Research Article

Evaluation of an Innovative Population Pharmacokinetic-Based Design for Behavioral Pharmacodynamic Endpoints

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Abstract. Pre-clinical behavioral pharmacology studies supporting indications like analgesia typically consist of at least three different studies; dose-finding, duration of effect, and tolerance-development studies. Pharmacokinetic (PK) plasma samples are generally taken from a parallel group of animals to avoid disruption of the behavioral pharmacodynamic (PD) endpoint. Our objective was to investigate if pre-clinical behavioral pharmacology studies in rats could be performed effectively by combining three studies into a single experimental design and using sparse PK sampling in the same animals as for PD. A refined dosing strategy was applied for a muscarinic agonist, AZD6088, using the rat spinal nerve ligation heat hyperalgesia model. PD measurements were performed on day 1, 3, 5 and 8. Two PK samples per day were taken day 2 and 4. In a separate control group, PD measurements were performed on rats without PK sampling. Data was analyzed using a population approach in NONMEM. The animals produced a consistent and reproducible response irrespective of day of testing suggesting that blood sampling on alternate days did not interfere with the PD responses. A direct concentration–effect relationship with good precision was established and no tolerance development was observed. The new design combining three studies into one and eliminating a satellite PK group realized substantial savings compared to the old design; animal use was reduced by 58% and time required to generate results was reduced by 55%. The design described here delivers substantial savings in animal lives, time, and money whilst still delivering a good quality and precise description of the PKPD relationship.

KEY WORDS: 3Rs; analgesia; muscarinic; PKPD.

INTRODUCTION

Applying pharmacokinetic–pharmacodynamic (PKPD) modeling principles pre-clinically is essential in understanding how the systemic exposure of a drug relates to the magnitude and time profile of the PD response in an animal model. The ultimate objective is to predict magnitude and duration of effect in man, and to guide the design of clinical studies.

In pre-clinical pharmacology studies in neuroscience, for example those directed towards the development of novel analgesics, at least three different types of studies are carried out; efficacy (dose finding), effect-duration, and tolerance development. The PD endpoints are often a measurement of a behavioral response, and these behavioral responses can be disrupted by the blood sampling required for PK measurements (1–5). Consequently, in such studies separate sets of animals are normally used to collect PK and PD data. Traditionally, PKPD modeling is applied to these existing data sets. However, the

precision in describing the PKPD relationships is reduced as the between individual variability in PK is not taken into account. Therefore, the inter-individual variability in PK will be represented by increased variability in the PD measures, resulting in increased uncertainty (lower precision) in the overall PD response. The effective concentration in animals is often used when predicting dose to man. In cases where PKPD relationships must be accurately established, for example, drugs with potentially narrow safety margins, emphasis on the precision is critical and often implies further exhaustive testing in animals.

The use of animals in research, teaching, or testing is a privilege that is extended when justifiable need is established. It is the responsibility of all animal users to comply with appropriate ethical guidelines and apply the principle of reduction, replacement, and refinement (3Rs) set forth by Russell and Burch (6). With this in mind, we aimed to develop an innovative PKPD study design which would meet the objectives of refining and reducing animal use while maximizing data quality and output. As a test case for development of a novel design, we chose to characterize the analgesic potential (efficacy, effect-duration, and tolerance development) of AZD6088 in the rat Spared Nerve Ligation (SNL) model of neuropathic pain. We measured the increase in sensitivity of the affected hindpaw to a heat stimulus as an index of neuropathic pain. AZD6088 (Fig. 1) is a muscarinic subtype 1 and 4 (M1/M4)-selective agonist. Muscarinic receptors are important in a number of physiological roles including cognitive, behavioral,

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Fig. 1. Chemical structure of AZD6088

sensory, motor, and autonomic processes. A substantial body of literature shows M1, M2, and M4 are involved in pain and analgesia (7–20) and a recent study by Sullivan *et al.* (16) demonstrates that an M1/M4-preferring agonist is effective in a number of rodent chronic pain models. In initial efficacy studies, we established that AZD6088 had a dose dependent effect in reducing the heat hyperalgesia with an EC₅₀ of 46.6 nM, making AZD6088 an ideal candidate for developing a novel design.

Firstly, based on our previous experience with other compounds (unpublished data), we proposed a time schedule whereby behavioral PD measures and the blood sampling for PK could be performed in the same animal without compromising the reliability of either the PD or the PK measures. To confirm the suitability of this schedule, we performed a pilot study. Having established a suitable time schedule, we then developed and applied a novel design that achieved the three different objectives of establishing the effective dose, the effect-duration and the potential to develop tolerance within a single experiment, using a much reduced number of animals compared to the traditional methods.

MATERIALS AND METHOD

Animals

Experiments were conducted in male Sprague–Dawley rats (125–200 g, Charles River, St. Constant, Canada and Harlan Inc., Indianapolis, USA). Rats were housed in groups of six in a temperature controlled environment (22±1.5°C, 30–80% relative humidity, 12-h light/dark) and were acclimatized in the animal facility for at least 3 days prior to use. This study was conducted under a protocol approved by the AstraZeneca Animal Care Committee. The animals were kept and experiments were performed at AstraZeneca R&D Montreal, which has accreditation from CCAC (Canadian Council on Animal Care) and AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care) is approved by the AstraZeneca Global Veterinary Council for study conduct. Experiments were performed during the light phase of the cycle. Food (Harlan Teklad, Montreal, Canada) and water was provided *ad libitum*.

Induction and Assessment of rat Spinal Nerve Ligation Model of Neuropathic Pain

As previously described in detail by Kim and Chung (21), under isoflurane anesthesia, an incision was made dorsal to the

lumbosacral plexus. The paraspinal muscles (left side) were separated from the spinous processes, the L5 and L6 spinal nerves isolated, and tightly ligated with (4-0 silk suture) distal to the dorsal root ganglion. The incision was closed in layers, and the skin was sealed with tissue adhesive (Vetbond). Rats were allowed to recover and then placed in cages with soft bedding. For the main study, a control group of rats (referred to as “naïve” animals) were randomly selected from the surgical cohort. These animals were not subjected to SNL surgery but otherwise were handled and housed in an identical manner to the SNL rats. The naïve rats served to establish the normal, non-pathological behavioral response, and thus set the target level for a 100% effect, or a complete return to normal.

In order to assess the degree of heat hyperalgesia, the rats were placed individually in Plexiglas boxes on the glass surface (maintained at 30°C) of the paw thermal stimulator system (IITC Life Science, Woodland Hills, USA, Model 390 Series 8), and allowed to acclimate for 30 min. A thermal stimulus, in the form of a radiant heat beam, was focused onto the plantar surface of the affected paw and the time to withdrawal of the paw was measured. An assay cut off was set at 20 s to avoid thermal injury. In each test session, rats were tested twice at approximately 5 min apart. A decrease in Paw Withdrawal Latency relative to naïve animals indicates a hyperalgesic state. Naïve animals responded with a mean latency of 10.50±0.5 s, while SNL animals responded with a mean of 5.87±0.2 s. SNL rats with thermal hyperalgesia, defined as a baseline paw withdrawal latency of 8 s or lower, were selected for study. Selected rats were randomly assigned to a treatment group. The anti-hyperalgesia effect of AZD6088 in rat SNL model was tested between days 20 and 27 after surgery.

Drug Treatment and Design

Initial Efficacy Study

This first experiment was designed to establish efficacy and consisted of a naïve group and six groups of SNL rats ($n=7$ –12/group) for behavioral testing. One SNL group was treated orally with vehicle (saline) and the remaining five groups with doses of AZD6088 (MW, 406.57) between 1 and 40 µmol/kg and behavioral testing performed at 1 h post dose. In parallel, five groups of satellite rats ($N=3$ /group) received doses of AZD6088 between 1 and 40 µmol/kg and blood samples were taken 1 h post dose.

Pilot Study

This study was designed to establish whether behavioral PD measures and the blood sampling for PK could be performed in the same animal without compromising the reliability of either the PD or the PK measures. The approximate EC₅₀ derived from the initial study was used for the pilot study. In this pilot study, two groups of SNL animals ($n=9$ /group) were treated with 2.5 µmol/kg of AZD6088 or vehicle (saline) once daily for 8 days. Behavioral testing was performed 1, 4, 7, and 24 h post dose on days 1, 3, 5, and 8. To minimize the potential effect PK sampling could have on PD measurements, blood samples for PK were withdrawn on alternate days to the PD measurements; thus, these same rats were sampled for blood at 2 and 6 h after administration days 2 and 4.

Main Study

The main study consisted of nine groups of animals, as follows: one group of naïve rats served as a baseline control for behavior ($n=6$). Another two groups of SNL rats served as vehicle controls ($n=6$ /group) while six further SNL groups were treated with drug. Three of these drug-treated groups ($n=6$ /group) were included in the behavioral testing, and the three remaining groups ($n=3$ /group) served as satellite rats to control for PK sampling; blood samples withdrawn but they were not subjected to any behavioral testing. The results from the initial efficacy study and the pilot study were used to decide dosing levels for the main study.

To be able to investigate potential tolerance development, full coverage of receptor occupancy is needed throughout the dosing period in at least one dose group. Therefore, the compound was administered twice daily. AZD6088 or vehicle was administered orally (8:00 AM and 4:00 PM) for 8 days and tested on days 1, 3, 5, and 8 for heat hyperalgesia 1, 2, 4, 6, 7, and 24 h after the first administration (Fig. 2). As established in the pilot study, rats were sampled for blood on alternate days to PD testing, that is at 2, 4, 6, and 7 h after administration on days 2 and 4. All behavioral testing and blood sampling was performed by experimenters blind to the drug treatment the animals received.

Collection of Plasma and Brain Tissue, and Determination of Drug Levels

Blood was collected by jugular vein puncture (under brief anesthesia) at the appropriate time points. Whole blood was transferred to heparinized tubes and centrifuged at $3,000\times g$ for 5 min. Plasma supernatant was then collected and frozen at -80°C . The determination of the total plasma concentration was performed by protein precipitation, followed by reversed-phase liquid chromatography and electrospray mass spectrometry.

Data Analysis

Data was analyzed using non-linear mixed effects modeling in NONMEM VII (Icon Development Solutions). PK samples

below lower limit of quantification ($0.0005\text{ }\mu\text{mol/L}$) were not included in the analysis. Model performance was assessed by evaluation of diagnostic plots, the objection function value, and the precision of the parameter estimates. To discriminate between nested models, the difference in the objective function value (OFV) was used. The OFV is approximately proportional to $-2\log$ likelihood. The criterion for inclusion of a parameter was a decrease in the objective function value of 6.63 ($p<0.01$) (22). Graphical evaluation was performed using the program Xpose version 4.3.3 (23).

Firstly, the pharmacokinetic model was developed. Thereafter, the PKPD model was developed where all PK and PD parameters were estimated simultaneously. Emax models with and without time delay was assessed, as well as models including tolerance development. Adding a sigmoidicity factor to the effect model as well as proportional or additive error models were also tested. Exponential models were used to describe inter-individual variability.

RESULTS

As expected, induction of the rat SNL model by tight ligation of spinal nerve L5 and L6 resulted in stable heat hyperalgesia. In the initial efficacy study, we established that AZD6088 was effective in reducing heat hyperalgesia in SNL rats with an EC_{50} of 46.6 nM (95% confidence interval of -14 to 107 nM). In the pilot study, we observed an analgesic effect of AZD6088 that was overall similar as that seen in the initial efficacy study. There was no significant change in the behavioral responses of the vehicle treated animals tested on days 3, 5, and 8 compared to the response on day 1, despite the blood samples taken on days 2 and 4. Likewise, the PD response to AZD6088 was consistent in magnitude and time course between day 1 and the subsequent days of testing.

The pilot study therefore established that the behavioral testing and blood sampling regime, with samples being taken on alternate days to the PD testing, was practicable and allowed blood samples to be taken from the same animals during an 8 day study without compromising the PD results. We therefore proceeded with the main study, designed to address three questions within a single study, namely (1) to

		Day 1								Day 3								Day 5								Day 8				
Treatment	n	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h	Drug	3 h	5 h	7 h	
4 μmol/kg	6	08:00	x		x	x		04:00	x	08:00		x	x		x	04:00	x	08:00	x		x	x		04:00	x	08:00	x	x	x/o	
10 μmol/kg	6	08:00		x	x		x	04:00	x	08:00	x		x	x		04:00	x	08:00		x	x		x	04:00	x	08:00	x	x	x/o	
70 μmol/kg	6	08:00	x		x	x		04:00	x	08:00		x	x		x	04:00	x	08:00	x		x	x		04:00	x	08:00	x	x	x/o	
Vehicle	6	08:00		x	x		x	04:00	x	08:00	x		x	x		04:00	x	08:00		x	x		x	04:00	x	08:00	x	x	x/o	
Vehicle	6	08:00	x		x	x		04:00	x	08:00		x	x		x	04:00	x	08:00	x		x	x		04:00	x	08:00	x	x	x/o	

		Day 2								Day 4							
Treatment	n	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h
4 μmol/kg	6	08:00		o		o		04:00		08:00			o		o	04:00	
10 μmol/kg	6	08:00			o		o	04:00		08:00		o		o		04:00	
70 μmol/kg	6	08:00		o			o	04:00		08:00			o	o		04:00	

		Day 1							
Satellite Treatment	n	Drug	1 h	2 h	4 h	6 h	7 h	Drug	24 h
4 μmol/kg	3	08:00	o	o	o	o	o	04:00	o
10 μmol/kg	3	08:00	o	o	o	o	o	04:00	o
70 μmol/kg	3	08:00	o	o	o	o	o	04:00	o

x = PD testing
o = PK sampling

Fig. 2. PKPD study design. Repeated, twice-daily oral administration of AZD6088. Rats were tested 1, 2, 4, 6, 7, and 24 h after drug administration on days 1, 3, 5, and 8. The same rats were sampled for plasma at 2, 4, 6, and 7 h after administration on days 2 and 4. Satellite animals were sampled at 1, 2, 4, 6, 7, and 24 h after drug administration on day 1

Fig. 3. Observed withdrawal latency vs. time after last dose conditioned on dose group

Fig. 4. Goodness-of-fit plots for final model. *Upper-left* observed concentration vs. population prediction. *Upper-mid* observed concentration vs. individual prediction. *Lower-left* observed withdrawal latency vs. population prediction. *Lower-mid* observed withdrawal latency vs. individual prediction. *Upper-right* is conditional weighted residuals for PK vs. time after dose. *Lower-right* conditional weighted residuals for PD vs. time after dose

Fig. 5. Observed withdrawal latency vs. individual predicted withdrawal latency by day

derive the effective concentration or EC₅₀ with high precision; (2) to investigate if there was a time delay between AZD6088 exposure and effect, and (3) to determine whether there was any development of tolerance (i.e., did the effect decrease if the receptor was fully occupied in a sustained manner as is seen for example with opiates).

As shown in Fig. 3, in the main study, over 900 data points were generated from a single experiment performed over 8 days. Five PK samples were below the lower limit of quantification. As observed in the previous studies, AZD6088 produced a time- and dose-related reversal of heat hyperalgesia in the rat SNL model. With respect to building a PK model for AZD6088, the concentration of AZD6088 over time was adequately described using a one-compartment model with inter-individual variability in clearance and volume of distribution. The highest dose group deviated in the plots. This might be due to higher bioavailability or saturation of elimination at the highest dose and after estimating different clearance values for this group OFV decreased 32 units and goodness-of-fit plots improved. Lack of plasma concentration data in the absorption phase made it impossible to estimate rate of absorption and k_a was therefore fixed to 5 times clearance. Changing the k_a to different values did not have an effect on OFV or other PK parameter estimates. The PK of satellite animals was tested separately as a covariate and it was concluded that the PK did not differ between satellite animals and animals with PD measurement. Goodness-of-fit plots for PK are shown in Fig. 4.

The pharmacodynamic response was adequately described using a direct effect with an E_{max} model. Goodness-of-fit plots for PD are shown in Fig. 4. Adding a time delay between plasma concentration and effect or adding a sigmoidicity factor did not

improve the model fit. Adding tolerance in the model (changing EC₅₀ or E_{max} over time) did not improve the model fit and goodness-of-fit plots conditioned on day were similar (Fig. 5). Further, no trends in the conditional weighted residuals could be seen. Parameter estimates with 95% confidence intervals based on bootstrap are shown in Table I. The estimated EC₅₀ of AZD6088 in the SNL model is 43.3 nM with a 95% confidence interval of 10 to 100 nM, an improvement in comparison to the original estimation prior to this study of 46.6 nM with a 95% confidence interval of -14 to 107 nM. Effect (withdrawal latency) *versus* concentration including prediction line is shown in Fig. 6. Thus, we achieved our three objectives of describing the effective concentration of EC₅₀ with high precision, establishing the duration of effect and testing whether AZD6088 at a high sustained exposure induced tolerance to the analgesic effect (the highest dose level maintained >EC₈₀ throughout the 8-day experiment).

We evaluated the difference in the number of animals required and the time spent by experimenters comparing our new study design and the traditional working model for a promising compound which involves three to four separate studies and separate groups of animals for PD testing and PK sampling. We found that overall the new design took 55% less time and used 58% fewer animals to deliver a similar data set (Table II).

DISCUSSION

In the present study, we found that behavioral analgesia testing and blood sampling can be performed on the same animals without compromising either the PD or PK results if

Table I. PK and PD Parameter Estimates

Parameter	Estimate	95% Confidence interval
Oral clearance (L/h/kg)	10.87	(9.06–13.11)
Oral clearance high dose (L/h/kg)	3.92	(2.86–6.02)
Oral volume (L/kg)	35.3	(28.5–43.8)
Proportional error	0.325	(0.270–0.375)
Baseline latency (s)	5.947	(5.87–6.01)
EC ₅₀ (μmol/L)	0.0433	(0.01–0.10)
E _{max} (s)	10.09	(9.44–11.4)
Additive error (s)	1.027	(0.93–1.12)

Fig. 6. Withdrawal latency vs. plasma concentration relationship. Observed withdrawal latency vs. predicted plasma concentration (*circles*) and modeled predicted withdrawal latency vs. plasma concentration (*solid line*)

blood samples were taken on alternate days to the PD testing. We used this testing and sampling regime to develop a novel experimental design that allowed us to determine the effective concentration with high precision, to establish the duration of effect and to test whether high sustained exposure induced tolerance to the PD effect in a single combined experiment. Here, we have described how we performed a pilot study before the main study in order to ensure that the sampling regime was suitable for our needs. However, once this was established for the specific animal model and behavioral endpoint in question,

we found that this design could easily be adapted to the study of other compounds with no need for a pilot study (unpublished observations). The only prior knowledge required is a simple PK description and a preliminary efficacy estimate derived from a single PD study or *in vitro* receptor occupancy results, as are commonly employed in drug discovery cascades.

Traditionally, a minimum of three to four animal PD studies are performed to progress a promising compound from the discovery phase through into development. Typically, in these studies different animals are used to collect PK and PD data, as a

Table II. Potential Benefits of Proposed Design

Study type	Typical # of animals ^a	Estimated time (h) ^b
Previous working model		
Dose-finding (efficacy)	98 (2 studies)	67
Effect-duration (time course)	38	37
Tolerance development	27	89
Additional study ^c	24	94
Total	187	287
New working model		
Dose-finding (efficacy)	49	34
Proposed new design	30	94
Total	79	128
Savings	108 (58%)	159 (55%)

^a includes animals for PK ($n=3/\text{group}$) and PD testing ($n=6-8/\text{group}$)

^b includes time for preparation, actual experiment, analysis, report

^c if necessary

result linking the systemic exposure of the compound to the magnitude and time profile of its PD response becomes limited and somewhat inaccurate as group means rather than individual values are used. Additionally, this type of data collection requires lots of resources and a large number of animals. In contrast, our new proposed design where PK and PD data are collected from the same animal but on alternate days, delivers good quality data, PKPD relationships and ultimately predictions to man. Moreover, if frontloaded earlier within a discovery phase, one can simultaneously evaluate the additional critical factors of effect-duration and tolerance development and therefore potentially save time and more importantly animal lives, as well as improve the quality of compounds being progressed into later stages.

It seems reasonable to assume that laboratory rats kept in uniform conditions have less inter-individual variability in pharmacokinetics compared with humans. However, as seen in the present study, clearance values do differ between animals, to a greater extent than the day-to-day variability in each individual rat. When using a separate group of animals for pharmacokinetic assessment, the variability due to exposure is unknown. By including this factor, the different sources of variability can be quantified and the exposure-response relationship better understood in addition to an improved precision in the parameter estimates. Nonetheless, to avoid disruption of the behavioral PD endpoint, we found that blood sampling was best performed on different days than the PD measures, thus the intra-individual day-to-day variability cannot be avoided. Thus, this approach may be less suitable for compounds showing very marked day-to-day variability in for example bioavailability.

The proposed new study design described here is an example of how combining population PK and PKPD analysis can improve the knowledge of the pharmacodynamics of a compound. In this example, three different studies addressing three different questions (effective concentration, duration of action and tolerance) were combined into one design. This reduced the animal number and time required for experimental work, but importantly still delivered good quality data. The PKPD of AZD6088 was described by a one-compartment PK model with an Emax function describing the PD effect. The estimate of primary interest in this study was EC50 since that value will be used for the dose to man predictions. The precision (e.g., confidence interval) of EC50 increased with this study compared to the prior knowledge of the compound. In addition, we were able to establish in the same experiment another important feature of the compound, namely the lack of tolerance or sensitization to the effect despite sustained occupancy of the receptor at levels approaching maximal.

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Conflict of Interest The authors state that there are no conflicts of interest in respect to the work reported in this paper.

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