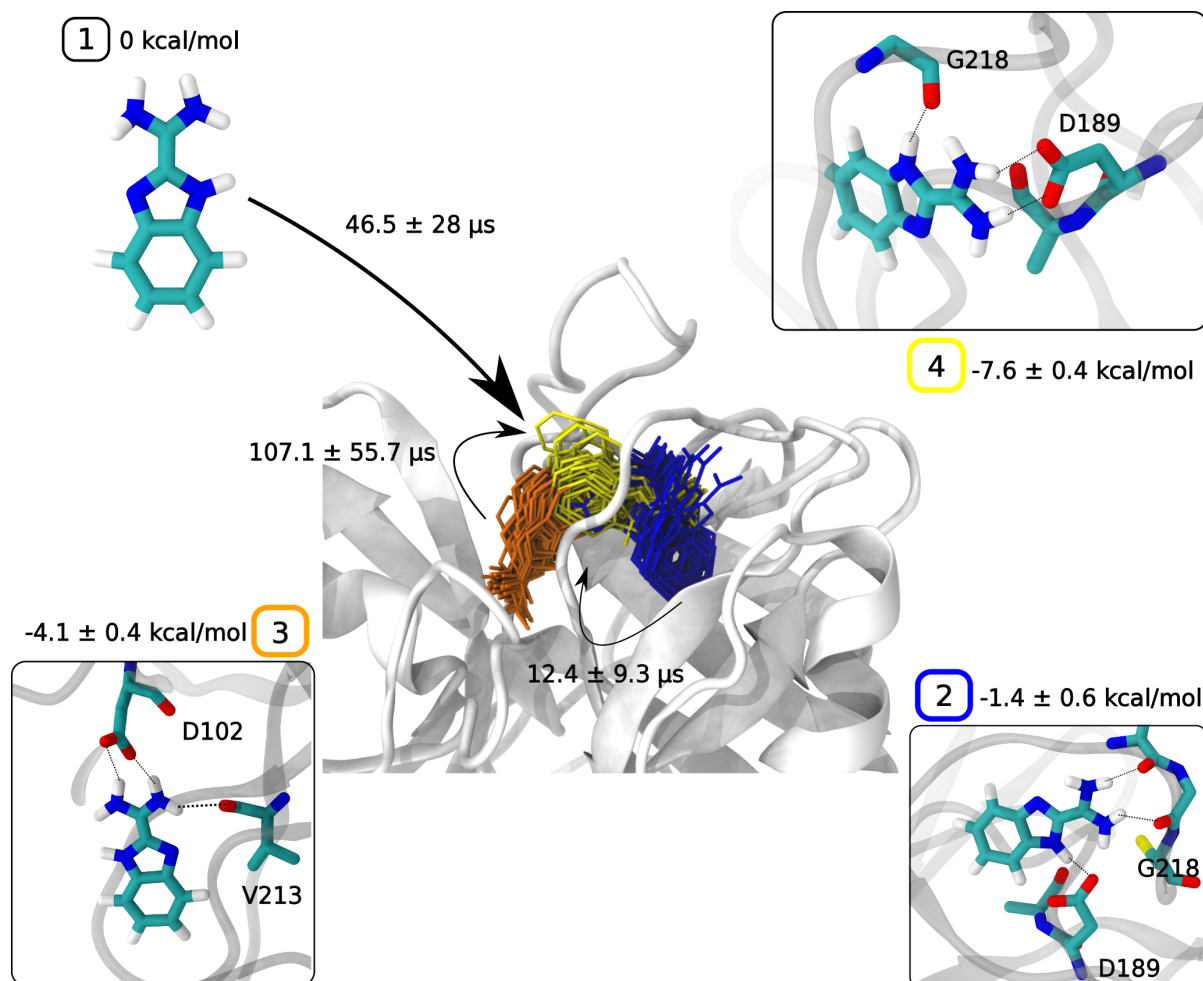# Binding kinetics in drug discovery

Noelia Ferruz,[a] Gianni De Fabritiis*[a, b]

Abstract: Over the last years, researchers have increasingly become interested in measuring and understanding drugs' binding kinetics, namely the time in which drug and its target associate and dissociate. Historically, drug discovery programs focused on the optimization of target affinity as a proxy of in-vivo efficacy. However, often the efficacy of a ligand is not appropriately described by the in-vitro measured drug-receptor affinity, but rather depends on the lifetime of the in-vivo drug-receptor interaction. In this review we review recent works that highlight the importance of binding kinetics, molecular determinants for rational optimization and the recent emergence of computational methods as powerful tools in measuring and understanding binding kinetics.

Keywords: binding kinetics, molecular dynamics, drug discovery, adaptive sampling.

1 Introduction

A drug is efficacious as long as it remains bound to its receptor.[1] As a consequence, most of the early-stage drug discovery projects have generally focused on optimizing the affinity and selectivity of the drug-receptor complex, based on the assumption that affinity is an appropriate proxy for *in vivo* efficacy.

For the purpose of understanding the drug-receptor complex formation from a theoretical point of view, we will consider the binding process in a simplistic single-step model in which the drug (D) reversibly binds its receptor (R), forming a complex (DR). Although real binding processes pass through much more complex mechanisms[2] –some of which will be presented later-, the following reversible two-state process suffices for an understanding of the theory. The rates at which this complex forms and dissolves are the so-called on (k_{on}) and off-rates (k_{off}) (eq. 2):

$$D + R \Leftrightarrow DR \tag{1}$$

$$\frac{d[DR]}{dt} = k_{on}[D][R] - k_{off}[DR] \tag{2}$$

The speed at which the complex forms depends on the rate at which it is made from association of the reactants ($k_{on}[D][R]$) and the rate at which the complex dissolves (-$k_{off}[DR]$). [D], [R] and [DR], which represent the molar concentrations of drug, receptor and complex, respectively, do not change once the system is in equilibrium. The affinity of this interaction is measured by the equilibrium dissociation constant (K_D) (eq. 3), which presents the units of concentration, and provides the extent to which the drug is bound in equilibrium.

$$K_D = \frac{[R]_{eq}[D]_{eq}}{[DR]_{eq}} \tag{2}$$

K_D directly relates to the Gibbs free energy difference (ΔG_D):

$$K_D = e^{-\Delta G_D / RT} \tag{3}$$

As observed in **Fig. 1**, ΔG_D only depends on the relative stability between free and bound states, initial and end points of the reaction coordinate regardless the pathway of binding. When less than zero, it is an indicative of reaction's

spontaneity at conditions of constant temperature and pressure such as the case of biological systems. However, affinity does not entirely determine whether a reaction will be fast or slow. Instead, the kinetics of binding, i.e., the association (k_{on}) and dissociation (k_{off}) rates, depend on the interactions along the binding pathway, and shape the energy profile of the binding reaction (**Fig. 1**). This way, stabilization or destabilization of the highest point in the energy barrier (the transition state) would modify both on and off-rates in the same direction without changing the affinity of the complex. As an example, **Fig. 2** shows a set of drugs which although all binding to HIV-1 with nanomolar affinity, present association rates differing by five orders of magnitude. The thermodynamics and kinetics of binding are related by the following equation in equilibrium:

$$K_D = \frac{k_{off}}{k_{on}} \tag{4}$$

Most commonly in the initial phases of the drug discovery pipeline, the goal is measuring the K_D of a series of compounds in a library by means of *in vitro* experiments, either by binding to its receptor directly or indirectly by measuring the biological activity of the target. These measurements are performed in closed-system conditions, where ligand and receptor concentration stay invariant through the course of the experiment allowing the equilibrium to be reached.[3] If that is the case and the process occurs in a single step then the reaction is fully explained by eq. 1 and 2. However, the *in vivo* scenario is very different. The drug's concentration fluctuates between doses, and may interact with other targets or diffuse out of the cell, as in an open system (**Fig. 3**). Because the drug's concentration is in continuous variation, the equilibrium condition does not hold and the K_D might not be an adequate descriptor of the *in vivo* efficacy.

In 2006, Robert Copeland coined the term residence time in the context of drug binding[3], [4] defined as the period of time the target is occupied by its drug. Interestingly, in this study Copeland *et. al.* showed by a theoretical model how

[a] Computational Biophysics Laboratory (GRIB-IMIM), Universitat Pompeu Fabra, Barcelona Biomedical Research Park (PRBB), Doctor Aiguader 88, 08003 Barcelona, Spain.

[b] Institució Catalana de Recerca i Estudis Avançats, Passeig Lluis Companys 23, 08010 Barcelona, Spain
*e-mail: : gianni.defabritiis@upf.edu

Supporting Information for this article is available on the WWW under www.molinf.com

the residence time can determine temporal selectivity for the target receptor despite the drug presented more affinity for other secondary targets. This method has been successfully applied to address muscarinic receptor subtype selectivity.[5], [6] The definition of residence time is independent of the environment of study, namely, the receptor-drug complex will have the same mean lifetime in the equilibrium or non-equilibrium scenario.

Mathematically, the residence time (τ) is quantified as the reciprocal of the k_{off}:

$$\tau = \frac{1}{k_{off}} \qquad (5)$$

It can be related to the pharmacokinetic half-life[4]:

$$t_{1/2} = \frac{Ln(2)}{k_{off}} \qquad (6)$$

There are a few works in which subtle structural changes affected drastically k_{on}, and were therefore an opportunity to tune potency.[7] However, although the rate of ligand association can have an important impact on pharmacology, it is interesting to focus attention on the dissociative process (and therefore the residence time). The association rate is weighted by the ligand concentration (the dose) and is ultimately limited by the diffusion limit or by the physicochemical steps such as absorption or distribution.[3] On the other hand, k_{off} is entirely determined by the target-drug interactions. Optimization of these interaction forces when linked to rational changes in the dissociation rate, could be an iterative process similar to that applied in SAR studies.[8]

In this review we review cases of kinetic optimization and summarize the emergence of computational methods in the last years.

2 Current biophysical techniques for kinetic measurements

Increased focus on the kinetics of binding has been supported by an improvement of the related instrumentation able to provide kinetic rates. To mention some exemplary techniques that appear to be the most frequently applied ones, we will focus in this section in techniques using a label for fluorescence (radioisotopes of fluorescence), label-free techniques (biosensors) and enzymatic experiments. Any comprehensive introduction to all the current methodology would be so extensive that is out of the scope of this review. A short summary of advantages and disadvantages for each technique is presented in **Table 1**.

Radioligand binding is the preferred technique for G-protein coupled receptors (GPCRs).[9], [10] There are two main ways to measure kinetics using radioligand binding: direct radiolabeling of the ligand of interest or indirectly by competition experiments.[11] In the direct method, the dissociation rates can be determined straightforwardly: the receptor is pre-incubated with a known concentration of the radioligand and the unbinding is measured in a washout phase by blocking the formation of new complexes. The

binding decay can then be fitted by a non-linear regression analysis. The k_{on} is computed by performing association experiments at different radioligand concentrations, or by performing a single experiment at a concentration when k_{off} is known. Alternatively, the binding properties of a set of unlabelled drugs are computed by competition displacement by a radioligand of known affinity as long ago proposed by Motulsky and Mahan.[12] More recently, dual-point competition assays have also shown being a fast high-throughput method.[9] Although the method permits direct measurement of rates, radiolabeling is expensive, laborious, time consuming and generates radioactive waste. An alternative to the use of radioligands are spectroscopic labels, the so-called fluorescence methods, like time resolved fluorescence resonance energy transfer (TR-FRET), fluorescence anisotropy and intrinsic fluorescence.[13], [14]

Label-free surface plasmon resonance (SPR) is a high sensitivity method which monitors refractive indexes changes when molecules absorb and desorb from a biosensor chip, dependent on the surface mass increase.[15] The receptor is immobilized on the solid surface and the drug (analyte) diluted in solution under continuous flow while the association is monitored in real-time. The method is particularly suitable for globular proteins.[16], [17] The method needs short development time, has low material consumption and is parallelizable. However, the immobilization of the receptor could affect the binding properties,[18] it has relatively low throughput and there are a limited range of rates which can be sensitively determined.[8]

Enzymatic activity assays can also determine the binding kinetics from enzyme activity. This approach has successfully been used to measure binding kinetics for many enzymes.[19]–[23] Jump dilution assays provide a format to highlight the dissociation kinetics by pre-incubating with high ligand concentration and diluting a hundredfold afterwards.[24]

Table 1: Summary of pros and cons of binding kinetics methods.

	Advantages	Disadvantages
Radioligand binding	- Suited for membrane systems - Direct determination of k_{on} and k_{off}	- Low throughput and labour-intensive - Radioactive waste - Requires high-affinity ligands. - Recombinant receptors in animal tissues
SPR	- No label required - Low material consumption - Medium throughput	- Usually only for soluble proteins - Immobilization of receptor.
Enzymatic assays	- No label required - Short development time	- Knowledge of mechanism required

3 Computational methods

The existence of theoretical methods to compute kinetic parameters has been available for long. Typically, efforts on designing and developing of computational methods have focused on the estimation of free energies. The reasons are both the increased greater attention on drug's affinities and the larger computational costs needed to sample pathways of binding or unbinding. While free energy estimations can be performed by studying one or a few of the reaction endpoints -such as in the case of empirical scoring functions, or in techniques using approximated thermodynamic cycles- and produce relatively fast results, estimation of kinetics relies upon the complete reconstruction of pathways of binding, the so called pathway-based methods. Two main techniques support the description of a system's motion at biologically relevant timescales.

On one side, the Brownian dynamics (BD) technique simulates macromolecules by following a Brownian motion, while the solvent molecules are considered to diffuse at a much faster timescales and can be coarse-grained. The ability to replace the solvent molecules by a stochastic force allows to sample timescales much larger than in molecular dynamics (MD). Compared to a MD of biomolecular encounters, BD requires less computation to sample an association event; in particular, millions of events can be simulated in 24h using moderate parallelization.[25] BD has been used to study protein-protein association kinetics in several studies during the last decade.[26], [27] In one of these applications, it was possible to show information about the mutation effects and the association and dissociation pathways of the electrostatically driven barnase-barstar complex. [28] More recently, the BD technique was used as a tool to provide kinetic quantitative estimates. In particular, the residence time of HSP90 and MDM2 inhibitors was computed along with the characterization of the drug-receptor encounter complexes.**[29]** A few studies on these systems followed this work using the BD methodology.**[30]–[32]**

However, the approximations made on BD can also infer inaccuracies, and in order to recover experimental observables additional models accounting for physical events such desolvation shells, polarization or entropic effects are needed.[33] On the other side, in MD the biomolecules are modelled following the Newton's law. The evolution of the system occurs by evaluating forces and updating velocities and coordinates at short timesteps. The sum of these forces is derived from a set of potentials termed as molecular forcefields, parameterized to capture the environment of all particles. MD is capable of providing quantitative binding estimates with high accuracy, but it has typically regarded as too computationally expensive. Typical simulation steps are usually less than 5 fs,[34] and therefore millions of steps must be produced to reach biologically relevant timescales. Fig. 4 presents approximate timescales for some biological events involving proteins or drugs. While the fastest protein motions and ligand binding events occur in the microsecond timescales, protein folding and ligand unbinding usually take milliseconds. Simulations in the last decade were not usually longer than a few dozens of nanoseconds. However, recent studies have reached the low-millisecond timescales. The reasons for this improvement in performance, are the advances in the software algorithms, parallelization of codes to run in high-performance supercomputers,[35] designing of specialized hardware[36] and development of intelligent MD protocols.[37]

Enhanced-sampling methods were generally used to reduce the MD's extensive computational needs when sampling binding pathways: conformational flooding,[38], [39] adaptive force bias[40], local elevation[41], metadynamics[42], and umbrella sampling[43] with weighted histogram analysis (WHAM) are some of these methods. While most of them were used to compute the free energy profile along the reaction coordinate (also known as potential of mean force (PMF), from where the binding affinities can be computed[44], [45]), in some recent applications it has been possible to reconstruct the kinetics of binding by combining them with other theoretical approaches. For instance, Laio et al. presented the bias-exchange metadynamics (BEMD) method, where it was possible to reconstruct the free energy profile of the miniprotein Trp-cage polypeptide.**[46]** The method was applied to indirectly estimate k_{on} and k_{off} in a couple of works: in the first, it allowed to quantify the binding rates of a series of peptides binding to HIV-1 protease**[47]**, while in the second the system comprised a drug binding cyclooxygenases COX-1 and COX-2. In this latter a new binding mode which explained the observed long residence time was identified.**[48]** The BEMD method by itself cannot provide absolute quantitative estimations. It can be used for comparative purposes among congeneric series of molecules, or alternatively, provide meta-stable states to be passed as input for a kinetic Monte Carlo simulation. Other biasing methods focus on the dissociative process. Random acceleration molecular dynamics (RAMD) has been used to identify unbinding pathways by applying a force vector with a random direction to the bound ligand.**[49]** It can be used to estimate relative estimates of dissociation kinetics or to predict the unbinding pathway and then use this information for an umbrella sampling or steered molecular dynamics (SMD).**[50], [51]** The PMF can be constructed from the latter and by use of the transition state theory (TST) one can compute absolute k_{on} and k_{off} values. It has recently been used in a wide range of problems, such as the unbinding of ligands from cytochrome P450cam**[52]** or the unbinding of carazolol from $\beta_2 AR$.**[53]**

Aside from the requirements on another technique to provide absolute kinetic values, these previous methods present other disadvantages, as they rely on a set of reaction coordinates (also called collective variables) chosen by human decision, requiring a previous knowledge of the system. In fact, the choice of the correct collective variables is the most crucial parameter for obtaining accurate PMF and the main drawback of pathway-based methods. Besides, the magnitude of the RAMD's pulling force must be carefully adjusted in order to allow the ligand to escape from the pocket in a short time while ensuring a minimal perturbation.

All previously mentioned techniques, despite being fast approaches with reasonable success, modify the system by applying forces that might alter the real dynamics to different extents. In the most recent years, the advent of new computing infrastructure has promoted a wealth of studies

making use of unbiased brute force simulations. In unbiased MD, ligand and receptor freely move in a solvated system. Often many replicas of the same processes are run, and these ensembles -which could vary from a few very long simulations or ensembles of thousands of short ones- contain all the needed information for reconstruction of the binding process. In 2008, a massively parallel supercomputer (ANTON)[54] was designed and built at D. E Shaw Research in New York, able to produce millisecond-long simulations Since then, ANTON, has been used to reconstruct the folding of proteins[55] and kinetics of binding.[56], [57] In 2011, extensive MD simulations were performed on the Src kinase in order to understand the binding of the drug dasatinib and the kinase inhibitor PP1.[57] From the four simulations -totalling 35 µs simulation time-, dasatinib correctly identified the kinase-binding site in one of them after 2.3 µs. 10 ligands were placed on each box giving a high concentration of 16.4 mM, on which aggregation was avoided by introducing a distance-dependent repulsive potential. Ligand PP1 bound to the same target in 3 out 7 simulations totalling 115 µs simulation time. 2 ligands were placed on each box in this case. The on-rate was obtained in both cases by computing the frequency of binding events and accounting its concentration. Despite the few binding events, the on rate was in good agreement with the experimental value.[57] In the same year Dror *et. al*, performed the binding analysis of four ligands to the beta-adrenergic receptor 1 and 2 (β_1AR and β_2AR) using long unbiased simulations.[56] 82 simulations ranging from 1 to 19µs were performed including 10 ligands per box. During the analysis it was found that the four ligands bound the to receptor via the same pocket, termed the 'extracellular vestibule', which conferred the largest energy barrier to the bound pose. Some other similar works on GPCRs followed this latter with interesting insights into allosteric mechanisms.[58], [59]

However, this highly specialized computing machinery, although have promoted an impressive contribution to the field and have settled MD as a well-recognized technique, is economically inaccessible to most researchers. Graphical processor units (GPUs) have also been demonstrated to be very efficient in MD. With the introduction of generalized GPU architecture like CUDA or OpenCL a GPU workstation is capable now of performing microsecond-length simulations. Still, most biological processes we are interested in occur in high-microsecond or millisecond timescales (Fig. 4), unaffordable times for a single GPU. However, it is possible to run multiple parallel simulations in GPU clusters, and posteriorly analyse them with probabilistic models. Nowadays, a single GPU is able to run around 125 ns of simulation time for a system sized 50000 atoms in a benchmark GPU like the GTX980. Assuming the case of a prototypical fast fragment binding event as benzamidine binding to trypsin protease -which occurs with an on mean first passage time of 6.9 µs at a concentration ~5 mM- we would need around 20 µs of aggregated simulation time to sample a more than an anecdotal binding event and obtain enough statistics for the construction of our model. Having an in-house cluster of 10 GPUs running uninterruptedly - currently affordable for many research groups- we could obtain our brute-force MD ensemble in about two weeks.

This specific example was a breakthrough work 5 years ago, when using GPUs volunteered from all over the work

(GPUGRID.net),[60] it was possible to completely reconstruct this binding event. 495 simulations of 100 ns each were performed leading to 197 binding events within 2 Å RMSD of the crystal structure. By using a Markov State Model (MSM) analysis (refs. [61]–[63] are suggested for a detailed description of MSMs), the simulations reconstructed binding intermediates, transition rates and affinities that occur at longer timescales, with remarkable accuracy.[64] The same technique was applied to the binding of carboxythiophene to AmpC B-lactamase,[65] by performing 148 µs of total aggregate time. In this work, it was possible to observe other secondary poses in agreement with the X-ray results. More interestingly, this study permitted to characterize the role of a loop in the vicinities of the pocket during the binding pathway: the loop was able to explore both open and closed conformations in absence of the ligand, however, the ligand stabilized the open conformer while entering the pocket, and the closed one once inside it.[65] More recently we focused on an larger library of 42 fragments against factor Xa, a serine protease with 2 known targetable pockets.[66] Using more than 2ms of total simulation time it was possible to obtain thermodynamic, poses and kinetic estimates in good agreement with experimental results. Interestingly, S1 fragments with amine or amidine groups showed slower on and off rates compared to S1 neutral binders (**Fig. 5**). The extension of this method to a full fragment library of around a thousand molecules is currently being developed. Some other studies are also using ensembles of MD trajectories in order to compute the kinetics of binding. For the specific computation of the dissociation rates, Huang and Caflisch[67] computed the unbinding times of a series of small molecules against FK506 binding protein. The compounds' low affinity allowed observing unbinding events despite the short sampling sizes.

In some of the mentioned cases above, the simulations were manually respawned from interesting states in order to enhance the sampling without biasing it. However, the states from which to respawn were selected by human decision. In 2014, a fast iterative protocol was published, that uses a Markov state model to decide where to respawn from automatically.[68] This intelligent approach had already been applied for folding[69] and is called adaptive sampling.

In the adaptive sampling, each epoch starts from a set of states discovered and selected by a simplified MSM constructed from the simulations produced in previous ones. Although adaptive sampling methods had been used in the past showing a decrease of 1 or 2 orders of magnitude of required simulation time,[69]–[73] this work was the first application for a ligand binding process. It was applied to the previously mentioned test case of trypsin-benzamidine and the model showed convergence needing only 600ns, compared to the 7.7 µs in brute-force simulations. In a similar fashion, Pande *et al*. performed an adaptive protocol for the binding of five ligands to the immunophilin protein FKBP12. By starting their simulations from bound, semi-bound and unbound poses and constructing a MSM they were able to provide pathway and kinetics of binding for the five ligands and getting association rates in the expected 10^6 s^{-1} M^{-1} range.[74]

While these cases were preliminary works showing the potential of the adaptive scheme in reducing the expensive

sampling time needs, many other similar works will predictably appear in the years to come. The theoretical reasons for this decrease in sampling time are sketched in **Fig. 6.** For the purpose of understanding, we will use a hypothetical drug binding to its receptor with a known activation energy (ΔG_{on}) of 10 kcal/mol, a similar value to those reported in literature.**[75]** The activation energy is related to the on-rate by the Arrhenius equation:

$$k_{on} = A\ e^{-\Delta G_{on}/RT} \qquad (7)$$

where A is the pre-exponential Arrhenius factor, ΔG_{on} is the activation free energy, T is the temperature and R is the universal gas constant. Assuming a slow binding on-rate of $10^4\ s^{-1}\ M^{-1}$ at usual computational concentrations, it would be needed a total of tens of milliseconds of classical high-throughput MD (HTMD) simulation time in order to sample one binding event, a timescale beyond the current computational capabilities. Let's suppose now one attempts to sample the binding process with an adaptive scheme, able to discretize the process into ten iso-energetic with heights of $\Delta G_{on,i}$ **=** 1 kcal/mol (**Fig. 6**). Therefore, the discretization gives $\Delta G_{on} = 10 \cdot \Delta G_{on,i}$, which translates to $k_{on,i}$ in the order of $10^{10}\ s^{-1}\ M^{-1}$ and consequently, a diffusion limited process in the nanosecond timescale. Summing up the ten jumps, the total binding process in this, difficult to achieve, perfectly discretized example could be reconstructed in a few hundreds of nanoseconds, several orders of magnitude faster than standard simulations.

The one-dimensional reaction coordinate is purely arbitrary in this example and MSMs usually use high dimensionality, self-discovered spaces to represent the slow degree of freedom of the process. However, it is still difficult to split the process into successive 1 kcal/mol barriers. In some cases, very complex rearrangement of the protein should be captured in order to obtain this. However, even if the speed-up were not of several orders of magnitude, it would still be significantly more efficient than running simulations from the same starting point.

Besides, two recent publications, although did not provide quantitative kinetic estimations, used other two techniques than the ones presented, provided binding and unbinding pathways and are worth mentioning. In the first, by using a robotics-inspired algorithm it was possible to explore the unbinding event of the insulin-phenol complex.[76] In the other, by using MC simulations combined with MSM models, the authors found meta-stable states and correctly estimated binding free energy for four benzamidine-derivative trypsin ligands.[77]

4 Discussion

Despite the recent advances in the related instrumentation, the increase in computer power and the design of specific hardware, there are some challenges that hinder computational kinetic measurements. Aside from the limitations we have mentioned when using biasing methods, the use of unbiased simulations, although theoretically more correct, still present some obstacles. We came across some of these challenges during the study of the large set of

fragments focused for the factor Xa protease.[66] For instance, one of the fragments, the largest in the library, lacked enough sampling to produce good statistics for a converged model. Assuming that compound size relates to association rates,[78] and seeing all other fragments had on-rates around 10^7-$10^8\ M^{-1}s^{-1}$, this fragment could bind with a high 10^6 on-rate. The simulations run with a concentration of 3.7 mM, and therefore, 54 μs of sampling time would be needed for a $k_{on} = 5 \cdot 10^6\ M^{-1}s^{-1}$. The binding set was composed of 63 μs of brute-force simulations, and perhaps sampled the bound pose too few times. Of course, there might be other reasons for the lack in accuracy, but serves as an example to take into account in other systems. A prior estimation of needed sampling times and the use adaptive sampling could reduce this problem. And although this case could potentially be solved, in more realistic scenarios, drug-like molecules will present real on-rates in the order of 10^4-$10^5\ M^{-1}s^{-1}$ and multi-second residence times. By using adaptive schemes and large-scale MD, binding poses and on-rates could likely be recovered, but multi-second residence times would need currently unaffordable sampling times.

In the same work, the specific parameterization of some chemical groups also revealed to have a great impact in accuracy. In particular, current parameterization protocols[79], [80] although permit the parameterization of large sets of molecules in seconds,[81] lack the accuracy needed in later stages of the drug discovery pipeline, when fewer compounds are tested and their specific binding modes and properties need to be reliable, even at the expenses of requiring larger times. Concretely, there are some determinants of ligand-receptor recognition which are currently not properly accounted: induced electronic polarizability,[82] changes in protonation states upon binding[83], the existence of tautomers,[84] or halogen bonding.[85] Finally, other intrinsic limitations in the methodology, are the accuracy of the forcefields and the clustering methods used in the MSM production.[86]

Another point to take into account is that real binding processes follow more complex mechanisms that the one presented in Fig. 1. [87], [88]. Fig. 7 summarizes three of them, being the first the single-step mechanism showed in Fig. 1. The complex reality is that the protein presents also different conformers in solution interconverting at larger scales than small molecule binding events, for which the ligands present different affinities. Therefore, starting the simulations from one of the conformers would bias binding estimates to that single process, although in the bigger *in vivo* picture the ligand might find the receptor in other conformations as well. Very recently, and using the trypsin-benzamidine test case, Noé *et. al.*[89] characterized the receptor conformational plasticity, and the relative affinities of the ligand for each of the conformers. It turned out that, for the case of trypsin-benzamidine, the main kinetic pathway is the direct binding to the active conformer, since it is mostly populated in solution. Therefore, previous works on this system were valid, but it could have impactful consequences when picking the least populated conformer. It is then necessary to perform a conformational analysis of the receptor and start the binding analysis from different receptor conformers following their equilibrium distribution.

Despite challenges and difficulties in accurately estimating drug-like kinetic rates, still the past years have

witnessed a wealth of computational works shedding light into binding processes. Deciphering the general links between structural features and binding kinetics is essential for drug discovery, and although some general factors have been proposed that might alter the binding kinetics, **[90]**,**[78]** it is hard to propose general rules.**[8] Fig. 1** shows that modification of kinetics could be performed in two ways: destabilizing or stabilizing the transition state (TS) while keeping bound and unbound species invariant, or stabilizing the bound state, which would increase the affinity and decrease k_{off}. However, although visualization of these ground-level species is experimentally plausible, determination of short-lived species, as meta-stable states, or the TS, is experimentally very complicated. To date, MD has already proven to be a powerful tool for the quantitative estimation of kinetics of fast associating fragments,**[56], [57], [64]–[66]** but it has also been very interesting for the understanding of binding pathways, intermediates and other kinetic determinants, difficult to characterize by experiments. Shaw *et. al.* have promoted an understanding of binding processes in GPCRs, [56], [58] and depicted some links between the structure of compounds and binding kinetics.[78] Molecular dynamics also helped to understand the responsible for slow kinetic behaviour, in particular, it has been proposed that the formation of 'water-shielded hydrogen bonds' play a role in extending residence times by hindering the access of water molecules and thus being unable to stabilize the transition state.[90].

There are, of course, besides the presented mentioned experimental techniques for measuring residence times, a few other emerging ones that might promise a detailed description of binding events, such as time-resolved Laue X-Ray crystallography[91] and kinetic isotopic effects (IE),[92] Still, With the reductions in sampling times provided by the adaptive sampling, awareness of the current limitations of the method (parameterization automation, more efficient sampling, clustering methods and receptor conformational changes) and continuous development of forcefields, we strongly believe that MD technique will become an accurate tool able to provide kinetics, pathways, intermediates of binding and molecular determinants.

Noelia Ferruz received her B.Sc. in Chemistry at the University of Zaragoza in 2011. After that, she accomplished the M.Sc of Bioinformatics for Health Sciences at the Pompeu Fabra University of Barcelona. Currently, Noelia is a Ph.D student of the Computational Biophysics Lab at the Research Group on Biomedical Informatics (GRIB) in the Pompeu Fabra University.

4 Acknowledgements

Gianni De Fabritiis received a degree in Mathematics from University of Bologna, a PhD in computational chemistry from University of London and accomplished a post-doctoral period at University College London. Currently, he is an ICREA Research Professor at Universitat Pompeu Fabra in Barcelona. His current research interests focus in computing in biology and biomedicine. Specifically, developing new computational physics methods and apply them to understand biological problems at the level of protein folding and ligand binding.
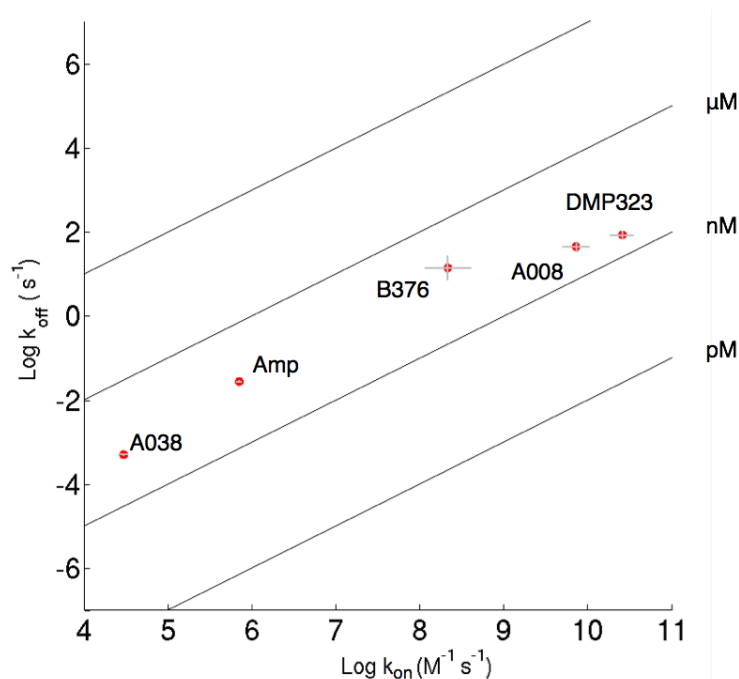
FIGURES

Figure 1: Energy profile of a drug (D) binding to its receptor (R) assuming a simple two-state model. The energy between unbound (D + R) and bound (DR) is the binding affinity of the process. The kinetics is governed by the energy of the transition state (TS), namely, the association rate (k_{on}) depends on the energy difference between unbound and the TS, (ΔG_{on}) and the dissociation rate on ΔG_{off}.
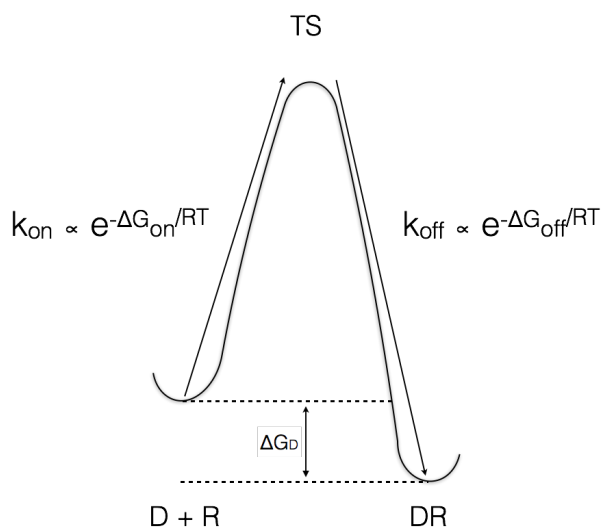

Figure 2: Representation of part of the data published in Markgren *et. al.*[16] X and Y axis represent the on and off-rates, respectively. Crosswise lines represent affinities (K_D). Although the five selected drugs bind to HIV-1 protease with similar nanomolar affinity, they can differ up to five orders of magnitude in the associative constant. (k_{on})
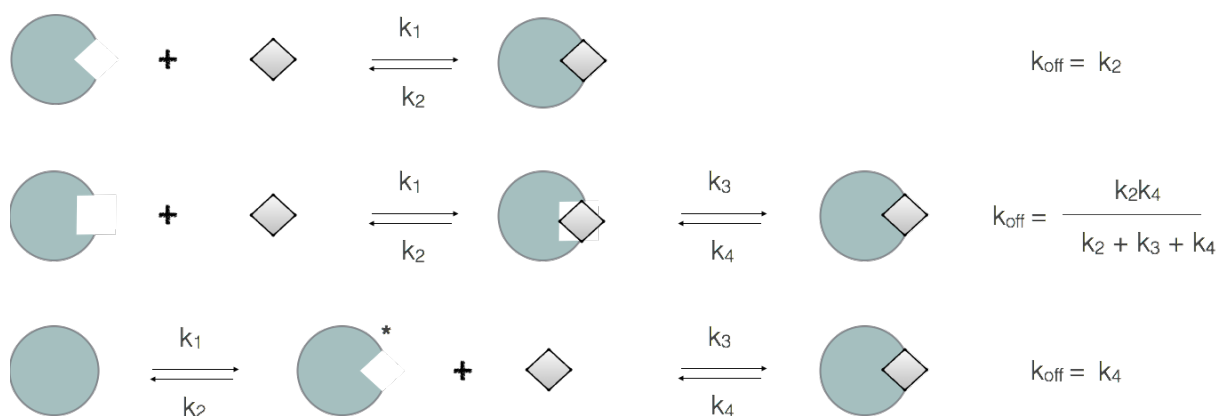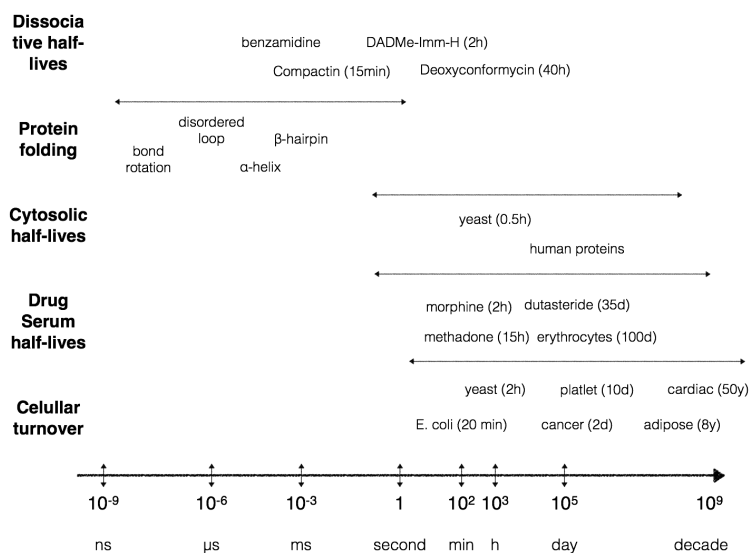
Figure 3: Simple schema showing some lateral processes that both receptor and drug can undergo *in vivo*. The drug could be affected by the affinity for secondary target, or psychochemical process as absorption, whereas the receptor could be in equilibrium with competing endogenous ligands.

absorption

$$D + R \leftrightarrow DR$$

$+ R_2 \quad + D_2$

$DR_2 \qquad RD_2$
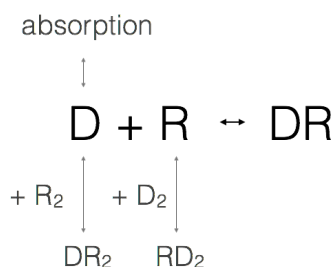
Figure 6: The three common mechanisms of inhibition and their respective dissociation rates: single-step, induced-fit and conformational selection mechanisms.

$k_{off} = k_2$

$$k_{off} = \frac{k_2 k_4}{k_2 + k_3 + k_4}$$

$k_{off} = k_4$

Figure 7: Example half-lives for some biological processes

Dissociative half-lives		benzamidine	DADMe-Imm-H (2h)	
		Compactin (15min)	Deoxyconformycin (40h)	

Protein folding: disordered loop, β-hairpin, bond rotation, α-helix

Cytosolic half-lives: yeast (0.5h), human proteins

Drug Serum half-lives: morphine (2h), dutasteride (35d), methadone (15h), erythrocytes (100d)

Celullar turnover: yeast (2h), platlet (10d), cardiac (50y), E. coli (20 min), cancer (2d), adipose (8y)

10^{-9}	10^{-6}	10^{-3}	1	10^2 10^3	10^5	10^9
ns	μs	ms	second	min h	day	decade

Figure 4: Figure taken from ref. 62. Interaction rate plot as obtained in for a set of small fragments against the serine protease factor Xa. Most potent and resident molecules contained amidine and basic moieties, whereas the rapidly dissociating fragments, encircled in orange, were neutral.
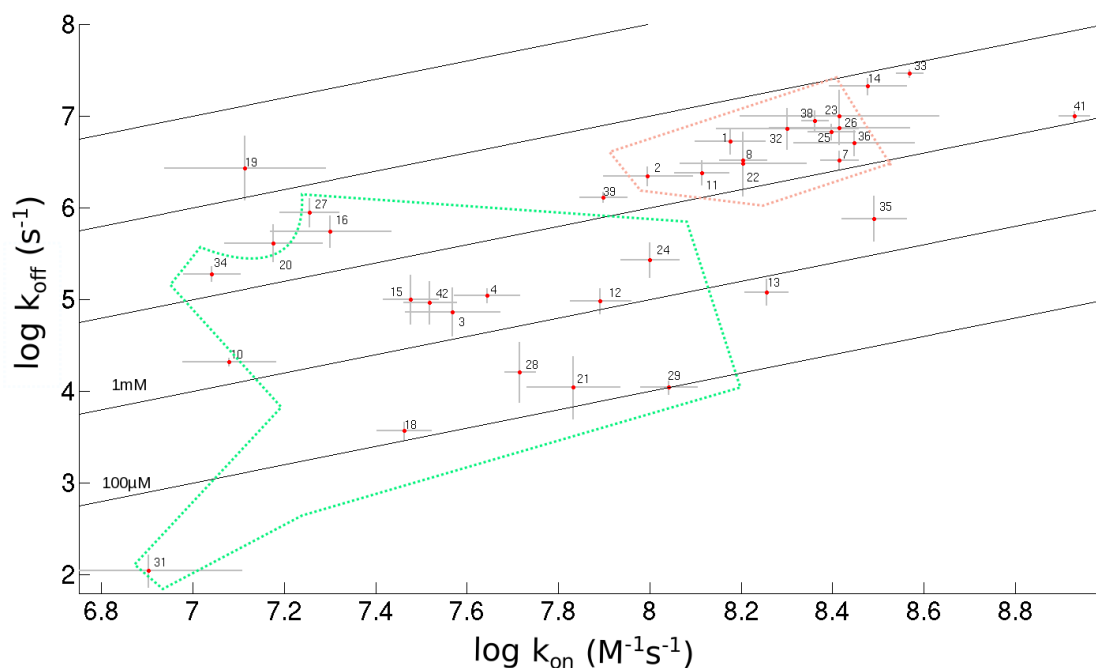
Figure 5: Energy profile of a drug-receptor binding pathway as sampled by the adaptive. By performing a Markov state modelling after each set of simulations and intelligently respawning from a selection of states, the adaptive scheme is able to discretize the activation energy (ΔG_{on}) into 10 epochs. By doing that, the needed sampling time can be reduced 1 or 2 orders of magnitude (see **Text**).

$$\Delta G_{on} = 10 \cdot \Delta G_{,i}$$
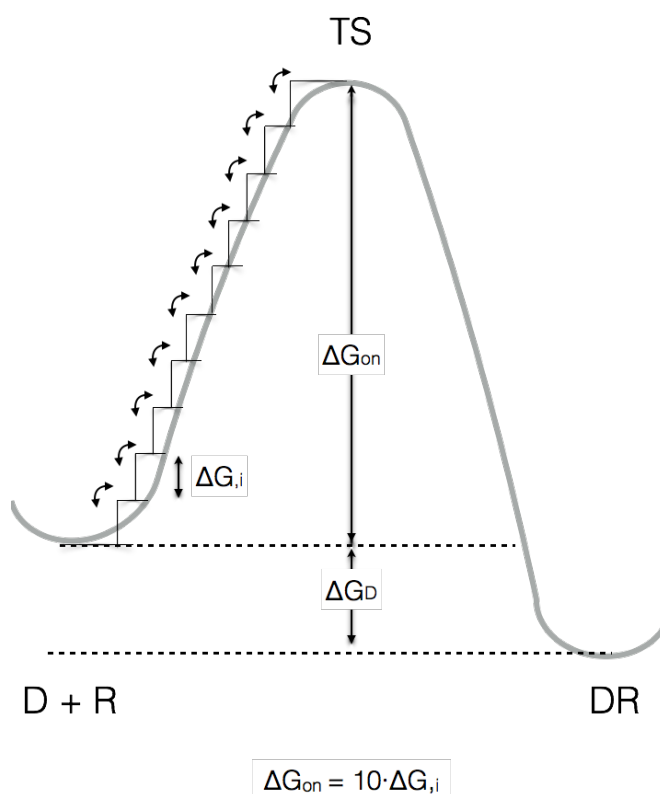
References

[1] J. N. Langley, "On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari," *J. Physiol.*, vol. 33, no. 4–5, pp. 374–413, Dec. 1905.

[2] H. Lu and P. J. Tonge, "Drug-target residence time: critical information for lead optimization," *Curr. Opin. Chem. Biol.*, vol. 14, no. 4, pp. 467–474, Aug. 2010.

[3] R. A. Copeland, D. L. Pompliano, and T. D. Meek, "Drug–target residence time and its implications for lead optimization," *Nat. Rev. Drug Discov.*, vol. 5, no. 9, pp. 730–739, Sep. 2006.

[4] R. A. Copeland, "Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists," *Methods Biochem. Anal.*, vol. 46, pp. 1–265, 2005.

[5] B. C. Moulton and A. D. Fryer, "Muscarinic receptor antagonists, from folklore to pharmacology; finding drugs that actually work in asthma and COPD," *Br. J. Pharmacol.*, vol. 163, no. 1, pp. 44–52, May 2011.

[6] A. Gavaldà, I. Ramos, C. Carcasona, E. Calama, R. Otal, J. L. Montero, S. Sentellas, M. Aparici, D. Vilella, J. Alberti, J. Beleta, and M. Miralpeix, "The in vitro and in vivo profile of aclidinium bromide in comparison with glycopyrronium bromide," *Pulm. Pharmacol. Ther.*, vol. 28, no. 2, pp. 114–121, Aug. 2014.

[7] J. Louvel, D. Guo, M. Agliardi, T. A. M. 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.*, vol. 57, no. 8, pp. 3213–3222, Apr. 2014.

[8] G. Keserü and D. C. Swinney, *Thermodynamics and Kinetics of Drug Binding*. John Wiley & Sons, 2015.

[9] D. Guo, E. J. H. van Dorp, T. Mulder-Krieger, J. P. D. 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.*, vol. 18, no. 3, pp. 309–320, Mar. 2013.

[10] A. P. Davenport, Ed., *Receptor Binding Techniques*, vol. 897. Totowa, NJ: Humana Press, 2012.

[11] G. Vauquelin, "Determination of drug–receptor residence times by radioligand binding and functional assays: experimental strategies and physiological relevance," *MedChemComm*, vol. 3, no. 6, p. 645, 2012.

[12] H. J. Motulsky and L. C. Mahan, "The kinetics of competitive radioligand binding predicted by the law of mass action," *Mol. Pharmacol.*, vol. 25, no. 1, pp. 1–9, Jan. 1984.

[13] Y. Fang, "Ligand-receptor interaction platforms and their applications for drug discovery," *Expert Opin. Drug Discov.*, vol. 7, no. 10, pp. 969–988, Oct. 2012.

[14] R. Sridharan, J. Zuber, S. M. Connelly, E. Mathew, and M. E. Dumont, "Fluorescent approaches for understanding interactions of ligands with G protein coupled receptors," *Biochim. Biophys. Acta*, vol. 1838, no. 1 Pt A, pp. 15–33, Jan. 2014.

[15] A. M. Giannetti, "From experimental design to validated hits a comprehensive walk-through of fragment lead identification using surface plasmon resonance," *Methods Enzymol.*, vol. 493, pp. 169–218, 2011.

[16] P.-O. Markgren, W. Schaal, M. Hämäläinen, A. Karlén, A. Hallberg, B. Samuelsson, and U. H. Danielson, "Relationships between structure and interaction kinetics for HIV-1 protease inhibitors," *J. Med. Chem.*, vol. 45, no. 25, pp. 5430–5439, Dec. 2002.

[17] W. Huber, "A new strategy for improved secondary screening and lead optimization using high-resolution SPR characterization

of compound–target interactions," *J. Mol. Recognit.*, vol. 18, no. 4, pp. 273–281, Jun. 2005.

[18] R. L. Rich and D. G. Myszka, "Survey of the year 2007 commercial optical biosensor literature," *J. Mol. Recognit. JMR*, vol. 21, no. 6, pp. 355–400, Dec. 2008.

[19] K. Anderson, Z. Lai, O. B. McDonald, J. D. Stuart, E. N. Nartey, M. A. Hardwicke, K. Newlander, D. Dhanak, J. Adams, D. Patrick, R. A. Copeland, P. 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," *Biochem. J.*, vol. 420, no. 2, pp. 259–265, Jun. 2009.

[20] R. A. Baron, Y. K. Peterson, J. C. Otto, J. Rudolph, and P. J. Casey, "Time-dependent inhibition of isoprenylcysteine carboxyl methyltransferase by indole-based small molecules," *Biochemistry (Mosc.)*, vol. 46, no. 2, pp. 554–560, Jan. 2007.

[21] A. Case and R. L. Stein, "Kinetic analysis of the interaction of tissue transglutaminase with a nonpeptidic slow-binding inhibitor," *Biochemistry (Mosc.)*, vol. 46, no. 4, pp. 1106–1115, Jan. 2007.

[22] P. A. Frantom, J. K. Coward, and J. S. Blanchard, "UDP-(5F)-GlcNAc Acts as a Slow-Binding Inhibitor of MshA, a Retaining Glycosyltransferase," *J. Am. Chem. Soc.*, vol. 132, no. 19, pp. 6626–6627, May 2010.

[23] S. R. Luckner, N. Liu, C. W. am Ende, P. J. Tonge, and C. Kisker, "A slow, tight binding inhibitor of InhA, the enoyl-acyl carrier protein reductase from Mycobacterium tuberculosis," *J. Biol. Chem.*, vol. 285, no. 19, pp. 14330–14337, May 2010.

[24] 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.*, vol. 416, no. 2, pp. 206–210, Sep. 2011.

[25] L. W. Votapka and R. E. Amaro, "Multiscale Estimation of Binding Kinetics Using Brownian Dynamics, Molecular Dynamics and Milestoning," *PLoS Comput Biol*, vol. 11, no. 10, p. e1004381, Oct. 2015.

[26] R. R. Gabdoulline and R. C. Wade, "Protein-protein association: investigation of factors influencing association rates by brownian dynamics simulations," *J. Mol. Biol.*, vol. 306, no. 5, pp. 1139–1155, Mar. 2001.

[27] R. R. Gabdoulline and R. C. Wade, "On the contributions of diffusion and thermal activation to electron transfer between Phormidium laminosum plastocyanin and cytochrome f: Brownian dynamics simulations with explicit modeling of nonpolar desolvation interactions and electron transfer events," *J. Am. Chem. Soc.*, vol. 131, no. 26, pp. 9230–9238, Jul. 2009.

[28] A. Spaar, C. Dammer, R. R. Gabdoulline, R. C. Wade, and V. Helms, "Diffusional encounter of barnase and barstar," *Biophys. J.*, vol. 90, no. 6, pp. 1913–1924, Mar. 2006.

[29] K. M. ElSawy, R. Twarock, D. P. Lane, C. S. Verma, and L. S. D. Caves, "Characterization of the Ligand Receptor Encounter Complex and Its Potential for in Silico Kinetics-Based Drug Development," *J. Chem. Theory Comput.*, vol. 8, no. 1, pp. 314–321, Jan. 2012.

[30] K. M. ElSawy, A. Sim, D. P. Lane, C. S. Verma, and L. S. Caves, "A spatiotemporal characterization of the effect of p53 phosphorylation on its interaction with MDM2," *Cell Cycle Georget. Tex*, vol. 14, no. 2, pp. 179–188, 2015.

[31] K. M. ElSawy, C. S. Verma, D. P. Lane, and L. S. D. Caves, "On the origin of the stereoselective affinity of Nutlin-3 geometrical isomers for the MDM2 protein," *Cell Cycle Georget. Tex*, vol. 12, no. 24, pp. 3727–3735, Dec. 2013.

[32] K. M. ElSawy, C. S. Verma, T. L. Joseph, D. P. Lane, R. Twarock, and L. S. D. Caves, "On the interaction mechanisms of a p53 peptide and nutlin with the MDM2 and MDMX proteins: a Brownian dynamics study," *Cell Cycle Georget. Tex*, vol. 12, no. 3, pp. 394–404, Feb. 2013.

[33] N. Greives and H.-X. Zhou, "BDflex: a method for efficient treatment of molecular

flexibility in calculating protein-ligand binding rate constants from brownian dynamics simulations," *J. Chem. Phys.*, vol. 137, no. 13, p. 135105, Oct. 2012.

[34] M. J. Harvey and G. De Fabritiis, "High-throughput molecular dynamics: the powerful new tool for drug discovery," *Drug Discov. Today*, vol. 17, no. 19–20, pp. 1059–1062, Oct. 2012.

[35] M. J. Harvey, G. Giupponi, and G. De Fabritiis, "ACEMD: Accelerating Biomolecular Dynamics in the Microsecond Time Scale," *J. Chem. Theory Comput.*, vol. 5, no. 6, pp. 1632–1639, Jun. 2009.

[36] D. E. Shaw, J. P. Grossman, J. A. Bank, B. Batson, J. A. Butts, J. C. Chao, M. M. Deneroff, R. O. Dror, A. Even, C. H. Fenton, A. Forte, J. Gagliardo, G. Gill, B. Greskamp, C. R. Ho, D. J. Ierardi, L. Iserovich, J. S. Kuskin, R. H. Larson, T. Layman, L.-S. Lee, A. K. Lerer, C. Li, D. Killebrew, K. M. Mackenzie, S. Y.-H. Mok, M. A. Moraes, R. Mueller, L. J. Nociolo, J. L. Peticolas, T. Quan, D. Ramot, J. K. Salmon, D. P. Scarpazza, U. Ben Schafer, N. Siddique, C. W. Snyder, J. Spengler, P. T. P. Tang, M. Theobald, H. Toma, B. Towles, B. Vitale, S. C. Wang, and C. Young, "Anton 2: Raising the Bar for Performance and Programmability in a Special-purpose Molecular Dynamics Supercomputer," in *Proceedings of the International Conference for High Performance Computing, Networking, Storage and Analysis*, Piscataway, NJ, USA, 2014, pp. 41–53.

[37] S. Doerr and G. De Fabritiis, "On-the-Fly Learning and Sampling of Ligand Binding by High-Throughput Molecular Simulations," *J. Chem. Theory Comput.*, vol. 10, no. 5, pp. 2064–2069, May 2014.

[38] B. G. Schulze, H. Grubmüller, and J. D. Evanseck, "Functional Significance of Hierarchical Tiers in Carbonmonoxy Myoglobin: Conformational Substates and Transitions Studied by Conformational Flooding Simulations," *J. Am. Chem. Soc.*, vol. 122, no. 36, pp. 8700–8711, Sep. 2000.

[39] H. Grubmüller and P. Tavan, "Molecular dynamics of conformational substates for a simplified protein model," *J. Chem. Phys.*, vol. 101, no. 6, pp. 5047–5057, Sep. 1994.

[40] J. Comer, J. C. Gumbart, J. Hénin, T. Lelièvre, A. Pohorille, and C. Chipot, "The Adaptive Biasing Force Method: Everything You Always Wanted To Know but Were Afraid To Ask," *J. Phys. Chem. B*, vol. 119, no. 3, pp. 1129–1151, Jan. 2015.

[41] T. Huber, A. E. Torda, and W. F. van Gunsteren, "Local elevation: a method for improving the searching properties of molecular dynamics simulation," *J. Comput. Aided Mol. Des.*, vol. 8, no. 6, pp. 695–708, Dec. 1994.

[42] A. Laio and M. Parrinello, "Escaping free-energy minima," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 20, pp. 12562–12566, Oct. 2002.

[43] G. M. Torrie and J. P. Valleau, "Nonphysical sampling distributions in Monte Carlo free-energy estimation: Umbrella sampling," *J. Comput. Phys.*, vol. 23, no. 2, pp. 187–199, Feb. 1977.

[44] I. Buch, S. K. Sadiq, and G. De Fabritiis, "Optimized Potential of Mean Force Calculations for Standard Binding Free Energies," *J. Chem. Theory Comput.*, vol. 7, no. 6, pp. 1765–1772, Jun. 2011.

[45] I. Buch, N. Ferruz, and G. De Fabritiis, "Computational modeling of an epidermal growth factor receptor single-mutation resistance to cetuximab in colorectal cancer treatment," *J. Chem. Inf. Model.*, vol. 53, no. 12, pp. 3123–3126, Dec. 2013.

[46] F. Marinelli, F. Pietrucci, A. Laio, and S. Piana, "A Kinetic Model of Trp-Cage Folding from Multiple Biased Molecular Dynamics Simulations," *PLoS Comput. Biol.*, vol. 5, no. 8, Aug. 2009.

[47] F. Pietrucci, F. Marinelli, P. Carloni, and A. Laio, "Substrate Binding Mechanism of HIV-1 Protease from Explicit-Solvent Atomistic Simulations," *J. Am. Chem. Soc.*, vol. 131, no. 33, pp. 11811–11818, Aug. 2009.

[48] V. Limongelli, M. Bonomi, L. Marinelli, F. L. Gervasio, A. Cavalli, E. Novellino, and M. Parrinello, "Molecular basis of cyclooxygenase enzymes (COXs) selective inhibition," *Proc. Natl. Acad. Sci. U. S. A.*,

vol. 107, no. 12, pp. 5411–5416, Mar. 2010.

[49] S. K. Lüdemann, V. Lounnas, and R. C. Wade, "How do substrates enter and products exit the buried active site of cytochrome P450cam? 1. Random expulsion molecular dynamics investigation of ligand access channels and mechanisms," *J. Mol. Biol.*, vol. 303, no. 5, pp. 797–811, Nov. 2000.

[50] S. Park and K. Schulten, "Calculating potentials of mean force from steered molecular dynamics simulations," *J. Chem. Phys.*, vol. 120, no. 13, pp. 5946–5961, Apr. 2004.

[51] B. Isralewitz, J. Baudry, J. Gullingsrud, D. Kosztin, and K. Schulten, "Steered molecular dynamics investigations of protein function," *J. Mol. Graph. Model.*, vol. 19, no. 1, pp. 13–25, 2001.

[52] P. J. Winn, S. K. Lüdemann, R. Gauges, V. Lounnas, and R. C. Wade, "Comparison of the dynamics of substrate access channels in three cytochrome P450s reveals different opening mechanisms and a novel functional role for a buried arginine," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 8, pp. 5361–5366, Apr. 2002.

[53] T. Wang and Y. Duan, "Ligand entry and exit pathways in the beta2-adrenergic receptor," *J. Mol. Biol.*, vol. 392, no. 4, pp. 1102–1115, Oct. 2009.

[54] D. E. Shaw, M. M. Deneroff, R. O. Dror, J. S. Kuskin, R. H. Larson, J. K. Salmon, C. Young, B. Batson, K. J. Bowers, J. C. Chao, M. P. Eastwood, J. Gagliardo, J. P. Grossman, C. R. Ho, D. J. Ierardi, I. Kolossváry, J. L. Klepeis, T. Layman, C. McLeavey, M. A. Moraes, R. Mueller, E. C. Priest, Y. Shan, J. Spengler, M. Theobald, B. Towles, and S. C. Wang, "Anton, a Special-purpose Machine for Molecular Dynamics Simulation," *Commun ACM*, vol. 51, no. 7, pp. 91–97, Jul. 2008.

[55] D. E. Shaw, P. Maragakis, K. Lindorff-Larsen, S. Piana, R. O. Dror, M. P. Eastwood, J. A. Bank, J. M. Jumper, J. K. Salmon, Y. Shan, and W. Wriggers, "Atomic-Level Characterization of the Structural Dynamics of Proteins," *Science*,

vol. 330, no. 6002, pp. 341–346, Oct. 2010.

[56] R. O. Dror, A. C. Pan, D. H. Arlow, D. W. Borhani, P. Maragakis, Y. Shan, H. Xu, and D. E. Shaw, "Pathway and mechanism of drug binding to G-protein-coupled receptors," *Proc. Natl. Acad. Sci.*, Luglio 2011.

[57] Y. Shan, E. T. Kim, M. P. Eastwood, R. O. Dror, M. A. Seeliger, and D. E. Shaw, "How Does a Drug Molecule Find Its Target Binding Site?," *J. Am. Chem. Soc.*, vol. 133, no. 24, pp. 9181–9183, Giugno 2011.

[58] R. O. Dror, H. F. Green, C. Valant, D. W. Borhani, J. R. Valcourt, A. C. Pan, D. H. Arlow, M. Canals, J. R. Lane, R. Rahmani, J. B. Baell, P. M. Sexton, A. Christopoulos, and D. E. Shaw, "Structural basis for modulation of a G-protein-coupled receptor by allosteric drugs," *Nature*, vol. 503, no. 7475, pp. 295–299, Nov. 2013.

[59] A. C. Kruse, J. Hu, A. C. Pan, D. H. Arlow, D. M. Rosenbaum, E. Rosemond, H. F. Green, T. Liu, P. S. Chae, R. O. Dror, D. E. Shaw, W. I. Weis, J. Wess, and B. K. Kobilka, "Structure and dynamics of the M3 muscarinic acetylcholine receptor," *Nature*, vol. 482, no. 7386, pp. 552–556, Feb. 2012.

[60] I. Buch, M. J. Harvey, T. Giorgino, D. P. Anderson, and G. De Fabritiis, "High-Throughput All-Atom Molecular Dynamics Simulations Using Distributed Computing," *J. Chem. Inf. Model.*, vol. 50, no. 3, pp. 397–403, Mar. 2010.

[61] G. R. Bowman, K. A. Beauchamp, G. Boxer, and V. S. Pande, "Progress and challenges in the automated construction of Markov state models for full protein systems," *J. Chem. Phys.*, vol. 131, no. 12, Sep. 2009.

[62] V. S. Pande, K. Beauchamp, and G. R. Bowman, "Everything you wanted to know about Markov State Models but were afraid to ask," *Methods San Diego Calif*, Jun. 2010.

[63] G. R. Bowman, V. S. Pande, and F. Noé, Eds., *An Introduction to Markov State Models and Their Application to Long Timescale Molecular Simulation*, vol. 797. Dordrecht: Springer Netherlands, 2014.

[64] I. Buch, T. Giorgino, and G. De Fabritiis, "Complete reconstruction of an enzyme-inhibitor binding process by molecular dynamics simulations," *Proc. Natl. Acad. Sci.*, vol. 108, no. 25, pp. 10184–10189, Jun. 2011.

[65] P. Bisignano, S. Doerr, M. J. Harvey, A. D. Favia, A. Cavalli, and G. De Fabritiis, "Kinetic Characterization of Fragment Binding in AmpC β-Lactamase by High-Throughput Molecular Simulations," *J. Chem. Inf. Model.*, vol. 54, no. 2, pp. 362–366, Feb. 2014.

[66] N. Ferruz, M. J. Harvey, J. Mestres, and G. De Fabritiis, "Insights from Fragment Hit Binding Assays by Molecular Simulations," *J. Chem. Inf. Model.*, Sep. 2015.

[67] D. Huang and A. Caflisch, "The Free Energy Landscape of Small Molecule Unbinding," *PLoS Comput Biol*, vol. 7, no. 2, p. e1002002, Feb. 2011.

[68] S. Doerr and G. De Fabritiis, "On-the-Fly Learning and Sampling of Ligand Binding by High-Throughput Molecular Simulations," *J. Chem. Theory Comput.*, Mar. 2014.

[69] G. R. Bowman, D. L. Ensign, and V. S. Pande, "Enhanced Modeling via Network Theory: Adaptive Sampling of Markov State Models," *J. Chem. Theory Comput.*, vol. 6, no. 3, pp. 787–794, Mar. 2010.

[70] N. S. Hinrichs and V. S. Pande, "Calculation of the distribution of eigenvalues and eigenvectors in Markovian state models for molecular dynamics," *J. Chem. Phys.*, vol. 126, no. 24, p. 244101, Jun. 2007.

[71] S. Pronk, P. Larsson, I. Pouya, G. R. Bowman, I. S. Haque, K. Beauchamp, B. Hess, V. S. Pande, P. M. Kasson, and E. Lindahl, "Copernicus: A new paradigm for parallel adaptive molecular dynamics," in *High Performance Computing, Networking, Storage and Analysis (SC), 2011 International Conference for*, 2011, pp. 1–10.

[72] J. K. Weber and V. S. Pande, "Characterization and Rapid Sampling of Protein Folding Markov State Model Topologies," *J. Chem. Theory Comput.*, vol. 7, no. 10, pp. 3405–3411, Oct. 2011.

[73] G. R. Bowman, X. Huang, and V. S. Pande, "Adaptive Seeding: A New Method for Simulating Biologically Relevant Timescales," *Biophys. J.*, vol. 96, no. 3, Supplement 1, p. 575a, Feb. 2009.

[74] M. Lawrenz, D. Shukla, and V. S. Pande, "Cloud computing approaches for prediction of ligand binding poses and pathways," *Sci. Rep.*, vol. 5, p. 7918, 2015.

[75] F. Bai, Y. Xu, J. Chen, Q. Liu, J. Gu, X. Wang, J. Ma, H. Li, J. N. Onuchic, and H. Jiang, "Free energy landscape for the binding process of Huperzine A to acetylcholinesterase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 11, pp. 4273–4278, Mar. 2013.

[76] D. Devaurs, L. Bouard, M. Vaisset, C. Zanon, I. Al-Bluwi, R. Iehl, T. Siméon, and J. Cortés, "MoMA-LigPath: a web server to simulate protein-ligand unbinding," *Nucleic Acids Res.*, vol. 41, no. Web Server issue, pp. W297–302, Jul. 2013.

[77] R. Takahashi, V. A. Gil, and V. Guallar, "Monte Carlo Free Ligand Diffusion with Markov State Model Analysis and Absolute Binding Free Energy Calculations," *J. Chem. Theory Comput.*, vol. 10, no. 1, pp. 282–288, Jan. 2014.

[78] A. C. Pan, D. W. Borhani, R. O. Dror, and D. E. Shaw, "Molecular determinants of drug–receptor binding kinetics," *Drug Discov. Today*.

[79] K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov, and A. D. MacKerell, "CHARMM General Force Field (CGenFF): A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields," *J. Comput. Chem.*, vol. 31, no. 4, pp. 671–690, Mar. 2010.

[80] J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, and D. A. Case, "Development and testing of a general amber force field," *J. Comput. Chem.*, vol. 25, no. 9, pp. 1157–1174, Jul. 2004.

[81] K. Vanommeslaeghe, E. P. Raman, and A. D. MacKerell, "Automation of the CHARMM General Force Field (CGenFF) II: Assignment of bonded parameters and

partial atomic charges," *J. Chem. Inf. Model.*, Nov. 2012.

[82] D. Jiao, P. A. Golubkov, T. A. Darden, and P. Ren, "Calculation of protein–ligand binding free energy by using a polarizable potential." [Online]. Available: http://www.pnas.org. [Accessed: 20-Jan-2016].

[83] H. Gohlke and G. Klebe, "Approaches to the description and prediction of the binding affinity of small-molecule ligands to macromolecular receptors," *Angew. Chem. Int. Ed Engl.*, vol. 41, no. 15, pp. 2644–2676, Aug. 2002.

[84] P. Pospisil, P. Ballmer, L. Scapozza, and G. Folkers, "Tautomerism in computer-aided drug design," *J. Recept. Signal Transduct. Res.*, vol. 23, no. 4, pp. 361–371, 2003.

[85] M. A. A. Ibrahim, "Molecular mechanical study of halogen bonding in drug discovery," *J. Comput. Chem.*, vol. 32, no. 12, pp. 2564–2574, Sep. 2011.

[86] F. Noe, H. Wu, J.-H. Prinz, and N. Plattner, "Projected and Hidden Markov Models for calculating kinetics and metastable states of complex molecules," *J. Chem. Phys.*, vol. 139, no. 18, p. 184114, 2013.

[87] G. A. Holdgate and A. L. Gill, "Kinetic efficiency: the missing metric for enhancing compound quality?," *Drug Discov. Today*, vol. 16, no. 21–22, pp. 910–913, Nov. 2011.

[88] P. J. Tummino and R. A. Copeland, "Residence time of receptor-ligand complexes and its effect on biological function," *Biochemistry (Mosc.)*, vol. 47, no. 20, pp. 5481–5492, May 2008.

[89] N. Plattner and F. Noé, "Protein conformational plasticity and complex ligand-binding kinetics explored by atomistic simulations and Markov models," *Nat. Commun.*, vol. 6, p. 7653, Jul. 2015.

[90] 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*, 2011.

[91] F. Schotte, J. Soman, J. S. Olson, M. Wulff, and P. A. Anfinrud, "Picosecond time-resolved X-ray crystallography: probing protein function in real time," *J. Struct.*

Biol., vol. 147, no. 3, pp. 235–246, Sep. 2004.

[92] H. Gu and S. Zhang, "Advances in kinetic isotope effect measurement techniques for enzyme mechanism study," *Mol. Basel Switz.*, vol. 18, no. 8, pp. 9278–9292, 2013.

[93] D. C. Swinney, "Biochemical mechanisms of New Molecular Entities (NMEs) approved by United States FDA during 2001-2004: mechanisms leading to optimal efficacy and safety," *Curr. Top. Med. Chem.*, vol. 6, no. 5, pp. 461–478, 2006.

[94] B. Disse, G. A. Speck, K. L. Rominger, T. J. Witek, and R. Hammer, "Tiotropium (Spiriva): mechanistical considerations and clinical profile in obstructive lung disease," *Life Sci.*, vol. 64, no. 6–7, pp. 457–464, 1999.

[95] M. Elg, D. Gustafsson, and J. Deinum, "The importance of enzyme inhibition kinetics for the effect of thrombin inhibitors in a rat model of arterial thrombosis," *Thromb. Haemost.*, vol. 78, no. 4, pp. 1286–1292, Oct. 1997.

[96] E. Lindström, B. von Mentzer, I. Påhlman, I. Ahlstedt, A. Uvebrant, E. Kristensson, R. Martinsson, A. Novén, J. de Verdier, and G. Vauquelin, "Neurokinin 1 receptor antagonists: correlation between in vitro receptor interaction and in vivo efficacy," *J. Pharmacol. Exp. Ther.*, vol. 322, no. 3, pp. 1286–1293, Sep. 2007.

[97] C. Napier, H. Sale, M. Mosley, G. Rickett, P. Dorr, R. Mansfield, and M. Holbrook, "Molecular cloning and radioligand binding characterization of the chemokine receptor CCR5 from rhesus macaque and human," *Biochem. Pharmacol.*, vol. 71, no. 1–2, pp. 163–172, Dec. 2005.

[98] X. Wang, K. Sarris, K. Kage, D. Zhang, S. P. Brown, T. Kolasa, C. Surowy, O. F. El Kouhen, S. W. Muchmore, J. D. Brioni, and A. O. Stewart, "Synthesis and evaluation of benzothiazole-based analogues as novel, potent, and selective fatty acid amide hydrolase inhibitors," *J. Med. Chem.*, vol. 52, no. 1, pp. 170–180, Jan. 2009.

[99] G. Tian, K. A. Paschetto, F. Gharahdaghi, E. Gordon, D. E. Wilkins, X. Luo, and C. W. Scott, "Mechanism of inhibition of fatty acid amide hydrolase by sulfonamide-

containing benzothiazoles: long residence time derived from increased kinetic barrier and not exclusively from thermodynamic potency," *Biochemistry (Mosc.)*, vol. 50, no. 32, pp. 6867–6878, Aug. 2011.

[100] S. Chackalamannil, Y. Wang, W. J. Greenlee, Z. Hu, Y. Xia, H.-S. Ahn, G. Boykow, Y. Hsieh, J. Palamanda, J. Agans-Fantuzzi, S. Kurowski, M. Graziano, and M. Chintala, "Discovery of a novel, orally active himbacine-based thrombin receptor antagonist (SCH 530348) with potent antiplatelet activity," *J. Med. Chem.*, vol. 51, no. 11, pp. 3061–3064, Jun. 2008.

[101] S. Chackalamannil, Y. Xia, W. J. Greenlee, M. Clasby, D. Doller, H. Tsai, T. Asberom, M. Czarniecki, H.-S. Ahn, G. Boykow, C. Foster, J. Agans-Fantuzzi, M. Bryant, J. Lau, and M. Chintala, "Discovery of potent orally active thrombin receptor (protease activated receptor 1) antagonists as novel antithrombotic agents," *J. Med. Chem.*, vol. 48, no. 19, pp. 5884–5887, Sep. 2005.

[102] S. Chackalamannil, "Thrombin receptor (protease activated receptor-1) antagonists as potent antithrombotic agents with strong antiplatelet effects," *J. Med. Chem.*, vol. 49, no. 18, pp. 5389–5403, Sep. 2006.

[103] J. Regan, C. A. Pargellis, P. F. Cirillo, T. Gilmore, E. R. Hickey, G. W. Peet, A. Proto, A. Swinamer, and N. Moss, "The kinetics of binding to p38MAP kinase by analogues of BIRB 796," *Bioorg. Med. Chem. Lett.*, vol. 13, no. 18, pp. 3101–3104, Sep. 2003.

[104] D. C. Miller, W. Klute, and A. D. Brown, "Discovery of potent, metabolically stable purine CRF-1 antagonists with differentiated binding kinetic profiles," *Bioorg. Med. Chem. Lett.*, vol. 21, no. 20, pp. 6108–6111, Oct. 2011.

[105] A. Basavapathruni, L. Jin, S. R. Daigle, C. R. A. 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.*, vol. 80, no. 6, pp. 971–980, Dec. 2012.

[106] M. Jin, B. A. Petronella, A. Cooke, M. Kadalbajoo, K. W. Siu, A. Kleinberg, E. W. May, P. C. Gokhale, R. Schulz, J. Kahler, M. A. Bittner, K. Foreman, J. A. Pachter, R. Wild, D. Epstein, and M. J. Mulvihill, "Discovery of Novel Insulin-Like Growth Factor-1 Receptor Inhibitors with Unique Time-Dependent Binding Kinetics," *ACS Med. Chem. Lett.*, vol. 4, no. 7, pp. 627–631, Jul. 2013.

[107] E. V. Schneider, J. Böttcher, R. Huber, K. Maskos, and L. Neumann, "Structure-kinetic relationship study of CDK8/CycC specific compounds," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 20, pp. 8081–8086, May 2013.

[108] D. Guo, L. Xia, J. P. D. van Veldhoven, M. Hazeu, T. Mocking, J. Brussee, A. P. Ijzerman, and L. H. Heitman, "Binding kinetics of ZM241385 derivatives at the human adenosine A2A receptor," *ChemMedChem*, vol. 9, no. 4, pp. 752–761, Apr. 2014.

[109] J. P. D. van Veldhoven, R. Liu, S. A. Thee, Y. Wouters, S. J. M. Verhoork, C. Mooiman, J. Louvel, and A. P. IJzerman, "Affinity and kinetics study of anthranilic acids as HCA2 receptor agonists," *Bioorg. Med. Chem.*, vol. 23, no. 14, pp. 4013–4025, Jul. 2015.

[110] M. Vilums, A. J. M. Zweemer, Z. Yu, H. de Vries, J. M. Hillger, H. Wapenaar, I. A. E. Bollen, F. Barmare, R. Gross, J. Clemens, P. Krenitsky, J. Brussee, D. Stamos, J. Saunders, L. H. Heitman, and A. P. Ijzerman, "Structure-kinetic relationships--an overlooked parameter in hit-to-lead optimization: a case of cyclopentylamines as chemokine receptor 2 antagonists," *J. Med. Chem.*, vol. 56, no. 19, pp. 7706–7714, Oct. 2013.