The need for high throughput kinetics early in the drug discovery process

Generally kinetics are only investigated late in a programme by which time many compounds, slightly less potent but with a much better kinetic profile, have already been discarded. This paper argues that having kinetic information at the early stages of drug discovery can bring numerous benefits to the process.

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The majority of drug discovery projects rely upon estimates of compound affinity to a target protein to guide lead seeking medicinal chemistry in early stages when high compound numbers are being screened. At this stage it is now quite common to test at IC_{50} level not only against the primary target but also against several selectivity and toxicity targets, so choosing compounds for progression against multiple criteria. Nevertheless any efforts to measure the on and off-rates that underlie the affinity (to either the primary target or selectivity target) are left to much later stages of the campaign when fewer compounds are tested. This is simply due to the high cost and low throughput of the specialist biosensor instruments typically used to determine binding kinetics.

However, evidence is plentiful that compounds with the same affinity but different on and off-rates can have a very different biological activity profile. The affinity-driven triage strategy currently used therefore results in superior compounds being rejected early in the discovery process. Improved methods and technologies enabling cost-effective, convenient and high throughput access to kinetic data would create opportunities for a much improved early drug discovery paradigm. The attributes of an ideal technology are discussed.

Affinity (K_D) for a target protein, derived from IC_{50}, is used regularly to rank compound performance. K_D is dependent on both the association (on) and dissociation (off) rates of the compound and it is not possible to predict these from the K_D as shown in **Table 1**.

So, are on and off-rates critical information or merely nice to know? The overwhelming answer is that they are critical. Andersson et al[1] recognise kinetic binding data as a decisive element in drug discovery, while the importance of drug-target residence time (as quantified by the dissociative half life of the drug-target binary complex) is emphasised for its potential impact on duration of effect and target selectivity by Copeland et al[2] and Zhang and Monsma[3]. Hopkins (www.hopkinslab.org) puts this firmly into the perspective of the patient 'understanding the binding kinetics can help shape the clinical profile of a drug that are important to patients: efficacy, safety, duration of action, greater tolerability, indication and therapeutic differentiation'. The following examples show why this is so.

Slow off-rates

Increased residence time gives better duration of action

A survey of the literature identifies a number of drugs, across a variety of target families, having exceptionally long duration of action, which can be ascribed to very slow off-rates when compared to other drugs of the same class. Examples include Aprepitant[4], Amlodipine[5], Maraviroc[6], Candesartan[7], Saquinavir[8] and Tiotropium[9].

In all of these cases, affinity would have been no

Table 1: For the same affinity, on and off-rates can vary enormously and cannot be predicted (illustrated here using mock data)

AFFINITY (K_D)	K-ON M^{-1} MIN^{-1}	K-OFF MIN^{-1}	SPEED
1×10^{-9}	10^5	0.0001 (6900 min)	Exceptionally slow on/off
1×10^{-9}	10^6	0.001 (690 min)	Slow on/off
1×10^{-9}	10^7	0.01 (69 min)	Fast on/slow off
1×10^{-9}	10^8	0.1 (6.9 min)	Very fast on/fast off
1×10^{-9}	10^9	1 (0.69 min)	Exceptionally fast on/off

guide to efficacy duration and it is a general finding that drugs with slow-off-rates are found by serendipity. We can see the impact of slow-off clearly in **Table 2** by comparing data from some M3 antagonists.

In the case of the Tiotropium, the molecule exhibits very long lasting effects with very little variation in bronchiodilatation between peak and trough (the time between administration and next administration) which rendered it the first truly once-a-day bronchodilator[10]. Plasma levels of Tiotropium at trough are in the low pg/ml range and hence very unlikely to explain the sustained effectiveness of the drug, which is instead ascribed to its slow dissociation from the M3 receptors in the lung[9]. In addition to long duration of action, the ability to retain effectiveness at lower blood levels endows a large therapeutic window. Additionally, since Tiotropium has a faster dissociation rate from M1 and M2 muscarinic receptors than from M3 receptors, an improved selectivity is apparent for this drug over other mixed M1/M2/M3 antagonists.

Table 2: Muscarinic M3 antagonists with similar affinities but very different off-rates[9,10]

	K_D/KI	ON RATE $X10^9M^{-1}$ MIN^{-1}	OFF-RATE MIN^{-1}
Atropium	0.2 nM	1.5	0.27 (2 mins)
Ipatropium	0.2 nM	0.5	0.07 (10 mins)
Clidinium	0.3 nM	10	0.02 (30 mins)
Tiotropium	8 pM	0.16	0.0015 (34.7 hrs)

Changes in off-rate leading to large differences in duration of action are most obvious where a compound series is subject to rapid clearance. Slowly dissociating antagonists, where the half life of the antagonist-receptor complex exceeds that of the free antagonist are likely to maintain a longer receptor protection *in vivo* than fast dissociating antagonists. Where the half life of the free antagonist is longer than the half life of the complex, longer occupation by the slowly dissociating antagonist is only relevant if the receptor is subject to large fluctuations in free agonist concentration[1].

Improved selectivity due to differential off-rates

Since many compounds bind to a number of proteins in addition to their intended target, improved selectivity for the chosen target can be a strong differentiator across a class of drugs. By monitoring not only the dissociation rate of the compound from its intended target but also from other potential selectivity or ADME targets, it should be possible to identify compounds with preferred characteristics where a faster off-rate from the unintended targets is observed in the presence of slow off-rate for the intended target. **Table 3** gives some examples of these findings.

Fast on rates

Improved *in vivo* efficacy where agonist concentrations are high

Despite a 50,000-fold difference in *in vitro* Ki values for two thrombin inhibitors, hirudin and malagatran, comparable plasma concentrations resulted in comparable antithrombotic effects *in vivo*. In contrast, comparable *in vitro* Ki values and *in vivo* EC_{50} scores were obtained when malagatran was compared with inogatran. These differing profiles have been explained by the concentration of thrombin in the thrombus and the rate of attaining an effective inhibitory concentration of antagonist drug. For the inhibitors tested (eight in total) there was an inverse relationship between k-on values *in vitro* and the slope of the dose response curves *in vivo* with inhibitors with k-on values of $<1x10^7$ M^{-1} s^{-1} having steep slopes with Hill coefficients >1 and better therapeutic index due to rapidly reaching critical concentration for efficacy. Contrarily, the association time for inhibition of thrombin by slow binding inhibitors is too long to give effective antithrombotic effects at low plasma concentrations. Where higher plasma concentrations are achieved, the association time decreases resulting

in a steeper dose response curve but at the price of a narrowed therapeutic interval[11]. In examples such as this, because the need to achieve critical concentration in a short time is the crucial factor in determining efficacy, on-rate is a much better predictor of efficacy than Ki.

How could we apply on and off-rate properties to enable better drug discovery?

***In vitro* screening**

Understanding of kinetics at early stages of Drug Discovery, even at the screening phase, ie before many compounds have been rejected, gives access to more diverse chemical space, more scope for intellectual property (IP) and patents, and better defined biology. Receptor selectivity can be driven by off-rate and not necessarily affinity and affinity and off-rate SAR may diverge with large differences in dissociation rates within a chemical series being not uncommon. In addition to kinetics at the primary target, this principle can apply equally to off target effects in respect of selectivity and safety issues. Where SAR diverges it may be possible to achieve a greatly improved safety profile by selecting compounds from the series based on their on/off-rates at proteins of concern.

***In vivo* screening**

Generally, better *in vivo* profiles are apparent with slow off compounds, less variation in receptor occupancy between peak and trough plasma concentrations leading to a smoother efficacy curve. Slow off-rates also are more likely to lead to a once-a-day dose projection, which can be an important differentiator.

Understanding on and off-rates may help us reconcile currently puzzling mismatches in efficacy, selectivity or safety between humans and animal laboratory species. If on/off-rates vary in human versus animal receptors, an expected margin of safety observed in one species could disappear due to different kinetic profiles in the human at the relevant receptors – or *vice versa*: a compound rejected due to poor margin of safety or selectivity in an animal species could be, in fact, adequately selective for the desired over the unwanted effects in human. Such variations would be particularly important where efficacy and safety studies are conducted in different species.

Is a fast K_{off} of use? It is possible to envisage that iv dosage forms of a drug with fast off-rate would enable a fast wash out of drug – short acting anaesthetic perhaps, or other use where tight control on the duration of effect would be valuable.

Manipulation of kinetic profiles

An understanding of kinetics, at an early stage in a lead seeking programme, could give real assistance by knowing in which areas to focus where a compound falls short of required effectiveness. Is there such a thing as a 'good kinetic profile'? Geitmann[12] describes a study of HIV-1 protease inhibitors where structural manipulation was able to produce a range of on and off-rates such that the compound properties could be used to map inhibitors into clusters that related their properties to their potential utility as drugs. Note here the different properties of association and dissociation rates. Association rates are related to the structure of the compound and to the dosage, while dissociation rates are solely dependent on the molecular structure. However, receptor kinetics are of even more use if we view them in conjunction with the pharmacokinetic properties of the compounds. Where *in vivo* performance is sub-optimal, by considering all of this data we would understand the reason for the poor efficacy and how this might be improved.

Table 3: Selectivity conferred by differing kinetics at receptor subtypes

COMPOUND	RECEPTORS	DATA	LITERATURE REFERENCE
Tiotropium	M3 versus M2, M1 muscarinic receptor	M3 off-rate much slower than M2 or M1 off-rate	Disse, 1999[9]
NPY/PYY	NPY1 versus NPY2	Y1 off-rate = 1 hour Y2> 24 hours	Dautzenberg, 2005[18]
ICI 137,798	Beta 1 versus Beta 2 adrenoceptor	Beta 2 off-rate 30 min Beta 1 > 6 hours	Keith, 1989[19]
SCH 527123	CXCR1 versus CXCR2	CXCR1 off-rate 10 min CXCR2 20 hours	Gonsiorek, 2007[20]

Table 4: What does a kinetic profile deliver?

PROFILE	HOW DOES THE COMPOUND BEHAVE?	PROS	CONS
Fast on/Fast off	Rapidly reaches effective concentration at receptor, rapidly dissociates	Low dose required for effect hence good therapeutic index. Could be preferable if bioavailability is poor	Duration is determined by clearance rate of free drug hence frequent dosing often required
Slow on/Slow off	Takes time to build to effective concentration at receptor, slow dissociation once bound	Long duration allows once daily dosing, stable effects between peak and trough; Long duration even if free drug is subject to rapid clearance	Slow on-rate, if plasma concentration is low, may mean that drug never achieves sufficient receptor occupancy for efficacy

An ability to modulate on and off-rates would be particularly valuable where poor pharmacokinetics are observed. Where bioavailability is low, a high on rate is essential to get the drug on to the binding site. Improving affinity by decreasing the off-rate will not help since dissociation rate is only important if there is a drug-target complex in the first place (in most cases, the drug will have to achieve some minimum receptor occupancy level to achieve efficacy). Contrarily, where the effectiveness of a drug series is compromised by rapid clearance, a compound with a very slow off-rate can still be efficacious, by maintaining receptor occupancy longer than the half life of the free drug would suggest. Decreasing the off-rate will be much more important than increasing the affinity (eg by increasing the on-rate) in this circumstance. Slow functional reversibility is associated with long lasting *in vivo* efficacy, whereas the efficacy of compounds with rapid reversibility is determined by their pharmacokinetics.

To summarise, there is no 'best ' kinetic profile; it depends on the objectives of the discovery programme, as illustrated in **Table 4**. Clearly if the kinetic profile of compounds are not known then the ability to select compounds on a rational basis is lost.

Why are kinetics not investigated earlier in drug discovery?

Because kinetics generally are investigated only late in a programme after a lot of decisions have been made and triage carried out, the fact is that many compounds slightly less potent than those selected for progression, but with a much better kinetic profile, may already have been discarded.

So, since having kinetic information would be really helpful to a discovery programme, why is it that kinetics based screening doesn't already happen on a regular basis?

A number of technologies are available to generate kinetic information, and can be broken down into two broad types: those utilising a label such as a radiochemical or fluorophor, and label free technologies which include surface plasmon resonance, biolayer interferometry and resonant acoustic profiling (for a more comprehensive review of available technologies see Comley[13]) Nonetheless, the fact that most laboratories use non kinetic measures to make an early selection decisions and make full kinetic measurements only on compounds that survive this pre selection process[14] is ample evidence that existing methodology does not currently enable a wider use of kinetic data. Some more accessible technologies, eg localised surface plasmon resonance (LSPR) currently used to obtain affinities have, in the principle, the capability of generating kinetic data but this is not yet developed.

Where laboratories generate kinetic data currently, it is likely that they will be using Biacore or a similar sensor-based system in their work, Biacore having by far the largest market share (29% in 2008 according to Comley, now likely to approach 50%). Until recently, Biacore enjoyed complete dominance of the kinetics-based label-free market due the virtual absence of credible alternatives and the Biacore name became synonymous with both label-free and SPR. Yet despite this market dominance, and clear market interest in label-free methods, most label-free screening has remained a niche and specialist approach. Label-free competitors to Biacore came to market over

the past several years and most are concentrating on an instrument-based approach – there are already many technical reviews covering new instruments (see, for example, Rich & Myszka[15]) which can be subdivided into Biacore-like Instrumentation and those with a substantively different approach. Corning, BioRad, ForteBio and TTPLabtech all have commercial offerings which require instrumentation that is expensive and dedicated to the purpose. Despite using different technologies, all the systems aim to address similar application areas as Biacore. Some offer higher throughput and improved ability to screen fragments whereas others provide benefits such as the use of crude samples. However, as a consequence of high instrument cost, the need for expert users (see Rich & Myszka[15] for the pitfalls that can catch the unwary and even expert users) and limited throughput, none of these dedicated instrument-based approaches has resulted in a high uptake of use of kinetic analyses early in the drug discovery process. Currently the ratio of cost to throughput compares unfavourably with other technologies and is the key limitation – the cost per data point in an average automated system is estimated at 15-30 times higher for SPR (excluding instrument cost) than traditional *in vitro* methods. High capital cost is an additional hurdle which inhibits many laboratories from entering this field and may have contributed to the withdrawal of the FujifilmPharma offering from the SPR market. Some companies, eg SensiQ, have chosen to reduce the level of automation in their equipment so as to reduce the cost to own, at the expense of throughput or convenience, although innovations in the way the equipment is used may mitigate this[16].

ForteBio has adopted an alternative approach to the Biacore-like implementation with the Octet family of instruments that dip optical microfibres directly into the wells of standard plates rather than requiring complex microfluidics. With similar flexibility to Biacore and a reasonable throughput, the Octet offers a real alternative at a reduced (but still substantial) capital outlay. As a further innovation, ForteBio has attempted to broaden the scope of use of its equipment so that it has utility

beyond kinetic measurements, thus mitigating the cost to own with increased versatility.

Looking back to label based-methods, the Motulsky Mahan method has been useful historically utilising competition between cold and radiolabelled compounds (although it would be equally applicable to fluorescence labels) to directly measure on and off-rates of compounds. This method has been especially applied to GPCR receptors. With Biacore-like methods, the protein of interest must be immobilised on the sensor chip and this has thus far proved difficult with GPCR receptors (previously achieved only for the more accessible case of the oestrogen receptor, strides are being made in screening of membrane bound GPCRs using SPR[17] but this still remains a challenging area). This methodology, although laborious, finds favour in some laboratories due to familiarity with the techniques employed and no requirement for additional specialist knowledge or equipment. Throughputs achieved can be substantial, limited only by the stamina of the scientist. A majority of laboratories using this methodology will rely upon radiochemical labelling due to the difficulties in achieving fluorescence labelling that does not induce steric hindrance to the interaction being measured. Custom production of radiolabelled ligands can be conducted by a number of specialist providers. Where an iodinated product is preferred, the relatively short half life limits users to local suppliers, hence in Europe PerkinElmer (covering the range of materials previously supplied by NEN) and Quotient Bioresearch Ltd (which has acquired the Amersham business from GE) would likely be first choice. Where a tritiated compound would be satisfactory, US-based suppliers such as RC TriTec, ViTrax, Tjaden and Moravek could be considered.

As a final comparator, a newer label-free technique, localised SPR (LSPR), can be considered alongside these more established methods. LSPR shares the advantages of the label-based methods and the ForteBio Octet in being microtitre plate based – a big advantage for a laboratory conducting a range of assay types, but unlike ForteBio and all other biosensor instruments, requires no specialised equipment (measurement is via a standard absorbance reader). To date LSPR has been used to measure affinities, but since the method can follow the association of compound with protein it should, in principle, be possible to derive the off-rate from the measured affinity and on-rate. While there is no evidence yet of proof of principle, such an approach would be very attractive particularly

Table 5: Key features of various kinetic measurement technologies

TECHNOLOGY	VENDOR	CAPITAL COSTS	CONSUMABLE COSTS PER DATA POINT**	FORMAT	THROUGHPUT DATA POINTS/DAY	SKILLS BASE REQUIRED	OTHER
Surface Plasmon Resonance	Biacore, SensiQ, Fujifilm Pharma and others	$0.5-1 Million (varies with extent of autom'n)	$1.5-$3 (mainly the cost of the sensor)	Flow cell based	Theoretical maximum 4,800 In practice, less than 200 per day	Specialist skill for data interpretation and for sensor regeneration	
Localised Surface Plasmon Resonance	Pharma-diagnostics	Nil*[1]	$0.2-$0.75 (throughput dependent)	Microtitre plate based		Any competent screening scientist	Kinetic data possible in theory – practical proof currently absent
Biolayer interferometry	ForteBio	$400-600K	$1.5-$3	Microtitre plate based		Specialist skills for data interpretation	
Radiochemical (Motulsky Mahan method)	Configure in house	Nil*[2]	Medium	Microtitre plate based	>5,000	Any competent screening scientist	Time consuming and laborious. Radioactivity usage

**assumes that the laboratory has access to general laboratory equipment. [1] absorbance reader, [2] scintillation counter. ** author's estimates*

to first time users and to third world laboratories due to the absence of requirement for major capital investment. This same advantage would allow a much broader application of kinetic measurements in laboratories currently hindered by throughput limitations.

Thus, issues with current methods and technologies include the requirement for major capital investment, consumables costs well in excess of those for 'traditional' screening technologies, relatively low throughput, lack of flexibility, specialist skills required to run equipment and interpret the data output, lack of body of evidence for fidelity (particularly true of the newer techniques). No one technology is free or guilty of all of these issues, as summarised in **Table 5**.

To enable a much wider use of kinetics in early project decision making what would an ideal technology look like?

So here's the challenge to innovators and vendors. We want to be able to generate kinetic data on the confirmed hits from HTS (so for a HTS of 1 million entities that is 1,000-10,000 compounds depending on selected hit threshold – typically 0.1-1%), and on all compounds synthesised in response to the HTS hits for all projects (ie lead seeking phase of a project), and on all members of a fragment library. Where we have a project that has a clear need for a definite kinetic profile (eg slow off for a long lasting bronchodilator) we want to conduct screening of large subsets or even of the whole file in kinetic format. Therefore, we would like a simple (any bio-scientist can do it), cost-effective (no more expensive than conventional screening technologies, say, $0.1 per data point) and reasonably high throughput (>10,000 data points per day – remember that each compound may require multiple data points!), assay technology to help us to achieve this. And we would like to be able to attempt this without recourse to specialist instrumentation requiring high capital outlay, and to be compatible with current compound storage formats. Is that too much to ask? DDW

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