REVIEW

[View Article Online](#)
[View Journal](#) | [View Issue](#)

Cite this: *Med. Chem. Commun.*,
2018, 9, 1426

Link between a high k_{on} for drug binding and a fast clinical action: to be or not to be?[†]

Georges Vauquelin

Review articles on binding kinetics essentially focus on drugs that dissociate slowly from their target since this is required for the successful treatment of many pathophysiological conditions. Recently, the therapeutic benefit of a high k_{on} (i.e. the second order association rate constant) has also been linked to fast association and to a fast clinical action. Other studies, however, called this assertion into question since additional factors, like the dosing paradigm and the binding mechanism, are important as well. The still ongoing reticence about integrating binding kinetics in lead optimization programs motivated us to critically review the link between the drug's kinetic rate constants and their *in vitro* and *in vivo* target occupancy profile, with special focus on k_{on} . The presented simulations tally with a positive link between a drug's effective/observed association rate (which is quite easy to determine *in vitro*) and the swiftness of its clinical action. On the other hand, the simulations show that the k_{on} -concept should not be confounded with the effective association process since increasing this parameter only enhances the drug's *in vitro* and *in vivo* association under certain conditions: the binding mechanism should be suitable, rebinding (and thus the factors within the target's micro-environment that favour this mechanism) should not be too prominent and the dosage should not be kept in par with the drug's affinity. Otherwise, increasing k_{on} could be ineffective or even be counter-productive.

Received 11th June 2018,
Accepted 15th August 2018

DOI: 10.1039/c8md00296g

rsc.li/medchemcomm

Department of Molecular and Biochemical Pharmacology, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. E-mail: gvauquel@vub.be
[†] Electronic supplementary information (ESI) available. See DOI: 10.1039/c8md00296g

Georges Vauquelin

Prof. Georges Vauquelin (now retired) led the Department of Molecular and Biochemical Pharmacology at the Free University of Brussels (VUB) in Belgium. His training in chemistry allowed him to perform the first purification of beta-adrenergic receptors (PhD, 1978). Subsequent research focused on ligand-GPCR interactions by radioligand binding and functional measurements, first on isolated cell membranes and

then on intact cell systems expressing endogenous and/or transfected receptors. Focus gradually moved to binding kinetics and micro-PK phenomena like rebinding and the participation of cell membranes. Simulations are now extensively used to further explore these topics.

Introduction

The efficacy and the duration of a drug's clinical action are governed by its pharmacokinetic (PK) and pharmacodynamic (PD) properties.¹ PK and PD have for a long time been considered to be separate but complementary disciplines. Based on the premise that the temporal fluctuation of a drug's free concentration suffices to determine the duration of its pharmacological effect, the PK approach was deemed sufficient to tackle this aspect. On the other hand, based on the premise that equilibrium equations are sufficient to describe drug-target interactions, past preclinical PD screening studies essentially aimed to optimize drug candidates in terms of their efficacy and potency/affinity. Yet, the seminal articles by Swinney in 2004 (ref. 2) and Copeland *et al.* in 2006 (ref. 3) paved the way for the present notion that the kinetic rate constants of drug binding (binding kinetics, BK, a sub-section of PD) are even more pertinent than its affinity. Indeed while equilibrium binding can be neared in a "closed" system such as in *in vitro* experiments in which the drug and the target are present at constant concentrations, it does not apply to an "open system" like the human body in where the concentration of the free drug changes constantly with time.^{4–6} The by now numerous other review articles on BK essentially focused on drugs that dissociate slowly from their target (often referred to as long

“residence time”, i.e. $\tau = 1/k_{\text{off}}$, where k_{off} stands for the first-order dissociation rate constant).⁷ This focus embodies the notion that long-lasting target occupancy is required for the successful treatment of many pathophysiological conditions and some slow dissociating drugs, such as the AT₁ angiotensin II receptor antagonist candesartan and the M₂ muscarinic receptor antagonist tiotropium, have regularly been cited to illustrate this concept.⁸

In contrast to k_{off} , it is only recently that attention has been paid to the impact of k_{on} (i.e. the second order association rate constant) on the drug's *in vivo*-like target occupancy *versus* time profile in PK-PD simulations. Most notably, the therapeutic benefit of a high k_{on} has repeatedly been linked to fast association and to a fast clinical action.^{9–12} Other studies, however, drew attention to the fact that this assertion should be appraised with caution since the results depend on additional factors like the adopted dosing paradigm, the binding mechanism and intervening micro-PK mechanisms.^{13–16} Alas, dissonant assertions in some of those studies may lead to confusion. Since there is still some reticence about integrating BK in lead optimization programs,^{17,18} we here review and compare the impact of the drug's kinetic rate constants on their *in vivo* occupancy profile, with special attention to the impact thereon of the dosing paradigm, the mode of drug-target interaction and drug rebinding. Special focus will be on k_{on} since the clinical pertinence of this BK parameter has only been tackled recently.

Background and one-step binding drugs

In compliance with principle of Occam's razor, drug (L)–target (R) binding is still primarily regarded to take place in a single step for analysing experimental data and for the vast majority of published PK-PD simulations. However, spectroscopic and molecular modelling studies increasingly disclose that the initially bound (RL) complexes go through multiple conformational adjustments for the drug to reach its orthosteric binding pocket.^{19–22} Here again for simplicity, two-step binding models are commonly adopted to represent such situations.^{2,4,11,23,24} The rate constants that govern these individual steps are commonly referred to as “microscopic” (and here denoted as k_1 to k_4) while the all-embracing ones, k_{on} for association and k_{off} for dissociation, are referred to as “macroscopic”.²⁵

The simplest binding model is a one-step reversible bimolecular process in which only one complex, RL, is produced (Fig. 1, top). Because of this single step, k_{on} and k_{off} can be used in synonymy with the second-order association rate constant k_1 and the first-order dissociation rate constant k_2 respectively. The RL complex will dissociate mono-exponentially with time in *in vitro* washout experiments (i.e. after removal of free drug in solution and/or by preventing it to (re)associate) (Fig. 1, bottom).²⁶ This allows a convenient calculation of k_{off} . In *in vitro* association experiments (i.e. in where targets are exposed to a fixed concentration of free

Fig. 1 Simplest one-step reversible binding model and *in vitro* kinetic data. R and L stand for the target and the drug, k_1 for the second-order association rate constant (in $\text{M}^{-1} \text{min}^{-1}$), k_2 for the first-order dissociation rate constant (in min^{-1}), k_{obs} for the pseudo-first order association rate constant (in min^{-1}) and K_D for the equilibrium dissociation constant (in M). k_{on} and k_{off} are the “macroscopic”/experimentally obtained rate constants.²⁵ Bottom from left to right: simulated dissociation experiments in where target is first incubated with drug. Then, free drug is removed at the onset of the wash-out phase and the time-wise decline in occupancy is recorded. Simulated association experiments in where target is incubated for increasing time periods with drug. While the effect of changing k_1 can be annihilated by an equivalent but opposite change of $[L]$, changing k_2 remains effective at all $[L]$. “c” stands for control. Parameters for control (c): $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{min}^{-1}$, $k_2 = 0.023 \text{ min}^{-1}$ (i.e. dissociation $t_{1/2} = 30 \text{ min}$). $[L] = K_D$ for association and, for dissociation, $[L] = 9 \times K_D$ for preincubation and set to 0 to start the washout. Simulations: data points were obtained by repeatedly, simultaneously implementing the differential equations describing all the intervening binding steps as previously described.^{28,93} Curves are generated by non-linear regression analysis by GraphPad Prism based on the simplest model relevant to each situation.

drug) target occupancy will also progress mono-exponentially with time (Fig. 1, bottom). This “observed” association process is delineated by the “pseudo first-order” association rate constant, k_{obs} . Of note is that k_{obs} is a composite parameter that is not only related to k_{on} but also to $[L]$ and even to k_{off} (*i.e.* $k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[L]$) (Fig. 1, top).^{4,27} Importantly, this equation reveals that k_{on} is not the sole factor that is able to delineate the observed association process. It is also noteworthy that, when comparing the association kinetics of different drugs, increasing k_{on} or $[L]$ alike produces the same outcome and that the effect of one such parameter can be annihilated by an equivalent but opposite change of the other one (Fig. 1, bottom). In this respect, the equivalence between k_{on} or $[L]$ can be placed in a more clinical context by substituting k_{on} by k_{off}/K_D to yield $k_{\text{obs}} = k_{\text{off}}(1 + [L]/K_D)$ (Fig. 1, top). This rearranged equation illustrates that changing k_{on} (and thus also K_D) will not affect k_{obs} if $[L]$ is adapted to keep the $[L]/K_D$ ratio of each drug constant.

Those binding parameters refer to a closed *in vitro* system (*i.e.* a system in where the total drug concentration remains constant⁶). However, living organisms are open systems in where drug concentrations vary constantly. Hence, drug dissociation will be more *in vivo*-like when $[L]$ is allowed to decline mono-exponentially with time (with rate constant k_e). Under such conditions $[RL]$ becomes likely to decline with an initial lag phase. This pattern also applies to theoretical instant equilibrium binding (*i.e.* $[RL]$ equals always $[R_{\text{tot}}]/(1 + K_D/[L])$ when the association and dissociation proceed infinitely fast) and thus merely reflects the hyperbolic relationship between $[RL]$ and $[L]$.^{14,28} Another important characteristic of such dissociation paradigm is that a low k_{off} only contributes to long-lasting occupancy if it drops below k_e .^{4,29,30}

Yet, to be even more relevant from the clinical perspective, $[L]$ should be allowed to vary with time in a bell-shaped fashion, with an initial rapid increase followed by a slower decline, such as after *in vivo* dosing (Fig. 2 to 5). Interestingly, the above-mentioned lag phase also shows up in therewith-corresponding pharmacokinetics–pharmacodynamics (PK–PD) simulations. Such simulations have hitherto mainly focussed on k_{off} and, here again, it was shown that slow dissociation only brings about longer-lasting occupancy when $[L]$ gets eliminated more swiftly (Fig. 2B).^{14,31} Additionally, very slow dissociation will eventually also “depress” the profile by delaying the appearance of peak occupancy and by decreasing its magnitude. Those effects are inappropriate when the goal is to procure high target occupancy straight on, but they do gradually fade after repeated dosings and/or can be alleviated by increasing the dosage.^{14,15} To better comprehend the impact of k_{on} and k_{off} on the *in vivo*-like occupancy profiles of different drugs, combinations thereof are presented in a “kinetic landscape”-format in Fig. 2B and C for the three prevailing dosing paradigms.

The “constant $[L_{\text{max}}]/K_D$ ratio” dosing paradigm (Fig. 2B) (*i.e.* in where the dosage varies in par with the K_D) is claimed to be the most relevant one from the clinical perspective³¹ but it has only been adopted for some of the PK–PD model-

ling studies (*e.g.* ref. 14, 16 and 31). Here, changing k_{on} does not impact the occupancy profile since its effect is cancelled out by an inverse change of $[L]$ at any time point.¹⁴ In contrast, decreasing k_{off} (*i.e.* from top to bottom) is still able to bring about longer-lasting occupancy and eventually even to depress the profile once it drops below k_e .

Alternatively, the dosage has also been kept constant irrespective of the drug's K_D in some of the published simulations.^{3,4,9,22} Such “constant $[L_{\text{max}}]$ ” conditions allow increasing k_{on} (Fig. 2C, from left to right) to speed up the initial ascending phase, to increase peak occupancy and to prolong the subsequent lag phase. Yet, those effects can simply be ascribed to the increased $[L_{\text{max}}]/K_D$ ratio since the same outcome is also observed for instant equilibrium situations. In contrast, changing k_{off} (Fig. 2C, from top to bottom) will not only impact the occupancy profile by affecting the $[L_{\text{max}}]/K_D$ ratio but also by itself once it drops below k_e (Fig. 2C, bottom).

Finally, occupancy profiles in diagonal order (marked with an asterisk in Fig. 2B and C) refer to the constant $[L_{\text{max}}]$ -constant K_D dosing paradigm (*e.g.* ref. 32) in where k_{on} and k_{off} need to change in par to keep K_D constant. Since this paradigm constitutes a special situation of the constant $[L_{\text{max}}]/K_D$ ratio paradigm,¹⁴ differences between the occupancy profiles can here also be attributed to differences in k_{off} only.

Two-step binding drugs

A two-step “induced fit” binding model, in where the initially formed RL complex isomerises into a more stable R'L state is considered to account for the binding of most of drugs with high affinity and clinical efficacy.^{7,21} On the other hand, a “bivalent drug” binding model,^{33–35} in where both binding domains/pharmacophores of an antibody/drug bind in succession to their cognate sites at the target, is commonly used to represent the binding of immunoglobulins and of increasingly popular synthetic bivalent constructs.^{36–39} Both models can be represented by the same simplified scheme that comprises four “microscopic” rate constants (k_1 to k_4) and whose values determine their composite “macroscopic” K_D and k_{off} (Fig. 3A).^{15,40}

For the “induced fit” model, k_1 and k_2 account for the initial association and dissociation, k_3 for the isomerisation of RL into R'L and k_4 for the reverse isomerisation.²⁵ It is the high stability of R'L (*i.e.* a low k_4) that is considered to be chiefly responsible for their slow dissociation and high affinity.²¹ The “bivalent binding” model rather stipulates that binding of the first pharmacophore (governed by k_1 and k_2) prompts the second one to stay close to its cognate binding site at the target and, because of its resulting very high local concentration, to associate very fast (*i.e.* with a high composite k_3).^{33–35} This also prompts a partly dissociated RL complex to progress back to the fully bound R'L state as long as one pharmacophore is still bound. Here, it is the repeated switches between RL and R'L that contributes to the high affinity (also denoted as avidity) and slow dissociation of such drugs.

Fig. 2 Simulated target occupancy vs. time curves by one-step binding drugs with different binding kinetics after a single *in vivo* drug administration according to different dosing paradigms. For more information, please see ref. 14. Panel A) Model in where $[L]$ evolves during 24 h according to the Bateman function⁹⁴ to yield $[L]_t$ at each post-dosing time: k_a and k_e are the first-order rate constants for drug's inflow into and elimination from the target-containing body compartment; ' f ' accounts for the drug's dose, bioavailability and volume of distribution, and is presently pre-set to obtain the maximal drug concentration ' $[L_{\max}]$ ' to yield desired maximal target occupancy ' $[RL_{\max}]$ ' for instant equilibrium binding (i.e. when the association and dissociation proceed infinitely fast, so that $[L_{\max}] = K_D / ([R_{\text{tot}}] / [RL_{\max}] - 1)$). Panel B) Simulated target occupancy vs. time profile for the constant $[L_{\max}]/K_D$ ratio dosing paradigm for the different k_1 - k_2 combinations shown. $[L_{\max}] = 9 \times K_D$, yielding 90% occupancy at theoretical instant equilibrium for all (shown as control). Other occupancy profiles refer to more realistic *in vivo*-like situations and differ because association and dissociation is not instantaneous. In practice, peak binding will be less and will be attained later on for actual *in vivo*-like situations. Please note that k_1 does not impact the occupancy profiles and that a low k_2 only impacts them sizably when it drops below k_e . Panel C) Simulated target occupancy vs. time profile for the constant $[L_{\max}]$ dosing paradigm for the same k_1 - k_2 combinations as for panel B. $k_a = 0.023 \text{ min}^{-1}$ ($t_{1/2} = 30 \text{ min}$) and $k_e = 0.00575 \text{ min}^{-1}$ ($t_{1/2} = 2 \text{ h}$). Control curves refer to theoretical instant equilibrium. Other curves refer to the *in vivo*-like situations. $[L_{\max}] = 20.7 \text{ nM}$ for all (this corresponds to $9 \times K_D$ for occupancy profiles marked with '*'). Those iso- K_D -based profiles are identical in panels B and C. Please note that changing k_1 can now impact the profiles by changing K_D (and thus also the $[L_{\max}]/K_D$ ratio).

Fig. 3 Two-step binding drug models (panel A), simulated dissociation curves (panel B), association curves (panel C) and target occupancy vs. time curves after a single *in vivo* administration (panel D) for drugs with different binding kinetics. Panel A) Overall, simplified two-step binding model and equations. $k_1 \dots k_4$ are microkinetic rate constants, k_{off} the composite macroscopic first-order dissociation rate constant and K_D the value of $[L]$ at which half of the targets are occupied at equilibrium (note that the occupancy includes both RL and R'L). RL stands for the initial, transient complex for induced-fit binding and the partially-bound target for bivalent binding while R'L stands for the final, long-lasting complex for induced-fit binding and the fully-bound target for bivalent binding. Both models can be differentiated based on the k_3/k_2 -ratio. For more information, please see ref. 15. Panel B) Simulated dissociation curves for induced fit-, heterobivalent- (i.e. with distinct pharmacophores) and homobivalent drugs (i.e. with identical pharmacophores). "c" stands for control. Dissociation is most affected by k_4 , equally affected by $k_2 \dots k_4$ and now also affected by k_1 (via k_3), respectively. Yet, this latter effect is still less than the combined effects of k_2 and k_4 . Parameters for control (c) of all: $[L] = 9 \times K_D$ for preincubation and set to 0 for washout. Induced-fit for control (c): $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, $k_2 = 4 \text{ min}^{-1}$, $k_3 = 0.25 \text{ min}^{-1}$, $k_4 = 0.0125 \text{ min}^{-1}$. Heterobivalent for control (c): $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, $k_2 = 4 \text{ min}^{-1}$, $k_3 = 64 \text{ min}^{-1}$, $k_4 = 0.2 \text{ min}^{-1}$. Homobivalent for control (c): $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, $k_2 = 1 \text{ min}^{-1}$, $k_3 = 64 \text{ min}^{-1}$, $k_4 = 1 \text{ min}^{-1}$. Panel C) Simulated association curves for the same drugs as in panel B. Parameters for control (c): $[L] = K_D$ for all, $k_1 \dots k_4$ same as for corresponding binding mechanisms in panel B. Please note that the effect of changing k_1 can only be annihilated by an equivalent but opposite change of $[L]$ for the induced fit- and heterobivalent binding models. Panel D) Simulated target occupancy vs. time profile for the constant $[L_{\text{max}}]/K_D$ ratio dosing paradigm for the same drugs as in panel B and peak occupancy at instant equilibrium amounts 75% of the targets for all (not shown). Similar as for panels B and C, k_1 is only able to impact the occupancy profile of the homobivalent drug (via k_3) but the combined effects of k_2 and k_4 are still more pronounced. Parameters for control curves (c): $k_a = 0.0115 \text{ min}^{-1}$ ($t_{1/2} = 60 \text{ min}$), $k_e = 0.00575 \text{ min}^{-1}$ ($t_{1/2} = 2 \text{ h}$), $[L_{\text{max}}] = 3 \times K_D$ to yield for all, $k_1 \dots k_4$ are the same as for corresponding binding mechanisms in panels B and C.

From the mathematical perspective, induced fit- and bivalent binders can be distinguished based on their k_3/k_2 ratio; *i.e.* <1 for the former and >1 for the latter (which is responsible for the repeated switches between RL and R'L before the drug finally fully dissociates) (Fig. 3A).¹⁵ The composite k_{off} of those drugs affects their *in vitro* dissociation- and *in vivo* occupancy profiles in closely the same way as for the simple one-step mechanism.¹⁵ Accordingly, the impact of changing k_2 , k_3 and k_4 on those profiles correlates with their relative contribution to k_{off} . This impact differs markedly between both mechanisms. On the one hand, k_4 clearly acts as the “kinetic bottleneck” for the dissociation of induced fit binders. Hence, changing k_4 impacts their profiles appreciably more than changing k_2 or k_3 (Fig. 3B–D, left). On the other hand, decreasing k_2 or k_4 or increasing k_3 depresses the occupancy profiles of the heterobivalent binders nearly equally (Fig. 3B–D, mid).

Differences between the induced fit- and bivalent mechanisms also apply to the k_{on} -concept. While k_{on} is still a constant (and reasonably close to k_1) for bivalent binding, this is no longer the case for the induced fit binding as well as for some other complex mechanisms.^{4,27} Indeed, k_{obs} vs. $[L]$ plots are well known to be linear for one-step binding and this offers a convenient method to calculate k_{off} and k_{on} (corresponding to the intercept with the ordinate and to the slope, respectively) and, hence, also to calculate K_D . Such as initially presented by Strickland *et al.*,²⁷ they are also quasi-linear for bivalent binding (allowing an approximation of k_1 and k_{off}) but become hyperbolic for induced fit binding. Hence, to deal with more complex binding mechanisms in general, it becomes more pertinent to focus on the impact of the microscopic rate constant of the initial binding event (here k_1) on the *in vitro*- and *in vivo* binding profiles. As shown in Fig. 3C, increasing k_1 or $[L]$ alike produces the same outcome in simulated *in vitro* association experiments with induced fit- and bivalent binders. Also, changing k_1 will not affect their *in vivo* occupancy profile when the PK–PD simulations are based on the constant $[L_{\text{max}}]/K_D$ ratio paradigm (Fig. 3D).¹⁵ Taken together, these findings comply with the formulation of k_{obs} as $k_{\text{off}}(1 + [L]/K_D)$ and with the notion that (here again) changing k_1 affects their association- and occupancy profiles by impacting K_D (defined as $[L]$ at which half of the targets are occupied at equilibrium).¹⁵

It is important to note that, while changing individual microkinetic parameters is pertinent for heterobivalent drugs (*i.e.* which carry distinct pharmacophores), k_1 and k_3 are (at least in theory) predisposed to change in par in case of homobivalent drugs (*i.e.* with identical pharmacophores) and the same interdependence may also apply to the k_2 – k_4 tandem. Simulations that are based on those premises show that a combined increase of k_1 and k_3 results in slower *in vitro* association than what can be obtained by an equivalent increase of the $[L]/K_D$ ratio by acting on $[L]$ only (Fig. 3C, right). This stems from the fact that increasing k_3 also decreases k_{off} . In the same vein, increasing k_1 (along with k_3) is now also able to dampen the *in vivo* occupancy profile, albeit to a lesser extent than when changing k_2 (along with k_4) (Fig. 3D, right).

Homotropic cooperativity for one-step binding drugs

The impact of allosteric interactions on drug binding are commonly represented by the allosteric ternary complex model (ATCM) in where two drug molecules alter each other's K_D by changing the conformation of a common target (or target complex) with two spatially separated binding sites.⁴¹ Those molecules may also be alike (*i.e.* “homotropic” cooperativity) such as for some GPCRs and other receptors that dimerize.^{42–45} The ATCM allows binding of the first drug molecule to impact the K_D of the second binding step and the cooperativity factor, α , defines the magnitude thereof (*i.e.* the K_D of the second step decreases for positive cooperativity ($\alpha > 1$) and increases for negative cooperativity ($\alpha < 1$)). At the more fundamental level, this cooperativity does affect the K_D of the second step by changing the association rate or the dissociation rate of the second molecule⁴⁶ (or both, not shown). Yet, saturation binding curves still overlap at equilibrium (Fig. 4A). While the curves without cooperativity are sigmoidal, those are shallower when $\alpha = 0.1$ and steeper when $\alpha = 10$. Such difference in shape is routinely quantified by the Hill coefficient (nH), with values of <1 and >1 , respectively.

To deal with non-equilibrium *in vivo* situations, we next compare the repercussions of changing the microscopic rate constants of such drugs on their *in vitro* association- and *in vivo* occupancy profiles. The four modes of cooperativity have in common that the *in vitro* association curves overlap when increasing k_1 or $[L]$ alike. The effect of increasing one of those parameters is also effectively cancelled out by an equivalent decrease of the other one (Fig. 4B). On the other hand, while association-based cooperativity does not affect the *in vitro* dissociation curves, dissociation-based cooperativity will decelerate (when $\alpha > 1$) or accelerate (when $\alpha < 1$) the dissociation and eventually even produce biphasic curves (data not shown).⁴¹ The *in vivo* occupancy profiles (Fig. 4C) comply with those *in vitro* observations. PK–PD simulations that are based on the constant $[L_{\text{max}}]/K_D$ ratio paradigm reveal that changing k_1 does not affect the occupancy profile, whatever the mode of cooperativity. On the other hand, decreasing k_2 is eventually able to depress the profile for all four cooperativity situations and this phenomenon is most pronounced for dissociation-based negative cooperativity.

Drug rebinding

Changing k_1 could also affect a drug's *in vivo* binding properties *via* “micro”-pharmacokinetic mechanisms like rebinding.⁴⁷ In this respect, *in vitro*- as well as in *ex vivo* wash-out experiments have shed light on the ability of rebinding to slow down drug dissociation in “infinite dilution” conditions (*i.e.* when only free drug was removed)^{28,48–51} and recent *in vivo* observations further illustrate the relevance of this phenomenon.^{52,53} Moreover, PK–PD simulations also suggest that this allows “borderline” drugs (*i.e.* those that dissociate slowly but still not as slow as

A) Homotropic cooperativity model

B) Effect of k_1 & k_2 on *in vitro* association C) Effect of k_1 & k_2 on *in vivo* occupancy (constant $[L_{max}]/K_D$)

Fig. 4 Homotropic cooperativity variants: models and simulated saturation curves (panel A), association curves (panel B) and target occupancy vs. time curves after a single *in vivo* drug administration (panel C) for drugs with different binding kinetics and/or cooperativity. Panel A) Overall, simplified model for drug binding to two sites at the target and equations. RL stands for the single-bound target and RL_2 stands for the double-bound target. The formation and dissociation of RL is governed by k_1 and k_2 and the formation and dissociation of RL_2 is governed by k_3 and k_4 . The magnitude of the latter is now also determined by the cooperativity factor, α , on top of k_1 and k_2 . For more information, please see the main text. The graph at the right side shows that, while cooperativity can act on the association and/or the dissociation of the second binding step,⁴⁶ this still yields overlapping shallow-(for $\alpha < 1$) or steep (for $\alpha > 1$) saturation binding curves at equilibrium. Parameters: $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ and $k_2 = 0.023 \text{ min}^{-1}$ (for dissociation $t_{1/2} = 30 \text{ min}$) for all. Incubation time: 1000 min. Panel B) Simulated association experiments in where target is incubated for increasing time periods with drug. The impact of changing k_1 (along with k_3) can be annihilated by an equivalent but opposite change of $[L]$ for all four cooperativity situations. "c" stands for control. Please note that curves for association- and dissociation based negative cooperativity nearly overlap and are not shown separately. Parameters for control (c) of all: $k_1 = 1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, $k_2 = 0.023 \text{ min}^{-1}$ and $[L] = k_2/k_1$ for all, $\alpha = 10$ for positive cooperativity and 0.1 for negative cooperativity. Dotted curves are for the absence of cooperativity (i.e. $\alpha = 1$). Panel C) Simulated target occupancy vs. time profile for the constant $[L_{max}]/K_D$ ratio dosing paradigm for the same drugs as in panel B. Changing k_1 (along with k_3) does not impact the occupancy profile for all four cooperativity situations. In contrast, a clear-cut effect is observed when decreasing k_2 (along with k_4) sufficiently. Such as in panel B, the curves for association- and dissociation based negative cooperativity overlap. Parameters for control (c) of all: $k_1 = 1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and $k_2 = 0.023 \text{ min}^{-1}$, $k_a = 0.023 \text{ min}^{-1}$ ($t_{1/2} = 30 \text{ min}$) and $k_e = 0.00575 \text{ min}^{-1}$ ($t_{1/2} = 2 \text{ h}$); $[L_{max}] = 2 \text{ nM}$. Hence, the dosage is only adapted to keep the $[L_{max}]/K_D$ ratio constant when changing k_1 or k_2 for each individual figure.

their *in vivo* elimination) like candesartan to transcend this boundary.^{14,15} The effect of rebinding on drug dissociation has been attributed to the ability of dissociated drug molecules to go through repeated encounters with the targets and/or those nearby before drifting further away.⁵⁴ This highly localised mechanism (which may also be regarded to produce an excess of drug molecules in the immediate vicinity of their targets) stems from hindered 3D drug diffusion by the presence of natural obstacles and/or confined spaces such as synapses and cells.^{6,55} Fast target association, a high local target concentration (such as inside cells) and/or a high local surface density thereof (such as in membrane microdomains) do contribute as well.^{15,16,51,54,56} Interestingly, those very same factors will also slow down the observed drug association because of their ability to deplete the drug concentration in the immediate vicinity of their targets.

The most common mathematical formulation of such “limited diffusion”-related rebinding¹⁵ requires k_1 and k_2 to be divided by $(1 + k_1[R]/k)$ to yield the ‘effective’ forward and reverse rate coefficients k_f and k_r (Fig. 5A). The $k_1[R]/k$ -factor accounts for the positive contribution of k_1 , the local concentration of free targets and/or their surface density on membranes. The parameter, ‘ k ’, depends on the free drug’s diffusion rate and the geometric characteristics of the target’s micro-anatomic environment.^{16,28,48,49,51,54,55} Of note is that rebinding is most effective at low target occupancy (Fig. 5A) and the $k_1[R_{\text{tot}}]/k$ product (denoted as the “rebinding factor”, F_r) constitutes a measure for the “firmness” of rebinding. Because the contribution of k_1 and k may differ from one drug to another, F_r may differ as well.¹⁴

Rebinding slows down the *in vitro* association and dissociation of one-step binding drugs and PK-PD simulations that include rebinding show that, quite alike decreasing k_2 , increasing k_1 is able to bring about longer-lasting occupancy and eventually also to “depress” the profile (Fig. 5B).¹⁴ Fig. S1 in the ESI† section further compares the impact of the different dosing paradigms on the effect of increasing k_1 for such one-step binding drugs in the presence of rebinding. This effect is clearly most outspoken for the constant $[L_{\text{max}}]/K_D$ ratio dosing paradigm. This may sound astonishing at first glance but it can be explained by the fact that an adjustment of the dosage compensates for the k_1 -mediated decrease of the drug’s K_D but not for the genuine effect rebinding (since it does not impact K_D). In contrast, the decreased K_D is no longer compensated for when the dosing proceeds according to the constant $[L_{\text{max}}]$ paradigm. The $[L_{\text{max}}]/K_D$ ratio thus increases and this effect now adds-up to the genuine effect of rebinding (Fig. 2B). Finally, the effect of rebinding is appreciably less pronounced when the dosing proceeds according to the constant $[L_{\text{max}}]$ -constant K_D dosing paradigm since it is largely offset by a higher k_2 to keep the K_D constant.

Interestingly, induced fit- and bivalent binders show different susceptibility to rebinding.¹⁵ Changing k_1 can impact the occupancy profile of both but for the induced fit binders its effect (like that of k_2 or k_3) is still less pronounced than when changing k_4 . For bivalent binders, however, k_1 is now

nearly as effective as changing the other three rate constants separately (Fig. 5C, left and mid). In the same vein, increasing k_1 for homobivalent drugs increases rebinding for the first step as well as k_3 for the second step. This combined action dampens the occupancy profile quite severely and nearly as much as by decreasing k_2 (along with k_4) (Fig. 5C, right). Of note is that, because the very high local concentration of the remaining free pharmacophore of bivalent drugs in general (giving rise to “forced proximity”-related rebinding)¹⁵ only the initial binding step is susceptible to (limited diffusion-related) rebinding. This is not so for homotopic cooperativity for which both steps are susceptible to (limited diffusion-related) rebinding because they embrace the binding of free drug molecules. In principle, both steps should be equally susceptible to rebinding for dissociation-based cooperativity while the second step should be more- or less susceptible to rebinding for association-based positive- or negative cooperativity, respectively (since rebinding is now acted on by $\alpha \cdot k_1$ instead of k_1). Yet, the present PK-PD simulations suggest that these differences may be rather subtle. More importantly, they reveal that increasing k_1 dampens the *in vivo* occupancy profiles nearly as efficiently as a decreasing k_2 for all four cooperativity situations in the presence of rebinding (Fig. 5D).

Discussion

Fast drug-target association and a corresponding swift clinical action have been called on to back the therapeutic benefit of a high k_{on} .^{9–12} Based on simulated *in vitro* and *in vivo* kinetic data, we here come up with a subtler picture or, in other words, that “conditions apply”. To begin with, it is also useful to recall two essential basic principles. First, whereas macroscopic (k_{on}) the microscopic- (k_1) association rate constants can be used in synonymy for one-step binding drugs, the association of two-step induced-fit binders is already too complex to be represented by such macroscopic rate constant.^{4,27} For this and other more complex binding mechanisms it is more relevant to focus on the microscopic rate constant that deals with the first association step (here k_1). Second, the k_{on} / k_1 -concept should not be confounded with the observed association process. Indeed, even for the simplest one-step binding model, this process is governed by k_{obs} , a composite first-order rate constant which depends on k_{on} , k_{off} as well as on free drug concentration, $[L]$.

Most importantly, past¹⁴ and present simulations call attention to the important role of the dosing paradigm when comparing the *in vitro* and *in vivo* association kinetics of different one-step binding drugs (*i.e.* the most widely used binding model to comply with principle of Occam’s razor). Indeed, whereas k_{obs} is traditionally expressed as $k_{\text{off}} + k_{\text{on}}[L]$,^{4,27} it can easily be reformulated as $k_{\text{off}}(1 + [L]/K_D)$. According to this alternative formulation, increasing k_{on} can only act on k_{obs} by decreasing the drug’s K_D . In agreement therewith, the PK-PD modelling simulations show that increasing k_{on} does indeed accelerate the initial occupancy and

Fig. 5 “Limited diffusion”-related rebinding is included in models for one-step binding drugs (panel A), two-step binding drugs (panel B) and homotropic cooperativity (panel C). For more information, please see ref. 14 and 15. Panel A) Impact of rebinding for one-step binding drugs.¹⁵ To invoke rebinding k_1 and k_2 are usually replaced by rate coefficients k_f and k_r . In accordance with those equations, the graph at the right side shows that rebinding is most effective when $[R] = [R_{tot}]$ and decreases when the occupancy increases. The therewith-corresponding “rebinding factor”, $k_1 \cdot [R_{tot}] / k$ (further denoted as F_r), provides a measure of the “firmness” of rebinding and may differ from one drug to another. Panel B) Simulated *in vitro* and *in vivo* occupancy profiles for one-step binding: impact of rebinding. “c” stands for control. Including rebinding delays the drug’s *in vitro* association (left side) as well as dissociation (mid) but without changing the drug’s K_D , and therefore also without changing the $[L_{max}] / K_D$ ratio for *in vivo* dosing (right side). In this respect, simulated target occupancy vs. time profiles show that including rebinding now permits an increase of k_1 to impact those profiles in the same way (and nearly as efficiently¹⁴) as decreasing k_2 . Parameters for control (c) of all: $k_1 = 1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, $k_2 = 0.023 \text{ min}^{-1}$ (for dissociation $t_{1/2} = 30 \text{ min}$). For *in vitro* association and dissociation: $F_r = 0$ (i.e. no rebinding) for control (c), $[L] = 9 \times K_D$ for association and set to 0 to start the washout. For *in vivo*: $[L_{max}] = 9 \times K_D$, $k_a = 0.023 \text{ min}^{-1}$ ($t_{1/2} = 30 \text{ min}$) and $k_e = 0.00575 \text{ min}^{-1}$ ($t_{1/2} = 2 \text{ h}$). Panel C) Simulated *in vivo* occupancy profiles for two-step binding: impact of rebinding. Rebinding only affects the initial binding step here and increasing k_1 10-fold will also increase F_r alike. Although the dosing is conducted according the constant $[L_{max}] / K_D$ ratio paradigm, increasing k_1 is now able to affect the occupancy profile *via* the intermediary of rebinding. For induced-fit binding drugs (left), this impact is quite similar a swith changing k_2 and k_3 but still appreciably less than with changing k_4 . For heterobivalent drugs (mid), the impact of k_1 is now nearly equal to that of three other microkinetic rate constants. For homobivalent binding drugs (right), increasing k_1 is now able to affect the occupancy profile *via* the intermediary of rebinding as well as *via* an equivalent increase of k_3 . This combined effect now allows k_1 to exert a substantial impact on the occupancy profile and nearly as much as the k_2 – k_4 combination. Parameters for controls (c): $F_r = 2$ for induced fit and heterobivalent binding, $F_r = 3$ for homobivalent binding; all other parameters are the same as for corresponding mechanisms in Fig. 3D. Panel D) Simulated *in vivo* occupancy profiles for homotropic cooperativity: impact of rebinding. Rebinding affects both binding steps in this model and the occupancy profiles are already perceptibly depressed out in the presence of even a modest level of rebinding (here $F_r = 3$). Owing to the contribution of k_3 and the uplifted rebinding, k_1 now exerts a similar substantial impact on the occupancy profile as the k_2 – k_4 combination for all four cooperativity situations. Please note that curves for association- and dissociation based negative cooperativity nearly overlap and are not shown separately. Parameters for control (c) of all: $F_r = 3$, all other parameters are the same as for corresponding mechanisms in Fig. 4C.

also to raise peak occupancy when the dosage of all the drugs is kept constant (*i.e.* the “constant $[L_{\max}]$ ” dosing paradigm, Fig. 2C).¹⁴ Likewise, this paradigm also applies when comparing *in vivo* target- to additional non-target binding but, in this respect, attention should be called on the fact that both k_{on} and k_{off} are likely to differ from those of the target of interest. On the other hand, increasing k_{on} becomes unable to affect k_{obs} when $[L]$ is adapted to keep the $[L]/K_D$ ratio constant (Fig. 1). In agreement therewith, the PK-PD modelling studies show that increasing k_{on} remains ineffective when the *in vivo* dosing complies with the “constant $[L_{\max}]/K_D$ ratio” dosing paradigm (Fig. 2B). Interestingly, this paradigm is claimed to be the most relevant one from the clinical perspective³¹ and it has also been adopted further on here to address more complex binding models (Fig. 3 to 5). Nevertheless, a drawback of this paradigm is that it may require the dosage to attain unworkable high levels for some of the drugs.¹¹ Finally, it is important to note that, in comparison to k_{on} , the *in vitro* and *in vivo* effects of k_{off} are less susceptible to the dosing (Fig. 2B and C). In this respect decreasing k_{off} has already been reported to bring about longer-lasting occupancy and eventually to depress the occupancy profile regardless of the dosing paradigm but provided that it drops below the k_e , the elimination rate constant of the drug.^{14,31}

Spectroscopic and molecular modelling studies^{19–22} indicate that drug-target binding is likely to involve complex multistep mechanisms. However, to comply again with principle of Occam’s razor, only the simplest two-step binding models have been considered here. For induced-fit, heterobivalent- and homotropic cooperative mechanisms, increasing k_1 is here again unproductive when this move is counterbalanced by an equivalent decrease of $[L]$ for *in vitro* association experiments or when the *in vivo* dosing complies with the constant $[L_{\max}]/K_D$ ratio paradigm (Fig. 3 and 4). Yet, such behaviour is not universal since, for homobivalent drugs, increasing k_1 is even able to slow down the initial ascending phase of the *in vivo* occupancy profile (instead of speeding it up) (Fig. 3D). Those drugs bear two identical pharmacophores and, as outlined above, k_3 (*i.e.* the composite association rate constant of their second binding step) will increase in par with k_1 . Since a higher k_3 results in a lower composite k_{off} , the occupancy profile will be impacted accordingly. Of course, the premise that k_3 changes in par with k_1 is based on the presuppositions that changing k_1 will not affect the distance between both pharmacophores, nor impose different translational or rotational constraints to the second binding step.^{35,36} Although all such conditions may not necessarily be met in practice, the merit of those simulations is that they draw attention to the concept that, even at the PD-level, k_1 could exert a counter-intuitive impact on the observed association of some drugs.

Obviously, it should be kept in mind that drug-binding models are only simplified representations of the actual *in vivo* situation. Indeed, most of them do not take account of phenomena like target turnover,¹¹ drug metabolism, micro-kinetic phenomena such as accumulation of the drug

into membranes,¹³ interference by metabolites and endogenous biomolecules, *etc.* Yet, although future refinement of the current models is certainly warranted, the present thereon-based *in vitro* and *in vivo* target occupancy profiles already provide preliminary insight into how and when increasing k_{on} / k_1 could trigger faster association and clinical action of a drug. In line with this contention, we here already also show that the impact of rebinding on the *in vivo* occupancy is preserved when target turnover is incorporated into the PK-PD model for 1-step binding (ESI† Fig. S2).

Drug rebinding has hitherto essentially been invoked for its ability to prolong target occupancy.^{6,27,28,47,57} Incidentally, this micro-kinetic mechanism has also been found to delay the association of drug molecules to their membrane-associated targets of plated cell systems⁵⁷ and in surface plasmon resonance (SPR)-based biosensor studies under the name of “mass transport limitation”.^{58–60} Admittedly, this latter terminology provides a more vivid rendition than “rebinding” of the fact that limited diffusion of a drug is also responsible for a drop in its concentration in the target’s micro-environment before the attainment of equilibrium binding. In the same way, “rebinding”-like phenomena inside the cell have also been referred to as “molecular crowding” (reviewed in ref. 28). Since the firmness of rebinding (corresponding to the rebinding factor $F_r = k_1[R_{\text{tot}}]/k$) is proportional to k_{on} / k_1 , rebinding thus provides another means for these rate constants to affect *in vitro* association and *in vivo* occupancy in roughly the same way as k_{off} / k_2 . By acting on the firmness of rebinding, increasing k_1 enough will prolong the *in vivo* occupancy of the targets and eventually even depress the occupancy profile. This effect also shows up for all the here-investigated binding models but it is only modest in case of induced-fit binding (Fig. 5).^{14,15} As shown in the ESI† Fig. S1 for 1-step binding drugs, the effect of increasing rebinding *via* k_1 is only limited when dosing proceeds according to the constant $[L_{\max}]$ -constant K_D ratio paradigm.

Rebinding can be conveniently attenuated by increasing the drug’s flow rate such as in SPR assays⁶¹ and, in this respect, a more pronounced exposure to the bloodstream has recently also been invoked to explain why dopamine receptors in pituitary lactotrophs experience less rebinding than those within the striatum.⁵² Likewise, most of the clinically relevant targets also reside in confined spaces within complex tissues with only limited convective stirring^{62–64} and/or also in the enclosed, crowded environment within cells.^{6,28,65,66} Those are therefore also likely to be quite susceptible to drug rebinding. In this respect, rebinding is often regarded to be beneficial from the clinical perspective because of the resulting longer-lasting post-dosing target occupancy. Yet, when the goal is to obtain swift association and clinical action, this may be rather regarded as counterproductive. It is therefore of interest to explore if the firmness of this micro-pharmacokinetic phenomenon can be modulated.

An eye-catching choice is to change k_{on} (*i.e.* most widely used denomination since drug-target interactions are most often presented as a 1-step process) and although “on-rates” only

vary little within many compound series, significant variation has actually also been observed for some others.¹⁰ In this respect, there is increasing awareness that (among others) the cell membrane is able to modulate the binding kinetics of drugs for their membrane-embedded targets. A most pertinent findings in this respect is that the accumulation of clinically relevant β_2 -adrenoceptor agonists within the membrane increases their observed k_{on} but not their k_{off} for the β_2 -adrenoceptors.¹³ This finding corroborates with the “diffusion microkinetic” model^{67,68} in where the membrane acts as a reservoir in where lipophilic drugs are able to accumulate (and, hence, to acquire a high local concentration). Moreover, even partial embedding of a drug within the membrane already promotes the release of bound water and reduces the number of conformations and orientations that it can adopt. In this respect, the early “membrane catalysis” model already stipulated that membranes may optimise the conformation and orientation of amphiphilic peptide ligands so that they may enter and fit their receptor's binding site with greater ease.^{69–71} There is increasing awareness that the destruction of the organized water network around hydrophobic drugs (*i.e.* desolvation) represents a major energetic barrier along their binding pathway and that this penalises their association rate.^{5,10,72} In this respect, simulations by Dror *et al.*⁷³ revealed that, when alprenolol approaches its β_2 -adrenoceptor from the aqueous phase, it first undergoes desolvation in a “vestibule” nearby the receptor's surface and then only migrates to its orthosteric binding pocket deeper within the receptor. Since the function of the membrane conforms well to that of the vestibule, it is conceivable that some hydrophobic drugs may bind swifter from inside the membrane than from the aqueous phase.

Moreover, several findings suggest that the membrane may also promote rebinding.^{47,74,75} For example, the lipophilic drugs spiperone and taranabant experience much firmer rebinding than less lipophilic ones in radioligand-washout experiments with plated cell systems.^{14,28} Very recently, it was also reported that the concentration of propranolol (a lipophilic β_2 -adrenoceptor antagonist) is significantly higher immediately adjacent to the plasma membrane of live β_2 -adrenoceptor expressing recombinant cells but not of the naïve cells.⁷⁶ These and other findings⁷⁷ corroborate with the earlier “unstirred layer”^{78,79} and “reduction in dimensionality” principles^{66,80,81} that are considered to promote (re)binding.^{75,82}

Drug-membrane interactions are now increasingly investigated by spectroscopic, biophysical and computational approaches^{76,83,84} and the need to establish thereon-based micro-PK-PD relationships becomes recognised as well.^{13,47,85} In this respect, diverse fundamental issues, still remain to be further clarified. For example, it needs to be further explored whether and/or when lipophilic drugs reach (and penetrate) their membrane-associated targets by a classical three-dimensional (3D) diffusion in water,^{67,85} by two-dimensional (2D) diffusion within an unstirred water layer^{76,80,81} or by 2D diffusion within the plane of the lipid bilayer⁸⁶ or even by mixed-type mechanisms.^{84,87,88}

From the technical side, it is obvious that the kinetic parameters are most relevant when the assays are performed in *in vivo*-like conditions; *e.g.* when the targets are still present in intact/living cells and when the measurements are made at 37 °C. Since the exposure of drugs to their targets at the outer cell membrane can be rapidly modulated, their binding kinetics are fairly easy to measure by making use of an increasing array of label- and label-free techniques.^{23,26,57,89} Intact cell experiments are also preferable for the study of intracellular targets since they often make part of extended molecular complexes, so that their isolation is likely to alter their behaviour.⁹⁰ Although the kinetics of drug-intracellular target interactions can be recorded indirectly when they produce a cellular response, recent studies^{90,91} suggest that bioluminescence resonance energy transfer (BRET) represents a convenient and broadly applicable approach to follow the very binding process in real time and without the need of cell lysis.

Conclusions

Increasing k_{on} / k_1 and decreasing k_{off} are both able to increase the drug's affinity for its target and, hence, to increase its selectivity *vs.* unrelated binding sites. The clinical benefit thereof is that the desired *in vivo* effect may be retained at lower dosage while the likelihood of undesired side-effects is reduced. Although it has been argued that this reasoning does not apply to an “open system” like the human body,^{4–6} Fig. 2C (which in fact also compares the *in vivo* occupancy profiles of drugs with different K_D) clearly illustrates that, at the same dosage, those with higher affinity bring forth higher occupancy (and are thus more selective) than those with lower affinity.

Decreasing k_{off} may offer additional benefit by conferring longer-lasting target occupancy⁴ regardless of the dosage. This mechanism has been referred to as “kinetic selectivity”.^{5,31,72,92} In the same vein, increasing k_{on} / k_1 may also offer additional benefit by enhancing the drug association so that the desired *in vivo* response is obtained earlier on after the dosing.^{9–12} Here we show that this move is indeed able to enhance drug association *in vivo* but only under certain conditions: it should not be counteracted by an equal decrease of $[L]$ such as when keeping the $[L_{\text{max}}]/K_D$ dosing ratio constant (*i.e.* the clinically most relevant dosing paradigm³¹), the binding mechanism should be suitable and rebinding (and thus the factors within the target's micro-environment that favour this mechanism) should not be too prominent. Otherwise, increasing k_{on} / k_1 may become ineffective and potentially even slow down drug's association and eventually even depress its occupancy profile.

The take home message is in fact very simple: while the swiftness of the clinical action can be tentatively linked to the rate of the experimental/observed association (which is quite easily determined *in vitro*), the latter should not be relegated to a single binding constant like k_{on} / k_1 since other parameters like k_{off} , the “firmness” of rebinding and the drug concentration also have a word to say. Since increasing k_{on} / k_1

may act as a double-edged sword, it may be preferable to focus more attention to the observed *in vitro* association rates.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

I am very grateful to Dr. David Swinney for enlightening discussions and critical reading.

References

- N. H. G. Holford and L. B. Sheiner, *Pharmacol. Ther.*, 1982, **16**, 143.
- D. C. Swinney, *Nat. Rev. Drug Discovery*, 2004, **3**, 801.
- R. A. Copeland, D. L. Pompliano and T. D. Meek, *Nat. Rev. Drug Discovery*, 2006, **5**, 730.
- P. J. Tummino and R. A. Copeland, *Biochemistry*, 2008, **47**, 5481.
- S. Núñez, J. Venhorst and C. G. Kruse, *Drug Discovery Today*, 2012, **17**, 10.
- R. A. Copeland, *Nat. Rev. Drug Discovery*, 2016, **15**, 87.
- R. A. Copeland, *Expert Opin. Drug Discovery*, 2010, **5**, 305.
- D. C. Swinney and J. Anthony, *Nat. Rev. Drug Discovery*, 2011, **10**, 507.
- N. Yin, J. Pei and L. Lai, *Mol. Biosyst.*, 2013, **9**, 1381.
- A. Schoop and F. Dey, *Drug Discovery Today: Technol.*, 2015, **17**, 9.
- R. H. A. Folmer, *Drug Discovery Today*, 2018, **23**, 12.
- I. Ramos, M. Aparici, M. Letosa, C. Puig, A. Gavalda, J. M. Huerta, S. Espinosa, D. Vilella and M. Miralpeix, *Eur. J. Pharmacol.*, 2018, **819**, 89.
- D. A. Sykes, C. Parry, J. Reilly, P. Wright, R. A. Fairhurst and S. J. Charlton, *Mol. Pharmacol.*, 2014, **85**, 608.
- G. Vauquelin, *Br. J. Pharmacol.*, 2016, **173**, 2319.
- G. Vauquelin, *Br. J. Pharmacol.*, 2017, **174**, 4233.
- W. E. A. de Witte, M. Danhof, P. H. van der Graaf and E. C. M. Lange, *Trends Pharmacol. Sci.*, 2016, **37**, 831.
- R. Zhang, *Nat. Chem. Biol.*, 2015, **1**, 382.
- G. K. Walkup, Y. Zhiping, P. L. Ross, E. K. H. Allen, F. Daryaei and M. R. Hale, *Nat. Chem. Biol.*, 2015, **4**, 16.
- E. P. Garvey, *Curr. Chem. Biol.*, 2010, **4**, 64.
- R. O. Dror, A. C. Pan, D. H. Arlow, D. W. Borhania, P. Maragakisa, Y. Shana, H. Xua and D. E. Shaw, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13118.
- R. A. Copeland, *Future Med. Chem.*, 2011, **3**, 1491.
- A. Strasser, H.-J. Wittmann and R. Seifert, *Trends Pharmacol. Sci.*, 2017, **38**, 717.
- D. Guo, J. M. Hillger, A. P. IJzerman and L. H. Leitman, *Med. Res. Rev.*, 2014, **4**, 856.
- G. Vauquelin, I. Van Liefde and D. Swinney, *Br. J. Pharmacol.*, 2016, **173**, 1268.
- R. Neubig, M. Spedding, T. Kenakin and A. Christopoulos, *Pharmacol. Rev.*, 2003, **55**, 597.
- G. Vauquelin, *Med. Chem. Commun.*, 2012, **3**, 645.
- S. Strickland, G. Palmer and V. Massey, *J. Biol. Chem.*, 1975, **250**, 4048.
- G. Vauquelin and S. Charlton, *Br. J. Pharmacol.*, 2010, **161**, 488.
- G. Vauquelin and I. Van Liefde, *Trends Pharmacol. Sci.*, 2006, **27**, 355.
- H. Lu and P. J. Tonge, *Curr. Opin. Chem. Biol.*, 2010, **14**, 467.
- G. Dahl and T. Akerud, *Drug Discovery Today*, 2013, **18**, 597.
- J. Gabrielsson, H. Dolgos, P.-G. Gillberg, U. Bredberg, B. Benthem and G. Duker, *Drug Discovery Today*, 2009, **14**, 358.
- A. Plückthun and P. Pack, *Immunotechnology*, 1997, **3**, 83.
- R. H. Kramer and J. W. Karpen, *Nature*, 1998, **395**, 710.
- G. Vauquelin and S. Charlton, *Br. J. Pharmacol.*, 2013, **168**, 1771.
- M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754.
- P. Holliger and P. J. Hudson, *Nat. Biotechnol.*, 2015, **23**, 1126.
- S. I. Rudnick and G. P. Adams, *Cancer Biother. Radiopharm.*, 2009, **24**, 155.
- C. Kroll, R. Mansi, F. Braun, S. Dobitz, H. R. Maecke and H. Wennemers, *J. Am. Chem. Soc.*, 2013, **135**, 16793.
- G. Vauquelin, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 2013, **386**, 949.
- A. Christopoulos and T. G. Kenakin, *Pharmacol. Rev.*, 2002, **54**, 323.
- T. E. Hebert and M. Bouvier, *Biochem. Cell Biol.*, 1998, **76**, 1.
- C. J. Robinson and S. E. Stringer, *J. Cell Sci.*, 2001, **114**, 853.
- U. Gether, *Endocr. Rev.*, 2000, **21**, 90.
- P. De Meyts, *Trends Biochem. Sci.*, 2008, **33**, 376.
- K. Mohr, C. Tränkle, E. Kostenis, E. Barocelli, M. De Amici and U. Holzgrabe, *Br. J. Pharmacol.*, 2010, **159**, 997.
- G. Vauquelin, *Expert Opin. Drug Discovery*, 2015, **10**, 1085.
- D. C. Perry, K. B. Mullis, S. Øie and W. Sadée, *Brain Res.*, 1980, **199**, 49.
- W. Sadée, D. C. Perry and J. S. Rosenbaum, *Eur. J. Pharmacol.*, 1982, **81**, 431.
- A. N. Gifford, S. J. Gatley and N. D. Volkow, *Synapse*, 1998, **28**, 167.
- G. Vauquelin, *Expert Opin. Drug Discovery*, 2010, **5**, 927.
- D. A. Sykes, H. Moore, L. Stott, N. Holliday, J. A. Javitch, J. R. Lane and S. J. Charlton, *Nat. Commun.*, 2017, **8**, 763.
- K. Petrov, I. Zueva, I. Kovyazina, I. Sedov, S. Lushchekina, A. Kharlamova, O. Lenina, S. Koshkin, Y. Shtyrlin, E. Nikolsky and P. Masson, *Neuropharmacology*, 2018, **131**, 304.
- B. Goldstein and M. Dembo, *Biophys. J.*, 1995, **68**, 1222.
- D. Coombs and B. Goldstein, *Biophys. J.*, 2004, **87**, 2215.
- M. Gopalakrishnan, K. Orsten-Williams, M. A. Nugent and U. C. Täuber, *Biophys. J.*, 2005, **89**, 3686.
- W. E. A. de Witte, Y. C. Wong, I. Nederpelt, L. H. Heitman, M. Danhof, P. H. van der Graaf, R. A. Gillsen and E. C. de Lange, *Expert Opin. Drug Discovery*, 2015, **11**, 45.
- C. E. Spivak, M. Oz, C. L. Beglan and R. I. Shrager, *Cell Biochem. Biophys.*, 2006, **45**, 43.
- P. Schuck and A. P. Minton, *Anal. Biochem.*, 1996, **240**, 262.
- L. L. H. Christensen, *Anal. Biochem.*, 1997, **249**, 153.
- R. Karlsson and R. Falt, *J. Immunol. Methods*, 1997, **200**, 121.
- A. Lovich, C. Creel, K. Hong, C. W. Hwang and E. R. Edelman, *J. Pharm. Pharmacol.*, 2001, **90**, 1324.

- 63 S. Hrabctová and C. Nicholson, *Neurochem. Int.*, 2004, **45**, 467.
- 64 A. Dokoumetzidis, V. Karalis, A. Iliadis and P. Macheras, *Trends Pharmacol. Sci.*, 2004, **25**, 140.
- 65 A. Lewandowicz, P. C. Tyler, G. B. Evans, R. H. Furneaux and V. L. Schramm, *J. Biol. Chem.*, 2003, **278**, 31465.
- 66 M. Oh, M. Ogiue-ikeda, J. A. Jadwin, K. Machida, B. J. Mayer and J. Yu, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 14024.
- 67 G. P. Anderson, *Life Sci.*, 1993, **52**, 2145.
- 68 M. Johnson, *Paediatr. Respir. Rev.*, 2001, **2**, 57.
- 69 D. F. Sargent and R. Schwyzer, *Proc. Natl. Acad. Sci. U. S. A.*, 1986, **83**, 5774.
- 70 R. Schwyzer, *Biopolymers*, 1995, **37**, 5.
- 71 R. Bader, A. Bettio, A. G. Beck-Sickinger and O. Zerbe, *J. Mol. Biol.*, 2001, **305**, 307.
- 72 A. C. Pan, D. W. Borhani, R. O. Dror and D. E. Shaw, *Drug Discovery Today*, 2013, **18**, 667.
- 73 R. O. Dror, A. C. Pan, D. H. Arlow, D. W. Borhani, P. Maragakis, Y. Shan, H. Xu and D. E. Shaw, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13118.
- 74 A. Szczuka, A. Packeu, M. Wennerberg and G. Vauquelin, *Br. J. Pharmacol.*, 2009, **158**, 183.
- 75 G. Vauquelin, *Br. J. Clin. Pharmacol.*, 2016, **82**, 673.
- 76 K. Gherbi, S. J. Briddon and S. J. Charlton, *Sci. Rep.*, 2018, **8**, 3479, DOI: 10.1038/s41598-018-21100-x.
- 77 F. L. P. Fierens, P. M. L. Vanderheyden, J.-P. De Backer and G. Vauquelin, *Eur. J. Pharmacol.*, 1999, **367**, 413.
- 78 T. Loftsson, *Pharmazie*, 2012, **67**, 363.
- 79 C. E. Spivak, M. Oz, C. L. Beglan and R. I. Shrager, *Cell Biochem. Biophys.*, 2006, **45**, 43.
- 80 G. Adam and M. Delbruck, in *Structural Chemistry and Molecular Biology*, ed. A. Rich and N. Davidson, WH Freeman & Co, San Francisco, CA, 1968, pp. 198–215.
- 81 M. Eigen, in *Quantum Statistical Mechanics in the Natural Sciences*, ed. S. L. Mintz and S. N. Widmayer, Plenum Press, New York, 1974, pp. 37–61.
- 82 C. E. Spivak, M. Oz, C. L. Beglan and R. I. Shrager, *Cell Biochem. Biophys.*, 2006, **45**, 43–58.
- 83 Y. N. Abdiche and D. G. Myszk, *Anal. Biochem.*, 2004, **328**, 233.
- 84 D. Fotakis, P. Christodouleas, E. Zoumpoulakis, E. Kristi, N.-P. Benetis, T. Mavromoustakos, H. Reis, A. Gili, M. G. Papadopoulos and M. Zervou, *J. Phys. Chem. B*, 2011, **115**, 6180.
- 85 C. J. Dickson, V. Hornak, C. Velez-Vega, D. J. McKay, J. Reilly, D. A. Sandham, D. Shaw, R. A. Fairhurst, S. J. Charlton, D. A. Sykes, R. A. Pearlstein and J. S. Duca, *J. Med. Chem.*, 2016, **59**, 5780.
- 86 M. A. Hanson, C. B. Roth, E. Jo, M. T. Griffith, F. L. Scott, G. Reinhart, H. Desale, B. Clemons, S. M. Cahalan, S. C. Schuerer, M. Germana Sanna, G. W. Han, P. Kuhn, H. Rosen and R. C. Stevens, *Science*, 2012, **335**, 851.
- 87 L. Moroder and R. Romano, *Pure Appl. Chem.*, 1994, **66**, 2111.
- 88 R. A. Coleman, M. Johnson, A. T. Nials and C. J. Vardey, *Trends Pharmacol. Sci.*, 1996, **17**, 324.
- 89 K. P. Cusack, Y. Wang, M. Hoemann, J. Marjanovic, R. G. Heym and A. Vasudevan, *Bioorg. Med. Chem. Lett.*, 2015, **15**, 2019.
- 90 M. B. Robers, M. L. Dart, C. C. Woodroffe, C. A. Zimprich, T. A. Kirkland, T. Machleidt, K. R. Kupcho, S. Levin, J. R. Hartnett and K. Zimmerman, *et al.*, *Nat. Commun.*, 2015, **6**, 10091, DOI: 10.1038/ncomms10091.
- 91 T. Machleidt, C. C. Woodroffe, M. K. Schwinn, J. Méndez, M. B. Robers, K. Zimmerman, P. Otto, D. L. Daniels, T. A. Kirkland and K. V. Wood, *ACS Chem. Biol.*, 2015, **10**, 1797.
- 92 D. C. Swinney and Curr Top, *Med. Chem.*, 2006, **6**, 461.
- 93 G. Vauquelin, P. Morsing, F. L. P. Fierens, J.-P. De Backer and P. M. L. Vanderheyden, *Biochem. Pharmacol.*, 2001, **61**, 277.
- 94 E. R. Garret, *J. Pharmacokinet. Biopharm.*, 1994, **22**, 103.