MAKING BETTER DRUGS: DECISION GATES IN NON-CLINICAL DRUG DEVELOPMENT

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Drug development is a risky business. Success or failure often depends on selecting one or two molecules for development from many choices offered by the engines of high-throughput discovery. A lead candidate needs to possess adequate bioactivity, appropriate physical–chemical properties to enable formulation development, the ability to cross crucial membranes, reasonable metabolic stability and appropriate safety and efficacy in humans. Predicting how a drug will behave in humans before clinical testing requires a battery of sophisticated *in vitro* tests that complement traditional *in vivo* animal safety assessments. This review discusses how to strategically identify which non-clinical studies should be performed to provide the required guidance and comfort to stakeholders involved in clinical drug testing.

A GUIDE TO DRUG DISCOVERY

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The process of drug development

The search for successful therapeutic interventions to treat disease and improve quality of life is evolving from managed serendipity to engineered selection. Recent advances in combinatorial chemistry, high-throughput screening, functional genomics and proteomics have fuelled an explosive proliferation of new chemical entities (NCEs) that possess promising pharmacological properties. The challenge is to select and advance one or two compounds with properties that are predictive of good efficacy and safety in humans. Despite improvements in the number and quality of potential drug candidates, drug development remains a highly difficult, costly and risky business. Success rests not only in the intrinsic qualities of the molecule, but also in how the development of the drug is planned and executed, and in the effective management of key resources: effort, time and expenditure.

Drug development is a process that proceeds through several key go/no-go 'decision gates', from the identification of a potential therapeutic candidate through to marketing a drug product (FIG. 1). A 'decision gate' is a useful analogy, because to pass through a decision gate, an NCE must successfully meet a series of predetermined criteria, followed by another, usually larger, set of studies to answer questions relevant to the next decision gate.

Two of the most important decision gates in the development of a therapeutic agent ask whether the drug works in humans, and whether the drug can be marketed. The first question is answered by a clinical proof-of-concept study in a small, but targeted, group of patients. Although it is not essential to establish statistical proof at this stage, sufficient evidence is needed to provide confidence that the therapeutic product is working as intended. Ultimately, the safety of human subjects is the predominant focus of early clinical research.

The decision to allow a new therapeutic entity to be given to human subjects in a clinical research setting, or to allow marketing of a drug to a large population of patients, is shared by many stakeholders, including government health regulators, clinical investigators, ethics review committees, healthcare providers and the

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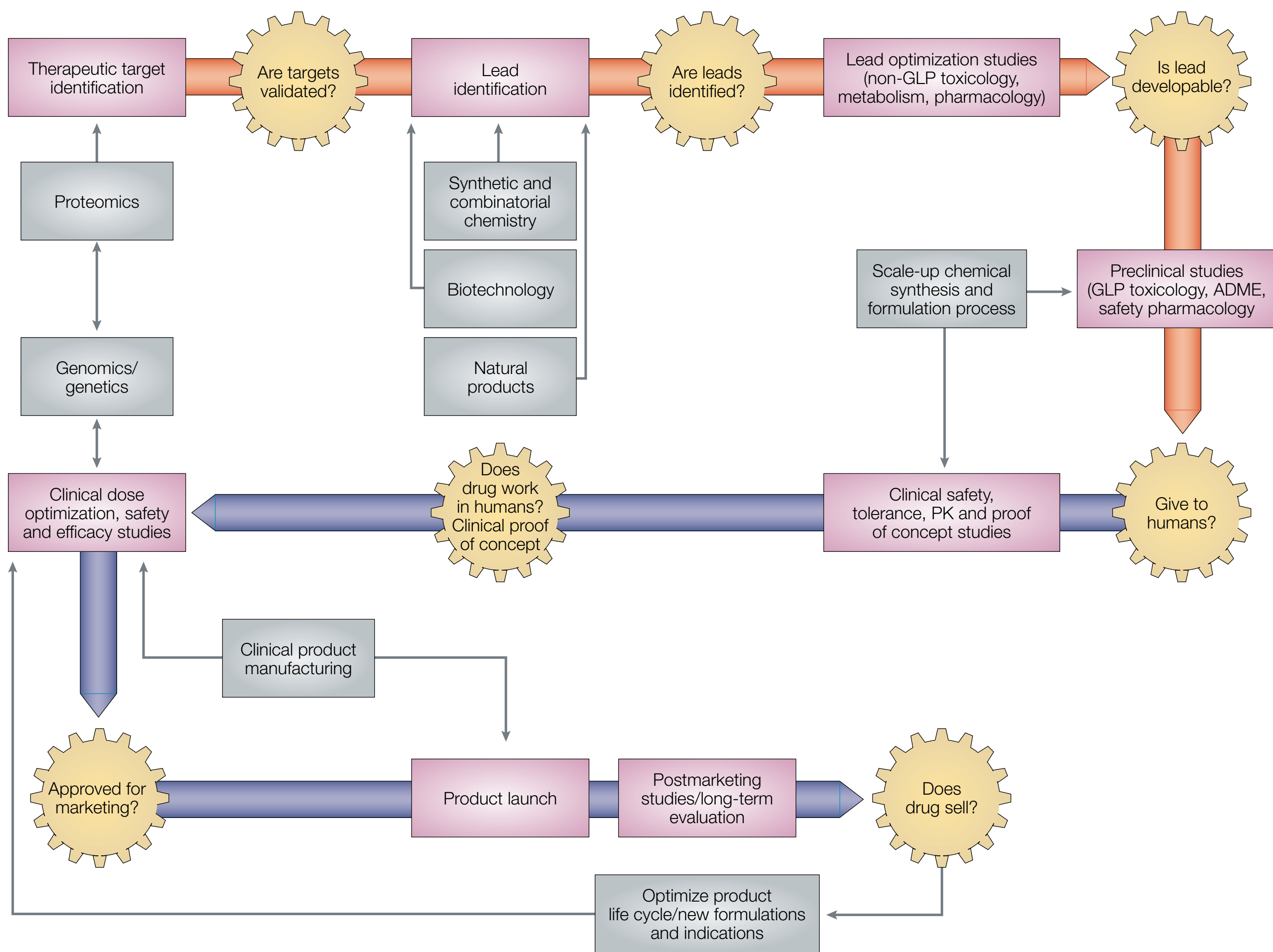

Figure 1 | **Key decision gates in drug development.** Gear symbols represent the key go/no-go decisions for development of a pharmaceutical product. Boxes represent key activities or disciplines involved in the research necessary to answer the questions generated at each decision gate. Asking the right questions at the right time is the key to strategic drug development planning. ADME, absorption, distribution, metabolism and excretion; GLP, Good Laboratory Practice; PK, pharmacokinetics.

sponsoring organization itself. The primary goal must be to establish what is best for the patient. Deciding if an NCE can be administered to humans for the first time is profoundly influenced by preclinical studies performed in animals that address the toxicology and exaggerated pharmacology of a drug product. During the latter stages of drug development, longer-term safety studies in animals are necessary before marketing approval can be obtained.

The science of non-clinical drug development has been dramatically affected by the emergence of sensitive analytical instrumentation and by the application of molecular genetics to enable the detection of changes in the expression of human proteins that could affect, or be affected by, new drug candidates. These new tools generate vast amounts of information and data that require active management. Increasingly, more knowledge about the characteristics of a drug is expected by decision makers at each phase of drug development in order to reduce risk to human subjects and to increase the chance of picking a winning therapeutic molecule. When resources are limited, the question of what knowledge is needed and when — the 'decision gate' analogy — becomes the principal strategic consideration in determining the priority of activities during early drug development.

Once a compound has been selected for development, regulatory guidances have a large influence on the conduct of non-clinical studies. As the administration of experimental drugs carries real risks to the human study subjects, certain non-clinical studies must be done according to government-regulated standards so that the data can be trusted by all of the stakeholders involved in clinical research[1]. In recent years, through the efforts of the International Conference on Harmonization (ICH), common global requirements have been largely agreed on[2]. Even though regulatory guidances define the normal expecations of what knowledge is needed to proceed to human testing or marketing, flexibility exists in the actual planning and

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design of a drug development programme. No one person can bring focus and expertise to all of the scientific and regulatory aspects of drug development. Therefore, the planning and execution of a programme of work is usually overseen by a team of committed scientists led by a 'champion' who is able to leverage the resources needed to complete the work in a timely way.

It is estimated that only 1 out of 5,000 screened compounds is approved as a new medicine[3]. A significant number of potential drugs in development fail because of unacceptable animal toxicity. Toxicity can be defined as any unwanted effect on normal structural or functional integrity, including unwanted or overly exaggerated pharmacological effects. Although most drugs will express unacceptable toxicity at some level of dosing, acceptability is determined by the degree of separation between the targeted pharmacological dose and the doses or exposures in animals when the first truly toxic signs develop.

It is not sufficient just to know what the drug does to the body (that is, its pharmacology and toxicology); it is also crucial to know what the body does to the drug. Knowledge of the absorption, distribution, metabolism and excretion (ADME) properties of the drug and its metabolites in humans, and animals used in toxicology assessments, is crucial to understanding differences in effect among species and for optimizing drug dosing[4,5]. For orally administered drugs, adequate absorption and bioavailability must be achieved. Absorption refers to the amount of total drug-derived material that crosses the gastrointestinal epithelium, whereas bioavailability addresses the amount of biologically active material that reaches the systemic circulation. Duration of drug action is often dependent on how rapidly the body eliminates the active molecules, either through chemical modification (metabolism) by the action of drug-metabolizing enzymes or by binding and transport away from the biologically active sites and excretion from the body.

Decision gates in drug development

The balance of this paper provides an overview of the types of approaches taken today to provide the right information for the decisions that need to be made in non-clinical drug development. Three decision gates are addressed: lead optimization, first-in-human studies and marketing decisions. In addition, the discussion focuses primarily on small-molecule drugs (that is, drugs with a molecular mass (MM) < 1,000), but also recognizes that larger therapeutic modalities alter the types of questions that need to be addressed. To be truly strategic in considering non-clinical drug development studies, regardless of the size of the molecule, the question that must always be addressed is what real predictive value do these studies add to our understanding of the efficacy and safety of drug candidates in humans?

Decision gate: can this drug be developed?

Smart drug development means eliminating potential drug candidates that are likely to fail in clinical trials early in their development. Typical questions addressed during the lead optimization phase are focused on determining whether there are any properties of the drug candidate that would make future development very difficult (TABLE 1). "Fail fast and cheap" are the watchwords at this stage. Although each drug development plan is unique, a typical flowchart of lead optimization activity is shown in FIG. 2, and key aspects of these studies are discussed below.

To this end, several approaches have been developed to help understand how drugs behave at the several barriers that exist between dosing a drug and the target of drug action[6,7]. These include permeation across epithelial membranes such as the gastrointestinal mucosa, drug metabolism, plasma protein binding and transport into and out of tissues, especially organs that eliminate drug products, such as the kidney

Table 1 | **Drug candidate selection: key questions and pivotal studies**

Questions	Studies that provide answers
Can the drug candidate be measured and is it stable in biological matrices?	Bioanalytical assay development.
Does the drug candidate have reasonable metabolic stability? What are the metabolites and are they active, possibly even a better drug candidates? Are there species differences in metabolism?	*In vitro* metabolism studies using animals and human hepatocytes, microsomes or human expressed enzyme systems and analysis of incubates using LC/MS/MS or LC/NMR/MS. Synthesis and pharmacology testing of metabolites.
Does the drug have sufficient oral bioavailability and persistence in the bodies of animal models?	Pharmacokinetic studies in rodent and non-rodent species after oral versus intravenous administration.
Is the drug mutagenic or cytotoxic *in vitro*?	Ames bacterial mutagenicity assay. Mammalian cell (for example, mouse lymphoma) mutagenicity assay.
What is the maximum tolerated dose (MTD) and dose-limiting toxicity?	Rising single- and repeat-dose escalation toxicology study in rodent and non-rodent species until limiting toxicity is observed.
Can active pharmaceutical ingredient (API) be synthesized at reasonable cost? Is the API stable after synthesis?	Chemical synthesis process assessment. Assay development for API purity. Chemical stability assessment. Generation of API certificate of analysis.
Can the drug be formulated for use in animal toxicology studies and early human studies?	Pre-formulation development testing. Assay development for purity and content formulated product.

LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance.

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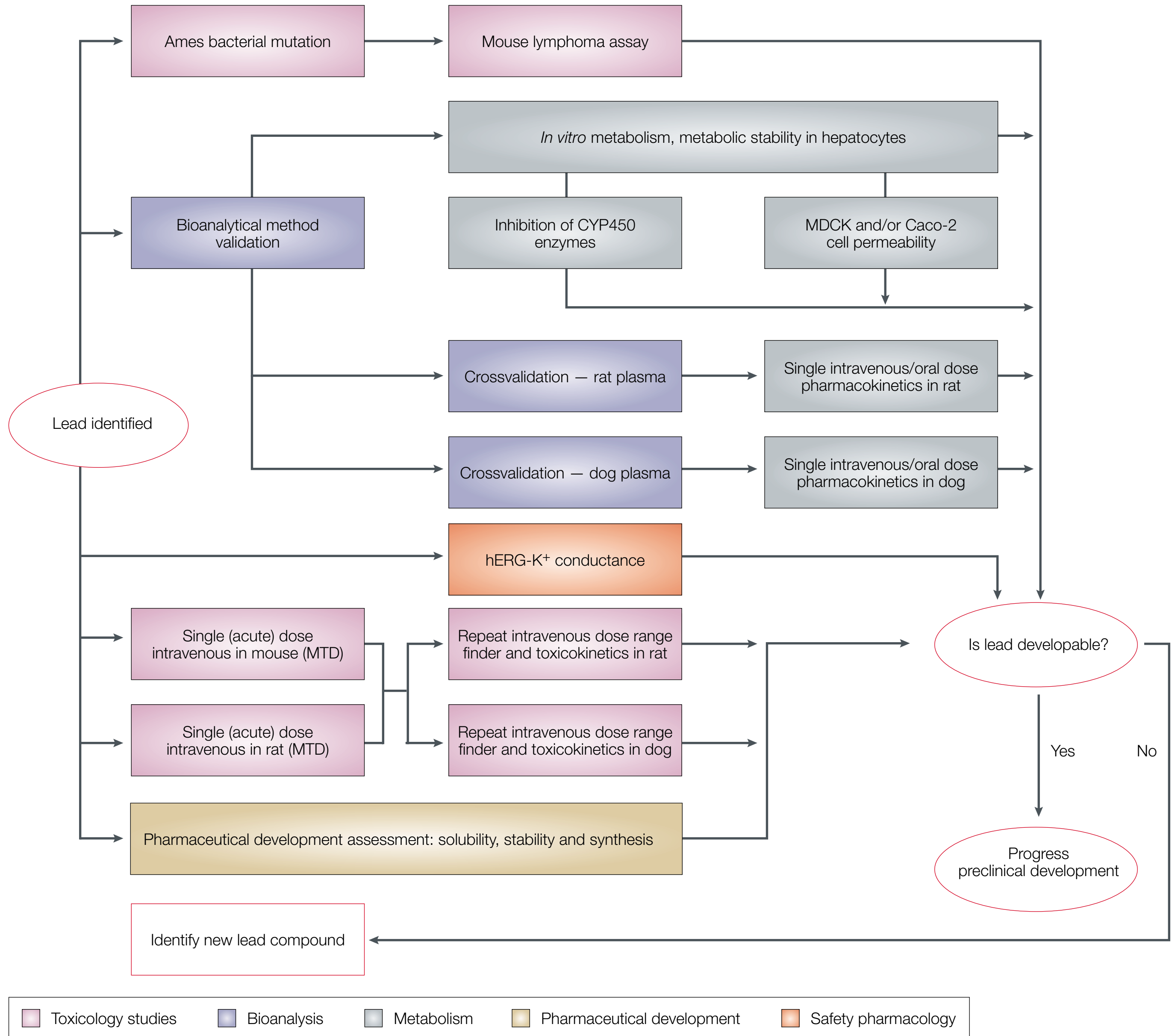

Figure 2 | **Potential flowchart for assessing whether a small-molecule new drug candidate is developable.** CYP450, cytochrome protein 450; hERG, human ether-a-go-go; MDCK, Madin–Darby canine kidney; MTD, maximum tolerated dose.

and liver. Arguably, the most important of these techniques in the prediction of the safety and efficacy of a drug are metabolism and oral absorption, whereas protein-binding characteristics are less significant[8]. *In vitro* ADME approaches are being applied to drug candidates both in the pre-selection (that is, discovery) process and earlier in development, as they are essential for identifying and eliminating compounds likely to present safety challenges in the later stages of drug development.

Is the drug absorbed?

The Caco-2 cell permeability assay is one approach that has been widely adopted for understanding the gastrointestinal drug absorption process[9–12]. Caco-2 cells are a human intestinal cell line that are grown to form a monolayer on a filter separating two microwell chambers. Drug candidates can be added to one side of the membrane and their permeation followed. Active and passive transport processes can be studied. The Madin–Darby canine kidney (MDCK) cell model is a practical alternative to Caco-2 cells, in that continuous monolayers of cells can be grown in a just a few days[13,14], rather than the three weeks required for Caco-2 cells. However, MDCK cells are less reflective of the intestinal epithelium and cannot be used for bi-directional transport studies. Increasingly, non-cell-based approaches, such as the parallel artificial membrane permeability assay (PAMPA), are gaining popularity for assessing passive permeability[15,16].

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Box 1 | LC/MS/MS analytical technologies enable early ADME testing

Liquid chromatography coupled with tandem mass spectrometric detection (LC/MS/MS) is the analytical method of choice in early absorption, distribution, metabolism and excretion (ADME) testing. The atmospheric pressure ionization interface now standard on modern LC/MS/MS systems enables rapid method development coupled with excellent sensitivity, specificity and high sample throughput. The quantitative selectivity afforded by selective reaction monitoring on a triple quadrupole instrument precludes the need for high chromatographic resolution or extensive sample clean up. Analytical throughput can be further maximized by using automated sample-processing techniques, such as on-line column switching[72], combined with high-sample-density microtiter plates. Modern LC/MS/MS also offers limits of detection extending down to the sub-nanogram per ml range using only minimal quantities of biological matrix (for example, 0.025 ml of plasma).

Not only does LC/MS/MS enable rapid and sensitive quantitation of new drug candidates, but it also provides important structural information on metabolites[73]. A full-scan LC/MS analysis can initially suggest possible oxidative and/or conjugative metabolic transformations on the basis of the ionic species observed. In the MS/MS mode, the instrument can be tuned to a selected precursor ion of interest, which is then further fragmented to form product ions that uniquely identify the metabolite (product ion scan).

Selectivity has been further enhanced by the quadrupole ion trap, a device that 'traps' ions in a space bounded by a series of electrodes[74]. The unique feature of the ion trap is that an MS/MS experiment (or, in fact, multi-step MS^n experiments) can be performed sequentially in time within a single mass analyser, yielding a wealth of structural information. Hybrid quadrupole-time-of-flight (Q-TOF) LC/MS/MS systems are also popular for the characterization of metabolite profiles[75]. The configuration of a Q-TOF results in an instrument capable of high sensitivity, mass resolution and mass accuracy in a variety of scan modes.

Looking ahead, liquid chromatography coupled with nuclear magnetic resonance spectroscopy (LC-NMR)[76] provides a way of confirming absolute molecular configurations. A new linear ion-trap mass spectrometer possessing significantly enhanced product ion-scanning capabilities, while retaining all of the scan functions of a triple quadrupole MS, has recently been introduced[77]. The ultra-high resolution and sensitivity of Fourier transform ion-cyclotron resonance MS (FT-ICR MS)[78] holds great promise for the analysis and characterization of biological mixtures. Finally, continued advancements in data processing and interpretation software packages should enable the analytical scientist to more efficiently identify and quantify metabolites throughout the drug development process.

Is the drug metabolized?

Metabolism is a key determinant of the *in vivo* fate of a drug candidate molecule. Too rapid or extensive metabolism to pharmacologically less active or inert metabolites and a compound will lose its therapeutic efficacy. Metabolism can lead to pharmacologically active metabolites, frequently with properties different from those of the parent drug. For example, the non-sedating antihistaminic drug terfenadine (Seldane; Marion Merrell Dow), was associated with TORSADES DE POINTES ventricular arrhythmias and was consequently withdrawn from the market, whereas its pharmacologically active metabolite fexofenadine (Allegra; Aventis), is devoid of cardiotoxicity. It is often important to define active metabolites early in development, to understand their relevance to toxicology and to fully protect the intellectual property value of the drug.

Owing to the importance of metabolism in interpreting toxicology findings[17], *in vitro* metabolism studies normally precede *in vivo* preclinical safety assessment[18,19]. Not surprisingly, inter-species differences exist in drug metabolism, in particular among the cytochrome P450 (CYP450) superfamily of drug-metabolizing enzymes[20,21]. *In vitro* preparations of human liver, including microsomes, hepatocytes and liver slices, as well as human complementary-DNA-expressed enzymes, now complement similar preparations for the principal toxicology species[22,23]. Therefore, metabolic stability can be readily tested *in vitro* across several species, enabling the comparison of the metabolism of the drug in animals with that in humans. In addition, by employing modern liquid chromatography/tandem mass spectrometry (LC/MS/MS) and LC/MS nuclear magnetic resonance (LC/MS/NMR) technology (BOX 1), metabolites can be quickly identified from the incubates of these experiments[24,25].

The next crucial question to be addressed early in drug development is which human drug-metabolizing enzymes are responsible for the metabolism of the drug. Many enzymes can contribute to the overall metabolism of a drug, and some are genetically polymorphic and/or associated with high inter-individual variability in expression levels[26–28]. From a safety perspective, it is important to identify, and perhaps eliminate, drugs from further development if they are subject to polymorphic metabolism or to extensive metabolism by key human enzymes, such as CYP3A4/5, CYP2D6, CYP2C9, CYP1A2 and CYP2C19 (REF. 29). These five enzymes are the predominant drug-metabolizing enzymes in human liver, and CYP3A4 is particularly significant as it has a role in the metabolism of approximately 50% of all small-molecule drugs in use today[30]. Extensive metabolism by a single enzyme such as CYP3A4 can lead to adverse drug–drug interactions when concomitant drug therapies require the same enzyme for their metabolic clearance. Individuals with inherited deficiencies in one or more key CYP enzymes are at increased risk for developing drug-related toxicities, or in the case of drugs requiring metabolic activation, experiencing a lack of therapeutic efficacy. Some of these genetic deficiencies in drug-metabolizing enzymes are relatively common: for example, 5–10% of Caucasians are poor metabolizers of CYP2D6, and 13–23% of people from South East Asia and Japan lack functional CYP2C19 enzyme. *In vitro* studies employing human liver microsomes or expressed enzymes, and selective chemical inhibitors or antibodies, are valuable tools for providing early information on human metabolism of a new drug that is crucial in predicting potential clinical drug–drug interactions and the impact of genetic polymorphisms.

Is the drug too toxic?

Not so long ago, the toxicologist would only become involved in drug development when the discovery research scientist handed over one precious candidate drug and got busy discovering the next. Nowadays, toxicology and safety pharmacology are involved much earlier and play an increasingly important role in the decision of whether to move a lead candidate into regulated *in vivo* toxicology studies[31].

In vitro genotoxicity assays are mandatory regulatory studies designed to detect potential mutagens and/or carcinogens. These assays are often performed during

TORSADES DE POINTES
This is a form of polymorphic ventricular tachycardia that is preceded by a prolongation of the QT interval. Although this condition is found in many clinical settings, it is mostly induced by drugs and drug interactions that prompt a long QT syndrome.

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lead optimization, but mini-versions of these have also been applied as early drug selection screens. Any statistically significant increase in mutation rate during such studies is often a reason to abandon further development of the drug candidate.

There is growing use of the human ether-a-go-go K^+ (hERG-K^+) conductance assay using Chinese hamster ovary (CHO) cells during lead optimization[32,33]. This *in vitro* electrophysiology study can be used to screen out those candidates likely to cause QT interval prolongation. Prolongation of cardiac repolarization has been associated with the specific and potentially fatal tachycardia Torsade de Pointes in humans, and usually precludes further development of the compound[34].

Increased understanding of individual differences and disease-related mechanisms will undoubtedly improve the selectivity of lead candidates. As relational databases reveal genomic and proteomic differences between the disease versus normal states, and between genomic and proteomic responses to different drugs and reference xenobiotics, this should lead to better biomarkers that will predict relevant human toxicities during non-clinical drug development. The term 'toxicogenomics' now refers to the application of genomics and proteomics to drug-safety assessment and has been the subject of several recent scientific workshops[35,36]. In the future, drug developers will attempt to match the genomic responses of lead candidates to the individual genotypes. For example, trastuzumab (Herceptin; Genetech) is only effective for women who carry multiple copies of the *ERBB2* (also know as *HER-2/neu*) gene[37].

What is the best species for toxicology?

The primary goal of early toxicology studies is to find a dose that produces toxicity in one or more rodent or non-rodent species[38–40]. Acute toxicity studies in two species (often mouse and rat) inform what happens after a single, very high dose. This knowledge is useful not only to help select a safe starting dose for humans, but also in providing worker-safety data for those employees faced with handling a new chemical substance. It also forms the basis for overdose information for future human trials.

Various dose-escalation study designs exist to quickly predict what would be the maximum tolerated dose (MTD) in the putative toxicology species. In addition, it is important to identify early on the target organs of toxicity, as this can affect future study design. Finally, there are often large inter-species differences in MTD. For example, the dog is known to be very sensitive to compounds that cause gastrointestinal irritation, and non-human primates are better predictors of effects in humans.

On the basis of studies of the metabolic profile of the drug across species, the rodent and large animal species are selected for formal toxicology evaluations under Good Laboratory Practice (GLP) procedures[41]. As a large body of data exists for the albino white rat, this species is virtually always chosen as the rodent species. The large animal species is typically the dog or a non-human primate, on the basis of relevant pharmacological knowledge (the species should respond pharmacologically to the drug), MTD, and metabolism information (as close as possible to human). The MINI-PIG is becoming a useful toxicology species for dermal products and cardiovascular agents, just as pig skin and heart are good models of the corresponding human organs.

MINI-PIG
A small species of pig weighing about 20–45kg.

What happens to the drug after dosing?

Oral bioavailability, plasma half-life, clearance and, to a lesser extent, volume of distribution and mean residence time, are pharmacokinetic parameters that are typically determined from plasma drug concentration profiles in a rodent and non-rodent species following oral and intravenous administration[42]. The area under the 'plasma concentration versus time' curve (AUC), which measures time-averaged systemic exposure of the body to the drug, is very helpful in comparing systemic exposure of animals used in toxicology testing with humans. Finally, measurement of a drug and its principal metabolites in urine can help identify the contribution that renal clearance and metabolic clearance make to the overall elimination of the drug from the body. Pharmacokinetic studies, properly planned and executed, are rich sources of information and are therefore among the first studies performed in drug development.

Pharmacokinetic analysis is dependent on the development of a reliable bioanalytical method to measure parent drug and relevant active metabolite(s) in plasma, and possibly urine, samples. As these assays are usually used to support systemic exposure relationships in the species used for toxicology studies, the methods must meet agreed guidelines that support GLP[43]. A fully validated assay has a defined quantitative analytical range, meets precision and accuracy expectations for the type of analysis chosen (for example, high-pressure LC (HPLC), LC/MS, radioimmuno assay (RIA), enzyme-linked immunosorbent assays (ELISA)), and has proven ruggedness across a period of repeated use.

In vivo pharmacokinetic studies are resource intensive. Therefore, to speed up the process during lead selection and optimization, methods such as 'cassette' dosing have been used[44,45]. This approach involves several potential lead compounds that are simultaneously administered to each animal, and pooling of samples to reduce analysis times using LC/MS/MS. Choice of compounds is important to avoid isobaric mass ions that would complicate bioanalysis. Also, results can be skewed by unintended drug–drug interactions. Nevertheless, this is a popular approach for gathering good pharmacokinetic information on several compounds quickly in early drug development.

Decision gate: safe for humans?

Timing and costs are always issues in drug development planning. Typically, the execution of the studies in FIG. 2 could take four to six months and cost US $500,000. However, costs can triple, and timing double, in order to answer the questions that need addressing before making the decision to proceed to human testing (TABLE 2, FIG. 3). The rate-limiting step is

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Table 2 | **Human safety indicators: key questions and pivotal studies**

Questions	Studies that provide answers
Can the drug be reliably prepared and formulated according to Good Manufacturing Practice (GMP) guidelines that regulate drug material prepared for human use?	Formulation development. Define release GMP testing criteria for content and purity. Define formulation stability.
What is the maximum no-effect dose following repeated dosing? Is there dose-related exposure? What organs are affected by repeated dosing?	Fourteen-day to three-month toxicology studies in a rodent and non-rodent species with toxicokinetics.
What is the safety margin?	Ratio of maximum no-effect dose or exposure over expected pharmacological dose or exposure.
What enzymes are involved in the drug's metabolism?	*In vitro* drug metabolism studies using human microsomes and/or cytochrome protein expression systems.
How long and by what routes is parent drug eliminated?	Pharmacokinetics in rodent and non-rodent species. Radiolabelled drug-excretion-balance studies
Does the drug produce any cardiovascular effects or cardiac conductance?	*In vivo* telemetry studies in animals evaluating alterations in cardiac electrophysiology and cardiovascular vital signs. Action potential duration using isolated rabbit Purkinje fibers.
Are there any effects on behavior or pulmonary function?	Irwin behaviour test in rats. Rat pulmonary function evaluation.

often the completion of the enabling toxicology studies; however, sourcing drug substance or preparing an appropriate formulation can also determine the pace of this phase of drug development. Full ADME characterization in the toxicology species is not required before human testing, although an understanding of the fate of the parent drug and known active metabolites and their distribution is helpful, and even crucial, for certain drugs. Toxicokinetics (pharmacokinetics applied to samples taken from animals dosed during GLP toxicology testing) can provide key evidence of exposure versus response that can sometimes be extrapolated to man[46].

There are several questions that are optional for this decision gate, depending on the intent of the early clinical research programme (TABLE 3). Before repeated doses can be administered to women of child-bearing age, Segment I and II reproductive toxicology studies need to have been completed[47]. *In vitro* studies that evaluate enzyme inhibition or induction potential can also be crucial elements of a plan, especially if drug–drug interactions of this nature plague other drugs in the same therapeutic class[48]. Finally, *in vivo* genotoxicity testing is done if there are any questions arising from earlier *in vitro* work.

What toxicity occurs following repeated dosing?

Repeated-dose toxicology studies are designed to mimic the human dosing regimen for the first repeat-dose clinical tolerance and efficacy trials, and, depending on the planned initial clinical programme, a minimal length of two weeks and a maximum of three months are required. Studies in both rodent and non-rodent species are usually required by regulatory reviewers. It is important to know how the toxicity changes with dose: does it change with time during the study, and does toxicity differ between male and female animals? Some studies include a design to establish whether the toxicity is reversible[49,50].

The ultimate question that must be answered by the toxicology studies is what the safety margin is. If the maximum no-effect level dose or exposure is at least tenfold that of the expected pharmacological exposure in rodents, and at least sixfold that in non-rodents, then it is generally believed that the compound can be safely managed in humans during clinical dose-escalation safety and tolerance testing. Related considerations are whether unique toxicological consequences of drug administration can be separated from exaggerated pharmacology produced by overdosing animals, and whether there are inter-species differences in the toxicological response. A final focus, especially for larger-molecule drugs, is whether the drug affects the immune system[51]. New technology (such as surface plasmon resonance and cell-based bioassays) is available to determine whether antibodies have formed or if immune system biomarkers have changed during toxicology testing.

Toxicology criteria can be relaxed if the new drug entity is designed to treat a previously unmet medical need or will be administered to a very ill patient population in which there is little, if any, hope for alternative therapy. Also, there is no point studying repeated-dose toxicity of a drug in male animals when it is a product for women's health and vice versa. Toxicology testing for products that are delivered directly to their site of action (for example, inhalation of an antibronchospasmodic agent, or dermal application of a treatment for a skin ailment such as psoriasis) require special thinking in order to design an appropriate test for both potential systemic as well as local exposure.

What is the systemic exposure of the drug?

Blood and sometimes urine samples are collected at various times during a study from either toxicology animals on study or from 'satellite' groups of animals dosed and housed with the toxicology animals. These are analysed for drug and any known active metabolites and pharmacokinetic parameters (such as C_{max}, AUC and half-life) determined as measures of systemic exposure over time. As these values are then associated with any observed

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toxicology findings, the term toxicokinetics is now applied to this form of analysis. C_{max} and AUC can be used instead of dose to estimate the safety margin and provide guidance for early dose-escalation studies in humans.

Toxicokinetic assessments can also show whether systemic exposure is affected by the presence of food at or near the dosing time, by the administration of drug relative to the light/dark cycle (rodents are nocturnal creatures) and by the duration of administration or by the route of dosing.

There is renewed interest in sparse sampling paradigms that can be applied to assess systemic exposure more intensively in the actual animals used in toxicology assessments. Usually, removal of anything but small amounts of blood will compromise the integrity of the toxicology study, hence the need for satellite groups of animals. Modern LC/MS methodology has dramatically improved the lower limits of assay sensitivity to low picogram per millilitre ranges, thereby making small sample volumes (10–50 μl) practical. Sparse sampling data sets fit nicely with population-based pharmacokinetic analyses.

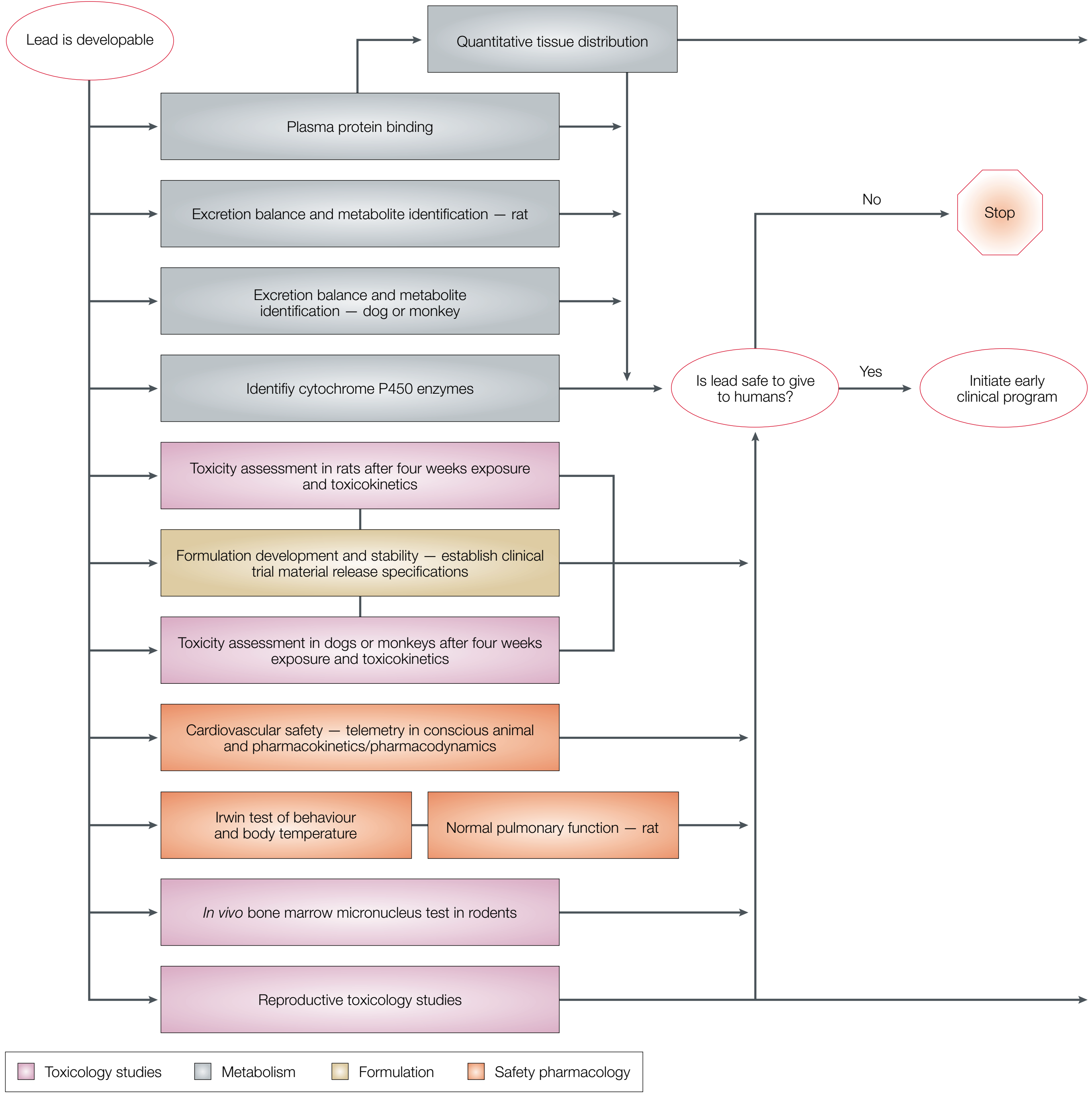

Figure 3 | **Potential flowchart for assessing whether a small-molecule new drug candidate is safe to give to humans.**

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Table 3 | **Requirements for additional work: key questions and pivotal studies**

Questions	Studies that provide answers
Where does the drug go in the body; how long does it or its metabolites stay there, and by which routes are it and its metabolites excreted?	Tissue-distribution studies in rats using radiolabelled drug. Whole-body autoradiography, usually in rats.
Is there a potential for untoward clinical drug-drug interactions (for example, enzyme inhibition or induction)?	Cytochrome protein inhibition kinetic studies. Induction studies with human cultured hepatocytes. Effects on reporter gene constructs.
Does the drug bind to plasma proteins and erythrocytes? If so, how does this affect interpretation of pharmacokinetic data derived from concentration measurements of parent drug in plasma?	*In vitro* studies in plasma or serum of relevant species including human that has been seeded with radiolabelled drug and free drug separated from bound drug using equilibrium dialysis, ultrafiltration, or chromatography. *In vitro* studies in blood seeded with radiolabeled drug and erythrocytes separated by centrifugation.
Does the drug affect reproductive performance in female rats?	Segment I reproductive toxicology studies in rats.
Is there evidence of teratogenicity, mutagenicity or embryo toxicity *in vivo* in rodents?	Segment II reproductive toxiciology studies in rats and rabbits.
Does the drug irritate the gastrointestinal tract or other sites of administration?	Injection site irritation studies. Gastrointestinal motility and gastric irritation studies in rats.

Does the drug affect vital functions?

Safety pharmacology studies are designed to assess any effect on vital functions. The three studies expected by regulators, review boards and investigators involved in approving early clinical studies address the cardiovascular, central and peripheral nervous systems, and the respiratory system. Recent regulatory consensus places a great deal of emphasis on the *in vivo* cardiovascular pharmacology safety study to provide assurance that the NCE does not cause QT interval prolongation[52]. Nevertheless, although *in vitro* screens are reasonably effective in eliminating compounds that change cardio-electrophysiological properties, these can no longer form the sole preclinical assessment of cardiovascular safety.

What are the ADME characteristics of the drug?

How does the body dispose of the drug? To collect information about how much and how fast a small-molecule drug product gets into and out of the body of animal species used in toxicology testing, an appropriately radiolabelled (and sometimes stable-labelled) drug is administered and the amounts of radioactive excreta and blood quantified[53,54]. Ideally, if >90% of the originally administered radioactive dose can be recovered, then the profile of how fast and by what routes a drug and its metabolites are excreted in that species will be known. The samples can also be further analysed for metabolites that would better define how much of the total radioactivity measured in each sample is attributable to the parent drug or its various metabolites. These data can be extremely useful in associating presence of parent drug or metabolite with toxicological or pharmacological effects.

Where is the drug distributed? An important feature to know for many new drug candidates is where the drug goes in the body and how long it stays in specific tissues or organs. These data can then be related to tissue-specific pharmacology or toxicology.

In vivo rodent models are usually used to quantify amounts of drug distributed into tissues, as results from such studies are generally believed to reflect tissue-distribution characteristics of a drug in humans. Although this information is not essential before early clinical studies, it is an expectation for marketing drug applications (for example, New Drug Applications or Marketing Authorization Applications). These studies are conducted by administering the radioactive drug, and the measurement of radioactivity in up to 60 tissues by either direct counting or sample combustion or by a whole-body scanning technique called whole-body autoradiography. GAMMA SCINTIGRAPHY and POSITRON EMISSION TOMOGRAPHY (PET) have also been applied to animal tissue distribution studies[55,56]. By including such radiosensitive tissues as gonads, thyroid and eyes, radioactive dosimetry is calculated that estimates the radiation exposure to each type of tissue. Dosimetry calculations are required before a radiolabelled drug can be given to humans, an approach that is often considered to define human ADME for market registration.

Plasma-protein and red-blood-cell binding is another key determinant of drug distribution and disposition, although it is rarely a factor in assessing safety and efficacy[9,57]. High plasma-protein binding is an issue for some drugs, such as anti-infective agents, for which a target concentration of unbound drug sufficient to kill the microorganism is the goal of therapy. However, high binding can also stabilize or solubilize a drug product in blood. Moreover, if the affinity for the receptor or enzyme target is higher than the affinity for the binding proteins, then an effective system exists for delivering the drug to the target without it dispersing throughout the entire body fluid compartment.

Efflux and uptake transporters present in many tissues of the body, including the intestine, liver, kidney and brain, can significantly influence the absorption and distribution of many drugs. One efflux protein in particular, P-glycoprotein (P-gp or MDR1), has been well studied, and *in vitro* models have been developed for identifying compounds that are P-gp substrates[58]. Because the multidrug resistance that develops towards many cancer chemotherapeutic agents is attributed to overexpression of P-gp in tumour cells,

GAMMA SCINTIGRAPHY
Gamma scintigraphy is a non-invasive method of examining the deposition of a compound in the test subject's body using standard radiolabelling techniques. A computerized image of γ-emissions displays overall product deposition, including differentiation of test article concentration.

POSITRON EMISSION TOMOGRAPHY
(PET). A method for imaging that measures changes in blood flow associated with brain function by detecting positrons emitted by radioactively labelled substances that have been injected into the body.

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carcinoma-derived cell lines such as Caco-2 can be used for examining the interaction of P-gp with drug candidate compounds[59]. Stably transfected cell lines expressing important efflux and uptake transporters are being developed for implementation in drug discovery and development screening programmes.

Will the drug affect or be affected by other drugs? Compounds that rely on only one metabolic pathway for elimination, or which are potent inhibitors (or, less importantly, inducers) of a key CYP enzyme, would be at high risk for adverse drug–drug interactions in human clinical studies. The kinetics of human CYP enzyme inhibition can be readily measured *in vitro*. Coupled with information on the putative therapeutic drug concentration range in plasma, the impact of CYP inhibition *in vivo* can be estimated[60].

Although a drug might inhibit metabolizing enzymes, it can also induce the synthesis of a new functional protein, resulting in increased clearance of co-administered drugs, which reduces their efficacy. For example, the herbal remedy St John's Wort contains hyperforin, a chemical that induces the CYP3A4 enzyme and which has been associated with reduced efficacy of many clinically important drugs[61].

Rat enzyme induction is not predictive of human induction, so *in vivo* or *ex vivo* animal studies are wasteful unless they explain changes in animal toxicity after repeated dosing. Human-based *in vitro* models designed to investigate enzyme induction use longer-term monolayer cultures of human hepatocytes, and the subsequent measurement of messenger RNA, protein expression or enzyme activity after exposure to the test drugs[62]. However, limited availability of fresh human liver, and complex isolation procedures, has led to poor reproducibility with this approach. Recent advances in the understanding of the induction mechanisms for the CYP3A and CYP1A families in particular have enabled the development of higher-throughput and more reliable induction assays that use reporter-gene constructs[63–66].

Are additional studies required?

An understanding of the safety margin in animals and of the pharmacokinetic properties of the drug in these species forms the rationale for identifying a suitably safe starting dose for first-time-in-human studies. This is also a good time to advise the clinician of any particular surrogate markers of toxicity to monitor during the clinical trials. For example, a common scenario is to pay particular attention to increases in circulating liver enzymes in the human studies on the basis of hepatotoxicity observed at high doses in animals. Once the clinical trials are underway, the animal toxicologist is often ignored in setting up and evaluating clinical safety databases for a new drug product. This crucial dialogue has the potential for significant learning and synergy between the non-clinical and clinical drug development scientists who specialize in drug safety assessment.

Once the compound enters into longer-term clinical studies, additional highly regulated toxicology studies become necessary (TABLE 4). These include longer-term repeat-dose studies lasting up to six months in rodents, and nine or twelve months in non-rodents, again mimicking the duration of dosing planned for clinical trials. Standardized reproductive toxicology tests (Segments I and II if not done earlier and then Segment III) are required to enable long-term administration to women of child-bearing potential.

Often, the significance of metabolites is not appreciated in early studies but is known by the time these long-term toxicology studies are initiated. It is expected that the contribution of any major and potentially active metabolite will be assessed during toxicokinetic evaluations of these studies and submitted as part of the marketing registration package[67].

Carcinogenicity studies examine whether the drug is carcinogenic when given for a lifetime[68,69]. These 'bioassay' studies typically involve rat and mouse species, dosed for their lifetime of around two years. These studies are large, long and expensive, and tend to be performed later in the life of a drug candidate. Despite best efforts at trying to eliminate potentially carcinogenic compounds as early as possible, some still occasionally prove positive at this stage. Recently, a compound targeting the peroxisome proliferator-activated receptor-γ (PPAR-γ) family was shown to be positive in the carcinogenicity bioassay study[70], causing the drug to be dropped during Phase III trials after many millions of dollars had been spent on the drug development programme. (In July 2002, Novo Nordisk suspended clinical development of its dual-acting insulin senzitiser ragaglitazar (NN622) because of findings of urine bladder tumours in mice and rats treated with the drug.)

Table 4 | **Market readiness: key questions and pivotal studies**

Questions	Studies That Provide Answers
What toxicology arises following long-term administration of the drug?	Six-month to one-year toxicology studies in a rodent and non-rodent species.
Is the drug carcinogenic?	Lifetime exposure studies in rat and/or mouse.
What is the complete metabolic fate of the drug? Is there a contribution to efficacy or toxicity by active drug metabolites?	Characterization of complete metabolic profile in humans and animal species used in toxicology testing. Radiolabelled drug often used to trace drug products.
What potential drug–drug interactions can be excluded based on knowledge of the drug's interaction with human drug-metabolizing enzymes or membrane transporters?	Identification of drug-metabolizing enzymes responsible for new chemical entity metabolism. *In vitro* drug–drug interaction studies using human enzymes.

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Drugs are getting bigger

In addition to the impact of technology on the process of building predictable and timely knowledge about a new drug candidate, new drug entities themselves have undergone a significant renaissance, for several reasons. First, as most relatively small molecules have already been patented, the majority of newer drug candidates have higher molecular masses, leading to greater challenges in formulation and bioavailability as a result of their inherent lower solubility. Second, advances in our knowledge of receptors and enzymes have made drug targets more specific; for example, many drugs targeting the central nervous system are designed to bind to a specific brain receptor subtype without affecting other similar receptors. Third, significant advances in genetics and proteomics have led to a wide variety of novel potential therapeutic products. Approximately half of the therapies in development, and over a quarter of new medicines approved for marketing in 2002, are macromolecules largely from biological sources, rather than small-molecule synthetic drugs[71].

Sometimes, the rationale for preclinical testing does not conveniently fit the properties of these larger molecular agents. For example, immunogenicity is a key safety issue for larger molecules, but how relevant are animal toxicity models when the antibody, peptide or recombinant protein has been specifically engineered to affect human targets? Moreover, the metabolism of protein products often results in the incorporation of amino acids into endogenous molecules. It is difficult to trace the fate of metabolites that themselves are normal body constitutents. Building a consensus on what predictive tests are appropriate at the preclinical stage for these molecules will be a future challenge for drug developers.

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