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A simple method for predicting serum protein binding of compounds from IC_{50} shift analysis for in vitro assays ☆

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Abstract—The shift in apparent IC_{50} that attends addition of serum proteins to in vitro cellular, enzymatic, and receptor binding assays can be used to determine the dissociation constant for compound–serum protein complexes. We show here that a simple linear relationship exists between the apparent IC_{50} in the presence of serum protein and the inverse of the apparent K_d for the compound–serum protein complex. Using a series of cell-active kinase inhibitors we demonstrate that the K_d value derived in this way can be used to predict the extent of protein binding in serum for various compounds. This method should provide a simple means of assessing the relative serum protein binding propensity of compounds early in the compound optimization phase of drug discovery campaigns.

The pharmacodynamic activity of most drugs is determined by the fraction of compound that is free in circulation, hence available for interaction with its molecular target. Complexation of drugs to serum proteins, most notably human serum albumin (HSA), depletes the free pool of compound in circulation and is therefore thought to diminish the pharmacological effectiveness of compounds. Traditionally, serum protein binding is assessed by equilibrium dialysis and ultrafiltration methods that define the fraction, or percentage, of compound that is protein bound and free when added to serum or plasma.[1] These methods provide good quantitation of serum protein binding, but can be relatively low throughput. Hence, assessment of serum protein binding is often deferred to late in the compound optimization process and data are usually generated for only a limited number of compounds. An additional limitation of methods that quantify serum protein binding in terms of fraction bound in whole serum or plasma is that this parameter is driven largely by the high protein content of serum so that even compounds with modest affinity display high fractional binding in serum. Because of this, small differences in binding affinity among compounds cannot be adequately assessed by these equilibrium methods. This concept is illustrated in Figure 1 (solid and dashed lines) where the percentage of compound bound (% Bound) to HSA in serum is plotted as a function of the K_d for the compound–HSA complex. For compounds with K_d values less than 30 μM there is little difference in the % Bound, even with a 30-fold change in compound affinity. Thus, if one's goal is to establish a structure–activity relationship (SAR) for serum protein binding within a compound series, better assessment of small, incremental improvements in reducing serum protein binding can be achieved by methods that define the apparent K_d value rather than simply the % Bound in serum or plasma.

Recently Copeland presented a theoretical treatment of the relationship between the IC_{50} determined by in vitro assays in the presence and absence of serum proteins (IC_{50} shift analysis) and the apparent K_d value for compound–serum protein binding.[2] This relationship is presented as Eq. 1.

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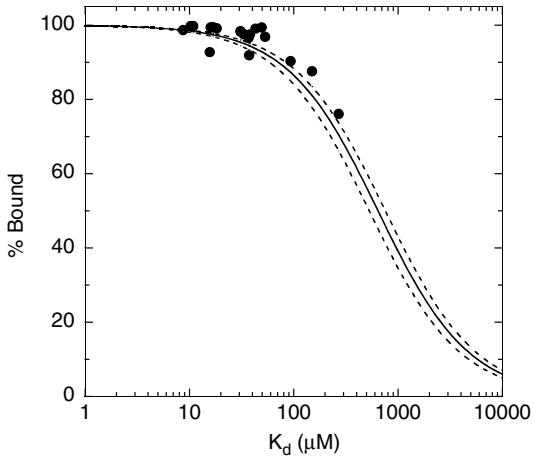

Figure 1. % Bound as a function of K_d for compound–HSA binding. The solid line represents the theoretically expected % Bound as a function of K_d when the total compound concentration is 4 μM and the serum HSA concentration is 644 μM (the midpoint of the normal clinical range of serum HSA concentrations). The dashed lines represent the theoretically expected % Bound when the serum HSA concentration is 530 and 758 μM (the low and high ends of the normal clinical range of serum HSA concentrations) and the total compound concentration is 4 μM. The solid circles represent the experimentally determined % Bound for the 19 compounds studied here.

$$\frac{v_i}{v_0} = \frac{1}{1 + \dfrac{[I_T] - \dfrac{([I_T] + n[P] + K_d) - \sqrt{([I_T] + n[P] + K_d)^2 - 4n[P][I_T]}}{2}}{IC_{50}}} \quad (1)$$

where v_i is the activity in the presence of inhibitor at total concentration $[I_T]$, v_0 is the activity in the absence of inhibitor, n is the number of binding sites per serum protein molecule, $[P]$ is the concentration of serum protein, and IC_{50} is the concentration of compound that produces a 50% reduction in activity in the in vitro assay in the absence of serum protein. Further simulations using Eq. 1 revealed that a linear relationship exists between the apparent IC_{50} in the presence of serum protein (IC'_{50}) and the amount of serum protein added to the in vitro assay. The form of this relationship is presented here in Eq. 2. The derivation of Eq. 2 from Eq. 1 is presented in the Supplemental Materials that accompany this communication.

$$IC'_{50} = IC_{50} + [P]\left(\frac{n}{1 + \frac{K_d}{IC_{50}}}\right) \quad (2)$$

Thus a plot of IC'_{50} as a function of added serum protein concentration ($[P]$) will produce a straight line with slope proportional to the inverse of the apparent K_d value for the compound–serum protein complex. The IC_{50} value for the compound is known from assays performed in the absence of added serum protein, and is also provided by the y-intercept of the linear fit of the IC'_{50} as a function of $[P]$ data set. Knowing the slope from this plot and the value of IC_{50} one can directly calculate the K_d value for the compound.[3] The K_d value thus determined provides a sensitive and accurate metric for comparing the relative serum protein binding propensity among compounds. When the majority of compound binding can be associated with a specific protein (e.g., HSA), one can also use the K_d value, together with knowledge of the protein concentration in serum, to calculate the % Bound expected in serum according to Eq. 3.

$$\%\ \text{Bound} = 100\left(\frac{[I_{\text{bound}}]}{[I_T]}\right) = 100\,\frac{([I_T] + n[P] + K_d) - \sqrt{([I_T] + n[P] + K_d)^2 - 4n[P][I_T]}}{2[I_T]} \quad (3)$$

A significant proportion of serum protein binding can be ascribed to drug interactions with HSA, due to the very high concentration of this protein in serum (35–50 g/L or 530–758 μM[4]). Thus, in many cases one could obtain a reasonable assessment of serum protein binding propensity from the K_d value obtained by measuring the IC_{50} of a compound, in an appropriate in vitro assay, in the absence of HSA, and in the presence of a few concentrations of HSA between 0 and ca. 600 μM. To test this method we have used archived data from a kinase inhibitor program for which the growth inhibitory activity (measured as remaining relative cellular protein after treatment) of compounds was measured for H460 cells in 5% fetal bovine serum (i.e., ~2 mg/mL bovine serum albumin) and in 5% fetal bovine serum supplemented with 341 μM HSA and 1 mg/mL α-acid glycoprotein (AAG). A data set was obtained for 19 compounds that included the IC_{50} values measured in the cellular assay under the above two conditions and the % Bound from equilibrium dialysis studies (Table 1). To calculate the K_d value for these compounds we have assumed that the shift in IC_{50} upon protein addition can be ascribed fully to the added HSA (i.e., we have ignored the influence of the low concentration of AAG added to the medium and the constant background of 5% fetal bovine serum). We further made the simplifying assumption that all of the compounds bind HSA with a 1:1 stoichiometry, so that the term n in Eqs. (1)–(3) is fixed at unity. With these assumptions in place we have calculated the K_d value for each compound from the changes in IC_{50} values (ΔIC_{50}) using a simplified version of Eq. 2 that is applicable when only two serum protein concentrations are tested (in this case $[P] = 0$, and $[P] = 341$ μM):

$$K_d = IC_{50}\left(\frac{n[P]}{\Delta IC_{50}} - 1\right) \quad (4)$$

The apparent K_d values for the 19 compounds are presented in Table 1, and span a relatively broad range from 8.7 to 269.4 μM. We used these values to calculate the expected % Bound in serum when the total concentration of compound is 4 μM[5] and assuming that the median concentration of HSA in serum is 644 μM.[4] The results of these calculations are presented in Table 1 along with the experimental values obtained from equilibrium measurements. The experimental values of % Bound span a range from 76.1% to 99.8%. The calculated % Bound values in every case match reasonably well with the experimental values; the percent error ranges from −5.3% to 7.5%, with an average absolute

Table 1. IC_{50} values for inhibition of intracellular kinase activity in H460 cells in the presence (IC'_{50}) and absence (IC_{50}) of added serum proteins and % Bound from equilibrium dialysis studies (% Bound exp.) for a series of experimental compounds

Compound ID #	IC_{50} (μM)	IC'_{50} (μM)	K_d (μM)	% Bound cal.	% Bound exp.	% Error[a]
250	0.51	19.29	8.7	98.7	98.7	0.0
682	0.36	11.81	10.3	98.4	99.8	1.4
907	0.59	17.88	10.9	98.3	99.8	1.5
657	0.89	19.20	15.7	97.6	92.7	−5.3
562	0.04	0.80	16.0	97.6	99.4	1.8
559	1.60	31.25	16.8	97.4	99.5	2.1
169	0.62	11.85	18.3	97.2	99.2	2.0
138	0.66	7.80	30.8	95.4	98.4	3.0
249	0.51	6.05	31.0	95.4	98.2	2.9
278	1.43	15.54	33.2	95.1	97.6	2.6
787	0.97	9.72	36.8	94.6	96.6	2.1
992	1.18	11.62	37.4	94.5	91.9	−2.8
840	1.67	15.99	38.1	94.4	97.5	3.2
937	0.22	1.95	43.1	93.7	99.1	5.5
754	0.40	3.15	49.2	92.9	99.4	6.5
017	1.04	7.57	53.3	92.3	96.9	4.7
018	2.91	13.19	93.6	87.3	90.3	3.3
800	1.17	3.81	149.9	81.0	87.6	7.5
372	1.95	4.40	269.4	70.4	76.1	7.5

The K_d and calculated % Bound (% Bound cal.) were calculated as described in the text.
[a] % Error was calculated as 100 (% Bound exp. − % Bound cal.)/% Bound exp.

percent error of 3.5%. A modest improvement in the fit is achieved by allowing the serum concentration of HSA to vary between the clinically observed extremes of 530 and 758 μM (Fig. 1).

In most cases the calculated % Bound slightly underestimated the experimental value. This trend may relate to the multiple assumptions made in our calculations (vide supra). We also note that the use of only two serum protein concentrations to define the value of K_d is suboptimal; in future studies we would recommend measurements at three or four serum protein concentrations (including zero) to better estimate the slope value from which the K_d value is derived. Nevertheless, the calculated values clearly track very well with the experimental data, providing an accurate rank ordering of the compounds with respect to serum protein binding propensity.

The data presented here suggest that the measurement of apparent K_d values for serum protein by IC_{50} shift analysis provides a simple and rapid means of quantifying the relative propensity of experimental compounds for binding to HSA and other serum proteins. There are, however, some limitations to the present method. First, as previously described by Copeland, the use of this method requires an in vitro assay that is tolerant to the addition of serum proteins without significant attenuation of signal. In some cases the assay format will not be amenable to such treatment, hence the present method will not be useful. Second, our assumption of 1:1 binding stoichiometry is clearly unrealistic for many compound interactions with serum proteins. Nevertheless, the slope of the plot of IC_{50} as a function of serum protein concentration should be directly proportional to the propensity for serum protein binding, especially when experiments are limited to measurements with a single serum protein (e.g., HSA) for a series of structurally related compounds (for which n may not be unity, but will nevertheless be constant). Finally, we have assumed that the bulk of serum protein binding can be modeled in terms of HSA binding alone. This also is clearly an unrealistic assumption, as many drugs are known to bind to additional serum proteins, especially AAG. Despite these limitations, the present method may find application in the development of SAR trends for serum protein binding as part of compound optimization efforts in preclinical drug discovery programs.

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References and notes

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2. Copeland, R. A. Determination of serum protein binding affinity of inhibitors from analysis of concentration–response plots in biochemical activity assays. *J. Pharm. Sci.* **2000**, *89*, 1000–1007.
3. The actual K_d value can only be calculated from the slope when $n = 1.0$. If n is >1 but constant for a specific chemical series, then an apparent value of K_d is obtained, which can still be used to rank-order compounds in terms of serum protein binding propensity. In some cases, serum proteins display multiple, nonequivalent binding pockets for drug molecules (e.g., HSA can display a high affinity binding

interaction and multiple low affinity interactions). In these cases the plot of IC'_{50} as a function of $[P]$ would deviate from linearity.
4. Case records of the Massachusetts General Hospital. Normal references values. *New England J. Med.* **1986**, *314*, 39–49.
5. Compound binding to human serum proteins was studied by equilibrium dialysis at a fixed concentration of 2 μg/mL. The average molecular weight of the compounds studied here was ~500 Da. Thus the average molar concentration of compound in the equilibrium dialysis measurements was 4 μM.