Supplemental Appendix 2:
Analysis of Off-Target *in vitro* Effects

Liu et al.

1 Introduction

Here we present an analytical framework for the steady-state solution of a competitive binding kinetics model. The model is constructed to analyze the deviation of drug-target binding *in vivo* from the *in vitro* case. It is an expansion of the model developed for Supplemental Appendix 1. The *in vivo* case includes three biochemical species: drug (D), its target (T), and human serum proteins (S). The *in vitro* case includes drug, its target, off-target binding partners such as those found in the FBS used in *in vitro* assays (B), and (optionally) human serum proteins (S) that may be included in *in vitro* assays. The purpose of this analysis is to use biochemical binding theory to determine how faithfully *in vitro* dose-response experiments (with and without human serum proteins) mimic *in vivo* conditions. We begin by considering the *in vivo* case, where drug can bind to either the intended target protein or human serum proteins according to the equation:

$$\mathrm{T + DS} \underset{\mathrm{k_{on,S}}}{\overset{\mathrm{k_{off,S}}}{\rightleftharpoons}} \mathrm{S + D + T} \underset{\mathrm{k_{off,T}}}{\overset{\mathrm{k_{on,T}}}{\rightleftharpoons}} \mathrm{DT + S} \qquad \{1\}$$

If *in vitro* drug, target, and human serum protein concentrations reflect those found *in vivo*, then the *in vitro* reaction is the same as Reaction {1}, with the added potential for drug to binding to *in vitro* off-target factors:

$$\mathrm{DB} \underset{\mathrm{k_{on,B}}}{\overset{\mathrm{k_{off,B}}}{\rightleftharpoons}} \mathrm{D + B} \qquad \{2\}$$

We solve for the steady-state solution of the model considering the three potential binding partners (T, S, and B). By fixing the initial concentration of certain binding partners to zero, we can use a single model to consider different cases of interest (*in vivo*: $B = 0$; *in vitro* without human serum proteins: $S = 0$; *in vitro* with human serum proteins: all nonzero.

2 Solution

We begin by solving for the steady-state concentration of unbound drug (D_{ss}). To do so, we first consider the differential equation governing drug-target complex concentration: DT. From the binding reaction {1}, we have:

$$\frac{dDT}{dt} = k_{on,T} \cdot D \cdot T - k_{off_T} \cdot DT \tag{1}$$

Given the principle of mass conservation, the total target concentration T_{tot} (bound and unbound) is $T_{tot} = DT + T$. Thus, we can substitute:

$$\frac{dDT}{dt} = k_{on,T} \cdot D \cdot (T_{tot} - DT) - k_{off_T} \cdot DT \tag{2}$$

Setting $\frac{dDT}{dt} = 0$, solving, and substituting $K_T = \frac{k_{off,T}}{k_{on,T}}$, we find the steady-state concentration of bound target DT_{ss}:

$$DT_{ss} = \frac{D \cdot T_{tot}}{D + K_T} \tag{3}$$

Using similar logic, we can determine the steady-state concentration for drug bound to human serum DS_{ss}

$$DS_{ss} = \frac{D \cdot S_{tot}}{D + K_S} \tag{4}$$

and drug bound to *in vitro* off-target factors DB_{ss}

$$DB_{ss} = \frac{D \cdot B_{tot}}{D + K_B} \tag{5}$$

From the principle of mass conversation, we have $D_{tot} = D+DT+DS+DB$. Thus,

$$D + \frac{D \cdot T_{tot}}{D + K_T} + \frac{D \cdot S_{tot}}{D + K_S} + \frac{D \cdot B_{tot}}{D + K_B} - D_{tot} = 0 \tag{6}$$

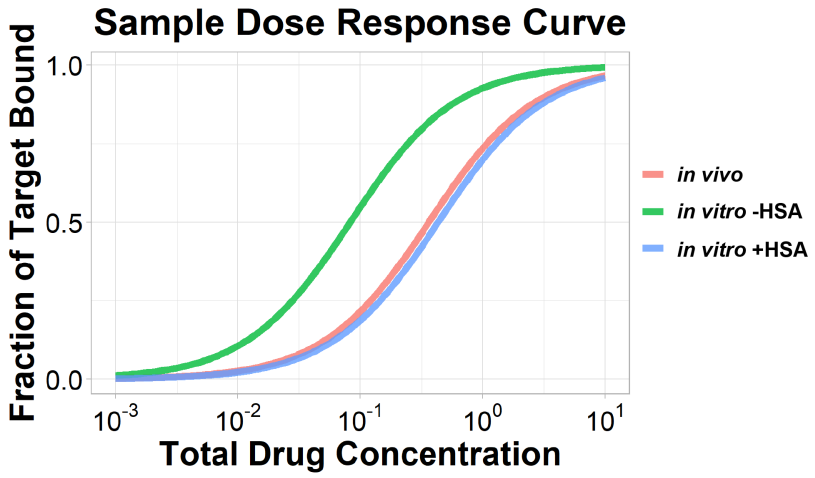
By multiplying each term by the denominators in Equation (6), we have:

$$\begin{aligned}
&(D_{ss} + K_T)(D_{ss} + K_S)(D_{ss} + K_B)(D_{ss} - D_{tot}) + \\
&DT_{tot}(D_{ss} + K_S)(D_{ss} + K_B) + \\
&DS_{tot}(D_{ss} + K_T)(D_{ss} + K_B) + \\
&DB_{tot}(D_{ss} + K_T)(D_{ss} + K_S) = 0
\end{aligned}$$

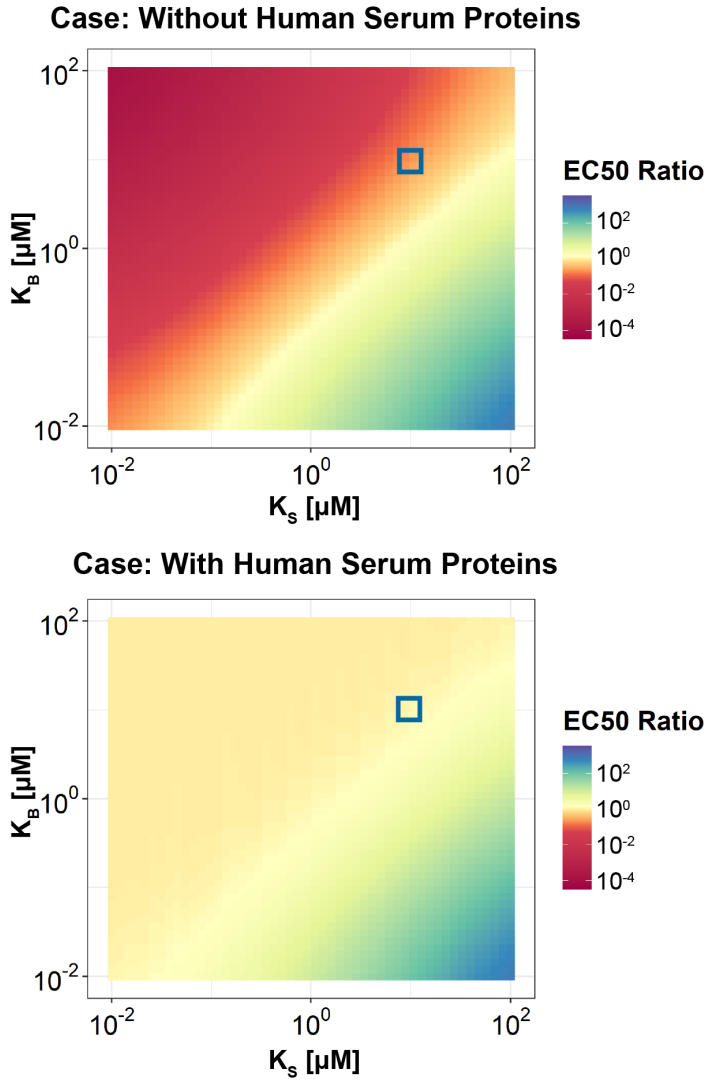
By expanding the binomials and solving for the zeroes using the MATLAB *roots* function, we can solve for D_{ss}. The other steady-state values are then identifiable from the mass conservation equations.

3 Analysis

The model solution can now be evaluated for a range of on- and off-target affinities. Justification for D_{tot}, T_{tot}, S_{tot}, and B_{tot} concentrations can be found in the corresponding MATLAB file on GitHub. The steady-state solution is evaluated for a range of affinities for off-target human serum proteins (K_S) and off-target *in vitro* factors (K_B). For each combination of affinities K_S and K_B, the dose response curve of fraction of target bound ($\frac{DT}{T_{tot}}$) by allowing D_{tot} to vary. This curve is calculated for the *in vivo* case ($B_{tot} = 0$), the *in vitro* case without human serum ($S_{tot} = 0$), and the *in vitro* case with human serum (all nonzero concentrations). An example dose-response curve is provided for $K_T = 0.01\mu M$ and $K_S = K_B = 10\mu M$.



We quantify the deviation of the two *in vitro* cases from the *in vivo* case by comparing the predicted EC_{50} values (defined as the amount of drug required for half target occupancy). The greater the ratio of *in vitro* EC_{50} to *in vivo* EC_{50} deviates from one, the larger the *in vitro* model deviates from the *in vivo* binding kinetics. These ratios are provided in the heat maps below for $K_T = 0.01\mu M$. The parameters corresponding to the above example curves is highlighted with a blue square.



4 Conclusion

The solution of our binding kinetics model mapped onto the $K_S - K_B$ parameter space reveals that *in vitro* dose-response assays that do not include human serum proteins are generally only faithful to *in vivo* drug binding kinetics for a very narrow region in the parameter space (yellow band in the upper heat map). This region represents a specific set of conditions where dose-responses *in vitro* and *in vivo* coincide, mostly by an accidental balancing of competing factors. In cases where drug affinity for human serum proteins is equal to or stronger than that for off-target *in vitro* factors (i.e. $K_B \geq K_S$) as in the above example, the *in*

vitro dose-response curve is left-shifted relative to the *in vivo* curve. However, the *in vitro* model that includes human serum proteins is generally faithful to *in vivo* competitive drug binding kinetics. The *in vitro* binding properties begin to deviate from the *in vivo* case only when drug affinity for off-target *in vitro* factors is orders of magnitude stronger than human serum proteins (i.e. $K_B << K_S$). We note that this set of conditions is rare, as systematic studies investigating drug binding with human and bovine serum proteins have found comparable binding affinities across species.[**1**]

Thus, we conclude that supplementing human serum proteins in *in vitro* dose-response assays faithfully recapitulates *in vivo* competitive drug binding kinetics, even in the presence of other off-target *in vitro* factors.

References

[1] Kosa T, Maruyama T, Otagiri M. Species Differences of Serum Albumins: I. Drug Binding Sites. *Pharmaceutical Research.* 1997;14(11):1607-1612.