



The HRN™ Mouse

**A new discovery platform for efficacy
screening, lead selection and lead
optimisation**

***The hepatic cytochrome P450
reductase null mouse model***

The HRN™ Mouse – A New Platform in Efficacy Screening, Lead Selection and Optimisation

CXR Biosciences has developed a unique mouse model to improve efficacy screening and to accelerate lead selection and optimisation. This paradigm will improve:-

- ✚ Demonstration of *in vivo* drug efficacy by eliminating the major confounding factor of hepatic cytochrome P450-mediated metabolism.
- ✚ Evaluation of the role of hepatic cytochrome P450 metabolism in drug bioavailability.
- ✚ Evaluation of the contribution of metabolites to efficacy or toxicity.
- ✚ Study of toxicokinetics, allowing informed decisions on route of administration, dose and dosing frequency for Phase 1 clinical trials.

Background

Drug targets are often identified by a combination of bioinformatics, *in vitro* biochemical studies or genetics. As a result, there is no guarantee that modulating the functions of a target will generate a therapeutic benefit. For this reason, *in vivo* target validation and demonstration of efficacy are playing an increasingly critical role in the drug development path. In addition, deciding which of a large number of compounds to take forward into development is a major challenge. The issue is how best to categorise compounds and backups generated from *in vitro* screening programmes with the greatest efficiency, with maximum speed and with the highest chances of success. This can involve studies using a combination of *in vivo* efficacy and *in vitro* and *in vivo* bioavailability and safety screens.

Some key questions to be addressed:-

- Is the *in vitro* target a therapeutic target *in vivo*?
- Are the compounds selected in screening studies efficacious against the target *in vivo*?
- Which of the compounds have the most favourable bioavailability characteristics?
- Which of the compounds have the most favourable safety properties?
- Are there any potential cytochrome P450 or drug transporter liabilities?

On the basis of these studies a decision can be reached on whether a programme should be modified or abandoned.

Compound selection is a process of attrition, where those with the most favourable characteristics are taken forward. Limitations in such studies are:-

- Difficulties in proving efficacy when bioavailability is low
- The need for multiple tests
- The time required to carry these out
- Limitations in their relevance and interpretation
- Limited amounts of compound to study
- Difficulties in making decisions if efficacy screens do not work.

We have developed a new paradigm to increase the speed and power of these studies by the application of a model where hepatic P450-mediated drug metabolism has been inactivated genetically. This improves the probability that efficacy tests work and also allows a wide range of important information targeted towards efficacy and lead selection to be obtained.

Rationale – The Cytochrome P450 System

The cytochrome P450 enzymes are a major determinant of drug bioavailability. They comprise a multigene family of proteins in animals and man, therefore the deletion of these genes individually is inconceivable. However, in order to function all the cytochrome P450 isozymes receive electrons from electron donor cytochrome P450 reductase. We have developed a model where P450 reductase in the liver (HRNTM) is conditionally deleted and, as a consequence, abrogates cytochrome P450 activity in these tissues (References 1 and 2).

We are proposing the application of this model early in drug discovery to increase the probability of demonstrating *in vivo* efficacy and to gain important insights into the pathways that affect drug bioavailability of drug candidates.

This model will markedly improve drug development decisions by:-

- Improving the probability of validating a target *in vivo*
- Facilitating decisions on whether to continue, modify, or stop a programme
- Facilitating lead selection by increasing knowledge on pathways of metabolism
- Reducing the number of experiments and animal use in lead selection
- Reducing costs.

Specific Applications of the HRNTM Mouse in Drug Discovery

More detailed technical information and experimental data on the HRNTM model is provided in the Appendix.

- Potential drug targets are identified by a wide range of *in vitro*, *in vivo* or genetic studies, often without knowing whether interaction with them will generate a useful pharmacological effect *in vivo*. Demonstration of *in vivo* efficacy is therefore a key early milestone in drug discovery. The ability to demonstrate efficacy can be severely compromised by poor compound bioavailability. This can be due either to the physiochemical properties of the compound or to metabolism by enzymes such as the cytochrome P450-dependent monooxygenase system. The HRNTM mouse model significantly increases the probability of demonstrating *in vivo* efficacy by essentially eliminating the P450 metabolic component. Such studies will also give indications as to whether a compound or compound series should be redesigned or abandoned.
- If *in vivo* proof of principle has been obtained, the HRNTM model can be applied to demonstrate the role of hepatic metabolism and, by default, gastrointestinal absorption on compound bioavailability and allow the selection of possible leads on this basis. Such information can also be used to design out metabolic liabilities. The knowledge that the P450 system is not a factor in compound pharmacokinetics is also very valuable information.
- The HRNTM model can also be applied to establish whether parent molecules and/or their metabolites have pharmacological activity by comparing the pharmacodynamic profile in wild type and HRNTM models. Information on whether metabolites exhibit biological activity is also important for patenting purposes.
- Phase II metabolism often plays a more important role in drug disposition in man than in laboratory animals, Phase II metabolites often not being observed in rodents because of high P450 activity. In this context the HRNTM mouse can therefore provide a more human-like model for drug metabolism than the wild type counterparts.
- Knowledge of the toxicokinetic and pharmacodynamic parameters of a drug candidate can be used to define the optimum mode and route of administration for *in vivo* and clinical studies. By careful consideration of these parameters, the risk of toxicity due

to an inappropriate dosing regimen can be greatly reduced. The use of the HRNTM mouse makes the design of these studies much simpler and provides information that cannot easily be obtained by any other means.

In addition to the application of the HRNTM model in drug discovery, there are several applications in problem solving or as a mechanistic tool in safety or metabolic studies. For example, the model can be used to establish whether toxic effects are due to parent compound or its metabolites.

Due to the flexibility of the gene knock-out system employed, the P450 system can be selectively inactivated in any tissue or cell type such as the brain, kidney, adrenal, skin, etc.

Intellectual Property and Commercial Availability

Patents have been filed on the applications of the HRNTM mouse model to drug discovery and development. CXR Biosciences will enter into non-exclusive collaborations and licensing arrangements with companies wishing to use these models in their internal programmes. CXR's philosophy is to maximise access to these models through two principal routes of collaboration - licensing and our drug development solutions service.

In the licensing approach, in return for an annual license fee we supply a small number of breeding animals to companies (or, if required, to third party breeding companies on the company's behalf) that can be used in internal drug research programmes.

In our drug development solutions programme client companies provide the compounds to be tested. CXR can carry out the entire work programme in its state-of-the-art facility or analytical work can be carried out by the client. This could range from performing only the *in vivo* dosing to providing a full analytical and interpretative service.

References

1. 'Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase'. Henderson, C.J., Otto, D.M., Carrie, D., Magnuson, M.A., McLaren, A.W., Rosewell, I. and Wolf, C.R. **J. Biol. Chem.** (2003) **278**:13480-13486.
2. 'Role of hepatic cytochrome P450s in the pharmacokinetics and toxicity of cyclophosphamide: Studies using the hepatic cytochrome P450 reductase null mouse'. Pass, G.J., Carrie, D., Boylan, M., Lorimore, S., Wright, E., Houston, B., Henderson C.J. and Wolf, C.R. **Cancer Res.** (2005) **65**, 4211-4217.
3. 'Relationship between hepatic phenotype and changes in gene expression in the cytochrome P450 reductase (POR) null mice.' Wang, X.J., Chamberlain, M., Vassieva, O., Henderson, C.J. and Wolf, C.R. **Biochem. J.** (2005) **388**, 857-867.

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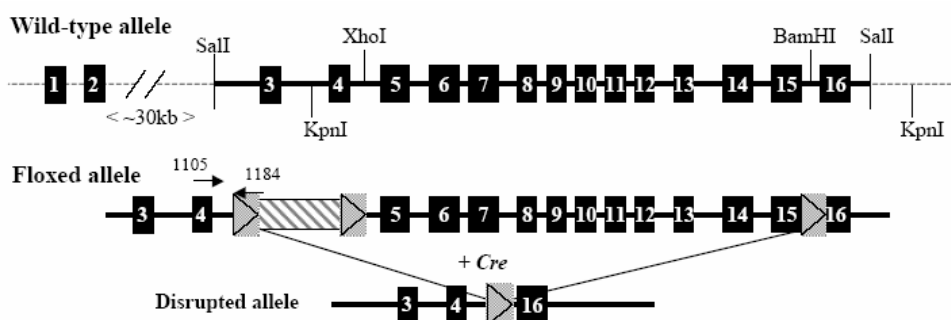
Appendix : Technical Information

The hepatic cytochrome P450 reductase null (HRNTM) mouse line (Patent application WOD4007708)

Design of the HRNTM Mouse Line

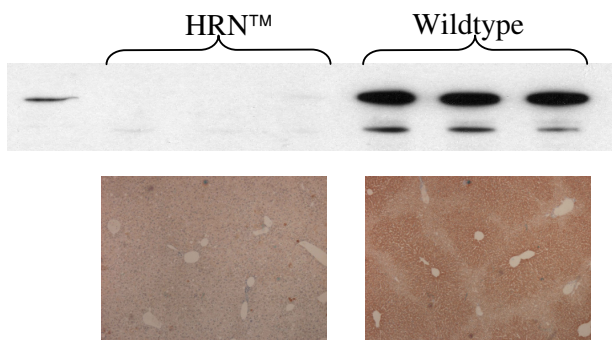
The HRNTM mouse was generated by introducing *LoxP* sites into intron 4 and intron 15 of the cytochrome P450 reductase (POR) gene (Figure 1). By crossing these mice into mice expressing Cre recombinase off the albumin promoter a liver-specific deletion of POR in hepatocytes was obtained after birth (Figure 2).

Figure 1: Modifications to the POR gene to allow P450 reductase deletion.



Maps of the wild-type, floxed and disrupted CPR alleles. The CPR gene is indicated by squares and *LoxP* sites are indicated by triangles. The activity of *Cre recombinase* recombines both *LoxP* sites and so excises the CPR gene between exons 5 and 15 (reproduced from reference 1).

Figure 2. Immunoblot and immunohistochemical analysis showing liver-specific deletion of POR.



POR is almost undetectable by Western blot or immunohistochemistry in the liver of HRNTM mice but is unchanged in other tissues (not shown). (taken from reference 2)

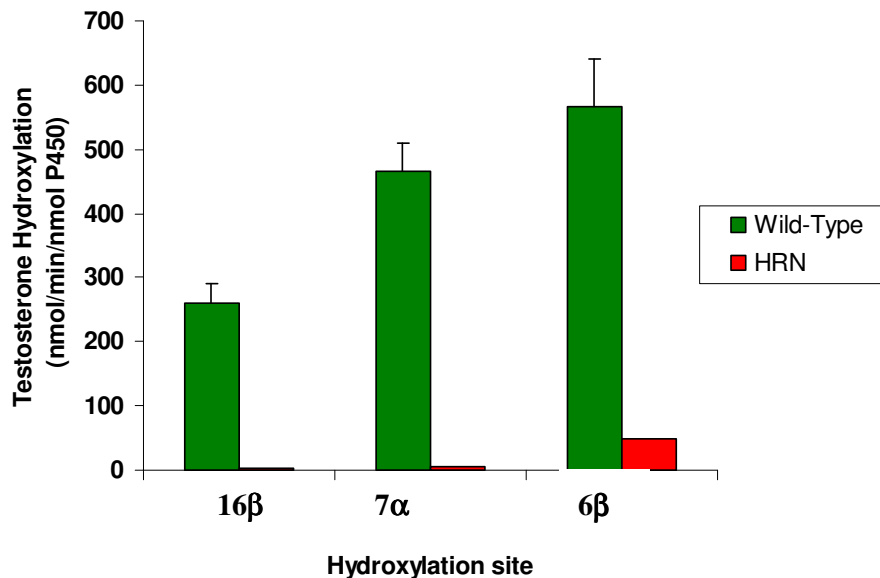
HRNTM mice are overtly normal and breed normally. However, some hepatic phenotypes such as enlarged liver and reduced bile acid production are observed.

In summary, the HRNTM mouse has a permanent knock-out of the POR gene which is limited to the liver alone. The HRNTM displays the phenotype of absence of P450-mediated hepatic drug metabolism constantly throughout adulthood.

In vitro validation of the HRN™ model

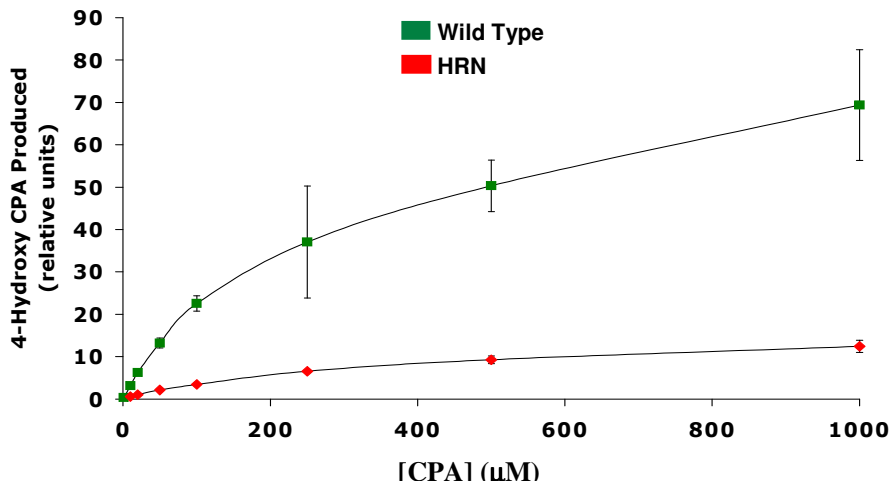
The HRN™ mice are currently commercially available and have a >95% reduction in *in vitro* hepatic P450 reductase activity. This is associated with a marked reduction in *in vitro* cytochrome P450 activity towards substrates such as testosterone and cyclophosphamide (Figures 3a and 3b). Experiments with the HRN™ mouse could either be on microsomal fractions or isolated hepatocytes. The latter will be a powerful way of assessing Phase II metabolism and hepatic drug transport in the absence of metabolism.

Figure 3a: Metabolism of testosterone in microsomal fractions from HRN™ and wild type mice.



NADPH-dependent oxidation of testosterone is dramatically reduced in microsomal fractions from HRN livers. The hydroxylation reflecting specific cytochrome P450 isozyme activity (expressed as percentages of control values) are: 6β-hydroxylation (CYP3A) <10%, 7α hydroxylation (CYP2A) <1%, 16β-hydroxylation (CYP2B) ~0%.

Figure 3b: Microsomal metabolism of cyclophosphamide (CPA) in HRN™ and wild type mice.



4-Hydroxy-cyclophosphamide formation was determined in microsomal incubations containing different concentrations of cyclophosphamide. (redrawn from reference 2)

Conclusion - *In vitro* validation

With the elimination of hepatic cytochrome P450 reductase a profound reduction in *in vitro* cytochrome P450 monooxygenase activity is observed. The HRNTM model can be used for *in vitro* metabolism and toxicology screens as well as to study the activity of drug transporters and Phase II enzymes in the absence of P450-mediated metabolism.

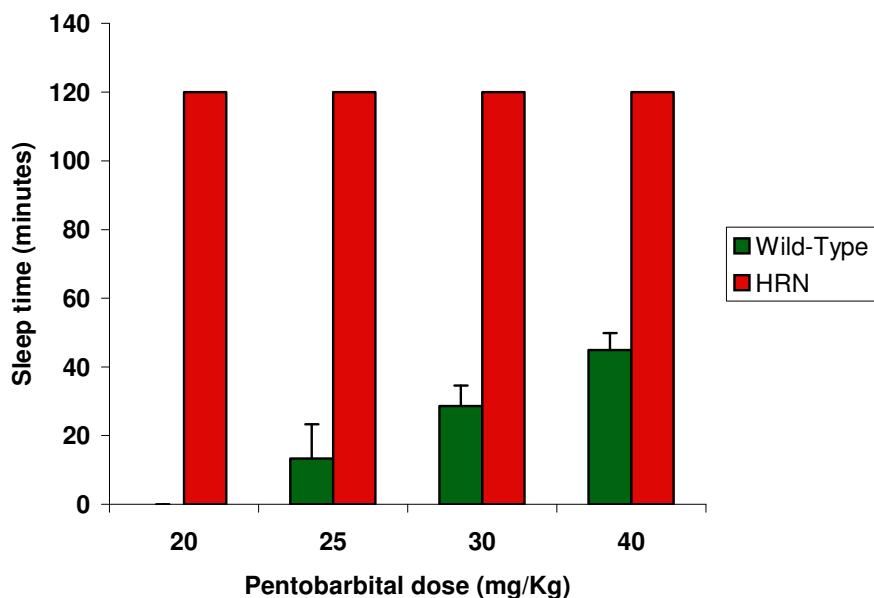
Applications of the HRNTM Mice

1) Drug Efficacy Screening

The success of an *in vivo* drug efficacy screen depends on many factors - the relevance of the drug target, the interaction of the candidate drug with the drug target and the bioavailability and pharmacokinetics of the compounds tested. On the basis that a significant number of compounds fail to show *in vivo* efficacy, it is rational to optimise the chances of success by eliminating bioavailability parameters as a confounding factor. Also, in the absence of hepatic metabolism and the resultant extended biological half-life of compounds, it is much easier to determine the effective circulating concentration of the compound and therefore what needs to be achieved clinically.

We have demonstrated the power of the HRNTM model for efficacy screening by studying the narcotic effect of the barbiturate pentobarbital (Figure 4). In HRNTM animals the narcotic effect of pentobarbital was greatly enhanced, and indeed the effects of the compound were observed at a dose (<20mg/kg) where no effect was seen in the wild type animals. These data demonstrate that a pharmacological effect was uncovered which would otherwise not have been observed and also that much lower amounts of compound can be used to demonstrate efficacy.

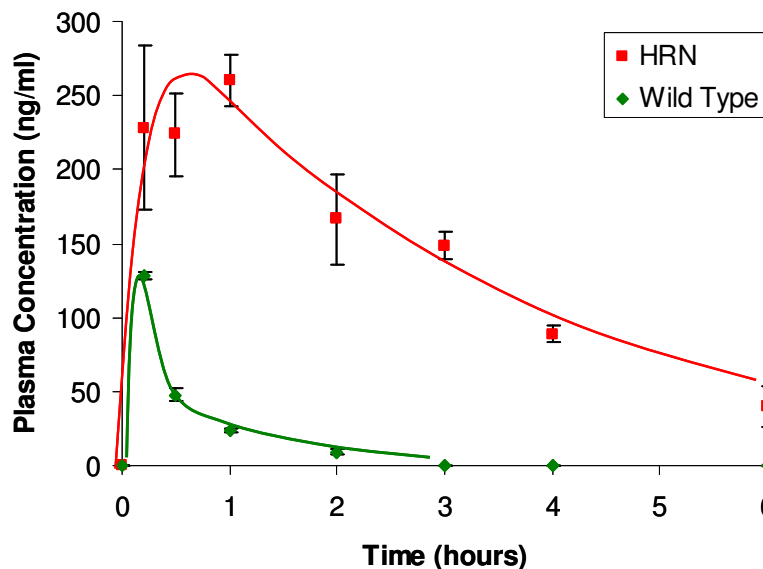
Figure 4: Induction of anaesthesia by pentobarbital in normal and HRN mice.



Pentobarbital was administered by intraperitoneal injection to groups of wild type and HRNTM mice (n=3) at four concentrations, and the time taken to regain the righting reflex was measured. HRNTM mice fail to regain consciousness even at concentrations (20mg/Kg) that are not efficacious in wild type animals.

Further demonstration of the value of HRNTM mice to improve bioavailability and pharmacokinetic parameters for drug efficacy screens has also been obtained from experiments with the analgesic, midazolam (Figure 5). These data show that the biological half life and overall exposure (AUC) were greatly increased in the HRNTM model (see also later).

Figure 5: Pharmacokinetics of midazolam in HRNTM and wild type mice.



Plasma concentrations of midazolam following single dose administration (2.5mg/Kg) to HRNTM and wild type mice were determined by mass spectrometry.

Parameter	HRN TM mouse	Wild type mouse
AUC 0-6hr (hr*ng/mL)	934	76
Tmax (hr)	0.85	0.09
Cmax (ng/mL)	244	185
CL-F (mL/hr)	2680	32900

Conclusion – Drug Efficacy Screening

In HRNTM mice *in vivo* efficacy was seen at much lower drug doses and was much more profound and longer lasting than in wild type animals due to the extended drug pharmacokinetics. Indeed, at low doses the *in vivo* pharmacological effects (i.e. efficacy) of pentobarbital would not have been observed. Such tests can be applied for oral, intraperitoneal or intravenous dosing regimens. Much less compound was needed to demonstrate *in vivo* efficacy than in wild type animals.

The HRNTM model increases the probability for a successful efficacy test and such experiments will demonstrate whether the parent compound and/or the metabolites are the effective pharmacological agent. This could be confirmed by determination of the pharmacokinetics in HRNTM vs. wild type mice.

2) Cytochrome P450/Bioavailability interaction studies

Interactions with the cytochrome P450 system can be a reason for lack of *in vivo* efficacy, tissue-specific toxicity and drug/drug interactions. In addition, certain drugs may only be efficacious following metabolism by these enzymes.

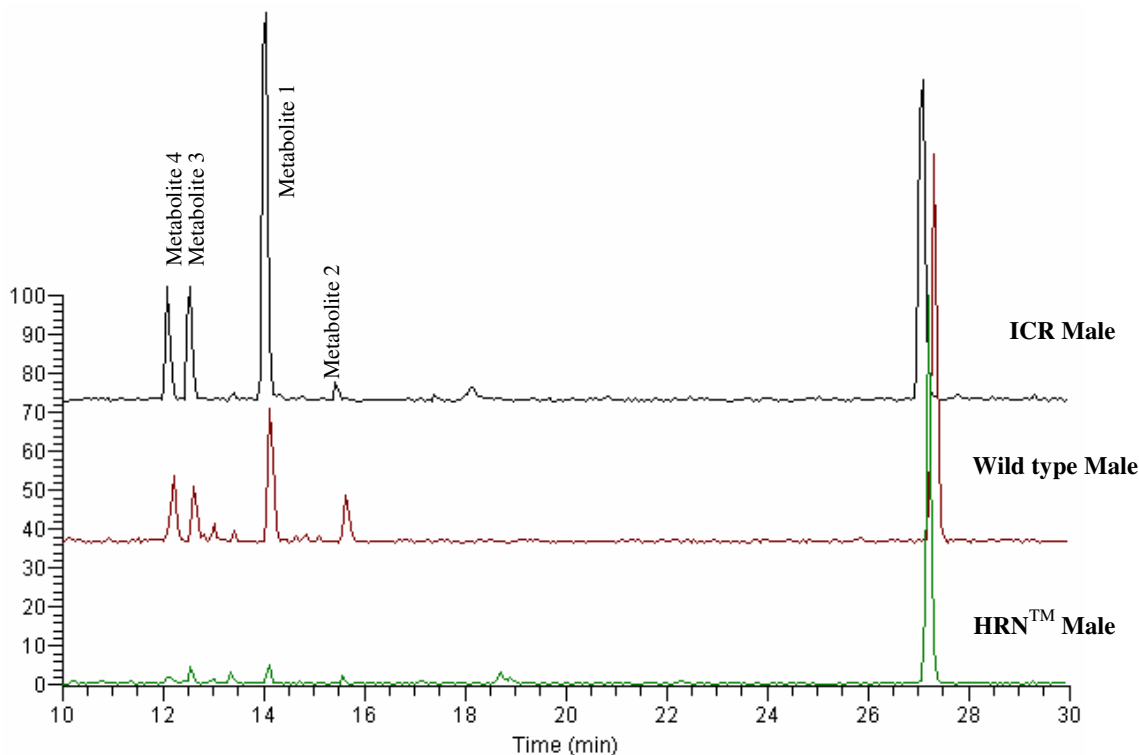
Comparison of drug bioavailability and pharmacokinetics in HRNTM and wild type mice will provide important insights into the role of the P450 system, Phase II metabolism and drug absorption as factors in drug bioavailability and elimination, allowing decisions to be made on lead selection, redesign or stopping a project. Indeed, this may identify pathways of disposition or metabolites found in man that may not be found in wild type animals, e.g. Phase II metabolites; as such, this could be considered a form of humanisation.

2a) Drug Absorption and Pharmacokinetics

Comparison of oral vs. iv drug pharmacokinetics using these models will establish whether hepatic or intestinal metabolism are important determinants in drug bioavailability. This will also establish whether hepatic or renal clearance is rate limiting.

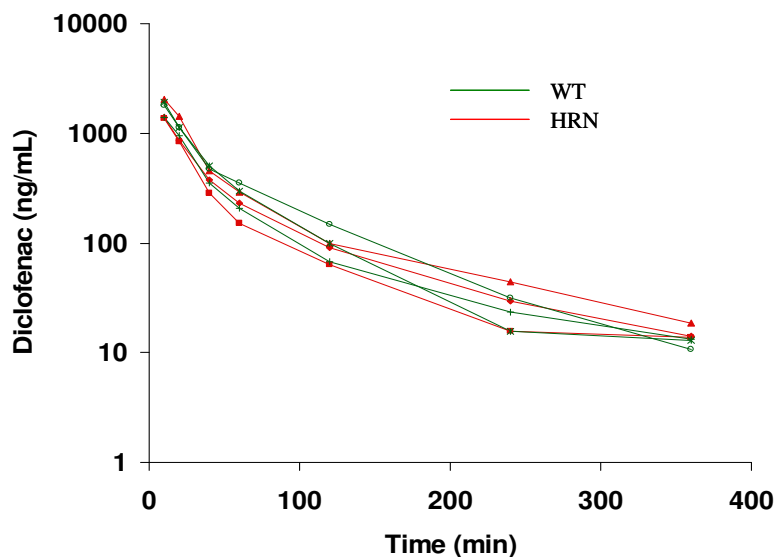
In addition to the validation studies discussed above, we have carried out a number of investigative studies using these models (Figures 6 and 7).

Figure 6: Mass chromatograms of urine samples showing parent and metabolites following administration of drug Y to HRNTM and two strains of control mice.



A profound reduction in urinary metabolites of greater than 90% in the HRNTM mouse was observed, demonstrating that this particular compound (drug Y) is a P450 substrate and largely cleared through metabolism by the hepatic P450 system.

Figure 7: Lack of cytochrome P450 involvement in the drug disposition of diclofenac.



Diclofenac plasma levels were measured in HRNTM and wild type mice following an oral dose. (Unpublished data kindly provided by Dr Georgia Pass)

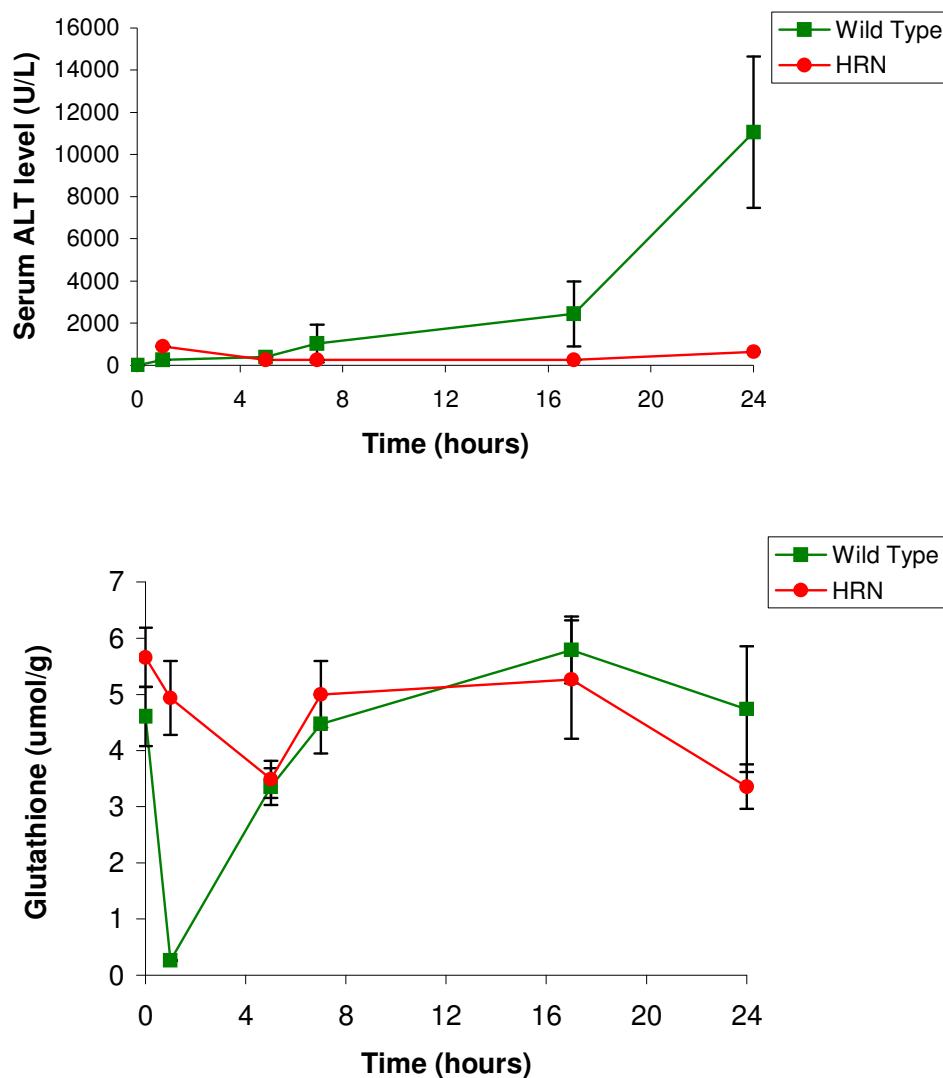
The lack of any difference in clearance between HRNTM and wild type mice shows that hepatic P450 metabolism is not a determinant of the pharmacokinetics of the non-steroidal anti-inflammatory drug, diclofenac. This is consistent with the known major pathway of disposition of diclofenac in man, which is through Phase II metabolism, namely glucuronidation. Although polymorphic human P450 CYP2C9 can oxidise this compound these data indicate that polymorphisms at this locus should not result in differential responses in patients.

2b) Cytochrome P450-mediated metabolic activation

Hepatotoxicity is a major reason for drug failure. We have demonstrated the value of the HRNTM model in establishing the role of the P450 system in this process using the hepatotoxin, acetaminophen.

The results shown in Figure 8 demonstrate that acetaminophen is metabolised in wild type mice to produce a toxic metabolite whereas the HRNTM mice are unable to metabolise the compound. This attribute of the HRNTM is extremely useful to determine if toxicity is due to exacerbated pharmacological effects or a toxic metabolite, and may allow such toxic liability to be designed out in lead optimisation studies.

Figure 8: Toxicity of acetaminophen (300mg/Kg i.p.) in HRNTM and wild type mice.

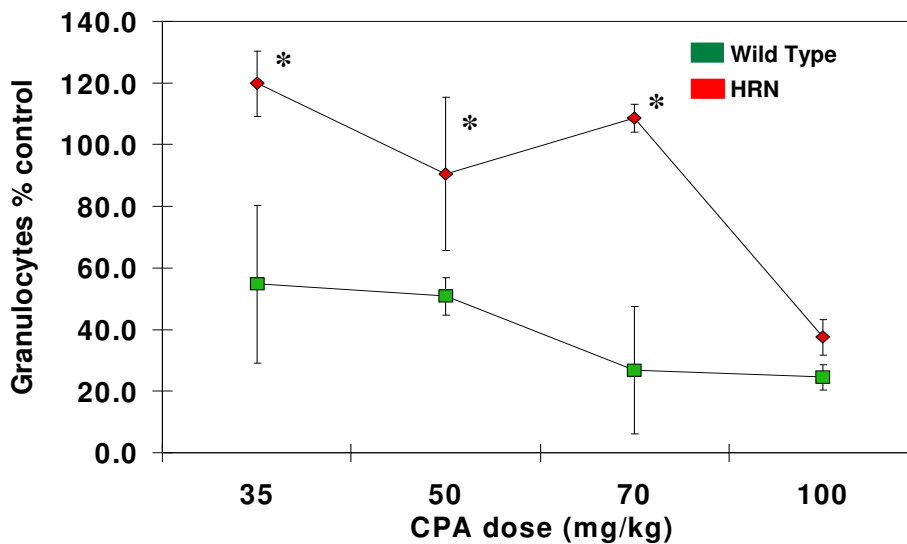


Serum alanine aminotransferase (top panel) and hepatic glutathione levels (bottom panel).

At a dose of 300mg/Kg, a large 90% drop in hepatic glutathione levels occurs within 1 hour following administration to wild type mice (top panel, Figure 8), and a subsequent large rise in serum alanine aminotransferase (ALT) levels, indicative of extensive liver damage (bottom panel), was observed. A similar treatment of HRNTM mice (red symbols) resulted in only a small change in hepatic glutathione levels and no increase in serum ALT, demonstrating an almost complete lack of toxic metabolite generation. (redrawn from reference 1)

Demonstration that toxic metabolites produced in the liver cause toxicity at a distant site has been exemplified using the myelotoxic antitumour agent, cyclophosphamide.

Figure 9: Effect of cyclophosphamide on bone marrow granulocyte levels in HRN™ and wild type mice.



Marked CPA-mediated myelotoxicity was seen at all doses in the wild type animals but only in HRN™ mice at the highest dose tested, demonstrating that hepatic metabolism is a major contributor to CPA's toxic effects. (data replotted from reference 2)

Conclusion - Cytochrome P450/Bioavailability interaction studies

The HRN™ model provides a powerful method for elucidation of the pathways of absorption, metabolism and disposition of compounds in a single experiment. Lack of oral bioavailability in the HRN™ indicates whether there are intestinal absorption, solubility or insurmountable problems associated with the oral administration of a compound or compound series. It also provides a simple method for evaluating the role of the P450 system in a toxic pathway and gives indications of potential drug/drug interaction liabilities.

3) Toxicokinetic Studies

The tolerability of drug side effects by drug regulators or by doctors is inevitably linked with a drug's therapeutic indication. For example, significant side effects are deemed acceptable for the treatment of life-threatening diseases such as cancer and in the treatment of psychiatric illness, but not in the chronic treatment of metabolic diseases or inflammatory conditions. The aspirations of all drug treatments are to maximise the therapeutic window by minimising the risk of side effects.

We define toxicokinetics as the relationship between drug kinetic parameters and toxic side effects. These are therefore a major determinant of drug developability. A number of different kinetic parameters can determine toxic response, for example maximum circulating drug (or metabolite) concentration (C_{max}), or the overall exposure (area under the curve, (AUC), or biodistribution, etc. Understanding the toxicokinetic properties of a compound can be a key factor in the appropriate design of clinical trials. Such studies are of particular importance in cancer chemotherapy since the therapeutic window is often narrow.

We have demonstrated the power of the HRNTM models for studies of this nature by investigating the toxicokinetics of the antitumour agent, cyclophosphamide (see Pass *et al*, Cancer Research (2005) **65**, 4211-4217). Cyclophosphamide (CPA) is metabolised to its active antitumour metabolite, 4-hydroxycyclophosphamide (4-OH-CPA), by the cytochrome P450 system and this metabolite is also responsible for the major side effect, myelosuppression. In wild type animals, the C_{max} and AUC for the production of the 4-OH-CPA at different doses were closely correlated with each other, so an analysis of which parameter is most important in the myelotoxicity of the compound was not possible. However, the change in metabolism in the HRNTM mouse has allowed these parameters to be clearly dissociated, i.e. in the HRNTM mice the C_{max} in relation to wild type was greatly reduced; however, the AUC remained very similar (Figure 10). (This also gives important insights into the kinetic parameters that determine metabolite elimination.)

Figure 10: Plot of AUC and C_{max} of 4-OH-CPA versus cyclophosphamide dose in HRNTM and wild type mice. (replotted from ref 2)

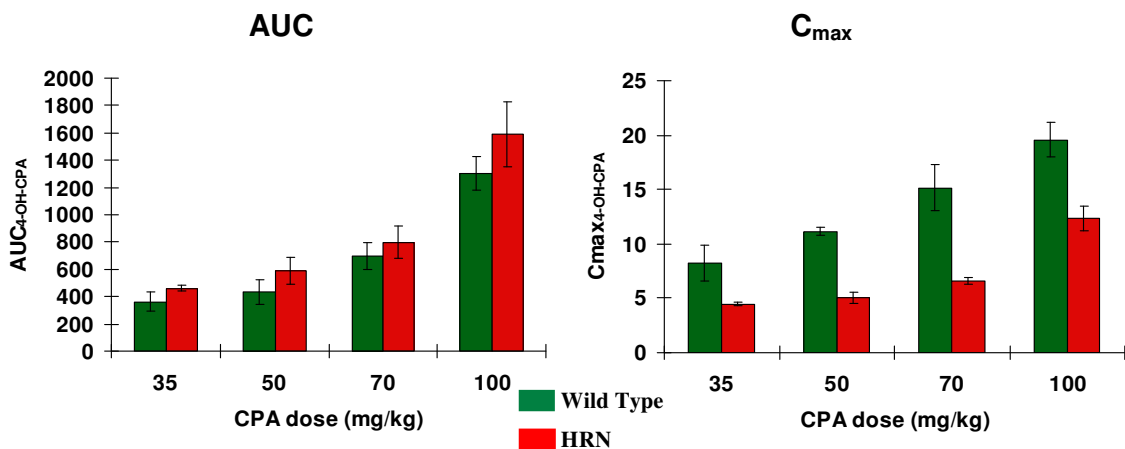
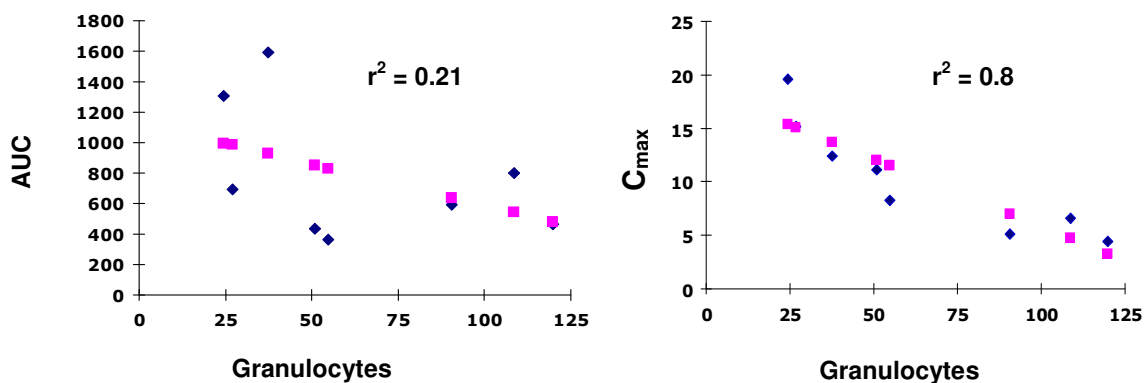


Figure 11: Correlation of C_{max} and AUC of 4-OH-CPA with myelosuppression following cyclophosphamide administration (replotted from reference 2)



Plotting AUC or C_{max} of 4-OH-CPA against the level of myelosuppression demonstrated a close correlation with the C_{max} (Figure 11) but not with AUC. Interestingly, the antitumour effects of cyclophosphamide are reported to correlate with the overall exposure, i.e. AUC. These data suggest that the way to maximise the therapeutic benefit of this compound is to use it as an infusion rather than a bolus injection. Quite clearly such information prior to the instigation of Phase I trials could be extremely valuable and could make the difference between drug success and failure.

Conclusion – Toxicokinetic Studies

The HRNTM allows a toxicokinetic evaluation of compounds to establish the optimal route and method of administration in Phase I trials. Such studies will also facilitate an evaluation of whether or not to shelve a programme.