174/138920108783497668

24. J. S. Warmus, C. L. Quinn, C. Taylor, S.

T. Murphy, T. A. Johnson, C. Limberakis, D. Ortwine, J. Bronstein, P. Pagano, J. D. Knafels, S. Lightle, I. Mochalkin, R. Brideau and T. Podoll: Structure based design of an *in vivo* active hydroxamic acid inhibitor of P. aeruginosa LpxC. *Bioorganic & Medicinal Chemistry Letters*, 22 (7), 2536-2543 (2012)
DOI: 10.1016/j.bmcl.2012.01.140

25. A. W. Barb, L. Jiang, C. R. Raetz and P. Zhou: Structure of the deacetylase LpxC bound to the antibiotic CHIR-090: Time-dependent inhibition and specificity in ligand binding. *Proc Natl Acad Sci U S A*, 104 (47), 18433-8 (2007)
DOI: 10.1073/pnas.0709412104

26. G. K. Walkup, Z. You, P. L. Ross, E. K. Allen, F. Daryaee, M. R. Hale, J. O'Donnell, D. E. Ehmann, V. J. Schuck, E. T. Buurman, A. L. Choy, L. Hajec, K. Murphy-Benenato, V. Marone, S. A. Patey, L. A. Grosser, M. Johnstone, S. G. Walker, P. J. Tonge and S. L. Fisher: Translating slow-binding inhibition kinetics into cellular and *in vivo* effects. *Nat Chem Biol*, 11 (6), 416-23 (2015)
DOI: 10.1038/nchembio.1796

27. F. van Leeuwen, P. R. Gafken and D. E. Gottschling: Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell*, 109 (6), 745-56 (2002)
DOI: 10.1016/S0092-8674(02)00759-6

28. Y. Okada, Q. Feng, Y. Lin, Q. Jiang, Y. Li, V. M. Coffield, L. Su, G. Xu and Y. Zhang: hDOT1L Links Histone Methylation to Leukemogenesis. *Cell*, 121 (2), 167-178 (2005)
DOI: 10.1016/j.cell.2005.02.020

29. S. R. Daigle, E. J. Olhava, C. A. Therkelsen, C. R. Majer, C. J. Sneeringer, J. Song, L. D. Johnston, M. P. Scott, J. J. Smith, Y. Xiao, L. Jin, K. W. Kuntz, R. Chesworth, M. P. Moyer, K. M. Bernt, J. C. Tseng, A. L. Kung, S. A. Armstrong, R. A. Copeland, V. M. Richon and R. M. Pollock: Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell*, 20 (1), 53-65 (2011)
DOI: 10.1016/j.ccr.2011.06.009

30. A. Basavapathruni, L. Jin, S. R. Daigle, C. R. Majer, C. A. Therkelsen, T. J. Wigle, K. W. Kuntz, R. Chesworth, R. M. Pollock, M. P. Scott, M. P. Moyer, V. M. Richon, R. A. Copeland and E. J. Olhava: Conformational adaptation drives potent, selective and durable inhibition of the human protein methyltransferase DOT1L. *Chem Biol Drug Des*, 80 (6), 971-80 (2012)
DOI: 10.1111/cbdd.12050

31. S. R. Daigle, E. J. Olhava, C. A. Therkelsen, A. Basavapathruni, L. Jin, P. A. Boriack-Sjodin, C. J. Allain, C. R. Klaus, A. Raimondi, M. P. Scott, N. J. Waters, R. Chesworth, M. P. Moyer, R. A. Copeland, V. M. Richon and R. M. Pollock: Potent inhibition of DOT1L as treatment of MLL-fusion leukemia. (2013)
DOI: 10.1182/blood-2013-04-497644

32. P. Schmidtke, F. J. Luque, J. B. Murray and X. Barril: Shielded hydrogen bonds as structural determinants of binding kinetics: application in drug design. *J Am Chem Soc*, 133 (46), 18903-10 (2011)
DOI: 10.1021/ja207494u

33. C. Bissantz, B. Kuhn and M. Stahl: A Medicinal Chemist's Guide to Molecular Interactions. *Journal of Medicinal Chemistry*, 53 (14), 5061-5084 (2010)
DOI: 10.1021/jm100112j

34. E. V. Schneider, J. Bottcher, M. Blaesse, L. Neumann, R. Huber and K. Maskos: The structure of CDK8/CycC implicates specificity in the CDK/cyclin family and reveals interaction with a deep pocket binder. *J Mol Biol*, 412 (2), 251-66 (2011)
DOI: 10.1016/j.jmb.2011.07.020

35. E. V. Schneider, J. Bottcher, R. Huber, K. Maskos and L. Neumann: Structure-kinetic relationship study of CDK8/CycC specific compounds. *Proc Natl Acad Sci U S A*, 110 (20), 8081-6 (2013)
DOI: 10.1073/pnas.1305378110

36. A. P. Kornev, N. M. Haste, S. S. Taylor and L. F. Eyck: Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc Natl Acad Sci U S A*, 103 (47), 17783-8 (2006)
DOI: 10.1073/pnas.0607656103

37. A. M. Aronov, Q. Tang, G. Martinez-Botella, G. W. Bemis, J. Cao, G. Chen, N. P. Ewing, P. J. Ford, U. A. Germann, J. Green, M. R. Hale, M. Jacobs, J. W. Janetka, F. Maltais, W. Markland, M. N. Namchuk, S. Nanthakumar, S. Poondru, J. Straub, E. ter Haar and X. Xie: Structure-guided design of potent and selective pyrimidylpyrrole inhibitors of

extracellular signal-regulated kinase (ERK) using conformational control. *J Med Chem*, 52 (20), 6362-8 (2009)
DOI: 10.1021/jm900630q

38. E. J. Morris, S. Jha, C. R. Restaino, P. Dayananth, H. Zhu, A. Cooper, D. Carr, Y. Deng, W. Jin, S. Black, B. Long, J. Liu, E. Dinunzio, W. Windsor, R. Zhang, S. Zhao, M. H. Angagaw, E. M. Pinheiro, J. Desai, L. Xiao, G. Shipps, A. Hruza, J. Wang, J. Kelly, S. Paliwal, X. Gao, B. S. Babu, L. Zhu, P. Daublain, L. Zhang, B. A. Lutterbach, M. R. Pelletier, U. Philippar, P. Siliphaivanh, D. Witter, P. Kirschmeier, W. R. Bishop, D. Hicklin, D. G. Gilliland, L. Jayaraman, L. Zawel, S. Fawell and A. A. Samatar: Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discov*, 3 (7), 742-50 (2013)
DOI: 10.1158/2159-8290.CD-13-0070

39. A. Chaikuad, E. M. Tacconi, J. Zimmer, Y. Liang, N. S. Gray, M. Tarsounas and S. Knapp: A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nat Chem Biol*, 10 (10), 853-60 (2014)
DOI: 10.1038/nchembio.1629

40. M. W. Karaman, S. Herrgard, D. K. Treiber, P. Gallant, C. E. Atteridge, B. T. Campbell, K. W. Chan, P. Ciceri, M. I. Davis, P. T. Edeen, R. Faraoni, M. Floyd, J. P. Hunt, D. J. Lockhart, Z. V. Milanov, M. J. Morrison, G. Pallares, H. K. Patel, S. Pritchard, L. M. Wodicka and P. P. Zarrinkar: A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol*, 26 (1), 127-32 (2008)
DOI: 10.1038/nbt1358

41. W. Bode: Structure and interaction modes of thrombin. *Blood Cells, Molecules, and Diseases*, 36 (2), 122-130 (2006)
DOI: 10.1016/j.bcmd.2005.12.027

42. T. Nilsson, Å. Sjöling-Ericksson and J. Deinum: The Mechanism of Binding of Low-Molecular-Weight Active Site Inhibitors to Human α-Thrombin. *Journal of Enzyme Inhibition*, 13 (1), 11-29 (1998)
DOI: 10.3109/14756369809035824

43. J. Winquist, S. Geschwindner, Y. Xue, L. Gustavsson, D. Musil, J. Deinum and U. H. Danielson: Identification of structural-kinetic and structural-thermodynamic relationships for thrombin inhibitors. *Biochemistry*, 52 (4), 613-26 (2013)
DOI: 10.1021/bi301333z

44. A. Biela, F. Sielaff, F. Terwesten, A. Heine, T. Steinmetzer and G. Klebe: Ligand binding stepwise disrupts water network in thrombin: enthalpic and entropic changes reveal classical hydrophobic effect. *J Med Chem*, 55 (13), 6094-110 (2012)
DOI: 10.1021/jm300337q

45. A. Sarkar and G. E. Kellogg: Hydrophobicity – Shake Flasks, Protein Folding and Drug Discovery. *Current topics in medicinal chemistry*, 10 (1), 67-83 (2010)
DOI: 10.2174/156802610790232233

46. P. Setny, R. Baron, P. Michael Kekenes-Huskey, J. A. McCammon and J. Dzubiella: Solvent fluctuations in hydrophobic cavity-ligand binding kinetics. *Proc Natl Acad Sci U S A*, 110 (4), 1197-202 (2013)
DOI: 10.1073/pnas.1221231110

47. A. C. Pan, D. W. Borhani, R. O. Dror and D. E. Shaw: Molecular determinants of drug–receptor binding kinetics. *Drug Discovery Today*, 18 (13–14), 667-673 (2013)
DOI: 10.1016/j.drudis.2013.02.007

48. J. F. Chen, H. K. Eltzschig and B. B. Fredholm: Adenosine receptors as drug targets--what are the challenges? *Nat Rev Drug Discov*, 12 (4), 265-86 (2013)
DOI: 10.1038/nrd3955

49. D. Guo, T. Mulder-Krieger, I. J. AP and L. H. Heitman: Functional efficacy of adenosine A (2)A receptor agonists is positively correlated to their receptor residence time. *Br J Pharmacol*, 166 (6), 1846-59 (2012)
DOI: 10.1111/j.1476-5381.2012.01897.x

50. P. A. Borea, A. Dalpiaz, K. Varani, S. Gessi and G. Gilli: Binding thermodynamics at A1 and A2A adenosine receptors. *Life Sci*, 59 (17), 1373-88 (1996)
DOI: 10.1016/0024-3205(96)00311-6

51. J. Singh, R. C. Petter, T. A. Baillie and A. Whitty: The resurgence of covalent drugs. *Nat Rev Drug Discov*, 10 (4), 307-17 (2011)
DOI: 10.1038/nrd3410

52. R. Mah, J. R. Thomas and C. M. Shafer: Drug discovery considerations in the development of covalent inhibitors. *Bioorganic & Medicinal Chemistry Letters*, 24 (1), 33-39 (2014)
DOI: 10.1016/j.bmcl.2013.10.003

53. J. R. Vane and R. M. Botting: The mechanism of action of aspirin. *Thrombosis Research*, 110 (5–6), 255-258 (2003)
[doi not found]

54. J. R. Vane: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol*, 231, 232-5 (1971)
DOI: 10.1038/newbio231232a0

55. P. J. Loll, D. Picot and R. M. Garavito: The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Biol*, 2 (8), 637-43 (1995)
DOI: 10.1038/nsb0895-637

56. R. B. Perni, S. J. Almquist, R. A. Byrn, G. Chandorkar, P. R. Chaturvedi, L. F. Courtney, C. J. Decker, K. Dinehart, C. A. Gates, S. L. Harbeson, A. Heiser, G. Kalkeri, E. Kolaczkowski, K. Lin, Y. P. Luong, B. G. Rao, W. P. Taylor, J. A. Thomson, R. D. Tung, Y. Wei, A. D. Kwong and C. Lin: Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. *Antimicrob Agents Chemother*, 50 (3), 899-909 (2006)
DOI: 10.1128/AAC.50.3.899-909.2006

57. J. B. Petro, S. M. J. Rahman, D. W. Ballard and W. N. Khan: Bruton's Tyrosine Kinase Is Required for Activation of Iκb Kinase and Nuclear Factor κb in Response to B Cell Receptor Engagement. *The Journal of Experimental Medicine*, 191 (10), 1745-1754 (2000)
DOI: 10.1084/jem.191.10.1745

58. S. Ponader, S. S. Chen, J. J. Buggy, K. Balakrishnan, V. Gandhi, W. G. Wierda, M. J. Keating, S. O'Brien, N. Chiorazzi and J. A. Burger: The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing *in vitro* and *in vivo*. *Blood*, 119 (5), 1182-9 (2012)
DOI: 10.1182/blood-2011-10-386417

59. J. M. Bradshaw, J. M. McFarland, V. O. Paavilainen, A. Bisconte, D. Tam, V. T. Phan, S. Romanov, D. Finkle, J. Shu, V. Patel, T. Ton, X. Li, D. G. Loughhead, P. A. Nunn, D. E. Karr, M. E. Gerritsen, J. O. Funk, T. D. Owens, E. Verner, K. A. Brameld, R. J. Hill, D. M. Goldstein and J. Taunton: Prolonged and tunable residence time using reversible covalent kinase inhibitors. *Nat Chem Biol*, advance online publication (2015)
DOI: 10.1038/nchembio.1817

60. W. D. Paton and H. P. Rang: The Uptake of Atropine and Related Drugs by Intestinal Smooth Muscle of the Guinea-Pig in Relation to Acetylcholine Receptors. *Proc R Soc Lond B Biol Sci*, 163, 1-44 (1965)
DOI: 10.1098/rspb.1965.0058

61. W. D. M. Paton and D. R. Waud: A quantitative investigation of the relationship between rate of access of a drug to receptor and the rate of onset or offset of action. *Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie*, 248 (2), 124-143 (1964)
DOI: 10.1007/BF00246668

62. W. D. M. Paton: Kinetic theories of drug action with special reference to the acetylcholine group of agonists and antagonists. *Annals of the New York Academy of Sciences*, 144 (2), 869-881 (1967)
DOI: 10.1111/j.1749-6632.1967.tb53816.x

63. G. Segre: Radioisotopes in the study of drugs kinetics. *J Nucl Biol Med*, 12 (1), 1-4 (1968)
[doi not found]

64. G. A. Weiland and P. B. Molinoff: Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. *Life Sci*, 29 (4), 313-30 (1981)
DOI: 10.1016/0024-3205(81)90324-6

65. N. Rietbrock and A. Lassmann: Stopped-flow studies on drug-protein binding. 1. Kinetics of warfarin binding to human serum albumin. *Naunyn Schmiedebergs Arch Pharmacol*, 313 (3), 269-74 (1980)
DOI: 10.1007/BF00505744

66. V. Maes, Y. Engelborghs, J. Hoebeke, Y. Maras and A. Vercruysse: Fluorimetric analysis of the binding of warfarin to human serum albumin. Equilibrium and kinetic study. *Mol Pharmacol*, 21 (1), 100-7 (1982)
[doi not found]

67. T. Endo, M. Nakanishi, S. Furukawa, F. J. Joubert, N. Tamiya and K. Hayashi: Stopped-flow fluorescence studies on binding kinetics of neurotoxins with acetylcholine receptor. *Biochemistry*, 25 (2), 395-404 (1986)
DOI: 10.1021/bi00350a019

68. M. L. Contreras, B. B. Wolfe and P. B. Molinoff: Kinetic analysis of the interactions of agonists and antagonists with beta adrenergic receptors. *J Pharmacol Exp Ther*, 239 (1), 136-43 (1986)
[doi not found]

69. J. E. Hawkinson and J. E. Casida: Binding kinetics of gamma-aminobutyric acidA receptor noncompetitive antagonists: trioxabicyclooctane, dithiane, and cyclodiene insecticide-induced slow transition to blocked chloride channel conformation. *Mol Pharmacol*, 42 (6), 1069-76 (1992)
[doi not found]

70. T. C. VanCott, L. D. Loomis, R. R. Redfield and D. L. Birx: Real-time biospecific interaction analysis of antibody reactivity to peptides from the envelope glycoprotein, gp160, of HIV-1. *J Immunol Methods*, 146 (2), 163-76 (1992)
DOI: 10.1016/0022-1759(92)90225-I

71. D. Altschuh, M. C. Dubs, E. Weiss, G. Zeder-Lutz and M. H. Van Regenmortel: Determination of kinetic constants for the interaction between a monoclonal antibody and peptides using surface plasmon resonance. *Biochemistry*, 31 (27), 6298-304 (1992)
DOI: 10.1021/bi00142a019

72. R. Karlsson: Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. *Anal Biochem*, 221 (1), 142-51 (1994)
DOI: 10.1006/abio.1994.1390

73. M. Hara, F. Tozawa, K. Itazaki, S. Mihara and M. Fujimoto: Endothelin ET (B) receptors show different binding profiles in intact cells and cell membrane preparations. *Eur J Pharmacol*, 345 (3), 339-42 (1998)
DOI: 10.1016/S0014-2999(98)00016-8

74. F. L. Fierens, P. M. Vanderheyden, C. Roggeman, P. Vande Gucht, J. P. De Backer and G. Vauquelin: Distinct binding properties of the AT (1) receptor antagonist (3)H) candesartan to intact cells and membrane preparations. *Biochem Pharmacol*, 63 (7), 1273-9 (2002)
DOI: 10.1016/S0006-2952(02)00859-6

75. A. Packeu, J. P. De Backer, I. Van Liefde, P. M. Vanderheyden and G. Vauquelin: Antagonist-radioligand binding to D2L-receptors in intact cells. *Biochem Pharmacol*, 75 (11), 2192-203 (2008)
DOI: 10.1016/j.bcp.2008.03.001

76. K. E. Luker, L. A. Mihalko, B. T. Schmidt, S. A. Lewin, P. Ray, D. Shcherbo, D. M. Chudakov and G. D. Luker: *In vivo* imaging of ligand receptor binding with Gaussia luciferase complementation. *Nat Med*, 18 (1), 172-7 (2012)
DOI: 10.1038/nm.2590

77. F. Schiele, P. Ayaz and A. Fernandez-Montalvan: A universal homogeneous assay for high-throughput determination of binding kinetics. *Anal Biochem*, 468C, 42-49 (2014)
DOI: 10.1016/j.ab.2014.09.007

78. L. A. Stoddart, E. K. Johnstone, A. J. Wheal, J. Goulding, M. B. Robers, T. Machleidt, K. V. Wood, S. J. Hill and K. D. Pfleger: Application of BRET to monitor ligand binding to GPCRs. *Nat Methods*, 12 (7), 661-3 (2015)
DOI: 10.1038/nmeth.3398

79. S. J. Ramsey, N. J. Attkins, R. Fish and P. H. van der Graaf: Quantitative pharmacological analysis of antagonist binding kinetics at CRF1 receptors *in vitro* and *in vivo*. *Br J Pharmacol*, 164 (3), 992-1007 (2011)
DOI: 10.1111/j.1476-5381.2011.01390.x

80. S. Kapur and P. Seeman: Antipsychotic agents differ in how fast they come off the dopamine D2 receptors. Implications for atypical antipsychotic action. *J Psychiatry Neurosci*, 25 (2), 161-6 (2000)
[doi not found]

81. N. Ginovart, W. Sun, A. A. Wilson, S. Houle and S. Kapur: Quantitative validation of an intracerebral beta-sensitive microprobe system to determine *in vivo* drug-induced receptor occupancy using (11C) raclopride in rats. *Synapse*, 52 (2), 89-99 (2004)
DOI: 10.1002/syn.20010

82. N. Thorne, D. S. Auld and J. Inglese: Apparent activity in high-throughput screening: origins of compound-dependent assay interference. *Curr Opin Chem Biol*, 14 (3), 315-24 (2010)
DOI: 10.1016/j.cbpa.2010.03.020

83. R. A. Copeland, A. Basavapathruni, M. Moyer and M. P. Scott: Impact of enzyme concentration and residence time on apparent activity recovery in jump dilution analysis. *Anal Biochem*, 416 (2), 206-10 (2011)

DOI: 10.1016/j.ab.2011.05.029

84. J. R. Simard, C. Grutter, V. Pawar, B. Aust, A. Wolf, M. Rabiller, S. Wulfert, A. Robubi, S. Kluter, C. Ottmann and D. Rauh: High-throughput screening to identify inhibitors which stabilize inactive kinase conformations in p38alpha. *J Am Chem Soc*, 131 (51), 18478-88 (2009)
DOI: 10.1021/ja907795q

85. J. R. Simard, M. Getlik, C. Grutter, R. Schneider, S. Wulfert and D. Rauh: Fluorophore labeling of the glycine-rich loop as a method of identifying inhibitors that bind to active and inactive kinase conformations. *J Am Chem Soc*, 132 (12), 4152-60 (2010)
DOI: 10.1021/ja908083e

86. J. Louvel, D. Guo, M. Agliardi, T. A. Mocking, R. Kars, T. P. Pham, L. Xia, H. de Vries, J. Brussee, L. H. Heitman and A. P. Ijzerman: Agonists for the adenosine A1 receptor with tunable residence time. A Case for nonribose 4-amino-6-aryl-5-cyano-2-thiopyrimidines. *J Med Chem*, 57 (8), 3213-22 (2014)
DOI: 10.1021/jm401643m

87. D. C. Swinney, P. Beavis, K. T. Chuang, Y. Zheng, I. Lee, P. Gee, J. Deval, D. M. Rotstein, M. Dioszegi, P. Ravendran, J. Zhang, S. Sankuratri, R. Kondru and G. Vauquelin: A study of the molecular mechanism of binding kinetics and long residence times of human CCR5 receptor small molecule allosteric ligands. *Br J Pharmacol*, 171 (14), 3364-75 (2014)
DOI: 10.1111/bph.12683

88. J. P. van Veldhoven, R. Liu, S. A. Thee, Y. Wouters, S. J. Verhoork, C. Mooiman, J. Louvel and I. J. AP: Affinity and kinetics study of anthranilic acids as HCA2 receptor agonists. *Bioorg Med Chem*, 23 (14), 4013-25 (2015)
DOI: 10.1016/j.bmc.2015.02.018

89. K. Okamoto, M. Ikemori-Kawada, A. Jestel, K. von Konig, Y. Funahashi, T. Matsushima, A. Tsuruoka, A. Inoue and J. Matsui: Distinct binding mode of multikinase inhibitor lenvatinib revealed by biochemical characterization. *ACS Med Chem Lett*, 6 (1), 89-94 (2015)
DOI: 10.1021/ml500394m

90. C. Meyners, M. G. Baud, M. J. Fuchter and F. J. Meyer-Almes: Kinetic method for the large-scale analysis of the binding mechanism of histone deacetylase inhibitors. *Anal Biochem*, 460, 39-46 (2014)
DOI: 10.1016/j.ab.2014.05.014

91. H. J. Motulsky and L. C. Mahan: The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol*, 25 (1), 1-9 (1984)
[doi not found]

92. N. Granqvist, A. Hanning, L. Eng, J. Tuppurainen and T. Viitala: Label-enhanced surface plasmon resonance: a new concept for improved performance in optical biosensor analysis. *Sensors (Basel)*, 13 (11), 15348-63 (2013)
DOI: 10.3390/s131115348

93. D. Guo, J. M. Hillger, I. J. AP and L. H. Heitman: Drug-target residence time--a case for G protein-coupled receptors. *Med Res Rev*, 34 (4), 856-92 (2014)
DOI: 10.1002/med.21307

94. C. E. Heise, S. K. Sullivan and P. D. Crowe: Scintillation proximity assay as a high-throughput method to identify slowly dissociating nonpeptide ligand binding to the GnRH receptor. *J Biomol Screen*, 12 (2), 235-9 (2007)
DOI: 10.1177/1087057106297362

95. D. Vanderpool, C. E. Grimshaw, J. D. Lawson and J. Ermolieff: Residence time and kinetic efficiency analysis of extracellular signal-regulated kinase 2 inhibitors. *Anal Biochem*, 473, 46-52 (2015)
DOI: 10.1016/j.ab.2014.12.008

96. D. Guo, E. J. van Dorp, T. Mulder-Krieger, J. P. van Veldhoven, J. Brussee, A. P. Ijzerman and L. H. Heitman: Dual-point competition association assay: a fast and high-throughput kinetic screening method for assessing ligand-receptor binding kinetics. *J Biomol Screen*, 18 (3), 309-20 (2013)
DOI: 10.1177/1087057112464776

97. L. Neumann, A. Ritscher, G. Muller and D. Hafenbradl: Fragment-based lead generation: identification of seed fragments by a highly efficient fragment screening technology. *J Comput Aided Mol Des*, 23 (8), 501-11 (2009)
DOI: 10.1007/s10822-009-9288-x

98. J. C. Uitdehaag, C. M. Sunnen, A. M. van Doornmalen, N. de Rouw, A. Oubrie, R. Azevedo, M. Ziebell, E. Nickbarg, W. J. Karstens and S. Ruygrok: Multidimensional profiling of CSF1R screening hits and inhibitors:

assessing cellular activity, target residence time, and selectivity in a higher throughput way. *J Biomol Screen*, 16 (9), 1007-17 (2011)
DOI: 10.1177/1087057111418113

99. R. A. Copeland: Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. *Methods Biochem Anal*, 46, 1-265 (2005)

100. A. C. Cheng and I. H. Tsai: Functional characterization of a slow and tight-binding inhibitor of plasmin isolated from Russell's viper venom. *Biochim Biophys Acta*, 1840 (1), 153-9 (2014)
DOI: 10.1016/j.bbagen.2013.08.019

101. A. Wang, C. Dorso, L. Kopcho, G. Locke, R. Langish, E. Harstad, P. Shipkova, J. Marcinkeviciene, L. Hamann and M. S. Kirby: Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin *in vitro* and *ex vivo* when compared to a rapidly-dissociating DPP4 inhibitor. *BMC Pharmacol*, 12, 2 (2012)
DOI: 10.1186/1471-2210-12-2

102. Q. Liu, S. Kirubakaran, W. Hur, M. Niepel, K. Westover, C. C. Thoreen, J. Wang, J. Ni, M. P. Patricelli, K. Vogel, S. Riddle, D. L. Waller, R. Traynor, T. Sanda, Z. Zhao, S. A. Kang, J. Zhao, A. T. Look, P. K. Sorger, D. M. Sabatini and N. S. Gray: Kinome-wide selectivity profiling of ATP-competitive mammalian target of rapamycin (mTOR) inhibitors and characterization of their binding kinetics. *J Biol Chem*, 287 (13), 9742-52 (2012)
DOI: 10.1074/jbc.M111.304485

103. R. A. Copeland: Tight Binding Inhibitors. In: *Enzymes*. John Wiley & Sons, Inc., (2002)
DOI: 10.1002/0471220639.ch9

104. R. A. Copeland: Time-Dependent Inhibition. In: *Enzymes*. John Wiley & Sons, Inc., (2002)
DOI: 10.1002/0471220639.ch10

105. R. Zhang and W. T. Windsor: *In vitro* kinetic profiling of hepatitis C virus NS3 protease inhibitors by progress curve analysis. *Methods Mol Biol*, 1030, 59-79 (2013)
DOI: 10.1007/978-1-62703-484-5_6

106. T. Kenakin, S. Jenkinson and C. Watson: Determining the potency and molecular mechanism of action of insurmountable antagonists. *J Pharmacol Exp Ther*, 319 (2), 710-23 (2006)
DOI: 10.1124/jpet.106.107375

107. J. Gatfield, C. Mueller Grandjean, T. Sasse, M. Clozel and O. Nayler: Slow receptor dissociation kinetics differentiate macitentan from other endothelin receptor antagonists in pulmonary arterial smooth muscle cells. *PLoS One*, 7 (10), e47662 (2012)
DOI: 10.1371/journal.pone.0047662

108. D. Hafenbradl, M. Baumann and L. Neumann: *In vitro* Characterization of Small-Molecule Kinase Inhibitors. In: *Protein Kinases as Drug Targets*. Wiley-VCH Verlag GmbH & Co. KGaA, (2011)
DOI: 10.1002/9783527633470.ch1

109. J. Borovska, V. Vyklicky, E. Stastna, V. Kapras, B. Slavikova, M. Horak, H. Chodounska and L. Vyklicky, Jr.: Access of inhibitory neurosteroids to the NMDA receptor. *Br J Pharmacol*, 166 (3), 1069-83 (2012)
DOI: 10.1111/j.1476-5381.2011.01816.x

110. B. Tillotson, J. Coco, N. Whitebread, K. West, K. Slocum, B. Thomas, J. Ge, J. Ali, E. Normant, J. Hoyt, J. MacDougall, J. Sydor, V. Palombella, J. Adams and C. Fritz: Abstract #1710: A novel pharmacodynamic assay to measure Hsp90 target occupancy by the small molecule inhibitors IPI-504 and IPI-493 in tumors. *Cancer Research*, 69 (9 Supplement), 1710 (2009)
[doi not found]

111. D. S. Auld, M. W. Farmen, S. D. Kahl, A. Kriauciunas, K. L. McKnight, C. Montrose and J. R. Weidner: Receptor Binding Assays for HTS and Drug Discovery. In: *Assay Guidance Manual*. Ed G. S. Sittampalam, N. P. Coussens, H. Nelson, M. Arkin, D. Auld, C. Austin, B. Bejcek, M. Glicksman, J. Inglese, P. W. Iversen, Z. Li, J. McGee, O. McManus, L. Minor, A. Napper, J. M. Peltier, T. Riss, O. J. Trask, Jr.&J. Weidner. Bethesda (MD) (2004)
[doi not found]

112. G. Vauquelin, S. Bostoen, P. Vanderheyden and P. Seeman: Clozapine, atypical antipsychotics, and the benefits of fast-off D2 dopamine receptor antagonism. *Naunyn Schmiedebergs Arch Pharmacol*, 385 (4), 337-72 (2012)
DOI: 10.1007/s00210-012-0734-2

113. N. Bocquet, J. Kohler, M. N. Hug, E. A. Kusznir, A. C. Rufer, R. J. Dawson, M. Hennig, A. Ruf,

W. Huber and S. Huber: Real-time monitoring of binding events on a thermostabilized human A2A receptor embedded in a lipid bilayer by surface plasmon resonance. *Biochim Biophys Acta*, 1848 (5), 1224-33 (2015)
DOI: 10.1016/j.bbamem.2015.02.014

114. D. E. Raines and N. S. Krishnan: Transient low-affinity agonist binding to Torpedo postsynaptic membranes resolved by using sequential mixing stopped-flow fluorescence spectroscopy. *Biochemistry*, 37 (3), 956-64 (1998)
DOI: 10.1021/bi971689w

115. O. Barauskas, A. C. Corsa, R. Wang, S. Hluhanich, D. Jin, M. Hung, H. Yang, W. E. t. Delaney and B. E. Schultz: Binding kinetics, potency, and selectivity of the hepatitis C virus NS3 protease inhibitors GS-9256 and vedroprevir. *Biochim Biophys Acta*, 1840 (12), 3292-8 (2014)
DOI: 10.1016/j.bbagen.2014.08.002

116. I. E. Andreeva, S. Nirthanan, J. B. Cohen and S. E. Pedersen: Site specificity of agonist-induced opening and desensitization of the Torpedo californica nicotinic acetylcholine receptor. *Biochemistry*, 45 (1), 195-204 (2006)
DOI: 10.1021/bi0516024

117. P. van der Sluijs, B. Postema and D. K. Meijer: Lactosylation of albumin reduces uptake rate of dibromosulfophthalein in perfused rat liver and dissociation rate from albumin *in vitro*. *Hepatology*, 7 (4), 688-95 (1987)
DOI: 10.1002/hep.1840070412

118. L. T. May, S. J. Briddon and S. J. Hill: Antagonist selective modulation of adenosine A1 and A3 receptor pharmacology by the food dye Brilliant Black BN: evidence for allosteric interactions. *Mol Pharmacol*, 77 (4), 678-86 (2010)
DOI: 10.1124/mol.109.063065

119. L. T. May, T. J. Self, S. J. Briddon and S. J. Hill: The effect of allosteric modulators on the kinetics of agonist-G protein-coupled receptor interactions in single living cells. *Mol Pharmacol*, 78 (3), 511-23 (2010)
DOI: 10.1124/mol.110.064493

120. G. Turcatti, H. Vogel and A. Chollet: Probing the binding domain of the NK2 receptor with fluorescent ligands: evidence that heptapeptide agonists and antagonists bind differently. *Biochemistry*, 34 (12), 3972-80 (1995)

DOI: 10.1021/bi00012a015

121. I. Nederpelt, V. Schuldt, F. Schiele, K. Nowak-Reppel, A. E. Fernandez-Montalvan, I. J. AP and L. H. Heitman: Characterization of 12 GnRH peptide agonists - a kinetic perspective. *Br J Pharmacol* (2015)
DOI: 10.1111/bph.13342

122. J. L. Mason, C. Spais, J. Husten, E. Prouty, M. S. Albom, S. L. Meyer, M. A. Ator and T. S. Angeles: Comparison of LanthaScreen Eu kinase binding assay and surface plasmon resonance method in elucidating the binding kinetics of focal adhesion kinase inhibitors. *Assay Drug Dev Technol*, 10 (5), 468-75 (2012)
DOI: 10.1089/adt.2012.453

123. D. Riester, C. Hildmann, A. Schwienhorst and F. J. Meyer-Almes: Histone deacetylase inhibitor assay based on fluorescence resonance energy transfer. *Anal Biochem*, 362 (1), 136-41 (2007)
DOI: 10.1016/j.ab.2006.12.019

124. T. Brauns, Z. W. Cai, S. D. Kimball, K. C. Kang, R. P. Haugland, W. Berger, S. Berjukov, S. Hering, H. Glossmann and J. Striessnig: Benzothiazepine binding domain of purified L-type calcium channels: direct labeling using a novel fluorescent diltiazem analogue. *Biochemistry*, 34 (10), 3461-9 (1995)
DOI: 10.1021/bi00010a039

125. K. D. Pfleger and K. A. Eidne: Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). *Nat Methods*, 3 (3), 165-74 (2006)
DOI: 10.1038/nmeth841

126. N. Boute, R. Jockers and T. Issad: The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol Sci*, 23 (8), 351-4 (2002)
DOI: 10.1016/S0165-6147(02)02062-X

127. E. Segala, J. C. Errey, C. Fiez-Vandal, A. Zhukov and R. M. Cooke: Biosensor-based affinities and binding kinetics of small molecule antagonists to the adenosine A (2A) receptor reconstituted in HDL like particles. *FEBS Lett*, 589 (13), 1399-405 (2015)
DOI: 10.1016/j.febslet.2015.04.030

128. T. Aristotelous, S. Ahn, A. K. Shukla, S. Gawron, M. F. Sassano, A. W. Kahsai, L.

M. Wingler, X. Zhu, P. Tripathi-Shukla, X. P. Huang, J. Riley, J. Besnard, K. D. Read, B. L. Roth, I. H. Gilbert, A. L. Hopkins, R. J. Lefkowitz and I. Navratilova: Discovery of beta2 Adrenergic Receptor Ligands Using Biosensor Fragment Screening of Tagged Wild-Type Receptor. *ACS Med Chem Lett*, 4 (10), 1005-1010 (2013)
DOI: 10.1021/ml400312j

129. M. J. Serrano-Vega, F. Magnani, Y. Shibata and C. G. Tate: Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proc Natl Acad Sci U S A*, 105 (3), 877-82 (2008)
DOI: 10.1073/pnas.0711253105

130. N. B. Shah and T. M. Duncan: Bio-layer interferometry for measuring kinetics of protein-protein interactions and allosteric ligand effects. *J Vis Exp* (84), e51383 (2014)
DOI: 10.3791/51383

131. R. G. Heym, W. B. Hornberger, V. Lakics and G. C. Terstappen: Label-free detection of small-molecule binding to a GPCR in the membrane environment. *Biochim Biophys Acta*, 1854 (8), 979-86 (2015)
DOI: 10.1016/j.bbapap.2015.04.003

132. O. P. Hamill, A. Marty, E. Neher, B. Sakmann and F. J. Sigworth: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch*, 391 (2), 85-100 (1981)
DOI: 10.1007/BF00656997

133. P. S. Hasenhuetl, K. Schicker, X. Koenig, Y. Li, S. Sarker, T. Stockner, S. Sucic, H. H. Sitte, M. Freissmuth and W. Sandtner: Ligand Selectivity among the Dopamine and Serotonin Transporters Specified by the Forward Binding Reaction. *Mol Pharmacol*, 88 (1), 12-8 (2015)
DOI: 10.1124/mol.115.099036

134. A. Chang, K. Kapilashrami, E. K. H. Allen and P. J. Tonge: The Kinetics and Thermodynamics of Staphylococcus aureus FabI Inhibition. In: *Thermodynamics and Kinetics of Drug Binding*. Wiley-VCH Verlag GmbH & Co. KGaA, (2015)
DOI: 10.1002/9783527673025.ch15

135. D. Tolkatchev, P. Xu and F. Ni: Probing the kinetic landscape of transient peptide-protein interactions by use of peptide (15) n NMR relaxation dispersion spectroscopy:

binding of an antithrombin peptide to human prothrombin. *J Am Chem Soc*, 125 (41), 12432-42 (2003)
DOI: 10.1021/ja021238l

136. D. F. Hansen, Z. Zhou, H. Feng, L. M. Miller Jenkins, Y. Bai and L. E. Kay: Binding kinetics of histone chaperone Chz1 and variant histone H2A.Z-H2B by relaxation dispersion NMR spectroscopy. *J Mol Biol*, 387 (1), 1-9 (2009)
DOI: 10.1016/j.jmb.2009.01.009

137. P. Tiwary, V. Limongelli, M. Salvalaglio and M. Parrinello: Kinetics of protein–ligand unbinding: Predicting pathways, rates, and rate-limiting steps. *Proceedings of the National Academy of Sciences*, 112 (5), E386-E391 (2015)
DOI: 10.1073/pnas.1424461112

138. Y. M. Huang, M. Kang and C. E. Chang: Switches of hydrogen bonds during ligand-protein association processes determine binding kinetics. *J Mol Recognit*, 27 (9), 537-48 (2014)
DOI: 10.1002/jmr.2377

139. J. R. Galvin, M. Mori and W. Stanford: High-resolution computed tomography and diffuse lung disease. *Curr Probl Diagn Radiol*, 21 (2), 31-74 (1992)
DOI: 10.1016/0363-0188(92)90022-8

140. L. S. Halperin and L. H. Schoch: Angle closure glaucoma after scleral buckling for retinopathy of prematurity. Case report. *Arch Ophthalmol*, 106 (4), 453 (1988)
DOI: 10.1001/archopht.1988.01060130495016

141. H. Deng, C. Wang, M. Su and Y. Fang: Probing biochemical mechanisms of action of muscarinic M3 receptor antagonists with label-free whole cell assays. *Anal Chem*, 84 (19), 8232-9 (2012)
DOI: 10.1021/ac301495n

142. R. E. Kunz and K. Cottier: Optimizing integrated optical chips for label-free (bio-) chemical sensing. *Anal Bioanal Chem*, 384 (1), 180-90 (2006)
DOI: 10.1007/s00216-005-0196-1

143. P. Kozma, A. Hamori, K. Cottier, S. Kurunczi and R. Horvath: Grating coupled interferometry for optical sensing. *Applied Physics B*, 97 (1), 5-8 (2009)
DOI: 10.1007/s00340-009-3719-1

144. D. J. Bornhop, J. C. Latham, A. Kussrow, D. A. Markov, R. D. Jones and H. S. Sorensen: Free-solution, label-free molecular interactions studied by back-scattering interferometry. *Science*, 317 (5845), 1732-6 (2007)
DOI: 10.1126/science.1146559

145. X. Duan, Y. Li, N. K. Rajan, D. A. Routenberg, Y. Modis and M. A. Reed: Quantification of the affinities and kinetics of protein interactions using silicon nanowire biosensors. *Nat Nanotechnol*, 7 (6), 401-7 (2012)
DOI: 10.1038/nnano.2012.82

146. R. Schroder, J. Schmidt, S. Blattermann, L. Peters, N. Janssen, M. Grundmann, W. Seemann, D. Kaufel, N. Merten, C. Drewke, J. Gomeza, G. Milligan, K. Mohr and E. Kostenis: Applying label-free dynamic mass redistribution technology to frame signaling of G protein-coupled receptors noninvasively in living cells. *Nat Protoc*, 6 (11), 1748-60 (2011)
DOI: 10.1038/nprot.2011.386

147. D. Burnouf, E. Ennifar, S. Guedich, B. Puffer, G. Hoffmann, G. Bec, F. Disdier, M. Baltzinger and P. Dumas: kinITC: A New Method for Obtaining Joint Thermodynamic and Kinetic Data by Isothermal Titration Calorimetry. *Journal of the American Chemical Society*, 134 (1), 559-565 (2012)
DOI: 10.1021/ja209057d

148. M. Kanoatov, L. T. Cherney and S. N. Krylov: Extracting kinetics from affinity capillary electrophoresis (ACE) data: a new blade for the old tool. *Anal Chem*, 86 (2), 1298-305 (2014)
DOI: 10.1021/ac4038976

149. R. C. Blake, 2nd, A. R. Pavlov and D. A. Blake: Automated kinetic exclusion assays to quantify protein binding interactions in homogeneous solution. *Anal Biochem*, 272 (2), 123-34 (1999)
DOI: 10.1006/abio.1999.4176

150. A. W. Drake, M. L. Tang, G. A. Papalia, G. Landes, M. Haak-Frendscho and S. L. Klakamp: Biacore surface matrix effects on the binding kinetics and affinity of an antigen/antibody complex. *Anal Biochem*, 429 (1), 58-69 (2012)
DOI: 10.1016/j.ab.2012.06.024

151. C. Bee, Y. N. Abdiche, D. M. Stone, S. Collier, K. C. Lindquist, A. C. Pinkerton, J. Pons and A. Rajpal: Exploring the dynamic range of the kinetic exclusion assay in characterizing antigen-antibody interactions. *PLoS One*, 7 (4), e36261 (2012)
DOI: 10.1371/journal.pone.0036261

152. T. Wunberg, M. Hendrix, A. Hillisch, M. Lobell, H. Meier, C. Schmeck, H. Wild and B. Hinzen: Improving the hit-to-lead process: data-driven assessment of drug-like and lead-like screening hits. *Drug Discov Today*, 11 (3-4), 175-80 (2006)
[doi not found]

153. L. M. Mayr and D. Bojanic: Novel trends in high-throughput screening. *Curr Opin Pharmacol*, 9 (5), 580-8 (2009)
DOI: 10.1016/j.coph.2009.08.004

154. J. Yang, R. A. Copeland and Z. Lai: Defining balanced conditions for inhibitor screening assays that target bisubstrate enzymes. *J Biomol Screen*, 14 (2), 111-20 (2009)
DOI: 10.1177/1087057108328763

155. D. Guo, L. H. Heitman and A. P. Ijzerman: The Role of Target Binding Kinetics in Drug Discovery. *ChemMedChem*, n/a-n/a (2015)
DOI: 10.1002/cmdc.201500310

156. B. Schwanhausser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen and M. Selbach: Global quantification of mammalian gene expression control. *Nature*, 473 (7347), 337-42 (2011)
DOI: 10.1038/nature10098

157. J. G. Cumming, M. R. Finlay, F. Giordanetto, M. Hemmerling, T. Lister, H. Sanganee and M. J. Waring: Potential strategies for increasing drug-discovery productivity. *Future Med Chem*, 6 (5), 515-27 (2014)
DOI: 10.4155/fmc.14.7

158. M. B. Robers, M. L. Dart, C. C. Woodroofe, C. A. Zimprich, T. A. Kirkland, T. Machleidt, K. R. Kupcho, S. Levin, J. R. Hartnett, K. Zimmerman, A. L. Niles, R. Friedman Ohana1, D. L. Daniels, M. Slater, M. G. Wood, M. Cong, Y.-Q. Cheng and K. V. Wood: Target engagement and drug residence time can be observed in living cells with BRET. *Nat Communications*, 6 (10091), 1-10 (2015)
DOI: 10.1038/ncomms10091

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