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Editorial

Free Drug Concepts: A Lingering Problem in Drug Discovery

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Free drug concepts underpin all pharmacokinetic−pharmacodynamic relationships and therefore should be a foundational element of drug discovery projects. The concepts are not new and have been widely accepted,[1−6] yet there remains misunderstanding of the principles, and confusion as to how best to apply them in drug design.

The lack of impact of plasma protein binding modulation on overall drug quality was discussed by Liu et al. in 2014[6] in this journal. However, the current authors' experience working within the industry, and reviewing drug discovery projects in academic and charitable research institutions, is that free drug concepts are not fully embedded in many organizations (Table 1). We also see that many published strategies aimed at "improved DMPK properties" are not founded on free drug principles and thus are flawed and misleading. This leads to the propagation of incorrect data interpretation and inefficient optimization strategies.

Table 1. Statements You May Hear When Free Drug Concepts Are Misunderstood

Free drug levels in plasma are just too low...we really need to reduce plasma protein binding.
Free drug levels in tissues are just too low...we really need to reduce tissue binding.
The low volume of distribution means the compound is just not accessing the target in tissues.
We do not work with acids because they are too highly bound in plasma and do not distribute into tissues.
The total brain to total plasma concentration ratio is above 1, meaning that the drug is pumped into the CNS.
We can assess brain penetration by measuring brain binding in vitro.
The concentrations of drug in tumor are very high relative to plasma meaning there is specific delivery to the tumor.
When we add protein to our in vitro potency assays the potency is reduced, so we need to lower plasma protein binding.

The aim of this editorial is to explore the reasons why this topic remains a challenge, to clearly describe the principles governing free drug concentrations and exposure, and to describe their utility in drug discovery projects.

WHY DOES THIS CONTINUE TO BE SUCH A PROBLEM?

Potential reasons include:

- The concepts, while not complex, can be counterintuitive.
- The processes governing free drug concentrations *in vivo* are different from those in *in vitro* assays but are often assumed to be the same.
- The most common calculation performed (conversion of total drug concentration to free drug concentration) requires an equation in a form that, while mathematically correct, has led to a mechanistic misunderstanding. This misunderstanding results in many published studies being incorrect in their use of pharmacokinetic data as drivers of compound optimization.
- Pharmacokinetic training focuses on total drug concentrations and parameters, and these also dominate clinical pharmacokinetics where the focus is on a single compound, and for which plasma binding is usually constant. Compound optimization in drug discovery is anchored on the understanding of unbound drug exposure and its relationship to target engagement; thus, comparisons between molecules requires the use of unbound exposure and unbound PK parameters.
- Even today, authors use the term "Free drug hypothesis", as though there is some doubt as to its validity. It is not a hypothesis; it is a fundamental principle.

WHAT ARE THE KEY FREE DRUG CONCEPTS?

- Unbound drug concentrations at the target site determine the degree of target engagement
- Unbound concentrations *in vivo* are not determined by the extent of plasma or tissue binding; instead, plasma and tissue binding determine the concentrations of bound drug
- Unbound drug concentrations *in vivo* are determined by the rate of drug input (absorption) and the rate of elimination (intrinsic clearance)
- At equilibrium, free drug concentrations in cytosol approximate those in plasma (with a few specific exceptions, described below

NOMENCLATURE

The nomenclature used to describe "intrinsic clearance" (CLint) is complex and the relationship between "intrinsic clearance" and "unbound clearance" (CLu) is not always clear. Figure 1 shows the clearance terms obtained from *in vitro* and *in vivo* pharmacokinetic experiments and their relationships

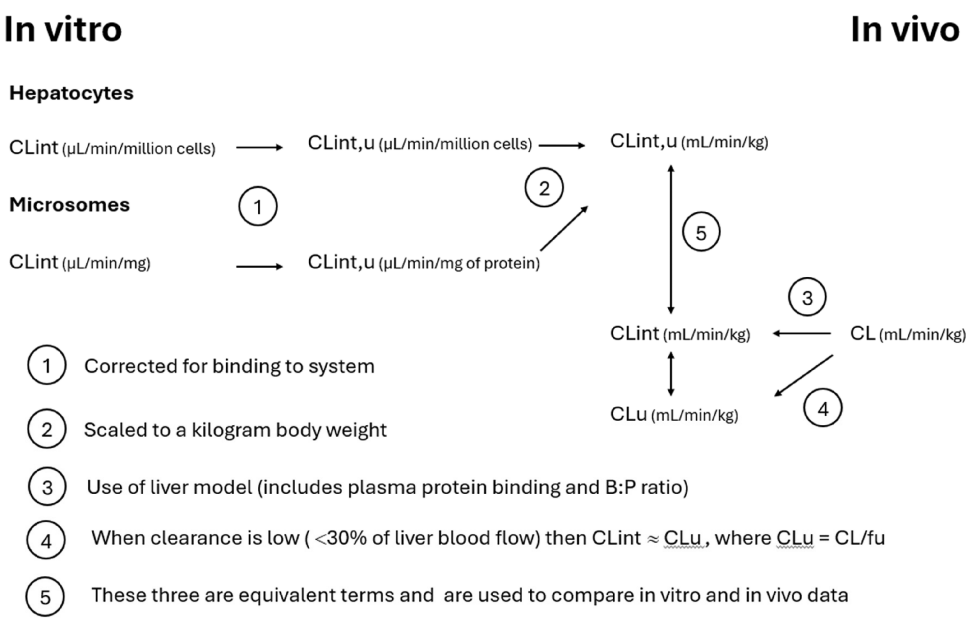

Figure 1. Clearance terms derived from in vitro and in vivo experiments and their relationships Note: CLint is, by definition, an unbound term and thus CLint measured *in vitro* is an apparent value until corrected for binding in the assay. However, for the avoidance of doubt, we have used CLint,u to represent the binding-corrected *in vitro* value.

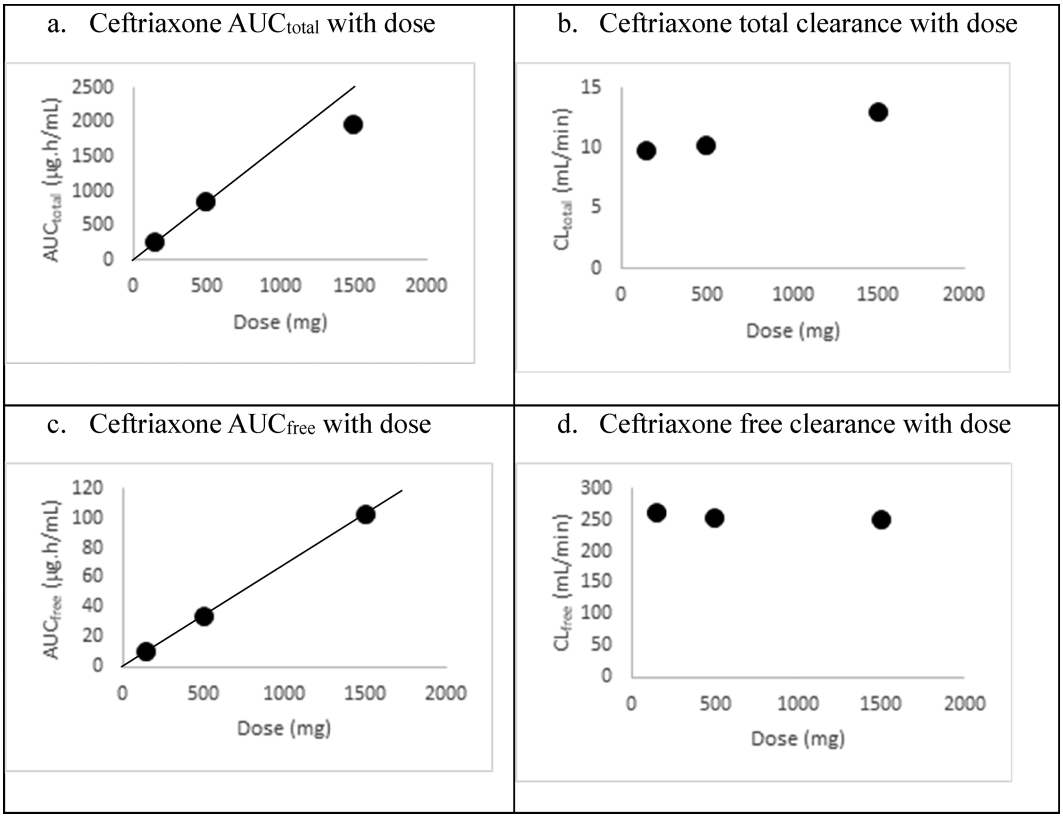

Figure 2. Effect of increasing dose on the total and free AUC and clearance for ceftriaxone.

WHY ARE FREE PLASMA CONCENTRATIONS NOT MODULATED BY PLASMA PROTEIN BINDING?

This is the key question. We seek to answer it by explaining what does control free drug concentrations, giving an example and showing some simulations.

The Pharmacokinetic Principles. What does control free plasma concentrations? A simple scenario best explains this, but the principle applies to all pharmacokinetic experiments. If we consider an intravenous infusion and ask what are the free plasma concentrations at steady-state? At steady-state the rate of input equals the rate of elimination.

$$\text{Rate of elimination} = \text{Rate of input (infusion rate)} \quad (1)$$

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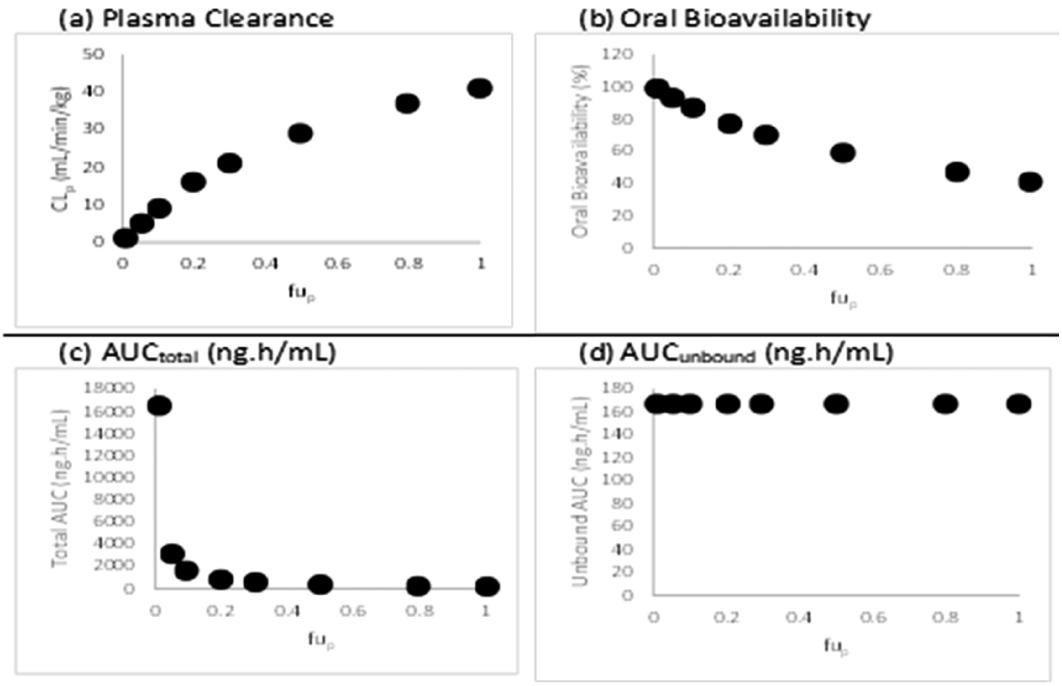

Figure 3. Simulations demonstrating influence of plasma protein binding on pharmacokinetic parameters.

What is the rate of elimination? Well, the fundamental definition of the pharmacokinetic parameter "clearance" is that it is the parameter that relates the concentration to the rate of elimination.

(The more familiar CL = Dose/AUC is just the integral$_{(0\text{-}\infty)}$ of this, where AUC is the integral of concentration, and dose is the integral of the input per unit time)

So

$$\text{Rate of elimination} = C \times CL \tag{2}$$

And, using unbound parameters

$$\text{Rate of elimination} = Cu \times CLu \tag{3}$$

So, at steady-state, where the input rate equals the elimination rate

$$Css,u \times CLu = \text{Rate of input} \tag{4}$$

Rearrangement of this equation for Css,u shows that it is defined by infusion rate and unbound (or intrinsic) clearance of the drug. There is no term for fu in this equation, showing that free drug concentration is independent of fraction unbound in plasma.

$$Css,u = \frac{\text{Rate of input}}{CLu} \tag{5}$$

It is also apparent that if this equation is converted back to reflect the total drug concentration, it is the total drug concentration that is dependent upon the fraction unbound.

$$Css = \frac{\text{Rate of input}}{CLu \times fu} \tag{6}$$

A Case Study Demonstrating the Principles. One difficulty with demonstrating the lack of effect of plasma protein binding on unbound concentration, is that it is extremely unlikely that within a chemical series, fu can be modulated without affecting CLu (or indeed other parameters as well) as both are highly dependent upon lipophilicity.[7] However, experimental confirmation of the lack of effect of fu on unbound exposure is available in a case where protein binding is saturated and CLu remains constant as doses increase.

An example of this occurred with ceftriaxone, which was administered to volunteers over a dose range 150−1,500 mg.[8] Ceftriaxone is a drug that shows increases in fu with concentration, due to saturation of plasma protein binding. In the clinical dose escalation study, total plasma AUC values increased subproportionally with dose (Figure 2(a)), suggesting that CL was increasing with dose (Figure 2(b)). When the total plasma AUC was corrected for the fu values across the concentration range, the free AUC values increased proportionally with dose (Figure 2(c)), suggesting that CLu was unchanged with increasing doses (constant value of approximately 255 mL/min). The only effect of reduction in plasma protein binding (increased fu) was to lower the dose-normalized total concentrations.

Simulations. Simulations can be used to illustrate the principles, building on the work of Liu et al.[6]

Assuming complete oral absorption (Fa = 1) and no gut metabolism (Fg = 1), with a fixed CLu of 100 mL/min/kg and doses of 1 mg/kg to rats (Liver blood flow, Q_H = 70 mL/min/kg), the CL, F, AUC, and AUCu can be calculated against a background of modulation of fu, using the Well-stirred liver model (Figure 3).

$$CL = \frac{Q_H \times fu \times CLint}{Q_H + fu \times CLint} \tag{7}$$

The simplest situation occurs when there is no binding to plasma proteins. Under these circumstances, the total and unbound clearances are equivalent, and the clearance reflects intrinsic clearance and the hepatic blood flow limitation. As the compounds become more highly bound, total clearance is reduced and oral bioavailability increases in line with the reduction in total clearance (decreasing hepatic extraction) of the compound. However, when corrected for the plasma protein

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binding, the unbound AUC remains consistent and dependent only on the fixed value of CLint. Consequently, modulation of plasma protein binding has no impact on the observed unbound plasma concentrations of a drug; only bound, and hence total, concentrations are changed with modulation of fraction unbound.

WHY DOES PLASMA PROTEIN BINDING CREATE SUCH CONFUSION?

Perhaps a major root cause of confusion is the equation:

$$Cu = fu \times C \tag{8}$$

This is universally and correctly used to calculate unbound concentration from the measured total concentration, using the unbound fraction (fu). However, it has unfortunately led to the mis-interpretation that *in vivo* free drug concentrations are driven by total concentration and the degree of binding, which is not the case. In its original form:

$$C = \frac{Cu}{fu} \tag{9}$$

Written in this form, the equation shows the correct dependency, i.e. that total drug concentrations are a function of the unbound concentration and the degree of protein binding.

Focus on unbound drug concentration actually simplifies oral drug pharmacokinetics, since the well-stirred model and first pass metabolism (eq 7) combine, to give a very simple equation[9]

$$AUCu = Fa \times Fgut \times \frac{Dose}{CLint} \tag{10}$$

Where Fgut is the fraction escaping gut metabolism and that the related Cav can be calculated by dividing by the dose interval,

$$Cav,u = \left(Fa \times Fgut \times \frac{Dose}{CLint}\right)/\tau \tag{11}$$

Equations such as these are commonly used in drug discovery, and incorporate familiar levers for optimization of pharmacokinetic properties, namely solubility, permeability, and intrinsic clearance; in addition, the exposure target for efficacy can be related to intrinsic potency. However, they are representative of the steady-state situation and, as such, are an oversimplification. Even when AUCu or Cav,u is the target, optimization toward a safe and effective drug also requires consideration of half-life and, by extension, distributional properties. Only when the half-life is appropriate for the intended dosing interval will a drug maintain target engagement while minimizing the potential for off-target or toxic effects. This therefore entails the optimization of intrinsic clearance in ratio to the unbound volume of distribution to achieve the desired half-life range.[7]

The incorrect focus on eq 1 to provide a mechanistic understanding of what drives free drug concentrations is analogous to the way that the equation

$$T_{1/2} = 0.693\frac{V}{CL} \tag{12}$$

should clearly not be used in the form

$$V = \frac{CL \times T_{1/2}}{0.693} \tag{13}$$

Despite being mathematically correct, as it would confound a key insight into drug disposition, that the half-life of a drug is function of its volume of distribution and its clearance.

BINDING IN IN VITRO ASSAYS

It is important to highlight that free drug concentrations in *in vitro* assays, such as potency and intrinsic clearance assays, are influenced by nonspecific drug binding, as the conditions are static. Nonspecific (low affinity) binding can occur with the components of the assay itself (cells, protein, media) as well as to the assay vessel. Nonspecific binding is dependent on lipophilicity and results in the free drug concentration being lower than the nominal drug concentration in the assay. Thus, observations made in *in vitro* assays are deemed "apparent" and require correction for the degree of nonspecific binding to yield the true value.

Unfortunately, the effect of binding in *in vitro* potency assays has led to misinterpretation of the effects of binding *in vivo*. Adding albumin or plasma to an *in vitro* potency assay will decrease apparent potency or affinity, if the compound binds to albumin. This type of assay has been widely used in drug discovery to guide synthesis and often termed a "shift assay". A low shift in potency has often been interpreted to mean a better compound. However, from the preceding discussion we can see that this interpretation is incorrect. Efficacy is determined by the unbound potency in relation to the unbound concentration *in vivo*, which is independent of protein binding. The principle here is determine the true unbound potency *in vitro* and compare this with unbound concentrations *in vivo*. Nonspecific binding can be readily measured using the same methodologies used to estimate plasma protein binding. This binding may also be calculated,[10] although measurements should be used initially to establish the accuracy of the calculations for the chemical series.

INTERPRETATION OF IN VIVO PHARMACOKINETIC STUDIES: EFFECTS OF PLASMA PROTEIN BINDING IN VIVO

Perhaps the greatest potential for misunderstanding with regards to the effect of plasma protein binding comes from in vivo PK studies. Bioanalytical methods on which PK evaluation is based, generally measure the total drug concentration (i.e., the sum of the free and protein bound drug) in plasma or blood. Consequently, all the descriptive PK parameters (C_{max}, AUC, CL, V_d) are based on total drug measurements. Independent estimation of the free fraction allows correction of these values to yield the unbound or "free" drug concentrations and unbound parameters. The unbound parameters CLu and Vdu are fundamental intrinsic properties of a drug, and (analogous to concentration and exposure) the total drug parameters CL and Vd are dependent on the unbound parameters CLu and Vdu, and the degree of plasma binding (fu).

Total drug concentrations are of limited value in themselves, in most cases they cannot be correlated with measures of affinity or activity obtained from in vitro assays. They can be used to calculate bioavailability, hepatic extraction and estimate fraction absorbed (Fa) by enabling the accounting for first pass effects. These applications are summarized in Table 2.

Free drug concentrations, in contrast, are highly valuable and directly comparable to in vitro measures of on-target and off-target potency, thus enabling explanation of the presence or absence of vivo effects. Moreover, the free concentrations in the circulation relate directly to those in most aqueous compartments in the body.[1] Exceptions to this can be observed in tissues or organs that have a barrier imposed by less permeable capillaries or where there is active transport (the CNS is the prime example). Other exceptions can be the excretory organs, if

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Table 2. Correct Use of Total and Free Data

Do not...

- Use total plasma concentrations and exposure to compare between compounds and between species
- Interpret changes in total drug parameters as indicators of compound quality (exception is total CL relative to hepatic blood flow as an indication of first pass extraction)
- Use microsome/hepatocyte CLint data for SAR without considering binding in those systems
- Target changes in plasma protein binding as a means to improve compound quality

Do...

- Correct for plasma binding to enable compound and species comparisons on the basis of unbound drug concentrations and unbound exposure
- Correct for binding in in vitro assays to reveal intrinsic values to guide SAR
- Interpret pharmacodynamic effects based on unbound exposure relative to unbound potency
- Use unbound parameters and parameter ratios to guide optimization of dose and dose regimen
- Correct Clearance and Volume data for protein binding to establish true changes in metabolic stability (CLint) and tissue affinity (Vss,u)
- Estimate free plasma concentrations in efficacy and toxicity studies; seek to link pharmacological effects to free drug concentrations

the rate of elimination is very high, or where there is pH-driven partitioning. The equivalence of free concentrations in cytosol and plasma has been demonstrated many times and is an accepted principle of pharmacology which we will return to later. Free drug concentrations also allow direct interspecies comparisons of pharmacodynamics since, by definition, species differences in protein binding are corrected for.

FREE DRUG CONCENTRATIONS IN TISSUES

In common with binding to constituents of blood/plasma, compounds also bind to components in tissues. Drugs bind to plasma proteins (principally albumin) by lipophilic and, for acidic drugs, ion pair interactions with positively charged lysines. Binding in tissues tends to be through lipophilic interaction with the triglyceride tails in membranes and, for basic drugs, ion pair interactions with negatively charged phospholipid head groups. Irrespective of the extent or mode of binding, the unbound concentrations of a drug are subject to the same equilibrium situation operating in blood/plasma. This is illustrated in a study by Mateus[11] looking at tissue partitioning from the aqueous media and measuring total drug (Kp) and free drug ratios (Kp_{uu}). The results are shown in Figure 4 and show that, despite high partitioning of total drug, Kp_{uu} is close to unity. The higher measured Kp_{uu} value for the basic compounds is consistent with the partitioning of positively charged compounds into acidic subcellular compartments such as endosomes and lysosomes. Clearly any attempt to relate pharmacological activity to total drug concentration would be in error, as for a neutral compound, for example, tissue total drug concentrations are on average 40-fold higher than free drug concentrations.

Studies such as this support the accepted principle that, at equilibrium (which is achieved rapidly in organs, for even moderately membrane-permeable compounds) the free drug concentrations in the plasma and cell cytosol are the same, i.e. the Kp_{uu} is 1. The huge benefit of this is that engagement of many intracellular targets can be simply estimated from the free plasma concentrations. There is no general need for measurement of drug concentrations in tissues. The exceptions to this are organs where cell access is modulated by drug transporter proteins, notably the brain, or where perfusion rates are very low,

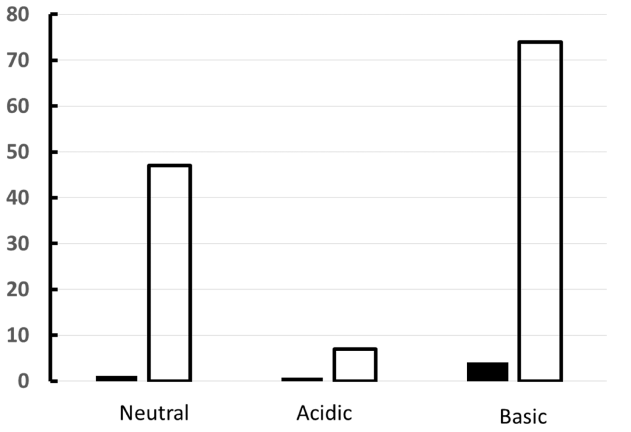

Figure 4. Mean Kp_{uu} (solid bars) and Kp for neutral, acidic (mainly ionised at pH 7.4) and basic drugs (mainly ionised at pH 7.4). Data replotted from Mateus et al.[11]

and study design means tissue concentrations do not reach steady-state.

There persists a view that compounds with low volume of distribution do not engage intracellular targets well, even though even for low volume of distribution compounds, the free concentration in plasma is the same as that in the cytosol, as stated previously. This false assertion comes from a misunderstanding of what the volume of distribution represents. It is just a measure of the relative affinity of a compound for tissues and plasma.

A useful model equation, based on the equivalence of free drug concentrations in plasma and tissues, is

$$V = \frac{fu}{fut}Vt + Vp \tag{14}$$

where fut is the fraction unbound in tissues and Vt is the volume of the tissues (nominal value 1 L/kg) and Vp is the volume of plasma (nominal value 0.04 L/kg. With these volumes, this equation approximates to

$$V = \frac{fu}{fut} \tag{15}$$

This equation shows how the volume of distribution is determined by a compound's relative affinity for tissues and plasma, but it can also be a source of confusion, as it could imply unbound tissue concentrations are controlled by tissue binding. This is not the case; they are determined by the unbound plasma concentration in equilibrium with the cell cytosol. The bound, and hence total cell concentration, is determined by fut.

This equation can also be used to show that the unbound volume of distribution is a measure of overall tissue affinity:

$$Vu = \frac{1}{fut} \tag{16}$$

However, it is important to reiterate that although the unbound volume depends on the tissue binding, the unbound concentration in tissues does not, as it equates to the plasma free concentration, modulated by any drug transporter effects in the tissues.

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MEDICINAL CHEMISTRY JOURNALS ARE PART OF THE PROBLEM, BUT NEED TO BE PART OF THE SOLUTION

Medicinal chemistry journals quite naturally focus on the chemistry and the logic used to achieve success in a particular project. Their editorial boards are populated with experts with considerable success in the area. As stated previously, the authors have observed many papers published in medicinal chemistry journals that claim to have improved drug-like properties by virtue of reduction in plasma protein binding. We ask that reviewers and editors be more diligent ensuring that both published strategies to achieve, and outcomes that are interpreted as "improved DMPK properties" are aligned with free drug principles, and do not propagate misguided assumptions that actually inhibit efficient compound optimization. Some guidelines are presented in Table 3.

Table 3. Some Guidelines for Use of PK Data in Publications

Do not...

- Claim reduced plasma protein binding as an achievement
- Claim reductions in plasma clearance are an indication that compounds are more metabolically stable
- Confuse fraction unbound with unbound concentration
- Express binding data as fraction% bound% unbound, or % fu—unbound fraction (a unitless value between zero and one) is preferred as it highlights differences between compounds and is used directly in calculation of unbound concentrations and parameters.
- Confuse fu and fu (%)—fu is the preferred parameter
- Use microsome/hepatocyte data for SAR without considering binding in those systems

Do...

- Characterize and compare compounds in terms unbound exposures achieved for a given dose, and their unbound PK parameters
- Express plasma, tissue, and in vitro assay binding data in terms of fraction unbound; fu—a unitless number between zero and one
- Use increases in free drug concentrations and exposure relative to unbound potency to indicate progress in optimization
- Focus on reductions in intrinsic clearance as indication that compounds are more metabolically stable
- Seek to link pharmacological effects to free drug concentrations
- Take care to use the correct units for pharmacokinetic parameters

We recognize that, often groups report significant progress in optimization of their chemical series toward more drug-like profiles. However, the goal has been achieved by modulation of important unbound parameters, and parameter ratios, as a byproduct of the stated strategy to lower plasma protein binding (e.g., lowering intrinsic lipophilicity).

Key Points.

- *In* vivo, protein binding and free fraction have no effect on free drug concentration for oral drugs at steady-state.
- For oral drugs, intrinsic clearance is the key determinant of free drug concentrations in plasma and tissues.
- *In vitro* potency assays represent a closed, static system in which nonspecific binding determines the free drug concentration, and should be corrected for.
- The only reason to measure fraction unbound in plasma is to convert total plasma concentrations/parameters to free concentrations/parameters.
- In most situations, free drug concentrations in plasma can be used as a surrogate for free drug concentrations at the site of action.
- Optimizing for low dose and acceptable dose frequency requires the appropriate balance between unbound exposure and unbound potency, and plasma protein binding has no impact on either of these.

Peter J. H. Webborn
Kevin Beaumont
Iain J. Martin
Dennis A. Smith

AUTHOR INFORMATION

Complete contact information is available at:
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Notes

Views expressed in this editorial are those of the authors and not necessarily the views of the ACS.

ABBREVIATIONS

CL	clearance
CLint	intrinsic clearance
CLint,u	intrinsic clearance corrected for binding to in vitro system
CLu	unbound clearance
fu	fraction unbound in plasma
fut	fraction unbound in tissues
Kp	ratio of concentrations in tissue and plasma
Kpuu	ratio of unbound concentrations in tissue and plasma
Vd	volume of distribution
Vss	volume of distribution at steady-state
Vss,u	unbound volume of distribution at steady-state

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