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The 'free drug hypothesis' : fact or fiction?

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Chapter 8

Challenging the 'free drug hypothesis' - summary, conclusions & perspectives

8.1 General background and scope of thesis

The objective of the investigations described in thesis was the development of a theoretical framework for prediction of the role of plasma protein binding as a determinant of *in vivo* drug effects.
Plasma protein binding may affect both the pharmacokinetics (PK) and the pharmacodynamics (PD) of drugs (Pacifici and Viani, 1992; Wright *et al*., 1996; Bergogne-Berezin, 2002; Colmenarejo, 2003). At present the theoretical basis of the influence of (alterations in) plasma protein binding on PK is well-established (Rowland and Tozer, 1995). Specifically, depending on the extraction ratio, the clearance of a drug can be restrictive or non-restrictive with regard to plasma protein binding (Rowland and Tozer, 1995). Clearance is nearly independent of the free fraction for high extraction ratio drugs (e.g. propranolol). In contrast, clearance is highly dependent on the free fraction for low extraction ratio drugs (e.g. diazepam). The other primary PK parameter, volume of distribution, is influenced by the free fraction as well in case of lipophilic drugs. For hydrophilic drugs, however, the free fraction in plasma is of little importance. As a result of the influence of plasma protein binding on clearance (*CL*) and volume of distribution (*V*), the secondary PK parameters (e.g. half-life) are affected by the free fraction as well. Due to the possible longer retention in the body (*CL*) and the decreased distribution throughout the body (*V*), plasma protein binding is considered to be quite positive with regard possibility of lower (optimal) frequency of drug administration. On the contrary, the influence of plasma protein binding on PD is considered to provide a negative outcome on drug action *in vivo.* However, the role of plasma-protein binding on PD has sofar never been examined in a systematic manner.
The 'free drug hypothesis' states that the pharmacological activity of a drug is correlated with its unbound drug concentrations in plasma. This hypothesis is based on the assumption that drug bound to plasma proteins cannot bind to the physiological target in the body. In drug discovery, non-specific binding to plasma proteins, is assumed to prevent the drug from binding to its physiological target (i.e. receptor or enzyme). In other words, high plasma protein binding (>95%) is considered a non-favorable property for a new chemical entity (NCE). For certain drugs like benzodiazepines, opiates, and neurosteroids experimental data indicate that it is indeed the free concentration that determines the intensity of the response (Mandema *et al*., 1991; Derendorf *et al*., 1993; Van Der Graaf *et al*., 1997; Cox *et al*., 1998; Visser *et al*., 2003). Interestingly, for other drugs such as A_1-adenosine agonists, it appears that it is the total rather than the free concentration that determines the response (Van Der Graaf *et al*., 1997). The 'free drug hypothesis' is, thus, subject to debate and, therefore, a number of investigations, both *in silico* and *in vivo*, were conducted to study the influence of PPB on pharmacodynamics. In this chapter, the results of the investigations are summarised and discussed. Furthermore the directions for future research are presented.

8.2 Development of a theoretical framework for prediction of the influence of plasma protein binding on PD

An *in silico* approach has the advantage that multiple hypothesis can be tested in a reasonably straightforward manner without the need of extensive experiments and can be combined with an experimental approach to provide understanding of the findings *in vivo*. In **chapter 1** an *in silico* approach was chosen to examine in a quantitative manner the influence of plasma protein binding on receptor binding. Meaningful parameter estimates of the binding affinities for the simulations were obtained from literature to provide insight in the "direct" competition between protein en receptor for the drug.

The pharmacological effect of a drug *in vivo* is the result of the direct competition between target binding and non-specific binding in the body. The differences between the binding at plasma proteins and at the biological target binding are the main determinant of the influence of plasma protein binding on pharmacodynamics.

There are important quantitative differences between the non-specific binding at plasma proteins and the specific binding at the biological target. Typically drugs bind with high affinity to their biological target, often a receptor. Pertinent information on the receptor binding affinities of drugs is readily available in the literature. For most drugs, receptor-binding affinities range between 10^{-12} and 10^{-5} M. As a consequence, receptor binding is readily saturable (drug concentration dependent). Moreover, as the binding capacity at the receptor is typically small, for small molecules the binding at the receptor does typically not influence the overall availability of the drug.

The major binding proteins in plasma are albumin and AGP. An important aspect of the binding of drugs at albumin is the high concentration of this binding protein and consequently the high binding capacity. Measures of the binding affinity of small molecules to albumin are not readily available. Yet calculations on basis of the albumin concentration and the free fraction of various small molecule drugs, as well as experimental observations on a limited number of drugs, yielded estimates of drug-protein binding affinities in the range between 10^{-7} and 10^{-3} M. The values of these protein binding affinity constants are much higher than the therapeutic concentrations of most drugs. As a result binding at albumin is typically linear with concentration (i.e. the free fraction is independent of the drug concentration). Estimates of the values of the binding affinity of drugs at AGP are typically in the rather wide range of 10^{-9} to 10^{-2} M. An important feature of AGP is that its plasma concentrations vary widely between and within subjects. Under normal physiological conditions saturation of drug binding to AGP binding may occur, while at elevated concentrations of AGP as occur in e.g. inflammation saturation is less likely.

In a series of computer simulations it was demonstrated that, in addition to the affinity for both protein and receptor, the plasma protein binding capacity is also a major determinant of the influence on the PD. More specifically, the difference in affinity and capacity for both target and protein determines the influence of plasma protein binding on receptor-occupancy of a compound under steady-state conditions. Therefore, capacity is a factor which should be taken into account when assessing the interaction between drug-target and drug-protein binding.

Regarding the differences in affinity it was found that the binding affinity of a drug for the receptor is likely to be higher than for the plasma proteins for a particular drug on individual basis. The ranges in binding affinities for plasma protein and receptor binding, however, overlapped. As a consequence, it is possible that the affinity of a drug for the protein approaches the affinity for the target. Although some studies indicate both restrictive and non-restrictive properties for the PD, it is generally assumed that only the free concentration in plasma is responsible for the pharmacological effect of drugs *in vivo* ('free drug hypothesis'). The simulations showed that under conditions of rapid equilibrium, plasma protein binding will indeed be restrictive for the PD of most drugs. Non-restrictive protein binding with regard to the PD is only observed for drugs with a very high affinity for the target compared to the affinity for the protein (>1000-fold difference).

8.3 Mechanism-based modelling of β-blockers

β-adrenoceptors antagonists as model drugs

β-adrenoceptors antagonists (β-blockers) were selected as model compounds, since they are

considered suitable drugs for the investigation of the relation between specific drug characteristics on drug action *in vivo*. As a class, the β-blockers are quite diverse, because they display a high range of values in plasma protein binding and also differ substantially in their affinity for binding to β-adrenoceptors (Johnsson and Regardh, 1976; Riddell *et al*., 1987; Mehvar and Brocks, 2001; Singh, 2005). In addition, a lot is known about the β-blockers and their mechanism of action. β-blockers are antagonists and as a consequence the pharmacological effect on heart rate is directly related to receptor occupancy. Heart rate can, hence, be used as a biomarker for receptor occupancy. A more practical advantage for the use of β-blockers is the readily available PD endpoint in both humans and laboratory animals (Wellstein *et al*., 1987; Piercy, 1988; Kendall, 1997). The β-blocker, S(-)-propranolol, has been frequently used in the research of plasma protein binding (PPB). The results of most studies seem to indicate that the free concentration is the main determinant of drug effect for this β-blocker (Yasuhara *et al*., 1985; Belpaire *et al*., 1986; Chindavijak *et al*., 1988; Terao and Shen, 1983). However the findings of these investigations have never been challenged in a systematic manner on the basis of mechanism-based PKPD modeling. This is important since plasma protein binding affects both the pharmacokinetic and the pharmacodynamics. By mechanism-based PKPD modelling not only changes in the pharmacokinetics are accounted for, but in addition, through the application of concepts from receptor theory, realistic estimates of the *in vivo* binding affinity are obtained. The aim of the currently presented investigations was therefore to challenge the 'free drug hypothesis' in a systematic manner by application of novel mechanism-based PKPD modelling concepts.

Isoprenaline-induced tachycardia as a continuous pharmacodynamic endpoint

Although readily available, the reduction in heart rate after β-blocker administration is small and difficult to distinguish from normal variations in heart rate. In clinical investigations the pharmacological response of β-blockers is for that reason commonly evaluated using isoprenaline-induced or exercise-induced tachycardia (Lipworth *et al*., 1991; Van Bortel *et al*., 1997; Schafers *et al*., 1999). Isoprenaline-induced tachycardia is obtained by short infusions of isoprenaline and the effect isoprenaline on heart rate is evaluated with and without β-blocker being present. A comparable methodology is used for exercise-induced tachycardia, in which the responsiveness of heart rate to exercise is evaluated with and without β-blocker.

For the development of mechanistic PKPD models a continuous measure of drug effect is preferable (i.e. heart rate, EEG, body temperature) (Dingemanse *et al*., 1988). For that reason, we investigated the use of a continuous intravenous infusion of isoprenaline (5 $\mu g\ kg^{-1}\ h^{-1}$) in the PD measurement of S(-)-atenolol in Wistar Kyoto rats. In **chapter 3**, heart rate under isoprenaline-induced tachycardia was validated as a continuous PD endpoint for the β-blockers in preclinical PKPD studies aiming at the development of mechanism-based PKPD models for β-blockers. Atenolol was used as a prototype β-blocker. A secondary objective was the establishment of a concentration-effect relationship for isoprenaline in Wistar Kyoto (WKY) rats as needed for the mechanism-based modelling approach (**chapter 4**). The results of this study showed that reduction of heart rate under isoprenaline-induced tachycardia is a reliable PD endpoint for β-blockers *in vivo* in WKY rats. Compared to exercise, induction of tachycardia with isoprenaline is easy achievable in experimental animals and the heart rate response is controllable. On top, the pharmacokinetics of isoprenaline in plasma are known (or can be measured) and this is an advantage for mechanism-based PKPD modelling in the sense that it constitutes a basis for the modelling of the competitive interaction with β-blockers.

To our knowledge, the PKPD of isoprenaline in rats have not been reported in literature. We constructed

a PK model and a concentration-effect relationship for this compound (**chapter 3**). The concentration-time profiles for isoprenaline were adequately described using a two-compartment model. Isoprenaline is considered an extremely potent β-adrenoceptor agonist in literature and this was confirmed in this study with an *in vivo* potency (EC_{50}) of 0.014 ng ml^{-1} (Waldeck, 2002).
A remarkable finding was the observed hysteresis observed in the concentration-effect relationship for S(-)-atenolol and the subsequent need for an effect compartment in the PKPD model. Although the use of an effect compartment for β-blockers is not uncommon in the literature for the effect on blood pressure, the effect on heart rate is assumed to be an direct effect since the β_1-receptor is present in the plasma compartment (Ritchie *et al*., 1998; Brynne *et al*., 2000; Hocht *et al*., 2004; Hocht *et al*., 2006).

Development of a mechanism-based PD interaction model
The estimation of *in vivo* affinity for 'silent' agonist is usually complicated, since under normal physiological conditions these drugs do not display large effects. The activity of a pure antagonist *in vivo* depends solely on binding and results from the displacement of the (endogenous) agonist from the target (Hardman *et al*., 2001). For that reason, estimation of the (*in vivo*) affinity of pure antagonists not only requires the presence of an (endogenous) agonist but also knowledge on the concentration of the agonist in the system, its target affinity and its intrinsic efficacy (Kenakin, 1993). Thus although the mechanism of action of antagonists is less complex than that of agonists, the characterization and quantification of pharmacological effect *in vivo* appeared to be quite challenging. In **chapter 4**, the development of a mechanism-based PD interaction model for an agonist and an antagonist, which can be used for the estimation of *in vivo* affinity of β-blockers, is described. The use of a mechanism-based interaction model requires an analysis of the PKPD relationship for isoprenaline on basis of the operational model of agonism (Leff *et al*., 2003). The affinity (K_A) of isoprenaline, $3.2x10^{-8}$ M (left atria WKY rats), was obtained from literature (Doggrell *et al*., 1998). The estimates including coefficients for variations for baseline (E_0), maximal effect (E_{max}) and efficacy (τ) using the operational model of agonism were 374 (1.9%) bpm, 130 (5.9%) bpm and 247 (33%) respectively. The obtained parameters estimates for isoprenaline served as an input to the interaction model. Isoprenaline is a very efficient agonist in a system with a large receptor reserve and this observation is in agreement with the high value obtained for τ in the system (Doggrell *et al*., 1998).
Subsequent to the PKPD model for isoprenaline, a mechanism-based PD interaction model, also based on the operational model of agonism, was developed. The interaction model adequately described the heart rate profiles in conscious rat, following administration of a combination of the agonist, isoprenaline, and the antagonist, S(-)-atenolol. The obtained estimate for *in vivo* affinity ($K_{B,vivo}$) of S(-)-atenolol, $4.62x10^{-8}$ M, is very close to values obtained from literature for the *in vitro* affinity ($K_{B,vitro}$) in functional assays. Nandakumur et al. reported a value of $3.26x10^{-8}$ M (*pA2* = 7.16) for the affinity of atenolol in spontaneous beating right atria obtained from Wistar rats (Nandakumar *et al*., 2005). Louis reported in 1999 a *pA2* value of 7.30 ($K_B = 2.5x10^{-8}$ M) for rat (Sprague Dawley) atria in a functional assay (Louis *et al*., 1999). Interestingly, the need for an effect compartment disappeared with the use of a mechanism-based interaction model. The observed hysteresis in the concentration-effect relationship for S(-)-atenolol (see **chapter 3**) was, thus, explained by the interaction at the β_1-adrenoceptor between isoprenaline and S(-)-atenolol.

8.4 Assessment of the role of plasma protein binding in PD

PKPD correlation for beta-blockers with varying degree of PPB

The goal of the investigations described in **chapter 5** was to examine in a systematic manner the influence of plasma protein binding on *in vivo* PD. To this end the role of plasma protein binding on the PD of four β-blockers with a varying extent of plasma protein binding was determined in comparative PKPD studies in conscious rats. The β-blockers S(-)-atenolol, S(-)-metoprolol, S(-)-propranolol and timolol were selected as model drugs since they vary widely in plasma protein binding, ranging from almost no plasma protein binding for atenolol (~3%) to high plasma protein binding for propranolol (~95%). The mechanism-based interaction model developed in **chapter 4** was applied to the PD interaction between isoprenaline and the individual β-blockers. The concentration vs. heart rate profiles were described on basis of both total and free drug concentrations and this yielded estimates of the $K_{B,vivo}$ which were compared to the $K_{B,vitro}$. The *in vitro-in vivo* correlation for receptor affinity on basis of the free drug concentration was linear (r^2 = 0.99) and approximated the line of identity. Moreover, on basis of the free drug concentration the $K_{B,vivo}$ (2.0 ± 0.71 nM) for S(-)-propranolol closely resembled the average $K_{B,vitro}$ (1.9 ± 0.48 nM). It was, thus, concluded that the free drug concentration in plasma is the main determinant of this β-blocker effect on heart rate *in vivo*. Although the relationship between the *in vivo* and the *in vitro* affinity constants based on free drug concentrations was linear and approximated the line of identity, the *in vivo* affinity on basis of free drug appeared to be systematically higher than the *in vitro* affinity for the four compounds. Model misspecification with regard to the efficacy of isoprenaline (τ) may partly explain this observation. The *in vivo* affinity estimate is related to the value of τ of the full agonist isoprenaline and as such this might explain the differences observed between the *in vitro* and *in vivo* for the β-blockers. On the other hand, the *in vitro* values of affinity might not be entirely representative for the *in vivo* values. The obtained *in vitro* affinities from literature also differ to some extent and this is an indication that the estimate of affinity is not totally independent of the experimental conditions.

The results of the simulations in **chapter 2** confirmed the findings of **chapter 5** in the sense that the active concentration is nearer to the free concentration than to the total concentration for compounds which rapidly interact with their receptor. The active concentration, however, does not always equal the free drug concentration and as a consequence the change in PD is not necessarily proportional to the shift in protein binding. For S(-)-propranolol, however, the shift is similar to the estimated change in free fraction as discussed in section 8.4.2. (**chapter 6**; **chapter 7** and figure 1).

Although the β-blockers were selected to minimise the distribution to the biophase, an effect compartment was needed to resolve hystereris for the lipophilic β-blockers (S(-)-metoprolol, S(-)-propranolol, timolol). Like for S(-)-atenolol, the hysteresis observed for S(-)-metoprolol was found to be significantly smaller than reported in literature (37 vs. 7 min, respectively) when using the interaction model (Hocht *et al*., 2006). The reduced hysteresis may to a certain extent be explained by the interaction with the (endogenous) agonist. The residual hysteresis, however, remains to be explained and could be due to target site equilibration, receptor association/dissociation and transduction (Tuk *et al*., 1997; Tuk *et al*., 1998; Cleton *et al*., 1999; Yassen *et al*., 2005; Danhof *et al*., 2007; 2008). Very limited evidence of slow binding kinetics has been reported in literature for isoprenaline and/or the β-blockers (Contreras *et al*., 1986). The validity of the assumption of instant binding to the receptor, thus, needs further investigation for the lipophilic β-blockers.

Influence of altered PPB on the PKPD relationship of S(-)-propranolol

For the purpose of investigations on the role of free drug concentrations as a determinant of the PKPD relationship of highly protein bound drugs determining the influence of altered plasma protein binding is an attractive approach. In this context it is important to be able modulate the free fraction *in vivo* in a controlled manner. Surgical implantation of permanent cannulas was shown to induce a ten- to fifteen fold increase in serum AGP concentrations at 2 days post-surgery (**chapter 6**). At 2 days (48 h) post surgery the AGP concentration was 1540 ± 122 μg/mL (~37 μM) and the elevated AGP levels returned back to baseline (85 ± 21 μg/mL (~2 μM)) within one week. Subsequently it was confirmed in *ex-vivo* experiments that an increase in AGP serum concentration from 55 to 675 μg/ml resulted in a profound decrease in the free fraction of S(-)-propranolol from 14 ± 0.6 to 1.9 ± 0.3 %. Cannulation, thus, modified plasma protein binding of S(-)-propranolol in a robust, reproducible and time-dependent manner leading to substantial alteration of plasma protein binding.

The objective of the study described in **chapter 7** was to challenge the 'free drug hypothesis', by investigating the influence of altered PPB on the PD or propranolol by *in vivo* mechanistic population PKPD modelling (**chapter 4**). The influence of altered serum AGP levels on heart rate effects of S(-)-propanolol was studied by comparison of PKPD correlations in rats at 2 and at 7 days post surgery, with elevated and normal plasma levels of AGP respectively. In the mechanism-based PKPD analysis the AGP concentration was found to be a covariate for the intercompartmental clearance (*Q3*) and the estimates $K_{B,vivo}$. The $K_{B,vivo}$ values decreased with increasing AGP concentrations. This indicated that the "free drug hypothesis" is indeed applicable to the PKPD correlation of S(-)-propranolol and this confirmed the findings in **chapter 5**.

The observed AGP levels in **chapter 6 and 7** under normal (~4 μM) and elevated conditions (~ 40 μM) were smaller than the concentration used in the simulations (9 – 72 μM) in **chapter 2**. As a consequence, plasma protein binding may be saturable with high drug concentrations. The maximal S(-)-propranolol plasma concentration, however, is approximately 0.4 μM (110 ng/mL). Saturable protein binding is, thus, not expected in the current experiments (**chapter 7**).

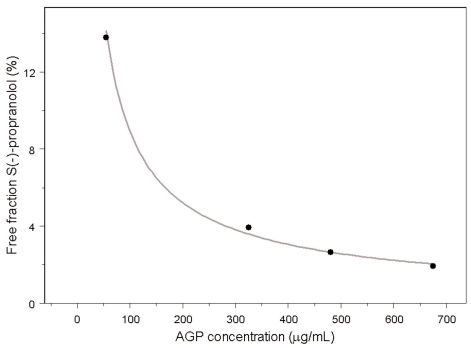

Figure 1, Curve fit of the free fraction of S(-)-propranolol versus the AGP concentration

In order to examine also the eventual change in the concentration-effect relationship on the basis of the alteration in plasma protein binding, it was also explored how changes in AGP levels would affect the free concentration of S(-)-propranolol (**chapter 6**). To this end the relationship between the free fraction S(-)-propranolol and the AGP concentration was characterised by means of a curve fit (figure 1). In **chapter 7**, the serum AGP concentration in the individual animals varied 10-fold with values ranging from 110 (day 7) to 1150 μg/mL (day 2). Consequently, the estimated percentages of free drug were 8.3% and 1.4% on day 2 and day 7 respectively. The estimates for $K_{B,\ vivo}$ on the basis of total plasma concentrations of S(-)-propranolol were 4.6 nM to 30 nM under conditions of normal and elevated plasma protein binding respectively. Consequently, the corrected estimates for $K_{B,\ vivo}$ on basis of free drug concentrations were 0.38 nM and 0.41 nM. Correcting for plasma protein binding, thus, resulted in nearly identical estimates of affinity and this further strengthens the conclusion that it is the free rather than the total concentration

is the main determinant of drug effect for S(-)-propranolol.

8.5 Conclusions and perspectives

The objective of the research described in this thesis was the development of a theoretical framework for the prediction of plasma protein binding on pharmacodynamics. The *in silico* and *in vivo* results indicate that under steady state conditions (assumption of rapid equilibrium with the receptor and protein) the free fraction in plasma is indeed the main determinant of drug effect for most drugs. On the other hand, the *in silico* investigations showed that non-restrictive protein binding with regard to PD is possible for drugs and can be explained by the saturable nature of target binding. Full receptor occupancy is obtained for high receptor-affinity compounds until the drug concentration becomes smaller than the concentration of receptors.

Mechanism-based modelling

Recently, mechanism-based PKPD modelling concepts have been introduced, which are based on concepts from receptor theory, and in which a strict distinction is made between drug-specific and biological system-specific parameters to characterise *in vivo* concentration-effect relationships (Van Der Graaf *et al*., 1997; Cox *et al*., 1998; Zuideveld *et al*., 2002; Visser *et al*., 2003). It is known from literature that the pharmacological effect of agonists is a combination between affinity (binding) and intrinsic efficacy (activation of the receptor) (Ariens, 1954; Furchgott, 1966; Kenakin, 1993). For the accurate estimation of *in vivo* affinity of the antagonist it is, therefore, essential to take into account the efficacy of the agonist (isoprenaline). The *in vivo* investigations described in this thesis, therefore, required a mechanism-based modelling approach in order to obtain meaningful estimates for the receptor binding affinity. The investigations clearly showed that a mechanism-based approach is essential for the identification of drug-specific parameters (*in vivo* affinity for β-blockers) which can be compared to parameters obtained *in vitro*.

Exact quantification of the $K_{B,vivo}$ of antagonist effects requires knowledge on the concentration of the agonist and this information is typically not obtained or is difficult to acquire in *in vivo* studies (Kenakin, 1993). On top, the concentration-effect relationship of the endogenous agonist should be quantified in terms of affinity and intrinsic efficacy. In the current investigations, the *in vivo* PD response of antagonists was quantified while taking into account the action of a non-endogenous agonist in the system. An alternative approach could be the identification of the PKPD relationship of the endogenous agonist (adrenalin) and the subsequent analysis of the PD interaction with the β-blockers. The influence of adrenalin on the β-adrenoceptor system under normal physiological conditions is small. As a consequence, the administration of β-blockers results in a small reduction in heart rate which is difficult to distinguish from normal variations in heart rate (**chapter 3**). As such the administration of an agonist is required for the proper identification of the β-blocker effect on heart rate. The use of a non-endogenous agonist was advantageous in the current investigations, because of the larger efficacy of isoprenaline at the β-adrenoceptor compared to adrenalin. Characterization of the concentration-effect relationship for endogenous agonists will, nonetheless, improve the understanding of PD effects of antagonists and the systems under investigation.

Although the β_1-adrenoceptor is located within the cardiovascular system for the β-blockers, a delay was observed between the concentration in plasma and the effect on heart rate. Hysteresis could be explained by the competition with isoprenaline (**chapter 3** and **chapter 4**) for the hydrophilic compound S(-)-atenolol.

The lipophilic β-blockers, however, still displayed a significant delay between concentration and effect, even though the interaction with isoprenaline was taken into account in the model (**chapter 5**). Target site equilibration, receptor association/dissociation and transduction are considered the potential causes for hysteresis (Tuk *et al*., 1997; Tuk *et al*., 1998; Cleton *et al*., 1999; Yassen *et al*., 2005; Danhof *et al*., 2007). Biophase equilibration is frequently considered the cause of hysteresis, but the study of the concentration at the target site *in vivo* is complicated. Mechanism-based PKPD modelling is considered a valuable tool in this matter (Yassen *et al*., 2005). Finally, research is needed to investigate the contribution of transduction processes to hysteresis. In the operational model of agonism distinct assumptions are made about the target binding and subsequent transduction process. These assumptions appear to be valid for the β-adrenoceptor system (**chapter 4**). For other systems, however, the transduction system might be more complex (Visser *et al*., 2003; Kenakin, 2005). It would, therefore, be of value to investigate the transduction process *in vivo* by combining the measurement of receptor occupancy with a relevant PD endpoint. Positron Emission Tomography (PET) is a technique which makes the measurement of receptor occupancy *in vivo* feasible (Liefaard *et al*., 2005).

Plasma protein binding and PD

The percentage plasma protein binding is dependent on the affinity of the drug for the protein, the concentration of plasma proteins and the number of binding sites (Paxton, 1985). The affinity of the drug for the protein is considered a drug-specific property, while the percentage binding (i.e. the free fraction) depends on both the protein binding affinity and the protein concentration. The affinity, although being a purely drug-specific property, has been reported only in a limited number of cases. It would, on the other hand, be of value to determine this parameter more often, since on this basis the percentage binding with changing plasma protein concentrations can be predicted on basis of affinity. In addition, for highly bound drugs the identification of the affinity is possibly more accurate than the percentage bound due to practical issues (e.g. LOQ).

As well as affinity, capacity is a key determinant in the interaction between plasma protein binding and receptor binding (**chapter 2**). Intuitively, one might expect that the drug-target and drug-protein binding is equal in case of an identical affinity for both receptor and protein. The capacity difference between receptor and protein, however, is the reason for the larger drug-protein binding in case of equal affinity. On top of that, the small capacity of the receptor is the cause for non-restrictive protein binding. Accordingly, a shift in the concentration-effect relationship will only be observed for compounds, which display a high affinity for the receptor when compared to the protein. In case of a large difference between the affinity for the binding protein and the receptor, a change in the plasma protein concentration will not affect the receptor occupancy. A number of reviews have addressed the clinical importance of drug protein binding (Sellers, 1979; Sparreboom *et al*., 2001; Benet and Hoener, 2002). The overall conclusion is that plasma protein binding of drugs and possible drug displacement interactions have little to no clinical significance. Benet concluded that for a large number of clinically used drugs (25 of 456) changes in plasma protein binding are not significant, since the AUC_{free} does not change with alterations in the free fraction in plasma (Benet and Hoener, 2002). This conclusion, however, is based on the general consensus that the pharmacological effect is related to exposure to unbound drug concentration ('free drug hypothesis') and as such does not assess the possible influence of plasma protein binding on PD.

Influence of target-site distribution

Distribution is required for many drugs to evoke effect *in vivo* and this might even be the case for β-

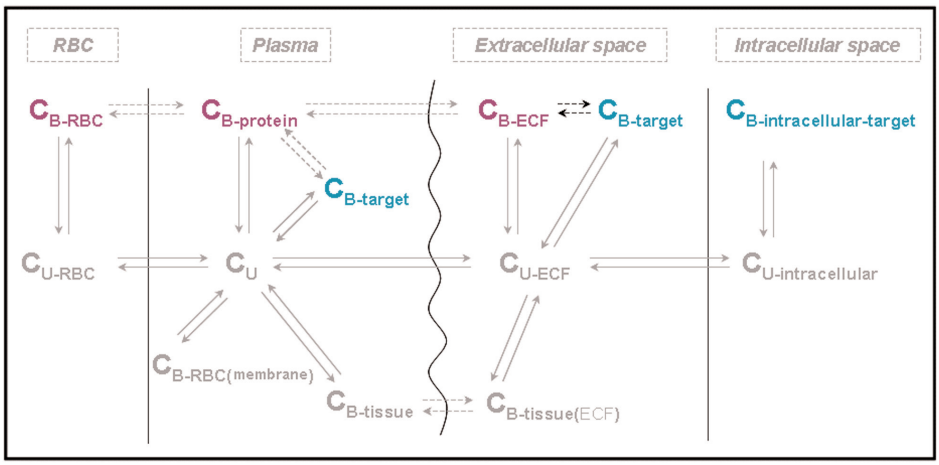

Figure 2, Schematic representation of distribution, specific (target) and non-specific binding (protein and tissue) of a drug in the body. The pharmacological effect of the drug is the result of binding to the target. Binding to protein is indicated in purple, binding to target in cyan.

blockers (**chapter 4** and **chapter 5**). In that situation the competing interaction between target and protein is indirect and the outcome of several steps when distribution is required for the PD (figure 2).
Certain drugs are distributed throughout the body by means of passive diffusion. Passive diffusion is the tendency of molecules to move down a concentration gradient (Rowland and Tozer, 1995). This type of distribution does not require any work of the system and is a non-specific process. As a consequence, passive diffusion is most probably dependent on the free concentration in plasma. Many drugs, however, are actively transported across epithelial membranes by means of transporters and this is considered a rather specific system dependent process. Active transporters may facilitate or limit access of drugs to certain organs and body compartments. Drug transporters are highly expressed in epithelia of the intestine, liver, kidney, placenta, and blood-brain barrier; they serve a major role in defining the pharmacokinetics of many drugs (Petzinger and Geyer, 2006). The role of active transport in distribution of drugs needs to be considered in drug discovery and development (Ayrton and Morgan, 2001). This applies not only with respect to drug disposition and the subsequent indirect influence on the response, but also with respect to the direct interaction between plasma proteins and transporters. Like for the competitive interaction between protein and target, the difference in affinity (and capacity) for both protein and transporter might determine the eventual effect of plasma protein binding on drug effect. The influence of plasma protein binding on the cascade leading to drug effect needs research. First, the direct influence of plasma protein binding on the target site distribution should be addressed. To this end, methods should be used which assess the drug concentration at the target site. Traditional methods for the investigation of drug target site distribution in humans include tissue biopsy, skin blister fluid sampling and saliva sampling (Brunner and Langer, 2006). More recently developed methods include imaging methods, like MRS or PET, and microdialysis. PET and microdialysis can also be used in preclinical investigations (Liefaard *et al*., 2005; Groenendaal *et al*., 2007). These techniques could for example be combined with the alteration of plasma protein binding *in vivo* in rats to evaluate the influence of plasma protein binding on distribution (**chapter 6**). In addition, the free drug concentration in blood can be assessed using blood microdialysis (Sarre *et al*., 1992; de Lange *et al*., 2000). Secondly, the target site concentration should be linked to the target binding *in vivo*. Liefaard and co-workers combined PET

scanning in rats with a mechanism-based modelling approach. They showed that it is feasible to characterise the binding of flumazenil to the $GABA_A$-receptor complex *in vivo* in rats. The main disadvantage of this approach is that the experiments are complex and labour intensive. To our knowledge, assessment of receptor binding *in vivo* remains difficult and new techniques have to be developed. The final step in the cascade leading to drug effect is the transduction process which follows target binding. As mentioned in section 8.4.1., it would be of value to combine the measurement of target site distribution and receptor occupancy with a relevant PD endpoint.

Target binding kinetics

The non-restrictive character of protein binding in the current simulations is caused by the fact that the receptor is saturable. In other words, the free concentration exceeds the concentration needed to obtain full receptor occupancy (active concentration). Thus for high affinity drugs, the receptor occupancy will only diminish when the receptor is depleted (receptor concentration < 0.001 μM).

A novel trend in drug discovery is the search for compounds which display both a high affinity for and a slow dissociating rate from the target. Compounds with a slow dissociation rate are believed to have a prolonged duration of action due to the slow dissociation from the target (Dowling and Charlton, 2006). Since NCEs are aimed to have a high affinity for the physiological target these drugs are likely to have high plasma protein binding because of their lipophilicity. Assessment of the influence on plasma protein binding on pharmacodynamics is, therefore, of high importance. In the present study, the influence of protein binding on pharmacodynamics was only investigated using the assumption of rapid equilibrium (steady state) for both drug-protein and drug-receptor binding. The influence of plasma protein binding might be different if we would take into account the kinetics of protein and target binding ("dynamic conditions"). To investigate this, we have performed a simulation which takes into account both protein and target binding kinetics. The differential equations describing the pertinent process are shown in equation 1, 2 and 3.

$$\frac{dA}{dt} = -k_{on}^{t} \cdot A \cdot T + k_{off}^{t} \cdot AT - k_{on}^{p} \cdot A \cdot P + k_{off}^{p} \cdot AP \quad (1)$$

$$\frac{dAT}{dt} = k_{on}^{t} \cdot A \cdot T - k_{off}^{t} \cdot AT \quad (2)$$

$$\frac{dAP}{dt} = k_{on}^{p} \cdot A \cdot P - k_{off}^{p} \cdot AP \quad (3)$$

In which A is the concentration of drug, T the concentration of the target ($T = T_{total} - AT$), P the concentration protein ($P = P_{total} - AP$), AT is the concentration of drug-target complex and AP is the concentration of drug-protein complex. In the simulation, the drug concentration at t=0 was 5 μM and the total target and protein concentration were 0.001 μM and 600 μM, respectively. The simulation was performed for a high affinity drug (*Kdr* = 1e-6 μM) with high protein binding (99.99%; *Kdp* = 0.01 μM). The association rate constant (k_{on}) was assumed to be 10000 μM^{-1} h^{-1} for both target and protein binding. Subsequently, the dissociation rate constant (k_{off}) was implemented in the model on basis of *Kd* (*Kdr* or *Kdp*) and k_{on} using equation 4:

$$k_{off} = K_D \cdot k_{on} \quad (4)$$

Simulations were performed using equation 1 to 4. First, target occupancy was simulated in absence of

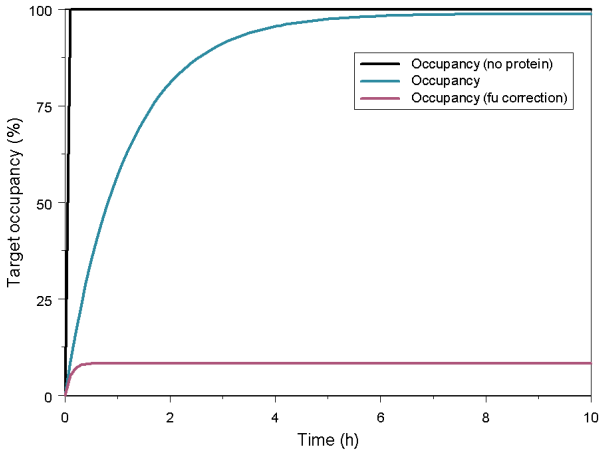

Figure 3, Simulation of target occupancy under dynamic conditions). The black line is the target occupancy in absence of protein binding. The cyan line is the time course of the simulated target occupancy for the dynamic interaction between target and protein. The purple line is the target occupancy on basis of the free drug concentratration (no dynamic interaction)

protein (P_{total} = 0). In this simulation target occupancy is fully dependent target binding rate constants, the concentration of target and the concentration of drug. Secondly, target occupancy was predicted on basis of the dynamic interaction between both protein and target. In this simulation, the target occupancy is not only dependent on the interaction between target and the drug, but also between protein and drug. Finally, a target occupancy simulation was performed in which the drug concentration was corrected for the percentage protein binding. In this simulation the free drug concentration (C_{fu} = fu * C_{drug}) was used to simulate target occupancy in absence of protein (P_{total}=0). In this last simulation the assumption was made that only the free drug concentration is able to bind the target and this, thus, reflects the 'free drug hypothesis'. The results are shown in figure 3.

In absence of protein, full receptor occupancy is obtained rapidly for a high affinity drug at a concentration of 5 μM. If the dynamic competition between target binding and protein binding is taken into account, the receptor occupancy increased more slowly reaching the maximal target occupancy of approximately 99% within 6-8 hours. The predicted target occupancy on basis of the free concentration without taking into account the dynamic interaction is approximately 10%. Target occupancy is underestimated if we assume that only the free drug binds to the target ('free drug hypothesis'). Although more extensive research is needed, we can conclude that the probability of non-restrictive protein binding with regard to the PD is potentially higher under dynamic conditions. Future research should, thus, determine the impact of plasma protein binding on the PD in relation to the binding kinetics at protein and receptor (Talbert *et al*., 2002).

Conclusions

In conclusion, the free drug concentration is the main determinant of the PD under equilibrium conditions for both target and protein. In case of a dynamic interaction, the probability of non-restrictive protein binding is (theoretically) higher with regard to the pharmacodynamics under certain conditions, but more extensive research is needed to confirm this statement.

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