Quantitative pharmacology or pharmacokinetic pharmacodynamic integration should be a vital component in integrative pharmacology

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Running title page

Running title: pharmacokinetic-pharmacodynamic integration

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Document statistics:

Number of text pages: 29

Number of tables: 0

Number of figures: 3

Number of references: 46 (agreed with editor)

Number of words in Abstract: 250

Non standard abbreviations:

C: Total plasma concentration; $C_b$: Bound plasma concentration; $C_u$: Unbound plasma or tissue concentration; $CL_u$: Unbound drug clearance; DMPK: Drug Metabolism and Pharmacokinetics; $f_u$: Unbound fraction in plasma; ip: Intraperitoneal; iv: Intravenous; NiAc: nicotinic acid; MDA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; NEFA: Non-esterified Free Fatty Acids; NXY-059: disodium 2,4-disulphophenyl-N-tert-butyl nitrone; PD: Pharmacodynamic(s); PK: Pharmacokinetic(s); PKPD: Quantitative pharmacology; sc: Subcutaneous; SSRI: Serotonin selective reuptake inhibitor
Abstract

Abstract: Pharmacodynamics (PD) examines the relationship between drug concentration and onset, intensity and duration of the pharmacological effect. Pharmacokinetics (PK) is the science of the time course of drugs in the organism. The quantitative pharmacological approach focuses on concentration-response and response-time relationships with special emphasis on the proposed impact of the drug on the disease. The review aims to raise awareness among pharmacologists as to why pharmacokinetic-pharmacodynamic (PKPD) integration is essential in basic pharmacology research to improve interpretation of data. Quantitative pharmacology is vital in drug discovery for target validation, optimizing the development of lead compounds, and scaling compounds to man and has become mandatory for regulatory bodies. However its use is still comparatively rare in experimental pharmacology and its absence diminishes the interpretative value of published experimental data and can allow the presentation of misleading information. A primary requirement for PKPD integration is establishing the inter-relationships between in vitro and in vivo PK and PD properties and extrapolation to the known or possible future clinical use of a compound. This review examines the use of PKPD in experimental pharmacology by reviewing drug exposure measurements, plasma protein binding, exposure-effect relationships and the measurement of active metabolites. It examines the significance of dosing schedules, the importance of target engagement and problems in examining time-response relationships. It shows how quantitative pharmacology adds significant value to study design and examines why ignoring pharmacokinetics can lead to misleading results and conclusions. Finally, a guide list of points to be considered when performing studies is provided.
Pharmacodynamics (PD) concerns the study of the time course of biological effects of drugs, the relationship to drug exposure, and drug mechanisms of action – that is, it examines what the drug does to the body. Pharmacokinetics (PK) investigates the time course of drugs in the organism – that is, it investigates what the body does to the drug. However, Levy (2006) wrote: “pharmacodynamics, as I see it, is concerned with the quantitative, temporal aspects of drug action, and particularly with the relationship between the concentration of drugs (and their active metabolites) in plasma or other biological fluids and their intensity and time course of their pharmacological effects”. We also believe that a better understanding of the inter-relationships between PK and PD and sometimes the lack of concordance between the two will improve many investigations and make their results more amenable to interpretations related to the current or future clinical use of the experimental compound (Levy 1993; 1998). Our contention is that in vivo pharmacokinetics must play a greater and more intelligent role in pharmacology research, thereby making the data obtained much more valuable.

The recent review detailing the rationale behind the pharmacokinetic-pharmacodynamic (PKPD or quantitative pharmacology) approach and its importance for rational drug discovery research and development was aimed primarily at scientists working in the pharmaceutical industry (Gabrielsson et al. 2009). In contrast, this review aims to raise awareness as to why PKPD integration should also be an essential element in the protocols of all in vivo pharmacologists in order to make their investigations relevant to the therapeutic mechanism of action of experimental compounds destined for clinical use and increase knowledge of drugs already marketed.
We are not suggesting that complex kinetic-dynamic relationship data be obtained by every pharmacologist, that should be left to pharmacokineticists; merely that studies are designed so that some basic information is obtained, or used, and certain ‘rules’ followed, to allow the integrative pharmacologist to maximize the value of every study. While the examples provided are weighted towards neuropharmacology because of our particular interests, the issues raised are relevant to all experimental pharmacology.

The PKPD approach and the consequences of ignoring it

It is common practice in experimental in vivo pharmacology to relate the pharmacological response to the administered dose. The danger is that factors like bioavailability, nonlinear concentration-dose relationships, active metabolites and concentration-dependent plasma protein binding differences may occur within or between different species and confound the interpretation.

It is rare for all the administered substance to be responsible for the observed pharmacological effect. Maybe only a fraction of the dose reaches the blood and eventually the target region. The rest may not be absorbed or is degraded during the first-pass through the liver or in the gastrointestinal tract. Figure 1 shows the concentration-response, dose-response and dose-concentration relationships for compounds exhibiting nonlinear pharmacokinetics at pharmacological concentrations. The schematically illustrated example in Figure 1 is, in our experience, a commonly encountered scenario, which is seldom correctly tackled by experimentalists (Gabrielsson et al, 2009). This means that doubling the dose may lead to more than doubling of the plasma concentration. If the dose-response relationship is plotted one obtains a steeper curve when replacing the dose on the x-axis with plasma concentrations (Figure 1a). The same distance on the response-axis $\Delta R$ covers two different ranges on the concentration ($\Delta C$, Figure 1a) and dose ($\Delta D$, Figure 1b) axes. The
relationship between dose and concentration is summarized in Figure 1c, which explains the different slopes of the two upper plots. The steep dose-response curve implies erroneously that drug causes an all-or-none response. Oral dosing may also give steep (or shallow) dose-response relationships due to an active metabolite or metabolites. What the dose-response relationship does not reveal is a shallower ligand-receptor-response relationship as well as the nonlinear pharmacokinetics. The administered dose, particularly in the case of extravascular administration, can never totally replace what is thermodynamically active, namely the biophase concentration, particularly in cases where drug uptake (absorption, bioavailability) into the systemic circulation is low and variable. In particular the unbound plasma concentration is a better marker of what is ‘driving’ the pharmacological effect as discussed below. The opposite situation may also occur with the concentration-response curve being steeper than the dose-response curve. Then the dose-concentration curve will display a hyperbolic relationship where a doubling of the dose leads to a less than doubling of the plasma concentration.

For these reasons, kinetic-dynamic reasoning should, whenever possible, be based on in vitro or in vivo concentration-time, response-time and concentration-response relationships, with an underlying ambition to couple this to the disease state. By failing to use an integrated PKPD approach to experimental design studies may be designed wrongly, thereby either overestimating or underestimating the efficacy of a compound as shown below.

**Simple PKPD considerations for experimental pharmacologists**

*Route of administration and drug exposure*

Many in vivo studies examining a physiological outcome measure (such as a blood pressure change or a behavioural response) involve animals being administered compounds at some rather arbitrary fixed dose or doses, with little (or no) consideration of the way the drug is, or
might be, given clinically. Drugs are often given intraperitoneally (ip), presumably because this is technically easier than oral or intravenous administration, and no attempt is made to measure absorption and exposure by measuring the plasma drug concentration. Consequently some studies must be reporting investigations where there is little exposure to the active substance because of low bioavailability.

One recent example is a study of the neuroprotective compound disodium 2,4-disulphophenyl-N-tert-butylnitrone (NXY-059) where the drug was given as a single ip bolus (Balogh et al., 2005). However NXY-059 has minimal absorption by that route (AstraZeneca, data on file) so its effect in the stroke model was minimal, in contrast to most studies where the drug was given by intravenous infusion (Bath et al., 2009), the route also used in clinical investigations (Diener et al., 2008).

In preclinical studies on marketed drugs there is often little consideration as to whether the administered dose produces clinically relevant exposure or, in the case of experimental drugs, exposure likely to be tolerated clinically. Consequently the biochemical mechanism being examined may either be subjected to a drug concentration too low to be relevant or one producing a response more reflective of toxicology than pharmacology. For example, there has been much discussion as to whether the neurotoxicity observed in experimental animals following administration of 3,4 methylenedioxymethamphetamine (MDMA or ‘ecstasy’) indicates that a similar change might occur in the brains of recreational users (see Green et al., 2003). Arguments generally focussed on doses given to rats versus those ingested by humans. Few preclinical studies measured plasma MDMA concentrations. Some investigators argued that the rat dose could be extrapolated to suggest that a similar dose was required to produce neurotoxicity in humans and that, for example, a 20 mg/kg dose in rats equated to a human dose of 1400 mg (20 mg/kg x 70 kg body weight) or around 20 ecstasy tablets (Sessa and Nutt, 2008). Others (McCann and Ricaurte, 2001) used the allometric
approach (Mordenti and Chappell, 1989, Boxenbaum and D'Souza, 1990) which proposes that using equation 1:

\[
D_{\text{human}} = D_{\text{animal}} \left( \frac{W_{\text{human}}}{W_{\text{animal}}} \right)^{0.7}
\]

(Eq. 1)

where D is dose (mg), W is body weight (kg), allows calculation of equivalent doses in animals and humans. This equation suggests that a 20 mg/kg dose to rats equates to 280 mg (4 mg/kg) or 4 ecstasy tablets in humans.

We recently took published data on dose versus the plasma MDMA concentration in rats and humans and calculated that injecting 7 mg/kg of MDMA into rats produced a similar exposure to a 2 mg/kg dose in humans (Green et al., 2009). Although measuring the peak plasma drug concentration fails to take into account plasma protein binding, the drug half-life and active metabolites (see later), it does take some account of systemic exposure and its value outweighs such limitations in its interpretation.

Plasma protein binding and unbound concentrations

Drug plasma protein binding is rarely taken into account in experimental pharmacology, but should be, particularly if studies involve more than one species or comparisons with different compounds, gender, disease states, or aims at assessment of the concentration-response relationship and safety margin. This is a commonly encountered situation where plasma protein binding changes and poses a problem, and therefore a brief background on how these issues can be resolved is provided.

If uptake of drug into blood cells is ignored, then a drug in plasma is either circulating unbound \((C_u)\) or bound \((C_b)\) to plasma proteins such as albumin and/or \(\alpha_1\)-acid-glycoprotein. For low molecular weight compounds, the equilibrium between \(C_u\) and \(C_b\) is determined by the concentration \(C_{ub}\), the concentration of the binding protein, the number of binding sites per
protein molecule and the affinity between drug and protein. $C_u$ is governed by the (oral) dosing rate and unbound clearance $CL_u$ (Equation 2).

$$C_u = \frac{Dose \ rate}{CL_u} \quad (Eq. 2)$$

The total plasma concentration ($C$) is determined by the ratio of unbound concentration-to-free fraction, and will therefore be affected by changes in binding protein or the affinity between the drug molecule and plasma protein for most small molecules.

$$C = \frac{C_u}{f_u} \quad (Eq. 3)$$

where $f_u$ is the proportionality factor between $C_u$ and $C$. The total plasma concentration $C$ is a consequence of $C_u$ and $f_u$. Consequently, a change in $f_u$, plasma protein concentration or affinity between drug and protein affects $C$.

The free fraction $f_u$ is generally constant at pharmacological concentrations, but when $C_u$ increases, $f_u$ also increases since the unbound concentration approaches the concentration of binding sites on the plasma protein. At low unbound concentrations, binding is constant and $f_u$ is independent of $C_u$. However at higher unbound concentrations, $f_u$ increases as more binding sites are occupied, while at high unbound concentrations, the binding proteins become saturated, $f_u$ approaches unity and $C$ and $C_u$ concentrations approach each other. Knowledge of plasma drug protein binding becomes vital when concentration-response studies involving drug concentrations outside the region of constant binding are undertaken.

Depending on the binding plasma protein, this nonlinearity starts to occur at different
plasma concentrations and at concentrations as low as 10% of the molar concentration of the binding protein. This is seldom an issue with albumin which occurs in plasma at about 500-700 µM. However, for α1-acid glycoprotein, a protein with a low and highly variable plasma concentration (9-22 µM in man), the risk for nonlinear binding begins in the 1-3 µM concentration range. Therefore it has been found that with any drug that binds extensively to this protein any change in the free fraction will markedly affect the slope of the total concentration-response relationship.

*Species-dependent exposure-effect relationships.* For pharmacologists primarily interested in the acute pharmacodynamics of a compound one principal problem can be the species variability of plasma protein concentration (see Gabrielsson et al., 2009). Data with NXY-059 illustrates this. Measurement of C and \( f_u \) in mice (5-10%), rats (30-35%) and marmosets (70% - a similar value to humans), allowed calculations of dosing required to achieve similar unbound concentrations. Consequently, dosing to produce a similar \( C_u \) was lower in marmosets (Marshall *et al.*, 2003) than rats (Sydserff *et al.*, 2002) due to differences in their unbound clearances. The results also allowed plots of neuroprotective efficacy versus plasma unbound drug concentration in stroke models (see Sydserff *et al.*, 2002) and assisted in selecting the human dose (Lees *et al.*, 2003; Green, 2008).

Confounding factors affecting plasma protein binding include species and strain differences, disease, concomitant use of other drugs, anesthetics, and surgical stress. Consequently *ex vivo* measurement from individual animals is better than using pooled samples or commercially available kits. If this is not possible then *in vitro* studies using plasma obtained from the relevant species should be used to confirm the general correctness of the dosing protocol.
Metabolism and active metabolites

Metabolism of compound under investigation

In addition to concerns about the ‘active’ concentration of a compound it is also important to be knowledgeable about its metabolic fate since it may have one (or more) pharmacologically active metabolites. Such activity may be similar to the parent drug, or sometimes an action at another ‘site’. If evidence has been gained for an active metabolite and its half-life, it is poor science to ignore such data when planning and interpreting new studies. For example, MDMA has, in rats, a major metabolite 3,4 methylenedioxyamphetamine (MDA) with similar acute pharmacological and neurochemical properties (Green et al., 2003). Approximately 2 h after MDMA administration plasma MDA concentrations are higher than the parent drug (Goni-Allo et al., 2008), so any sustained acute effect of MDMA may be due to MDA. Since the rate of formation of MDA and other metabolites is influenced by the ambient temperature of the animal housing (Goni-Allo et al., 2008), this knowledge may assist in better understanding MDMA-induced neurotoxicity. MDMA is not neurotoxic when injected into the brain (Esteban et al., 2001) so neurotoxicity probably results from the action of a peripherally formed metabolites. Since the severity of MDMA-induced neurotoxicity is exacerbated by high ambient temperature it is probable that this phenomenon results, in part, from alterations in the rate of formation of neurotoxic metabolites (Goni-Allo et al., 2008).

While MDMA induces serotonin neurotoxicity in most species (Green et al., 2003) it induces dopamine neurotoxicity in mice (O’Shea et al., 2001). The metabolic fate of MDMA differs in mice and rats (Easton and Marsden, 2006), primarily because of different metabolizing enzymes (de la Torre and Farré, 2004) which presumably accounts for the different neurotoxicity profile (de la Torre et al., 2009). This problem of a drug being metabolized in different ways by different species also occurs with methamphetamine which rats metabolize by ring hydroxylation, but monkeys and humans metabolize primarily by
side-chain deamination (Caldwell, 1976). Non-human primates are therefore likely to be more reflective of human neurotoxicity mechanisms. These examples emphasise that knowledge of metabolism of a drug in one species does not necessarily allow translation to another.

Studies on MDMA metabolism revealed another problem in translating data from rats to humans. In rats there is a linear relationship between dose and plasma concentration of the drug (Green et al., 2009) but in humans there is an increased gradient in the slope with a four-fold increase in plasma concentration with only a two-fold increase in dose from 1 mg/kg to 2 mg/kg because MDMA inhibits CYP2D6, one of its major metabolizing enzymes (Tucker et al., 1994), and this occurs within 1 h (Yang et al., 2006). Assuming that it is exposure to MDMA that produces the acute adverse event of hyperthermia, but metabolite formation that induces neurotoxicity then auto-inhibition could have significant consequences. Binge dosing is sometimes used by recreational users to try and lessen acute adverse events. However, with hindsight, we suggest that the preclinical studies mimicking binge dosing (e.g Green et al., 2004) may be flawed in terms of modelling both the acute and long-term consequences in humans since binge dosing will both enhance the acute effects and slow the formation of toxic metabolites in humans, but not rats.

Some drugs have one or more active metabolites and the active metabolite may be part of a therapeutic strategy by the use of a prodrug. L-DOPA and omeprazole are both examples of this approach. L-DOPA, but not its active metabolite dopamine, readily penetrates the blood-brain-barrier and is then decarboxylated to form dopamine in the brain. The H⁺, K⁺-ATPase inhibitor omeprazole is trapped and bioactivated in the parietal cells of the gastric mucosa, resulting in a very selective antacid action (see Andersson and Weidolf, 2008). The opiate addict uses the prodrug principle (and incidentally other pharmacokinetic principles) when injecting heroin. Oral administration of heroin results in the compound being extensively
metabolized to morphine (during the first-pass through the liver) which has relatively poor brain penetration. By injecting heroin intravenously the first-pass metabolism is avoided, and the high lipophilicity of heroin ensures its rapid entry to the brain where it is then hydrolyzed to morphine and another active metabolite 6-acetylmorphine (see Reisine and Pasternak, 1996). Measurement of the active metabolite or metabolites in the target organ therefore provides additional and possibly more meaningful concentration-response information.

Another example is the muscarinic acetylcholine receptor antagonist tolterodine, developed for treatment of urinary incontinence. The hepatic enzyme CYP2D6, for which there is genetic polymorphism, is an important metabolic pathway for this compound resulting in its active equipotent metabolite 5-OH-tolterodine. In poor metabolizers, bioavailability of tolterodine is large and there are low or negligible amounts of 5-OH-tolterodine, whereas in rapid metabolizers plasma levels of 5-OH-tolterodine are high and levels of the parent compound correspondingly low. Clinical efficacy is maintained under both conditions (Brynne et al., 1998).

More often, the active metabolite is an unwanted aspect of drug treatment since the metabolite can cause unexpected effects or drug interactions in at least two ways. Firstly, an active metabolite may display a similar pharmacodynamic profile as the parent compound, but display a different pharmacokinetic behavior. Current examples of this are the metabolites norfluoxetine and 6-hydroxy-risperidone the respective metabolites of the antidepressant fluoxetine and the antipsychotic risperidone (Preskorn, 1993; van Beijsterveldt et al., 1994; Borison et al., 1994). In both cases the active metabolite has a longer half-life in plasma and thereby produces unexpected interactions, due to genetic polymorphism in hepatic cytochrome P450 enzyme activity. Secondly, the active metabolite may have a different pharmacological profile from the parent compound. The 5-HT\textsubscript{1A} receptor agonist buspirone has a major metabolite, 1-(2-pyrimidinyl)-piperazine, with prominent $\alpha_2$-
adrenoceptor blocking properties (Blier, 1991), so the pharmacodynamic profile of the metabolite is an important factor for the mechanism of action of this anxiolytic agent. Furthermore, potential drug interactions will be different for the parent compound and metabolite.

Figure 2 illustrates procedures used to detect the pharmacodynamic effects of a possible metabolite of the 5-HT$_{1A}$ receptor agonist ebalzotan (Hammarberg et al., 2000). It was assumed that the metabolite possessed the same pharmacodynamic profile as its parent compound, albeit with possible differences in potency and/or efficacy. By examining suppression of rearing behavior which results from 5-HT$_{1A}$ receptor activation as the functional index (Hillegaart, 1991), together with plasma measurements of ebalzotan and its major metabolite and utilizing subcutaneous and oral routes of ebalzotan administration to minimize and optimize, respectively, possible hepatic bioactivation of ebalzotan the metabolite was shown to be more potent than the parent compound.

Metabolism of a ‘challenge’ drug

By ‘challenge’ drug we mean a compound used to investigate the action of the primary compound under investigation. A challenge drug could therefore be an agonist, antagonist or one known to modulate the action of the primary compound in some way. Knowledge of the metabolic fate of the challenge drug can assist in interpreting results.

Research on MDMA illustrates this point. Fluoxetine prevents MDMA-induced neurotoxicity in rats when given 4 days prior to MDMA (Sanchez et al., 2001). There was a good correlation between neuroprotection and the brain concentration of fluoxetine plus norfluoxetine, the active major metabolite of fluoxetine (Sanchez et al. 2001). Such a correlation would not have been found if only fluoxetine had been measured. These combined pharmacological and pharmacokinetic data thus allowed confidence in concluding
that fluoxetine had a sustained neuroprotective effect because it, and its major metabolite, inhibited the serotonin uptake site and prevented the entry of MDMA or a neurotoxic metabolite and not because it either inhibited CYP2D6 or down regulated the serotonin transporter.

**Dosing schedules, acute and sub-acute**

Beuters *et al.* (2009) recently emphasised the importance of correct timing of the dose when investigating any animal model of a disease. Most studies examining putative neuroprotective drugs administered the compound 10 min (range -60 to 360 min) after initiation of an experimental stroke (Van der Worp *et al.* 2005). Since stroke patients may take 3-6 h to present clinically this fact demands that compounds are examined in stroke models using the same time frame (Bath *et al.* 2009). No assumption can be made that damage occurs more rapidly in rats than humans since studies have shown that for a thrombolytic drug the ‘window of opportunity’ (the time after the onset of ischemia in which a compound is effective) for neuroprotection is the same in both rats and patients with a stroke (see Green 2008). Similar considerations are considered to apply in other studies using animal models of a disease (Bueters *et al.*, 2009).

With acute dosing, the main requirement is to achieve plasma concentrations with translational validity to the clinic. Sub-acute, or what is sometimes called chronic dosing (although in most preclinical studies treatment only lasts a few days) requires more. Just giving (for example) two doses daily is not satisfactory, and administering the drug in drinking water or food and calculating dose from water or food consumption can be misleading as it may not produce a steady-state concentration due to a diurnal drinking/feeding pattern (Yuan, 1993) or poor oral bioavailability (Gabrielsson and Weiner, 2006). There can also be the problem of confirming that every animal is dosed adequately
and similarly if they are group-housed. If the drug is marketed, pharmacokinetic data in both rodents and humans will likely have been published, allowing simple calculations on the doses required in animals to produce steady-state plasma levels that are reflective of clinical administration. If the drug is in development then the investigator may be able to obtain information from the pharmaceutical company. Steady-state plasma concentrations can be produced by intravenous infusion but this can be problematical; an in-dwelling line can get kinked or pulled out. An alternative is to use osmotic mini-pumps. However pump implantation requires a brief surgical operation under anesthesia. If extended dosing requires removal of empty pumps and insertion of new pumps this both complicates the study and may have implications for the legally acceptable care of the animals.

**Target engagement**

A topic commonly arising in experimental pharmacology is target engagement, that is, whether the compound exhibits a pharmacological effect or not. Target engagement is a conglomerate of the compound dose size, the systemic exposure to the compound (pharmacokinetics), interaction with the target (affinity and efficacy, pharmacology) and the physiological (system) reaction to the target-drug interaction.

Figure 3 illustrates schematically how different doses (upper left), plasma clearances (upper right), potency values (lower left) and changes in the physiological system parameters (lower right) affects the time course of a biomarker. In light of these profiles it is unsurprising that even compounds with a strong potential of giving a clear response may lack the possibility of showing a response due to sub-optimal design. The experimentalist relies on measurements at a single time point (often at maximum plasma concentration) or inadequate time points which lacks sufficient information about potency and efficacy. In other situations the dose may be too high and therefore hide the pharmacological effect because of Bell- or U-
shaped concentration-response relationships, which has been reported for chemotherapeutics, immunosuppressants and hormones (Calabrese and Baldwin, 2003).

Target engagement is not only a pharmacological event. A compound may have excellent target binding properties, but fails due to low bioavailability or being cleared rapidly from plasma due to a high systemic clearance. The system may also need time to react to the drug-receptor interaction, so in order to establish a good pharmacological response, the compound needs to be given repeatedly. An example of this is the body weight reduction (fat reduction) effect of CB1-inverse agonists. To observe a clinical effect, these compounds must be given repeatedly, due to the slow turnover of body fat in experimental animals. The half-life of turnover of fat in obese mice fall is in the region of weeks, which suggests that 3 to 4 weeks dosing is required to establish pharmacodynamic steady-state. The pharmacokinetic steady-state is reached much earlier due to a shorter half-life of test compound in plasma. In this specific example, neither dose, nor clearance, nor receptor affinity/efficacy poses a problem. It is the slow system properties that require special attention for establishment of target engagement.

Nicotinic acid (NiAc) has been used clinically for decades as an antilipolytic drug reducing the plasma concentrations of non-esterified free fatty acids (NEFA). In pre-clinical models, NiAc has a very short half-life in plasma, a rapid concentration-NEFA lowering equilibrium, combined with both acute functional adaptation followed by rebound of the NEFA concentrations upon cessation of NiAc dosing (Iwaki et al, 1996, Isaksson et al 2009). In this case it is neither the affinity/efficacy of the NiAc-receptor interaction nor the turnover of NEFA (rapid system) that confounds target engagement, but rather the high doses combined with short half-life of NiAc. Sampling time of both NiAc systemic exposure and NEFA concentrations become crucial.
These examples show that a single point approach (measuring the pharmacology at $C_{max}$) is not as informative as a time-series, if a correct documentation of target engagement is to be done.

Temporal differences between concentration and response

Numerous drugs have been developed using an acute response measurement. However many are then administered to patients requiring long-term treatment. In some notable cases evidence has emerged that the therapeutic mechanism of action is probably not the mechanism used to assist selection in the discovery process (although that mechanism might be responsible for initiating or sustaining the long-term mechanism). Many tricyclic antidepressants, serotonin selective reuptake inhibitors (SSRI) and monoamine oxidase inhibitors were developed on the basis of an acute action on monoamine neurotransmitters. However the time-delay in their antidepressant effect (2-3 weeks) and subsequent biochemical pharmacology studies resulted in neuroscientists suggesting that the drugs produced neuroadaptive effects and that it was these longer-term changes that were crucial to their therapeutic effect (Grahame-Smith, 1997). Studies on neuroadaptation are difficult since longer-term treatment of experimental animals is seldom straightforward and pharmacokinetic modelling and selection of appropriate dosing schedules becomes vital (Gabrielsson et al., 2009) if we are not to get meaningless data that has resulted from animals merely being injected twice daily to hopefully “mimic” human long term administration. For a review of models that capture temporal differences see Gabrielsson and Weiner (2006).

An example of temporal difference between plasma concentration and pharmacological response has recently been in studies on a compound that has an inhibitory action on gamma secretase in the brain. The compound inhibits the synthesis rate of gamma secretase which indirectly reduces the formation of neurofibrillary tangles. The peak plasma concentration
$C_{\text{max}}$ occurred at 0.5 h, whereas the inhibitory activity on gamma secretase was seen at 2 h; a temporal difference of 1.5 h. By only sampling the pharmacological response at 0.5 h after different doses of the compound, little information would have been obtained about the onset of action, maximum intensity and duration of response. The $C_{\text{max}}$ approach would have resulted in biased estimates of potency of test compound.

An interesting example of a proper experimental design to elucidate the synergistic action of a peroxisome proliferator-activated receptor agonist on the turnover of plasma triglycerides in rats is the study of Oakes et al. (2005). A combination of chronic dosing, coupled to measurements of systemic exposure to tesaglitazar, and manipulation of the basic turnover (inhibition) of triglycerides, demonstrated the multiplicative effect that results from simultaneous inhibition of release of triglycerides into plasma and stimulation of removal of triglycerides from plasma into tissues. The authors studied the rise in triglycerides in both tesaglitazar-treated and control animals at steady-state after blocking hepatic clearance of triglycerides with TritonWR1135. Combining turnover data of triglycerides in treated and control animals, with the rate of increase in triglyceride levels after blocking its clearance, the authors could dissect the individual contribution of inhibition of production (50%) and stimulation of loss (83%) and the combined effect (93%).

Again, these examples demonstrate how pharmacodynamic complexities may be tackled by also including the time course of plasma exposure to test compound into the analysis, and thereby avoiding the misconception that maximum information of response occurs at $C_{\text{max}}$.

**Proposals for the experimental pharmacologist**

From the discussion and examples above it can be seen that certain basic studies should be considered mandatory when examining drug action in vivo.
First, make an assessment of drug exposure in the animal, preferably by measurement of plasma drug concentrations at several time points, and after several different doses.

Second, perform a measurement of the degree of plasma protein binding of the drug, *ex vivo* if possible, and if not, then *in vitro*. This is vital if comparisons are to be made of a drug action in more than one species.

Third, obtain knowledge on the metabolism of the drug and determine whether active metabolites could be influencing the results being obtained.

Fourth, ensure that the dose-schedules to be used are relevant to the way the drug will be used in humans to allow translational value to the clinic.

Fifth, consider whether there is a temporal mismatch between the exposure information being gained and the outcome measure being investigated, since this might give insight into the mechanism of action of the compound under investigation.

Sixth, consider the determinants of target engagement whenever the information above has been gathered.

Of course, some of this information may be available in the published literature. It is not necessary for every investigator to collect it first-hand. However if it is available, it becomes obligatory to incorporate the information into the study design.

**Perspectives and Conclusions**

Quantitative pharmacological reasoning focuses on concentration-response and response-time relationships with special emphasis on the impact of drugs on disease (Levy, 1993) and this perspective was written to raise awareness among pharmacologists as to why PKPD integration is essential in their studies.

A common misconception among scientists is that ‘sampling for PK’ is done in the pharmacological experiment. When blood samples are drawn in PD experiments they are
used primarily as exposure samples, i.e., samples for support and guidance of the *in vivo* response. Again, exposure samples will help to assess the onset, intensity and duration of response. However, actual ‘PK samples’ are taken in separate experiments aimed at the characterization of the pharmacokinetic properties of a compound, such as clearance, volume and half-life. ‘PK samples’ serve as a basis of the inter-species extrapolation and/or prediction of an appropriate dosing regimen in animals and man.

To overcome the lack of a quantitative holistic understanding of experimental design issues and interpretation of *in vivo* data, we need to educate today’s experimentalist in PKPD awareness. There is, at present, limited undergraduate education in the area of quantitative pharmacology with few courses in the USA and even fewer in Europe. Courses tend to favor clinical pharmacokinetics and those trained are predominantly recruited by the pharmaceutical industry. We feel that integrative pharmacologists must receive increased basic training in this area, as they do in statistics and experimental design, in order to undertake animal experimentation in the most appropriate way. This, in turn, should ensure that the information obtained from every experiment is useful and relevant to the questions being asked.
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Tesaglitazar, a dual PPARα/γ agonist, ameliorates glucose and lipid intolerance in obese

and fluoxetine on the acute and long term changes induced by MDMA (“ecstasy”) on the


Yang J, Jamei M, Heydari A, Yeo KR, de la Torre R, Farré M, Tucker GT, and Rostami-

Footnotes

Conflict of interest: Johan Gabrielsson is an employee of AstraZeneca and A. Richard Green is a former employee of AstraZeneca. Some of the compounds they are or have previously worked on are discussed in the text. JG uses the pictures in his textbook and teaching.

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**Legends to figures**

**Fig. 1.** Schematic illustration of the concentration-response curve (a, upper left), dose-response curve (b, upper right) and dose-concentration plot (c, bottom). The dose-concentration relationship demonstrates nonlinear pharmacokinetics in that doubling of the dose leads to a more than proportional increase in plasma concentration.

**Fig. 2.** Left: concentration of ebalzotan versus rearing after subcutaneous (sc) and oral (po) dosing of ebalzotan to rats. Note the apparent shift in slopes of the two datasets. Right: Metabolite plasma concentration versus response (rearing) after subcutaneous (sc) and oral (po) dosing of ebalzotan to rats. Note how the two datasets superimpose nicely. In this case the metabolite has a higher potency than the parent compound.

**Fig. 3.** Target engagement including for different simulation of changes in *dose, plasma clearance, potency* and *turnover time*, respectively, everything else kept constant: **Upper left:** Three simulations of different doses given to demonstrate the behaviour of an indirect response system everything else kept constant. **Upper right:** Three simulations with changing values of clearance of a drug exhibiting an indirect response. **Lower left:** Three simulations of an indirect response system with different values of the drug potency. **Lower right:** Three simulations of the impact of changing the physiological system properties (turnover time).
Figure 1
Figure 2