# Explicit Treatment of Non Michaelis-Menten and Atypical Kinetics in Early Drug Discovery

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**Abstract:** Biological systems are highly regulated. They are also highly resistant to sudden perturbations enabling them to maintain the dynamic equilibrium essential for sustenance of life. This robustness is conferred by regulatory mechanisms that influence the activity of enzymes/proteins within their cellular context, to adapt to changing environmental conditions. However, the initial rules governing the study of enzyme kinetics were tested and implemented for mostly cytosolic enzyme systems that were easy to isolate and/or recombinantly express. Moreover, these enzymes lacked complex regulatory modalities. Now, with academic labs and pharmaceutical companies turning their attention to more complex systems (for instance, multi-protein complexes, oligomeric assemblies, membrane proteins and post-translationally modified proteins), the initial axioms defined by Michaelis-Menten (MM) kinetics are rendered inadequate and the development of a new kind of kinetic analysis to study these systems is required. The current review strives to present an overview of enzyme kinetic mechanisms that are atypical and, oftentimes, do not conform to the classical MM kinetics. Further, it presents initial ideas on the design and analysis of experiments in early drug-discovery for such systems, to enable effective screening and characterisation of small-molecule inhibitors with desirable physiological outcomes.

**Abbreviations:** MM-Michaelis-Menten; HTS-High-throughput screening; CRC-concentration response curves; MoA- Mechanism of Action; MWC- Monod-Wyman-Changeux; SAR-structure activity relationship; PAINS-Pan assay interfering compounds; MSS: Multiple stationary states; DRE- Deterministic rate equations; CME-Chemical master equation; QSSA- Quasi steady state assumption; CYP- Cytochrome p450; FLA- Free ligand approximation; RSA- Reactant stationary assumption;

**Keywords:** Non Michaelis-Menten Kinetics; High-throughput screening; allostery; cooperativity; processive kinetics; distributive kinetics; single-molecule; auto-catalytic;  drug discovery

## 1. Introduction:

Mechanistic enzymology plays a pivotal role in rational drug discovery efforts by providing critical insights into the nature of the target enzyme that is inhibited. Understanding the course of an enzyme-catalysed reaction in terms of the order of substrate binding, generation of intermediates and the release of products can help to conceptualise different types of inhibitor and to inform the design of screens to identify desired mechanisms. Furthermore, an enzyme molecule is an ensemble of dynamic conformations that can get locked into unique conformational subspaces when interacting with substrates, cofactors or inhibitors. Information on these conformational subpopulations, provided by mechanistic enzymological studies, will further guide the design principles for early drug discovery screening and characterization efforts, increasing the potential for success in identifying efficacious molecules with clearly differentiated mechanisms of action.

Traditional kinetic studies carried out on enzymes have relied on the availability of the protein in substantial amounts. Before the advent of recombinant DNA (rDNA) technology, enzymes were purified from native sources like animal tissues, plant extracts or microbes. This resulted in purification of predominantly cytosolic proteins in miniscule quantities with significant batch-to-batch variation in both the yields and the behavior of enzymes. rDNA technology was introduced in 1973 ushering in an era of heterologous protein/enzyme production[1]. Though rDNA technology helped solve the problems associated with the yield and behavioral
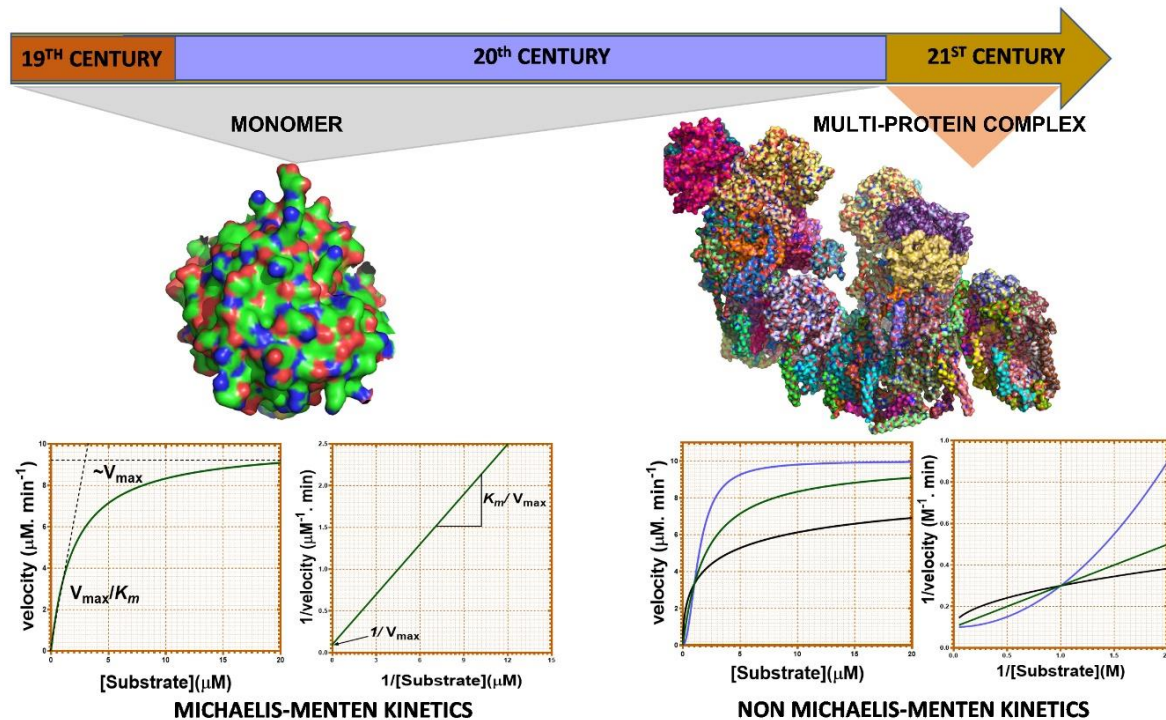
*Figure 1. Timescale of protein purification and the resultant elucidation of kinetic behaviour. Late 19th century and most of 20th century signified a period where predominantly monomeric/dimeric soluble proteins were studied and the axioms of MM kinetics was perfected. However, it was only in the 21st century that complex macromolecular assemblies were studied and instances of non-MM behaviour became more common. The curves were simulated with the respective equations representing the models. The $K_m$ and $V_{max}$ were arbitrarily fixed at 2 µM and 10 µM min$^{-1}$, respectively.*

variability of proteins purified across batches, it took a couple more decades to perfect the methodology for expressing complex macromolecular assemblies, post-translationally modified variants, and membrane proteins. It was only in the 21st century that the latter classes of proteins, which are close mimics of the physiological form of an enzyme/protein in the reductionist sense, were successfully expressed and saw some success with purification (Figure 1).

In parallel, there were only a limited number of substrate-to-product transitions that could be assayed in the early 20th century. rDNA technology also contributed towards overcoming this bottleneck by speeding-up the institution of coupled assays. Furthermore, aspects of labelling and derivatization, enabling the detection of product formation or substrate consumption in an enzymatic reaction, accelerated in the last two decades of the 20th century facilitating the assaying of a broader subset of enzymes.

However, the principles that govern the understanding of enzyme kinetics were developed in the early 20th century based on studies performed predominantly with monomeric/dimeric enzymes that could be easily isolated and purified at the time. The researchers were also limited in studying only those enzymes for which a substrate to product transition could be assayed. These enzymes lacked complex regulatory behavior leading to the institution of enzyme kinetic methodologies with overly simplified assumptions and approximations guiding the initial axioms. As the 21st century progressed and researchers were successful in recombinantly expressing, purifying and studying complex oligomeric assemblies of post-translationally modified proteins widely, they repeatedly found that the traditional treatment of enzyme kinetics was inadequate to model the observed behavior of these enzyme systems (Figure 1). Even an old survey of enzymes studied during the period 1965–1976 showed that more than 800 enzymes displayed highly complex curves not compatible with the traditional Michaelis-Menten (MM) hyperbola[2]. Although no recent survey exists, it is expected that the number of such enzymes would have increased with the ability to study more complicated enzyme systems[3–7]. Furthermore, traditionally ingrained beliefs such as enzyme catalysis decoupled

from protein dynamics and diffusion of reactants in solution are challenged by new findings[8]. This has led to a resurgence of interest in orthogonal approaches like full time-course analysis using simulations and molecular dynamics to gain a complete understanding of enzyme kinetics[9,10].

The current review summarizes the basic Michaelis-Menten (MM) assumptions, scenarios where they break down and suggests initial approaches that would allow the assessment of such non-Michaelis-Menten (non-MM) behavior. This will serve to guide and inform approaches for effective high-throughput screening (HTS) and subsequent characterization of the hits in early drug discovery.

2. **Michaelis-Menten assumptions and instances where they break-down:**

Leonor Michaelis and Maud Menten, expanding on the quantitative methods pioneered by Victor Henri, are credited with instituting the principles that govern our understanding of enzyme kinetics[11]. To extract kinetic parameters from time-course measurements, Michaelis and Menten derived the rate of product formation from initial velocity data (where less than 5% of substrate is converted to product). Although they had performed full time-course analysis of substrate to product conversion in their work on invertase[12], they are extensively known for their analysis of the initial velocity data. They plotted the rate (extracted from initial velocity phase) as a function of substrate concentration, fitted that to a single-site binding hyperbola and extracted the kinetic parameters $k_{cat}$ and $K_s$ ($k_{-1}/k_1$), respectively. Derivation of the equation for the single site binding hyperbola, popularly known as the MM equation (Equation 1 below), was undertaken for multiple turnover enzymes and involved several approximations and assumptions[13,14]. Differential equations for the four species (E, S, P and ES), in the simple kinetic scheme shown below, were solved using analytical methods and quasi steady state (QSS) approximation to obtain equation 1
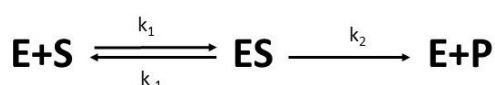
$$v = \frac{V_{max} \times [S]}{K_m + [S]} \ldots \ldots \ldots \ldots \ldots (1)$$

The assumptions involved in deriving the above equations are: (1) Enzyme is a catalyst and is not consumed in the reaction, (2) enzyme and substrate react rapidly to form the enzyme-substrate complex, (3) only a single substrate and a single enzyme-substrate complex are involved and the enzyme-substrate complex breaks down directly to form free enzyme and product, and (4) enzyme, substrate and enzyme-substrate complex are at equilibrium, i.e., the rate at which ES dissociates to E and S is much faster than the rate at which ES breaks down to form E + P (This was revised by George Edward Briggs and John Burdon Sanderson Haldane, who treated the system as "quasi-steady state" where the ES complex accumulates to a constant concentration under initial velocity conditions. Under such conditions, the $K_s$ ($k_{-1}/k_1$) term changes to $K_m$ ($k_{-1}+k_2/k_1$), the latter being a pseudo equilibrium constant). To achieve the above specified conditions, it was essential to maintain the substrate in far excess of the enzyme employed in an assay so that the free substrate concentration is equal to the total substrate concentration (in a typical assay setup, the enzyme is in nanomolar range while the substrate is in micromolar range). This assumption is called free ligand approximation (FLA) (also known as reactant stationary assumption). Additionally, this condition ensures that the QSS approximation is met, whereby the concentration of the ES complex reaches a steady state. This is usually achieved under conditions when $s_0/e_0 > 100$ [15] or $e_0/s_0 \ll 1$ [16] or $e_0/K_m \ll 1$ [17,18], where $s_0$ and $e_0$ are initial substrate and initial enzyme concentrations, respectively. Schnell[19] has beautifully argued that the reactant stationary assumption (RSA) is distinct from the steady state assumption (SSA) and the former is the necessary condition for the validity of the MM equation. He defines RSA as the implicit assumption in-built in the SSA that there is an initial transient during which the substrate concentration remains unchanged and is equal to the initial substrate concentration, as the concentration of the ES complex increases[19]. An outcome of the assumptions, which restrict the observations to the initial transient, is that the product formed as a

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P$$

function of time and enzyme concentration is usually linear.

In many instances the MM assumption breaks down, especially with increasing complexity of the system under study[20,21]. In this review, non-Michaelis Menten kinetics is an umbrella term used to denote enzyme systems whose kinetics do not lend themselves to analysis within MM assumptions elaborated above[22–25]. In other words, the term non-MM is exclusively used

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### BOX1: Enzymological terms

**Rapid-equilibrium Assumption:** *This assumption implies that the rates of complex formation (E+S=ES) and complex dissociation (ES=E+S) are faster than the rate at which ES breaks down to form product/s (ES=E+P). Thus E+S and ES are treated as being in a thermodynamic equilibrium.*

**Quasi Steady-state Assumption:** *The concentration of the Enzyme-Substrate complex (ES) remains constant under initial velocity conditions. Stated slightly differently, the ES intermediate is consumed ( ES=E+P; ES=E+S) as quickly as it is generated (E+S=ES) in an Uni-Uni enzyme reaction.*

**Initial velocity conditions:** *The rate of an enzymatic reaction at its inception at well-defined concentration of substrates and products. Practically, initial velocity conditions represent velocity measured at less than 5 % substrate to product conversion.*

**Free ligand approximation/ Reactant stationary assumption:** *The free substrate concentration is close to the total substrate concentration in the reaction vial. This assumption is valid only if the total enzyme concentration is significantly below the $K_m$ of the enzyme for its substrate.*

**Uni-Uni/Bi-Uni/Bi-Bi:** *The notation/s are used to show enzyme-catalysed reactions that involve a single or more than one kinetically significant substrate and product species (reactancy), respectively. Uni signifies one and Bi signifies two with the first term signifying the substrate species and the second term signifying the product species.*

**Positive/Negative homotropic cooperativity**: *A prior ligand association event altering the affinity of a subsequent binding event for the same ligand, either positively or negatively, in a, predominantly, multimeric organization.*

**Distributive mechanism:** *This refers to an enzyme catalyzed reaction where, for a substrate with multiple modification sites or multiple consecutive chemical alterations, every catalytic cycle results in dissociation and reassociation of the substrate/s and intermediate/s.*

**Processive mechanism:** *In an enzyme catalyzed reaction where multiple rounds of catalysis happens on a substrate without releasing it at each intermediate stage. Processivity is quantified by the number of cycles of catalysis that happens prior to complex dissociation.*

**Tight binding conditions**: *Conditions that signify significant depletion of the free inhibitor whereby the total inhibitor concentration cannot be approximated as being equal to the free inhibitor concentration. This is seen when the dissociation constant of the inhibitor is very close to (either equal or less than) the total concentration of enzyme in the assay.*

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to represent enzyme kinetic systems that are complex and require increased mathematics than that used to model the reaction of, for example, invertase in the early days of classical MM theory. A few examples of systems that can be modelled as non-MM are enumerated here. Some of them will be elaborated below under individual headings. (1) Self-catalyzing enzymes are a prominent instance where the MM assumption breaks down because the substrate cannot be maintained in far excess of the enzyme (enzyme is the substrate) apart from the fact that enzyme is consumed in the reaction. (2) Enzymes displaying complex regulatory modalities such heterotropic allosteric regulation (activation and inhibition) and homotropic positive or negative cooperativity usually do not follow MM

kinetics because of linked equilibria (some exceptions to this exist). (3) Free-ligand approximation usually breaks down in cases where the $K_m$ of the substrate is less than the total enzyme employed in an assay. This also holds true for tight-binding inhibitors whose $K_i$ is less than the enzyme concentration used in the assay. (4) Covalent irreversible inhibitors, where inhibition is time-dependent, and the fraction of active enzyme is depleted subsequent to each catalytic cycle. (5) Intracellular kinetics and interfacial enzyme catalysis are constrained by crowding effects (former) and diffusion in 2-dimensions (latter). (6) Single molecule kinetics because of its non-deterministic stochastic nature. (7) Enzymes with processive mechanism with more than one intermediary complex. (8) Enzymes with distributive mechanism that have equal likelihood of accepting the initial substrate or the altered substrate depending on their respective affinities and the law of mass action. (9) Enzymes showing substrate inhibition because of the likelihood of more than one unique enzyme-substrate complex. (10) Multi-substrate enzyme mechanisms, especially ones that follow ordered mechanism. (11) Single-turnover enzyme because of enzyme depletion. Finally, as an important case pertinent to drug discovery, (12) cytochrome P450 family of enzymes that are involved in oxidation and clearance of xenobiotics.

## 3. MM kinetics and its limitations in early drug discovery

Early drug discovery involves hit identification, hit-validation and an iterative process of chemical optimization as a function of desired biochemical and/or physiological outcome. HTS, which is often the first step in the process, is a multidisciplinary exercise involving the screening of a large library of chemical molecules to identify pharmacophores/leads for an appropriate drug target. A strong biochemical design for HTS and subsequent hit validation/characterization exercise is important for identifying hits that could be subsequently optimized in a Design Make Test Analyse (DMTA) Cycle[26]. It is well documented that effective HTS experimental design can enable the selective enrichment of small-molecules with a particular mode of action[26–29] (Figure 2 A). Post HTS, concentration-response curves (CRC) are commonly used to validate the hits to assess and

rank compound potency. The parameter $IC_{50}$, obtained from a CRC and indicating the concentration of an inhibitor where the activity of the target protein is reduced by 50 %, is dependent on the concentration of substrate used in the assay and the affinity of the substrate for the enzyme ($K_m$) in the case of linear inhibition. This relationship is well-known as the Cheng-Prusoff relationship and is different depending on the mechanism of inhibition[30–32] (Equation 2,3,4,5).

### *Competitive*

$$IC_{50} = K_i \left( 1 + \frac{[S]}{K_m} \right) \dots (2)$$

If S=$K_m$, $IC_{50}$=2$K_i$; If S<< $K_m$, $IC_{50}$~ $K_i$; If S>> $K_m$, $IC_{50}$>> $K_i$

### *Uncompetitive*

$$IC_{50} = K_i \left( 1 + \frac{K_m}{[S]} \right) \dots (3)$$

If S=$K_m$, $IC_{50}$=2$K_i$; If S<< $K_m$, $IC_{50}$ >> $K_i$; If S>> $K_m$, $IC_{50}$~ $K_i$

### *Pure non-competitive*

$$IC_{50} = K_i \dots (4)$$

Unlike in equation (2) and (3), the relationship in (4) remains the same for [S]=$K_m$ or [S]>>$K_m$ or [S]<<$K_m$. Further, in the above equations, it is assumed that the inhibitor dissociation constants to the free enzyme ($K_{ia}$) or enzyme-substrate complex ($K_{ib}$) are equal. In mixed-type inhibition, the Cheng-Prusoff relationship is as shown below

### *Mixed-type*

$$IC_{50} = \frac{[S] + K_m}{\frac{[S]}{\alpha K_i} + \frac{K_m}{K_i}} \dots (5)$$

$K_{ia} < K_{ib}$ or $K_{ia} > K_{ib}$ is determined by the magnitude of α. α is the factor that
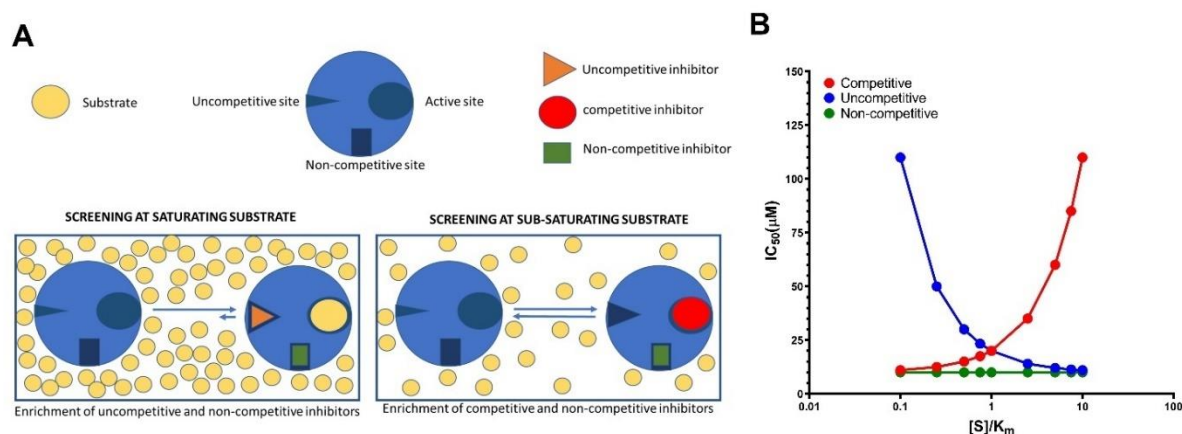
*Figure 2: Biochemical design of High-throughput screening leads to enrichment of compounds with different modalities of inhibition. (A) At saturating substrate, competitive inhibitors would be underrepresented in the hits obtained, whereas uncompetitive and non-competitive inhibitors could be enriched. At subsaturating substrate, inhibitors with competitive, uncompetitive and non-competitive inhibition modalities would be equally likely to get enriched. (B) IC$_{50}$ values as a function of substrate concentration for different modalities of inhibition. The plot is generated for an arbitrary K$_i$ value of 10 and a representation of x-axis two log units about the K$_m$ ,where IC$_{50}$ and K$_i$ values are related by Cheng-Prusoff equation[30].*

modulates the affinity of the enzyme for the inhibitor as a function of substrate binding or vice versa. A large α value signifies that substrate and inhibitor binding are mutually exclusive events resembling competitive inhibition. On the other hand, an infinitesimally small α value signifies that substrate binding is conditional upon inhibitor binding or vice-versa resembling uncompetitive inhibition.

In Figure 2 B, the Cheng-Prusoff relationship is plotted graphically for an inhibitor with $K_i$ value of 10 nM at a hypothetically low enzyme concentration ( << 10 nM). It would have to be appreciated that the relationship of IC$_{50}$ values with [S]/$K_m$ remains qualitatively the same irrespective of the absolute value of $K_i$. Further, uncompetitive inhibitors are highly sensitive to minor perturbations in [S] concentrations below the $K_m$ of the enzyme, gaining in apparent potency as [S] approaches the $K_m$. On the contrary, competitive inhibitors are highly sensitive to minor perturbations in [S] concentrations above the $K_m$ of the enzyme, losing apparent effective potency linearly for [S] concentration increase above $K_m$. For non-competitive inhibitors, the potency remains unchanged as a function of substrate concentration change for α=1, while it increases or decreases non-linearly for α<1 or α>1, respectively [33].

The Cheng-Prusoff relationship changes when the inhibitor $K_i$ value is equal to or less than half the enzyme concentration used in the assay. Here, IC$_{50}$ becomes dependent on

the enzyme concentration too[31–34] (equation 6,7,8). This is commonly known as the tight-binding limit (TBL) and is discussed extensively in section 4.3 as a special case of non-MM kinetics.

**Competitive**

$$IC_{50} = K_i^{app} + \frac{[E_t]}{2}; \quad K_i^{app} = K_i\left(1 + \frac{[S]}{K_m}\right) \dots (6)$$

**Uncompetitive**

$$IC_{50} = K_i^{app} + \frac{[E_t]}{2}; \quad K_i^{app} = K_i\left(1 + \frac{K_m}{[S]}\right) \dots (7)$$

**Pure non-competitive**

$$IC_{50} = K_i^{app} + \frac{[E_t]}{2}; \quad K_i^{app} = K_i \dots (8)$$

In the above equations, it is assumed that the inhibitor dissociation constants to free enzyme ($K_{ia}$) or enzyme-substrate complex ($K_{ib}$) are of the same magnitude. Mixed inhibition, where $K_{ia} < K_{ib}$ or $K_{ia} > K_{ib}$, is not considered here.

Hence, depending on the mechanism of action of the inhibitor, the apparent potency can

vary considerably depending on how a HTS assay is designed. For monosubstrate reactions, a screening exercise carried out at the $K_m$ of the substrate for the enzyme would lead to the enrichment of small-molecule binders that could be either competitive, non-competitive, uncompetitive or mixed (Figure 2 A and B). However, the same exercise carried out at a substrate concentration that is 10 times the $K_m$ would preferentially enrich uncompetitive and non-competitive inhibitors (Figure 2 A and B). Choosing the right substrate concentration and experimental design becomes more complicated when bisubstrate enzymes are involved[35]. However, HTS exercises and subsequent characterization stop short of efforts to determine balanced conditions when faced with enzymes that show complex regulations and non-MM kinetics. That is because searching for optimal conditions may be non-trivial given the current state of knowledge on the non-MM kinetics vis-à-vis early drug discovery. There are no well-defined guidelines for defining the order of binding of inhibitors vis-à-vis substrate for these systems and this review does not aspire to serve as a step-by-step guide to address those lacunae. However, it is an attempt at presenting a select set of kinetic behaviours that could be classed as non-MM, and provide some initial considerations for design of a HTS exercise and subsequent mechanism of action (MoA) studies in such situations.

## 4. Selective examples of enzymes showing non-MM kinetics

### 4.1. Self-catalyzing or auto-modifying enzymes:

***4.1.1. Introduction:*** Self-catalyzing enzymes are those that act on themselves. Self-catalyzing enzymes can operate in either a positive feedback loop or a negative feedback loop (Figure 3). In positive feedback, self-catalysis leads to activation as is demonstrated for zymogen[36] and certain kinases[37,38]. In negative feedback loops, the enzymes show inactivation as a result of self-catalysis (Figure 3). The latter is commonly seen in a few kinases that self-phosphorylate[39] and poly ADP-ribosylating enzymes such as PARP1 and PARP2[40]. In the case of self-catalyzing enzymes, the catalyst binds a cofactor and transfers a group from the latter onto itself. In the case of kinases, the

cofactor is ATP and for PARP1/2, it is $NAD^+$. The transfer could be *in cis-* (unimolecular or intramolecular) or *in trans-* (bimolecular or intermolecular) or a combination of both, though *in trans* mechanisms have been more extensively characterized. An exception to this generalization is PARP1, where the unimolecular versus bimolecular self-catalysis debate is still unresolved with proof for and against both[40]. Self-modification can lead to changes in stability of protein tertiary or quaternary structure or can affect the conformational isomerism of the protein[41]. All self-catalyzing enzymes fall under the category of non-MM enzymes since the enzyme acts as the substrate and hence, cannot be at a concentration lower than that of substrate in the assay mix. Furthermore, with each round of catalysis, the concentration of a unique unaltered enzyme species also decreases as is usually the case with substrate. Depending on several parameters such as rates and equilibrium constants for the various intermediates, the time courses would become non-linear even within conditions of initial velocity. This can be quickly assessed by employing a Selwyn's test[42]. The non-linear progress curves would lead to difficulty in estimating kinetic parameters because of constantly changing enzyme (a.k.a. substrate) concentration, the possibility of undesirable interactions and population of several intermediates apart from and in addition to the naïve ES complex. Deterministic curves usually have sigmoidal shapes that are indicative of non-MM kinetics[43]. Further, an autocatalysis-induced activated or inhibited state gives rise to heterogeneous population of protein subspecies, with variable catalytic fitness. This complicates the landscape for estimation of association and dissociation rate constants and usually results in ensemble averaged estimates that are grossly inaccurate.

***4.1.2. Self-catalyzing enzymes and approaches in drug discovery:*** Carrying out HTS and MoA studies with self-catalysing enzymes is a complicated and nontrivial exercise (Table 1). Heterogeneity in protein population arising due to self-modification is hardly treated explicitly, and appropriate kinetic framework for analysis is lacking[44]. Further, there is paucity of literature correlating protein modifications with enzyme activity loss (inhibition) or gain (activation) that are quantified by Tsau plots[41].

Often, the non-linearity in the initial time course measurement is either ignored or is approximated to linearity to make analysis simpler. However, this might lead to erroneous interpretation of the HTS, downstream validation and MoA outcomes for such enzymes. The first approach when dealing with auto-modifying enzymes, if there is a unique modification, is to establish that the protein modification (dependent variable) is a linear function of time (independent variable). This could be achieved by starting the reaction at several different protein concentrations and looking for possible differences in the overall
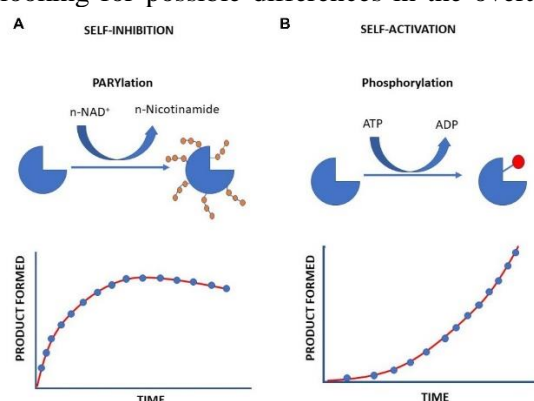


**Figure 3.** *Auto-catalysing enzymes **(A)** Auto inhibition of the enzyme PARP1/2 as a function of self PARylation and the resultant time-course behaviour resembling an initial burst and constant reduction in activity. **(B)** Auto-activation is often seen in kinases and the time-courses look like an initial lag followed by continuous increasing slope.*

behaviour of the enzyme (apart from magnitude effects)[41]. Another approach is to perform a full time-course simulation with appropriately defined models to understand the reaction and approximate it to experimental outcome (Table 1). Additionally, due to constant change in the concentration of catalytically competent enzyme as a result of self-activation or self-inhibition, rate of catalysis, or its lack thereof, should be treated as an acceleration or deceleration term rather than as velocity[45](Table 1). However, from a practical perspective, the most appropriate approach in treating non-MM self-catalysing enzymes is to ensure that under the conditions of analysis, the non-MM behaviour is suppressed as best as can be realistically achieved and conditions should be optimized for them to behave as apparent MM enzymes. Auto-catalytic enzymes, apart from being involved in modifying themselves, in most cases have other accessory substrates

on which they act to bring about their physiological outcome. This aspect must be integrated in designing the HTS and MoA studies for optimal outcome. For instance, although PARP1 is itself a major acceptor of PAR (poly ADP ribose) chains, other substrates for PARylation include core histones[46,47]. It would have to be ensured that the HTS effort and subsequent MoA studies starts with a homogenous population of enzymes with identical modification status. For self-activating enzymes, this could be achieved by pre-incubating the cofactor with the enzyme for a substantially long period before starting the HTS with the non-self-substrate. For self-inhibitory enzymes, the assay should be initiated with cofactor under an excess of non-self-substrate to prevent any self-catalysis mediated inactivation. However, the HTS initiative should be aware that this approach will decrease the probability of obtaining competitive inhibitors of the enzyme because of the unique HTS design with excess non-self substrate. Having said that, these steps will help to achieve the necessary resolution vis-à-vis inhibition of self catalysis versus non-self-substrate catalysis. Furthermore, the affinity or potency term would be the apparent value of differential $k_{on}$ and $k_{off}$ for the activated/inhibited versus the unmodified enzyme. i.e., the inhibitor might have differential affinity (affecting $K_i$) and potency (affecting $k_{cat}$ of the enzyme for its cognate substrate normalized for differential activity) for the modified enzyme vis-à-vis the unmodified variant. Hence, if there is a time-dependent element in the progress-curves of self-activating or inhibiting enzymes (i.e. burst or lag), it would have to be factored into the experimental design. Another approach, which could be exclusively employed for enzyme systems where the trans-model of self catalysis has been established, is to create an enzyme variant with active site mutant. This variant could be used as substrate with catalytic amount of the non-mutated enzyme to return the system to MM conditions (Table 1). The above suggested solutions are tailored to ensure that self-modifying enzymes behave analogously to those that follow MM kinetics, enabling the investigator to extract as much information as

possible, given the complexity (Table 1). In parallel, biophysical methods could be employed to assess inhibitor affinity for both the unmodified and modified form of the enzyme and light scattering based approaches used to ensure that the sample is homogenous as a function of modification.

## 4.2. Allosteric regulation and homotropic cooperativity.

### 4.2.1. Introduction:
Biological systems have evolved to maintain network robustness to overcome random disruptions of metabolic gradients[48]. Allosteric regulation of protein molecules constitutes one of the many factors that confer this robustness to biological networks. Allosteric control is a mode of regulation whereby a ligand, which is either similar (homotropic) or dissimilar (heterotropic) vis-a-vis the substrate, binds to a site distal from the active site and modulates the activity at the latter (Figure 4 A). As we will see below, the distal site could be another active site on an oligomer. Further, allosteric sites can either be explicit or cryptic depending on whether they are visible in the apo-enzyme or are only revealed in the holo-enzyme co-crystallized with the allosteric regulator[49]. Two distinct attributes define the interaction of an allosteric modulator with its receptor. The first one is the inherent affinity of the binding site on the receptor for the allosteric ligand and the second one is the way the binding gets transduced to another pocket and modulates the binding at the orthosteric site[29].

Traditionally, allosteric sites have not been typical targets of rational drug discovery efforts because of the perception that shallow or transient allosteric pockets can lead to low affinities of inhibitor interaction at that site versus active site binders. However, recently there has been a resurgence of interest in targeting allosteric pockets because of potential disadvantages in targeting orthosteric sites. For instance, targeting of orthosteric pockets could result in issues of selectivity in a family of homologous enzymes sharing high structural similarity in the binding pocket[50]. Further, the high intracellular concentration of substrate, which goes up in the vicinity of the enzyme in the event of inhibition, can make the task of targeting the substrate binding pockets quite daunting due to law of mass action and one would require inhibitors of extremely high
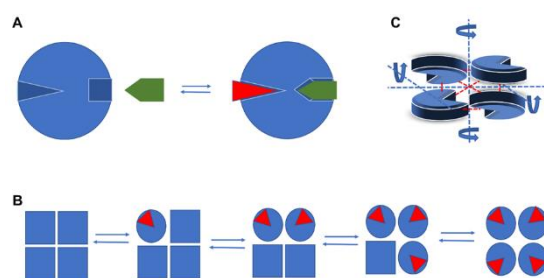


**Figure 4.** (**A**) *Heterotropic allostery in a monomeric enzyme. Binding of allosteric modulator renders the enzyme receptive to substrate molecules. Dark blue square and triangle: Substrate binding pocket in the absence of allosteric modulator and allosteric modulator binding pocket, respectively. Dark blue pentagon: Substrate binding pocket in the presence of allosteric modulator; red triangle: allosteric modulator; Green pentagon: substrate. (**B**) homotropic cooperativity in a tetrameric enzyme. Cooperativity is a special case of allostery and is found both in monomeric and oligomeric enzymes. (**C**) Cooperativity is mostly seen in enzymes with dihedral symmetry (rather than circular symmetry unlike the way it is depicted in panel B for simplicity's sake). The dimer symmetry axes are shown in dotted blue lines and the subunit interactions are shown in dotted red lines*

potency. However, a prominent exception to this are allosteric inhibitors which are nevertheless competitive vis-à-vis substrate[33]. Thus, targeting allosteric sites is becoming increasingly attractive by helping achieve higher selectivity and lower toxicity. It can also serve to help in modulating the target rather than completely shutting it down as is the case with active site binding molecules (i.e. partial allosteric regulation can offer control with respect to efficacy in addition to potency)[51]. Several allosteric modulators have been successfully identified employing HTS[52]. A few examples include sulphonamide inhibitors of LIM2 kinases, urea based selective allosteric modulators for p38α MEP, dibenzodiazepine inhibitors for PAK1, urea and acrylodan based-modulator of A172C p38alpha and so forth. Additionally, common drugs such as valium and benzodiazepines act by allosteric modulation of ionotropic GABA receptors[52].

Cooperativity is a special case of allostery that is seen frequently in homooligomeric organization of protein molecules (Figure 4 B)[29]. It has been hypothesized that approximately 25% of protein with oligomeric organization show cooperativity [2,53–59](Figure 5). Moreover, from the symmetry perspective, it has been demonstrated that most enzymes displaying cooperativity are packed into space groups with dihedral symmetry (Figure 4 C)

(rather than circular symmetry as depicted in the schematic of Figure 4 B for simplicity's sake). This is attributed to the network of subunit cross-talk in dihedral symmetry that is absent in organisations with circular symmetry[60]. However, it has been argued that some monomeric enzymes may also show non-MM cooperativity[61] resulting from substrate-induced slow structural transition in enzymes resulting in non-attainment of equilibrium between E and ES on the timescale of catalysis[61]. Additionally, ligand-mediated subunit association/dissociation could also reveal itself as cooperative deterministic curves especially for enzymes with interfacial active site pockets[56]. Predominantly, cooperative
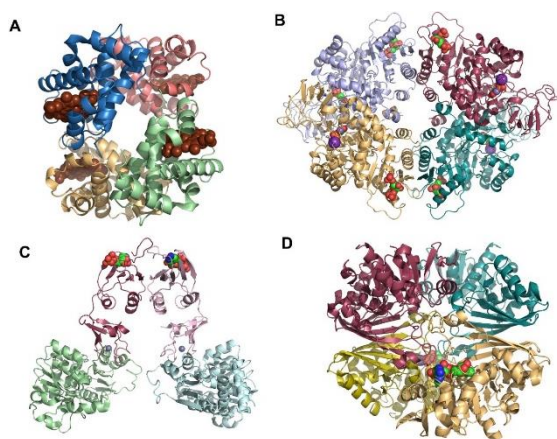


**Figure 5.** *Oligomeric enzymes showing cooperativity **(A)** Deoxy haemoglobin tetramer from Homo sapiens **(B)** Saccharomyces cerevisiae pyruvate kinase complexed with fructose-1,6-bisphosphate **(C)** Escherichia coli aspartate transcarbamoylase with CTP **(D)** rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. The protein is shown in cartoon representation with the individual monomers coloured differently. The ligand moieties are shown as ball representation. The figures were rendered using Open source PyMOL*

effects arise because of binding of similar ligands (homotropic) across a homooligomeric organization of protein subunits. Cooperativity in oligomeric systems is classified as either positive or negative. In positive cooperativity, binding of the first ligand increases the binding affinity of the second ligand and so on and so forth depending on the number of subunits that the homooligomeric assembly has ($K_{D-initial} > K_{D-final}$). On the other hand, negative cooperativity leads to high-affinity of first ligand binding followed by sequential decreasing affinity for subsequent binding events ($K_{D-initial} < K_{D-final}$).

Positive cooperative binding results in sigmoidal MM and Klotz curves (Figure 6 A & B) and non-linear Lineweaver-Burk (LB) plots bending upwards (Figure 6 C)[62]. On the other hand, negative cooperative effect could give rise to biphasic primary curves (Figure 6 A) and non-linear LB plots bending downwards[58,63–65] (Figure 6 C). Data fit to Equation 9 with Hill coefficient (n) greater than or less than 1 is indicative of positive cooperative effects or negative cooperative effects, respectively. Secondary replots such as Hill plot of log $v/(V_{max}-v)$ on the y-axis and log [S] on the x-axis (Figure 6 D), Eadie-Scatchard plot of $v$/[S] on the y-axis and $v$ on the x-axis (Figure 6 E), Hoffstee-Augustinsson plot of $v$ on the y-axis and $v$/[S] on the x-axis and Hanes plot of [S]/$v$ on the y-axis and [S] on the x-axis (Figure 6 F) all have diagnostic patterns indicative of either positive or negative cooperativity[66]. Despite having their own set of limitations[67–69], diagnostic plots have proven quite useful in assessing cooperative effects (Figure 6). This is especially true for understanding the mechanism of inhibition of the inhibitor vis-à-vis cooperative substrate/s (Figure S1) (Table 1).

$$v = \frac{V_{max} \times [S]^n}{(K_{0.5}^n + [S]^n)} \dots \dots \dots (9)$$

where, $K_{0.5}$, also referred to as $K_{half}$, is the substrate concentration that results in reaction velocity that is 50% of $V_{max}$. $n$ is unitless and is known as the Hill coefficient. If $n = 1$, there is no cooperativity and the Hill equation is reduced to the MM equation.

Cooperativity was originally detected and modelled for oxygen binding by haemoglobin[70] (Figure 5 A). Subsequently, many enzymes and receptor molecules have been shown to be cooperative[70]. A few prominent examples of metabolic enzymes displaying cooperative binding (both positive and negative) for a substrate or cofactor include yeast pyruvate kinase[71] (Figure 5 B), aspartate transcarbamoylase from *Escherichia coli*[72] (Figure 5 C), liver glucokinase, bovine 5' nucleotidase, porcine fumarase and rabbit glyceraldehyde 3-phosphate dehydrogenase[70] (Figure 5 D). However, caution should be exercised in modelling cooperativity since it can be a consequence of artefactual enzyme activation by solvents/buffer components and/or entropy-driven non-specific interaction
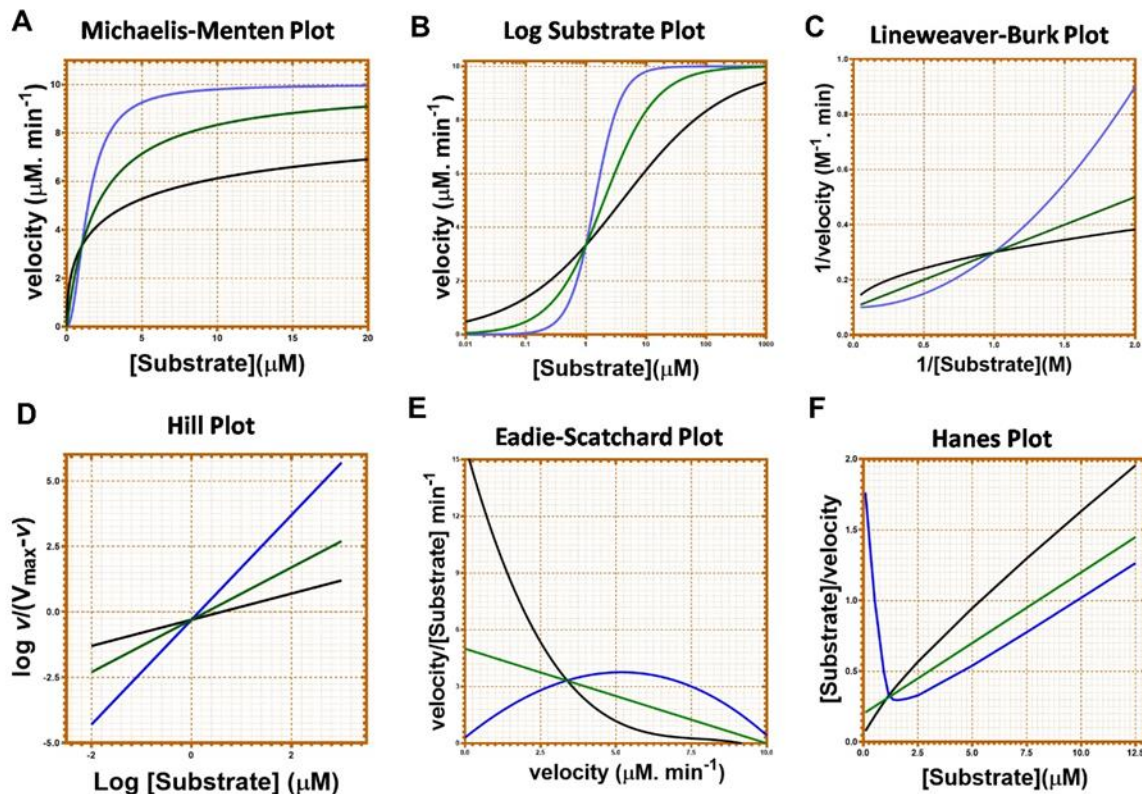
**Figure 6:** *Assessment of non-MM cooperative behaviour by secondary replots of the primary data. Each panel shows a schematic representation of the diagnostic plot for positive, negative and non-cooperative enzyme system. In each plot, the conventional MM behaviour is shown in olive-green line, the positive cooperative behaviour in blue line and the negative cooperative behaviour in black line, respectively. Note that heterogenous binding sites on the enzyme would behave the same as negative cooperative systems and would yield the same diagnostic plots. (A) Classical Michaelis-Menten plot, (B) MM plot with the X-axis shown in log scale (Klotz plot) (C) Lineweaver-Burk plot (D) Hill's plot (E) Eadie-Scatchard plot and (F) Hanes plot. The plots were generated from the respective models and their transformations with $V_{max}$ kept invariable arbitrarily at 10 μM. min$^{-1}$ and $K_m$ at 2 μM.*

of substrates with proteins[73]. A strong biological rationale should be presented for suspecting cooperativity. Increasing the parametric space without adequate rationale for doing so could result in overfitting the model. As John von Neumann has famously stated *"With four parameters I can fit an elephant, and with five I can make him wiggle his trunk"*[74]. However, once cooperativity is suspected and validated, the regulatory potential is very important to model since all cooperative effects have been shown to possess physiological significance. For instance, it has been traditionally believed that positive cooperativity gives rise to a multimeric receptor's sharp response to ligand gradient changes while negative cooperativity gives a far more graded response. Further, recent literature suggests that under excess receptor concentration and high affinity of the first ligand binding event ($K_1$), negative cooperativity can function as a ligand sink

depleting the ligand. This can result in binary sensitivities to changes in ligand concentration by filtering out small stimuli while acting sharply at high concentration of the ligand beyond a threshold. This is akin to the hook effect or the prozone effect seen in the case of antibody(Ab)-antigen (Ag) interactions, where, at either high concentration of the antibody (Ab) or the antigen (Ag), there will be no detection of the Ab-Ag complex formation. This can lead to complex system-level behavior with decisive bimodal switches that can give rise to bistability and oscillations[75].

***4.2.2. Cooperative enzymes and approaches in drug discovery:*** Once an allosteric or cooperative modulator is identified, interpreting their mechanism of action can oftentimes be complicated by behaviours that do not conform to MM kinetics[76]. This is because of the following reasons that were not so well formulated at the time of derivation of

the original MM assumptions (1) The enzyme molecule is an ensemble of different conformations and, as per the model put forth by Monod-Wyman-Changeux (MWC)[77] and Koshland-Nemethy-Filmer (KNF)[78], a conformational selection or induced fit event could be at play when allosteric interactions are involved[77]. This aspect becomes even more pertinent for allosteric enzymes as compared to non-allosteric ones because of the cross-talk inherent in the former that can modulate affinities and rates (linked equilibria). This ties the timescales of conformational fluctuations with the catalytic cycle[79–84]. (2) There are more than one unique intermediate in the catalytic pathway and the pathway can adopt different trajectories depending on the order of ligand interaction with the enzyme and ligand-independent receptor conformational isomerism[85]. (3) Aspects like catalysis-mediated bursts of superdiffusive enzyme motion that enhances their diffusivity over longer timescales[8] can have important implications for modelling a system that has multiple interacting partners and intra-protein signal propagation events. This will result in non-equilibrium models that deviate substantially from the behaviour of a conventional MM enzyme. Additionally, it is important to appreciate the scaling in cooperative enzymes as compared to MM enzymes. It is customary to come across terms like binary and ternary to signify enzyme-substrate/s complex for uni-uni enzymes and bi-uni/bi-bi enzymes, respectively. However, the rate equations for any complex that are higher than ternary become increasingly complex and are hardly treated in conventional enzyme kinetics textbooks or literature[86]. For a tetrameric enzyme (the most common form of homooligomeric organization encountered), the least complex complexation in cooperative systems would be quinary complex for uni-uni reactions! This necessitates a completely different treatment of such systems[21].

There are multiple challenges associated with instituting a HTS and carrying out downstream validation that exclusively strives to identify cooperative compounds[87,88]. Apart from the traditional focus on potency, cooperativity could be useful in drug discovery from two different perspectives. Small-molecules that show positive cooperativity will ensure that the receptor activity follows a two-state model, i.e., the receptor is either active or inactive and the transition from the inactive to active state is sharp (sigmoidal primary curves). An analogy would be the on and off state of an electric switch that represents binary state. This prevents leaky activity and ensures complete inhibition with a sharp transition as a function of inhibitor concentration. Likewise, negative cooperative system ensures an extremely shallow gradient before inhibition takes effect (biphasic primary curves). Both these scenarios are optimum depending on whether one wants to effect complete shutdown or mild modulation of the receptor. Additionally, inhibitor binding can increase the positive or negative cooperativity of substrate binding to the receptor making the landscape of regulation rich and prone to exploitation vis-à-vis drug discovery. From the HTS and subsequent hit validation perspective, if cooperativity is the desired trait that needs selection, looking for non-tight binding inhibitors with Hill slope of more than one in the CRC curves from initial round of screening is the way forward[89]. However, care should be exercised since nuisance inhibitors can also show steep Hill slopes and overreliance on Hill slopes alone might lead to misleading interpretation of the data. Once it is ensured that the steep Hill slope is exclusively because of cooperativity, SAR-mediated optimization could be used to improve their potency in the subsequent rounds. Another approach would be the simultaneous application of a selection criterion that weighs both potency and cooperativity[90](Table 1). However, a distinction would have to be drawn between quantification of inhibitor binding to the protein target and the resulting response (inhibition) indicated by potency. Unlike in cases with MM kinetics, the Cheng-Prusoff relationship translating affinity of inhibitor binding ($K_i$) to potency ($IC_{50}$) is not a simple relationship and is modulated by the cooperativity coefficient and slope factor, among other things[91,92]. A general equation that relates $IC_{50}$ and $K_i$ for competitive inhibition with cooperativity index $K$ (slope parameter of the agonist response curve) and $n$ (slope parameter of the inhibitor curve) less than, equal to, or greater than unity is given by the following Equation 10[92]

$$K_i = \frac{(IC_{50})^n}{\left(1 + \frac{[S]^K}{K_m}\right)}$$

$$= \frac{(IC_{50})^n}{\left[1 + \left(\frac{[S]}{EC_{50}}\right)^K\right]} \dots \dots \dots (10)$$

where [S] is the concentration of the agonist against which the $IC_{50}$ is determined, $K_m$ is the apparent equilibrium dissociation constant of the agonist and $K_i$ is the equilibrium dissociation constant of the antagonist for the receptor.

On similar lines, for gradual modulation of a receptor, optimizing for inhibitors yielding shallow CRC curves with Hill slope of less than 0.7 is desirable[89]. Though selecting for this Hill coefficient will not be able to discriminate between differential affinity of the inhibitors for the different sites ("*preexisting heterogeneity of binding sites*")[66] versus negative cooperativity, it is worthwhile to treat this explicitly. An extreme case of negative cooperativity is half-site reactivity. Inhibitors that can bind to one protomer of an oligomeric target molecule with half-site reactivity can shut down the other site preventing complete inhibition. This could potentially get revealed as partial inhibition[51,93] and is an important aspect to explore during inhibitor characterization (Table 1). If the cooperative behaviour is a result of oligomeric equilibration between different oligomeric forms as a result of either dilution or concentration of the stabilizing substrate, caution should be exercised in the way the enzyme is diluted into the final assay mix and on the way a constant substrate concentration is maintained. Experimental methods would have to be kept invariable for consistency of results and their eventual interpretation. Several studies have tried characterizing cooperativity in the same enzyme as a function of different substrates or cofactors. As a specific example, inhibitors of P2X7 receptors show positive cooperative binding to the target protein while metabolites like ATP and vanadate (phosphate mimic) show negative cooperative binding[94].

A detailed treatment of HTS considerations for identifying and treating allosteric ligands is presented in[90,95]. For MoA studies, Table 1 lists some guidelines in characterizing cooperative enzyme systems.

Appreciating the regulatory landscape that could be exploited in cooperative systems and incorporating them up in HTS and subsequent characterization can pay rich dividends in drug discovery.

### 4.3. Tight-binding and the free-ligand approximation:

***4.3.1. Substrate and Inhibitor tight-binding, an introduction:*** Tight binding behaviour is a special case of atypical kinetics with aspects resembling non Michaelis-Menten behaviour though it is most often not thought of in those terms. It occurs when the FLA, an important assumption in deriving the MM equation, becomes invalid and the total substrate is no longer equal to the free substrate. This can arise when the $K_m$ of the substrate is less than the total enzyme $[E_T]$ that can be employed in an assay to achieve effective signal to noise ratio. For such cases, the kinetic equation is derived without resorting to the FLA, is quadratic in nature[96] and is commonly known as the Morrison equation (Equation 11).

$$v = V_{max} \frac{([E_T]+[S_T]+K_m) - \sqrt{([E_T]+[S_T]+K_m)^2 - 4[E_T][S_T]}}{2[E_T]} \dots (11)$$

Where, $v$ is the velocity, $V_{max}$ is the maximum velocity, $E_T$ is the total enzyme concentration, $S_T$ is the total substrate concentration and $K_m$ is the Michaelis-Menten constant assuming multiple-turnover steady-state conditions.

The equation becomes the hyperbola described by Michaelis and Menten when the $K_m$ is larger than total enzyme. However, as the $K_m$ reduces vis-à-vis the total enzyme concentration, the curve edge becomes sharper and sharper (Figure 7 A). The higher the affinity with respect to the total enzyme concentration, the sharper the inflection when transitioning from the first order phase to the zero-order phase (vis-à-vis substrate) of the curve (Figure 7 A). When tight-binding is suspected, it is advisable to collect highly confluent data points around the point of inflection to fit the equation and obtain statistically reliable parameters. As a representative general case, the tight binding equation can always be employed irrespective of whether one suspects tight binding of substrate or otherwise because it avoids the assumption that the free ligand concentration is equal to the total ligand concentration. This will
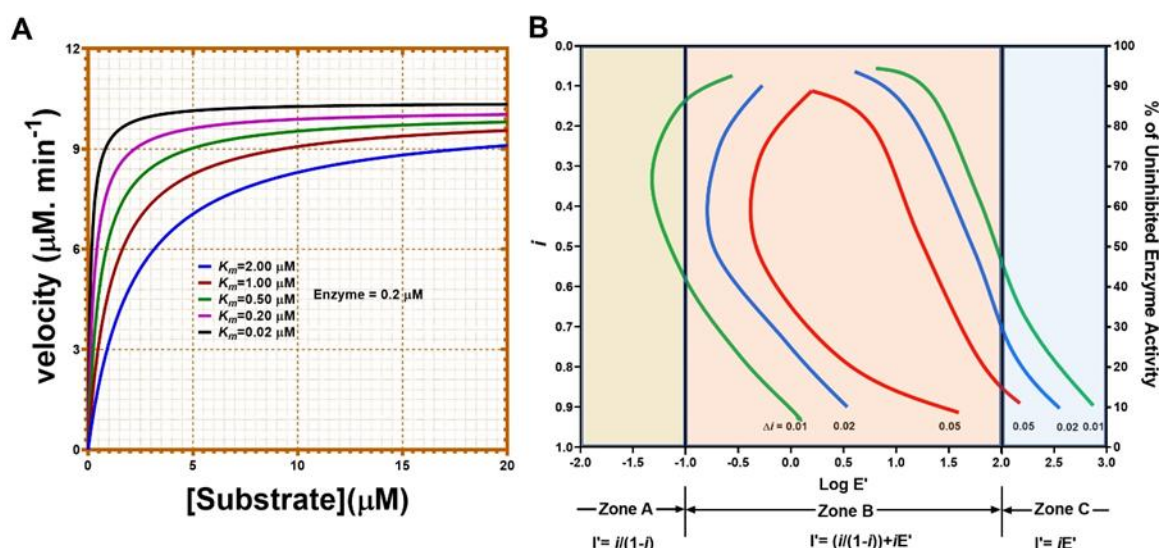
**Figure 7.** *Tight binding inhibition **(A)** Schematic representation of the shape of the substrate versus velocity plot as a function of relative value of enzyme concentration and affinity of the enzyme for the substrate. The family of curves were simulated using the Morrison's equation at various dissociation constant of the substrate for the enzyme. **(B)** Schematic representation of the Strauss and Goldstein zone at various enzyme concentrations vis-à-vis inhibitor $K_i$. The zones are shown in different coloured hues. I is total molar concentration of inhibitor, i is fraction of total enzyme combined with inhibitor(EI/E or $1-v/V_{max}$) ( Range, i will vary from 0 to 1 as v will vary from $V_{max}$ to 0), I' is specific concentration of inhibitor expressed as I/K (where, K is the equilibrium constant), Δi is maximum acceptable error in I, E' is specific concentration of the enzyme expressed as E/K. The panel was redrawn from [99]*

also help estimate reasonably accurate $K_m$ values within the limitations of the errors introduced by the "double fitting approach". The double fitting approach refers to linear regression (or non-linear regression) of progress curve data to extract initial velocity slopes and, subsequently, fitting the slope versus substrate concentration to MM or tight-binding equation to extract kinetic parameters. From an HTS or MoA perspective, an accurate estimate of $K_m$ is critical to ensure that the $IC_{50}$ estimations for lead compounds are reliable and can shed light on the mechanism of inhibition when assessed as a function of substrate concentration (Figure 2 B). However, it would have to be appreciated that most enzymes have evolved for catalytic perfection over millions of years of evolutionary timescale and do not show tight-binding behaviour vis-à-vis their substrate because that would not be conducive for effective catalysis[27].

The term Tight-binding, especially in drug-discovery settings, is most often used in the context of inhibitors. Tight binding inhibitor behaviour, where the affinity of the hits approach the least feasible enzyme concentration that can be used for effective signal to noise in the assay, is usually seen in

late stages of drug discovery. When the affinity of the inhibitor is higher (lower $K_i$ value) than the total enzyme employed in an assay, the total inhibitor concentration is not the same as the free inhibitor concentration. The enzyme binding site acts as a ligand sink and titrates the latter. A typical trait displayed by such inhibitors are steep Hill slopes of a CRC curve due to stoichiometric binding. This means that the inhibition increases sharply over less than a 10-fold concentration range of the inhibitor[97]. Note that, usually for classical inhibitors, the inhibition increases from 10% to 90% over an 81-fold concentration range. However, one should exercise restraint in differentiating steep CRC arising out of tight binding from those that are the result of cooperative interactions or protein aggregators (See section above). Another method to assess tight-binding is by employing Ackermann-Potter plots ( plotting velocity as a function of $E_T$, at various concentrations of I) that is used to distinguish reversible linear inhibition from stoichiometric tight-binding inhibitor (under conditions where $E_T >>> K_i$)[34,98]. For reversible inhibition, the slopes would decrease as a function of inhibitor concentration intersecting at the origin (Figure S2 A). However, for tight-binding inhibitors, the curves would appear parallel but would be

non-linear curving to intersect at the origin (Figure S2 C).

***4.3.2 Tight-binding and approaches in drug discovery:*** Often, the term tight-binding is interchangeably used with high-affinity inhibitors. Care should be exercised in doing that since "tight-binding" behaviour can be shown by low affinity inhibitors at high protein concentration that is employed in an assay for obtaining effective signal/noise. Having said that, most often the enzyme concentration used in an assay is in the low nanomolar or picomolar range and hence, the terms can be used interchangeably after exercising proper caution. Estimation of $IC_{50}$ values, which often forms the backbone of SAR-mediated medicinal chemistry efforts, must be carefully undertaken in circumstances where the $K_i$ is lower than the total enzyme employed in an assay. This is because the $IC_{50}$ value is a function of $K_i^{app}$ and $[E_T]$. The equation that defines the relationship between $IC_{50}$ and $K_i^{app}$ for all ratios of $K_i^{app}/[E_T]$ is shown in Equation 6, 7, and 8 for different mechanisms of inhibition. Morrison's quadratic equation, similar to the one employed for substrate, could also be employed here to get an exact estimate of $K_i^{app}$ (Equation 12) (Table 1)

$$v_i = v_0 \frac{-([I_T]-[E_T]+K_i^{app}) + \sqrt{([I_T]-[E_T]+K_i^{app})^2 - 4[E_T]K_i^{app}}}{2[E_T]} \dots (12)$$

Where, $v_i$ is the inhibited rate, $v_0$ is the uninhibited rate, $[I_T]$ is the total inhibitor concentration, $[E_T]$ is the total enzyme concentration and $K_i^{app}$ is the apparent equilibrium dissociation constant of the inhibitor for the enzyme.

Strauss and Goldstein define three distinct zones depending on the ratios of $K_i^{app}/[E_T]$ (Figure 7 B ). A value of $K_i^{app}/[E_T] >$ 10 (Zone A) indicates that the 1/2 $[E_T]$ term becomes insignificant and, for all practical purposes, it is safe to assume that $IC_{50} = K_i^{app}$. A value of $K_i^{app}/[E_T]$ between 0.01 and 10 (Zone B) shows that both the $K_i^{app}$ and $[E_T]$ terms in equations (5,6 and 7) contributes to the $IC_{50}$ estimation. Zone C represents values of $K_i^{app}/[E_T]$ <0.01 and the principal term that determines (and limits) the estimation of $IC_{50}$ is the total enzyme concentration since the inhibitor is essentially titrating the enzyme in the absence of EI dissociation[27,99]. Researchers

would have to incorporate this knowledge in their routine interpretation of $IC_{50}$ values. Further, an enzyme concentration dependent $IC_{50}$ estimation would be an essential exercise to undertake with tight binding inhibitors (Table 1). More accurately, researchers are encouraged to move "beyond" $IC_{50}$ analysis and define parameters such as $K_i^{app}$, wherever possible (Table 1).

The take home messages for HTS and MoA studies is to employ quadratic equation for substrate/inhibitor affinity estimations, understand the zonation defined by Strauss and Goldstein to weigh the parameters shown in equation (9) appropriately for $IC_{50}$ estimations and to be aware that steep dose response curves could be possibly indicative of tight binding conditions (Table 1).

**4.4. Time-dependent covalent irreversible inhibitors:**

***4.4.1. Introduction:*** Several marketed drugs are covalent irreversible inhibitors of their respective enzyme targets[100]. These inhibitors do not equilibrate with the enzyme rapidly on the timescale of the reaction (Figure S3 A). Thus, it is very difficult to use steady-state kinetic approaches to understand the MoA of such compounds. Hence, time-dependent covalent irreversible inhibition represents another unique case of atypical kinetics since: (1) full time-course measurements will be necessary to capture the time-dependence of inhibition (not the initial velocity alone), (2) ES or EI complex may not be the only unique intermediate but additional species such as E*-I (where the hyphen represents covalent bond formation between E and I and asterisk represents inactivated enzyme, respectively) may be present and (3) active [E] will be depleted as a function of time not because of complexation but permanent inactivation. Covalent inhibitors are broadly classified as: (1) affinity labels that modify a functional group based on their activity or reactivity (activity refers to specific binding interactions followed by modification while reactivity refers to modification alone) (2) quiescent affinity labels, which are similar to affinity labels but bind and modify the enzyme in a two-step process and use off-pathway mechanism for activation or (3) mechanism-based inhibitors, which are modified by the enzyme using its catalytic mechanism to produce a reactive

species, resulting in covalent bond formation and enzyme inactivation[27,101].

### 4.4.2. Covalent inhibitors and approaches in drug discovery:

Covalent inhibitors are either rationally designed or are identified by running an $IC_{50}$ estimation exercise with and without preincubation of the inhibitor with the enzyme. A preincubation-dependent increase in potency is a strong indication of either reversible slow-binding or irreversible covalent inhibition. Covalent irreversible inhibition can be understood graphically by seeing parallel lines on an Ackermann-Potter plot (Figure S2 B)[34,98]. As explained above, specific covalent inhibition involves two distinct steps, binding and inactivation. The binding event is implicit in the term $K_I$ (with uppercase I subscript) that should not be confused with $K_i$ (with lowercase i subscript). $K_i$ represents the reversible equilibrium constant between E and I while $K_I$ represents inhibitor concentration at half maximum rate of covalent bond formation. The inactivation step is the first order rate constant $k_{inact}$, representing the rate constant at maximum rate of covalent bond formation between inhibitor and enzyme once all the enzyme is complexed to the inhibitor reversibly. $K_I$ can approximate to $K_i$ when the first order rate constant for inactivation ($k_{inact}$) <<< $k_{off}$. However, care should be taken with such an interpretation because enzyme depletion occurs as a function of inactivation, however small the rate constant is. The overall enzyme inactivation, brought about by a covalent inhibitor, is thus expressed by a second order inactivation constant $k_{inact}/K_I$ (akin to the specificity constant $k_{cat}/K_m$ with the sole difference that the $E_T$ remains constant in the latter case). $k_{inact}/K_I$ can be estimated by the slope of the linear fit for one-step inhibition ( Figure S3B). However, individual $k_{inact}$ and $K_I$ values can be estimated from the fit of $k_{obs}$ versus [inhibitor] data to the two step model (Figure S3C) (equation 13).

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \ldots \ldots \ldots \ldots \ldots (13)$$

It is a common mispractice to employ $IC_{50}$ values as a metric to rank order covalent compounds. Given the variability of $IC_{50}$ for covalent inhibitors (depending on time and other factors), it is important to use $k_{inact}/K_I$ as a metric for covalent potency[102,103] (Figure S3 B

& C) (Table1). This metric is estimated by plotting the rate of transition of initial velocity to steady-state velocity ($k_{obs}$) as a function of inhibitor concentration for both one step and two-step models (Figure S3 B and C)[27]. The magnitude of the implicit binding aspect in $K_I$ will be important in guiding SAR by providing a relative measure of on-target and off-target interaction strengths. Furthermore, a potent $K_I$ can help in driving lower, less frequent dosing, depending on the half-life of the target protein. This is because the strength of interaction, in most instances, correlates with potency (median effective concentration/dose) and efficacy (the ability of the small-molecule to elicit the desired pharmacological outcome)[104].

A HTS program instituted to detect and, later, rank covalent compounds must strive to estimate $k_{inact}/K_I$ as a metric rather than relying on $IC_{50}$. Where possible, complete dissection of the parameters like $k_{inact}$, $K_I$ and $K_i$ should be undertaken, including through the use of pre-steady kinetic methods such as stopped-flow spectrophotometry[105]. These values for key compounds is critical for providing maximal information to aid molecular design, and also dose prediction. Explicit treatment of the above-mentioned non-MM behavioural aspects are essential for appropriate characterization of covalent inhibitors in a drug discovery program (Table 1).

### 4.5. Interfacial enzyme catalysis:

### 4.5.1. Introduction:

With increasing complexity of the enzyme behavior, obtaining systems level understanding is essential to complement the output of reductionist *in vitro* studies[106]. Cellular enzyme kinetics is a special case of non-MM kinetics where the behavior of the enzyme is modulated, to a large extent, by compartmentalization, crowding and diffusion limited substrate-enzyme interactions[107,108]. These result in behaviors such as multiple stationary states (MSS), interfacial effects, threshold effects and temporal patterns, homeostatic regulation, amplification, and irreversible differentiation[107–110]. It has been demonstrated that, because of the cumulative effect of all the above discussed attributes, the $k_{cat}$ of the enzyme can be slower and the $K_m$ can be higher than what is obtained with *in solution* studies[111,112]. Further, Thiele modulus, which is the relationship that equates particle size with catalytic activity in heterogenous catalysis, starts influencing the behaviour of

compartmentalized or interfacial enzymes[109]. When the Thiele modulus (also represented by the Damköhler number) is large, internal diffusion usually limits the overall rate of reaction; when it is small, the surface reaction is usually rate-limiting. Since most of the enzyme kinetic studies in the field of drug discovery are undertaken with the aim of understanding cellular and organismal effects of small-molecule inhibitors, the kinetics of the enzymes within the cellular milieu is equally relevant as their *in vitro* behaviour. A special case of intracellular enzymes are the interfacial enzymes, which are discussed in detail below.

Enzymes that function at interfaces are involved in diverse biological functions such as inflammation, membrane modeling, endocytosis and signal transduction[113]. They are ubiquitous *in vivo,* and it has been speculated that almost half of all the proteins that are intracellular are interfacial in nature. They function within the two-dimensional matrix that they are embedded in rather than three-dimensional bulk solvents (Figure 8 A). Most interfacial enzymes undergo activation upon membrane binding. The extent of activation is determined by the strength of binding and mode of interaction (angular orientation and membrane insertion) of the enzyme with the interface[114]. Many of these interfacial enzymes/proteins that are activated upon membrane anchorage are important drug-targets. A few examples include secreted phospholipase A$_2$ (PLA$_2$)[114], lipoxygenase (LO)[114], catechol-O-methyltransferase[115] and G-protein coupled receptors (GPCR)[116].

***4.5.2. Interfacial enzyme kinetics and approaches in drug discovery:*** Despite their importance in drug discovery, analysis of their kinetic behaviour is not straightforward. This is mainly because the bulk-concentration of substrate or inhibitor concentration is just one of the parameter that influences the behaviour of interfacial enzyme. Rather than absolute concentrations, the fraction of the concentration that interacts with the lipid anchored interfacial enzyme system to form the Michaelis complex becomes more relevant. The reason interfacial systems behave in non-MM manner are two-fold. The enzyme interacts with the bulk concentration of substrate to partition into the interface and, subsequently, the partitioned enzyme interacts with substrate that has partial mobility by virtue of being immobilised. This

necessitates modelling several parameters for a complete description of the fraction of active enzyme in the interface, its residence time vis-à-vis bulk solvent and the concentration of ligands the enzyme is capable of interacting[117,118]. Prominent examples include the kinetics of PLA$_2$ and hydrolysis of insoluble cellulose[119]. However, there are several approaches espoused to understand their kinetics. It has been demonstrated that the enzyme, depending on its affinity for the vesicle, can either adopt a scooting mode of catalysis or a hopping mode of catalysis. The former indicates persistent anchoring at the interface of a vesicle for multiple rounds of catalysis while the latter indicates frequent exchange across aqueous media onto multiple vesicles. It is recommended and argued that condition should be optimized to minimize hopping and constrain the system in the scooting mode for effective interpretation of catalysis[118]. Additionally, fluorescence-based equilibrium-binding methods have also been shown to assist in interpreting the kinetics of interfacial enzymes in scooting method[120–122]. Another approach, that has been pioneered by Edward Dennis, is called surface dilution kinetics and involves the dilution of the surface phospholipid concentration (in the case of PLA$_2$) with an inert detergent and assess for linear decrease in the activity as a function of reduced interfacial concentration[123]. Recently, an inverse-MM approach is also suggested to readily model the kinetics of interfacial system. The inverse-MM approach is defined as the validity of QSS approximation when the concentration of the enzyme is far greater than the molar concentration of the substrate sites[119].

Any HTS effort that aims to discover hits against these interfacial enzymes would have to make sure that they are anchored to their interfaces mimicking their physiologically active state. Attempts to truncate them for solubilization, with an eye on carrying out HTS and subsequent validation, may represent a form that is not relevant for the diseased state. Further, for effective interpretation of the potency of small molecules, the approaches espoused above should be used and efforts should be invested in running the assay at saturating enzyme and sub-saturating substrate concentrations in line with inverse-MM approach.

Having presented cellular enzyme kinetics and interfacial enzyme kinetics as a

complementary approach enriching conventional reductionist approaches, it would have to be appreciated that implementation in routine drug discovery is lagging in spite of the technology and the theoretical framework guiding data analysis. A viable alternative, which has emerged and is proving to be a powerful approach towards the realization of the above stated objective, is the use of cell-based assays as a system level reporter on inhibition of a target. This technique is gaining in popularity because of its ability to reflect on the *ex-vivo* system level behavior of inhibitor action on the target of interest coupled to the ease of its adaptation in the high-throughput plate-based format. Cell-based assay is an umbrella term coined for assay formats that measure outcomes as diverse as cell proliferation, toxicity, marker expression, signalling pathway activation, motility and morphological changes as a function of inhibitor administration[124,125]. Thus, the output from cell-based assays likely reports on the non-MM behaviour of intracellular or interfacial enzymes.

### 4.6. Single molecule kinetics:

***4.6.1. Introduction:*** Enzyme molecules are highly dynamic. Bulk measurement studies, by virtue of reporting average effects, ignore the distributions in molecular properties that are pivotal to understanding the effect of drug interaction with enzymes. Until recently, the only technique to visualize this dynamic nature was by means of molecular dynamic (MD) simulation[126]. However, the principal limitation of MD simulation is that they are restricted to timescales shorter than many biologically meaningful conformational changes[127]. Accelerated developments with patch clamp techniques, atomic force microscopy, optical tweezers, and fluorescence microscopy have allowed observation of single-molecule events at the millisecond to second timescale[128]. These techniques have shed light on the conformational changes that are slow and hence are potentially masked in ensemble averaged studies. Furthermore, enzyme turnover transformations can be monitored at the level of a single-molecule, as has been reported for a few motor proteins, a nuclease, a flavoenzyme[126], to mention a few examples. As we have seen above, for close to a century, deterministic rate equations (DRE) have aided in the estimation of rate constants and mechanisms from reaction kinetic experiments carried out on a population of molecules. The deterministic equation derived from the rate
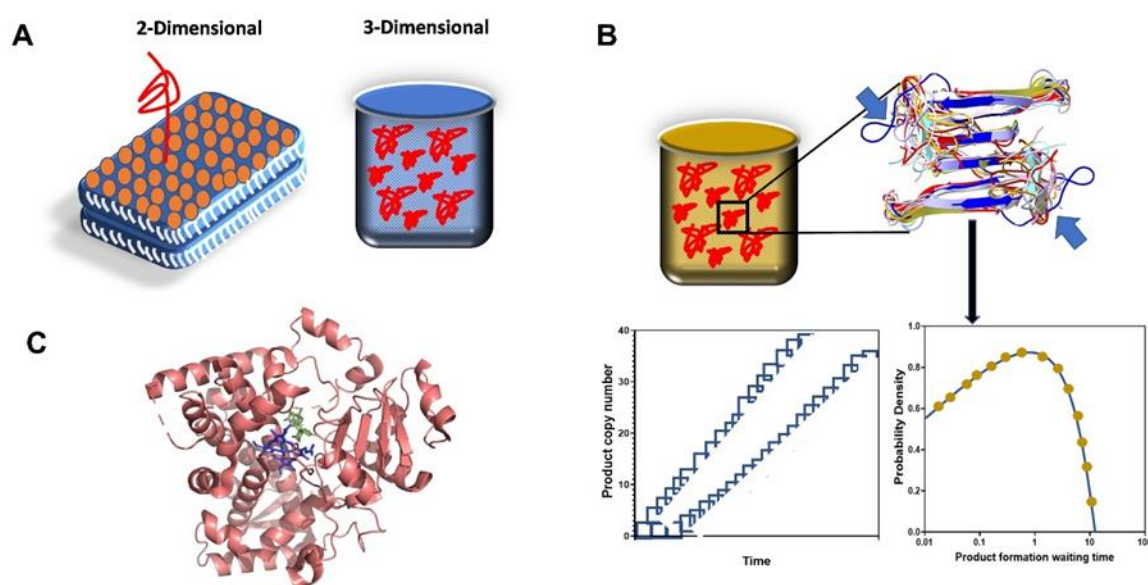


**Figure 8.** *Other specific examples of enzymes displaying non Michaelis-Menten kinetics.* **(A)** *Interfacial enzymes embedded in the lipid matrix are constrained in 2-dimensional space unlike enzymes in solution* **(B)** *Single molecule enzymology, protein dynamics and stochasticity of substrate to product conversion. The figure shows the zoom of a highly dynamic enzyme that catalyses stochastic product formation as a function of time (left panel). Probability estimates for the time duration between successive substrate turnovers is an additional parameter that can be obtained by CME (right panel). The figure has been partly redrawn from* [148] **(C)** *Human drug metabolizing Cytochrome P450 3A4 (PDB ID: 6OOA) that displays atypical non-MM kinetics.*

perspective by Michaelis and Menten have dominated the discourse in modelling enzyme kinetics. However, the interpretation and analysis of single molecule data requires stochastic methods utilizing the time-perspective since it deviates substantially from the common Markovian description employed in traditional kinetic analysis[129] (Figure 8 B) using DRE. The latter implicitly assumed a macroscopically large number of enzyme and substrate molecules.  This is in contrast with intracellular concentrations which, in some instances, could be as few as 10s of molecules with large in-built variability and noise[130]. The noise arises because of several different reasons including stochastic fluctuations such as the Brownian motion of substrate and enzyme molecules. However, the noise becomes irrelevant in bulk situations since it scales as the inverse square root of the total number of molecules present. Single molecule enzyme kinetics is handled with the chemical master equation (CME) while DRE is used in traditional enzyme kinetics analysis. CME provides an accurate estimate for both the mean concentration of the various species involved and noise (or fluctuation about the mean). It also gives probability estimates for the time duration between successive substrate turnovers, the latter being highly useful in assessing enzyme mechanism[131] (Figure 8 B). Prediction of the mean substrate, enzyme and enzyme-substrate complex concentration as a function of time could be different when employing either CME or DRE to model the kinetics. The differences are usually small for enzymes displaying MM kinetics while they can get considerably larger when substrate inflows, especially in bursts, is a feature of the system[131].

Enzymes are dynamic entities. Their fluctuations (in terms of catalysis and conformational diversity) are relatively fast enabling the MM assumption to hold. However, in scenarios where the fluctuations become extremely slow, as revealed by single molecule experiments for some enzymes, it has been questioned whether the MM assumptions would still be relevant[84]. Min *et al* have analysed three distinct conditions namely (1) when the ES complex has slower conformational sampling vis-à-vis the free enzyme and the catalytic rate (quasi-state conditions), (2) when the rate of substrate dissociation is faster than the rate of catalysis

independent of the speed of conformational sampling (quasi equilibrium conditions) and (3) when substrate dissociation and catalytic rate is dependent on the rate of conformational sampling (conformational equilibrium conditions)[84].  They conclude that these are all scenarios where the enzyme does not follow MM kinetics, though they acknowledge that it is difficult to assess this experimentally especially if one is sampling a large parameter space[84].

### 4.6.2. *Single-molecule kinetics and approaches in drug discovery:*
Though there are no explicit specifications that have been either outlined or optimized for HTS or validation studies for single molecule enzymology, there are a few papers that elaborate on this concept. An exemplary overview of inhibition at single molecule level is presented by Robin *et al*[132]. Additionally, a few studies describe the use of double-stranded DNA linkers[133] and single molecule force spectroscopy[134] for single molecule enzyme-drug interaction assessments. Another study[135] describes a high-throughput single-molecule/single cell imaging screen that was used to identify and validate small molecules that alter transcription kinetics. However, it would have to be appreciated that implementation of single molecule enzyme kinetics has not come to realization in early drug discovery, except in the area of bespoke experimentation, and will take substantial time to be a regular part of the pipeline.

## 4.7. Other examples of enzymes showing atypical and non-Michaelis-Menten kinetics:

### 4.7.1. *Processive enzymes*:
Processive enzymes are those that perform sequential catalysis of substrates without dissociating from them. The substrates for processive enzymes are usually polymeric in nature, e.g. cellulose, polypeptides or DNA, and the enzyme undertakes sliding, hopping, or intersegmental transfer as a means of carrying out the reaction[136]. However, sequential catalysis on the same substrate locus is also treated as a special case of processive mechanism, as in the case for arginine methyltransferases that carry out symmetric or asymmetric dimethylation on the same arginine. Another special case of processivity is the multisite phosphorylation and dephosphorylation of proteins by kinases and phosphatases, respectively[137]. A distinct

characteristic of enzymes showing a processive mechanism is that the concentration of any intermediate substrate/product species is undetectable and can be never greater than the enzyme concentration employed. Though attempts have been made to formulate kinetic principles based on deterministic kinetic models with QSS assumption for modelling processive enzymes, parameters like 'kinetic processivity coefficient', must be built in to mirror the experimental outcome. 'kinetic processivity coefficient' is a probability term for dissociation of enzyme from the substrate before the completion of n sequential catalytic steps (n is the mean processivity number assessed experimentally)[138]. Additionally, simplifying assumptions that the catalysis and dissociation rate constants are independent of the length of the substrate chain are in-built into the derivation. However, as has been clearly indicated by the authors examining the enzymology of Cellulase, such a treatment has significant limitations and in-built assumptions that can complicate the interpretation of applying deterministic MM kinetics to processive enzymes. A few of the limitations of the treatment include (1) A deterministic treatment of the kinetic model with QSS assumption is an overly simplified treatment since there are several steps like association, diffusion and acquisition of the substrate in kinetically competent mode that are either ignored or treated implicitly. This results in the parameters being composite rate constants rather than stringent ones. (2) In a deterministic model, the $k_{on}$ for the substrate and the enzyme is treated based on law of mass action despite the implicit case that the substrate could be essentially treated as solid and non-diffusible, especially for polymeric substrates. This makes the specification of initial substrate concentration inaccurate and hence, unreliable for modelling purposes. (3) As a direct extension of the discussion above, because of inaccurate estimates for the initial substrate, it might not be always possible to assess whether the substrate is in excess of the enzyme. (4) Further, processive enzymes are known to display non-linear kinetics (constant reduction in the rate of reaction) even in the absence of substrate depletion and product inhibition. This prevents treating the system as a true steady state where the rate of the reaction remains constant or the amount of product formed is linear as a function of time. Early drug

discovery efforts need to treat processivity in enzyme catalysis explicitly to understand the enzyme form that is targeted by inhibitor molecules. This may not be a straightforward exercise and needs extensive basic characterization of the system before initiating inhibitor discovery.

***4.7.2. Distributive enzymes:*** A related system is known as distributive enzyme mechanism. A distributive enzyme is one that releases the substrate in solution and the substrate would have to compete with a heterogenous pool of variably modified substrate molecules for the enzyme active site again. This entails that both the $k_{on}$ and $k_{off}$ values are important determinants of the kinetic behavior. It has been posited in literature that though the role of $k_{on}$ has been explored extensively in conventional MM kinetics, the role of $k_{off}$ has largely been assumed as being inversely related to $k_{cat}$[139]. This has been recently contradicted in literature[139]. Additionally, this kind of catalysis has distinct aspects of non-MM behavior as enumerated below (1) There is a heterogenous mix of substrates and hence a unique ES is not the only intermediate present in the reaction vial and (2) the initial concentration of a unique substrate/intermediate species among the heterogenous mix of substrate/intermediate molecules could be equal to or marginally greater than the enzyme concentration used in the assay. However, the maximum concentration formed of the heterogenous mix of substrate/s is not limited by the amount of enzyme. The above scenarios are contradictory of the MM assumptions reinforcing that enzymes displaying distributive mechanisms would have to be treated distinctly and explicitly in early drug discovery initiatives.

***4.7.3. Multisubstrate enzymes:*** Certain categories of multi-substrate reaction mechanisms such as sequential ones where a ternary or higher complex is formed can result in complex rate equations that would have to be treated appropriately for effective characterization of enzyme-inhibitor interactions. This is a non-trivial exercise as has been demonstrated in literature[35]. This scenario becomes yet more complex when there is linked equilibria as is seen in ordered mechanisms where binding of one substrate facilitates the binding of the next substrate in a sequential reaction. Additionally, formation of multiple

products in a reaction can also result in potential hurdles for unambiguous assignment of inhibitor mode of binding depending on the differential binding affinity, if any, of the various products. Furthermore, in multi-substrate reactions, specificity, defined as the preference of an enzyme for a particular substrate, and selectivity, defined as the preference of the enzyme for one substrate over another in a mixture of substrates, become important determining factors in assessing inhibitor interaction vis-à-vis its affinity and potency[140]

***4.7.4. Single-turnover catalysis:*** Single-turnover enzymes are yet another special case of non-MM systems where the enzyme is inactivated after one round of catalysis. The inactivation could be the result of interaction with the substrate[141], in which case it is called suicide substrate, or an inherent property of the enzyme whereby it binds to the products tightly. Though the system could be approximated by steady state kinetics, time-dependent loss of enzyme activity makes this uniquely non-MM kinetics. A prominent example is *Streptococcus pyogenes* CRISPR-Cas9. spCas9 is a single-turnover enzyme that interacts with substrate DNA reversibly and, subsequent to catalysis, binds to the DNA cleavage products tightly[142].

**4.7.5. *Substrate inhibition:*** Yet another case of atypical kinetics are enzymes showing substrate inhibition[143,144], where the enzyme achieves maximum rate of catalysis until a particular substrate concentration threshold is reached, beyond which substrate starts inhibiting the enzyme. Presence of substrate inhibition necessitates the determination of equilibrium dissociation constant of the substrate for the inhibitory site and the catalytic dissociation constant of the substrate for the active site both in the presence and absence of the substrate binding at the inhibitory site. Further, the $k_{cat}$ of the ES and the ESS complexes would be different and would have to be treated explicitly if the inhibitor interaction with the enzyme would need to be quantified under catalytic conditions. However, it should be noted that the substrate $K_i$ values tend to be significantly higher than their respective $K_m$ values. Hence, under most HTS conditions that utilise substrate at its $K_m$ value, this will not be a major issue. However, this behaviour would have to be

explicitly treated when carrying out bespoke work or when the substrate is saturating.

**4.7.6. *Cytochrome p450 kinetics:*** Deviating from the norm of clustering examples under a non-MM behavioral category, below we present an example of an enzyme family with atypical kinetics that has an extensive role in drug metabolism and clearance. Cytochrome p450s (CYPs) are enzymes that bring about this transformation of metabolizing the drugs and clearing it from the system (Figure 8 C). However, it is well known that these enzymes show non-MM kinetics[145],[146],[147]. Several CYP isoforms such as 3A4, 1A2, 2E1, 2D6, and 2C9, show non-MM kinetics if assessed *in vitro*. Since non-MM behaviour is usually associated with oligomeric organization, it is noteworthy that both monomeric CYP, and CYP as a heterooligomer with reductases or Cyt b5 shows atypical kinetics. Moreover, the fact that multiple ligands can bind to the CYP active site further complicates the landscape of CYP-mediated kinetics of drug metabolism and clearance. CYP, in catalysing the oxidation of its substrates, shows hyperbolic, sigmoidal, biphasic and substrate-inhibition patterns in various forms of substrate versus velocity plots[145]. Modelling of *in vivo* hepatic clearance for drug molecules based on *in vitro* data obtained by MM treatment of CYP kinetics needs to be carefully analysed in order to avoid the generation of misleading outcomes. For example, it has been posited that inaccurate application of MM kinetic model to enzyme systems showing substrate inhibition will result in gross underestimation of the $K_m$ and $V_{max}$. On the other hand, MM kinetics applied to sigmoidal model will overestimate $K_m$ and $V_{max}$ especially at therapeutically relevant lower substrate concentrations[73].

What emerges clearly from the above specified examples is that a significant number of non-MM kinetics displaying enzymes, which are classified under distinct subheadings, show overlapping behavior. For example, there are aspects of overlap in the non-MM treatment of interfacial enzymes, processive enzymes and self-modifying enzymes. Most of these enzymes show non-linear progress curves and deviate from hyperbolic substrate versus velocity plots. It would have to be appreciated that the above classification was exclusively instituted in order to facilitate the appreciation of different non-MM behavior displaying

systems and is not meant to convey that they are exclusive domains of behavioral uniqueness that do not share common aspects with other enzyme systems.

## 5. Conclusions and perspectives

The purpose of a drug discovery initiative is to obtain as many specific hits as possible with diverse mechanism of action and with the potential for pre-clinical optimization leading to desirable outcome in the clinic. This goal can remain unrealized if the HTS and the downstream validation steps do not incorporate the atypical kinetics of an enzyme's behavior in the design of the screen or the downstream validation of the hits. Although, one would obtain hits that might show high affinity, they may fail later because of their optimization on potency criterion alone rather than on a non-MM parameter such as, say, Hill coefficient or processivity coefficient. With increasing complexity of the enzyme systems under study, it would be prudent to incorporate key aspects

of non-MM behavior displayed by enzymes when selecting compounds with the desirable mode(s) of action. Early, detailed understanding of the target enzymes' behavior, and appropriate analysis of their interactions with ligands, will aid translation into a cellular context with more definition. This will, in the long run, help to reduce the attrition rate that is a reality of current drug discovery pipeline.

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**Table 1**. HTS and MoA guidelines and role of equilibrium binding studies in characterizing representative non-MM behaviour.

| Non-MM behaviour | HTS characterization and MoA guidelines | Opportunity for equilibrium binding studies |
|---|---|---|
| Self-catalysis | • Employ excess of catalytic mutant of the enzyme as substrate to mimic MM conditions (for trans).<br>• Treat rate as an acceleration or deceleration term.<br>• Use non-self substrate to mimic MM conditions.<br>• Non-linearity in progress curves should be explicitly modeled by full-time course simulation.<br>• Account for protein heterogeneity leading to activity loss or gain.<br>• Assess the relationship between protein modification and time. | • Assess inhibitor affinity ($K_D$) for both unmodified and modified form of the enzyme.<br>• Factor linked equilibria between substrate/cofactor and [I] binding by the relative magnitudes of $K_D$ and $K_i^{app}$ ($K_i^{app} < K_D$ indicates ordered binding).<br>• Light-scattering based approaches to assess sample homogeneity as a function of modification. |
| Cooperativity | • Rank compounds for both potency and Hill coefficient.<br>• While using LB plot, raise the power of the substrate to the Hill term to get linearity and assess MoA.<br>• For negative cooperativity, exclude pre-existing heterogeneity and explore half-site reactivity & oligomeric equilibration. | • Tease apart substrate binding cooperativity from inhibitor binding cooperativity.<br>• Factor linked equilibria between [S] and [I] binding by the relative magnitudes of $K_D$ and $K_i^{app}$. |
| Tight-binding inhibition | • Use Morrison's quadratic equations to estimate the $K_m$ and $K_i^{app}$ for substrate and inhibitor, respectively.<br>• Non-linearity in LB plots at high [S] should be considered before assigning MoA. Competitive inhibition can be mistaken for non-competitive inhibition.<br>• Treat non-linearity in progress curve (possible slow-onset) explicitly.<br>• Try reducing the [Enzyme] taking into account S/N of the assay.<br>• Look for stoichiometric binding and high Hill slopes.<br>• Employ the modified Cheng-Prusoff relationship for $IC_{50}$-$K_i$ conversions and be aware of Strauss-Goldstein zonation.*<br>• Estimate $IC_{50}$ as a function of enzyme concentration.*<br>• Overcome tight-binding for competitive inhibitors by increasing [S]. | • Use of techniques like SPR to estimate the $K_D$, $k_{on}$ and $k_{off}$ of the inhibitor binding to the target. This will indicate how slow is the association rate. |
| Covalent irreversible inhibition | • Estimate $k_{inact}/K_I$ rather than $IC_{50}$ to rank order the hits.<br>• Where possible, estimate parameters like $k_{inact}$, $K_I$ and $K_i$, including through the use of pre-steady kinetic methods.<br>• Model the non-linearity in progress curves explicitly.<br>• Carry out substrate-mediated inhibition protection for understanding site of binding.<br>• Be aware of PAINS that inhibit by reactive affinity groups. | • Estimating $K_I$ by a biophysical direct binding method (i.e. ITC, DSC or SPR or a combination of these methods)<br>• Trapping the covalent intermediate using mass-spectrometry<br>• Determining $K_D$ of first reversible binding step employing site-directed mutants. |

*[E]- Enzyme concentration, [S]-substrate, HTS- High-throughput screening, MM-Michaelis-Menten, $IC_{50}$- Concentration of inhibitor required for 50 % inhibition, $k_{inact}/K_I$- A second order parameter representing maximum rate of inactivation normalized by concentration of inhibitor at half maximum rate of covalent bond formation, $K_D$- equilibrium constant for dissociation, S/N- signal to noise, PAINS- Pan-assay interfering compounds, ITC- isothermal titration calorimetry, DSC- Differential scanning fluorimetry, SPR- Surface plasmon resonance, MoA-Mechanism of Action, $k_{on}$-association rate constant, $k_{off}$-dissociation rate constant. *Morrison's $K_i$ estimation is preferable.