COMMENTARY

Nonspecific Binding Considerations in the Rational Design and Development of Small Molecule COVID-19 Therapeutics

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In their recent paper, Boffito *et al.*[1] caution readers not to forget the fundamentals of pharmacology in the rush to identify antiviral therapeutics to combat the ongoing coronavirus disease 2019 (COVID-19) pandemic. Emphasis on the appropriate use of nonspecific binding data is warranted because the underlying, oft-misunderstood concepts are critically important to effective research and development. Confusion over the appropriate way to consider nonspecific plasma and tissue binding has persisted to this day and it is clear from emerging literature that it is potentially confounding effective pharmaceutical research on severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2). However, many of the pharmacokinetic and pharmacodynamic implications of such binding have been known for decades, reviewed in very practical terms,[2–4] and incorporated into antiviral guidance documents from both regulatory agencies[5] and scientific congresses.[6] Admittedly, the implications can be confusing or even counterintuitive because nonspecific plasma and tissue binding affects many aspects of pharmacology simultaneously. Further complicating matters, even among subject matter experts, there is a tendency to conflate issues of nonspecific binding with other relevant aspects of pharmacology and experimentation, leading to largely unproductive and invalid challenges to the free drug hypothesis. For those that are engaged in such work, it is worth reading the paper by Boffito *et al.* as well as the aforementioned reviews and keeping in mind some of the following concepts that have been formulated through years of experience across many therapeutic areas.

UNBOUND DRUG CONCENTRATION AND PHARMACOLOGICAL RESPONSE

The first issue that one must address when considering the appropriate interpretation of nonspecific binding in plasma and other biological matrices is whether the pharmacological response is driven by unbound concentration (referred to as the free drug theory by Boffito *et al.*). For many small molecule drugs, unbound fraction is determined by low-affinity interactions with high-capacity nonspecific binding partners like albumin or alpha-1-acid glycoprotein in plasma and phospholipids in tissues. In contrast, the pharmacological response is typically determined by high-affinity interactions with very low-capacity proteins. In this circumstance, the unbound fraction in both the plasma and tissues is typically independent of target binding and constant in the pharmacologically relevant range, with unbound drug concentration driving the pharmacological response. Of course, exceptions do exist where the balance of these interactions leads to an unbound fraction that is determined by target binding which, in turn, can contribute to distribution and drug clearance. In these cases, unbound concentration may indeed provide a false sense of target coverage. However, this is relatively uncommon among small molecules as compared with large molecule biologics that have exquisite potency for the molecular target and limited nonspecific binding in plasma and tissues. A hallmark of this situation is target-mediated disposition, where nonlinearities in unbound fraction, clearance and/or distribution will exist over the pharmacologically relevant range.[7] However, with regard to small molecule antivirals, the general utility of the protein binding adjusted ratio of trough plasma

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concentration-to-*in vitro* antiviral potency (IQ) in predicting efficacy and the absence of reports regarding target-mediated pharmacokinetics suggests that unbound concentrations are commonly relevant for antivirals. However, because each molecule and molecular target pair represents a potentially unique scenario, the potential for target-mediated disposition should considered on a case-by-case basis.

UNBOUND PLASMA AS A SURROGATE OF EFFECT SITE CONCENTRATION

Assuming that unbound concentration is the driver of pharmacological response, the next issue is how best to determine unbound drug concentration at the site of action. Because it is seldom practical to measure unbound concentration at key sites of action within tissues, unbound plasma concentrations are commonly used as a "best estimate" of unbound tissue exposure. Of course, there are several mechanisms by which unbound concentrations between plasma and sites of action within tissue can differ dramatically (e.g., drug transporters, pH partitioning, slow permeation, and bulk flow clearance). In these cases, measures of unbound plasma concentration may dramatically over or underestimate unbound concentrations at the effect site. Again, whereas the possibility of exceptions exists, the general utility of the protein binding adjusted ratio of trough plasma concentration-to-*in vitro* antiviral potency (IQ) in predicting efficacy suggests that unbound plasma concentrations typically provide a reasonable estimate of unbound concentrations at the site of action for antivirals.

EFFECT SITE CONCENTRATION

When considering effect site concentrations, it is important to understand the mechanisms by which drugs distribute to the site of action since some have implications for unbound effect site concentration and some do not. For SARS-CoV-2, lung tissue is clearly an important site of action for efficacy and prevention of infection. Like the plasma, lung concentration represents a composite measure of unbound and bound drug (i.e., total concentration). Differences in the unbound fraction between tissues like the lung and plasma is common, representing the major driver for total lung-to-total plasma ratios (K_p) and the basis of distribution volume (Eqs. 1, 2).

$$K_p \sim \frac{fu_{plasma}}{fu_{tissue}} \quad (1)$$

$$V_{ss} = V_p + \sum K_{p,tissues} \times V_{tissues} \quad (2)$$

Generally, total tissue concentration, K_p, and V_{ss} do not have implications for unbound tissue concentrations because they represent relative partitioning of drug that is unavailable to the molecular target.[8] As such, approaches that use total lung concentration or computational methods for predicting lung K_p are typically inappropriate for estimating pharmacologically relevant target site exposure of potential COVID-19 therapies. In cases where mechanisms relevant to unbound drug accumulation or impairment in lung exist (e.g., transport and metabolism), methods that provide unbound tissue concentration or unbound lung-to-unbound plasma ratio (lung $K_{p,uu}$) may provide more pharmacologically relevant indicators of exposure. However, the quantitative contribution of such mechanisms to unbound lung concentration is currently not well-understood.[9] Furthermore, as Boffito *et al.*[9] point out, methods for determining unbound exposure in the lung have yet to be qualified in support of developing such an understanding. One significant limitation to such methods is that they typically lump together different cell types present in the lung (e.g., mucosal epithelial, alveolar macrophages, endothelial, and interstitial) and fail to discern unbound concentrations at distinct subcellular sites of action. This issue may be particularly relevant to SARS-CoV-2, where some weakly basic molecules accumulate in acidic endo-lysosomes via pH partitioning and subsequently affect viral endo-lysosome trafficking and mRNA release.[10] Given these complexities, assessments of the potential for accumulation or impairment in unbound concentration at the site of action within lungs are typically qualitative in nature and largely serve to highlight potential caveats to the utility of unbound plasma concentrations on a case-by-case basis.

TRANSLATION OF *IN VITRO* SYSTEMS

Antiviral potency, particularly the 90% inhibitory concentration estimated using cells infected with SARS-CoV-2 *in vitro*, is a key parameter underwriting drug design and repurposing. As such, it is critical to understand the factors that affect the translation of *in vitro* potency to the *in vivo* scenario. Differences in biological context between *in vitro* and *in vivo* systems can create apparent disparities in potency and confound effective pharmaceutical research. The examples of lopinavir and remdesivir provided by Boffito *et al.* serve as a good reminder for how differences in biological context between *in vitro* and *in vivo* systems may affect the drug concentration available at the site of action. Some aspects, like differences in the unbound fraction of lopinavir *in vitro* and *in vivo*, are common and straightforward to measure. Other aspects, like the rate and extent of intracellular remdesivir activation, are less common and more difficult to measure. Beyond those covered in these examples, many other mechanisms of drug disposition may differ between *in vitro* and *in vivo* systems. One that may be particularly relevant, given the widespread use of SARS-CoV-2 infected VeroE6 cells, is efflux via P-gp. Boras *et al.* have very recently reported a decrease in antiviral half-maximal inhibitory concentration of > 100-fold for the investigational protease inhibitor, PF-00835231, in SARS-CoV-2 infected VeroE6 cells in the presence of a P-gp inhibitor.[11] Consistent with relatively low expression of P-gp in the lungs, this potency was very similar to that determined in more physiologically relevant cell lines (e.g., A549-ACE2 and polarized human airway epithelial cells). Interestingly, the lopinavir example of Boffito *et al.* is a known P-gp substrate, and this has been shown to affect intracellular lopinavir distribution cell lines expressing P-gp. Although it would seemingly contradict recent clinical results[12] and the reported potency in more physiologically relevant Calu-3 cells, a significant P-gp related shift in lopinavir potency in SARS-CoV2 infected VeroE6 cells could paint a more optimistic picture of potential antiviral efficacy in the lungs than that previously provided. Of course, biological context can affect antiviral

potency in ways that are independent of drug disposition (e.g., cell type, multiplicity of infection, duration of infection, and timing of treatment relative to infection). Although this aspect receives comparatively less focus, much can be learned from other therapeutic areas regarding the biological factors that affect potency between *in vitro* and *in vivo* systems.[13] Given the number of potentially confounding factors, it is of little surprise that clinically effective concentrations across a wide range of therapeutics are often not simply related to apparent *in vitro* measures of potency without consideration of differences in biological context between systems.[14]

IMPLICATIONS FOR DRUG DESIGN AND CLINICAL OUTCOMES

Finally, perhaps the most counterintuitive aspect of binding to plasma proteins and other biological matrices is that, although it is critically important to understand, it is generally not considered to be a valid property for optimization. Fundamentally, this is related to the fact that unbound fraction affects multiple aspects of pharmacokinetics and drug action simultaneously, often resulting in offsetting effects for clinically relevant end points, such as dose and half-life.[2,3] For these same reasons, as Boffito *et al*. indicate, differences in unbound fraction in disease usually have negligible implications for the therapeutic or safe dose.[4] As reviewed previously, there are exceptions that should be considered on a case-by-case basis.[2-4]

CONCLUSION

As Boffitio *et al*. state, no *in vitro* assay or prior knowledge of pharmacokinetic/pharmacodynamic can guarantee success in drug design or repurposing for SARS-CoV-2 or any other therapeutic. However, experience derived from decades of antiviral and other therapeutic areas of research has revealed key factors that, if appropriately considered, inform good decision making and ultimately increase the likelihood of success. Of these, nonspecific binding in plasma and other biological matrices has proven to be among the most generally relevant and facile aspects to consider. As such, this aspect should be considered in any comprehensive analysis of a molecule's potential to treat SARS-CoV-2 infection.

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CONFLICT OF INTEREST

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