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INTRODUCTION: HISTORY OF METABOLITE SAFETY IN DRUG DEVELOPMENT

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1.1 PEOPLE, EVENTS, AND REACTION

Change, whether social or technological, often is catalyzed by a convergent set of events and influences. Drug testing using animals became important in the twentieth century. In 1937 a preparation of sulfanilamide, using diethylene glycol (DEG) as a solvent and called the preparation "elixir sulfanilamide," was marketed. DEG is highly toxic and the preparation led to the deaths of more than a hundred people. No animal testing had been conducted, a step that would have highlighted the risks. The public outcry caused by this incident led to the passing of the 1938 Federal Food, Drug, and Cosmetic Act requiring safety testing of drugs on animals before they could be marketed. This event would have remained perhaps as a footnote, with drug metabolism remaining an academic pastime of minor note, but further events and people would catalyze dramatic change.

Richard Tecwyn Williams was one of the founding scientists in the systematic study of the metabolism of chemicals including drugs. He detailed this in a major book *Detoxication Mechanisms* which was published in 1947. His work led to the widely adopted phase 1 (oxidation, hydrolysis, etc.) and phase 2 (conjugation) divisions of drug metabolism. Following his appointment in 1949 to Chair of Biochemistry at St Mary's Hospital Medical School in London, a growing group of

researchers studied in the field. The group focused on many aspects of drug metabolism, but species differences in metabolism became a key topic, building on observations described in the aforementioned book. For instance, in 1956 together with Parke, he published a paper describing "species differences in the ortho-hydroxylation and para-hydroxylation of aniline." As part of his later research activities (Schumacher et al. 1965), he examined the metabolism of thalidomide (a key component in the changes; see as follows) showing hydrolysis products and hydroxylated metabolites in various species, but a constant theme was species differences. Typical findings of Williams' group are exemplified by studies with sulphamethomidine (Bridges et al. 1969). In the rat, rabbit, and monkey, the main metabolite was the N^4-acetyl derivative. In man, the major metabolite was the sulphamethomidine N^1-glucuronide, which was also excreted by the monkey but not by the rat or rabbit. Many other drugs were examined, all leading to descriptions of species differences of a major or minor form such as amphetamine, methamphetamine, etc. (Caldwell et al. 1972). In many of these publications, the use of ^{14}C labeled drugs was used to obtain detailed results. The studies created an awareness that the products of metabolism excreted by animals could differ markedly from those excreted by man. Again this course of research could have remained an academic pursuit, but events dictated otherwise.

The drug thalidomide was first marketed in 1957 in West Germany under the trade name Contergan as a sedative. Generally very well tolerated it was prescribed for a number of CNS indications, but crucially it became commonly administered to counteract nausea and alleviate morning sickness in pregnant women. The drug was licensed out to other distributors and was widely used. A terrible consequence of its wide acceptance was the realization that thalidomide was a human teratogen. Throughout the world, about 10 000 cases were reported of infants with phocomelia (a malformation in which the limbs were absent or present as stumps), with a high morbidity (50% survival). Deformities were also observed in the eyes, hearts, and alimentary and urinary tracts. At this time there was no legal requirement for animal studies to evaluate risk in pregnancy, although they were already established, albeit without rigorous guidelines or protocols on species selection, duration, and size of dosing.

This drug tragedy led to a complete change in the way drugs could be marketed. For instance, in the United Kingdom in 1963, Sir Derrick Dunlop set up a committee to investigate the control and introduction of new medicines which resulted in the Committee on Safety of Drugs being established. This evolved into the Committee on Safety of Medicines. These moves paralleled the Kefauver Harris Amendment in the United States and Directive 65/65/EEC1 in the EU. The principal change was the enforcement that applicants needed to prove efficacy and to disclose all side effects encountered in testing prior to marketing approval.

1.2   THE RISE OF INDUSTRIAL DRUG METABOLISM

The metabolic fate of drugs in animals and man, in these times, was not a major focus or priority research topic for the innovator company. Illustrative of this period is propranolol, the first full antagonist of β-adrenoceptors which was discovered in 1962, first

marketed in 1964. Analytical methods for propranolol were first published in 1965 with techniques relying on the optical (fluorescence) properties of the drug, while it was not until 1967 (Bond 1967) that preliminary reports on its metabolic fate first appeared.

Williams' book title implied that metabolism was primarily a detoxification step, a concept that is still mainly correct. However growing evidence indicated that metabolism could also lead to toxicity. These observations concerned metabolites which were intrinsically reactive. Pioneering work on the carcinogenicity of polycyclic aromatic hydrocarbons and other planar heterocyclic aromatic compounds (Boyland 1969; Ames et al. 1972) had shown that the reactive metabolites were the ultimate toxin. This finding was broadened by early studies of liver necrosis in rodents. Many studies demonstrated that enhanced toxicity was associated with induction of liver enzymes by agents such as phenobarbital and attenuated toxicity with the inhibition of drug-metabolizing enzymes by agents such as SKF525A. Radiolabeled studies showed that this toxicity was accompanied by the irreversible covalent binding of drug-related material. By the 1970s Gillette et al. (1974) was able to establish that cellular necrosis, hypersensitivity, and blood dyscrasias could result from the formation of reactive metabolites.

The increased focus on drug safety, the realization that species differences could occur, and the concept that drugs could be metabolized to reactive metabolites and bind to organs, combined to lead to the decision that part of drug safety should have a drug metabolism component. Thus toxicology species and eventually human would be examined for overall excretion of the drug and its products, evidence that the drug had not been sequestered in the body (of animals) and a view as to what metabolites were produced. Metabolite profiling, where urine and feces extracts were compared quantitatively between species and eventually human, by techniques such as thin-layer chromatography became common practice. These experiments were conducted after the synthesis of a ^{14}C- or ^{3}H-labeled version of the candidate drug of interest. These early studies lacked sophistication as methodology was not advanced. Identifying metabolites required considerable workup of fairly large quantities. *In vitro* reagents such as human microsomes and hepatocytes were not available to allow early species comparisons. The principal focus was on excreted metabolites as evidenced with work on propranolol. The determination that 4-hydroxypropranolol, a major circulating metabolite of propranolol, had pharmacological activity equivalent to the parent was not made until 1971 (Fitzgerald and O'Donnell, 1971), 7 years after its launch.

Studies tended to report the identity of excreted metabolites. Circulating drug-related material was often reported as the proportion of parent present in the total radioactivity measured in plasma. Regardless of dose, results were quoted as percentage and not absolute amounts. Typical of this is isoxepac (6,11-dihydro-11-oxodibenz[be]oxepin-2-acetic acid) which Illing and Fromson reported in 1978. The disposition was studied in rat, rabbit, dog, rhesus monkey, and human. Fecal excretion of radioactivity occurred in the rat (26–37%) and dog (33–49%), whereas in the other species elimination was mainly urinary (<83%). Biliary excretion accounted for 18–52% of the dose in the rat and dog. Enterohepatic circulation was demonstrated in both species. Plasma of all species was found to contain mainly unchanged isoxepac.

The compound was rapidly eliminated from plasma of dog, rhesus monkey, and man but was more slowly eliminated in rat and rabbit. In the rabbit and dog the principal metabolites were the glycine and taurine conjugates of isoxepac, respectively, whereas in the rhesus monkey and man, isoxepac was excreted unchanged or as the glucuronide. This species difference was highlighted even though the metabolites were conjugates. It is arguable that emphasis was put on species differences to justify publication. The emphasis from excreted metabolites to both circulating metabolites and excreted metabolites was a gradual process over the next decade, and authors began to state more implications for their findings. For instance, the disposition of amlodipine, R,S,2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine, was reported in 1998 by Beresford et al. and showed renal elimination was the major route of excretion. Only pyridine metabolites of amlodipine were excreted in urine (apart from a small amount of parent). The major excreted metabolite was 2-([4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-2-pyridyl] methoxy) acetic acid representing 33% of urinary radioactivity. The data indicated that oxidation of amlodipine to its pyridine analog is the principal route of metabolism with subsequent metabolism by oxidative deamination, de-esterification, and aliphatic hydroxylation. In the circulation amlodipine concentrations in plasma declined with a mean half-life of 33 h, while slower elimination of total drug-related material from plasma was observed. Importantly only amlodipine and pyridine metabolites were found in the circulation. As these pyridine derivatives have minimal calcium antagonist activity, the authors were able to conclude that the efficacy of amlodipine in man was due solely to the parent drug.

1.3   THE APPEARANCE OF MIST

Debate continued as to which was the more important matrix to study for metabolism, a debate often confused by the scientific objectives versus the technological challenges. The ad hoc approach to metabolism studies and comparison with animals continued until a unifying force appeared. In 2002 a report (Baillie et al. 2002) was published under the title *Drug Metabolites in Safety Testing* summarizing the deliberations of a multidisciplinary committee, sponsored by the Pharmaceutical Research and Manufacturers of America, on current "best practices" within the US pharmaceutical industry in assessing the role of drug metabolites as potential mediators of the toxicity of new drug products. A workshop was held in 2000 and the collective advice of members of the pharmaceutical industry, academic investigators, and representatives of regulatory was assembled. The paper set out to define practical and scientifically based approaches to the use of metabolite data that responded to issues in the safety evaluation of drug candidates. The report stated that consensus was lacking on how best to deal with several aspects and still case by case analysis was vital. Importantly the document did propose situations in which metabolites should be further studied to help define risk assessment for the parent drug. The primary trigger suggested was if the metabolite is present in humans at 25% or more of the total drug-related material in the circulation, it would require further investigation as a potential contributor to safety findings.

This figure was based on the need for a defined limit and on pragmatic considerations of the technical feasibility of radiometric methods of quantitation, the results of which were almost always presented as percentage rather than absolute amounts. The title of the paper led to this branch of pharmaceutical sciences being branded metabolites in safety testing or more usually MIST.

In responding to this paper, the FDA produced a draft guidance, while a number of authors produced independent manuscripts. The FDA's initial draft focused on 10% of the amount of drug. This was based on the toxicity of drugs such as halothane and paracetamol where the toxic metabolite was around 10% of the dose. Dialog on this and other aspects were presented by Smith and Obach in 2005 who proposed that absolute abundance criteria be used rather than relative abundance. The absolute abundance of a metabolite in circulation or excreta in humans should be combined with other information regarding the chemical structure of the metabolite (e.g., similarity to the parent drug, presence of chemically reactive substituents, or downstream products of reactivity) and potential mechanisms of toxicity (e.g., suprapharmacological effects or secondary pharmacological effects). Various redrafts that were discussed with the International Conference on Harmonization: Guideline on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (ICH M3[R2]) describe the threshold as 10% of total drug-related exposure. Metabolites above this threshold need to be qualified in preclinical toxicity testing by demonstrating similar exposure based on absolute amount (Robison and Jacobs, 2009). The case by case analysis now includes consideration of the metabolite type (e.g., *N*-glucuronides which are known to be preferentially formed in human but have no association with toxicity), protein binding, and actual concentrations observed in the clinic and safety testing rather than pure reliance on percentage.

1.4   THE JOURNEY TRIGGERED BY THALIDOMIDE: WOULD PRESENT SCIENCE HAVE MADE A DIFFERENCE?

At the outset of this chapter, we discussed the impact of thalidomide (Figure 1.1) on drug regulation and the trail to MIST. The question can be asked as to how well placed we are now to avoid another thalidomide and how present thinking would have impacted its development.
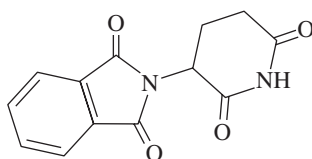

FIGURE 1.1 Structure of thalidomide. Source: http://chemwiki.ucdavis.edu/Organic_Chemistry/Organic_Chemistry_With_a_Biological_Emphasis/Chapter_03%3A_Conformations_and_Stereochemistry/Section_3.3%3A_Stereoisomerism_%E2%80%93_chirality,_stereocenters,_enantiomers. http://creativecommons.org/licenses/by-nc-sa/3.0/us/.

Thalidomide shows species-specific effects on the developing embryo. Rodents rarely show birth defects even at high oral doses. Rabbits show teratogenic effects and their sensitivity to thalidomide is a major reason for their adoption as a pivotal species in reproductive studies. Clinically the drug was used as a hypnotic and sedative at dose from 25 to 200 mg (0.3–3 mg/kg). Even a single administration was thought to be sufficient to elicit malformations. To illustrate the range of effects in animals, the most sensitive species (Fratta et al. 1965), New Zealand White rabbits, showed dose-dependent fetal anomalies after administration orally of 50, 150, and 300 mg/kg (dosed from 4 to 16 days of gestation). In contrast CF_1, ICR, SJL, CBA, and C_{57} mice did not produce malformed fetuses after oral dosing of 200 mg/kg. Rats (Long–Evans and Dunning–Fischer) and hamsters show no effects on fetuses at 150 mg/kg. At the same dose arachnodactyly was noted in 8% of the Dunning–Fischer inbred rats. Species differences such as this may suggest metabolism differences.

The hypothesis that thalidomide causes birth defects by being metabolized to a toxic electrophilic intermediate was advanced by Gordon et al. (1981). Supporting data that showed hepatic microsomes from pregnant rabbits mediated the production of a metabolite that was toxic to lymphocytes. Toxicity was enhanced by inhibitors of epoxide hydrolase and abolished by adding the purified enzyme to the incubation medium. The toxic metabolite of thalidomide was not produced by rat liver microsomes but was produced by hepatic preparations from monkey and human fetuses.

A more specific mechanism emerged a decade later. A study in 1994 (D'Amato et al. 1994) showed that orally administered thalidomide was an inhibitor of angiogenesis in a model using a rabbit cornea micropocket assay. The finding that this effect was only produced after oral administration indicated to the authors that an active metabolite was the causal agent. If confirmed the possibility of an unqualified human metabolite or one disproportionate to amounts formed in rodents would be a possibility. This route specificity and metabolite formation are not borne out by studies comparing the teratogenic potency of thalidomide has in rabbits and rats (Schumacher et al. 1968) after intravenous administration. After oral administration thalidomide was markedly teratogenic in rabbits but not rats. After intravenous injection of the drug, teratogenic effects were observed both in rabbits receiving daily doses as low as 2.5 mg/kg and in rats receiving doses of 10 mg/kg.

The evolution of technology allowed detailed metabolism studies to be performed. Metabolism studies (Chung et al. 2004) performed *in vivo* indicate that hydrolysis products are observed in humans and these are present in rabbit and mice. These hydrolysis products are formed spontaneously as well as enzymatically. Rabbits and mice also form hydroxylated metabolites which can also form glucuronides in mice. Mice appear more capable of oxidation than rabbits (Chung et al. 2004). This *in vivo* finding is also confirmed in *in vitro* studies (Lu et al. 2004) in which 5-hydroxy thalidomide was readily formed by mice and rabbit microsomes but only formed in trace quantities by human microsomes. The low rate of oxidation of thalidomide had been shown previously by Eriksson et al. (1998) who showed after incubation of thalidomide, with human liver S9, slow formation of the 5-hydroxy and 5′-hydroxy metabolites. In human volunteers who had received thalidomide, only the 5′-hydroxy metabolite was found in plasma samples. These detailed studies strongly indicate

that metabolic differences do not account for species sensitivity toward thalidomide, and with the abundance of the unchanged drug in human, thalidomide itself is the causal agent.

In 2010 Ito and colleagues demonstrated that thalidomide itself initiated its detrimental effects on the embryo by binding to a protein (cereblon) and inhibiting the associated ubiquitin ligase activity. Cereblon forms an E3 ubiquitin ligase complex which ubiquitinates a number of other proteins resulting in reduced levels of fibroblast growth factor 8 (FGF8). FGF8 regulates a number of developmental processes, such as limb and auditory vesicle formation. Thus thalidomide binding to cereblon results in deleterious effects on limb outgrowth in embryos.

This important finding did not deter continued focus on reactive toxic metabolites, even using very sophisticated animal models (Yamazaki et al. 2011). For instance, in experiments with chimeric NOD–*scid* IL2Rg[null] mice with humanized livers, after oral administration of racemic thalidomide, plasma concentrations of 5-hydroxythalidomide were significantly higher in humanized mice than in control mice. A glutathione conjugate was detected in the plasma. The results were interpreted as thalidomide activation occurring and of significance to its toxicity. It is likely that continued experiments will be published like this, even though cereblon binding of the drug itself seems to be a compelling mechanism.

Assuming that it is the parent drug which is the toxic species and the various reactive metabolite findings are not part of the mechanism, three possible scenarios could explain the species sensitivity. The species differences in teratogenicity could be explained by species differences in cereblon expression or structure leading to different affinities for thalidomide, species differences in the pharmacokinetics of thalidomide, or the downstream consequences of cereblon expression on the growing fetus.

Much more is now known about the receptor for thalidomide cereblon (Lopez-Girona et al. 2012; Fischer et al. 2014; Hartmann et al. 2014). This has been stimulated by its efficacy in diseases such as cancer. There is very strong conservation of the cereblon sequence between species. The actual ligand-binding domain, an aromatic cage of three tryptophan residues, has a high degree of conservation in both bacteria and eukaryotes. Thalidomide mimics and competes for the binding of uridine. It has binding affinity (K_d) of 250 nM. This suggests that the expression or structural differences in cereblon do not explain the species differences. The various pharmacokinetic properties for mouse, rabbits, and humans are compared in Table 1.1. Plasma protein binding of the drug is moderate with around 60% bound. While human could be rationalized as the most sensitive species with a single dose (200 mg) potentially inhibiting cereblon for 24 h, the data does not discriminate between teratogenic doses in rabbit (50 mg/kg) and nonteratogenic doses in mouse (200 mg/kg).

At this stage it suggests that there are differences between rodents, rabbits, and primates in the importance of cereblon and/or protein substrates modified by cereblon activity during embryonic development at different stages. The previous text indicates the difficulty in interpreting animal safety data. A detailed discussion of thalidomide and the value and limitations of animal testing, in particular reproductive studies, has been authored by Greek et al. (2011) and suggests the thalidomide disaster has not

TABLE 1.1   Pharmacokinetic Parameters Calculated from the Data Presented by Chung et al. (2004)

	Dose (mg/kg)	$T_1/2$ (h)	C_{max} (µM)	C_{av} (0–24 h) (µM)	Time above K_d (h)
Man	3	5	5	3.3	>24
Mouse	200	1	120	18	12
Rabbit	50	1.5	45	8	11

been repeated because of extreme caution in administering drugs to pregnant women rather than extensive animal testing. The review highlights the need to put into context the data from complex systems with different evolutionary systems. The authors suggest revision of the Federal Food, Drug, and Cosmetic Act including the Kefauver Harris Amendment based on current scientific knowledge. What exact revision is required is not stated, but it raises the question that if animal safety studies have high degrees of uncertainty in human prediction, does making them more complex add or diminish the uncertainty? Clearly thalidomide would have been detected in New Zealand rabbits under present protocols, but for another mechanism it is possible that similar species differences in response, but with different species, could give negative findings in reproductive testing, and the sole barrier is the aforementioned extreme caution in administering drugs to pregnant women. The focus on metabolism has continued for over 20 years and probably will continue even though a mechanism implicating the parent drug is established. Most drugs will form reactive metabolites, and with the sensitive systems employed today, they will be detected under the right conditions. In an analysis of the top 200 US prescribed drugs, approximately half contained one or more alerts in their chemical architecture to the possibility of generating reactive metabolites and in subsequent *in vitro* studies were found to form them (Stepan et al. 2011). Clearly drug metabolism can contribute to drug safety assessment, but it has to ensure that its results are not misleading or distracting from true mechanisms and species sensitivity. These are highlighted by comparison of pharmacokinetic parameters such as unbound drug concentrations across species as shown in Table 1.1.

1.5   KEY EVENTS FROM THALIDOMIDE TO MIST

A rapid advancement of understanding oxidative metabolism and in particular cytochrome P450 (CYP) allowed the variability in pharmacokinetics of drugs and their pharmacodynamics to be rationalized. The pivotal catalyst was the discovery (Mahgoub et al. 1977) of poor metabolizers (PMs) and extensive metabolizers (EMs) of debrisoquine, which was linked to the presence or absence of its hydroxylated metabolite in urine. Because not all drugs were affected in this way in the individuals, the concept of multiple human CYPs was developed. Concerted efforts to purify the enzyme(s) were initiated. The emerging clinical data and the purified enzymes revealed isoforms with broad substrate selectivity. Genetic sequences led to the

multiple forms of CYP450 being classified into families (Nebert and Gonzalez 1987). Family 1 (CYP1) enzymes catalyzed the metabolism of many carcinogens and drugs. CYP2 and CYP3 catalyzed the metabolism of drugs; CYP4 lipids and other forms were involved in steroidogenesis.

The metabolism of debrisoquine was shown by an isoform now termed CYP2D6 which also metabolized the hydroxylation of beta-adrenoceptor blockers, class 1 antiarrythmics, tricyclic antidepressants, etc. These drugs all were ionized cations at physiological pH, and the position of hydroxylation was positionally similar in all the molecules. PM status was conferred by several "loss of function" alleles of the CYP *CYP2D6* gene. "Ultrarapid" metabolizers were also identified, caused by duplication or amplification of the active *CYP2D6* gene. Therapeutic failure changes, or toxicity to metabolites (see later), of CYP2D6 substrates could be associated with the ultrarapid status. Study of CYP2C9 revealed substrates such as losartan, phenytoin, tolbutamide, and torsemide. Again polymorphisms in the coding region of the *CYP2C9* gene produced protein variants. Individuals with one of the variants (Leu359) have markedly diminished metabolic capacities for most CYP2C9 substrates and again can explain side effects but, equally important, diminished pharmacological response to drugs which are converted to active metabolites such as losartan (Miners and Birket 1998). CYP3A4 was identified as the most abundant and the isoform metabolizing the most marketed drugs and although highly variable in expression did not show the same polymorphisms.

Such advances helped to predict and understand drug variability *per se* and that caused by conmeds. The characterization of the isoforms allowed a rationalization of many drug–drug interactions. Warfarin often had raised concentrations and enhanced pharmacodynamics when administered with some nonsteroidal anti-inflammatory drugs (NSAIDs). The conventional belief was this was due to protein binding displacement. Enzymological studies conclusively proved it was inhibition of CYP2C9 (a major clearance enzyme for warfarin and NSAIDs) that caused the interaction.

A turning point in the science of drug interactions was created by terfenadine which was marketed as a nonsedating antihistamine. Until the problems with this drug were resolved, defining route of clearance and enzymology, if clearance was by metabolism, were not major priorities. In addition the impact of conmeds was not studied in a concise and detailed manner. Terfenadine would have been sedating (a lipophilic amine), but the pharmacologically active species was its zwitterionic metabolite fexofenadine, which had high selectivity for the H1 receptor and poor brain penetration. Terfenadine was very rapidly converted to this metabolite, and concentrations of the parent drug in the circulation were very low. Like many lipophilic amines terfenadine exhibited polypharmacology, and in addition to activity at the H1 receptor, the drug was a potent inhibitor of the I_{Kr} channel. Conversion to fexofenadine was by CYP3A4 (Yun et al. 1993), and coadministration with potent inhibitors of CYP3A4 such as ketoconazole or erythromycin (Honig et al. 1993) leads to elevated concentrations of terfenadine, which led to prolongation of QT in some individuals and in a small proportion of these fatal cardiac arrhythmias.

The biological response to a drug can be viewed in terms of on-target in which the drug or its metabolites interact with the desired target and off-target in which the

drug or its metabolites interact with undesired receptors or enzymes. This concept of receptors includes reversible interactions with proteins and lipid bilayers and irreversible interactions where a protein is adducted, which may in itself be critical or be part of an immune response or a region of DNA critical to a cellular process. In this definition drug interactions triggered by enzyme or transporter inhibition are part of the off-target pharmacology of a drug. Both on- and off-target interactions can elicit toxicities. On-target side effects (suprapharmacological effects) arise as receptor occupancy or enzyme inhibition becomes too great or occupancy or inhibition occurs for too extended a period of time or affecting a receptor in a tissue that is not the tissue targeted for therapy. Factors that can cause this are poor dose selection and individual variability including drug metabolism enzyme or transporter polymorphism.

The example of tramadol illustrates how inter-individual variability can lead to on-target side effects (Gleason et al. 1997). The drug is a centrally acting analgesic which inhibits monoamine transporters and also acts as an agonist of μ-opioid receptors. The parent molecule has both activities, but the μ-opioid receptor agonist activity principally resides in its *O*-demethylated metabolite formed by CYP2D6. Structural comparison with morphine shows that the demethylated hydroxyl group is spatially identical to the essential 3-hydroxy group of opiate agonists such as morphine. Normally the drug is well tolerated; however, a patient, following a 100 mg dose of tramadol, rapidly developed ataxia, dilation of the pupils, limb numbness, tremulousness, and dysphoria. The patient was phenotyped for CYP2D6 activity and was found to be an extensive (super)metabolizer, with very high CYP2D6 activity.

Off-target toxicity is elicited by drugs or metabolites that do not have sufficient selectivity over the target receptor at therapeutic concentrations. An example of this is binding to the I_{Kr} channel and resultant QT interval prolongation and possible cardiac arrhythmias as evidenced by the aforementioned terfenadine. A further example is provided by the appetite suppressants fenfluramine and dexfenfluramine (single enantiomer of fenfluramine). These drugs caused pulmonary hypertension and valvular heart disease in patients, leading to their withdrawal from the market. Fenfluramine and dexfenfluramine are not the active principle. The major circulating metabolite norfenfluramine (nordexfenfluramine) is responsible for the pharmacological activity. The metabolites of the drug bind and activate 5-HT_{2C} receptors, thereby reducing appetite. Norfenfluramine (nordexfenfluramine) is equally potent activating 5-HT_{2B} receptors. Activation of 5-HT_{2B} receptors on heart valves and pulmonary artery interstitial cells leads to the formation of proliferative fibromyxoid plaques that cause the toxicity (Fitzgerald et al. 2000; Rothman et al. 2000). This finding is shared by other drugs which activate at 5-HT_{2B} receptors such as ergotamine and methysergide. Both these drugs are implicated in valvular heart disease.

The period saw increasing findings about the role of reactive metabolites in toxicity. Despite the large amount of work conducted, results were sometimes conflicting. In almost all cases the results were circumstantial without a full link to the toxic event. For example, work was conducted on paracetamol which showed species differences in hepatic effects. Different studies produced conflicting results. The drug and its putative toxic reactive metabolite, NAPQI, were investigated in

hepatocytes from different species (Tee et al. 1987). Results from these studies showed acetaminophen triggered cell blebbing and loss of viability in the cells from mouse and hamster, whereas human and rat hepatocytes were much more resistant to these effects. When NAPQI, itself, was tested, there were no significant differences in the sensitivity of the cells, from any species. The conclusion reached in these studies was that species differences in sensitivity to the hepatotoxicity of acetaminophen were due to differences in the rate of formation of NAPQI and not to any intrinsic differences in sensitivity or in any difference in the fate of NAPQI once formed. In contrast monitoring downstream NAPQI metabolites (activation) including glutathione, cysteinylglycine, cysteine, and mercapturate conjugates and detoxification pathways such as paracetamol glucuronide and sulfate conjugates indicated species sensitivity being determined by the balance between toxification and detoxication metabolic pathways (Gregus et al. 1988).

It was said that the only difference between a drug and a poison is the dose. Paracetamol is hepatotoxic if a sufficient amount of the drug is taken. Of equal concern was low-frequency toxicity, which was also being ascribed to reactive metabolites possibly involving effects on the immune system. Furthermore such toxicity does not occur in most people taking the drug at any dose level, thus making these types of drug reactions virtually undetectable during the course of clinical trials as well as representing an exception to the previously mentioned drug/poison/dose anecdote. Halothane is one example. Massive liver cell necrosis can occur in 1 in 20 000 patients normally having past experience of halothane anesthesia, frequently leading to fulminant hepatic failure. Halothane is metabolized (Cohen et al. 1975) to three main excreted metabolites: trifluoroacetic acid (TFA), *N*-trifluoroacetyl-2-aminoethanol, and to a lesser extent *N*-acetyl-*S*-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine. The latter two metabolites are downstream products of reactive metabolites. They are formed by hydroxylation of halothane with spontaneous loss of HBr to form trifluoroacetyl chloride (TFAC). TFAC can acylate the lysine residues of proteins. CYP2E1 is the major catalyst in conversion of halothane to the reactive metabolite TFAC. High levels of autoantibodies that recognized CYP2E1 (Eliasson and Kenna 1996) were detected by an enzyme-linked immunosorbent assay in 14 of 20 (70%) sera from patients with halothane hepatitis. In contrast only very low levels of such antibodies were detected in sera from healthy controls, from patients anesthetized with halothane without developing hepatitis or from patients with other liver diseases.

A convincing piece of circumstantial evidence from the halothane story that supports the role of reactive metabolites in this type of toxicity is the relative amounts of TFAC formed after structural analogs of halothane are administered (Njoku et al. 1997). Isoflurane produces considerably less TFAC than halothane upon metabolism by CYP2E1 and is associated with significantly less liver failure. Desflurane, lacking a chlorine atom, produces little of the fluorine analog of TFAC, and hepatic toxicity is virtually eliminated (see Table 1.2).

Another example is clozapine-induced agranulocytosis. This example is interesting from the standpoint that the toxicological target are neutrophils or granulocytes (neutrophil precursors in the bone marrow) and not the liver. Furthermore, the

TABLE 1.2   The Metabolism of Halothane and Its Analogs to a Trifluoroacetyl Chloride Metabolite and the Relative Association of Each Anesthetic with Hepatic Failure

		Metabolism (%)	Toxicity
F Cl F−C−CH $\xrightarrow{\text{P4502El}}$ F Br Halothane	$\begin{array}{c} F \\ F-C-C{\Large\diagup}^{O}_{Cl} \\ F \end{array}$ Trifluoroacetyl chloride	20–50	Reversible and irreversible hepatotoxicity
F H F F−C−C−O−CH $\xrightarrow{\text{P4502El}}$ F Cl F Isoflurane	$\begin{array}{c} F \\ F-C-C{\Large\diagup}^{O}_{Cl} \\ F \end{array}$ Trifluoroacetyl chloride	<1	Rare hepatotoxicity
F H F F−C−C−O−CH $\xrightarrow{\text{P4502El}}$ F F F Desflurane	$\begin{array}{c} F \\ F-C-C{\Large\diagup}^{O}_{F} \\ F \end{array}$ Trifluoroacetyl fluoride	≪1	Even rarer hepatotoxicity

agranulocytosis caused by clozapine can cause death if the depletion of neutrophils is not detected in time, usually by an overwhelming infection stemming from the absence of bactericidal neutrophils. However in contrast to the hepatocyte, or other liver cells, the number and function of neutrophils are easily monitorable by a simple blood test which, if conducted regularly, leads to a favorable risk/benefit profile in susceptible people. In fact the drug remains on the market due to a successful (but costly) mandatory screening program that one must undergo while taking clozapine. If a reactive metabolite of clozapine is a necessary feature of the mechanism of clozapine-induced agranulocytosis, then the reactive metabolite, due to their short half-lives by definition, would need to be formed at or near the site of toxicity. While a number of oxidative enzymes are present in the neutrophil and its granulocytic precursors in the bone marrow, the quantitatively most important is myeloperoxidase (MPO) which is the key enzyme involved in generating the so-called oxidative burst. Upon stimulation (i.e., molecular recognition by cell-surface receptors pathogens, cytokines, etc.) the cell-surface NADPH oxidase converts oxygen to superoxide (O_2^-). This undergoes spontaneous and enzymatic conversion to hydrogen peroxide (H_2O_2) which then acts as a cosubstrate along with chloride ion for the production of the indiscriminant oxidant hypochlorous acid (HOCl) by MPO. Circulating drugs can be oxidized either by HOCl or act as a substrate to MPO. Although it is not proven that reactive metabolites are a necessary feature of drug-induced agranulocytosis, drug-modified MPO was detected from neutrophils of people taking clozapine as well as animals on a course of clozapine treatment (Gardner et al. 1998). Interestingly, olanzapine, a structurally similar molecule to clozapine, which generates a similar nitrenium ion reactive intermediate to a similar extent as clozapine *in vitro*, is associated with a significantly lower frequency of agranulocytosis (Uetrecht 1999; Iverson et al. 2002). The therapeutic dose in milligrams of olanzapine is about

100 times less than clozapine which means that there would be significantly less of the nitrenium ion reactive metabolite formed and thereby significantly less adduction to MPO. Consistent with this, olanzapine-modified MPO was not detected in the neutrophils or bone marrow precursors of people on a course of olanzapine treatment (Gardner et al. 1998).

The examples described earlier of halothane and clozapine are representative of the type of adverse drug reaction that is virtually impossible to predict with today's knowledge and are called idiosyncratic drug reactions due to their unpredictability. Quantitating metabolites generated by these drugs in the circulation does not offer predictive power since these reactions do not have a dose/exposure relationship in the vast majority of persons taking the drug. In other words, the reaction appears to be more dependent on the individual than on the amount of reactive metabolite generated. Furthermore, reactive metabolites are reactive and as such not detected in the circulating compartment. Reactive metabolites generally fall outside of the scope of a so-called MIST assessment; however they are nonetheless a subject of regulatory concern. Drug safety following marketing has continued to be a problem. Both the previously mentioned fenfluramine and terfenadine were highlighted as being withdrawn from the marketplace. Other drugs withdrawn over the period where metabolites (reactive) may have contributed to their toxicity include benoxaprofen, bromfenac, pemoline, ticrynafen, troglitazone, tolcapone and trovafloxacin (hepatotoxicity), nomifensine, remoxipride and temafloxacin (hemolytic and aplastic anemia), and zomepirac (anaphylaxis).

1.6   THE PURPOSE OF THIS BOOK

From the time of the enhanced role of regulatory authorities following thalidomide to the introduction of MIST guidelines, the role of drug metabolism had grown considerably, and a variety of drug side effects could be ascribed to not fully understanding the route of clearance (metabolism), the enzymology, the metabolic products, and the interactions that could occur in different patients on differing comeds. Not surprisingly regulatory bodies wished to ensure that metabolism should be considered from every standpoint in developing new medicinal products and be reinforced by guidelines. While MIST guidelines ask certain important questions, they provide guidance for only circulating, stable metabolites.

The aim of this book is to provide the reader with a comprehensive overview of why and how metabolites are studied during drug development in the pharmaceutical industry. There are several published resources describing what drug metabolites are and which enzymes generate them. However there seems to be a lack of practical "how to" compilations for the researcher in pharmacology and toxicology. Furthermore, this book attempts to give explanations for why the characterization and quantitation of metabolites are a necessary part of drug development, not only as a consequence of the mistakes of the past but also why a certain study is conducted as part of the regulatory package in one drug project but not the other. The chapters of this book will take the reader from a historical account of metabolites including

the emergence of regulatory guidance to all the way into the future, where metabolites and our remaining collective gaps in knowledge about them are described. The chapters between describe the examination of metabolites using *in silico*, *in vitro*, and *in vivo* systems in addition to the analytical instruments that aid in these investigations. The liver is not the only organ involved in metabolism, and drugs can be administered by other routes than intravenous and oral. The book includes a chapter on the study of metabolites generated in the skin, as topical medicinal agents are a growing market and skin is the largest organ of the body with the capacity to metabolize drugs.

Finally, actual cases from the course of drug development are presented. These cases demonstrate the thinking behind why certain studies are conducted while others are not for a particular drug project and furthermore why certain analytical tools are used while others are not used. These cases demonstrate that there is no one way to conduct an assessment of the safety of drug metabolites.

In short, this book is intended to provide a reference work covering all these topics in a comprehensive manner with an aim to providing clear guidance and consensus on the issues of the present day.

REFERENCES

B. N. Ames, P. S. Sims, and P. L. Grove. Epoxides of carcinogenic polycyclic hydrocarbons are frameshift mutagens. *Science*, 1972, 176, 47–49.

T. A. Baillie, M. N. Cayen, H. Fouda, R. J. Gerson, J. D. Green, S. J. Grossman, L. J. Klunk, B. LeBlanc, D. G. Perkins, and L. A. Shipley. Drug metabolites in safety testing. *Toxicol. Appl. Pharmacol.*, 2002, 182, 188–196.

A. P. Beresford, D. McGibney, M. J. Humphrey, P. V. Macrae, and D. A. Stopher. Metabolism and kinetics of amlodipine in man. *Xenobiotica*, 1998, 18, 245–254.

P. A. Bond. Metabolism of propranolol ("Inderal"), a potent, specific β-adrenergic receptor blocking agent. *Nature*, 1967, 213, 721.

E. Boyland. The correlation of experimental carcinogenesis and cancer in man. *Prog. Exp. Tumor Res.*, 1969, 11, 222–223.

J. W. Bridges, S. R. Walker, and R. T. Williams. Species differences in the metabolism and excretion of sulphasomidine and sulphamethomidine. *Biochem. J.*, 1969, 111, 173–179.

J. Caldwell, L. G. Dring, and R. T. Williams. Metabolism of [14 C] methamphetamine in man, the guinea pig and the rat. *Biochem. J.*, 1972, 129, 11–22.

F. Chung, J. Lu, B. D. Palmer, P. Kestell, P. Browett, B. C. Baguley, M. Tingle, and L. M. Ching. Thalidomide pharmacokinetics and metabolite formation in mice, rabbits, and multiple myeloma patients. *Clin. Cancer Res.*, 2004, 10, 5949–5956.

E. N. Cohen, J. R. Trudell, H. N. Edmunds, and E. Watson. Urinary metabolites of halothane in man. *Anesthesiology*, 1975, 43, 392–401.

R. J. D'Amato, M. S. Loughnan, E. Flynn, and J. Folkman. Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.*, 1994, 91, 4082–4085.

E. Eliasson and J. G. Kenna. Cytochrome P450 2E1 is a cell surface autoantigen in halothane hepatitis. *Mol. Pharmacol.*, 1996, 50, 573–582.

T. Eriksson, S. Björkman, B. Roth, H. Björk, and P. Höglund. Drug metabolism: hydroxylated metabolites of thalidomide: formation in-vitro and in-vivo in man. *J. Pharm. Pharmacol.*, 1998, 50, 1409–1416.

E. S. Fischer, K. Böhm, J. R. Lydeard, H. Yang, M. B. Stadler, S. Cavadini, J. Nagel, F. Seluca, V. Acker, G. D. Lingaraju, R. B. Tichkule, M. Schebests, W. C. Forrester, M. Schirle, U. Hassiepen, J. Otti, M. Hild, R. E. J. Beckwith, W. C. Forrester, J. Harper, J. L. Jenkins, and N. H. Thomä. Structure of the DDB1-CRBN E3 ubiquitin ligase in complex with thalidomide. *Nature*, 2014, 512, 49–53.

J. D. Fitzgerald and S. R. O'Donnell. Pharmacology of 4-hydroxypropranolol, a metabolite of propranolol. *Br. J. Pharmacol.*, 1971, 43, 222–235.

L. W. Fitzgerald, T. C. Burn, B. S. Brown, J. P. Patterson, M. H. Corjay, P. A. Valentine, J. H. Sun, J. M. Link, and B. L. Largent. Possible role of valvular serotonin 5-HT$_{2B}$ receptors in the cardiopathy associated with fenfluramine. *Mol. Pharm.*, 2000, 57, 75–81.

I. D. Fratta, E. B. Sigg, and K. Maiorana. Teratogenic effects of thalidomide in rabbits, rats, hamsters, and mice. *Toxicol. Appl. Pharmacol.*, 1965, 7, 268–286.

I. Gardner, S. Leeder, T. Chin, N. Zahid, and J. Uetrecht. A comparison of the covalent binding of clozapine and olanzapine to human neutrophils in vitro. *Mol. Pharmacol.*, 1998, 53, 999–1008.

J. R. Gillette, J. R. Mitchell, and B. B. Brodie. Biochemical mechanisms of drug toxicity. *Annu. Rev. Pharmacol.*, 1974, 14, 271–288.

P. P. Gleason, R. F. Frye, and T. O'Toole. Debilitating reaction following the initial dose of tramadol. *Ann. Pharmacother.*, 1997, 31, 1150–1152.

G. B. Gordon, S. P. Spielberg, D. A. Blake, and V. Balasubramanian. Thalidomide teratogenesis: evidence for a toxic arene oxide metabolite. *Proc. Natl. Acad. Sci. U. S. A.*, 1981, 78, 2545–2548.

R. Greek, N. Shanks, and M. J. Rice. The history and implications of testing thalidomide on animals. *J. Philos. Sci. Law*, 2011, 11, 1–32.

Z. Gregus, C. Madhu, and C. D. Klaassen. Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. *J. Pharmacol. Exp. Ther.*, 1988, 244, 91–99.

M. D. Hartmann, I. Boichenko, M. Coles, F. Zanini, A. N. Lupas, and B. H. Alvarez. Thalidomide mimics uridine binding to an aromatic cage in cereblon. *J. Struct. Biol.*, 2014, 188, 225–232.

P. K. Honig, D. C. Wortham, K. Zamani, D. P. Conner, J. C. Mullins, and L. R. Cantilena. Terfenadine–ketoconazole interaction: pharmacokinetics and electrocardiographic consequences. *JAMA*, 1993, 269, 1513–1518.

H. P. Illing and J. M. Fromson. Species differences in the disposition and metabolism of 6, 11-dihydro-11-oxodibenz [be] oxepin-2-acetic acid (isoxepac) in rat, rabbit, dog, rhesus monkey, and man. *Drug Metab. Dispos.*, 1978, 6, 510–517.

T. Ito, H. Ando, T. Suzuk, T. Ogura, K. Hotta, Y. Imamura, Y. Yamaguchi, and H. Handa. Identification of a primary target of thalidomide teratogenicity. *Science*, 2010, 327, 1345–1350.

S. Iverson, N. Zahid, and J. P. Uetrecht. Predicting drug-induced agranulocytosis: characterizing neutrophil-generated metabolites of a model compound, DMP406, and assessing the relevance of an in vitro apoptosis assay for identifying drugs that may cause agranulocytosis. *Chem. Biol. Interact.*, 2002, 142, 175–199.

A. Lopez-Girona, D. Mendy, T. Ito, K. Miller, A. K. Gandhi, J Kang, J. Kang, S. Karasawa, G. Carmel, P. Jackson, M. Abbasian, A. Mahmoudi, B. Cathers, E. Rychak, S. Gaidarova, R. Chen, P. H. Schafer, H. Handa, T. O. Daniel, J. F. Evans, and R. Chopra. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia*, 2012, 26, 2326–2335.

J. Lu, N. Helsby, B. D. Palmer, M. Tingle, B. C. Baguley, P. Kestell, and L. M. Ching. Metabolism of thalidomide in liver microsomes of mice, rabbits, and humans. *J. Pharmacol. Exp. Ther.*, 2004, 310, 571–577.

A. J. Mahgoub, J. R. Idle, L. G. Dring, R. Lancaster, and R. L. Smith. Polymorphic hydroxylation of debrisoquine in man. *Lancet*, 1977, 2, 584–586.

J. O. Miners and D. J. Birket. Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br. J. Clin. Pharmacol.*, 1998, 45, 525–538.

D. W. Nebert and F. J. Gonzalez. P450 genes: structure, evolution and regulation. *Annu. Rev. Biochem.*, 1987, 56, 945–993.

D. Njoku, M. J. Laster, D. H. Gong, E. I. Eger, G. F. Reed, and Martin J. L. Biotransformation of halothane, enflurane, isoflurane, and desflurane to trifluoroacetylated liver proteins: association between protein acylation and hepatic injury. *Anesth. Analg.*, 1997, 84, 173–178.

D. V. Parke and R. T. Williams. Species differences in the ortho-hydroxylation and para-hydroxylation of aniline. *Biochem. J.*, 1956, 63, 12.

T. W. Robison and A. Jacobs. Metabolites in safety testing. *Bioanalysis*, 2009, 1, 1193–1200.

R. B. Rothman, M. H. Baumann, J. E. Savage, L. Rauser, A. McBride, S. J. Hufeisen, and B. K. Roth. Evidence for possible involvement of 5-HT2B receptors in cardiac valvulopathy associated with fenfluramine and other serotonergic medications. *Circulation*, 2000, 102, 2836–2841.

H. Schumacher, R. L. Smith, and R. T. Williams. The metabolism of thalidomide: the fate of thalidomide and some of its hydrolysis products in various species. *Br. J. Pharmacol. Chemother.*, 1965, 25, 338–351.

H. Schumacher, D. A. Blake, J. M. Gurian, and J. R. Gillette. A comparison of the teratogenic activity of thalidomide in rabbits and rats. *J. Pharmacol. Exp. Ther.*, 1968, 160, 189–200.

D. A. Smith and R. S. Obach. Seeing through the mist: abundance versus percentage. Commentary on metabolites in safety testing. *Drug Metab. Dispos.*, 2005, 33, 1409–1417.

A. F. Stepan, D. P. Walker, J. Bauman, D. A. Price, T. A. Baillie, A. S. Kalgutkar, and M. D. Aleo. Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem. Res. Toxicol.*, 2011, 24, 1345–1410.

L. B. Tee, D. D. S. Davies, C. E. Seddon, and A. R. Boobis. Species differences in the hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. *Biochem. Pharmacol.*, 1987, 36, 1041–1052.

J. P. Uetrecht. New concepts in immunology relevant to idiosyncratic drug reactions: the "Danger Hypothesis" and innate immune system. *Chem. Res. Toxicol.*, 1999, 12(5), 387–395.

H. Yamazaki, H. Suemizu, S. Igaya, M. Shimizu, N. Shibata, M. Nakamura, G. Chowdhury, and F. P. Guengerich. In vivo formation of a glutathione conjugate derived from thalidomide in humanized uPA-NOG mice. *Chem. Res. Toxicol.*, 2011, 24, 287–289.

C. H. Yun, R. A. Okerholm, and F. P. Guengerich. Oxidation of the antihistaminic drug terfenadine in human liver microsomes. Role of cytochrome P-450 3A(4) in N-dealkylation and C-hydroxylation. *Drug Metab. Dispos.*, 1993, 21, 403–409.