The Kinetic Component in Drug Discovery: Using the Most Basic Pharmacological Concepts to Advance in Selecting Drugs to Combat CNS Diseases

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Abstract: To reach the central nervous system (CNS), drugs must cross the brain-blood barrier and have appropriate pharmacokinetic/dynamic properties. However, in early drug discovery steps, the selection of lead compounds, for example, those targeting G-protein-coupled receptors (GPCRs), is made according to i) affinity, which is calculated in in vitro equilibrium conditions, and ii) potency, a signal transduction-related parameter, usually quantified at a fixed time-point in a heterologous expression system. This paper argues that kinetics must be considered in the early steps of lead compound selection. While affinity calculation requires the establishment of a ligand-receptor equilibrium, the signal transduction starts as soon as the receptor senses the agonist. Taking cAMP production as an example, the in vitro-measured cytoplasmic levels of this cyclic nucleotide do not depend on equilibrium dissociation constant, \( K_D \). Signaling occurs far from the equilibrium and correlates more with the binding rate \( k_{on} \) than with \( K_D \). Furthermore, residence time, a parameter to consider in lead optimization, may significantly vary from in vitro to in vivo conditions. The results are discussed from the perspective of dopaminergic neurotransmission and dopamine-receptor-based drug discovery.

Keywords: Agonist binding, association, dissociation, equilibrium constant, GPCR, rate constants.

1. INTRODUCTION

The chances of suffering from Alzheimer’s (AD) or Parkinson’s (PD) diseases raise as life expectancy increases. Apart from genetic factors occurring in cases of early-onset debut of clinical symptoms, age is the main risk factor for idiopathic AD and PD cases. The unmet needs in neurodegenerative diseases include interventions to stop disease progression; however, there are tools to address symptoms, for instance in PD, whose patients have lost a significant number of dopamine-producing neurons in the substantia nigra. One of the most notorious is levodopa, a long-lasting successful therapeutic drug, which was discovered decades ago and it is still prescribed [1-4]. The development of new CNS-acting drugs requires drug design aided by in vitro assays in cells and the measurement of efficacy in animal models. Due to lead optimization and pharmacokinetic/pharmacodynamic characterization, a potential neuroprotective drug may enter into clinical trials.

Neurotransmission is fast and the action potential is extraordinarily quick. It may be argued that such high speed is due to the action of neurotransmitters on ionotropic receptors, i.e., on ion channels that are activated by neurotransmitters. Interestingly, dopaminergic transmission is dependent on (only) GPCRs, occurs quickly and participates in a myriad of higher functions, ranging from motor control to emotion control [5-7]. Kara et al., 2010 suggested cooperativity in the binding of N-n-propylnorapomorphine from D₂ receptors using both the competition and the dilution procedures. These findings were compatible with cooperative dissociation of two molecules from a homodimer. The different dissociation rates due to the occurrence of two species (different conformation states) of the receptor cannot be ruled out. Interestingly, the authors were able to estimate the dissociation constants of dopamine itself and the results were important. The percentage of the fast dissociation component was around 30, irrespective of the methods used and the half time of fast dissociation was 5.5 or 9.4 min (depending on the experimental approach), while the half time of slow dissociation was 145 or 430 min (depending on the experimental approach) [8]. In other words, dopamine acts almost instantly but residence time (RT) in receptors, calculated as the inverse of in vitro-measured \( k_{off} \) lies in the minute range. Would these in vitro-calculated elevated RTs be similar to those occurring in vivo? Recent data obtained with antipsychotic drugs show high-affinity binding to dopamine D₂ receptors, which is important for their success in vivo.

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receptors (D₂Rs) with pKᵦ values in the range: 7.1 to 10 and (monophasic) slow dissociation obtained in competition assays; kₑff values were in the range of 0.113 to 0.026 min⁻¹ [9]. In the most extreme case, spiperone with a Kᵦ in the sub-nanomolar range has an in vitro calculated RT of 35 min. Using the dilution method, the half dissociation of two of the assayed compounds, aripiprazole and cariprazine, occurred in >10 min. Half dissociation time for the same compounds in the D₃ receptor (D₃R) was similar despite the fact that they presented biphasic dissociation curves, similar to those reported by dopamine in D₂Rs. Interestingly, the affinity of the compounds in the D₃R was about one order of magnitude lower. The biphasic dissociation curves may represent two different forms of the receptor that would display significant differences in kₑff from 0.02 to 0.04 in one case and from 0.18 to 0.63 min⁻¹ in the other [9]. The thermodynamic and kinetic parameters were used to demonstrate allosteric behavior of SB269,652, which has the potential of a drug to combat dyskinesias of Parkinson’s disease chronically subjected to anti-symptomatic pharmacological therapy. A non-selective radiolabeled antagonist, nemonapride, with sub-nanomolar Kᵦ values, was used in both D₂ and D₃ receptors. kₑff values (min⁻¹) were about two-fold higher in the case of D₃ receptors, and the resulting kₒff was 0.20 for D₂ (D₂Rs) and 0.36 nM⁻¹ min⁻¹ for D₃ receptors (D₃Rs). kₑff was 0.023 for D₂Rs and 0.062 min⁻¹ for D₃Rs. Accordingly, kₒff/kₑff ratios are 0.11 (D₃R) and 0.17 (D₂R) nM [10], and the RT of nemonapride in D₃/D₃ receptors is in the 16-43 min range. The results show that upon binding of a ligand the RT of agonists/antagonists of D₂/D₃ receptors, measured in in vitro conditions is fairly high, especially in the case of dopamine, the endogenous ligand. In summary, in vitro measures that are centered on the Kᵦ and equilibrium assumptions may not be appropriate for what in vivo occurs quickly and far from any equilibrium.

The usual way of selecting drug candidates is to perform binding assays to calculate receptor-drug affinity and signaling assays to calculate drug potency. Usually, the kinetic component is not taken much into consideration to obtain the parameters that will be used to select drug candidates. In line with the recent and detailed review of Peter J. Tonge [11] it was argued that kinetics should be considered from the very beginning, way before a given drug is selected for study tissue/blood/brain distribution, kinetics of brain accumulation/disappearance and/or in vivo metabolism. Kinetics must already be considered in the in vitro assessment of receptor-ligand interactions and signal transduction.

2. THE KINETIC COMPONENT IN GPCR PHARMACOLOGY

35-45% of current medicines target G-protein-coupled receptors (GPCRs) [12-14]. They consist of i) 7 transmembrane domains, ii) N-terminal and C-terminal domains and iii) extracellular and intracellular loops. They interact with heterotrimeric G proteins and also with other GPCRs, scaffolding molecules and/or with proteins of the signal transduction machinery. Basic concepts of GPCR biochemistry and pharmacology should be considered to revisit the factors affecting any signaling arising from agonist activation. Gs-coupled GPCRs are considered, whose activation leads to an increase in adenylyl cyclase (AC) activity and cytoplasmic cAMP levels. cAMP, second messenger produced by AC using ATP as a substrate, is degraded to AMP by phosphodiesterases. At a theoretical level, the real signaling output is the initial rate of production of cAMP. In practice, however, researchers use assays of cAMP level determination at fixed time points in the presence of phosphodiesterase inhibitors. In real physiological conditions, i.e., assuming degradation by phosphodiesterases, the order of potency of 3 different agonists depends on the time after GPCR activation (Fig. 1). Recent technological developments allow obtaining cytosolic cAMP levels in real-time from which AC activity may be determined using the linear part of the graph of [cAMP] versus time (between 0 and t₁ in Fig. 1). Determining the variation of initial enzymatic rate as a function of the agonist concentration would give accurate estimates of the maximal value of AC enzyme activity and of agonist EC₅₀. Unfortunately, such measurements are technically challenging.

3. THE AGONIST-RECEPTOR ASSOCIATION CONSTANT (kₒ) SETS THE RHYTHM

We have adopted two mechanistic approaches to link GPCR agonist binding to AC activation. All the possible equilibria involving ligand/receptor interactions, G protein activation and AC activation are provided in Fig. 2.

Fig. (1). End-time point affects potency. Time-response of second messenger production upon the activation of a GPCR with different agonists (blue, red and green). The simulation of cAMP levels upon the activation of a Gs-coupled GPCR in the absence of phosphodiesterase inhibitors, i.e., in physiological-like conditions. The values are normalized and the rate of production of cAMP is different in each curve. It is assumed that cAMP is degraded in a linear fashion (i.e., proportional to the [cAMP]) by phosphodiesterases. Inset: Rank order of potency at the 5 fixed time points (t₁ to t₅). (A higher resolution / colour version of this figure is available in the electronic copy of the article).
Fig. (2). Scheme of models linking agonist (Ago) binding to a Gs-coupled GPCR to adenylyl cyclase activation. α, β and γ are the subunits of Gs and AC indicates adenylyl cyclase. The coupled model is shown in panel A and the uncoupled model in panel B. In both schemes the disappearance of the ligand upon time is not shown but was considered in the simulations; it was assumed as proportional to the concentration of the agonist (Fig. 3 legend). (A higher resolution / colour version of this figure is available in the electronic copy of the article).
Fig. (3). The time course of the activation of the AC (A) using either the coupled model displayed in Fig. 2A or the uncoupled model displayed in Fig. 2B. $K_D$, $k_{on}(k_{i})$ and $k_{off}(k_{j})$ values are indicated in the insets. A common assumption is the lack of cAMP degradation; in fact, phosphodiesterase inhibitors are always included in in vitro assays of cAMP level determinations. Panel A: Coupled model. The values of the remaining kinetic constants were: $k_1 1,E6 M^{-1} s^{-1}$, $k_2 1E6 M^{-1} s^{-1}$, $k_3 1E3 s^{-1}$, $k_4 1E7 M^{-1} s^{-1}$, $k_5 1E10 M^{1} s^{-1}$, $k_6 1 s^{-1}$, $k_7 1E13 M^{1} s^{-1}$, $k_{on} 1 s^{-1}$, $k_{off} 5 M^{1} s^{-1}$. Steps 3, 5 and 7 are virtually irreversible; accordingly, $k_3$ and $k_7$ are taken as zero. [GDP], [GTP] and [ATP] = $1E-4 M$; [L] = $5E-8 M$. Panel B: Uncoupled model. The values of the remaining kinetic constants were: $k_1 1,E6 M^{-1} s^{-1}$, $k_2 1E-1 s^{-1}$, $k_3 5E6 M^{1} s^{-1}$, $k_4 1E4 M^{1} s^{-1}$, $k_5 1E3 s^{-1}$, $k_6 1M^{3} s^{-1}$, $k_7 1E16 M^{1} s^{-1}$, $k_8 1 s^{-1}$, $k_9 1E10 M^{1} s^{-1}$, $k_{on}GTP 2E1 M^{1} s^{-1}$. Step 7 is virtually irreversible; accordingly, $k_7$ is taken as zero. [GDP], [GTP] and [ATP] = $1E-4 M$; [L] = $5E-8 M$. Panels A-B: The disappearance/degradation of agonist was considered in both cases. To simplify the reaction rate, it was considered to be proportional to [L] (the agonist) with a $k_{off} = 5 M^{1} s^{-1}$. *RU: relative units. (A higher resolution / colour version of this figure is available in the electronic copy of the article).
The models displayed were used as a framework to simulate the degree of enzyme activation as a function of $K_D$, the equilibrium dissociation constant of the agonist/receptor interaction. $K_D$, a measure of the affinity, may be readily determined by means of radioligand binding or by a recently developed homogeneous assay, based in time-resolved FRET and whose benefits outclass those based on radioactivity [15]. The main difference between the two schemes in Fig. 2 is the more or less the coupling capacity of the heterotrimeric G protein to the GPCR. Importantly, it is considered that the agonist progressively disappears (by metabolism, clearance, etc.), as it would occur in any in vivo situation (see Fig. 2 and 3 legends).

GPCR operation involves basal and active states, binding of an agonist (Ago), coupling to the G protein, the GTP/GDP cycle exchange and the G-protein-mediated regulation of AC activity. Using each of the two models, the relative activity of the cyclase was calculated by varying $K_D$ values and fixing $K_D$ value and varying $k_i$ ($k_{s1}$ being deduced from the equation $K_D = k_{on}/k_i$). The kinetic constants for the binding of the agonist to the receptor are usually denoted $k_{on}$ (equal to $k_i$ in Fig. 2) and $k_{off}$ (equal to $k_{i}$ in Fig. 2).

The first scenario considers that the G protein is pre-coupled to the receptor (Fig. 2A) and the second considers a further equilibrium: $R^+G = RG$ (Fig. 2B). On the other hand, pre-coupling has been demonstrated for adenosine and dopamine heteroreceptor complexes [16]. On the other hand, the uncoupled model does not necessarily mean lack of physical RG contact but structural changes that lead to two conformations of the G protein, one prone and another refractory to activation [17, 18]. The output in terms of GS-coupling should be, ideally, in the form of enzyme activity. However, technical challenges impede direct measurement of GS engagement and, to overcome the issue, AC activation is addressed by measuring cytosolic cAMP levels. For this purpose, both the activity of the cyclase upon time and the accumulated cAMP levels have been simulated (in conditions mimicking in vitro pharmacological assays, i.e. in the presence of phosphodiesterase pharmacological assays). Remarkably, the results obtained using the coupled model indicate that the $K_D$ value does not correlate with the potency of agonists to activate AC. In fact, agonists with different affinities may lead to similar potencies when the activity of the cyclase is theoretically calculated using the constraints of the model, in particular considering the action mass law (Fig. 3A). When accumulated levels of cAMP are considered, the conclusion is the same, i.e. the higher the $k_i$ the quicker the rise in the levels of accumulated cAMP. Early determined accumulated cAMP levels depend on $k_i$, even if $K_D$ values differ in two orders of magnitude (Table 1). When time is considered, the effect resulting from quick agonist binding (high $k_{on}$) is a transient peak of cyclase activity that correlates with the $k_i$ ($k_{on}$) value (Fig. 3A). In this model however, irrespective of the height of the peak, which correlates with $k_i$, AC returns to basal activity and, accordingly, accumulated cAMP values stabilize. This would obviously occur in the absence of cAMP degradation (only in vitro). Simulation was performed assuming the presence of phosphodiesterase inhibitors, which are always present in in vitro pharmacological assays of cAMP level determination. In summary, the coupled model predicts that the potency does not change with measurement at different times. As an example, three compounds with the same $K_D$ (10 nM) would have this order of potency according to the $k_i$ (M$^{-1}$s$^{-1}$) values: 2.8E8 >> 2.8E7 > 2.8E6. Obviously, $k_i$ and RT would vary depending on the $K_D$ and $k_i$ values ($K_D$=k$_{on}$/k$_i$; RT=1/k$_i$).

In the RG “uncoupled” model displayed in Fig. 2B, the results in terms of AC activity are similar to those described above for the coupled model, i.e. the height of cyclase activity peak correlates with $k_i$ and not with $K_D$, albeit with different timings of return to basal activity, which was very rapid in the case of high $k_i$ (2.8E8) and very slow for the lowest $k_i$ value (2.8E6) (Fig. 3B). Accumulated cAMP in the presence of phosphodiesterase inhibitors depends, again, in

### Table 1. Accumulated cAMP (in relative units) calculated using the indicated values of $K_D$ (M), $k_{s1}$ (M$^{-1}$s$^{-1}$) and $k_{off}$ (s$^{-1}$) for both coupled and uncoupled models.

| Coupled Model (Fig 2A) | Uncoupled Model (Fig 2B) |
|------------------------|--------------------------|
| $k_i$ | $K_D$ | $k_{s1}$ | cAMP | $k_i$ | $K_D$ | $k_{s1}$ | cAMP |
| 2.9E+8 | 1.0E-10 | 2.9E-2 | 1.66E-15 | 2.9E+8 | 1.0E-10 | 2.9E-2 | 1.71E-16 |
| 1.0E-9 | 2.9E-1 | 1.66E-15 | | 1.0E-9 | 2.9E-1 | 1.71E-16 | |
| 1.0E-8 | 2.9 | 1.65E-15 | | 1.0E-8 | 2.9 | 1.68E-16 |
| 2.9E+7 | 1.0E-10 | 2.9E-2 | 3.85E-16 | 2.9E+7 | 1.0E-10 | 2.9E-2 | 3.7E-17 |
| 1.0E-9 | 2.9E-1 | 3.84E-16 | | 1.0E-9 | 2.9E-1 | 3.69E-17 |
| 1.0E-8 | 2.9 | 3.74E-16 | | 1.0E-8 | 2.9 | 3.56E-17 |
| 2.9E+6 | 1.0E-10 | 2.9E-2 | 4.28E-17 | 2.9E+6 | 1.0E-10 | 2.9E-2 | 4.13E-18 |
| 1.0E-9 | 2.9E-1 | 4.27E-17 | | 1.0E-9 | 2.9E-1 | 4.11E-18 |
| 1.0E-8 | 2.9 | 4.15E-17 | | 1.0E-8 | 2.9 | 3.95E-18 |
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4. DISCUSSION

The results show that the AC activity, and accordingly, the rate of cAMP production correlates with the rate \( k_1 \) of agonist/GPCR binding. Remarkably, once \( k_1 \) is fixed, the variation of \( K_D \) values, even by two orders of magnitude, has a negligible effect on the cyclase activity. The signaling output in terms of initial AC enzymatic rate is roughly proportional to the value of \( k_1 \). These results are based on two reliable models that fulfill the law of mass action and use reported values for kinetic constants; the only assumption is that AC activity depends on the amount of Gs-GTP (active G protein) in a linear fashion. A second assumption, the lack of cAMP degradation, is due to the fact that \textit{in vitro} protocols for [cAMP] determination always include phosphodiesterase inhibitors.

In \textit{in vitro} assays, the dissociation of a hormone, like epinephrine, or of a neurotransmitter, like dopamine, has a negligible effect because it occurs slowly; the RT of hormones in the orthosteric centers of GPCRs may go from several seconds to minutes. Considering recent and sound reports on the kinetics of dopamine receptors, it is confirmed that \textit{in vitro}-measured dissociation occurs in scales of time that cannot be compared with the rate at which neurotransmission takes place. When dopamine, which is very labile and prone to oxidation [19], is released from the nerve terminal of a dopaminergic neuron, dopamine receptors are activated and cAMP levels change. In the case of D1 and D2-like receptors, dopamine leads to AC activation via Gi coupling, while the activation of D2 and D2-like receptors decrease cAMP levels via Gi coupling [20]. Taken together, it appears that \( k_1 \) and anything that depends on \( k_1 \) have little impact in early signal transduction/neurotransmission events. Furthermore, by definition, \( K_D \) (or \( K_i \)) refers to equilibrium and it is often forgotten that signal transduction occurs far from any ligand/receptor equilibrium. The value of \( K_D \) (the affinity constant) in the two most relevant dopamine receptors (D1 and D2) lies in the micromolar range [21, 22] thus suggesting that, if association is relatively rapid, the \( k_{off} \) constant should be fairly low (see Introduction). Hence, the results in this paper suggest the relevance of quick determination of events happening when a molecule enters and activates a given GPCR all the way until the agonist leaves the orthosteric site. In the minutes the receptor molecule keeps the GPCR in inactive conformation, unsolved questions remain, e.g., is signaling occurring all the time? Is occupation per se a desensitization mechanism? How internalization is affected by ligands with different structures and different interaction kinetic parameters?

It is worth mentioning that, among the different steps needed for therapeutic drug approval, several time-dependent aspects should be considered. In contrast to pharmacokinetics and pharmacodynamics, there are kinetic issues related to drug selection in initial screening and kinetic issues related to the time a compound remains in the orthosteric site in a physiological set-up. The results of this paper focus on \( k_1 \), which may be reliably obtained only in \textit{in vitro} experiments. \( k_1 \) values are often obtained when dissociation is boosted by adding an enormous amount of a “free/unlabeled” ligand (usually at 1,000-fold higher than that of the bound ligand). Such difference in concentrations does not occur \textit{in vivo}. Accordingly, RT deduced from \( k_1 \) obtained in \textit{in vitro} assays, is not equivalent to \textit{in vivo} measured RT of a drug whose free concentration would be low and would progressively decrease with time. Surely both are of value in drug discovery. During early drug screening stages, the association rate is relevant for quick selection of compounds whereas “global RT” is relevant for \textit{in vivo} acting therapeutic drugs.

For many years, the developments brought by Copeland have shown the relevance of RT in drug discovery. He and his colleagues distinguish between closed and open systems in relationship to the conditions by which a drug meets the target. A closed system is the one occurring at drug screening and in many experimental \textit{in vitro} set-ups in which the target and the drug remain virtually unchanged. In open systems, the receptor changes its conformation and function with time while the administered drug has to be distributed (usually via blood) to the different tissues, metabolized and cleared away. RT measured in closed systems relies on \( k_{off} \) governing dissociation, which only depends on the receptor target and the bound ligand; the authors argue that the drugs with similar equilibrium (\( K_D/K_i \)) parameters may have quite different \( k_{off} \) values and that it is necessary, at the screening stage, to carefully assess the kinetics of binding to estimate the duration of action, or in authors’ words: “the temporal components of efficacy” (see [23, 24] for review). Apart from pointing out that different mechanisms of drug-target interactions impact on the equation describing \( k_{off} \) the RT in a closed system is \( 1/k_{off} \) whereas, the half time of dissociation is 0.693/\( k_{off} \) and, therefore, the half time of dissociation is 69.3% of RT.

\textit{In vivo} conditions are milder than those used in \textit{in vitro} assays because the dissociation of the ligand bound to a receptor is not forced to occur “rapidly” by adding an excess of unlabeled compound. Therefore, it is assumed that “physiological” RT of a given compound in the orthosteric center could eventually be higher than the \textit{in vitro}-calculated dissociation time. These considerations are more relevant for therapeutic drugs than for endogenous ligands since the dwelling time of a given drug is important for many reasons, in particular to fix the dosage. As an example, a complex model developed to analyze \textit{in vivo} receptor occupancy of atypical drugs, paliperidone and risperidone, to D2Rs, concluded that: “Receptor affinities and brain-to-plasma ratios should be considered before choosing the best PK-PD model for centrally active drugs” [25]. Therefore, the model, related to drugs that act as D2R antagonists, does not attribute the effect of kinetic but thermodynamic receptor parameters in the time course of brain and of plasma concentration of antipsychotics. A more recent study have used pharmacological parameters obtained \textit{in vitro} to perform an \textit{in silico} analysis using an \textit{ad hoc} model for D2R antagonists operation. The majority of the 17 antagonists used, had \( k_{off} \) around 0.003 s\(^{-1}\) [26]. The value for dopamine was 0.028 s\(^{-1}\) (RT= 35 s), i.e., substantially higher than that reported [8] (see
above). The huge discrepancies in $k_{\text{off}}$ (but also in $k_{\text{on}}$) for dopamine and D$_2$R antagonists may be due to the labeled ligand used as reference, and to the experimental approach for binding measurement (radiolabeled versus homogeneous non-radioactive assay). Importantly, the conclusions using the model were: “the $D_2$ receptor antagonist dissociation rate constant ($k_{\text{off}}$) is limited to the maximal rate of fluctuations in dopamine signaling as determined by the dopamine $k_{\text{off}}$ and the cAMP turnover” [26]. Exceptions may occur due to the convergence of a variety of circumstances. In fact, the JNJ-37822681 Janssen compound has fast dissociation kinetics that seem to be responsible for its potential to combat schizophrenia. The dopaminergic system participates in several central processes and dopamine receptors are therapeutic targets in different diseases. A given disease may target a specific receptor but, also, two different diseases may target a specific receptor but with ligands displaying different pharmacology. Drugs blocking D$_2$ receptors act as antipsychotics but extrapyramidal side-effects need to be minimized and this is achieved by fast dissociation compounds. Even at low doses (per oral 0.39 mg/Kg) JNJ-37822681 occupies brain dopamine D$_2$ receptors and it is effective in psychosis models. According to the fast dissociation kinetics, this compound would not have been selected for further development based on $K_i$ (or $K_D$) values; in fact the compound’s $K_i$ is 158 nM [27, 28]. It seems that schizophrenia may benefit from the atypical pharmacology, i.e. fast dissociation rate, of JNJ-37822681. The authors of these reports indicate that in vitro (binding) studies would not be appropriate to select compounds with the properties of JNJ-37822681 and suggest that: “fast dissociation from the D$_2$ receptor may result in more flexible levels of D$_2$ receptor blockade, allowing D$_2$ receptors to react rapidly to rising dopamine levels in response to environmental stimuli”.

CONCLUSION

As a conclusion, and with some exception as the one detailed in the previous paragraph, it is shown that $k_{\text{on}}$ appears crucial for selecting drug candidates in early screening stages, whereas $K_D$ would serve to calculate a reference $k_{\text{off}}$ value to address in vivo RT and/or drug latency. As pointed out by Copeland, in vivo calculated RT would fit with a balanced action, i.e., a compromise between a long effect but not too long as to lead to undesired side effects [24].

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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