5. La Revue Prescire – Enquête. Médicaments pour les maladies rares: bilan contrasté en Europe — Synthèse élaborée collectivement par la Rédaction. *La Revue Prescire* **26**, 781 (2006) (in French).
6. European Medicines Agency. COMP report to the Commission in relation to article 10 of regulation 141/2000 on orphan medicinal products. *European Medicines Agency website* [online], http://www.ema.europa.eu/docs/en_GB/document_library/Report/2010/04/WC500089638.pdf (2005).
7. Heemstra H. H. *From Research on rare diseases to new orphan drug development*. Thesis, Utrecht Univ., Netherlands (2009).
8. Wellman-Labadie, O. & Zhou, Y. The US Orphan Drug Act: rare disease research stimulator or commercial opportunity? *Health Policy* **95**, 216–228 (2010).
9. CRA International. The current state of innovation in the pharmaceutical industry. *European Federation of Pharmaceutical Industries and Associations website* [online], http://www.efpia.org/content/default.asp?PageID=559&DocID=4896 (2008).
10. Paul, S. M. *et al.* How to improve R.&D productivity: the pharmaceutical industry's grand challenge. *Nature Rev. Drug Discov.* **9**, 203–214 (2010).
11. Joppi, J. *et al.* Orphan drug development is not taking off. *Br. J. Clin. Pharm.* **67**, 494–502 (2009).
12. Hirschhorn, R. & Reuser A. J. J. in *The Metabolic & Molecular Bases of Inherited Diseases* 8th edn (eds Scriver C. R. *et al.*) 3395 (McGraw-Hill, New York, 2001).
13. Genzyme Corporation. Genzyme annual report 2004. (Genzyme, Cambridge, Massachusetts, 2004).
14. Council of the European Union. Council Recommendation on action in the field of rare diseases. *Council of the European Union website* [online], http://www.consilium.europa.eu/uedocs/cms_data/docs/pressdata/en/lsa/108383.pdf (2009).
15. The European Agency for the Evaluation of Medicinal Products. Status report on the implementation of the European Parliament legislation on orphan medicinal products. (EMEA, 2001).
16. European Commission. Register of designated Orphan Medicinal Products. *The European Commission website* [online], http://ec.europa.eu/enterprise/sectors/pharmaceuticals/documents/community-register/html/orphreg.htm (2010).
17. Alcimed. Study for the European Commission: Study on Orphan Drugs, Part I and Part II. *European Commission website* [online], http://ec.europa.eu/health/files/orphanmp/doc/pricestudy/final_final_report_part_1_web_en.pdf; http://ec.europa.eu/health/files/orphanmp/doc/pricestudy/final_final_report_part_2_web_en.pdf (2004).
18. Eichler H. -G. *et al.* Relative efficacy of drugs: an emerging issue between regulatory agencies and third-party payers. *Nature Rev. Drug Discov.* **9**, 277–291 (2010).
19. Commission of the European Communities. Commission staff working document on the experience acquired as a result of the application of Regulation (EC) No 141/2000 on orphan medicinal products and account of the public health benefits obtained (document on the basis of Article 10 of Regulation (EC) No 141/2000). *The European Commission website* [online], http://ec.europa.eu/health/files/orphanmp/doc/orphan_en_06-2006_en.pdf (2006).
20. The European Platform for Patients' Organisations, Science and Industry. The 8th workshop on partnering for rare disease therapy development: the reality of orphan medicines. *The Platform for Patients' Organisations, Science and Industry website* [online], http://www.epposi.org/upl/1/default/doc/8th_Workshop_Partnering_for_Rare_Diseases_Report.pdf (2007).
21. US Food and Drug Administration. New resources for drug developers: the rare disease repurposing database. *US Food and Drug Administration website* [online], http://www.fda.gov/ForIndustry/DevelopingProductsforRareDiseasesConditions/HowtoapplyforOrphanProductDesignation/ucm216147.htm (2010).
22. Commission of the European Communities. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the regions on Rare Diseases: Europe's challenges. *The European Commission website* [online], http://ec.europa.eu/health/ph_threats/non_com/docs/rare_com_en.pdf (2008).
23. The Pharmaceutical Forum. Improving access to orphan drugs for all affected EU citizens. *The European Commission website* [online], http://ec.europa.eu/pharmaforum/docs/pricing_orphans_en.pdf (2008).
24. Commission of the European Communities. Guideline on aspects of the application of Article 8(2) of Regulation (EC) No 141/2000: review of the period of market exclusivity of orphan medicinal products. *The European Commission website* [online], http://ec.europa.eu/health/files/orphanmp/doc/c_2008_4051_en.pdf (2008).

Acknowledgements
The author would like to thank M. Dooms, Pharmacist, University Hospital of Leuven, Belgium, for helpful suggestions and C. De Bie of Genzyme Corporate Communications for editing the text.

Competing interests statement
The author declares competing financial interests: see web version for details.

FURTHER INFORMATION
EPPOSI (European Platform for Patients' Organizations, Science and Industry): http://www.epposi.org
European Commission (The Orphan drugs strategy): http://ec.europa.eu/health/rare_diseases/orphan_drugs/strategy/index_en.htm
EURORDIS: www.eurordis.org
ICORD: http://www.icord.se
National Institutes of Health, United States: http://rarediseases.info.nih.gov
NORD: http://www.rarediseases.org
The Orphanet database: www.orpha.net

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

A GUIDE TO DRUG DISCOVERY — OPINION

The effect of plasma protein binding on *in vivo* efficacy: misconceptions in drug discovery

Dennis A. Smith, Li Di and Edward H. Kerns

Abstract | Data from *in vitro* plasma protein binding experiments that determine the fraction of protein-bound drug are frequently used in drug discovery to guide structure design and to prioritize compounds for *in vivo* studies. However, we consider that these practices are usually misleading, because *in vivo* efficacy is determined by the free (unbound) drug concentration surrounding the therapeutic target, not by the free drug fraction. These practices yield no enhancement of the *in vivo* free drug concentration. So, decisions based on free drug fraction could result in the wrong compounds being advanced through drug discovery programmes. This Perspective provides guidance on the application of plasma protein binding information in drug discovery.

Drug molecules *in vivo* are either bound to proteins and lipids in plasma (termed plasma protein binding (PPB)), to proteins and lipids in tissues, or are free (that is, unbound) and diffuse among the aqueous environment of the blood and tissues. There are differences in the types and the amounts of proteins and lipids that are present in plasma compared with tissue, which result in different levels of drug binding in the two compartments, depending on the properties of a compound. In most cases, only free drug molecules interact with the therapeutic target — for example, a receptor — to produce efficacy.

Unfortunately, there is a lack of knowledge and no consensus approach in the pharmaceutical industry of how the PPB of a drug relates to its *in vivo* efficacy. A range of divergent concepts on PPB have appeared in the modern medicinal chemistry literature, suggesting that in order to optimize *in vivo* efficacy it is necessary to reduce PPB[1,2], that PPB reduces pharmacokinetic clearance[3] or that it is advantageous to find an optimum PPB range (referred to as the PPB sweet spot)[3–5].

These concepts are based on the common practice of predicting the *in vivo* efficacy of a compound using data from *in vitro* activity assays that incorporate plasma proteins into the assay. It is assumed that such *in vitro* assays are able to predict the concentration of a compound that is available to interact with the therapeutic target *in vivo* and produce efficacy. An example of such an assay is the 'shift assay', which is frequently carried out to select compounds for advancement or to guide structure optimization. This assay involves additions of serum proteins, which often decrease the apparent efficacy of the drug, with the assumption that PPB similarly attenuates

Box 1 | **The free drug hypothesis**

Drug molecules *in vivo* either bind reversibly to proteins and lipids in plasma (that is, plasma protein binding) and in tissues, or they are free (that is, unbound) and diffuse across membranes among the aqueous environment of the blood and tissues to interact with the intended therapeutic target or with other biomolecules (for example, enzymes, transporters or receptors). The results of these interactions are embodied in two key concepts, which form the free drug hypothesis:

- Part I: at steady state, the free drug concentration is the same on both sides of any biomembrane
- Part II: the free drug concentration at the site of action, the therapeutic target biophase, is the species that exerts pharmacological activity

The free drug hypothesis is widely applied in drug discovery and development to establish pharmacokinetic–pharmacodynamic relationships, to predict the therapeutically relevant dose and to monitor drug concentration in clinical studies. It has been confirmed by numerous studies in various therapeutic areas (for example, infectious disease[8], neuroscience[9,10,12,13], cardiovascular[14], oncology and general pharmacology[11,15]) and for different therapeutic target types (for example, the cell surface, intracellular enzymes, receptors and ion channels).

the efficacy of the drug *in vivo*. Compounds that are the most efficacious *in vitro* in the presence of serum proteins are then selected for advancement to *in vivo* testing[2,5].

Another common practice for a discovery project team is to obtain data on the free drug fraction (f_u) of a compound using an *in vitro* PPB assay, and then to modify the compound's chemical structure to try to reduce the PPB, thus increasing f_u, assuming that this will result in a higher average *in vivo* free drug concentration in the plasma (defined as $C_{av,\,u}$).

However, we suggest that there is an error in extrapolating such *in vitro* serum shift data and PPB data to predict *in vivo* efficacy. This is because living systems are dynamic, with many simultaneous physiological actions on the drug molecules — for example, binding to the target, PPB, metabolism, transport, movement between cellular and tissue compartments — whereas *in vitro* experiments are static, with a limited number of simultaneous actions on drug molecules. *In vitro* serum shift assays and PPB experiments lack the metabolism, the transport and the movement among compartments of the dynamic *in vivo* systems. Therefore, it is insufficient to use an *in vitro* experiment, such as serum shift assays or PPB measurements, containing a limited number of actions to accurately predict *in vivo* efficacy.

In practice, for most compounds, PPB has minimal effects on $C_{av,\,u}$ and on the efficacy of the compound. Although this concept is practiced in clinical settings[6,7], it is often misunderstood in drug discovery. We believe that changing the focus from reducing the PPB of a compound to enhancing the concentration of the free (unbound) compound should result in more successful clinical candidates. The aims of this Perspective are to provide a framework for understanding how drugs behave *in vivo* with regard to PPB, to clarify misconceptions about PPB and to provide guidance for applying the information on free drug concentration to inform the structural design of compounds so that they have optimal characteristics for producing clinical efficacy.

Absorption and distribution of oral drugs

This Perspective focuses on oral drugs that are cleared by the liver. However, the principles we present also apply to most situations involving non-oral delivery routes (for example, all intravenous drugs, except those for which a high proportion of the total drug administered is extracted when blood flow passes through the liver).

Oral drugs are administered on a schedule that achieves a steady state (or a pseudo steady-state) in which a general equilibrium between all the processes of drug distribution and elimination is achieved. Before reaching the systemic circulation, an oral drug must dissolve in the aqueous contents of the gastrointestinal tract — a process known as dissolution — before crossing the membranes of the gastrointestinal tract to reach the portal vein. The passage through the gastrointestinal tract membranes may be attenuated by the presence of transporters and drug metabolizing enzymes. Additional barriers (that constitute first-pass metabolism and hepato-biliary extraction) are usually encountered as the portal vein flows through the liver before returning blood to the heart, lungs and arterial system.

Once systemically available, the drug will distribute to tissues, and the process of non-first-pass elimination begins. Most oral drugs are lipophilic — a physicochemical property that is associated with higher PPB and hepatic clearance. By contrast, renally cleared drugs are hydrophilic and usually have moderate to low PPB. Here, we discuss specific aspects of plasma and tissue binding to help drug discovery scientists make appropriate and informed applications of binding data.

The free drug hypothesis and exceptions

In this section, we first discuss the free drug hypothesis (BOXES 1,2), which emphasizes the central role of free drug concentration at the therapeutic target in determining *in vivo* efficacy and *in vivo* pharmacokinetics, to provide a basis for applying PPB data in

Box 2 | **Exceptions to the free drug hypothesis**

Free drug hypothesis part I

At steady state, the free drug concentration is the same on both sides of any biomembrane. This is represented by $C_{plasma,\,u} = C_{tissue,\,u}$ and $C_{extracellular,\,u} = C_{intracellular,\,u}$, where $C_{plasma,\,u}$ is the unbound plasma concentration; $C_{tissue,\,u}$ is the unbound tissue concentration; $C_{extracellular,\,u}$ is the unbound extracellular concentration; $C_{intracellular,\,u}$ is the unbound intracellular concentration.

Exceptions

- When a drug has low passive permeability
- When efflux of the drug occurs from the tissue of the therapeutic target by P-glycoprotein and other efflux transporters
- When influx of the drug occurs into the tissue of the therapeutic target, mediated by active transporters
- When the drug encounters tissues with low discontinuous blood flow

Free drug hypothesis part II

Free drug concentration affects pharmacological activity.

Exceptions

- When the action of the drug results in irreversible inactivation of the target, for example, with covalent binding
- When the action of the drug involves multiple mechanisms and the activation of target-mediated events
- When *in vitro* assays are used for which the therapeutic target concentration of drug differs from that in the *in vivo* environment

drug discovery. We then expand on parts of the free drug hypothesis and explain the recognized exceptions that drug discovery researchers should be aware of.

The free drug hypothesis part I — the free drug concentration is the same on both sides of biomembranes. At steady state, drugs that have a high rate of membrane permeation have a free drug concentration that is the same in plasma, in biophases (for example, the brain), in extracellular fluid, in cerebrospinal fluid (CSF) and in intracellular fluid[8–10]. It should be noted that only the free drug concentration — not the total concentration or the bound drug concentration — is the same on both sides of biomembranes. The concentration of bound drug in a particular tissue or fluid depends on the amount and the binding capacity of the proteins and the lipids there, and on the affinity of the drug molecules for them.

Even for drugs with a slow rate of membrane permeation, access to the extracellular fluid of most tissues, such as the heart and the gut, is not restricted, due to leaky pores in the blood capillaries that make transcellular permeation unnecessary. Many drug targets, such as G protein-coupled receptors and ion channels, are on the external surface of cells and thus are in direct contact with the extracellular fluid. So, the free drug concentration at these targets is the same as that in the extracellular fluid and in plasma. For intracellular targets residing within the cell membranes, such as nuclear receptors and enzymes, compounds need to have a rapid rate of membrane permeation for the free drug concentration that surrounds the intracellular target to reach steady-state equilibrium with the free drug concentration in plasma.

An example to illustrate the principle of equilibrium throughout the various fluids of the body is provided by the disposition of fluconazole, a drug that has a rapid rate of membrane permeation and a f_u of 88% in plasma[11]. Fluconazole had similar free drug concentrations in various body fluids, such as vaginal secretions, breast milk, saliva, sputum, prostatic and seminal vesicle fluid, CSF, and plasma, after single or multiple doses[12,13]. To reach each of these fluids, fluconazole had to cross different membranes. The same equilibrium principle applies regardless of the f_u in plasma. For example, naproxen, a compound with a rapid rate of membrane permeation and a f_u of <1% in plasma (a relatively low free drug faction compared with many other drugs; greater than 99% of naproxen is bound to protein in plasma), shows identical free drug concentrations in synovial fluid (deep tissue fluid) and in plasma either at steady state or after a single dose of drug[14].

Table 1 | ***In vitro* potency and *in vivo* free drug concentrations at mean efficacious dose**

Drug target	Compound	*In vitro* measure	*In vitro* concentration (nM)	Average free drug concentration (nM)
Ca^{2+} channel	Nifedipine	IC_{50}	4	6
Ca^{2+} channel	Amlodipine	IC_{50}	2	1
5-HT transporter	Sertraline	K_i	7	4
K^+ channel	Dofetilide	EC_{15}	7	3
M3 muscarinic receptor	Darifenacin	K_b	4	10
M3 muscarinic receptor	Zamifenacin	K_b	10	20
M3 muscarinic receptor	UK-112,166	A_2	1	3
β-adrenergic receptor	Propranolol	K_i	4.5	3
β-adrenergic receptor	Alprenolol	K_i	8	18
α_{1A}-adrenergic receptor	Tamsulosin	K_i	0.04	0.03–0.16
α_{1A}-adrenergic receptor	Terazosin	K_i	1	1–9
A_{2A} adenosine receptor	2-chloroadenosine	K_i	80	202–225
PDE5 inhibitor	Sildenafil	K_i	4	10
Thromboxin receptor antagonist	UK-147,535	A_2	0.1	0.5
CYP51	Fluconazole	MIC	2,600	Exceeds MIC for 8 hours; $C_{av,u}$ over this period is 4,000
CYP51	Ketoconazole	MIC	20	Exceeds MIC for 8 hours; $C_{av,u}$ over this period is 200

5-HT, 5-hydroxytryptamine (serotonin); A_2, a value obtained from a Schild plot analysis, which calculates antagonist potency in a functional assay; $C_{av,u}$, the average free drug concentration; IC_{50}, half-maximal inhibitory concentration; K_b, the equilibrium binding constant, a measure of drug potency (affinity) in a receptor binding assay; K_i, equilibrium inhibition constant; a measure of the potency of enzyme inhibition; MIC; minimum inhibitory concentration; PDE5, phosphodiesesterase 5. All data previously published in REFS 85–89.

These are examples of how drugs with a fast rate of membrane permeation diffuse freely across cell membranes and rapidly reach equilibrium on both sides of the membranes, regardless of their f_u in plasma. The driving force for the rate of membrane permeation by passive diffusion is the gradient of the free drug concentration across the membrane. Drugs with a fast rate of membrane permeation reflect this by reaching the same concentration throughout all the aqueous pools in the body.

Exceptions to the free drug hypothesis part I. There are several reasons (summarized in BOX 2) why access of free drug molecules to tissues *in vivo* may be limited. This results in a lower free drug concentration in the biophase than in plasma, and steady-state equilibrium may not be reached across the membrane[15,16].

First, the concentration of drugs with a slow rate of membrane permeation takes a long time to reach equilibrium across membranes[17]. When this occurs, the free drug concentration is typically higher in the compartment outside the membrane than in the compartment inside the membrane. For example, the blood–brain barrier may limit the penetration of the drug with a slow permeation rate to the biophase in the central nervous system[18,19] that contains the therapeutic target. Moreover, processes that clear the drug molecules from the brain, for example, CSF turnover, can be greater than the low flux of drug into the brain, owing to limited

Box 3 | **Variables that influence free drug concentration after oral administration**

Free drug concentration after oral dosing is affected by the dose, the fraction of a dose absorbed, intrinsic clearance and the dose interval, but not by plasma protein binding.

- $C_{av,u} = (F_a \times dose) \div (Cl_{int} \times T)$
- Where $C_{av,u}$ is the average free (unbound) plasma concentration; F_a is the fraction of a dose absorbed; Cl_{int} is intrinsic clearance; T is the dose interval
- Compounds that are cleared predominantly by non-hepatic clearance are exceptions to this equation

membrane permeability. For example, cimetidine has a slow rate of membrane permeation, as indicated by physicochemical parameters (log $D_{7.4}$ of –0.3). In dogs, the drug concentration in the CSF, which is indicative of free drug concentration in the brain, is only 12.5% of the free drug concentration in plasma. At equilibrium, the ratio would be approximately 1. Furthermore, elimination from the brain by clearance into the CSF and by active transport is greater than the slow flux of cimetidine into the brain[20,21]. Data from human studies give similar values of 10–20% CSF to free drug concentration in plasma[22]. So, the equilibrium of free drug concentrations on both sides of the blood–brain barrier membrane might not be established for cimetidine. Another example of not establishing equilibrium across a membrane is when the therapeutic target of a compound with a slow permeation rate is inside the cell. In this case, the cell membrane may limit penetration of the drug to the intracellular target biophase. In these two examples, the *in vivo* pharmacological activity is related to the concentration of free compound inside the cell rather than in the plasma.

A second cause of not establishing equilibrium of free drug concentration on both sides of the membrane is when drug molecules have low distribution to cells, owing to either low blood flow to the tissue or long distances between the target cells and the blood capillaries. In these cases, drug molecules are not effectively delivered to the target cells. For example, in a solid tumour, blood vessels are not well developed and distributed, leading to a large intercapillary distance[23]. This can result in parts of the tumour that are more remote from capillaries having reduced exposure to the drug. Therefore, the free drug concentration in tissues that have low perfusion may not be the same as the concentration in plasma.

Compounds that are substrates of efflux transporters (for example, P-glycoprotein, multidrug resistance-associated protein 2 and breast cancer resistance protein) or of influx transporters (for example, L-amino acid transporter 1, peptide transporter 1, organic anion transporting polypeptide, organic cation transporter and organic anion transporter) are also exceptions to the free drug hypothesis. The rate of membrane permeation of a drug consists of its rate of permeation as a result of passive diffusion, which is driven by the free drug concentration gradient, plus its transporter-assisted permeation rate, which is energy-driven. For transporter substrates, the free drug concentration on one side of the membrane will be higher than on the other side. Drugs that are substrates for efflux transporters (for example, P-glycoprotein on the blood–brain barrier[8]) have reduced free drug concentration in the therapeutic target biophase (for example, the brain). By contrast, uptake transporter substrates, such as statins, which are substrates of organic anion transporting polypeptide 1B in the liver[24], have increased free drug concentration in the biophase (that is, the liver).

Free drug hypothesis part II — the free drug concentration affects the pharmacological activity. The free drug hypothesis states that for most drugs it is the free drug concentration at the site of action (that is, the biophase) that affects the biological activity, such as *in vivo* efficacy and toxicity, not the total drug concentration or the concentration of drug that is bound to plasma or tissue proteins. This hypothesis is well established in pharmacology[25–28] and is exemplified in the cases we discuss in this section.

TABLE 1 summarizes the *in vitro* and *in vivo* activity of 16 drugs with diverse therapeutic targets and mechanisms of action. The average free drug concentration that is present *in vivo* at the mean efficacious dose (last column in the table) is in good agreement with the *in vitro* potency, consistent with the hypothesis that the free drug is the active species that exerts pharmacological activity. For example, the calcium channel blocker nifedipine has a half-maximal inhibitory concentration (IC_{50}) of 4 nM *in vitro* and an *in vivo* average free drug concentration of 6 nM at the efficacious dose. A study of seven opioids, which determined the *in vivo* concentrations of free drug in the brain and the binding affinities from brain homogenate binding studies, showed that the half-maximal effective concentration (EC_{50}) values based on free drug concentration, rather than on total serum concentration, were highly correlated to the *in vitro* K_i (the concentration of drug that will bind to half the binding sites)[29].

These examples indicate that free drug concentration at the target is correlated with *in vivo* efficacy. Therefore, we believe it is important to use free drug concentration in the biophase when assessing the pharmacokinetic and pharmacodynamic relationships of a drug. For example, this is important when making comparisons between compounds in the discovery stage of research because differences in compound structure can lead to differences in the average free drug concentration, even with the same dose, owing to differences in absorption and clearance, as discussed later (BOX 3).

The free drug concentration also affects toxicity, which is caused by unwanted *in vivo* effects at sites other than the therapeutic target. For example, for a set of drugs of diverse structures, there was a good correlation ($R^2 = 0.81$) between the free drug concentration in plasma that was associated with considerable clinical QT prolongation, (also known as torsades de pointes) and the *in vitro* IC_{50} values for activity at the human ether-a-go-go-related (HERG) potassium channel (also known as KCNH2)[30]. Thus, it is common practice to estimate a drug's therapeutic index for HERG-mediated toxicity as the ratio between the free drug concentration in plasma at the efficacious dose divided by the *in vitro* IC_{50} value for HERG activity[31,32].

Exceptions to the free drug hypothesis part II. For some drugs, the free drug concentration that is efficacious *in vivo* is different from the *in vitro* potency (for example, the IC_{50} or the EC_{50}). These exceptions are discussed in the following paragraphs. It is worth commenting on the fact that in many *in vitro* assay systems, the concentration of free drug is actually lower than that assumed (that is, lower than the added concentration). This is because drug molecules can bind to the apparatus, the assay reagents and the surrounding media. Cell culture methods usually contain albumin at a concentration of around 500 μM, so even after substantial washing, high concentrations of albumin may remain.

One exception to the free drug hypothesis part II is when the *in vitro* assay for the therapeutic target differs greatly from the conditions found *in vivo*[33–35]. For example, certain *in vitro* assays use cells that overexpress the

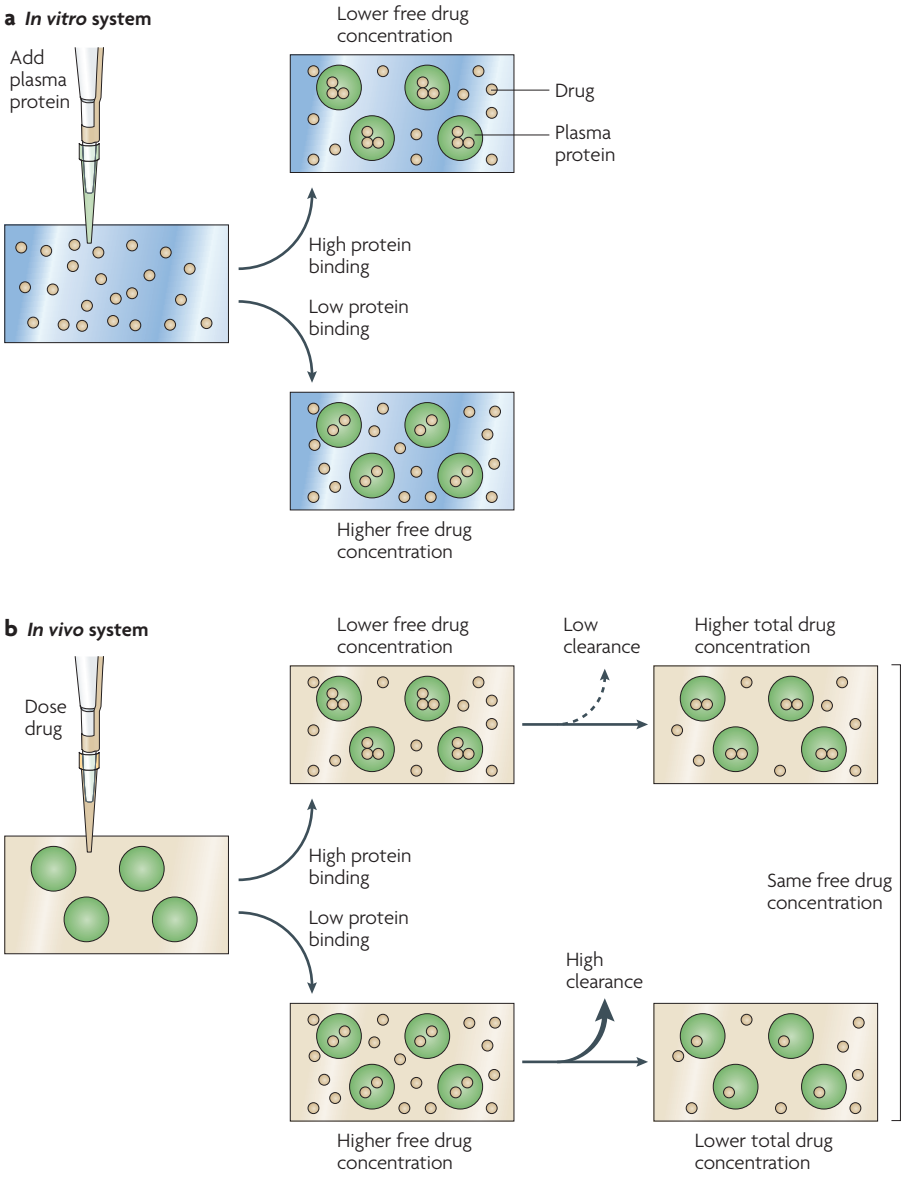

Figure 1 | **Effects of plasma protein binding on *in vitro* and *in vivo* systems. a** | In an *in vitro* system, a compound with high plasma protein binding (PPB) (top; free drug fraction (f_u) = 12 ÷ 24 = 0.5) will have a lower free drug concentration (12 free molecules). A compound with low PPB (bottom; f_u = 16 ÷ 24 = 0.67) will have a higher free drug concentration (16 free molecules). **b** | In an *in vivo* system, a compound with high PPB (top; f_u = 12 ÷ 24 = 0.5) will have a lower free drug concentration (12 free molecules), which leads to lower clearance and a higher total drug concentration, which results in the same final free drug concentration as a compound with low PPB (8 free molecules). A compound with low PPB (bottom; f_u = 16 ÷ 24 = 0.67) will have a higher free drug concentration (16 molecules), which leads to higher clearance and a lower total drug concentration and results in the same final free drug concentration as a compound with high PPB (8 free molecules).

target proteins in order to enhance the assay signal and its sensitivity. However, the overexpressed *in vitro* target requires a higher drug concentration to inhibit or to activate the larger amount of target protein, resulting in a higher IC_{50} or EC_{50} values. In this case, the free concentration of drug that is efficacious *in vivo* can be substantially lower than the *in vitro* activity, owing to the much lower expression of the target *in vivo*. The kinetics of the drug binding to the receptor when the receptor is overexpressed are complex, but as a generalization, the correlation between the *in vitro* IC_{50} or EC_{50} values and the *in vivo* values may still hold but there will be an offset shift of the exact values (that is, non-zero intercept on the correlation graph) and a correction factor may be required by calibrating *in vitro* data against *in vivo* results[30]. In the extremes, the potency values of (tight binding) potent inhibitors may reflect purely the concentration of the receptor.

Another exception, albeit partial, is drugs that modulate the target through a complex mechanism of action. The free drug concentration will always determine the initial binding kinetics of the drug to the target or the site of action, but the subsequent time course of receptor occupancy and the pharmacodynamic events that the drug triggers do not follow the time course of free drug concentration. For example, the activity of a compound is sometimes dependent on the cumulative concentration of irreversibly bound drug or metabolite to the target. Selegiline and rasagiline irreversibly and selectively inhibit monoamine oxidase B (MAO-B), which increases the half-life of dopamine by reducing its metabolism by MAO-B. The efficacy of the inhibitors is related to the deactivation of MAO-B and the time delay for the body to resynthesize MAO-B, rather than the free drug concentrations of selegiline or rasagiline in plasma[36]. Another example is the antiplatelet drug clopidogrel, which relies on a short-lasting reactive metabolite that must first be formed by a cytochrome P450 enzyme[37] before it binds to the target to produce the pharmacological activity. Further examples include the anti-ulcer drug omeprazole[38], which first forms a sulphenic acid under acidic conditions before interacting with the target. Thus, drugs that form active metabolites and display non-equilibrium binding do not obey the free drug hypothesis part II.

Misconceptions and clarifications on PPB

Based on the free drug hypothesis, we believe that there are several common drug discovery practices involving the measurement of the binding of a drug to plasma proteins that are counterproductive. These are discussed below.

The shift assay is not beneficial. An *in vitro* serum shift assay measures the change (shift) in potency of compounds when serum is added to the biological assay medium. Shift assays are used to differentiate compounds and to guide structure modification[39], and in some cases, they are considered important enough to automate into a high-throughput mode[40].

When plasma protein is added into an *in vitro* assay, drug molecules bind to proteins[41], such as human serum albumin or α1 acid glycoprotein, and to lipids, which decreases the free drug concentration (FIG. 1a). The IC_{50} curve shifts to the right,

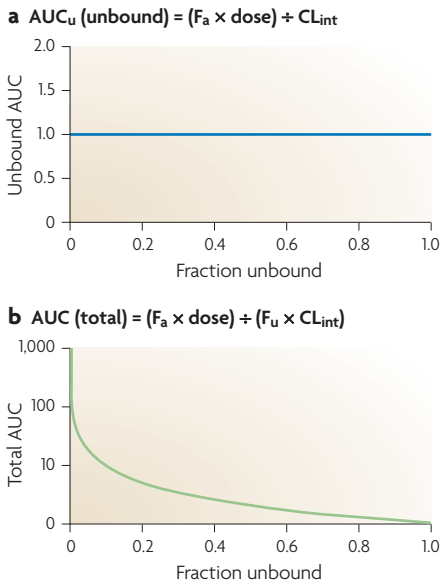

Figure 2 | **Effects of free drug fraction on unbound and total area under the plasma concentration–time curve for an orally administered drug. a** | Change of free drug fraction (f_u) from 0 (complete binding) to 1 (no binding) has no effect on the unbound area under the plasma concentration–time curve (AUC_u) for an orally administered drug. **b** | Change of f_u from 0 (complete binding) to 1 (no binding) affects the total AUC_u for an orally administered drug. Higher plasma protein binding results in higher total AUC owing to lower clearance. The lower the plasma protein binding the lower the total AUC owing to higher clearance. Cl_{int}, intrinsic clearance. F_a, the fraction of a dose absorbed.

indicating weaker biological activity (that is, higher IC_{50})[1]. Assuming that two compounds have similar potency, the shift assay predicts that the compound with the higher PPB has lower *in vitro* activity. However, selecting compounds for further studies based on the shift assay can be misleading because PPB has different effects for *in vitro* and *in vivo* systems, and so high PPB and low potency on addition of serum in the shift assay do not necessarily translate to poor *in vivo* efficacy.

In vivo, the binding of a drug to plasma proteins does not usually change the concentration of free drug[6] (FIG. 1b). Based on *in vitro* shift assays, compounds with lower PPB would be expected to have a higher free drug concentration *in vivo*. However, multiple processes *in vivo* — for example, protein binding, metabolism, permeation through membranes and excretion — occur dynamically (that is, the processes can be interactive, competing or reinforcing) and simultaneously. This is very different to the *in vitro* environment, which is static and does not include all these processes. A change in drug concentration as a result of one of these processes causes a change in another process *in vivo*. For example, after bolus intravenous administration, the early free drug concentrations may be higher. However, the higher free drug concentration *in vivo* that occurs when a compound has a higher f_u results in an increased flux of free drug into clearance organs (that is, the liver and the kidney). This results in increased total drug clearance, which directly counteracts the higher free drug concentration in plasma. Thus, over the time course of a drug — that is, the concentration of drug plotted against time after administration — the average concentration of free drug (or area under the plasma concentration–time curve of a the free drug (AUC_u) is the same. This is the case for chronic dosing of a drug, in which steady-state equilibria are established and the free drug concentration depends, throughout the time course, on the intrinsic clearance rather than the f_u (REFS 6,42,43). Therefore, we believe that using the shift assay to try to identify drug candidates might not be appropriate. Additional examples from the literature suggest a similar conclusion[44,45].

Optimization of the free drug fraction is scientifically unsound. Another practice, which has similar misconceptions to the shift assay, uses the f_u to develop a structure–activity relationship, which is then used to guide the structural modification of a chemical series. The goal of this strategy is to produce greater *in vivo* activity by having a higher free drug concentration, which is achieved through an increased f_u. The f_u is measured by an *in vitro* PPB assay[2,46,47], for example, using equilibrium dialysis between plasma and buffer, and it is common practice to include this in the primary screening strategy. Structure–PPB relationships are then developed from these data and used in research decisions.

However, we believe that this strategy does not reflect the *in vivo* effects of PPB and can result in the selection of lower-quality compounds. As discussed above, reducing the f_u or decreasing PPB has no effect on the free drug concentration *in vivo* for most drugs[6,42,43]. The exposure of the therapeutic target *in vivo* to the concentration of free drug, as measured by the AUC_u, which is the exposure or measurement of the quantity of unbound drug in the body is independent of the f_u (FIG. 2a) for most orally administered drugs[6,42,43]. The total AUC (AUC_{total}, bound plus unbound) decreases as the f_u increases, owing to increasing clearance (FIG. 2b). However, the average free drug concentration is unchanged with PPB.

Prioritizing compounds based on their PPB profile sometimes produces good clinical candidates, but for reasons other than reduced PPB. Increasing the f_u of a compound in plasma or in tissues often requires changes in several structural variables, such as lipophilicity (log D), pK_a and the polar surface area[48,49]. Such structural variables also affect metabolic clearance, renal clearance, absorption and dissolution. Substituting a compound that has high lipophilicity with one that has moderate lipophilicity will often reduce PPB, but it will also often reduce intrinsic clearance[50]. It is the reduced intrinsic clearance that increases the free drug concentration in plasma, not the change in PPB as illustrated in FIG. 3. As it is unproductive to optimize compounds by increasing the f_u, we suggest that PPB should not be measured until *in vivo* evaluation of the compounds, and then only to convert total drug concentrations into unbound concentrations (that is, $C_u = C_{total} \times f_u$ or $AUC_u = AUC_{total} \times f_u$).

The goal of a drug discovery programme should be to enhance the free drug concentration rather than the f_u. An example that illustrates the irrelevance of f_u and PPB on *in vivo* efficacy is provided by preliminary data of two anticholinergic drug development candidates, one that reached Phase II trials (zamifenacin) and one that reached registration (darifenacin). Darifenacin was found to be 2–4 times more potent *in vitro* against the M3 muscarinic receptor than zamifenacin. Both drugs are metabolically cleared by cytochrome P450 oxidation, giving similar intrinsic clearance values in human liver *in vitro* systems; zamifenacin is 300 times more highly protein bound (f_u = 0.02%) than darifenacin, (f_u = 6%). Despite these major differences in f_u, the daily clinical dose of zamifenacin is only twofold higher than darifenacin and both achieve similar pharmacodynamic responses. This reflects the receptor affinity and the intrinsic clearances of the compounds and not the large difference in PPB (D.A.S., unpublished observations).

Moderately or highly lipophilic drugs, particularly acids, all have high PPB (>99%) and yet many appear in the 100 most prescribed drugs for 2005 (FIG. 4; see Further information). This indicates the lack of a consensus in the industry with regard to PPB, with some compounds progressing in development despite having a low f_u, whereas others are discarded. Drugs with high PPB include diclofenac, ibuprofen, losartan (and its metabolite, EXP3174), naproxen, pioglitazone, rosiglitazone and montelukast. Clinical doses of pioglitazone, rosiglitazone and

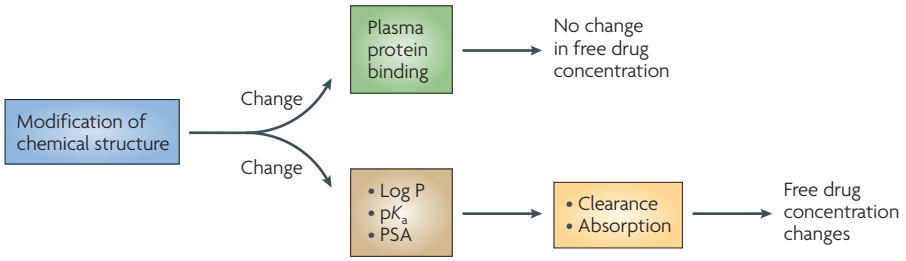

Figure 3 | **Effects of structure modification on *in vivo* exposure.** Structure modification can change plasma protein binding and other physiochemical parameters, such as log P, the dissociation constant pK_a and the polar surface area (PSA). Changes in plasma protein binding do not change the free drug concentration *in vivo*, whereas changes in other physicochemical parameters can modulate clearance and absorption, and change the free drug concentration.

montelukast are in the low milligram range, illustrating that f_u is not a factor in the effectiveness of these drugs.

Confusion between free drug fraction and free drug concentration. Misunderstandings about PPB also result from the imprecise use of the terms 'free drug fraction' (f_u) and 'free drug concentration' ($C_{av,u}$)[51]. Many scientists use these terms interchangeably. However, the f_u is the ratio between free drug concentration and total drug concentration, which has no units (TABLE 2) and is expressed as a decimal or as a percentage, whereas the free drug concentration is the concentration of free drug *in vivo*, with units of ng per ml or nM. The f_u increases as the amount of drug bound to plasma proteins decreases, whereas the free drug concentration is determined by the intrinsic clearance and the dose of drug, and usually is not changed by PPB[10].

On–off rate versus free drug concentration. It is often misconceived that if a compound binds to plasma proteins with a fast on-rate and a fast off-rate, then the dissociation constant (K_d, which is equal to the off rate divided by the on rate)[52] does not determine the free drug concentration, and the free drug concentration is equal to the total concentration, because the free drug can be readily replenished from the bound drug[44]. Actually, the binding of a compound to plasma proteins reaches equilibrium in milliseconds[53] and once equilibrium is reached, the free drug concentration is dependent on K_d, rather than on the on–off rate[52]. In almost all cases, the free drug concentration is not affected by the on rates or the off rates.

Increasing brain to plasma ratio can be counterproductive. For neuroscience projects, the brain/plasma ratio (the ratio of the drug in the brain to that in the plasma, a measure of partitioning of the drug between the brain tissue and the blood compartment; calculated as (brain maximal concentration C_{max} ÷ plasma C_{max} or brain AUC_{total} ÷ plasma AUC_{total}) or the log BB (log_{10} brain/blood) are used to guide structural modification or to select compounds for *in vivo* efficacy studies[54–57]. In such studies, the total drug concentration (C_{total}, equal to bound plus unbound drug concentrations) in blood samples and in the entire brain are each measured and used to calculate the brain/plasma ratio. Drug candidates with a high brain/plasma ratio are commonly prioritized over those with a low brain/plasma ratio for central nervous system projects because it is assumed that this indicates strong exposure of the brain to the compound.

In our opinion, this approach can be misleading; additional measurements of the f_u in *ex vivo* brain samples could greatly improve the understanding of free drug concentration at the target. The data produced in studies using brain/plasma ratios is the amount of drug in the extracellular fluid plus that in the intracellular fluid (the latter dictates pharmacological activity), plus that bound to lipids and proteins in the brain. A high brain/plasma ratio or a low brain/plasma ratio cannot be interpreted as high or low free drug concentrations in the brain *per se*. A high brain/plasma ratio is often the result of a high level of nonspecific binding to brain tissue proteins and lipids, whereas a low brain/plasma ratio can occur in the absence of nonspecific binding, and neither are an indication of free drug concentration

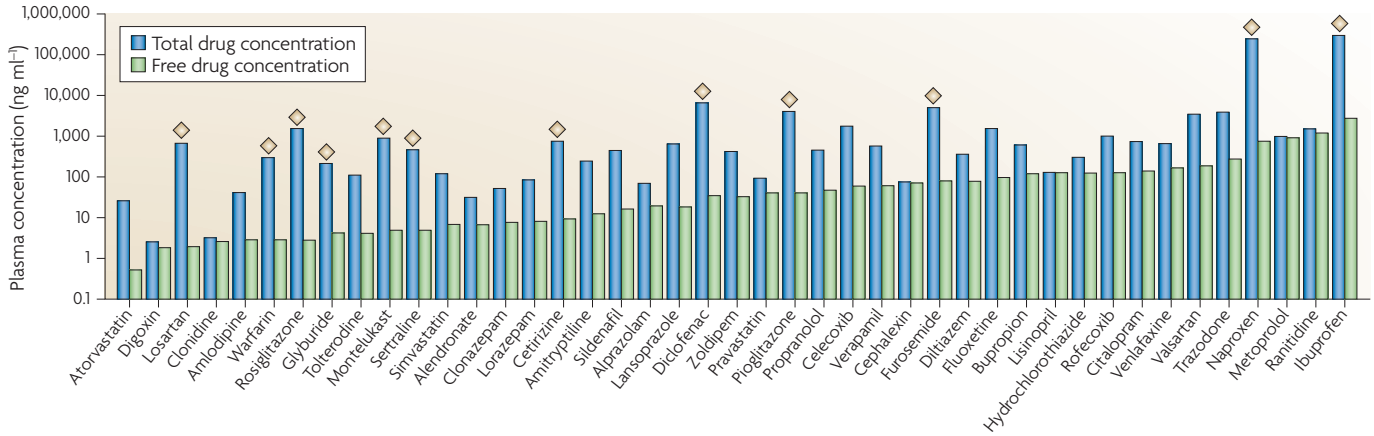

Figure 4 | **The plasma protein binding of some of the top 100 most prescribed drugs.** Many of the top 100 most prescribed drugs have greater than 98% plasma protein binding, as shown by approximately 2 log units difference in unbound and total plasma concentrations (indicated by the diamonds). Data are represented as plasma concentrations of drug at the mean efficacious dose. These data highlight that plasma protein binding has no effect on the success of drug candidates; drugs with high plasma protein binding occur in different therapeutic areas, such as cardiovascular disease (losartan, warfarin and furosemide); pain (diclofenac and naproxen); metabolic diseases (rosiglitazone, glyburide and pioglitazone); allergy and respiratory conditions (cetirizine and montelukast); and central nervous system disorders (sertraline).The information about the most prescribed drugs was obtained from the RxList website. Further data on an individual compound was obtained from the label information using the US FDA Center for Drug Evaluation and Research website (see Further information).

Table 2 | **Free drug fraction and free drug concentration**

Free drug fraction (f_u)	Free drug concentration (C_u)
Definition	
Free drug concentration divided by the total drug concentration	Unbound drug concentration
Units	
None	ng per ml
Plasma protein binding effect	
Increases with decreasing plasma protein binding	No effect *in vivo* for most compounds
In vivo *efficacy*	
No correlation	Strong correlation

in the brain[15]. Using the free drug concentration in the brain is a sounder strategy for comparing drug candidates and for developing pharmacodynamic–pharmacokinetic relationships. Such data can be derived from *in vitro* equilibrium dialyses between brain homogenate and buffer (to measure f_u in the brain), then multiplying this by the total drug concentration in the brain from an *in vivo* study in which the brain is removed from a dosed animal and the total drug concentration is measured.

An example of such a study is the investigation of KA-672 (anseculin), a lipophilic acetylcholinesterase inhibitor[58]. The compound achieved concentrations in total brain (free concentration plus bound concentration) of 0.39 μM at a dose of 1 mg per kg, which is equivalent to the IC_{50} determined *in vitro* of 0.36 μM. Despite this apparently encouraging data, doses of up to 10 mg per kg had no pharmacological effect *in vivo*. In CSF (in which a drug concentration is a surrogate measure of the free drug concentration in the brain[8] and is close to the concentration to which receptors or enzymes in the brain tissue are exposed to), the concentrations of KA-672 were less than 0.01 μM, which is far below the IC_{50} of 0.36 μM and explains why no *in vivo* activity was observed. As the drug is lipophilic and so able to cross membranes, as demonstrated by the high total brain concentration, it is likely that these low concentrations are due to a high intrinsic clearance of the free drug, which results in low concentrations of free drug in the plasma, the brain and the CSF.

In such a study[58], the use of different experimental values, such as the total brain concentration of a drug, the brain concentration of the free drug, the CSF concentration or the brain/plasma ratio, would each lead to different conclusions regarding the lack of pharmacological efficacy. The total brain concentration or brain/plasma ratio would not be correlated with the *in vivo* efficacy and so would not provide useful guidance for further decisions. However, the use of the free drug concentration or the CSF concentration values provides an explanation for the lack of pharmacological efficacy. In our view, the selection or the optimization of compounds with higher brain/plasma ratio can be counterproductive[8,18,43,59], and so discovery teams should focus on the selection of compounds that have a higher concentration of free drug in the brain.

Using total exposure to develop pharmacokinetic–pharmacodynamic relationships in drug discovery is misleading. Many pharmacokinetic studies measure the total drug concentration. Therefore, it is common to correlate AUC_{total} or total C_{max} to *in vivo* efficacy when developing pharmacokinetic–pharmacodynamic relationships. This approach is successful if an individual compound is being evaluated, such as a clinical development candidate, or if the compounds in the series have very similar plasma protein or tissue binding (in this situation, although the f_u in plasma will be different to that in tissue because of the dissimilarities between the proteins and lipids, the free drug concentration is the same in both compartments at steady state). However, for compounds with diverse structures, this approach is erroneous, as they have different f_u values when the compound is bound to plasma or bound to tissue. In many cases, when total drug concentration was used to develop pharmacokinetic–pharmacodynamic relationships, no correlations were observed between *in vitro* potency and *in vivo* efficacy[29]. Instead, free drug concentration (AUC_u or $C_{max,u}$) should be used to correlate *in vitro* potency with *in vivo* efficacy[60].

Box 4 | **Duration of drug action (plasma half-life) and plasma protein binding**

The half-life of a drug is dependent on its volume of distribution and on its clearance. The volume of distribution (V) is governed by the equation: $V = [f_u \div f_{uT}] \times V_T + V_P$. Where f_u is the free drug fraction in the plasma; f_{uT} is the unbound drug fraction in the tissue; V_T is the volume of the tissue and V_P is the volume of the plasma.

As V_T usually greatly exceeds V_P, by a factor of 10 or more, changes in f_u will directly affect V. But similarly f_u will also affect total clearance and the two will cancel each other out, thus leading to no change in the half-life as shown below. V_T and f_{uT} are largely governed by lipophilicity and ionization[84], which dictates the affinity for tissue membranes and proteins, such that:

- Bases have high affinity for phospholipid membranes owing to interactions with acidic head groups and show the highest values of V_T and f_{uT}
- Neutral compounds interact to a lesser extent with the lipid portion of membranes
- Acids have low membrane affinity

Most drug discovery programmes will aim to attenuate clearance and to produce drugs that have moderate to low metabolic clearance. These are termed moderate to low extraction drugs, that is, the total drug extracted is moderate or low when blood flow passes through the liver. For these drugs half-life is independent of the fraction unbound and governed by the simplified equation: $T_{½} = [0.693 (V_T \div f_{uT})] \div Cl_{int}$. Where V_T is the volume of the tissue; Cl_{int} is the intrinsic clearance and $T_{½}$ is the half-life.

Thus for most drug discovery programmes the important pharmacokinetic parameters relating to the exposure and to the duration of unbound drug are unaffected by plasma protein binding.

This simplified equation also applies when the effect of plasma protein binding attenuates the effects of high intrinsic unbound clearance and renders highly bound drugs in the moderate to low extraction category. Thus, the simplified equation applies broadly in drug discovery. An example of this is diclofenac, which in the absence of protein would demonstrate high extraction[71].

In the case of some acidic drugs, the V_T becomes small (less than fivefold higher) relative to V_P and so the major factor that controls f_{uT} is the affinity for extravascular albumin; the volume becomes less dependent on f_u and approximates to a constant. In these cases, protein binding will attenuate total clearance but will not affect total volume. Thus, the plasma half-life of the drug will be increased. It should be stressed that the exposure to unbound drug remains constant (BOX 3) and the daily dose size constant. However, the drug may be administered less frequently and still maintain similar peak to trough ratios.

Do membrane concentrations of drug correlate with* in vitro *and* in vivo *activity?
There is debate surrounding whether it is the membrane concentration rather than the free drug concentration in plasma or other fluids that influences *in vivo* or *in vitro* activity[61]. Drug within a membrane is in direct equilibrium with free drug in the plasma (or other fluid). When a drug is at steady state (or pseudo steady-state), membrane concentrations parallel free drug concentrations and the distinction of one from the other is practically unimportant, in most cases, for drug activity. There are situations for which the role of membrane-bound drug has still not been fully clarified and these have been recently reviewed[62]. These include situations in which the concentration of the compound in the membrane also alters the conformation of the target protein to change its ligand-binding properties (allosteric effects); situations in which the active site of the target protein is accessed from the membrane rather than the aqueous face of the protein; situations in which access to the receptor for high localized concentrations of the drug in the aqueous phase results from a local equilibrium with high concentrations of drug in the membrane (which are in greater than the free drug concentration at more distant sites).

However, lipophilic drugs with high membrane affinity usually behave as more hydrophilic variants and access the active site of receptors and enzymes from the aqueous phase rather than from the membrane, so even for compounds with high membrane affinity the evidence as to how a compound accesses its target is not conclusive. For example, amlodipine (TABLE 1) has high affinity for membranes but has an *in vitro* and an *in vivo* potency similar to nifedipine (TABLE 1), which has much lower membrane affinity[63]. The high membrane affinity has been associated with the long duration of amlodipine. However, this is not supported by studies with UK-118,434-05 (quaternary amlodipine), which is permanently charged and cannot penetrate the membrane, indicating that access to the binding site must be through the aqueous channel pore[64]. Moreover, UK-118,434-05 shows the same slow offset kinetics as amlodipine, highlighting that binding is a property of the ligand–protein interaction that is independent of the membrane concentrations.

Salmeterol is a β_2-adrenergic receptor agonist with high membrane affinity and a long duration of action. Exosite models, membrane accumulation models and rebinding models have all been postulated to explain salmeterol's long duration of action[65]. All of these explain the long duration of action, but studies using forms of the β_2-adrenergic receptor modified by site-directed mutagenesis to replace the amino acids 149–173 in the transmembrane domain D4 with the corresponding β_1-adrenergic receptor sequence suggest that the long duration of action is due to a protein–ligand interaction without a contribution from the membrane. The modification removes the putative protein exosite (a second binding site for the lipophilic tail group of salmeterol, which is distinct from the active site of the adrenergic receptor *per se*) and eliminates the slow dissociation of salmeterol without changing salmeterol's affinity[66].

There is analogous debate concerning how compounds access binding sites of the proteins that influence drug clearance. For instance, various models of membrane and aqueous access are postulated to explain how drugs access the efflux transporter P-glycoprotein[67]. Although the existence of various access channels in the membrane has been postulated, these do not explain the transport of quaternary compounds, such as derivatives of propafenone[68]. Thus, the concentration of a drug in the membrane may in certain cases influence drug activity, but these cases are exceptions. In those cases frequently cited in the literature, the evidence is far from conclusive. Moreover, the equilibria between the free drug in the aqueous phase and the drug in the membrane attenuate the impact on the free drug hypothesis.

Duration of drug action (plasma half-life)
The half-life of a drug is dependent on its volume of distribution and on its clearance. In BOX 4 we discuss the interplay between the fraction unbound, clearance and the volume of distribution, and highlight that the half-life is independent of the fraction unbound.

Box 5 | **Guidance for applying principles of plasma protein binding in drug discovery**

Advance drug candidates based on free drug concentration
Lead optimization and candidate advancement should focus on optimization of the free drug concentration in the biophase of the therapeutic target. The biophase may be in simple equilibrium with plasma or reside in a barrier-limited biophase. Almost all drug discovery programmes will benefit from improving absorption, such as by increasing solubility or membrane permeation, or by reducing clearance, for example, by increasing metabolic stability or by reducing the uptake and excretion by clearance organs through transporters. All programmes will benefit from understanding the relationship between unbound drug in the plasma and the pharmacodynamic effect. Membrane permeation of a drug is a key factor in reducing uptake and excretion by clearance organs through transporters.

For a limited range of targets, such as those in the liver, the active uptake and excretion by clearance organs through transporters may predominate in determining unbound drug exposure in the biophase and unbound drug in plasma may be less helpful in understanding primary pharmacodynamics.

Avoid structural modification to reduce the free drug fraction for plasma protein binding
Data from the *in vitro* plasma protein binding assay, in which the free drug fraction (f_u) of a compound is measured, should not be used to guide compound design. This is because the resulting increase in free drug concentration is counterbalanced *in vivo* by increased clearance, resulting in no net improvement in free drug concentration.

Do not use shift assays
Serum shift assays, which measure the *in vitro* activity of compounds in the presence of serum protein, should not be used to rank discovery compounds for expected *in vivo* activity because they do not incorporate other properties that can affect the free drug concentration *in vivo*. Shift assays occasionally relate to *in vivo* activity, when they indirectly measure properties that affect clearance, such as lipophilicity.

Avoid the trap of total drug concentration and brain/plasma ratio
For targets in the brain, a focus on maximizing total (bound plus free) drug exposure ($C_{max,total}$ or AUC_{total}) in brain tissue or on the brain to plasma ratio (brain/plasma, defined as the total compound exposure in the brain divided by the total compound exposure in plasma) to guide structure modification should be changed, whenever possible, to measure the unbound concentration in the brain. This can be estimated by the f_u in the brain (obtained from an *in vitro* assay) multiplied by the total drug concentration in the brain from an *in vivo* dosing experiment.

Discover the missing link between pharmacokinetics and pharmacodynamics
Frequently, the missing link between pharmacokinetics and pharmacodynamics, or the *in vitro–in vivo* correlation, is the free drug concentration. When developing pharmacokinetic–pharmacodynamic relationships, project teams should use free drug concentrations. Where the target exists in a biophase that is separate from plasma, the relationship between free drug in the plasma and free drug in the biophase needs to be explored.

Enhancing free drug concentration

For oral drugs, the free drug concentrations will not be altered by PPB (with the exception of compounds that are predominantly cleared from the body by non-hepatic clearance[6]). The free compound concentration following an oral dose is affected by the dose, the dose interval, the fraction of a dose absorbed (*Fa*) and by intrinsic clearance, as indicated by the equation in BOX 3 (REFS 6,43).

Although, as previously discussed, some researchers have tried to increase the free drug concentration *in vivo* by increasing the f_u, the average free drug concentration *in vivo* after oral dosing is independent of PPB, as indicated by the equation in BOX 3 (REFS 6,43). Based on this equation, the free drug concentration in plasma can be enhanced by improving the *Fa* (by increasing the solubility or the rate of membrane permeation) in the intestine and by reducing clearance (by increasing metabolic stability and by decreasing efflux)[6,42,43,69,70]. The principles of how to improve solubility, membrane permeation, metabolic stability and to reduce efflux, together with practical examples, are found in other references[71,72].

Potential drug–drug interactions

There has been concern among some drug discovery scientists that drug–drug interactions could occur through the displacement of the molecules of one drug from plasma proteins by the molecules of another drug[73–75]. However, because the free drug concentration is determined by intrinsic clearance, few clinical drug–drug interactions occur as a result of this mechanism[6,76]. *In vitro* experiments, usually using high concentrations of drugs, show that displacement of drug molecules from plasma proteins can occur; however, this seldom happens at the concentrations achieved clinically. Even if displacement did occur, the $C_{av,u}$, and AUC_u would be unchanged and any acutely raised concentration of free drug would lead to more rapid overall elimination until steady state was restored[6,77,78].

Although drug–drug interactions were originally thought to occur by the displacement from plasma proteins, a premise that stemmed from observed clinical drug–drug interactions between non-steroidal anti-inflammatory drugs and oral anticoagulant drugs, this is now known to be incorrect. The actual mechanism of these drug–drug interactions is the inhibition of metabolic enzymes that are primarily responsible for metabolizing one or more drugs. This is exemplified by studies on lornoxicam[79].

This non-steroidal anti-inflammatory drug undergoes 5-hydroxylation by cytochrome P450 2C9, the metabolizing enzyme also responsible for the intrinsic clearance of the oral anticoagulants warfarin, phenprocoumon and acenocoumarol. Using *in vitro* metabolism experiments, the increases in steady-state plasma concentrations or AUC of the oral anticoagulants caused by concomitant lornoxicam medication were predicted, including the 1.6-fold increase in the steady-state plasma concentration for (S)-warfarin.

This study concluded that the degree of pharmacokinetic interactions exhibited by oral anticoagulants and lornoxicam were dependent on the respective contribution of cytochrome P450 2C9 to their intrinsic clearance and not on the displacement of the anticoagulant from plasma protein. This is an example of the general finding that changes in PPB have little clinical relevance, including drug–drug interactions[80–83].

Conclusions

Based on the examples outlined in this article, we conclude that the binding of a drug to plasma proteins has little effect on the *in vivo* efficacy of that drug. Rather, it is the free drug concentration at the therapeutic target — which can be enhanced by improving solubility, membrane permeation, metabolic stability and by reducing efflux — that is crucial for *in vivo* activity. We suggest guidance for applying the principles of PPB in drug discovery, summarized in BOX 5, which will hopefully result in improved processes for compound optimization and in drug candidates that have greater therapeutic efficacy being prioritized in drug discovery programmes.

Dennis A. Smith is at the Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9UJ, UK.

Li Di is at the Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research and Development, Eastern Point Road, Groton, Connecticut 06340, USA.

Edward H. Kerns is at Discovery ADME, 20 Hampton Court, Skillman, New Jersey 08558, USA.

Correspondence to L.D.
e-mail: li.di@pfizer.com

doi:10.1038/nrd3287

1. Molla, A. *et al.* Human serum attenuates the activity of protease inhibitors toward wild-type and mutant human immunodeficiency virus. *Virology* **250**, 255–262 (1998).
2. Velaparthi, U. *et al.* Discovery and evaluation of 4-(2-(4-chloro-1*H*-pyrazol-1-yl)ethylamino)-3-(6-(1-(3-fluoropropyl)piperidin-4-yl)-4-methyl-1*H*-benzo[*d*] imidazol-2-yl)pyridin-2(1*H*)-one (BMS-695735), an orally efficacious inhibitor of insulin-like growth factor-1 receptor kinase with broad spectrum *in vivo* antitumor activity. *J. Med. Chem.* **51**, 5897–5900 (2008).
3. McKerrecher, D. *et al.* Design of a potent, soluble glucokinase activator with excellent *in vivo* efficacy. *Bioorg. Med. Chem. Lett.* **16**, 2705–2709 (2006).
4. Leach, A. G. *et al.* Matched molecular pairs as a guide in the optimization of pharmaceutical properties; a study of aqueous solubility, plasma protein binding and oral exposure. *J. Med. Chem.* **49**, 6672–6682 (2006).
5. Boros, E. E. *et al.* Synthesis and antiviral activity of 7-benzyl-4-hydroxy-1,5-naphthyridin-2(1*H*)-one HIV integrase inhibitors. *J. Med. Chem.* **52**, 2754–2761 (2009).
6. Benet, L. Z. & Hoener, B.-A. Changes in plasma protein binding have little clinical relevance. *Clin. Pharmacol. Ther.* **71**, 115–121 (2002).
7. Pang, K. S. & Rowland, M. Hepatic clearance of drugs. I. Theoretical considerations of a "well-stirred" model and a "parallel tube" model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *J. Pharmacokinet. Biopharm.* **5**, 625–653 (1977).
8. Lin, J. H. CSF as a surrogate for assessing CNS exposure: an industrial perspective. *Curr. Drug Metab.* **9**, 46–59 (2008).
9. Liu, X. *et al.* Evaluation of cerebrospinal fluid concentration and plasma free concentration as a surrogate measurement for brain free concentration. *Drug Metab. Dispos.* **34**, 1443–1447 (2006).
10. Hammarlund-Udenaes, M., Friden, M., Syvanen, S. & Gupta, A. On the rate and extent of drug delivery to the brain. *Pharm. Res.* **8**, 1737–1750 (2008).
11. Brammer, K. W., Farrow, P. R. & Faulkner, J. K. Pharmacokinetics and tissue penetration of fluconazole in humans. *Rev. Infect. Dis.* **12**, S318–S326 (1990).
12. Schramm, P., Wildfeuer, A. & Sarnow, E. Determination of fluconazole concentrations in the prostatic and seminal vesicle fluid (split ejaculate). *Mycoses* **37**, 417–420 (1994).
13. Debruyne, D. Clinical pharmacokinetics of fluconazole in superficial and systemic mycoses. *Clin. Pharmacokinet.* **33**, 52–77 (1997).
14. Day, R. O., Francis, H., Vial, J., Geisslinger, G. & Williams, K. M. Naproxen concentrations in plasma and synovial fluid and effects on prostanoid concentrations. *J. Rheumatol.* **22**, 2295–2303 (1995).
15. Maurer, T. S., DeBartolo, D. B., Tess, D. A. & Scott, D. O. Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. *Drug Metab. Dispos.* **33**, 175–181 (2005).
16. Summerfield, S. G. *et al.* Improving the *in vitro* prediction of *in vivo* central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. *J. Pharmacol. Exp. Ther.* **316**, 1282–1290 (2006).
17. Avdeef, A. Physicochemical profiling (solubility, permeability and charge state). *Curr. Top. Med. Chem.* **1**, 277–351 (2001).
18. Di, L., Kerns, E. H. & Carter, G. T. Strategies to assess blood–brain barrier penetration. *Expert Opin. Drug Discov.* **3**, 677–687 (2008).
19. Liu, X., Tu, M., Kelly, R. S., Chen, C. & Smith, B. J. Development of a computational approach to predict blood–brain barrier permeability. *Drug Metab. Dispos.***32**, 132–139 (2004).
20. Ziemniak, J. A., Shank, R. G. & Schentag, J. J. The partitioning of cimetidine into canine cerebrospinal fluid. *Drug Metab. Dispos.***12**, 217–221 (1984).
21. Suzuki, H., Terasaki, T. & Sugiyama, Y. Role of efflux transport across the blood–brain barrier and blood–cerebrospinal fluid barrier on the disposition of xenobiotics in the central nervous system. *Adv. Drug Deliv. Rev.* **25**, 257–285 (1997).
22. Somogyi, A. & Gugler, R. Clinical pharmacokinetics of cimetidine. *Clin. Pharmacokinet.* **8**, 463–495 (1983).
23. Minchinton, A. I. & Tannock, I. F. Drug penetration in solid tumours. *Nature Rev. Cancer* **6**, 583–592 (2006).
24. Lau, Y. Y., Huang, Y., Frassetto, L. & Benet, L. Z. Effect of OATP1B transporter inhibition on the pharmacokinetics of atorvastatin in healthy volunteers. *Clin. Pharmacol. Ther.* **81**, 194–204 (2007).
25. Kang, J., Wang, L., Chen, X.-L., Triggle, D. J. & Rampe, D. Interactions of a series of fluoroquinolone antibacterial drugs with the human cardiac K^+ channel HERG. *Mol. Pharmacol.* **59**, 122–126 (2001).
26. Webster, R. *et al.* Pharmacokinetic/pharmacodynamic assessment of the effects of E4031, cisapride, terfenadine, and terodiline on monophasic action potential duration in dog. *Xenobiotica* **31**, 633–650 (2001).

27. Miyazaki, M., Maekawa, C., Iwanaga, K., Morimoto, K. & Kakemi, M. Bioavailability assessment of disopyramide using pharmacokinetic–pharmacodynamic (PK–PD) modeling in the rat. *Biol. Pharm. Bull.* **23**, 1363–1369 (2000).
28. Bischoff, U., Schmidt, C., Netzer, R. & Pongs, O. Effects of fluoroquinolones on HERG currents. *Eur. J. Pharmacol.* **406**, 341–343 (2000).
29. Kalvass, J. C., Olson, E. R., Cassidy, M. P., Selley, D. E. & Pollack, G. M. Pharmacokinetics and pharmacodynamics of seven opioids in P-glycoprotein-competent mice: assessment of unbound brain EC50, u and correlation of *in vitro*, preclinical, and clinical data. *J. Pharmacol. Exp. Ther.* **323**, 346–355 (2007).
30. Webster, R., Leishman, D. & Walker, D. Towards a drug concentration effect relationship for QT prolongation and torsades de pointes. *Curr. Opin. Drug Discov. Dev.* **5**, 116–126 (2002).
31. Redfern, W. S. *et al.* Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. [Erratum to document cited in CA139:172923]. *Cardiovasc. Res.* **63**, 186–187 (2004).
32. Redfern, W. S. *et al.* Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc. Res.* **58**, 32–45 (2003).
33. Zhang, J. Y. *et al.* Development of an improved IP1 assay for the characterization of 5-HT2C receptor ligands. *Assay Drug Dev. Technol.* **8**, 106–113 (2010).
34. Zhu, P. J. *et al.* A miniaturized glucocorticoid receptor translocation assay using enzymatic fragment complementation evaluated with qHTS. *Comb. Chem. High Throughput Screen.* **11**, 545–559 (2008).
35. van Steeg, T. J. *et al.* Influence of plasma protein binding on pharmacodynamics: estimation of *in vivo* receptor affinities of beta blockers using a new mechanism-based PK–PD modelling approach. *J. Pharm. Sci.* **98**, 3816–3828 (2009).
36. Jankovic, J. & Tolosa, E. *Parkinson's Disease and Movement Disorders* 5th edn (Lippincott Williams & Wilkins, Philadelphia, Pennsylvania; 2007).
37. Savi, P., Nurden, P., Nurden, A. T., Levy-Toledano, S. & Herbert, J. M. Clopidogrel: a review of its mechanism of action. *Platelets* **9**, 251–255 (1998).
38. Puscas, I., Coltau, M., Baican, M. & Domuta, G. A new concept regarding the mechanism of action of omeprazole. *Int. J. Clin. Pharmacol. Ther.* **37**, 286–293 (1999).
39. Moss, N. *et al.* A new class of 5-HT2B antagonists possesses favorable potency, selectivity, and rat pharmacokinetic properties. *Bioorg. Med. Chem. Lett.* **19**, 2206–2210 (2009).
40. Ratliff, K. S. *et al.* An automated electrophysiology serum shift assay for Kv channels. *Assay Drug Dev. Technol.* **6**, 243–253 (2008).
41. Trainor, G. L. The importance of plasma protein binding in drug discovery. *Expert Opin. Drug Discov.* **2**, 51–64 (2007).
42. Lin, J. H. & Lu, A. Y. H. Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol. Rev.* **49**, 403–449 (1997).
43. Liu, X. & Chen, C. Strategies to optimize brain penetration in drug discovery. *Curr. Opin. Drug. Discov. Devel.* **8**, 505–512 (2005).
44. Livingston, D. J. *et al.* Weak binding of VX-478 to human plasma proteins and implications for anti-human immunodeficiency virus therapy. *J. Infect. Dis.* **172**, 1238–1245 (1995).
45. Bilello, J. A. & Drusano, G. L. Relevance of plasma protein binding to antiviral activity and clinical efficacy of inhibitors of human immunodeficiency virus protease. *J. Infect. Dis.* **173**, 1524–1526 (1996).
46. Geng, B. *et al.* Potent and selective inhibitors of *Helicobacter pylori* glutamate racemase (MurI): pyridodiazepine amines. *Bioorg. Med. Chem. Lett.* **19**, 930–936 (2009).
47. Coleman, P. J. *et al.* Nonpeptide αvβ3 antagonists. Part 11: discovery and preclinical evaluation of potent αvβ3 antagonists for the prevention and treatment of osteoporosis. *J. Med. Chem.* **47**, 4829–4837 (2004).
48. Fessey, R. E., Austin, R. P., Barton, P., Davis, A. M. & Wenlock, M. C. in *Pharmacokinetic Profiling in Drug Research: Biological, Physicochemical, and Computational Strategies* [LogP2004, Lipophilicity Symposium], (ed. B. Testa) 119–141 (Verlag Helvetica Chimica Acta, Zurich, Switzerland, 2006).
49. Wan, H., Rehngren, M., Giordanetto, F., Bergstroem, F. & Tunek, A. High-throughput screening of drug–brain tissue binding and *in silico* prediction for assessment of central nervous system drug delivery. *J. Med. Chem.* **50**, 4606–4615 (2007).
50. Lewis, D. F. V., Jacobs, M. N. & Dickins, M. Compound lipophilicity for substrate binding to human P450s in drug metabolism. *Drug Discov. Today* **9**, 530–537 (2004).
51. Toutain, P. L. & Bousquet-Melou, A. Free drug fraction vs free drug concentration: a matter of frequent confusion. *J. Vet. Pharmacol. Ther.* **25**, 460–463 (2002).
52. Boxenbaum, H. in *Pharmacokinetic, Pharmacokinetic & Pharmacodynamic Data Analysis: Concepts and Applications* 4th edn Vol. 32 (eds Gabrielsson, J. & Weiner, D.) (Swedish Pharmaceutical Press, 2007).
53. Peletier, L. A., Benson, N. & van der Graaf, P. H. Impact of plasma-protein binding on receptor occupancy: an analytical description. *J. Theor. Biol.* **256**, 253–262 (2009).
54. Ogino, Y. *et al.* Syntheses and structure-activity relationships of novel, potent, and selective trans-2-[3-o xospiro[isobenzofuran-1(3*H*),1-cyclohexan]-4-yl] benzimidazole NPY Y5 receptor antagonists. *Bioorg. Med. Chem. Lett.* **18**, 4997–5001 (2008).
55. Zheng, G. Z. *et al.* Correlation between brain/plasma ratios and efficacy in neuropathic pain models of selective metabotropic glutamate receptor 1 antagonists. *Bioorg. Med. Chem. Lett.* **16**, 4936–4940 (2006).
56. Hattori, K. *et al.* Rational approaches to discovery of orally active and brain-penetrable quinazolinone inhibitors of poly(ADP-ribose)polymerase. *J. Med. Chem.* **47**, 4151–4154 (2004).
57. Gilligan, P. J. Corticotropin-releasing factor receptor antagonists. *Expert Opin. Ther. Pat.* **16**, 913–924 (2006).
58. Hilgert, M., Noldner, M., Chatterjee, S. S. & Klein, J. KA-672 inhibits rat brain acetylcholinesterase *in vitro* but not *in vivo*. *Neurosci. Lett.* **263**, 193–196 (1999).
59. Jeffrey, P. & Summerfield, S. G. Challenges for blood–brain barrier (BBB) screening. *Xenobiotica* **37**, 1135–1151 (2007).
60. Gabrielsson, J. & Green, A. R. Quantitative pharmacology or pharmacokinetic pharmacodynamic integration should be a vital component in integrative pharmacology. *J. Pharmacol. Exp. Ther.* **331**, 767–774 (2009).
61. Coleman, R. A. On the mechanism of the persistent action of salmeterol: what is the current position? *Br. J. Pharmacol.* **158**, 180–182 (2009).
62. Vauquelin, G. & Packeu, A. Ligands, their receptors and. plasma membranes. *Mol. Cell. Endocrinol.* **311**, 1–10 (2009).
63. Mason, R. P., Moiesey, D. M. & Shajenko, L. Cholesterol alters the binding of calcium channel blockers to the membrane lipid bilayer. *Mol. Pharmacol.* **41**, 315–321 (1992).
64. Kwan, Y. W., Bangalore, R., Lakitsh, M., Glossmann, H. & Kass, R. S. Inhibition of cardiac L-type calcium channels by quaternary amlodipine: implications for pharmacokinetics and access to dihydropyridine binding site. *J. Mol. Cell. Cardiol.* **27**, 253–262 (1995).
65. Szczuka, A., Wennerberg, M., Packeu, A. & Vauquelin, G. Molecular mechanisms for the persistent bronchodilatory effect of the β_2-adrenoceptor agonist salmeterol. *Br. J. Pharmacol.* **158**, 183–194 (2009).
66. Green, S. A., Spasoff, A. P., Coleman, R. A., Johnson, M. & Liggett, S. B. Sustained activation of a G protein-coupled receptor via "anchored" agonist binding. Molecular localization of the salmeterol exosite within the beta 2-adrenergic receptor. *J. Biol. Chem.* **271**, 24029–24035 (1996).
67. Lee, C. A., Cook, J. A., Reyner, E. L. & Smith, D. A. P-glycoprotein related drug interactions: clinical importance and a consideration of disease states. *Expert Opin. Drug Metab. Toxicol.* **6**, 603–619 (2001).
68. Schmidt, S., Gonzalez, D. & Derendorf, H. Significance of protein binding in pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.* **99**, 1107–1122 (2010).
69. Hussein, Z., Evans, A. M. & Rowland, M. Physiologic models of hepatic drug clearance: influence of altered protein binding on the elimination of diclofenac in the isolated perfused rat liver. *J. Pharm. Sci.* **82**, 880–885 (1993).
70. Wood, A. & Armour, D. The discovery of the CCR5 receptor antagonist, UK-427, 857, a new agent for the treatment of HIV infection and AIDS. *Prog. Med. Chem.* **43**, 239–271 (2005).
71. Smith, D. A., van de Waterbeemd, H. & Walker, D. K. *Pharmacokinetics and Metabolism in Drug Design* 2nd edn Vol. 13 (Wiley-VCH, Weinheim 2006).
72. Kerns, E. H. & Di, L. *Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization* 56–167 (Elsevier, London; 2008).
73. McNamara, P. J., Trueb, V. & Stoeckel, K. Ceftriaxone binding to human serum albumin. Indirect displacement by probenecid and diazepam. *Biochem. Pharmacol.* **40**, 1247–1253 (1990).
74. Pedersen, L. E. & Bonde, J. Displacement of disopyramide from human plasma proteins. *Acta Pharmacol. Toxicol.* **57**, 223–226 (1985).
75. Mungall, D. R. *et al.* Effect of diltiazem on warfarin plasma protein binding. *J. Clin. Pharmacol.* **24**, 264–266 (1984).
76. Sparreboom, A., Nooter, K., Loos, W. J. & Verweij, J. The (ir)relevance of plasma protein binding of anticancer drugs. *Neth. J. Med.* **59**, 196–207 (2001).
77. Sellers, E. M. Plasma protein displacement interactions are rarely of clinical significance. *Pharmacology* **18**, 225–227 (1979).
78. Bjornsson, T. D., Meffin, P. J., Swezey, S. & Blaschke, T. F. Clofibrate displaces warfarin from plasma proteins in man: an example of a pure displacement interaction. *J. Pharmacol. Exp. Ther.* **210**, 316–321 (1979).
79. Kohl, C. & Steinkellner, M. Prediction of pharmacokinetic drug/drug interactions from *in vitro* data: interactions of the nonsteroidal anti-inflammatory drug lornoxicam with oral anticoagulants. *Drug Metab. Dispos.* **28**, 161–168 (2000).
80. DeVane, C. L. Clinical significance of drug binding, protein binding, and binding displacement drug interactions. *Psychopharmacol. Bull.* **36**, 5–21 (2002).
81. Sansom, L. N. & Evans, A. M. What is the true clinical significance of plasma protein binding displacement interactions? *Drug Saf.* **12**, 227–233 (1995).
82. Rolan, P. E. Plasma protein binding displacement interactions — why are they still regarded as clinically important? *Br. J. Clin. Pharmacol.* **37**, 125–128 (1994).
83. MacKichan, J. J. Protein binding drug displacement interactions: fact or fiction? *Clin. Pharmacokinet.* **16**, 65–73 (1989).
84. Smith, D. *Absorption and Distribution as Factors in Drug Design, in Medicinal Chemistry — Into the Millennium* Vol. 264 (eds Campbell M. M. &, Blagbrough, I. S.) 331–344 (Royal Society of Chemistry, Cambridge, UK, 2001).
85. Yamada, S., Kato, Y., Okura, T., Kagawa, Y. & Kawabe, K. Prediction of alpha 1-adrenoceptor occupancy in the human prostate from plasma concentrations of silodosin, tamsulosin and terazosin to treat urinary obstruction in benign prostatic hyperplasia. *Biol. Pharm. Bull.* **30**, 1237–1241 (2007).
86. Yamada, Y., Ito, K., Nakamura, K., Sawada, Y. & Iga, T. Prediction of therapeutic doses of beta-adrenergic receptor blocking agents based on quantitative structure–pharmacokinetic/pharmacodynamic relationship. *Biol. Pharm. Bull.* **16**, 1251–1259 (1993).
87. Mathot, R. A. A., Soudijn, W., Breimer, D. D., Ijzerman, A. P. & Danhof, M. Pharmacokinetic–hemodynamic relationships of 2-chloroadenosine at adenosine A1 and A2a receptors *in vivo*. *Br. J. Pharmacol.* **118**, 369–377 (1996).
88. Troke, P. F., Andrews, R. J., Pye, G. W. & Richardson, K. Fluconazole and other azoles: translation of *in vitro* activity to *in vivo* and clinical efficacy. *Rev. Infect. Dis.* **12**, S276–S280 (1990).
89. Smith, D. A. in *Microsomes Drug Oxidation* (ed. Kaminsky, L. S.) 31–36 (Monduzzi Editore, Bologna, Italy, 2008).

Acknowledgements
The authors would like to thank B. Harrison, G. Carter, O. McConnell, J. Butera, T. Mansour, M. Troutman, J. Liras and T. Liston for their support and leadership.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Top 300 Prescriptions for 2005: http://www.rxlist.com/script/main/art.asp?articlekey=79509
The FDA Center for Drug Evaluation and Research: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm

ALL LINKS ARE ACTIVE IN THE ONLINE PDF