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Nonlinear Protein Binding: Not What You Think

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Abstract

Nonlinear protein binding is traditionally thought of as an increasing fraction unbound with increasing total drug concentration. In the past several years, research into the protein binding of several tetracyclines has shown that an unexpected and counterintuitive phenomenon has been observed. Specifically, that of decreasing unbound drug fraction with increasing total concentrations of drug over certain concentration ranges. While several studies of tigecycline have shown the importance calcium and its chelation may play in the protein-drug interaction, the potential clinical implications and relevance have not been explored. Here we define typical and atypical nonlinear protein binding, overview protein binding theory, and discuss theoretical implications on pharmacokinetics. Using tigecycline as an example, *in silico* simulations and calculations show how when atypical nonlinear protein binding is not accounted for free drug exposure and drug tissue penetration may be overestimated. It is important to revisit the impacts of nonlinearity in protein binding on clinical pharmacokinetics and pharmacodynamics, and ultimately, clinical efficacy. While this phenomenon could potentially warrant clinical dose adjustment for certain compounds, it also presents a potential opportunity to exploit underlying mechanisms to develop new therapies and better understand molecular interactions of xenobiotics within the physiological system.

Keywords

protein binding; clinical pharmacokinetics; nonlinear pharmacokinetics

1. Introduction

For nearly a century, the protein binding of drugs has been depicted by a simple saturable binding of drug to protein binding sites. Very often, due to the large binding capacities of proteins or low binding affinities of drugs for proteins, saturation does not occur clinically and the unbound drug fraction is therefore independent of its total concentration. This is referred to as linear protein binding. Saturation of proteins results in concentration dependency and can occur and have clinical relevance for some drugs in general, or in specific clinical scenarios or populations. Until recently the saturation behavior was the only nonlinear phenomenon widely known. The recent development of several tetracycline derivatives (i.e. tigecycline, eravacycline) has revealed atypical nonlinear protein binding i.e.

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the counterintuitive trend of increased binding to protein as total drug concentrations increase[1–4]. Implications of atypical nonlinear protein binding on clinical efficacy or safety were not assessed.

This review aims to describe the concepts surrounding protein binding, while giving a more in depth view into nonlinear protein binding than presented previously. The theoretical pharmacokinetic (PK) impacts of nonlinear protein binding including typical and atypical will be discussed. And finally, tigecycline is used as an example to explore potential effects of atypical nonlinear plasma protein binding on clinical PK.

2. Basics of Protein Binding

2.1. A BRIEF HISTORY

The techniques by which protein binding of drugs are characterized were first developed in the early 1900s[5]. The idea of blood as transport organ was described by Bennhold in the late 1930s[5]. Langmuir's isotherm established the chemical interaction basis for these protein-drug interactions, which is still used today[6]. Over the years there has been intense discussion regarding the clinical significance of protein binding and corresponding alterations[7–9]. One could argue that for the majority of drugs, alterations in PK caused by changes in protein binding are clinically insignificant[9]. Even if true only for a particular population, it should be part of the clinical reasoning to consider the effects of altered drug-protein binding in different disease states, age groups, acute inflammatory conditions, and polypharmacy.

If the efficacy of a drug is known to be potentially impacted by changes in plasma protein binding, investigation of such potential alterations should be performed. Protein binding is an important consideration in drug design, discovery and development [10]. Protein binding is evaluated in *in vitro* and *in vivo* pre-clinical settings and ideally is used to predict unbound (active) concentrations in clinical studies, allowing for lead optimization[10]. Protein binding may be determined in clinical samples, but this is not always the case. Often protein binding values from previous *in vitro* determination in human plasma are used to estimate binding of clinical samples. For accurate extrapolation or prediction of free clinical drug exposure, differences in interspecies physiological, metabolic, and elimination processes should be considered.. Furthermore, any expected pharmacodynamic effect, be it drug efficacy or toxicity, should be predicted based on free exposure. Given the increased use of pharmacometrics to predict clinical dosing, mathematical models may be developed to predict the effects of such alterations in a clinical setting. In the future, clinical models may be developed and implemented at the bedside to deliver optimized therapy to patients for drugs requiring adjustment in the presence of more complex PK phenomena, such as nonlinear protein binding.

2.2. THEORY OF NONLINEAR PROTEIN BINDING

The expected binding behavior for almost all drugs is that the fraction unbound (f_u) is constant over a concentration range (i.e. linear protein binding) until a point at which protein binding sites begin to be saturated, leading to increasing unbound fraction with increasing total concentrations, typically referred to as nonlinear protein binding. This behavior is

described as part of the Langmuir model[5,6] (illustrated in Equation 1 and Figure 1), where A_{max} is the maximum binding capacity for substrate, K_d is the dissociation constant, C_u is the unbound drug concentration, and C_b is the bound drug concentration.

$$C_b = \frac{A_{\max} \times C_u}{K_d + C_u}$$

Technically speaking, nonlinear protein binding is defined as any pattern of protein binding which is not linear (or at a concentration range at which the f_u is not constant). Until recently, nonlinear behaviors other than the Langmuir model had not been well described. During the development of tigecycline, a unique protein binding behavior was observed and not pursued further until recent investigations linked the nature of the behavior to divalent metal ion chelation[1,11,12]. Other tetracyclines (i.e. eravacycline[2,13], TP-271[4], minocycline and doxycycline[3]) have displayed similar behavior, but further mechanistic investigations have not been performed. The nonlinear protein binding of tigecycline has been described as "U-shaped" with a counterintuitive decrease in f_u with increasing total concentrations and an eventual return to the appearance of the more typical saturation behavior (Figure 2). The evidence of increasing binding with increasing total concentrations of eravacycline in several species is shown in Figure 3. Similar trends have been observed for minocycline, doxycycline, and TP-271.

Any behavior deviating from a typical Langmuir, saturation-related, nonlinear behavior, has been defined as "atypical nonlinear protein binding"[11], while the former will be referred to as "typical nonlinear protein binding". These definitions have been listed in Table 1 for quick reference and clarity.

2.3. FACTORS IMPACTING PLASMA PROTEIN BINDING DETERMINATION

Various methods for plasma protein binding determination are available, including equilibrium dialysis, ultrafiltration, ultracentrifugation, charcoal adsorption, chromatographic methods, and solid phase microextraction. Table 2 briefly describes advantages and disadvantages of commonly used protein binding methods. Equilibrium dialysis is often considered the gold standard but the ease of use and fast processing makes other methods like ultrafiltration attractive in many settings[14]. Protein binding should be determined under appropriate physiological conditions, over a clinically meaningful concentration range[15], and with great methodological care for experimental factors such as pH[15], buffers[15] and solvents used, device[16], temperature[16], animal species[17], protein concentration[15], proteins or endogenous substances present[15], and sample volume. Regardless of the method used, each method has its own caveats and considerations that have been reviewed and investigated extensively elsewhere[14].

3.0 Theoretical Implications and Examples

3.1 THEORETICAL IMPLICATIONS

The impacts of changing f_u in linear scenarios have been previously discussed[18]. In summary, any changes in protein binding may affect the clearance (CL) and/or volume of distribution (V_d) of a drug, which may or may not significantly impact PK parameters (i.e. half-life, and free and total steady state, minimum and maximum concentrations), PK in the tissues of interest, or bioavailability. These changes may be significant based on route of administration (oral versus parenteral), low versus high extraction, and original tissue distribution (or the magnitude of V_d). Given that changes may affect elimination processes, for orally administered drugs subject to first pass metabolism, bioavailability may be impacted for high extraction drugs but not for low extraction drugs. Conversely total clearance would be significantly affected for low extraction drugs for either route of administration. In the case of drugs with low V_d, changing f_u may not significantly impact V_d, while for high V_d, changes in f_u are more likely to impact distribution. Changes in V_d also depend on tissue binding relative to changes in plasma protein binding.

3.1.1 Typical Nonlinear—Similar to linear protein binding, effects of changing protein binding can be anticipated based on V_d, extraction ratio, and administration route. Given the time- and concentration-dependent nature of changing PK parameters in the presence of nonlinear binding, predicting PK is complicated. As concentration-time profiles may not explain much about changes in distribution, it is important to assess the implications of these alterations at clinically relevant doses/concentrations and be aware of underlying mechanisms. Several simulation and modelling exercises have been performed to investigate the effects of nonlinear binding behavior on clinical PK.

Martin evaluated dissociation constants (K_d) a main driver of the potential for albumin saturation stating that K_d less than 1×10^{-4} was conducive with potential saturation especially at higher doses[19]. He noted that calculating elimination rate constants (k_e) from apparently linear total concentration-time curves would underestimate elimination[19,20]. More drug is present in plasma as concentrations decline due to a decrease in saturation of proteins and decrease in f_u. As time goes on, this decline in f_u leads to a continued decrease in k_e and an increase in half life ($t_{1/2}$). k_e as related to free drug concentration over time remains linear. Given the expected increase in protein saturation at high doses, k_e at high concentrations is especially underestimated, while at later time points, when concentrations are lower and saturation decreases, k_e is overestimated. Log(concentration)-time nonlinearities may be mistaken for slower elimination at higher doses, rather than nonlinear protein binding.

øie and colleagues recognized that earlier simulations assumed linear tissue binding and thus explored saturable plasma and/or tissue binding in a one compartment model after intravenous (IV) administration for drugs with various intrinsic clearance (CL_{int}) and V_d values[21]. Their model also assumed binding in the extracellular space when saturable plasma protein binding was present and therefore defined tissue binding as that intracellular binding. Table 3 summarizes their simulated results for eight scenarios: Typical nonlinear plasma protein binding with low or high V_d, and low or high CL_{int}, linear plasma protein binding

with typical nonlinear tissue binding, with low or high CL_{int}, and finally, typical nonlinear plasma and tissue protein binding, with low or high CL_{int}. After a single IV administration, when typical nonlinear binding was present in either plasma or tissues, as expected, unbound fraction in plasma and/or tissue (f_{uP} and f_{uT}) decreased with total concentration. Decreases in f_{uP} results in decreased V_d over time, while decreases in f_{uT} led to increased V_d over time. When both f_{uP} and f_{uT} change (in the case of typical nonlinear binding in plasma and tissue), V_d may increase, decrease or remain similar, depending on the relative magnitude of the unbound fraction changes. In these simulations, f_{uP} and f_{uT} changed proportionally and the subsequent changes in k_e, $t_{1/2}$, and PK profiles reflected no change in V_d. As per theory, only changes in f_{uP} affect low extraction (low CL_{int}) drugs, while high extraction drugs are unaffected. In the case of low Vd and low extraction with typical nonlinear plasma binding only, since the V_d decreases with concentration, the concavity of the log-concentration time curve is less striking. For high V_d, low extraction drugs with only saturable plasma binding, the approximate changes in V_d and CL were similar, leading to no apparent change in k_e. This is interesting as the log-total concentration versus time curve exhibits convexity, which would lead one to assume nonlinear elimination. By examining the log-free concentration versus time profile, linear elimination is revealed. While these explorations by øie and colleagues did not address all possible scenarios, especially those where simultaneous changes are not proportional, they serve as a good starting point for predicting expected alterations in PK resulting from changes in f_{uP} and f_{uT} under various PK scenarios. It should not go without emphasis that without considering saturable binding these curves may be misinterpreted as nonlinearities in drug metabolism or multiple compartmental distribution.

McNamara and colleagues concurred with øie and colleagues model and derived new V_d terms to describe the changing PK terms and applied this to ceftriaxone[22,23]. They assumed no intracellular/tissue binding given the properties of ceftriaxone and included typical nonlinear binding of proteins in the extracellular space based on the expected concentration of albumin in the interstitium as compared to plasma. In their simulations they observed concave concentration-time profiles for ceftriaxone at higher doses.

3.1.2 Atypical Nonlinear—With these models of typical nonlinear protein binding, one could predict PK alterations for atypical nonlinear protein binding. The complexity arises from when the drug potentially reverses back to the typical nonlinear phenomenon at higher concentrations, which is the case for tigecycline. In these situations, use of pharmacometric modelling to simulate complex changes, is a better approach.

Currently the only class of drugs which appears to have atypical nonlinear PPB is the tetracyclines. While it is important to note that this behavior has not been observed or characterized for all tetracyclines, it has been recently well-described for minocycline[3], doxycycline[3], tigecycline[12,24] , eravacycline[2,13], and TP-271[4]. Of note, the plasma protein binding of omadacycline, a new tetracycline derivative in clinical development, was found to be linear over the investigated concentration range[25]. This may be due to the overall lower binding of omadacycline (21.3%[25]) as compared to other tetracyclines exhibiting atypical nonlinear binding (73–93%[26]). Clinical PK effects of these atypical phenomena have not been studied in clinical trials or modeled with the changing f_u in mind. One study has investigated the use of the f_u in clinical breakpoint determination for tigecycline[27], which

has also been adopted by others for eravacycline[28]. There is a clear gap in work examining the expected PK alterations due to increase binding with total concentration.

3.2 POTENTIAL IMPLICATIONS ON TIGECYCLINE

3.2.1 Pharmacokinetic Effects—Current pharmacokinetic models do no account for the atypical nonlinear protein binding of tigecycline[29–32], eravacycline[33], doxycycline[34] or minocycline[35]. Based on what we know from drugs with typical nonlinear protein binding, the free drug will follow linear PK, while total drug will have nonlinear PK. Since most population PK models assume linear processes, the modelling of potentially nonlinear processes using linear models may mischaracterize the existing nonlinearity as additional compartments or multiple elimination processes[36]. Use of these models in predictions may lead to error in PK profiles and expected exposures. Our group modified a population PK model of tigecycline in healthy volunteers[29] to incorporate nonlinear protein binding based on a previously developed model[27]. Fourteen-day dosage regimens were simulated in NONMEM (Version 7.3) for linear and nonlinear scenarios for doses ranging from 25 to 200 mg every 12 hours. As shown in Figure 4, CL and V_d varied widely for nonlinear binding as compared to linear binding scenarios with predicted CL of 4.98–26.5 L versus 16.8 L/h and predicted V_d of 14.8–48 L versus 27.8 L for nonlinear versus linear binding, respectively. The resulting fluctuation in $t_{1/2}$ was less substantial. This change could be naively overlooked as parameter variability. Simulated total exposures (AUC) were higher for the nonlinear scenario (up to 202% of linear binding) and free exposures (fAUC) were up to 30% lower for higher doses (Figure 5). Trends were maintained when CL_{int} or tissue binding were changed. Overall, these simulations suggest that at higher doses free drug exposure may be overestimated by current models when atypical nonlinear binding is unaccounted for, which could lead to subtherapeutic dosing and possibly clinical failure. In addition, PK variability across doses may result from nonlinear binding.

This analysis has some limitations that should be noted: Lacking the original data, a new model was not constructed, instead original model parameters were used as reference points in implementing alterations. There is little known about the tissue binding and nonlinear binding was only incorporated into the central compartment, a point also brought up by øie and colleagues[21]. With this these simulations should only be regarded as hypothesis generating and as motivation to examine free exposure of tigecycline at the site of action (infected tissue) to truly understand and develop an accurate model of the active concentration-time profiles.

3.2.2 Calculating Penetration—When determining pharmacological activity, free concentrations at the site of action need to be considered. Often predicted plasma concentrations are extrapolated to the tissues with the use of penetration ratios (i.e. AUC_{tissue}:AUC_{plasma}). In our analysis of free tigecycline penetration, we found that penetration ratios may also be overestimated if linear binding is assumed. Two studies[37,38], examining the penetration of tigecycline into ELF fluid utilized quantified total plasma concentrations. If the fAUC in plasma or serum are calculated by simply multiplying AUC by a constant f_u of 0.21, then the ratio of $fAUC_{ELF}$ to $fAUC_{central}$ were 5.04 and 7.94 for each study. If the clinical protein binding model developed by Bulik and colleagues[39] is used

to calculate free concentrations and subsequently fAUCs, the resulting penetration ratios are much lower (1.86 and 2.32). In a subcutaneous microdialysis study[39], $fAUC_{tissue}$:$fAUC_{plasma}$ ratios were 0.99 and 1.00 in the thigh and wound, respectively, closer to these values than those calculated based on linear binding.

The gross overestimation of drug penetration calculated based on linear binding for drugs exhibiting atypical nonlinear binding could lead to overestimation of pharmacodynamic effects and subsequent underdosing. In the case of tigecycline, inadequate treatment of an infection could lead to prolonged and progressive illness, the need for intubation, additional antibiotics, or surgical intervention, or death. Subtherapeutic concentrations from underdosing may also result in increased antibiotic resistance, eventually leading to further development of superbugs against which few antibiotics are effective.

4 Conclusion

Assessment of protein binding in determining pharmacologically active drug concentrations at the site of action is critical in drug development and clinical practice. The PK effects of linear and typical nonlinear binding have been well studied for a variety of compounds. Given the discovery of the novel atypical nonlinear phenomenon, it is important to revisit the impacts of nonlinearity in protein binding on clinical pharmacokinetics and pharmacodynamics, and ultimately, clinical efficacy. The presented analyses using tigecycline as an example to demonstrate the need for further studies into this phenomenon, its underlying mechanism and impact on dosing across patient populations. The discovery of the atypical protein binding phenomenon also presents the promise of potential opportunity to exploit underlying mechanisms to develop new therapies and better understand molecular interactions of xenobiotics within the physiological system.

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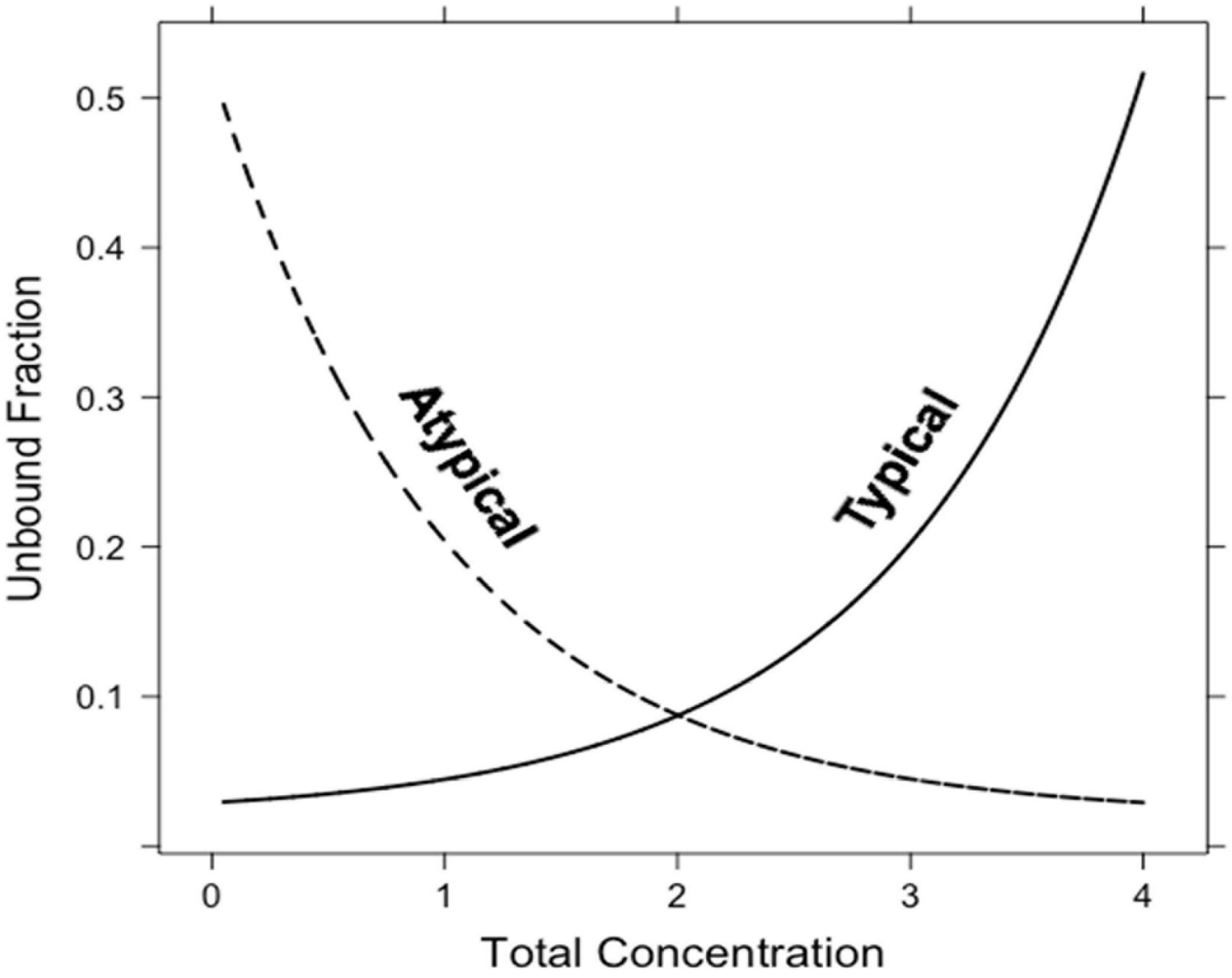

Figure 1: Illustration of different types of nonlinear protein binding.

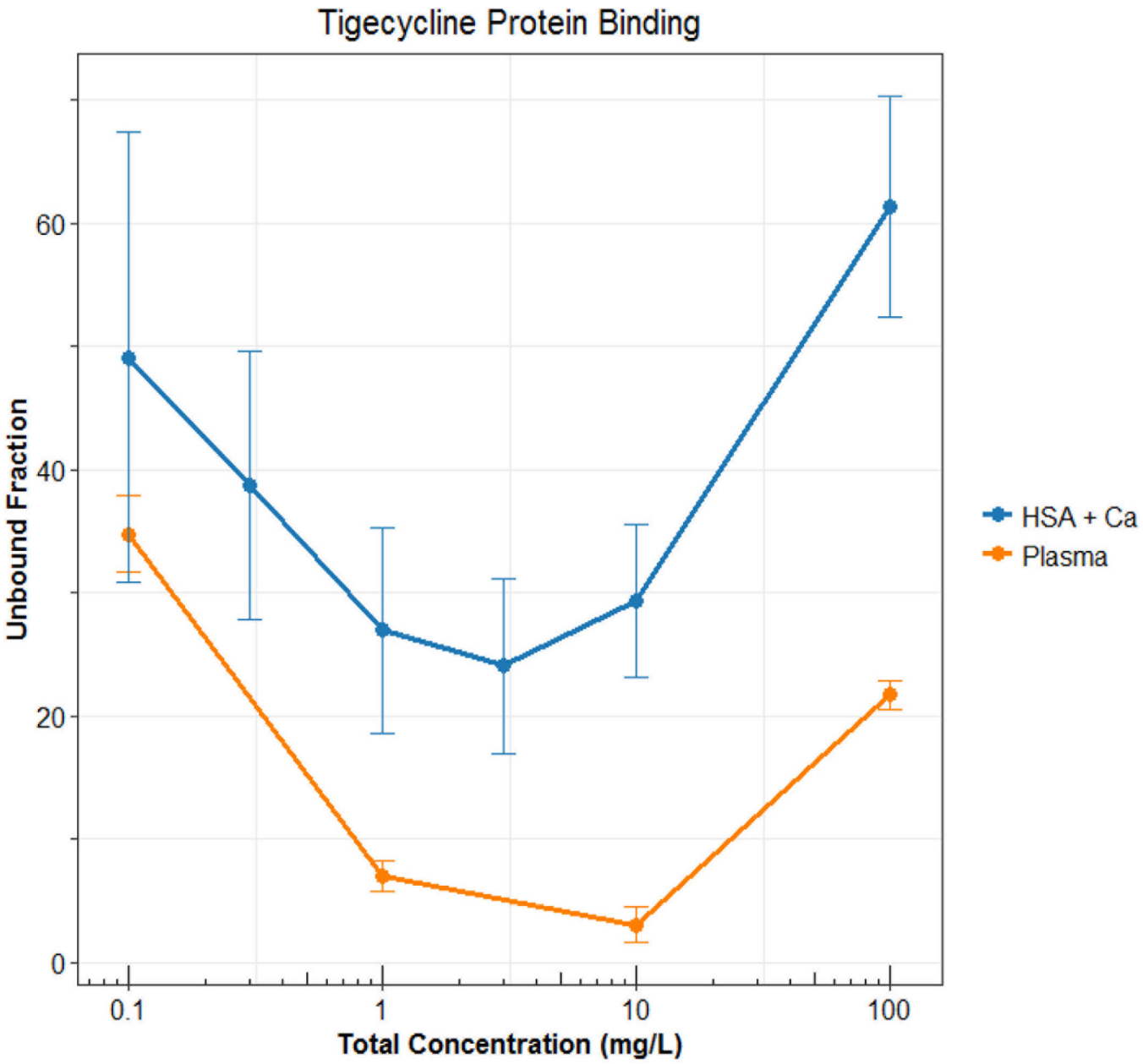

Figure 2: Observed "U-shaped" protein binding behavior of tigecycline (Mean±SD). Data from Mukker *et al.*[24] and Dorn *et al.*[12] HSA: human serum albumin

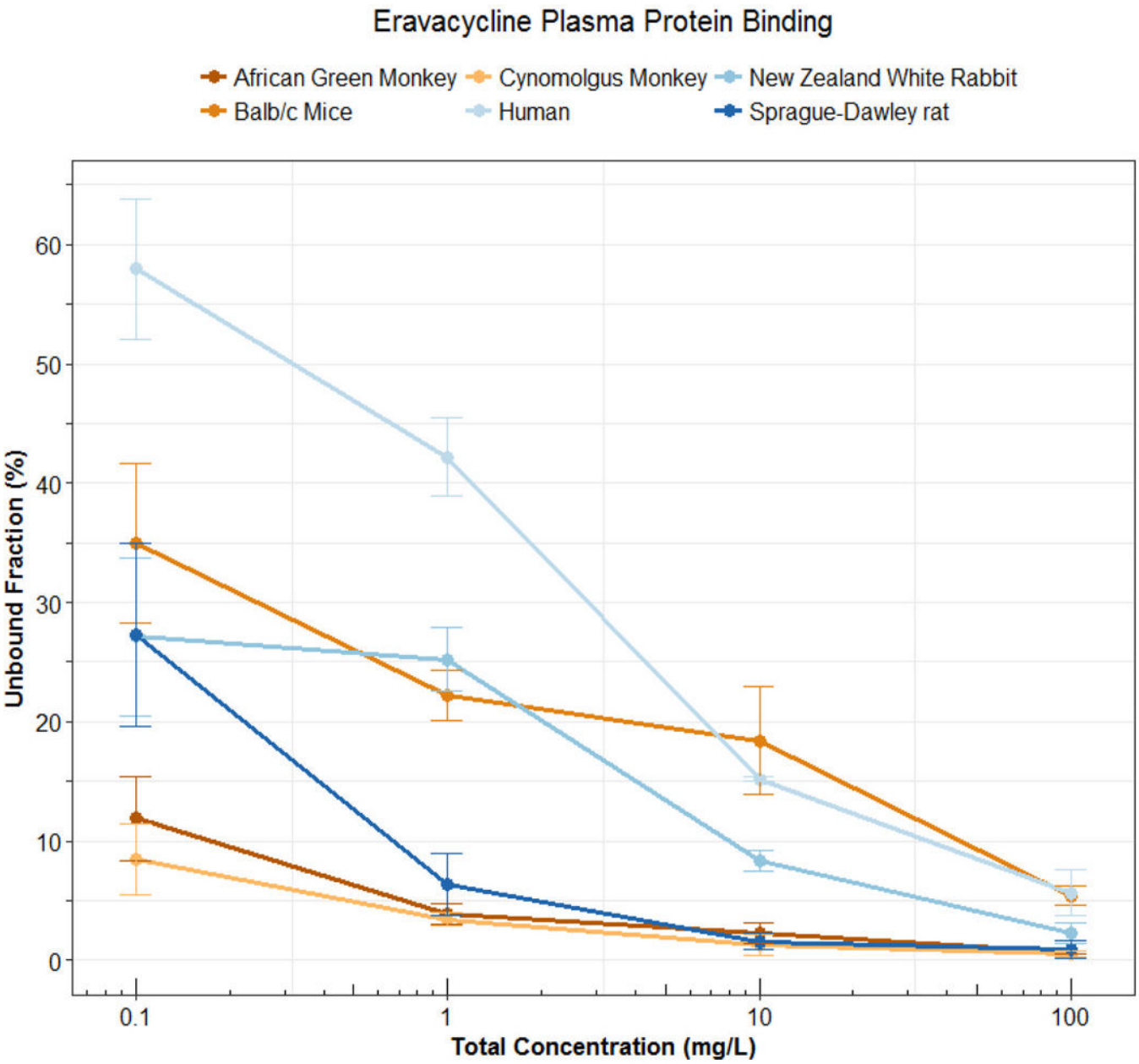

Figure 3: Protein binding (Mean±SD) of eravacycline in pooled plasma of 6 different species. Data previously presented by Singh *et al.*[2]

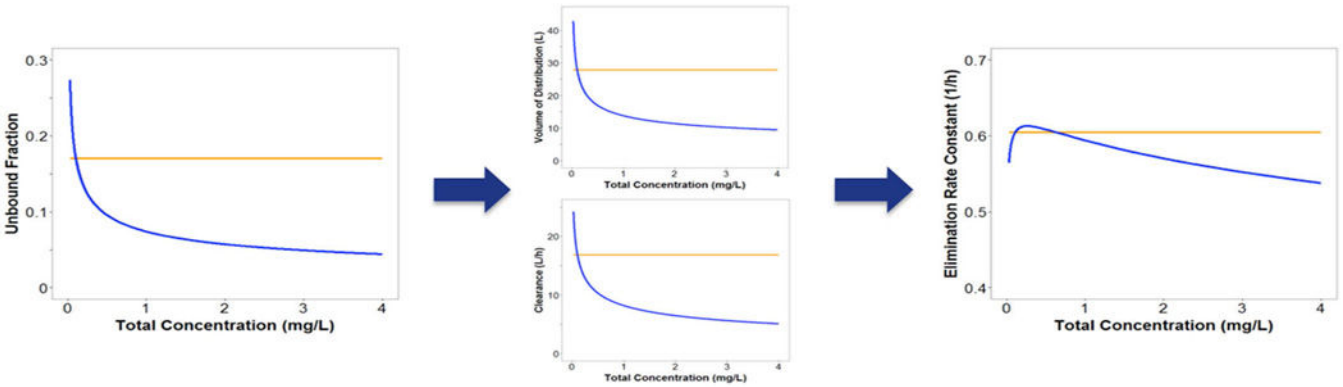

Figure 4: Effects of changing plasma protein binding on different PK parameters over a total concentration range with atypical (blue) and linear (orange) plasma protein binding.

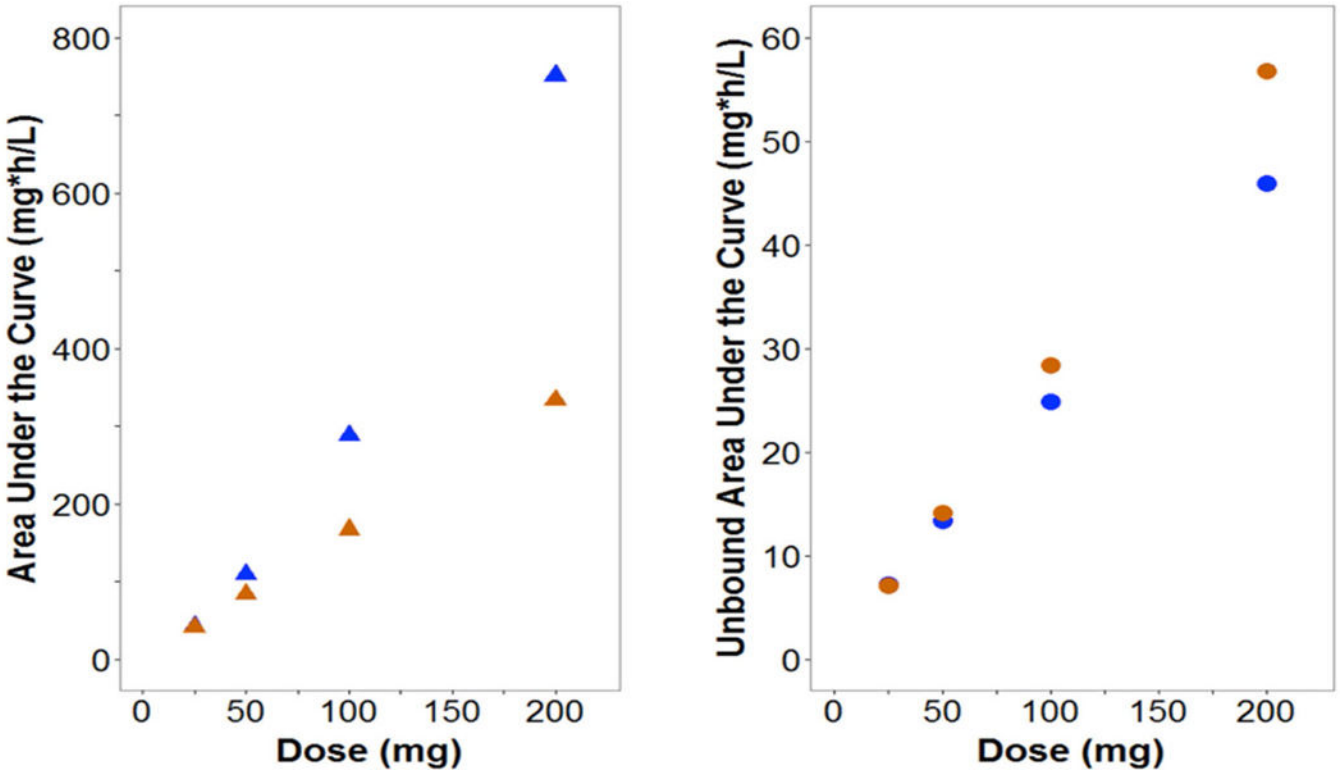

Figure 5: Cumulative total and free exposure (CAUC and fCAUC) for each dosing regimen under atypical (blue) and linear (orange) plasma protein binding scenarios.

Table 1:
Pertinent definitions and examples of different binding phenomenon.

Term	Definition	Examples
Clinical concentration range	A concentration range which is clinically observable or expected for a given regimen	When X mg of drug A is given to a patient of interest, observable *in vivo* concentrations range from 0 to XX mg/L at the site where protein binding is being assessed (typically, the plasma)
Linear protein binding	- f_u remains constant over a clinical concentration range	- Most drugs
Nonlinear protein binding	- f_u does not remain constant over a clinical concentration range	
Typical Nonlinear protein binding	- A subclassification of nonlinear binding - Protein is saturated and f_u increases with total concentration (Langmuir model) - Often what is being referred as “nonlinear protein binding” or “concentration-dependent protein binding”	- Most drugs exhibit this behavior at some concentration range (may or may not be clinical) - disopyramide[41] - ceftriaxone[42] - valproic acid[43] - eplerenone[44] - linagliptan[45] - trandolaprilat[46] - prednisolone[47]
Atypical Nonlinear protein binding	- A subclassification of nonlinear binding - Any nonlinear behavior which does not follow typical saturation/Langmuir model	- doxycycline[3] - minocycline[3] - tigecycline[1,12,24] - eravacycline[2,13] - TP-271[4]

Table 2:

Summary of advantages and disadvantages of commonly used protein binding determination methods.[10,]

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Method	Advantages	Disadvantages
Equilibrium dialysis	- High throughput - Relatively straight forward method - Reliable results, gold standard	- Experiment duration (drug stability) - Less physiological - Technical concerns (volume shifts)
Ultrafiltration	- High throughput - Rapid - Relatively straight forward method - Ease of use	- Technical considerations (binding to membrane, leakage, volume shifts)
Ultracentrifugation	- Simple - No membrane-related technicalities	- Low throughput - May overestimate binding - Not favorable for large molecules
Microdialysis	- Can use in nearly any tissue of interest - Versatile - Can use *in vivo* - No concern of volume shifts - Ease of continuous sampling	- Semi-invasive - Low throughput - Technically more complex (binding to membrane/tubing, equilibration and assay development)
Charcoal adsorption	- No membrane adsorption concerns - Good for drugs bound to lipoproteins - Characterize full binding profile	- Binding underestimation - Extensive sampling
Chromatographic methods	- Accurate - Fewer practical considerations (binding to membrane, volume gradients, membrane leakage) - Rapid - Small sample volume needed	- Less physiological - More complex development and execution - Low sensitivity
Solid phase microextraction	- Simple - High throughput - Highly sensitive	- Experiment duration - Technically sensitive

Table 3:

Simulated PK changes caused by nonlinear plasma and tissue protein binding.

				Change with Time							
f_{uP}	**f_{uT}**	**CL_{int}**	**V_d**	**f_{uP}**	**f_{uT}**	**V_d**	**CL**	**k_e**	**$t_{1/2}$**	**Cp**	**Cu**
Typical Nonlinear	Linear	Low	Low	↓	-	↓	↓	↓*	↑*	Concave*	Concave*
			High	↓	-	↓	↓	-*	-*	Convex*	Linear*
		High	Low	↓	-	↓	-	↑	↓	Convex	Sigmoidal
			High	↓	-	↓	-	↑	↓	Convex	Convex
Linear	Typical Nonlinear	Low	-	-	↓	↑	-	↓	↑	Concave	Concave
		High	-	-	↓	↑	-	↓	↑	Concave	Concave
Typical Nonlinear		Low	-	↓	↓	-*	↓	↓*	↑*	Concave*	Concave*
		High	-	↓	↓	-*	-	-*	-*	Linear*	Sigmoidal*

*During the simulations of øie and colleagues[21] changes in influential parameters impacted subsequent parameters differently depending on the relative changes in other parameters. For those with an asterisks, simulated results are listed but theoretically under different conditions the results may differ.