Characterization of drug-plasma protein interactions using surface plasmon resonance

Binding to plasma proteins is a key parameter in evaluating candidate compounds during the lead optimization and early ADME phases of the drug development process. Surface plasmon resonance (SPR) technology is ideally suited for the analysis of multiple aspects of drug-plasma protein interactions. Here we describe how a Biacore™ SPR based system was used to analyze a panel of low molecular weight drug compounds for binding to human plasma proteins, using a two-stage assay strategy. Compounds were first ranked in terms of protein binding using a rapid, single-concentration assay. High-affinity binders were then selected for a more comprehensive assay, in which compounds were analyzed over a range of concentrations and from which equilibrium constants were calculated. The results demonstrate that high-affinity plasma protein binders can be efficiently discriminated by SPR-derived affinity analyses and that these data can be simply converted to %-bound values.

Introduction

The clinical potential of drug compounds is greatly affected by the nature of their interactions with circulating plasma proteins, such as human serum albumin (HSA) and α1-acid glycoprotein (AGP). The effects of plasma protein binding by drugs are varied and can influence factors such as the free concentration of drug compound in the circulation, transport and distribution around the body, and the duration of drug action. An understanding of how candidate drug compounds interact with plasma proteins is becoming increasingly important during lead optimization and early ADME (absorption, distribution, metabolism and excretion) studies. Assays that can provide high quality information about drug-protein binding as early as possible in the drug development process could, therefore, greatly increase the efficiency and reduce the costs of taking a pharmaceutical product to market.

HSA is the most abundant plasma protein and is known to be the principle drug transporter in the human circulatory system. Although AGP is present at a much lower serum concentration than HSA, it is also an important modulator of drug action, since these two plasma proteins have a somewhat different binding profile. AGP predominantly binds neutral and basic compounds, whereas HSA preferentially binds acidic compounds. Therefore, by measuring binding to both HSA and AGP, a good understanding of the plasma protein binding properties of drug compounds can normally be obtained.

A broad range of approaches have been used to study drug-protein binding, including techniques based on dialysis, filtration, centrifugation, chromatography, electrophoresis, spectrometry and calorimetry. Although many of these methods can deliver reliable binding constants, they frequently require a high sample consumption and/or provide a relatively low throughput. The SPR-based biosensor technology utilized by Biacore systems provides an alternative approach and has a number of intrinsic advantages over the traditional methods. These include the ability to run rapid, automated assays with high sensitivity and relatively low sample consumption.

Non-SPR techniques also require some form of labeling step, or the involvement of additional technology to achieve detection. The absence of any labeling requirement for the interaction partners in a Biacore assay is therefore a major advantage and means that in principle any compound may be analyzed in an unmodified state using this technology.

This Application Note describes the characterization of plasma protein binding for a test panel of eight low molecular weight drug compounds. The following approaches were used:

- A rapid throughput assay using single drug concentrations, designed to rank the compounds according to their binding response to HSA and AGP.
- A more detailed characterization of compounds ranked as high affinity HSA or AGP binders, using concentration series to generate equilibrium dissociation constant (K_D) values. These were subsequently converted into %-bound figures, which are frequently used in the ADME field.

From a drug development perspective, the requirement for this type of high-resolution characterization exists mainly for strong binders, since the importance of precise measurement is greatest here. Although plasma proteins often play an important role in the transport of drug compounds and can provide a buffering function with major consequences for drug retention in the circulation, compounds that exhibit very high binding may be so strongly associated with plasma proteins that they are unable to reach their target molecules. Many candidate drugs have failed in clinical trials due to excessively strong plasma protein binding.

It is important to stress that the %-bound figures associated with ADME studies are only one component of a very complex system. Although a %-bound figure is important information such data must always be considered in the context of the affinity of the drug for its therapeutic target molecule. For example, a compound that shows a 1 µM binding affinity to a plasma protein may still be a good lead candidate providing it has a target affinity in the low nanomolar range. While many in the ADME field work in terms of %-bound figures for drug-plasma protein binding, it is nevertheless important to consider this type of data in the context of the relative affinities (as expressed in terms of K_D) of drug-target and drug-ADME target interactions.

Methods

Immobilization

HSA and AGP were immobilized to Sensor Chip CM5 with 10 mM phosphate buffered saline (PBS), pH 7.4, as running buffer. HSA was diluted to 15 µg/mL in 10 mM sodium acetate buffer pH 5.2 and immobilized with amine coupling. Prior to immobilization AGP was modified with 2-(2-pyridinyldithio) ethaneamine (PDEA) hydrochloride. PDEA modified AGP at 200 µg/mL in 10 mM Na-citrate, pH 3.6 was then immobilized using the surface thiol coupling procedure. Immobilization levels ranged from 8000-13 000 RU for HSA and from 7000 to 10 000 RU for AGP.

Running buffer

The running buffer was 10 mM phosphate buffered saline (PBS), pH 7.4, containing 5% dimethyl sulfoxide (DMSO).

Sample preparation

Stock solutions of the anonymous test compounds (designated here as S1-S8) and the marker drugs, quinine, pyrimethamine, warfarin and naproxen, were diluted to 30 µM in running buffer for binding analysis. Appropriate concentration series were made for compound characterization assays, resulting in top concentrations for warfarin, S1 and S2 of 200 µM, 5 µM, and 1 µM, respectively.

Assay conditions

Pre-conditioning of the sensor surface and flow system was performed using three injections of 50 mM NaOH, followed by three start-up cycles of running buffer injections at the start of each run. Sample cycles consisted of a 60 s sample injection, a 30 s buffer flow (= dissociation phase), a needle and tube wash with 50% DMSO, a 30 s regeneration pulse of 10 mM glycine-HCl, pH 1.5, a 30 s stabilization period and finally, a 60 s buffer injection to check for carry-over. The known HSA-binder, warfarin, was run at intervals to monitor HSA activity during the assay. All injections within the sample cycle were made at a flow rate of 30 µL/min. Solvent correction cycles (1) using eight correction points (4.5% to 5.8% DMSO) were run at intervals during the assays. All assays were carried out at 25°C.

Data evaluation

Solvent correction was applied to compensate for small differences that may arise in the bulk contribution of DMSO to the SPR response between the reference and sample surfaces, due to mismatches between sample and running buffer composition and the excluded volume effect of immobilized ligand on the sample surface (1).

For ranking analysis binding responses were divided with compound molecular weights for better comparison of response levels.

Data from concentration series were used in K_D analysis. Response values were fitted either to a one-site model:

Equation 1: $Req = Conc. \times R_{max}/(Conc. + K_D)$

or to a two-site model:

Equation 2:
$Req = Conc. \times R_{max}/(Conc. + K_D) + Conc. \times R_{max}2/(Conc. + K_D2)$

By using the MW adjusted response, R_{max} was considered identical for all samples in the fitting procedure.

For single protein assays, K_D values were converted to a %-bound figure using the equation:

Equation 3: $K_D = \frac{((y - xp) \times (x - xp))}{xp}$

Here, x is the total concentration of the compound, y is the total concentration of the protein in the blood stream, and p is the fraction bound (%-bound/100) of the drug.

In the examples calculated, the drug concentration was set to 10 µM and serum concentrations of HSA and AGP were assumed to be 0.68 mM and 24 µM respectively.

Results

Ranking of drug binding to HSA

The first step in the study was to rank the binding responses of the test compounds S1-S8 to immobilized HSA using the known binders: quinine, pyrimethamine, warfarin, and naproxen as controls. Using 30 µM duplicate injections of these compounds, binding responses were obtained and ranked according to their molecular weight-adjusted values (Fig 1). The test compounds were classified as low, medium, or high affinity binders using the values for pyrimethamine and warfarin to set the borders of these groups. Samples S1-S8 produced a good distribution of binding responses across the range of the four control drug compounds with even representation among the three assigned affinity classes.

Combined affinity ranking to HSA and AGP

Although HSA is a good single protein model for ADME studies, a more complete picture may be gained by including the important plasma protein AGP in the analysis. As shown in Figure 2, several compounds showed significantly higher ranking to one of the two plasma proteins. For example, compounds S3 and S5 ranked highest for binding to AGP but ranked relatively low for binding to HSA. By contrast, naproxen ranked as the third strongest binder to HSA but last in binding to AGP.

One important feature of SPR is that binding reactions are monitored in real time and this data is used to automatically produce information-rich sensorgrams. The sensorgrams themselves are a valuable source of direct information and

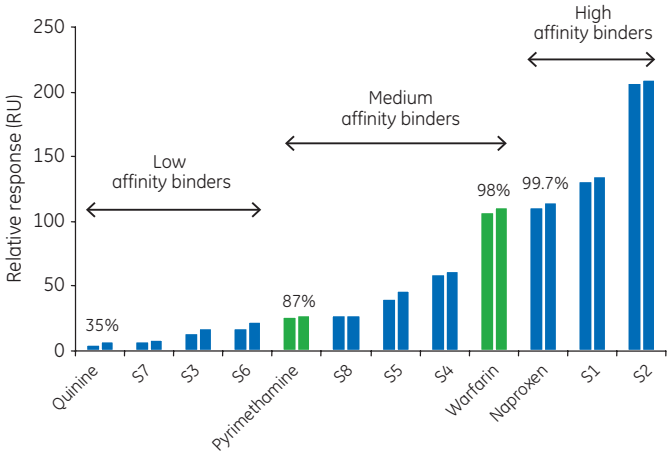

Fig 1. Ranking of drug compound binding to HSA. Molecular weight-adjusted binding responses from the duplicate sample injections are presented in ascending order, left-to-right. A relative binding level value of 100 represents the mean response from the multiple control warfarin injections used to monitor HSA activity during the assay. Compounds are grouped into low, medium and high affinity HSA-binders using pyrimethamine and warfarin (shown in green) to define the low-to-medium and medium-to-high borders respectively. For reference purposes, published %-bound values are indicated for quinine (2), pyrimethamine (3), warfarin (4) and naproxen (5).

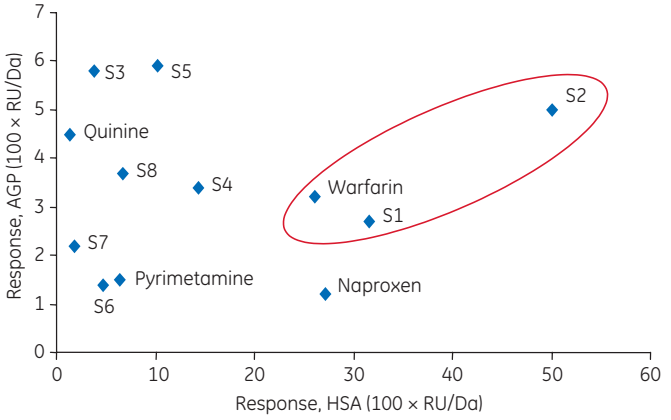

Fig 2. Combined HSA/AGP affinity map. Test and control drug compounds (30 µM) were injected over immobilized HSA and AGP. Responses were adjusted for molecular weight and expressed as 100 × response units (RU)/ molecular weight (Da). The three compounds showing the highest combined ranking for HSA and AGP binding (S1, S2, and warfarin) are ringed in red.

in addition to the ranking tools available, visual inspection of the binding response and curve shape may also be used to assess drug compound binding to plasma proteins. Individual compound binding curves can be combined into a single overlay plot to facilitate a comparative inspection. This can be used as a tool for selecting compounds for further characterization, potentially providing information that a ranking of binding level alone may miss. For example, a compound may give only a very moderate response during the sample injection but if a distinct dissociation phase can be seen on the sensorgram this can be used as a selection criterion. Figure 3 shows overlay plots of the sensorgrams obtained from binding analysis experiments using immobilized HSA and AGP.

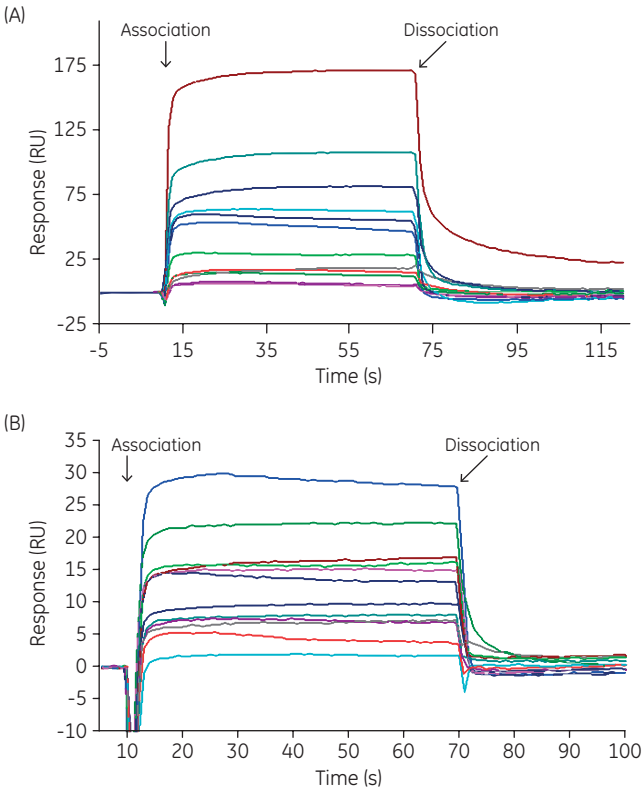

Fig 3. Overlay plots from a single concentration assay. The sensorgrams show the real-time SPR response over time for each binding reaction. Separate overlay plots are shown for compound binding to (A) HSA and (B) AGP. The start and end points of the sample injections, which define the association and dissociation phases respectively, are indicated by the arrows.

The figures presented here are typical of screening data. Most of the compounds bind rapidly, a steady-state binding level is reached during injection and dissociation is almost instantaneous. For these compounds, the binding level during injection is used as ranking criteria. Despite having very rapid dissociation, these compounds can still have affinities in the low micromolar range and some of them can therefore be tightly bound to plasma proteins. For a few compounds, a clear dissociation phase is visible. These compounds normally have sub-micromolar affinities and a visible dissociation phase is therefore a useful selection criterion.

For the highest-ranking plasma protein binders, relatively small differences in affinity may be of crucial importance. From an ADME perspective, therefore, these high binders are most likely to require high-resolution affinity data. Consequently, the usual procedure following a single concentration assay would be to proceed to affinity analysis only with those lead compounds that fall within the high-ranking group. Of the twelve compounds analyzed here, S1, S2, and warfarin ranked the highest in combined HSA/AGP binding with S2 as the strongest binder by some considerable margin. These three compounds were consequently chosen for a more detailed affinity analysis.

Characterization of high affinity HSA binders

For HSA binding, compound S1 and warfarin produced characteristic square-wave binding curves indicating a rapidly formed but unstable complex (Fig 4A). Compound S2 on the other hand, produced sensorgrams with clearly discernible association and dissociation phases (Fig 4B).

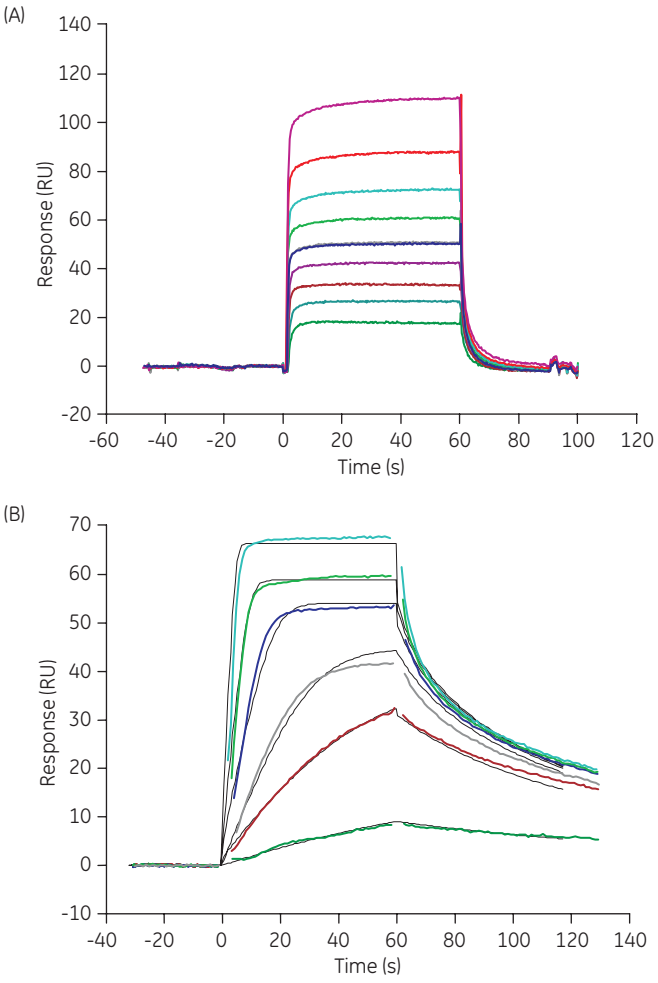

Fig 4. Compound characterization of HSA binding. The sensorgrams show the real-time SPR response over time for each binding reaction and are displayed as overlay plots containing the data from the entire concentration series used. (A) Warfarin exhibits a "square wave" plot over the entire range of concentrations used. (B) Compound S2 displays readily visible association and dissociation phases.

The concentration series were fitted to a suitable model for determination of K_D values. The S2/HSA data (Fig 4B) were fitted using a kinetics model, whereas the S1/HSA and warfarin/HSA data were plotted as response against concentration and evaluated using a one-site (Fig 5A) and multiple-site (Fig 5B) steady-state model, respectively. The K_D values obtained from these evaluations are shown in Table 1. The K_D obtained for warfarin agreed closely with that expected for a binding reaction involving both the high- and low-affinity sites on HSA (6, 7).

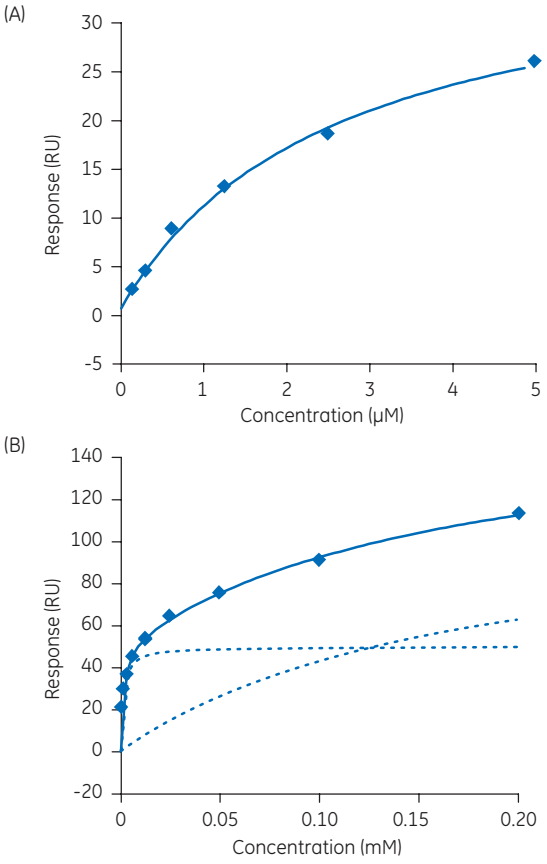

Fig 5. Equilibrium dissociation constant derivations for drug binding to HSA. The K_D values for S1 and warfarin were calculated from plots of binding response against concentration. (A) Compound S1, evaluated with a one-site model, and (B) warfarin, evaluated using a multiple site model. The dotted lines represent the calculated contributions from the two sites in the model.

Table 1. Equilibrium dissociation constants for drug binding to HSA. The fitting model used to calculate the K_D values for each compound is also indicated

Compound	K_D	Model
S1	2.5 µM	One-site steady state
S2	34 nM	Kinetic 11 model
Warfarin	1.2 µM	Multiple-site steady state

Calculation of %-bound values for high affinity binders

Although a K_D value is normally used to describe the strength of binding between two molecules, the ADME field most frequently expresses this data in terms of %-bound, since the relative amount of free versus protein-bound drug in the circulation is a key factor in its clinical application. If serum concentrations of the drug compound and plasma protein(s) are known, the affinity data can be used to calculate a %-bound figure.

K_D values were determined for the binding of S1, S2, and warfarin to HSA and AGP. As shown in Table 2, these affinity data were then transformed to %-bound values using equation 3.

The figure of 99.8% for warfarin binding to HSA alone is in good agreement with published figures for this interaction (7). For all three drug compounds, the %-bound figures for HSA were much higher than for those to AGP. This reflects both the higher affinities of all three drugs towards HSA and the much lower serum concentration of AGP compared to HSA.

Table 2. Calculation of %-bound values for binding of drug compounds to HSA and AGP

	% bound (individual proteins)	
Compound	HSA	AGP
S1	99.6	26.3
S2	>99.9	43.1
Warfarin	99.8	39.3

The figures in the column headed "%-bound" are those expected for exclusive binding to one plasma protein. Serum concentrations of drug compounds, HSA and AGP were assumed to be 10, 680, and 24 µM respectively.

For drug compounds that bind HSA with medium-to-low affinity, however; other plasma proteins may play a much more significant role. By considering the relationship between protein concentrations and drug affinities the following rules of thumb may be useful.

- When the compound affinities for HSA and AGP are comparable, HSA dominates due to its almost 30-fold higher serum concentration.
- If the compound affinity for HSA is 10-fold lower than for AGP, then the combined effect of the two plasma proteins begins to have an impact on the total %-bound.
- If the compound affinity for HSA is 100-fold lower than for AGP, then the latter dominates the combined protein contributions to the total %-bound.
- When the compound affinity to HSA is 1000-fold lower than to AGP the total %-bound is controlled almost entirely by the contribution of AGP.

Discussion and conclusions

The use of Biacore systems for drug-target and drug-ADME target studies during both hit-to-lead and lead optimization phases of drug development can provide important insights on how plasma protein binding may impact drug dose and/or drug efficacy.

The data produced from single concentration analyses permit the ranking of compounds in terms of a binding response to single or multiple plasma proteins. Based on this ranking assay, compounds for which a high-resolution analysis is required can be selected for affinity analysis and for determination of K_D values that can be transformed to simulate %-bound figures.

The importance of K_D analysis was illustrated with S2 binding to HSA, whereas a K_D of 34 nM translates to a %-bound figure of 99.995% (Table 2). This would leave a theoretical free drug concentration of only 0.5 nM, which may be too low for the drug to assert the desired effect.

While the examples given serve an introduction to plasma protein binding, this application can be extended and examples from literature demonstrate:

- SPR technology offers the potential to produce high quality kinetic data as an extension of affinity and %-bound values (7, 8).
- Plasma protein binding assays can be run in competition mode to provide detailed information on the site specificity of drug binding to albumin (9).
- Plasma protein binding may be species-specific and may impact the choice of animal model for drug development (9) or for explaining differences of drug effects in humans and in animals (10).
- The Biacore system is being recognized for in depth analysis of strong interactions with HSA (11).

References

1. Karlsson, R. *et al.* Biosensor analysis of drug–target interactions: direct and competitive binding assays for investigation of interactions between thrombin and thrombin inhibitors. *Anal Biochem.* **278 (1),** 1–13 (2000).
2. Wanwimolruk, S. *et al.* Plasma protein binding of Quinine: Binding to Human Serum Albumin, α1-Acid Glycoprotein and plasma from patients with malaria. *J Pharm Pharmacol.* **44**, 806–811 (1992).
3. Rudy, A. *et al.* Binding of Pyrimethamine to human plasma proteins and erythrocytes. *Pharm Res.* **7**, 1055–1060 (1990).
4. Ferrer, J. *et al.* The binding of benzopyranes to Human Serum Albumin. A structure-affinity study. *J Protein Chem.* **17**, 115–119 (1998).
5. Cheruvallath, V. *et al.* A quantitative circular dichroic investigation of the binding of the enantiomers of Ibuprofen and Naproxen to Human Serum Albumin. *J Pharm Biomed Anal.* **15**, 1719–24 (1997).
6. Frostell-Karlsson, A. *et al.* Biosensor analysis of the interaction between immobilized Human Serum Albumin and drug compounds for prediction of Human Serum Albumin binding levels. *J Med Chem.* **43**, 1986–92 (2000).
7. Rich, R. L. *et al.* High-resolution and high-throughput protocols for measuring drug/Human Serum Albumin interactions using Biacore. *Anal Biochem.* **296**, 197–207 (2001).
8. Shim, Y. Y. and Reaney, M.J., Kinetic interactions between cyclolinopeptides and immobilized Human Serum Albumin by surface plasmon resonance. *J Agric Food Chem* **63 (4)**, 1099–1106 (2015).
9. Day, Y. S.N. and Myszka, D. G. Characterizing a drug's primary binding site on Albumin. *J Pharm Sci.* **92**, 333–343 (2003).
10. Giannetti, A. M. *et al.* Identification, characterization, and implications of species-dependent plasma protein binding for the oral hedgehog pathway inhibitor Vismodegib (GDC-0449). *J Med Chem.* **54 (8)**, 2592–2601 (2011).
11. Vuignier, K. *et al.* Global analytical strategy to measure drug–plasma protein interactions: from high-throughput to in-depth analysis. *Drug Discovery Today*, **18 (21-22)**, 1030–1034 (2013).

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

gelifesciences.com/biacore

29253246 AA 02/2017