1
Origin and Historical Perspective on Reactive Metabolites

Abbreviations

AFB_1	Aflatoxin B_1
BQ	Benzoquinone
BSA	Bovine serum albumin
CYP	Cytochrome P450
GSH	Glutathione
GSSG	Glutathione disulfide
NAPQI	*N*-Acetyl-*p*-benzoquinoneimine
TCPO	1,2-Epoxy-3,3,3-trichloropropane
TFA	Trifluoroacetic acid
TFAC	Trifluoroacetyl chloride

1.1
Mutagenesis and Carcinogenesis

The concept that chemicals, including drugs, could exert harmful effects on living organisms by their conversion into reactive metabolites probably dates back to the 1950s. The most compelling evidence began to be drawn in the 1960s from the area of carcinogenicity and studies looking at processing by metabolism of compounds to unstable, reactive metabolites. These studies drew from the human occupational exposure and animal experiments, which linked polycyclic aromatic hydrocarbons and certain other planar heterocyclic aromatic compounds, containing one or more nitrogen, sulfur, or oxygen atoms, to carcinogenic effects in humans. Human exposure was via coal tars, soot, pitch, dyes, adhesives, oil products, and tobacco smoke. Detailed examination showed that these compounds required bioactivation to electrophilic metabolites to exert their mutagenic or carcinogenic effects [1, 2]. In most cases, oxidation by cytochrome P450 (CYP) enzymes was seen as the rate-limiting step in the activation process to produce the reactive electrophilic species. Among the metabolic pathways identified for polycyclic aromatic hydrocarbons was the bay-region dihydrodiol epoxide pathway [3]. It involves three enzyme-mediated

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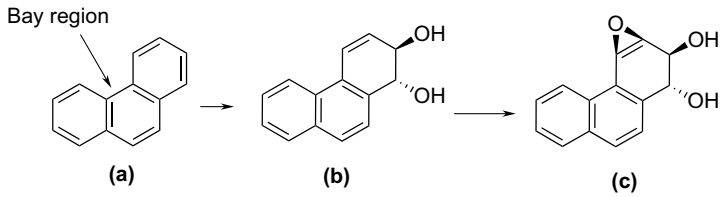

Figure 1.1 Conversion of phenanthrene (a) to its diol (b) by cytochrome P450 and epoxide hydrolase and its ultimate carcinogen/mutagen (c) bay-region diol epoxide by cytochrome P450.

reactions (Figure 1.1): first, oxidation of a double bond catalyzed by CYP enzymes to unstable arene oxides; second, hydrolysis of the arene oxides by microsomal epoxide hydrolase to dihydrodiols; and, finally, a second CYP-catalyzed oxidation at the double bond adjacent to the diol function to generate a vicinal diol epoxide [3]. The vicinal diol epoxide was formed in the sterically hindered bay region. The bay-region diol epoxides are electrophiles capable of covalently binding to DNA. The formation of bay-region diol epoxides has been demonstrated with several polycyclic aromatics such as benzo[*a*]pyrene, chrysene, 5-methylchrysene, benzo[*c*] phenanthrene, benz[*a*]anthracene, and phenanthrene. Other potentially reactive metabolites with possible mutagenic and carcinogenic activity were being discovered at the same time via studies on known carcinogens (e.g., *N*-hydroxylation of 2-acetylaminoflourene) [4].

Functional *in vitro* tests for mutagens (and ultimately carcinogens) were pioneered in the 1970s by Ames and coworkers (see Chapter 2 for utility of the Ames test in drug discovery). Their work examined a set of carcinogens, including aflatoxin B_1 (AFB_1), benzo[*a*]pyrene, acetylaminofluorene, benzidine, and *N,N*-dimethylamino-*trans*-stilbene, and used a rat or human liver homogenate to form reactive metabolites and colonies of *Salmonella* histidine mutants for mutagen detection. These experiments demonstrated that for the set of carcinogens there was a ring system sufficiently planar for a stacking interaction with DNA base pairs and a chemical functionality capable of being metabolized to a reactive species [5].

The work on mutagenesis and carcinogenesis has evolved continuously so that the effects of DNA binding are far more understood. For instance, AFB_1 is present in certain foodstuffs, particularly in developing mold, and is recognized as a major contributor to liver cancer in parts of the developing world (also see Chapter 9) (Figure 1.2).

AFB_1 forms an epoxide (the *exo* isomer is ~1000 times more genotoxic than the *endo* form) that reacts with DNA to form a guanine–AFB_1 DNA conjugate [6].

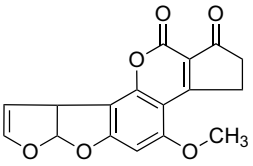

Figure 1.2 Structure of aflatoxin (AFB_1).

This difference in DNA reactivity and toxicity is believed to be due to DNA intercalation (affinity) and reactivity via an S_N2 pathway. The *exo* epoxide has an aqueous half-life of 1 s but is still stable enough to migrate into the cell nucleus and modify DNA [6]. Different CYPs produce different amounts of the *exo* and *endo* forms, but the major human CYP isozyme, that is, CYP3A4, produces exclusively the *exo* form. The damage caused to DNA is specific and the effects on the ultimate protein coded for have been researched. The activation of proto-oncogenes and inactivation of tumor suppressor genes in cells are considered as major events in the multistep process of carcinogenesis. p53 is a tumor suppressor gene, which is mutated in about half of all human cancers. About 80% of these mutations are missense mutations that lead to amino acid substitution and alter the protein conformation and stability of p53. These changes can also alter the sequence-specific DNA binding and transcription factor activity of p53. Thus, the role of p53 in DNA repair, cell cycle control, and programmed cell death can be substantially altered. AFB_1 exposure is correlated with a G:C to T:A transversion that leads to a serine substitution at residue 249 of p53, resultant altered activity, and ultimately hepatocellular carcinoma [7].

1.2 Detection of Reactive Metabolites

Reactive metabolites exist in aqueous solution for a short finite time. Their detection *per se* is difficult. The work in carcinogenicity established ways through which the problem could be tackled. Urinary products may reflect the presence of a reactive metabolite in downstream products (see Section 1.4) with stable metabolites formed by conjugation, hydration, or rearrangement of the reactive species. Sometimes, the conjugates include hydrolysis products of proteins (or genetic material), which are the target for covalent modification by the reactive metabolite. Often, *in vitro* systems such as liver microsomes with exogenously added nucleophilic trapping agents such as the endogenous antioxidant glutathione (GSH) provide further substantive chemical clues. Some form of functional tests (such as the *Salmonella* Ames test in Section 1.1) is invaluable, but broader toxicity findings are usually unavailable. Benzene is a toxin carcinogen that also causes certain blood dyscrasias including acute myeloid leukemia and aplastic anemia. It is thus carcinogenic and also myelotoxic. The principal site of toxicity is the bone marrow. The toxicity of the chemical was recognized by epidemiology and associated with, among others, the shoe industry, where it was a major constituent of glues used to bond the soles to the shoe upper. While metabolism of benzene to phenol was known before the nineteenth century, detailed investigations into the bioactivation mechanism revealed the existence of the electrophilic benzene oxide as the putative carcinogenic entity (similar to examples of polycyclic hydrocarbons in Section 1.1). The identification of the epoxide was through the isolation of the stable dihydrodiol metabolite of benzene [8]. It would be tempting to conclude that the toxicity could be explained with this finding.

In vivo studies [9] sampling urine have over the years revealed different evidence for reactive metabolite formation. These include

I) ring-hydroxylated metabolites including phenol, catechol, hydroquinone, and 1,2,4-trihydroxybenzene;
II) *trans*,*trans*-muconic acid, a ring-opened metabolite formed from muconaldehydes;
III) *N*-acetyl-5-(2,5-dihydroxyphenyl)-L-cysteine, a downstream conjugate of benzene, phenol, and hydroquinone;
IV) *S*-phenylmercapturic acid, a GSH-derived metabolite.

Detailed *in vitro* studies [8] have indicated that the complex pathway derives from a single enzymatic step involving the formation of benzene oxide primarily by CYP2E1 in the liver. Benzene oxide equilibrates spontaneously with the corresponding oxepine valence tautomer, which can open the ring to yield a reactive α,β-unsaturated aldehyde, *trans*,*trans*-muconaldehyde. Reduction or oxidation of *trans*,*trans*-muconaldehyde gives rise to 6-hydroxy-*trans*,*trans*-2,4-hexadienal and *trans*,*trans*-muconic acid. Both *trans*,*trans*-muconaldehyde and the hexadienal metabolites are myelotoxic in animal models. Alternatively, benzene oxide can undergo conjugation with GSH, resulting in the eventual formation and urinary excretion of *S*-phenylmercapturic acid. Benzene oxide is also a substrate for epoxide hydrolase, which catalyzes the formation of benzene dihydrodiol, itself a substrate for dihydrodiol dehydrogenase, producing catechol. Finally, benzene oxide spontaneously rearranges to form phenol, which subsequently undergoes conjugation (glucuronic acid or sulfate) or oxidation to hydroquinone and 1,2,4-trihydroxybenzene. The two diphenolic metabolites of benzene, catechol and hydroxyquinone, undergo further oxidation to the corresponding *ortho*-(1,2)- or *para*-(1,4)-benzoquinones (BQs) that can by myelotoxic. The 1,2- and 1,4-BQs are highly electrophilic and capable of reacting with DNA.

Benzene oxide is surprisingly stable and has a half-life of around 8 min when added to rat blood and 34 min in aqueous buffer. The metabolite therefore can perfuse out the liver to reach all the organs of the body. The toxicity of benzene in bone marrow may be due to benzene oxide formed in the liver being further oxidized in the bone marrow [8]. Benzene illustrated the complex nature of many investigations, revealing multiple possible chemical alternatives that may contribute to the toxicity in different ways.

1.3 Induction and Inhibition: Early Probes for Reactive Metabolites and Hepatotoxicants

Undoubtedly this focus on chemically reactive metabolites and cancer helped scientists to postulate and experimentally test if drug toxicity could also be initiated by such metabolites. Gillette [10] in the 1970s, among others, suggested cellular necrosis, hypersensitivity, blood dyscrasias, and fetotoxicities could be the result of reactive metabolites. Much of the early work focused on rodent toxicants at high

doses and used metabolic inducers and inhibitors. Thus, bromobenzene was shown to be metabolized via a reactive metabolite pathway (epoxide) and was recovered as mercapturic acid (GSH conjugate) and dihyrodiol derivatives in the excreta. In studies utilizing identical doses of bromobenzene, pretreatment of rats with phenobarbital, which induced the metabolism of bromobenzene, increased liver cell necrosis, while SKF525A, which inhibited bromobenzene metabolism, decreased liver cell necrosis. Similar studies implicated reactive metabolites in the hepatotoxicity of acetaminophen and furosemide. Acetaminophen research helped define a number of important areas of reactive metabolite research as detailed below. Acetaminophen has remained a focus because the drug when given or ingested in large doses causes hepatotoxicity in all species, albeit with different sensitivities (in humans the toxic dose is around 20–40 g), although some changes in hepatic function have been observed in daily doses as low as 2 g. The toxicity is related to dose size, and *in vitro* and *in vivo* models can be readily established.

1.4 Covalent Binding and Oxidative Stress: Possible Mechanisms of Reactive Metabolite Cytotoxicity

Early investigations lacked many of the physicochemical measures available today, but the 1980s were critical in the identification of covalent binding *per se* and/or redox recycling and oxidative stress as possible toxic outcomes of reactive metabolite formation. Identifying the reactive species actually binding was problematic. Different target proteins were often added to incubations and synthetic metabolites used. Acetaminophen was a major focus. The compound when radiolabeled bound to hepatic microsomes from phenobarbital-pretreated mice [11]. Cysteine and GSH inhibited this binding, whereas several non-thiol amino acids did not. Bovine serum albumin (BSA), when used as an alternative target protein, inhibited covalent binding to microsomal protein in a concentration-dependent manner. BSA has a single thiol group and the binding now occurred to BSA. When α-s1-casein was substituted (a nonfree protein), little binding to the protein occurred. In duplicate experiments synthetic *N*-acetyl-*p*-benzoquinoneimine (NAPQI) (the two-electron oxidation product of acetaminophen) reproduced the results, identifying it as the reactive arylating metabolite of acetaminophen and suggesting that downstream effects of interactions with thiol groups may be responsible for the toxicity.

The reaction with thiol groups may be important but other aspects of reactive metabolite formation began to be examined [12]. The reaction of NAPQI with GSH in aqueous solution forms an acetaminophen–GSH conjugate and acetaminophen and glutathione disulfide (GSSG). Similar reactions occur in hepatocytes, but the GSSG is rapidly converted back to GSH by the NADPH-dependent glutathione reductase and a rapid oxidation of NADPH. Inhibitors of glutathione reductase prevent this and enhance cytotoxicity without changing the extent of covalent binding. Dithiothreitol added to isolated hepatocytes after maximal covalent binding of NAPQI, but preceding cell death protects cells from cytotoxicity and regenerates

protein thiols. Thus, the toxicity of NAPQI to isolated hepatocytes may not result simply from covalent binding but from its oxidative effects on cellular proteins.

1.5
Activation and Deactivation: Intoxication and Detoxification

As work on reactive metabolites has progressed, so the balance between competing pathways, detoxifying reactions (such as GSH conjugate formation), and actual inherent species or individual sensitivity has become progressively more important. Without this knowledge it is tempting to conclude that it is the formation rate and amount of a particular metabolite that is important.

Acetaminophen showed varying toxicity when tested in different species. The drug and its toxic reactive metabolite, NAPQI, were, therefore, investigated in hepatocytes from different species [13]. Clear conclusions were drawn from these early studies in which acetaminophen triggered cell blebbing and loss of viability in the cells from mouse and hamster in contrast to human and rat hepatocytes, which 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, to the toxic effects. 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 due to any intrinsic differences in sensitivity or any difference in the fate of NAPQI once formed. Later studies [14] have seen some correlations but without such clear-cut conclusions, observations often concluded that the actual test system, or conditions, produced significant variation. In these studies all the metabolites were quantified. These were separated into those downstream of NAPQI (GSH, cysteinylglycine, cysteine, and mercapturate conjugates) and alternative pathways (metabolites, such as the glucuronide and sulfate conjugates). The ratio of downstream NAPQI/alternative pathway metabolites excreted was 2.2, 1.0, 0.25, 0.1, and 0.08 for hamsters, mice, rabbits, rats, and guinea pigs, respectively, and inversely related to the hepatotoxic dose reported for these species. This is supportive of species sensitivity being determined by the balance between toxification and detoxification metabolic pathways [15].

It is likely that species differences in acetaminophen toxicity are due to a combination of pharmacokinetic (metabolism) differences and other biological variations. There is a natural inclination to believe that metabolism differences explain different responses, but the evidence in many cases is lacking. This is particularly true when the products of metabolism differ along with species responses.

1.6
Genetic Influences on Reactive Metabolite Formation

Early focus, even in the 1980s, was to look for at-risk populations. Understanding of enzymology and genetic variation was at an early stage but some links were

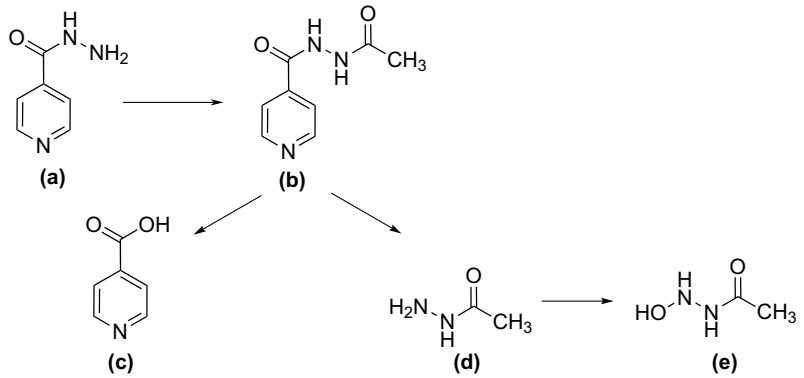

Figure 1.3 Pathway of isoniazid (a) metabolism forming *N*-acetylisoniazid (b) by acetylation, hydrolysis to isonicotinic acid (c) and *N*-acetylhydrazine (d), and subsequent *N*-hydroxylation (e).

established. Isoniazid, an antitubercular drug, caused hepatotoxicity in around 1% of the population. Increased risk was observed in fast acetylators of the drug that formed comparatively more of the metabolite *N*-acetylisoniazid. This hepatotoxicity could also be observed in animal studies together with covalent binding of radioactivity in the liver. Radiolabeled (^{14}C) versions of the metabolite bound covalently only when the acetyl group was labeled and not when the ^{14}C was incorporated into the pyridine ring [16]. Pathways suggested involved the formation of an *N*-hydroxy derivative, which dehydrated to a diazene that could fragment in the presence of oxygen to radical species (Figure 1.3).

1.7
Halothane: the Role of Reactive Metabolites in Immune-Mediated Toxicity

Halothane anesthesia may be followed by changes in liver function. For 25–30% of patients there is a minor degree of disturbance of liver function shown by increased serum transaminases or glutathione-*S*-transferase. With this mild change subsequent reexposure to halothane is not necessarily associated with evidence of liver damage. In 1 in 20 000 patients normally having past experience of halothane anesthesia, massive liver cell necrosis can occur, frequently leading to fulminant hepatic failure. This type of liver damage has clinical, serological, and immunological features strongly indicating an immune-mediated idiosyncratic reaction [17].

Halothane metabolism [18] produces three main excreted metabolites: trifluoroacetic acid (TFA), *N*-triflouroacetyl-2-aminoethanol, and, to a lesser extent, *N*-acetyl-*S*-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine. The last two metabolites are downstream products of reactive metabolites. They are formed by hydroxylation of halothane with spontaneous loss of hydrogen bromide to form trifluoroacetyl chloride (TFAC), which would form TFA by hydrolysis or *N*-triflouroacetyl-2-aminoethanol by reaction with intracellular products. TFAC is also known to acylate lysine

residues on proteins. Dehydrofluorination to 2-bromo-2-chloro-1,1-difluroethylene is the likely route to the cysteine conjugate.

CYP2E1 is the major catalyst in conversion of halothane to the reactive metabolite TFAC [19] and formation of trifluoroacetylated proteins. Trifluoroacetylated CYP2E1 was detected immunochemically in livers of rats treated with halothane and high levels of autoantibodies that recognized purified rat CYP2E1 but not purified rat CYP3A were detected by 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. The intracellular location of trifluoroacetyl adducts was predominantly in the endoplasmic reticulum and also, to a lesser extent, on the cell surface. Thus, halothane metabolism by CYP2E1 results in the cell surface expression of acetylated CYP2E1 that could be important as an antigen in halothane hepatotoxicity.

1.8
Formation of Reactive Metabolites, Amount Formed, and Removal of Liability

Other inhaled anesthetics such as isoflurane and desflurane [20] also have TFAC as a metabolic product as evidenced by recoveries of TFA. However, the degree of biotransformation of these anesthetics is much less than that of halothane. This lower degree of exposure to the reactive metabolite may be an important factor in a much lower immune response and hepatotoxic risk. A later gaseous anesthetic sevoflurane [20] is biotransformed to a lesser degree than halothane (3–5% versus 18–25%), and much less total mass of metabolites is formed. The primary organic metabolite of sevoflurane is hexafluoroisopropanol, not TFAC. This metabolite is not chemically reactive in comparison to TFAC. Hexafluoroisopropanol does not accumulate, being rapidly cleared by glucuronidation that in turn is rapidly excreted in the urine. This is in contrast to halothane, where TFA (from TFAC) is detectable in urine for up to 12 days after 75 min of anesthesia (Figure 1.4).

1.9
Antibodies: Possible Clues but Inconclusive

Similar to halothane, tienilic acid forms antibodies that are associated with hepatotoxicity. In this case the circulating antibodies recognize CYP2C9, the principal CYP isozyme in the metabolism of tienilic acid. Moreover, tienilic acid is a very potent mechanism-based inhibitor of CYP2C9. Again the haptenized protein appears on the surface of the hepatocyte [21]. Other covalently altered enzymes are apparently the hapten for antibody production. For instance, iproniazid, an irreversible monoamine oxidase-B inhibitor, causes antibodies to be formed against monoamine oxidase-B. While it is enticing to link outcome to an immunological

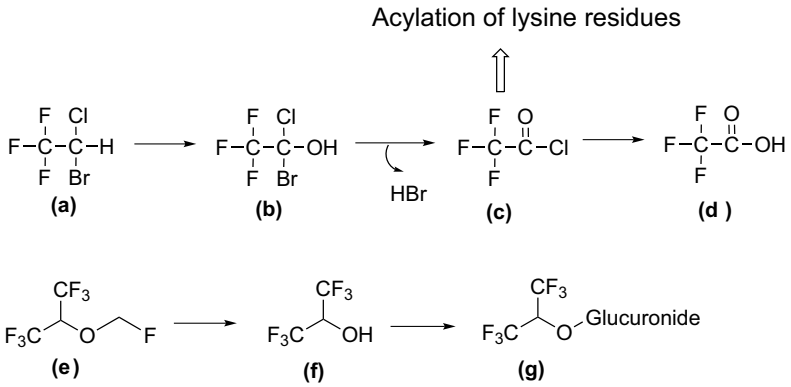

Figure 1.4 Structure of halothane (a), its hydroxylated metabolite (b), the reactive trifluoroacetyl chloride (c, TFAC) formed by HBr loss, and the downstream product trifluoroacetic acid (d, TFA). The metabolism of sevoflurane (e) is shown in comparison, where the principal stable metabolite is hexafluoroisopropanol (f), rapidly converted to a glucuronic acid conjugate (g).

mechanism, many patients have circulating antibodies with no sign of toxicity. Practolol, a β-adrenoceptor antagonist with an acetylated aniline template, was responsible for oculomucocutaneous syndrome. An antibody specific to a practolol-reactive metabolite, most probably formed by oxidation of the aniline nitrogen, was found in the plasma of practolol-treated patients with or without a history of adverse reaction to the drug. No antibodies were detected in patients treated with other β-blocking drugs. Titer of the antibody was highly variable [22].

1.10
Parent Drug and Not Reactive Metabolites, Complications in Immune-Mediated Toxicity

Sulfonamide antibacterials are one of the earliest examples of chemotherapy against infection and are inhibitors of tetrahydropteroic acid synthetase. The natural substrate for this enzyme is *para*-aminobenzoic acid. Sulfonamides mimic the natural substrate with the *para*-aminobenzene (aniline) being retained but with the carboxylic acid being replaced by the isostere sulfonamide. The drugs cause serious skin toxicities including erythema multiforme, Stevens–Johnson syndrome, and toxic epidermal necrolysis. The *N*-4-hydroxylamine metabolite of these drugs, which can be formed in the skin, was initially identified and a rationale of covalent binding to proteins and induction of specific adverse immune response was developed. Anilines can be oxidized to more than one reactive metabolite and the nitroso intermediate has been shown to be a potent antigenic determinant [23, 24]. Surprisingly and contrary to the theories on reactive metabolites, T cell responses against the parent drug have also been detected. These T cells are low in proportion and highly selective, being responsive only to the particular drug used in the treatment

such as sulfamethoxazole. Thus, they do not react to related sulfonamide antibacterial agents such as sulfapyridine or sulfadiazine. In contrast, those generated from reactive metabolites of sulfamethoxazole can be stimulated by other structurally related drugs such as sulfapyridine and sulfadiazine.

1.11
Reversible Pharmacology Should not be Ignored as a Primary Cause of Side Effects

Phenytoin is responsible for "fetal hydantoin syndrome," a defined set of side effects on the embryo, which include embryonic death, intrauterine growth retardation, mild central nervous system dysfunction, and craniofacial abnormalities. Original theories as causes included reactive metabolite formation. Phenytoin teratogenesis was postulated to result from epoxide formation [25] and covalent binding of the epoxide, the ultimate teratogen, to constituents of gestational tissue. Some experimental evidence was obtained in which Swiss mice were given teratogenic doses of phenytoin with and without a nonteratogenic dose of 1,2-epoxy-3,3,3-trichloropropane (TCPO), an epoxide hydrolase inhibitor. TCPO significantly increased the incidence of I-induced cleft lip and palate and enhanced the embryolethality twofold compared to phenytoin alone. The covalent binding of phenytoin-derived radioactivity in fetuses and placenta was enhanced by TCPO.

Further experiments looking at other causes have shown that the syndrome is unequivocally linked to phenytoin's reversible secondary pharmacology, namely, its blockade of the IKr delayed rectifier K^+ channel. In rodents the expression of this channel is age-specific, making the fetal heart especially sensitive compared to the mature animal. IKr blockers (which include phenytoin) initiate concentration-dependent embryonic bradycardia/arrhythmia resulting in hypoxia, explaining embryonic death and growth retardation, and episodes of severe hypoxia, followed by generation of reactive oxygen species within the embryo during reoxygenation, causing orofacial clefts and distal digital reductions and alterations in embryonic blood flow and blood pressure, inducing cardiovascular defects [26].

1.12
Conclusions: Key Points in the Introduction

This historical review serves as a stepwise introduction to the book. The important topics that have been introduced in this chapter will be explored in much greater detail in subsequent chapters and include the following:

1) Genotoxicity of many carcinogenic compounds could be linked to reactive metabolites.
2) These reactive metabolites covalently bind to DNA and lead to misreading of the message.
3) The genotoxins have a shape determined by their receptor (DNA) being planar and able to intercalate.

4) Similar reactive metabolites could also interact with proteins and cause other forms of toxicity.
5) Effects such as cellular necrosis, hypersensitivity, blood dyscrasias, and fetotoxicities could be due to reactive metabolites.
6) Cell necrosis, such as hepatotoxicity, could be caused by interaction with protein targets and alteration of protein function or by redox recycling and oxidative stress.
7) Other toxicity mechanisms could be triggered by generation of antigens and an autoimmune response.
8) The amount of metabolite formed may be critical in determining the outcome; moreover, structural changes that limit or prevent metabolite formation mitigate the risk.
9) Compounds can produce multiple reactive metabolites, the effects of which could be accumulative.
10) The identification of reactive metabolites may disguise other toxicity mechanisms such as reversible pharmacology or even autoimmune roles of the parent compound.

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