Advances in Molecular Toxicology

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ADVANCES IN MOLECULAR TOXICOLOGY

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Editor's Preface

Toxicology is said to be the study of poisons, but this suffers from considerable imprecision that emanates from the broad interpretation of the meaning of the word 'poisons'. Certainly in the mind of the public at large this encompasses a relatively limited set of agents that enter the consciousness from time to time via the media – chemical weapons, insecticides or the occasionally recalled pharmaceutical. As a consequence, this editor much enjoys delivering a first lecture in Toxicological Chemistry on the dangers of oxygen consumption – an eye-opening subject generally received by students with a good deal of surprise.

'Molecular' too carries considerable imprecision. Signal transduction pathways, multi-protein complexes, and gene expression are in the domain of 'molecular' biologists. By contrast, chemists often eschew the complexity and uncertainties inherent in such systems, preferring to focus on issues and problems that are quantitatively more tractable and about which more intimate 'molecular' details can be more definitively understood.

For the purpose of this series then, Molecular Toxicology can be viewed as a wide-ranging field that not only straddles the chemistry–biology interface but also makes use of experimental approaches and techniques firmly embedded in either of the two core disciplines. This diversity is to be recognized and in honor of it, a given volume is rarely to be focused on a narrow area of the field. Contributions will summarize progress in both new and mature areas. Those with an interest in contributing a chapter are encouraged by the editor to discuss a proposal preferably by electronic means.

The widespread use of hormone replacement therapy to ameliorate symptoms in menopausal women presents a compelling justification for understanding the metabolism, and consequences of metabolism, of these compounds and preparations; this is the subject of Chapter 1. The author, Judy Bolton, is among the foremost researchers in elucidating

metabolism of, and the chemistry of metabolites of, estrogenic compounds. Until, relatively recently, the known carcinogenic activity of estrogenic compounds and preparations had largely been presumed to be due to their ''hormonal'' activities – their ability to promote cell growth and proliferation. Bolton and others have demonstrated a constellation of metabolites with toxic potential. Indeed these studies have spawned the formulation of new agents with similar estrogenic potency, but that are appropriately functionalized to evade formation of certain of the more toxic metabolites.

The awareness that there are a large number of endogenous modes by which nucleobases, in both DNA and RNA, can undergo deamination has dawned only recently and this still-developing story is reviewed by Peter C. Dedon in Chapter 2. Such lesions can be highly persistent and this makes them a likely significant source of promutagenic lesions in DNA. In RNA, such bases can give rise to proteins of aberrant function. While deamination is accomplished by normal metabolic pathways, and even this may contribute to the level of deaminated nucleobases in the polymers, other enzymatic and non-enzymatic modes of deamination have been demonstrated and are summarized in detail. Attention is also paid to the numerous experimental pitfalls and artifacts that have hindered accurate quantification of the extent and nature of deamination *in vivo*.

Evolution has imbued cells with an array of enzymology by which to protect against electrophilic and/or oxidative assault by toxins and the molecular interactions that give rise to the upregulation of such enzymes is the subject of Daniel C. Liebler's contribution in Chapter 3. Transcription of the genes encoding for many of these enzymes is enhanced transcription factor Nrf2. The ability of Nrf2, which is mainly found in the cytosol in unchallenged cells, to enter the nucleus and thereby initiate transcription, is controlled by a chaperone protein – Keap 1. Keap 1 is a cysteine-rich protein that is thought to be an electrophile/oxidant sensor, by virtue of its sulfhydryls, and a number of groups have begun to focus on dissecting which of the candidate thiols on Keap 1 are crucial for permitting nuclear translocation of Nrf2. Results of studies from different groups with overlapping and different reaction systems are not in perfect agreement. Beyond issues of simple chemical reactivity, Liebler summarizes complimentary data from more biochemical approaches and points out some consistencies and importantly some remaining disagreements and conundrums.

The genotoxicity of chlorophenols and the mycotoxin ochratoxin A is extensively summarized by Richard Manderville and Annie Pfohl-Leszkowicz in Chapter 4. Here, as well, the metabolism of these agents, central to their genotoxic activities, is detailed. There is compelling evidence of carcinogenicity for a number of these compounds. The polychlorophenols have been widely employed as insecticides and fungicides and so they have wide distribution in the human environment. Ochratoxin is produced in temperate and tropical climes by a range of fungi that inhabit human and domestic animal foodstuffs, in particular many grains. Polychlorophenols exhibit a metabolism rich in reactive intermediates that result in a bifurcated assault involving both reactive oxygen species, derived from redox cycling, and a variety of electrophiles. A number of these lead to novel nucleobase adducts, as has been described by the authors and others most recently. Ochratoxin is the 'special case', a highly elaborated monochlorophenol. It has been the subject of extensive study regarding metabolism, which is complex and is thoroughly reviewed here, and the molecular basis of its toxicity is presently controversial. It has been claimed to act indirectly, not through the formation of DNA adducts. The authors summarize recent evidence to the contrary.

Jack Uetrecht summarizes, in Chapter 5, a view of a particularly problematic area of toxicology that of idiosyncratic drug reactions. Such reactions typically are unrelated to the therapeutic mode of action of the drug and typically occur infrequently. Thus they are difficult to predict and it is equally difficult to get at a mechanistic basis for etiology. As pointed out, more than 1 in 10 pharmacologic agents that have recently come to market have been recalled or subsequently received 'black box' labeling due to adverse events in individuals that went undetected in clinical trials. Uetrecht examines two leading notions regarding what is largely causal to such reactions and summarizes the evidence for each. The case that there is immune response underlying these complications is clearly layed out in the case of liver, skin and blood. The notion that reactive metabolites initiate idiosyncratic drug reactions is considered in detail. A series of cases is examined and the equivocal basis of some of these is emphasized.

It is hoped that readers enjoy the timely and detailed reviews in this founding volume.

CHAPTER 1

Bioactivation of Estrogens to Toxic Quinones

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Abbreviations: 2-OHE, 2-hydroxyestrone, 2, 3-dihydroxy-1, 3, 5(10)-estratrien-17-one; 4-OHE, 4-hydroxyestrone, 3, 4-dihydroxy-1, 3, 5(10)-estratrien-17-one; estrone, 3-hydroxy-1, 2, 5-(10)-estratrien-17-one; 4-OHEN, 4-hydroxyequilenin, 3, 4-dihydroxy-1, 3, 5(10), 6, 8-estrapentaen-17-one; equilenin, 1, 3, 5(10), 6, 8-estrapentaen-3-ol-17-one; equilin, 1, 3, 5(10), 7-estratetraen-3-ol-17-one; 9(11)-dehydro-4-OHE, 9(11)-dehydro-4-hydroxyestrone, 3, 4-dihydroxy-1, 3, 5(10), 9(11)-estratetraen-17-one; NQO1, NAD(P)H:quinone oxidoreductase, DT-diaphorase; 8-oxo-dG, 8-oxodeoxyguanosine; 8-oxo-dA, 8-oxodeoxyadenosine; P450, cytochrome P450.

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1. INTRODUCTION

1.1. The risk/benefits of traditional estrogen replacement therapy

Recently, the National Toxicology Program of NIEHS declared that steroidal estrogens, both of endogenous nature and as components of hormone replacement therapy (HRT) formulations, are ''*known to be human carcinogens*'', causing breast and endometrial cancers [1]. In July 2002, the Data and Safety Monitoring Board prematurely terminated a major clinical Women's Health Initiative trial on the long-term risks and benefits of estrogen plus progestin therapy, a form of HRT for postmenopausal women that have an intact uterus [2,3]. This decision was based, in part, on the significantly increased risk (24%) of invasive breast cancer, as well as a higher incidence of heart disease and stroke in women undergoing estrogen replacement therapy as compared to those receiving placebos. Even those without tumors were often found to have more abnormal mammograms than those on placebo [3–5]. Indeed, it has been known for some time that estrogens and estrogens plus progestin [6] can contribute significantly to the development of cancers [7,8], especially of the breast [4,9–13] and other hormone-sensitive tissues [14] such as the ovary and uterus. These are some of the major types of cancers that afflict women in the United States [12].

Nevertheless, there are also significant benefits since estrogen replacement therapy relieves symptoms of menopause such as sleeplessness, hot flashes, and mood swings, protection against early menopausal bone loss, and a lower risk of colon cancer [2,3]. For these reasons, women continue to use hormone replacement formulations [15] in spite of the well-recognized risk [16]. Although, the sales of standard dose Premarin prescriptions (0.625 mg/day) have decreased by 33%, since the NHLBI terminated the clinical trial on the long-term risks and benefits of estrogen plus progestin therapy in July 2002, the sales of low-dose Premarin preparations (0.45 mg/day) have been rising [15].

2. MECHANISMS OF ESTROGEN CARCINOGENESIS

The molecular mechanisms of steroidal estrogen carcinogenesis are still not well understood [8,12,13,17]. Malignant phenotypes arise as a result of a series of mutations, most likely in genes associated with

tumor suppressor, oncogene, DNA repair, or endocrine functions [14]. At least two major pathways are being considered to be important and include the extensively studied hormonal pathway, by which estrogen stimulates cell proliferation through estrogen receptor (ER)-mediated signaling pathways, thus resulting in an increased risk of genomic mutations during DNA replication ([18–20]; *Hormonal mechanism*, Fig. 1). The second pathway involves estrogen metabolism, mediated by cytochrome P450, which generates reactive electrophilic estrogen *o*-quinones and reactive oxygen species (ROS) through redox cycling of these *o*-quinones (*Chemical mechanism*, Figs. 1 and 2). Studies have shown that a constitutive and TCDD-inducible P450 isozyme, P4501B1 selectively catalyzes hydroxylation at the 4-position of estrone and 17β-estradiol [21–23] suggesting that excessive exposure to

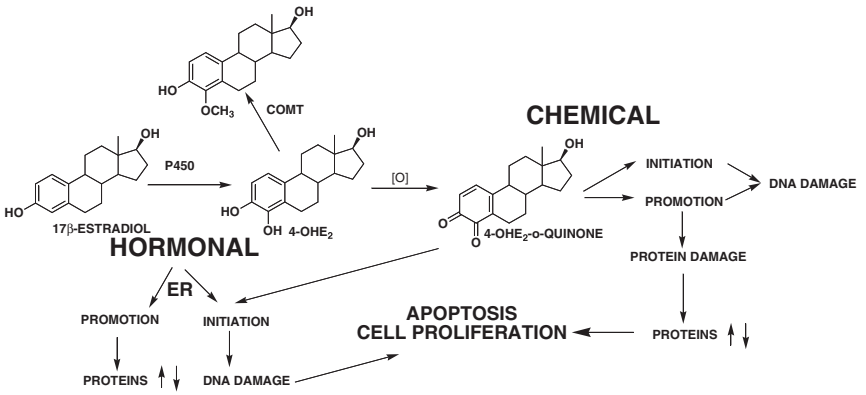

Fig. 1. Mechanisms of estrogen carcinogenesis.

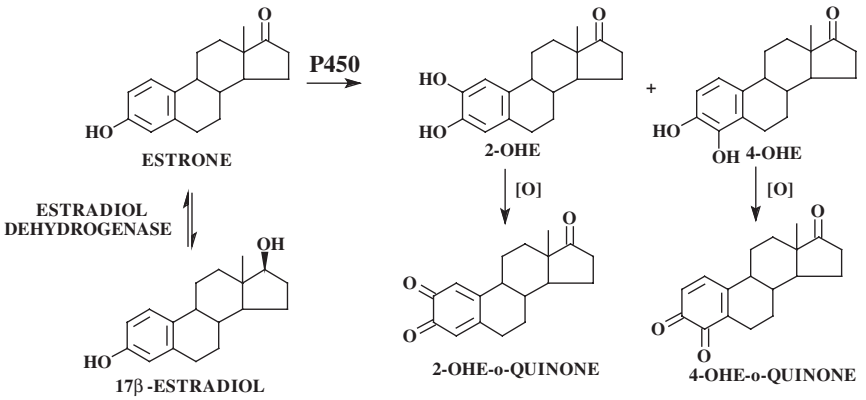

Fig. 2. Phase I metabolism of endogenous estrogens.

environmental pollutants could lead to enhanced production of this metabolite. This is particularly significant, since only 4-hydroxyestrone was found to be carcinogenic in the male Syrian golden hamster kidney tumor model, whereas, 2-hydroxyestrone was without activity [24,25]. Similarly, Newbold and Liehr [26] have shown that 4-hydroxyestradiol induced uterine tumors in 66% of CD-1 mice, whereas, mice treated with 2-hydroxyestradiol or 17β-estradiol had a total uterine tumor incidence of 12% and 7%, respectively. Finally, epidemiological studies have suggested a link between genetic polymorphism in the estrogen 4-hydroxylase (P4501B1 and/or 1A1) and a risk for developing breast cancer [27,28]. These data suggest that *metabolism* of estrogens is required for the development of cancer.

Most of the epidemiological studies on estrogen replacement therapy and cancer risk have been conducted with Premarin® (Wyeth-Ayerest) which remains the estrogen replacement treatment of choice and one of the most widely prescribed drugs in North America [29]. Since Premarin® was approved by the Food and Drug Administration in the 1940s, very little is known about the metabolism and potential toxic metabolites that could be produced from the various equine estrogens which make up ~50% of the estrogens in Premarin® (Fig. 3; [30–33]). It is known that treating hamsters for 9 months with either estrone, equilin + equilenin, or sulfatase-treated Premarin®, resulted in 100% kidney tumor incidences and abundant tumor foci [34]. We have shown that a major phase I metabolite of both equilenin and equilin (4-hydroxyequilenin, 4-OHEN) can act as a complete carcinogen and tumor promotor *in vitro*, whereas the endogenous catechol estrogen

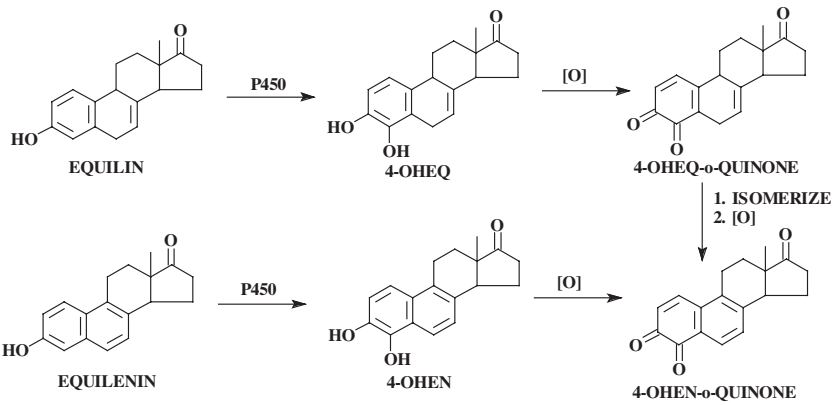

Fig. 3. Primary phase I metabolism for equine estrogens.

metabolite, 4-hydroxyestrone was much less effective [35]. As a result, it is quite possible that the B-ring unsaturated equine estrogens have very different biological properties *in vivo* compared to the endogenous catechol estrogens [36–38].

Interestingly, increasing unsaturation in the B-ring leads to a change in metabolism from predominately 2-hydroxylation for estrone to mainly 4-hydroxylation for equilin and exclusively 4-hydroxylation for equilenin (Fig. 3). This could be problematic, since 2-hydroxylation of endogenous estrogens is regarded as a benign metabolic pathway, whereas 4-hydroxylation could lead to carcinogenic metabolites [33,34]. In fact, based on our progress to date, it is our *strong belief* that metabolism of equilenin or equilin (and their 17β-hydroxylated metabolites) to 4-OHEN and 4,17β-dihydroxyequilenin represents a major carcinogenic pathway for equine estrogens.

3. OXIDATION OF CATECHOL ESTROGENS TO *O*-QUINONES

Once formed, the endogenous catechol estrogens can be oxidized by virtually any oxidative enzyme and/or metal ion giving *o*-quinones [39]. The *o*-quinone formed from 2-hydroxyestrone has a half-life of 47 s, whereas the 4-hydroxyestrone-*o*-quinone is considerably longer lived ($t_{1/2} = 12$ min; [40]). Interestingly, the 4-hydroxylated equine catechol estrogens (4-OHEN and 4-OHEQ) both autoxidize to *o*-quinones without the need for enzymatic or metal ion catalysis (Fig. 4; [41]). The *o*-quinone formed from 4-OHEN is much more stable than the endogenous catechol estrogens ($t_{1/2} = 2.3$ h; [41]). It appears that the

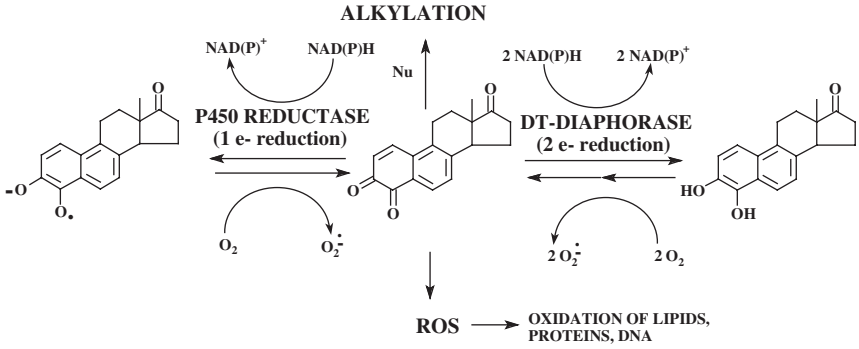

Fig. 4. Potential reactions of catechol estrogen *o*-quinones *in vivo* using 4-OHEN as an example.

adjacent aromatic ring stabilizes 4-OHEN-*o*-quinone through extended π-conjugation [41]. In support of this it has been shown that the catechol metabolite of benzo[a]pyrene rapidly undergoes air oxidation to yield a very stable *o*-quinone, benzo[a]pyrene-7,8-dione [42,43].

The 4-OHEQ-*o*-quinone readily isomerizes to 4-OHEN-*o*-quinone (Fig. 3). As a result, most of the biological effects caused by catechol metabolites of equilin are likely due to 4-OHEN-*o*-quinone formation [41]. Finally, although 2-hydroxylation does occur with equilin producing 2-hyroxyequilin, which will isomerize to 2-hydroxyequilenin, the latter catechol does not autoxidize to an *o*-quinone at any appreciable rate [44]. This suggests that similar to what has been observed with endogenous catechol estrogens, 2-hydroxylation is likely a benign metabolic pathway for equilin.

4. DNA DAMAGE INDUCED BY CATECHOL ESTROGENS

4.1. Oxidative damage

o-Quinones are also potent redox-active compounds [45,46]. They can undergo redox cycling with the semiquinone radical generating superoxide radicals mediated through cytochrome P450/P450 reductase (as shown in Fig. 4 for 4-OHEN). The reaction of superoxide anion radicals with hydrogen peroxide, formed by the enzymatic or spontaneous dismutation of superoxide anion radical, in the presence of trace amounts of iron or other transition metals gives hydroxyl radicals. The hydroxyl radicals are powerful oxidizing agents that may be responsible for damage to essential macromolecules. Biomarkers for oxidative damage to DNA include the formation of the mutagenic lesion, 8-oxo-2′-deoxyguanosine (8-oxo-dG) [47].

The excessive production of ROS in breast cancer tissue has been linked to metastasis of tumors in women with breast cancer [48,49]. The source of ROS has been suggested to be the result of redox cycling between the *o*-quinones and their semiquinone radicals generating superoxide, hydrogen peroxide, and ultimately reactive hydroxyl radicals, which cause oxidative cleavage of the phosphate–sugar backbone as well as oxidation of the purine/pyrimidine residues of DNA [50]. In support of this mechanism, various free radical toxicities have been reported in hamsters treated with 17β-estradiol including DNA single-strand breaks [51,74], 8-oxo-dG formation [75], and chromosomal abnormalities [52,53]. Recently, it has also been shown

that 4-hydroxyestradiol also induces oxidative stress and apoptosis in human mammary epithelial cells (MCF-10A), although the concentrations used in this study (25 μM) have questionable physiological relevance [54]. We have shown that 4-OHEN is also capable of causing DNA single-strand breaks and oxidative damage to DNA bases both in breast cancer cells as well as in the rat mammary gland after treatment with 4-OHEN [55,56]. Treating λ phage DNA with 4-OHEN resulted in extensive single-strand breaks that were due to concentration and time dependence. By including scavengers of ROS in the incubations, DNA could be completely protected from 4-OHEN-mediated damage. In contrast, nicotinamide adenine dinucleotide (NADH) and $CuCl_2$ enhanced the ability of 4-OHEN to cause DNA single-strand breaks presumably due to redox cycling between 4-OHEN and the semiquinone radical generating hydrogen peroxide, and ultimately copper peroxide complexes. It was confirmed that 4-OHEN could oxidize DNA bases, because hydrolysis of 4-OHEN treated calf thymus DNA and high-performance liquid chromatography (HPLC) separation with electrospray-mass spectroscopy (MS) detection revealed oxidized deoxynucleosides including 8-oxo-dG and 8-oxo-dA. 4-OHEN also caused a dose-dependent increase in the mutagenic lesion 8-oxo-dG in breast cancer cells as determined by LC–MS–MS [57]. In support of this, previous reports have shown that incubations with 4-OHEN-*o*-quinone, DNA, and hamster liver microsomes also enhanced 8-oxo-dG formation [58]. Using the single cell gel electrophoresis assay (Comet assay) to measure DNA damage, we found that 4-OHEN causes concentration-dependent DNA single-strand cleavage in breast cancer cell lines and this effect could be enhanced by NADH or diethyl maleate [79]. Finally, we have shown that injection of 4-OHEN into the mammary fat pads of Sprague–Dawley rats resulted in dose-dependent increase in single-strand breaks and oxidized bases as analyzed by the Comet assay [52]. In addition, extraction of mammary tissue DNA, hydrolysis to deoxynucleosides, and analysis by LC-MS-MS showed the formation of 8-oxo-dG as well as 8-oxo-dA. These and other data are evidence for a mechanism of estrogen-induced tumor initiation/promotion by redox cycling of estrogen metabolites generating ROS, which damage DNA.

4.2. Formation of catechol estrogen DNA adducts

Some of these metabolites, especially the 3,4-catechols of endogeneous and equine estrogens lead to direct genotoxic effects by

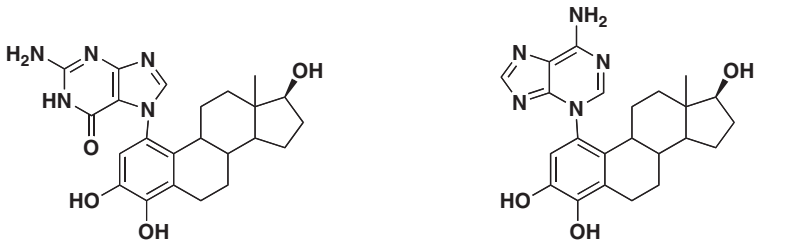

Fig. 5. Structures of the DNA adducts of 4-OHE_2.

damaging cellular DNA [17,59–67]. Cavalieri's group has shown that the major DNA adducts produced from 4-hydroxyestradiol-*o*-quinone are depurinating N7-guanine and N3-adenine adducts both *in vitro* and *in vivo* (Fig. 5; [60,68,69]). Interestingly, they have recently concluded that only the N3-adenine adduct is likely to induce mutations since this adduct depurinates instantaneously, whereas the N7-guanine adduct takes hours to hydrolyze [70]. Similarly, our *in vivo* experiments with rats treated with 4-OHEN as described above, showed the formation of an alkylated depurinating guanine adduct following LC-MS-MS analysis of extracted mammary tissue [52]. However, extraction of mammary tissue DNA, hydrolysis to deoxynucleosides, and analysis by LC-MS-MS also showed the formation of stable cyclic deoxyguanosine (Fig. 6) and deoxyadenosine adducts as well as the above-mentioned oxidized bases. Interesting, the ratio of the diasteriomeric adducts detected *in vivo* differs from *in vitro* experiments suggesting that there are differences in the response of these stereoisomeric lesions to DNA replication and repair enzymes. This is the first report showing that 4-OHEN is capable of causing DNA damage *in vivo*. In addition, the data showed that 4-OHEN induced four different types of DNA damage that must be repaired by different mechanisms. This is in contrast to the endogenous estrogen 4-hydroxyestrone where only depurinating adducts have been detected *in vivo*. Finaly, in a recent report, highly sensitive nano liquid chromatography–nano electrospray tandem mass spectrometry techniques were used to analyze the DNA in five human breast tumor and five adjacent tissue samples, including samples from donors with a known history of Premarin-based HRT [71]. While the sample size is small, and the history of the patients is not fully known, cyclic 4-OHEN-dC, -dG, and -dA stable adducts were detected for the first time in four out of the 10 samples. These results suggest that

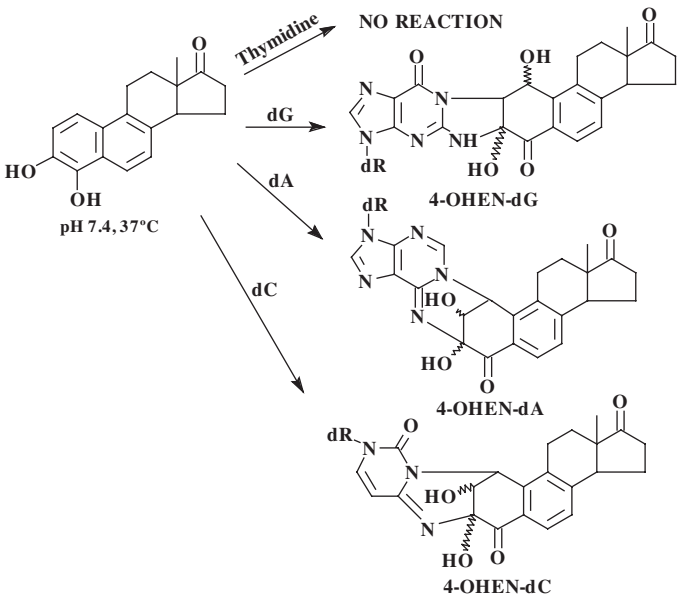

Fig. 6. Stable adducts formed from reaction of 4-OHEN with DNA.

4-OHEN has the potential to be a potent carcinogen through the formation of variety of DNA lesions *in vivo*.

5. PROTEIN DAMAGE INDUCED BY CATECHOL ESTROGENS

5.1. Glutathione *S*-transferase

In addition to DNA damage, it is quite likely that targets for catechol estrogen *o*-quinones could be crucial cellular proteins. Modification of cysteine sulfhydryl groups either through akylation or oxidation is most likely; however, reaction of *o*-quinones with nitrogen nucleophiles on proteins has also been reported. For example, Michael-type nucleophilic reactions of quinones with amines and thiols have been implicated in the antitumor activity of ellipticines [72] and some substituted catechols [73], the neurotoxicity of serotonine [74], and the hypersensitivity reaction of poison ivy [75]. With estrogens, chemical or enzymatic activation of estrone or 17β-estradiol and their catechol metabolites leads to protein alkylation, which may be responsible for the toxic effects of estrogen *o*-quinones (Fig. 7; [76–78]). For the equine estrogens, we have found that both 4-OHEN and 4-hydroxyequilin (4-OHEQ)

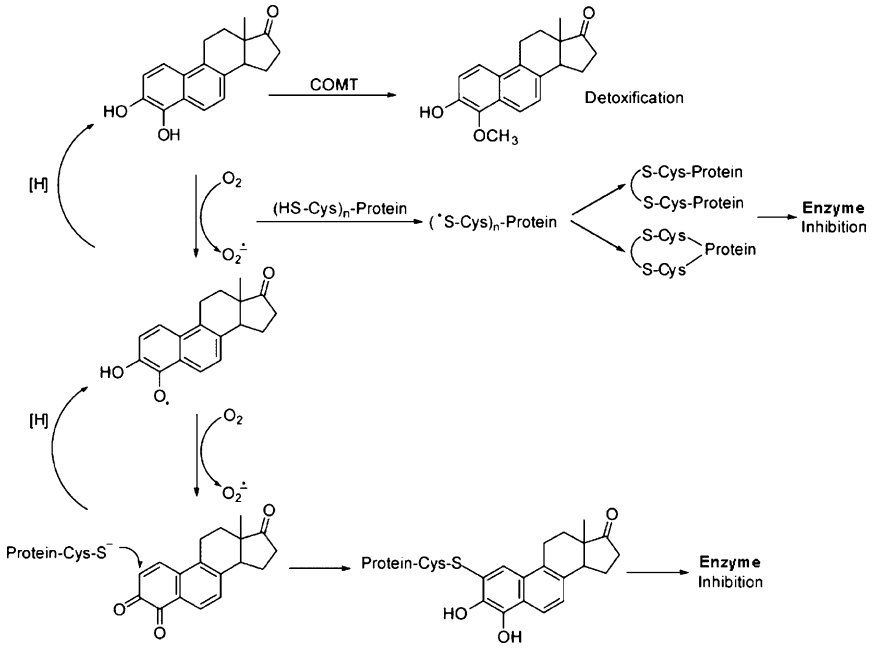

Fig. 7. Types of protein damage induced by 4-OHEN.

are potent irreversible inactivators of glutathione *S*-transferase [79,80]. This could be particularly significant to the mechanism of estrogen carcinogenesis, since GST polymorphisms have been associated with increased breast cancer risk [81–83]. to investigate the role of cysteine residues in the 4-OHEN-mediated inactivation of this enzyme, one or a combination of cysteine residues was replaced by alanine residues (C47A, C101A, C47A/C101A, C14A/C47A/C101A, and C47A/C101A/C169A mutants). Electrospray ionization mass spectrometric analyses of wild-type and mutant enzymes treated with 4-OHEN showed that a single molecule of 4-OHEN-*o*-quinone attached to the proteins, with the exception of the C14A/C47A/C101A mutant where no covalent adduct was detected. 4-OHEN also caused oxidative damage as demonstrated by the appearance of disulfide-bonded species on non-reducing SDS–PAGE and protection of 4-OHEN-mediated enzyme inhibition by free radical scavengers. The studies of thiol group titration and irreversible kinetic experiments indicated that the different cysteines have distinct reactivity for 4-OHEN; Cys 47 was the most reactive thiol group, whereas Cys 169 was resistant to modification. These results demonstrate that hGST P1-1 is inactivated by 4-OHEN through two possible mechanisms: (1) covalent modification of cysteine residues and (2)

oxidative damage leading to proteins inactivated by disulfide bond formation. Kinetic inhibition experiments with 4-OHEN showed that GST P1-1 had a lower K_i value (20.8 μM) compared to glyceraldehyde-3-phosphate dehydrogenase (52.4 μM), P450 reductase (77.4 μM), pyruvate kinase (159 μM), glutathione reductase (230 μM), superoxide dismutase (448 μM), catalase (562 μM), GST M1-1 (620 μM), thioredoxin reductase (694 μM), and glutathione peroxidase (1410 μM) [84]. We also found that 4-OHEN significantly decreased GSH levels and the activity of GST within minutes in both ER-negative (MDA-MB-231) and ER-positive (S30) human breast cancer cells. In addition, 4-OHEN caused significant decreases in GST activity in non-transformed human breast epithelial cells (MCF-10A), but not in the human hepatoma HepG2 cells, which lack GST P1-1. We also showed that GSH partially protected the inactivation of GST P1-1 by 4-OHEN *in vitro*, and depletion of cellular GSH enhanced the 4-OHEN-induced inhibition of GST activity. In contrast to the significant inhibition of total GST activity in these human breast cancer cells, the other cellular enzymes including P450 reductase, pyruvate kinase, glutathione reductase, superoxide dismutase, catalase, thioredoxin reductase, and glutathione peroxidase were resistant to 4-OHEN-induced inhibition. These data suggest that GST P1-1 may be a preferred protein target for catechol estrogens *in vivo*. It is possible that the reason for the specificity for GST P1-1 over other cellular enzyme targets is that 4-OHEN could be a substrate for this enzyme; however, to date the rapid kinetic experiments necessary to provide evidence for this hypothesis have not been done and it is unclear that the already very rapid non-enzymatic reaction of the catechol estrogen *o*-quinones with GSH would be enhanced by enzymatic catalysis *in vivo*.

The inhibition of GST activity in cells may be associated with early events that trigger apoptosis. Not only is GST a detoxification enzyme providing protection against products of oxidative stress, it has also been shown to be involved in cellular regulation through the JNKs signaling pathway [85]. Adler *et al.* [85] have demonstrated that human GST P1-1 is an endogenous inhibitor of JNKs, which belong to the multi-member family of stress kinases and play a role in cell growth, apoptosis, and transformation [78]. It has been reported that GST inhibitors (*S*-hexylglutathione and ethacrynic acid) caused significant male germ cell apoptosis and that apoptosis, induced by H_2O_2, could be enhanced by the presence of GST inhibitors [86]. Baez *et al.* [87] have also showed that GST can protect cells against apoptosis induced

by *o*-quinones derived from catecholamines. It should be noted that the endogenous catechol estrogen 4-OHE, did not decrease the intercellular GSH levels nor inhibit GST activity in human breast cancer cells and it has been shown to be ineffective at inducing apoptosis in these cells [92]. It will be the subject of future work to study the signal transduction pathways activated after the decrease in cellular GSH levels and the inhibition of GST activity by catechol estrogens in cells.

5.2. Catechol-*O*-methyltransferase

Unlike GSTs where it is not known if catechol estrogens are substrates, both endogenous catechol estrogens and the equine catechol estrogens are catechol-*O*-methyltransferase (COMT) substrates [88,89]. COMT catalyzes the transfer of a methyl group from the donor SAM to the catechol estrogen and thus represents a crucial detoxification pathway for catechol estrogens. Similar to GST, genetic polymorphism in COMT has also been associated with increased risk for developing breast cancer, although some epidemiological studies have not shown a correlation [78,90–92]. 4-OHEN was found to be an irreversible inhibitor of COMT-catalyzed methylation of the endogenous catechol estrogen 4-hydroxyestradiol (4-OHE_2) with a K_i of 26.0 μM and a k_2 of 1.62×10^{-2}/s. 4-OHEN *in vitro* not only caused the formation of intermolecular disulfide bonds as demonstrated by gel electrophoresis, but electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) also showed that 4-OHEN alkylated multiple residues of COMT. Peptide-mapping experiments further indicated that Cys 33 in recombinant human soluble COMT was the residue most likely modified by 4-OHEN *in vitro*. Furthermore, we have also shown that the variant form of COMT, where valine has been substituted for methionine, is more susceptible to 4-OHEN-mediated inhibition [93,94]. These data suggest that inhibition of COMT methylation by 4-OHEN might reduce endogenous catechol estrogen clearance *in vivo* and further enhance toxicity. Experiments are in progress to determine if COMT is a target for 4-OHEN *in vivo*.

6. ROLE OF THE ESTROGEN RECEPTOR IN ESTROGEN CARCINOGENESIS

It is well known that the ER plays a major role in the mechanism of estrogen-induced carcinogenesis ([20,95]; *Hormonal mechanism*,

Fig. 1). The theory is that excessive binding to the ER leads to an increase in cell proliferation in hormone-sensitive target tissues such as the breast and endometrium. In these rapidly dividing cells, the chances for mutations to occur increases dramatically leading to initiation/promotion of the carcinogenic process. Recently, we have hypothesized that the ER could also play a role in catechol estrogen/*o*-quinone-induced carcinogenesis. It is quite possible that the catechol estrogen and/or *o*-quinone bind to the ER, which carries it directly to estrogen-sensitive genes, where DNA damage occurs resulting in mutations.

We have preliminary data that this mechanism may play a role in catechol estrogen-induced toxicity, DNA damage, and apoptosis [96,97]. We have examined the effect of ER status on the relative ability of 4-OHEN and 4,17β-OHEN to induce DNA damage in ER-negative cells (MDA-MB-231), ERα-positive cells (S30), and ERβ-positive cells (β41). The ER-positive cell lines are stable transfectants derived from the MDA-MB-231 cells [98,99]. The data showed that both 4-OHEN and 4,17β-OHEN induced concentration-dependent DNA single-strand cleavage in all three cell lines (Fig. 8). However, cells containing ERα or ERβ had significantly higher DNA damage, although there was no significant difference between the two ER containing cell lines. In addition, the more estrogenic 4,17β-OHEN generated an increased amount of DNA single-strand breaks as compared to 4-OHEN consistent with its enhanced estrogenic activity [93]. Very similar results were obtained when apoptosis was examined in that ER-positive cells were

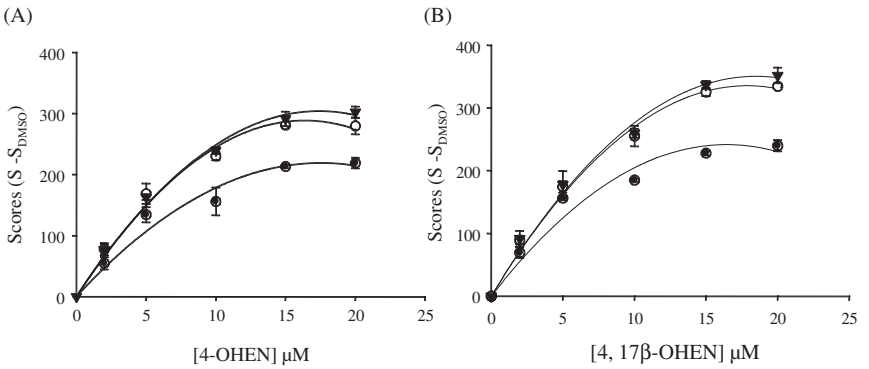

Fig. 8. Induction of DNA single-strand breaks induced by (A) 4-OHEN and (B) 4,17β-OHEN in ER-negative and ERα- or ERβ-positive breast cancer cell lines. Cells were treated with compounds or vehicle for 90 min. Closed circles, MDA-MB-231 cells; open circles, β41 cells; closed triangles, S30 cells.

much more sensitive to 4-OHEN-mediated induction of apoptosis compared to ER-negative cells. Finally, the endogenous catechol estrogen metabolite 4-hydroxyestrone was considerably less effective at inducing DNA damage and apoptosis in breast cancer cell lines as compared to 4-OHEN. Our data suggest that the cytotoxic effects of 4-OHEN could be related to its ability to induce DNA damage and apoptosis in hormone-sensitive cells *in vivo,* and these effects may be potentiated by the ER.

7. SUBSTITUTION OF THE 4-HYDROXY GROUP ON CATECHOL ESTROGENS WITH FLUORINE ABOLISHES QUINOID FORMATION WHILE MAINTAINING ESTROGENIC ACTIVITY

Chemical modification of estrogens in order to prevent oxidation to toxic quinoids while maintaining the beneficial effects of estrogens would be highly desirable for new hormone replacement therapies. It has been reported that fluorination of the A-ring of estradiol in the 4-position dramatically reduces catechol estrogen formation without loss of estrogenic activity [100]. In addition, 4-fluoroestradiol is much less carcinogenic compared to estradiol *in vivo*. In order to block catechol formation from equilenin, 4-halogenated equilenin derivatives were synthesized (Fig. 9; [101]). These derivatives were tested for their ability to bind to the ER, induce estrogen-sensitive genes, and their potential to form catechol metabolites.

We found that the 4-fluoro derivatives were more estrogenic than the 4-chloro and 4-bromo derivatives as demonstrated by a higher binding affinity for ERs α and β, an enhanced induction in alkaline phosphatase activity in Ishikawa cells, pS2 expression in S30 cells, and PR expression in Ishikawa cells. Incubation of these compounds with tyrosinase in the presence of GSH showed that the halogenated

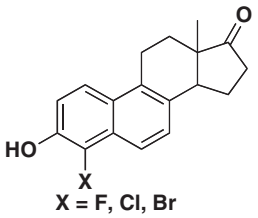

Fig. 9. Structures of halogenated equilenin derivatives.

equilenin compounds formed less catechol GSH conjugates than the parent compounds, equilenin and 17β-equilenin. In addition, these halogenated compounds showed less cytotoxicity in the presence of tyrosinase than the parent compounds in S30 cells. Also as stated above, the 4-fluoro derivatives showed similar estrogenic effects as compared with parent compounds; however, they were less toxic in S30 cells as compared to equilenin and 17β-equilenin. Since 17β-halogenated equilenin derivatives showed higher estrogenic effects than the halogenated equilenin *in vitro*, we studied the relative ability of the 17β-halogenated equilenin derivatives to induce estrogenic effects in the ovariectomized rat model. The 4-fluoro derivatives showed higher activity than 4-chloro and 4-bromo as demonstrated by inducing higher vaginal cellular differentiation, uterine growth, and mammary gland branching. However, 17β-hydroxy-4-fluoroequilenin showed a lower estrogenic activity as compared to 17β-hydroxyequilenin and estradiol, which is probably due to the alternative metabolic and distribution pathways in different tissues. These data suggest that the 4-fluoroequilenin derivatives have promise as alternatives to traditional estrogen replacement therapy due to their similar estrogenic properties with less overall toxicity.

8. SUMMARY

The roles of quinones in mediating the adverse effects of estrogens have not been investigated in detail. It is possible for these electrophilic/redox active quinones to cause damage within cells by a variety of different pathways. Oxidative enzymes, metal ions, and in some cases molecular oxygen can catalyze quinoid formation; so alkylation of cellular nucleophiles (GSH, proteins, DNA) by these species may occur to a significant extent in many tissues. In addition, the formation of ROS especially through redox cycling between the quinones and semiquinone radicals, could contribute to the adverse properties of the parent compounds. Redox cycling can cause lipid peroxidation, consumption of reducing equivalents, oxidation of DNA, and DNA strand breaks. DNA binding occurs, but the sites of alkylation and relationships to cytotoxicity are dependent on the chemical structure of the quinones and the cellular environment in which they are formed. Finally, ERα and/or ERβ may play a role in potentiating the deleterious effects of catechol estrogens and/or *o*-quinones. Given the direct link

between excessive exposure to estrogens, metabolism of estrogens, and increased risk of breast cancer, it is crucial that factors, which affect the formation, reactivity, and cellular targets of estrogen quinoids, be thoroughly explored.

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CHAPTER 2

Diverse Mechanisms of Endogenous Nucleobase Deamination in DNA and RNA

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1. INTRODUCTION

The past decade has witnessed an emerging appreciation for the complexity of DNA damage caused by agents arising as consequence of the normal physiology of human cells. These so-called endogenous DNA lesions span the full range of chemical reactions, including oxidation, reduction, halogenation, alkylation, nitrosation and hydrolysis, with damage affecting both the nucleobase and deoxyribose moieties of DNA. A recent review by De Bont and van Larebeke addresses many of the endogenous base lesions with a thorough compilation of estimates of the quantities of lesions in mammalian cells and tissues [1]. While lesions derived by oxidation of purines and pyridines have received significant attention over the past 20 years, it is only recently that lesions derived from lipid peroxidation products and nucleobase deamination have emerged as both complex and multifaceted. This review will focus on nucleobase deamination (Fig. 1) in light of recent technical advances in analytical methods to quantify nucleobase deamination products, as well as several recent observations on the stability of these DNA lesions and the diverse mechanisms of their formation.

Nucleobase deamination by any mechanism results in the formation of xanthine, hypoxanthine and uracil, with nitrosative mechanisms leading to the formation of oxanine, abasic sites and DNA cross-linking. The confusing nomenclature for nucleobase deamination products is presented in Table 1 and their structures in Fig. 1. This review addresses the diversity of mechanisms involved in nucleobase deamination in DNA and RNA, with an update on the currently known mechanisms and discussion of several novel pathways that may influence the cellular burden of these toxic and mutagenic DNA lesions.

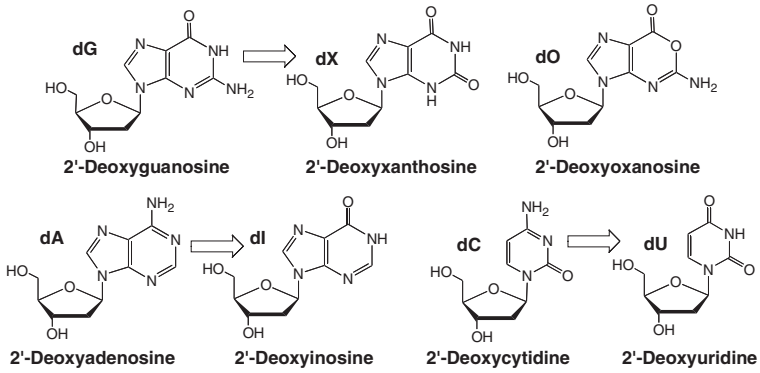

Fig. 1. Nucleobase deamination products as 2′-deoxyribonucleosides.

Table 1. Nucleobase nomenclature

Free base	2′-Deoxyribonucleoside	Ribonucleoside
Xanthine (X)	2′-Deoxyxanthosine (dX)	Xanthosine (rX)
Oxanine (O)	2′-Deoxyoxanosine (dO)	Oxanosine (rO)
Hypoxanthine (I)	2′-Deoxyinosine (dI)	Inosine (rI)
Uracil (U)	2′-Deoxyuridine (dU)	Uridine (rU)

1.1. Stability of dX to depurination

One factor contributing to the relatively slow progress in studies of nucleobase deamination has been the presumed [2,3], though unsubstantiated at the time, instability of dX toward depurination. Two recent studies, however, have confirmed that, at neutral pH, dX is a relatively stable lesion capable of contributing to the mutagenic burden of cells. Suzuki and coworkers observed that dX was approximately 40-fold less stable to depurination than dG at pH 4 and 37 °C [3], but without knowledge of the temperature- and pH-dependence of dX depurination, it is not possible to extrapolate the data to biological conditions. In more rigorous studies, Termini and coworkers observed that, at acidic pH, depurination of dX occurred an order of magnitude more rapidly than dG in a single-stranded oligodeoxynucleotide, but found depurination to occur at a rate similar to that of dG at neutral pH [4]. Vongchampa *et al*. determined the pH-dependence of dX depurination kinetics in the form of a 2′-deoxynucleoside, and as single- and double-stranded oligodeoxynucleotides [5]. At neutral pH, dX in double-stranded DNA has a half-life of ~2 years, with the depurination rate constant inversely proportional to pH. This behavior is similar to the pH-dependence for depurination of dG and dA in DNA observed by Lindahl and Nyberg [6] and it is consistent with the two depurination processes shown in Fig. 2 [7–9]: an acid-catalyzed protonation of dX at pH <7 with a depurination rate constant of $2.6 \times 10^{-5}\,s^{-1}$ and a pH-independent hydrolysis at pH >7 with a rate constant of $1.3 \times 10^{-8}\,s^{-1}$. The latter value is ~100-fold greater than the analogous rate constant of $\sim 10^{-10}\,s^{-1}$ for depurination of dG and dA in DNA at 37 °C and pH 7 [6]. Though Vongchampa *et al.* observed a 5-fold increase in dX stability upon incorporation into a single-stranded oligo, they observed a much smaller reduction in the rate of dX depurination in double-stranded oligodeoxynucleotide than the expected 3- to 4-fold reduction based on depurination of dG and dA in DNA [6]. One explanation for the lack of a stabilizing effect may be

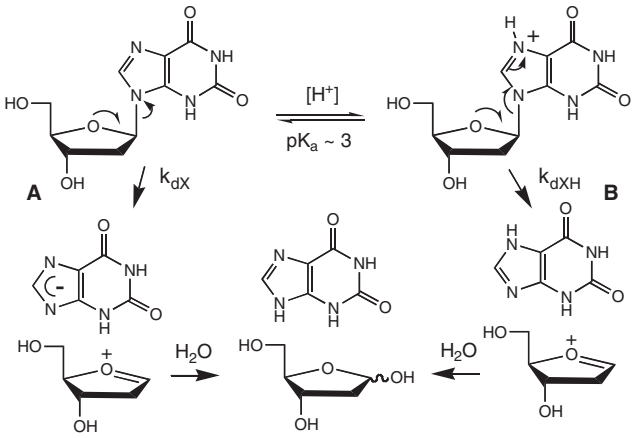

Fig. 2. Pathways for depurination of dX.

related to the relatively low pK_a of ~5.6 for the N^3 of dX [10], which, at pH 7, would result in a negative charge for ~95% of the xanthine bases and destabilization of the helix.

These studies point to a role for dX in the mutagenicity of base deamination products. With a half-life of 2 years, endogenously formed dX residues, if not repaired, can be expected to undergo depurination to the extent of only 3% and 11% in one and four months, respectively. Given the relatively slow rate of cell division in tissues such as the human colon (3 divisions per year; Ref. [11]), the bulk of dX residues in a cell will be present during cell replication and may thus contribute to mutagenesis.

1.2. Analytical methods and artifacts

A variety of methods have been developed to quantify nucleobase deamination products, but few have the necessary sensitivity and reproducibility required to measure DNA lesions occurring with frequencies below 1 per 10^6 nucleotides (nt) and all are subject to artifact due to the activity of nucleobase deaminase enzymes that are ubiquitous in prokaryotic and eukaryotic cells. Several efforts to measure uracil have employed high-performance liquid chromatography (HPLC) quantification [12], ^{32}P post-labeling of 2′-deoxyribonucleosides released from DNA [13], and uptake and labeling of DNA with ^{3}H-uridine [14] or ^{3}H-2′-deoxyuridine [15]. These methods suffer from artifacts of cytosine deamination [14], RNA-derived uracil contamination [15] and high

variability [13], and all are not sensitive enough to detect background levels of dU in DNA.

As with other DNA and RNA lesions, the most sensitive and accurate methods for the quantification of nucleobase deamination products employ a gas or liquid chromatograph coupled to a mass spectrometer (GC/MS, LC/MS, LC/MS/MS). This approach combines the resolving power of chromatography with the sensitivity and rigor of mass spectrometry for identifying specific chemical species, with sensitivities to 5 lesions per 10^8 nt in 50 μg of DNA [16]. GC/MS approaches have been used to quantify uracil [17,18], xanthine [19,20] and hypoxanthine [19,20] as free bases. LC/MS methods have been developed for oxanine and dO [16,21–23], inosine in RNA [24], dX, dI and dU in DNA [16].

The major hurdle to accurate quantification of nucleobase deamination products in DNA and RNA is the activity of deaminase enzymes present in all cells. The most notable recent example is the dC deaminase activity termed activation-induced cytidine deaminase (AID) [25,26]. As will be discussed in Section 4.1, this enzyme performs a critical function in immunoglobulin diversification by apparently causing sequence selective cytidine deamination either in DNA or RNA. Other nucleobase deaminase activities include the adenosine deaminase involved in RNA editing [27–29] and purine nucleotide metabolism, and guanine deaminase involved in purine base salvage pathways [30,31]. These enzymes have the potential to cause deamination of nucleobases in DNA during cell manipulations, DNA isolation and during DNA processing. We encountered significant dA deaminase activity as a contaminant of commercial sources of alkaline phosphatase [16], as well as adventitious dC deaminase activity in human B-lymphoblastoid TK6 cells [32], which is not surprising given the expression of AID protein in B cells [25,26].

The solution to the problem of undesirable deaminase activity is the use of deaminase inhibitors. As shown in Fig. 3, 3,4,5,6-tetrahydrouridine (THU) is a specific and potent inhibitor of C/dC deaminase [33] that decreases the detected level of dU when present during processing of TK6 cell DNA for LC/MS analysis [32]. Coformycin is a specific inhibitor of adenosine deaminase [34] that effectively inhibits dI formation without interfering with alkaline phosphatase activity when added during DNA processing (Fig. 3). While we have not yet encountered adventitious dG deaminase activity in *Escherichia coli*, yeast, mouse or human cells (Pang, Dong and Dedon, unpublished observations), the

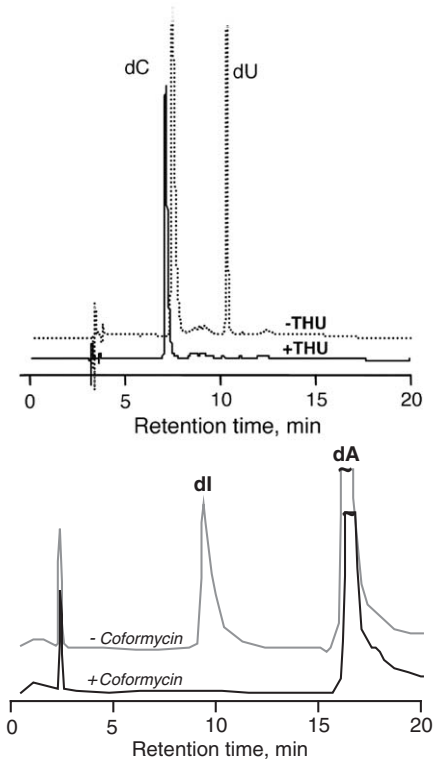

Fig. 3. HPLC analysis of THU inhibition of dC deaminase activity (upper) and coformycin inhibition of dA deaminase activity (lower). Dashed line: no inhibitor; solid line: presence of inhibitor.

availability of guanine deaminase inhibitors, azepinomycin and 4,6-diamino-8H-1-hydroxyethoxymethyl-8-iminoimidazo[4,5-e][1,3]diazepine [35,36], suggests that its emergence would not pose an insoluble problem. It is important to consider that these inhibitors have not seen widespread use in published analytical methods for nucleobase deamination products, so there is some question about the accuracy of the quantities of dX, dI and dU determined *in vitro* and *in vivo*.

2. SIMPLE HYDROLYTIC MECHANISMS

In addition to exogenous agents such as bisulfite [37], there are three major endogenous mechanisms for nucleobase deamination. As illustrated in Fig. 4, the simplest is hydrolytic deamination that occurs in all aqueous environments and that has been the subject of numerous

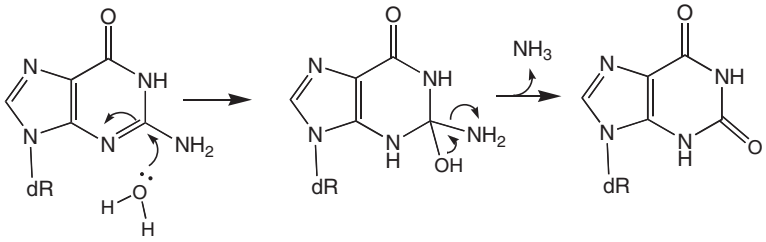

Fig. 4. Hydrolytic deamination of dG to form dX.

reviews that obviate repetition here [38–40]. The propensity for hydrolytic deamination of DNA occurs in the order 5-methyl-dC > dC > dA > dG [41,42], with a half-life for dC in the range of 10^2–10^3 years for single-stranded DNA and 10^4–10^5 years in double-stranded DNA [43–45]. C^5-Methylation of dC increases the rate of deamination by 2- to 20-fold [44,45]. The high rate in dC and 5′-methyl-dC has been proposed to account for the fact that C → T mutations at CpG sites represent the most common human mutation [26], though CpG motifs have also been observed to be hotspots for reactions by a variety of genotoxic chemicals [46,47].

3. NITROSATIVE DEAMINATION AND INFLAMMATION

While there are established environmental sources of nitrosating agents [48], endogenous agents may represent the major source of nitrosative stress in humans. Epidemiological studies demonstrate an association between chronic inflammation and increased cancer risk [49–52], such as the links between inflammatory bowel disease and colon cancer [53,54], *Helicobacter pylori* infection and gastric cancer [55,56] and *Schistosoma haematobium* infection and bladder cancer [57,58]. The strongest evidence for a mechanistic link between inflammation and cancer involves the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by macrophages and neutrophils in response to cytokines and other signaling processes arising at sites of inflammation (Fig. 5). These chemical mediators of inflammation cause damage to surrounding host tissue by oxidation, nitration, halogenation and deamination of biomolecules of all types, with the formation of toxic and mutagenic products leading to somatic mutations associated with malignant transformation [59,60], with additional contribution mechanisms that are independent of genotoxicity [61,62]. Of

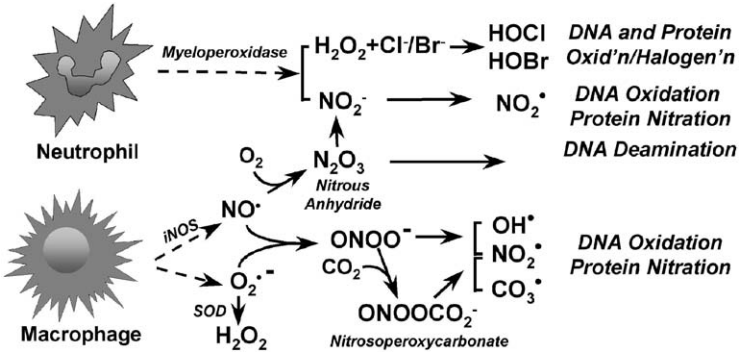

Fig. 5. Generation of reactive oxygen and nitrogen species at sites of inflammation.

particular importance here are the RNS arising from nitric oxide (NO) production by activated macrophages, the chemistry of which leads, along one pathway, to nitrosating species capable of nucleobase deamination in DNA and RNA [62].

3.1. $NO^\bullet$ biochemistry

With a host of biological functions, $NO^\bullet$ has become one of the most highly studied molecules and its biochemistry has been thoroughly covered in several review articles [64–70]. The major source of $NO^\bullet$ at sites of inflammation is the inducible form of nitric oxide synthase (iNOS), one of three NOS isoforms with homology to cytochrome P450 reductase. While physiological production of $NO^\bullet$ (e.g., neurotransmission, blood pressure control) occurs at nanomolar concentrations, higher levels of $NO^\bullet$ are associated with inflammation and host defenses against microbes. Macrophage cultures activated with lipopolysaccharide and INF-γ *in vitro* have been shown to produce $NO^\bullet$ at a rate of $\sim$6 pmol s^{-1} per 10^6 cells or 4×10^6 molecules of $NO^\bullet$ per cell [71]. The data regarding biologically relevant levels of $NO^\bullet$ are not firm, but it is generally assumed that steady-state concentrations of $NO^\bullet$ do not exceed $\sim$1 μM at sites of inflammation [71–73]. With inflammation lasting months or years [74–76], local epithelial cells are thus exposed to significant quantities of ROS and RNS. While $NO^\bullet$ is released from macrophages in combination with many other species (e.g., hydrogen peroxide, tumor necrosis factor, cytokines), studies with $NO^\bullet$

scavengers point to $NO^{\bullet}$ as the key mediator of macrophage-induced cytostasis [67,77,78].

In chronically inflamed tissue, high local levels of $NO^{\bullet}$ are available for reaction with oxygen or superoxide to generate a multitude of reactive species, as shown in Fig. 5. The biological effects of $NO^{\bullet}$ ultimately depend on the complexity of the cellular milieu, including concentrations of superoxide and oxygen, enzymes such as catalase and superoxide dismutase, antioxidants such as glutathione, and the diffusion distances between generator cells and target cells [79]. In general terms, however, the reactions of $NO^{\bullet}$ fall along three major pathways in biological systems: (1) diffusion and intracellular consumption; (2) auto-oxidation to form nitrous anhydride, N_2O_3 (Fig. 5); and (3) reaction with superoxide to form peroxynitrite, $ONOO^-$ (Fig. 5) [80]. $ONOO^-$ can oxidize and nitrate DNA and may potentially cause strand breaks through attack on the sugar–phosphate backbone.

3.2. Background on N_2O_3-induced nucleobase deamination

The oxidation of $NO^{\bullet}$ by molecular oxygen leads to the formation of N_2O_3 and a number of other nitrosating agents, including the nitrosonium ion (NO^+), nitrous acidium ion ($NO\text{-}OH_2^+$), NOX and N_2O_4, that are favored in gas-phase reactions or under highly acidic conditions. However, while acidified solutions of NO_2^- will generate nitrosating agents [81–84], deamination of DNA bases at physiological pH in cells is likely mediated primarily by N_2O_3 [71]. In addition to forming *N*-nitrosamines [85], this powerful nitrosating agent will react with the primary and heterocyclic amines in DNA bases to cause conversion of cytosine to uracil (2′-deoxyuridine, dU), guanine to xanthine (2′-deoxyxanthosine, dX) and oxanine (2′-deoxyoxanosine, dO; observed with HNO_2 at low pH), 5-methylcytosine to thymine and adenine to hypoxanthine (2′-deoxyinosine, dI), as well as abasic sites and inter- or intra-strand G–G cross-links [18,20,81,86–88] (Fig. 1). The reactivity of DNA bases with N_2O_3 is reversed relative to hydrolysis [18] and the abasic sites arise by nitrosation of the N^7-dG and N^7 or N^3 of dA that destabilizes the glycosidic bond and leads to depurination [21,22]. While nitrosative deamination of dC and dA appear to involve straightforward chemistry, the formation of dO and dX has been the subject of some controversy.

3.3. Unusual chemistry of N_2O_3-induced deamination of dG

The chemistry of nitrosative deamination of nucleobases in DNA has proven to be both complicated and experimentally challenging to define. The simplest mechanism, and the one likely to account for formation of dU from dC and dI from dA, involves nitrosation of exocyclic amines in the nucleobases with subsequent nucleophilic substitution of N_2 by water, as shown in Fig. 6. The observed formation of abasic sites likely involves similar *N*-nitrosation of the N7 positions of dG and dA [16,21,22]. However, it is now clear that the decades-old assumption of a simple guaninediazonium ion intermediate common to xanthine and G–G cross-links is incorrect and that a more complicated mechanism exists to account for chemistry. The major challenge to this simple model is the observed formation of two deamination products of dG: dX and dO in reactions involving nitrosative chemistry. Suzuki *et al*. originally described the formation of dO in reactions of nucleosides and

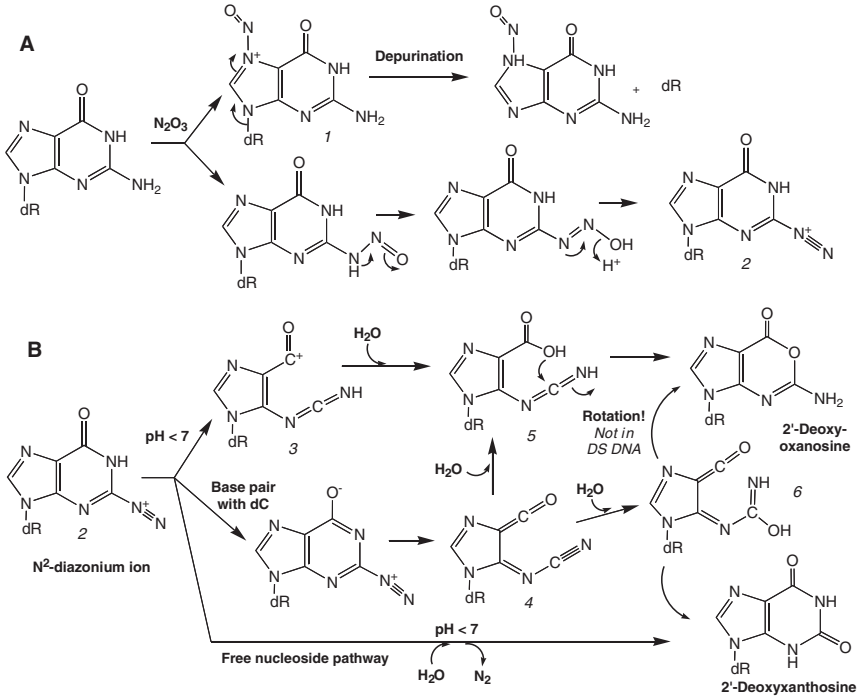

Fig. 6. Chemistry of nitrosative deamination of nucleobases in DNA. (A) Basic *N*-nitrosation leading to depurination and deamination. (B) Glaser's dG deamination model to account for the formation of dO and dX [104].

DNA with nitrite under acidic (pH < 4) conditions [23,81] and subsequently described the physicochemical and biological properties of dO [3,89–99]. Shuker and coworkers also observed the formation of oxanine base in reactions of 2′-deoxyribonucleotides and calf thymus DNA with millimolar concentrations of the mutagenic nitrosating agent, 1-nitrosoindole-3-acetonitrile (NIAN) [21,22].

These observations stand in contrast to our inability to detect dO in DNA exposed to $NO^{\bullet}$ and O_2 under conditions approaching those thought to exist at sites of chronic inflammation, as discussed shortly. Using a sensitive LC/MS method and the addition of deaminase inhibitors, we quantified dX, dI, dO and dU in DNA that was exposed to steady-state concentrations of 1.3 μM $NO^{\bullet}$ and 190 μM O_2 (calculated steady-state concentrations of 40 fM N_2O_3 and 3 pM NO_2) in a recently developed reactor [100] that avoids the anomalous gas-phase chemistry of $NO^{\bullet}$; the presence of a headspace containing air in earlier delivery systems alters the chemistry of $NO^{\bullet}$ by biasing the formation of N_2O_3 and allowing formation of N_2O_4 [101,102]. Under these conditions, dX, dI and dU were formed at nearly identical rates ($k = 1.2 \times 10^5\,M^{-1}\,s^{-1}$) to the extent of ~80 lesions per 10^6 nt after 12 h exposure to $NO^{\bullet}$ in the reactor (Table 2). However, dO was not detected in $NO^{\bullet}$-exposed DNA at a level of > 6 lesions per 10^8 nt, except when the DNA was exposed to nitrite at pH 3.8. This result and the observations of Suzuki *et al*. [23,81] suggested a pH-dependent partitioning of the reaction intermediate leading to either X or O. Indeed, it is likely that low pH accounts for Shuker's observation of O formation in DNA exposed to NIAN [21]. In those studies, the observation of extensive depurination of dG, dA and dO suggests an acidic

Table 2. Spectrum of DNA lesions produced by exposure to steady-state levels of 1.3 μM $NO^{\bullet}$ and 190 μM O_2

	Lesions per 10^6 nt				
Dose (μM min)	AP[a]	dX	dI	dU	dO
0	5	12	11	11	< 0.06
234	9	27	41	37	< 0.06
468	13	45	49	58	< 0.06
936	15	99	91	88	< 0.06
1872	22	131	143	109	< 0.06

[a] AP: abasic sites; values for all lesions represent mean for four determinations in plasmid pUC19 DNA.

condition arising from weak buffering (0.5 mM Tris) in the presence of millimolar concentrations of NIAN and its degradation products, conditions that would favor the formation of dO in DNA. What has not been explained yet is the observation of dO formation in nucleosides and nucleotides at near-neutral pH by Suzuki and coworkers [81] and possibly by Shuker and coworkers [21], though the latter studies were again performed under weakly buffered conditions.

To explain these observations, as well as to account for the sequence specificity of dG–dG cross-links formed during nitrosative deamination [88,103] and the predominance of dX over dO under most conditions, Glaser and coworkers recently proposed an attractive model [104] based on currently available computational data [104–113] and the experimental results discussed above. As shown in Fig. 6A, the model begins with the basic diazotization of the N2 position of dG to form diazonium ion **2**. In environments not involving double-stranded DNA (e.g., free nucleoside, nucleotide, single-stranded DNA), the diazonium ion can undergo nucleophilic displacement by a water molecule to form dX (lower pathway in Fig. 6B) or it can lose N_2 and undergo pyrimidine ring opening to form cation **3**. The latter pathway is the only one leading to dO. In double-stranded DNA, however, base pairing with cytosine is proposed to provide base catalysis for a rapid deprotonation of the dG diazonium ion, with subsequent rapid ring opening to form cyanoimine **4**. Perhaps the weakest point in the model is that subsequent addition of water to **4** in double-stranded DNA leads to formation of **6** rather than **5**, with consequential formation of dX rather than dO due to limited C–N bond rotation of **6**. Nonetheless, the model [104] adequately accounts for most if not all of the observed deamination products under different conditions. Of course, the model is easily tested by defining the complete product spectra in nucleoside, single- and double-strand DNA forms of dG at a range of pH values. The model would predict that, while dX will always predominate, the proportion of dO should be highest in nucleoside reactions of dG and lowest in double-stranded DNA under biological conditions of pH.

3.4. N_2O_3-induced nucleobase deamination *in vitro* and *in vivo*

In addition to dX, both dI and dU have been observed in bacterial and mammalian cells exposed to $NO^{\bullet}$ *in vitro* [20,86] and in activated

macrophages [114]. The major problem with most previous studies of nitrosative DNA damage is that the delivery of $NO^{\bullet}$ and O_2 occurred under conditions not considered to be biologically relevant. For example, Nguyen *et al*. exposed DNA and TK6 cells to a ~20 mM total bolus (syringe) dose of $NO^{\bullet}$ and found that dX and dI were formed to the extent of 3 and 10 lesions per 10^3 nt, respectively, for isolated DNA, with 3-fold lower levels in cells [20]. Yields of dI and dX in DNA were found to be 15- to 100-times higher, respectively, than those observed with free dA and dG [20]. Similarly, Wink *et al*. found 5 dU per 10^3 nt in DNA exposed to $NO^{\bullet}$ by bubbling the gas into solution until 1 mol l^{-1} had been absorbed [87]. Caulfield *et al*. used a delivery system in which Silastic tubing allowed controlled diffusion of $NO^{\bullet}$ into solution (with headspace!) at a rate of 10–20 μM min^{-1} and found that xanthine was formed at twice the rate of uracil and single-stranded DNA oligonucleotides were nearly 10-fold more reactive than double-stranded DNA [18].

To avoid the anomalous gas-phase chemistry of $NO^{\bullet}$, Deen and coworkers developed a Silastic tubing-based reactor [100] without headspace. As mentioned earlier, a recent study by Dong *et al.* employed this reactor to define the spectrum of DNA lesions arising under conditions approaching physiological relevance [104]. The resulting spectrum of nitrosatively induced abasic sites and nucleobase deamination products is shown in Table 2. While dX, dI and dU were formed at nearly identical rates, dO was not detected in $NO^{\bullet}$-exposed DNA ($<$6 lesions per 10^8 nt). Another important observation was the $NO^{\bullet}$-induced production of abasic sites, which likely arise by nitrosation of the N7 positions of guanine and adenine with subsequent depurination, to the extent of ~10 per 10^6 nt after 12 h of exposure in the $NO^{\bullet}$ reactor. In conjunction with other studies of nitrosatively induced dG–dG cross-links [86], these results suggest the following spectrum of nitrosative DNA lesions in inflamed tissues: ~2% dG–dG cross-links, 4–6% abasic sites and 25–35% each of dX, dI and dU.

4. ENZYMATIC DEAMINATION OF NUCLEOBASES

While hydrolysis and nitrosation have been recognized as the major mechanisms of nucleobase deamination for decades, a growing body of literature points to a variety of enzymatic mechanisms with the potential to contribute to the cellular burden of DNA and RNA deamination products.

4.1. AID protein

The most notable recent example of an enzymatic DNA deamination mechanism is the dC deaminase activity termed AID, an enzyme subject to extensive review in the past few years [25,26,29,115–118]. This enzyme, originally discovered by Honjo and coworkers by subtractive RNA hybridization of transcripts differentially expressed in activated B lymphocytes [119], performs critical functions in three facets of immunoglobulin diversification: class switch recombination, somatic hypermutation and gene conversion (reviewed in [25,26,29,115–118]). However, it does not appear to be involved in V(D)J recombination [120]. Whereas class switch recombination requires exchange of heavy chain, class-specific DNA fragments between genes, and thus requires double-stranded DNA cleavage, somatic hypermutation entails generation of numerous point mutations in the variable region exons of Ig heavy and light chain genes to cause combinatorial selection of high affinity antibodies against a novel antigen [121,122]. As covered in the excellent review by Chaudhuri and Alt [117], these diverse mechanisms probably require all of the different DNA repair pathways and a host of ancillary proteins in addition to AID. While the biological endpoints are clearly defined, the biochemical mechanism underlying these functions are yet to be clearly defined, with evidence pointing to cytidine deamination in both DNA and RNA.

The DNA target hypothesis involves AID-mediated conversion of dC to dU followed, in the case of somatic hypermutation, by mutagenic responses to dU. These include abasic site formation as a result of the action of uracil *N*-glycosylase, with subsequent polymerase errors at the resulting abasic site; error-prone mismatch repair of the dU:dG pair [123,124] or polymerase bypass of unrepaired dU [124,125]. In the case of class switch recombination, the conversion of dC to dU by AID initiates a cascade of repair activities leading to highly controlled, sequence-specific recombination events [117]. The recent claim by Honjo and coworkers that uracil glycosylase is not required for somatic hypermutation is problematic in several regards, the most important of which is that they used DNA double-strand breaks, as inferred from histone H2Ax phosphorylation, as an endpoint for somatic hypermutation [126]. While double-stranded DNA breaks can occur by a statistical coalescence of single-strand breaks, double-strand breaks are not required for somatic hypermutation, so this endpoint seems inappropriate for studying uracil glycosylase involvement in this process.

Several other studies have established a role for uracil glycosylase in somatic hypermutation [124,125,127].

The RNA hypothesis, which more narrowly affects class switch recombination and is not supported by experimental evidence, involves the action of AID on one or more hypothetical mRNAs that code for nuclease activities that actually cause the recombination-inducing DNA cleavage. The basis for this hypothesis is the sequence and biochemical similarity of AID to apolipoprotein B mRNA editing catalytic subunit 1 (APOBEC-1) [128–131]. This model does not account for somatic hypermutation, but it is quite possible that multiple mechanisms of AID action exist in light of the multiple physiological functions affected by AID and the evidence for multiple activities present in the single AID molecule [131].

One of the major obstacles to defining the precise mechanism of action of AID is the highly selective substrate and sequence selectivity of AID. While the earliest studies of AID revealed no activity against nucleic acid substrates [119], Goodman and coworkers clearly defined the substrate selectivity of AID as involving single-strand DNA, with no apparent activity with double-strand DNA, single-strand RNA or RNA–DNA hybrids [132]. To define sequence selectivity of AID, Goodman and coworkers took the approach of quantifying mutations as an index of AID activity on single-strand DNA substrates and on an actively transcribed circular DNA model [133]. They observed that mutations initially occurred at hotspot sequence motifs for somatic hypermutation $A\frac{A}{G}C$, with a 15-fold preference for the non-transcribed over the transcribed strand of DNA [132], and expanded to neighboring dCs to create larger clusters of mutated regions [133]. These *in vitro* results with AID are consistent with observed traits of somatic hypermutation, including the sequence selectivity and transcriptional dependence.

4.2. Enzymatic editing of RNA

The identification of DNA deaminase activity was preceded by several decades by the discovery of numerous chemical modifications of nucleobases in tRNA, including *N*-methylation of rG and rA, 2′-*O*-methylation, *N*-isoprentenylation of rA, formation of pseudouridine, transglycosylation of guanine with the base analog queuine and, of course, nucleobase deamination products [134,135]. The latter phenomenon is now known to involve conversion of both rA to rI and rC to rU.

As reviewed elsewhere [136,137], the enzymatic deamination of RNA bases was first described in the South African clawed toad, *Xenopus laevis,* with the conversion of adenosines to inosines within double-stranded regions of RNA [138–141]. This led to the identification of three recognized adenosine deaminases that act on RNA (ADARs) and are found only in higher eukaryotes [29,142–144]. The enzymes act on unspliced transcripts at rA in an exon that is base paired with an adjacent intron, with the resulting rI being read as rG during translation [29,142,145]. Currently known genes affected by A-to-I editing include the calcium-gated glutamate receptor [146–148], the 5-hydroxytryptamine receptor [149,150], the potassium channel KCNA1 [137,151] and several identified by comparative genomics, including filamin A protein (FLNA), bladder cancer-associated protein (BLCAP), insulin-like growth factor binding protein 7 (IGFBP7) and cytoplasmic FMR1 interacting protein 2 (CYFIP2) [152,153].

The first example of site-specific editing was observed in a pre-mRNA encoding the vertebrate calcium-gated glutamate receptor subunit (GluR-B) of the aminohydroxymethylisoxazole propionate (AMPA) class of glutamate receptors [146–148]. Editing by ADAR2 converts a glutamine to an arginine within the ion pore of the channel and occurs in virtually all transcripts of the gene under normal conditions. The unedited form of the GluR-B subunit causes the associated AMPA receptor to be more permeable to calcium ions. With glutamate as the major excitatory neurotransmitter in the mammalian brain, it is not surprising that loss of function mutations in ADAR2 causes a variety of neurological symptoms, including death in infancy with seizures and neurodegeneration in the hippocampus [154]. Interestingly, these symptoms only occur when the GluR-B transcript is expressed in its unedited form, since mice with complete deletion of the GluR-B gene are viable due to overlapping function of other subunit genes [155].

The 5-hydroxytryptamine receptor 2C is a member of the rhodopsin family of G protein-coupled receptors and is the only known G protein-coupled receptor known to undergo RNA editing [150]. The editing phenomenon involves positions 157, 158, 159 and 161 in an intracellular loop of the receptor that functions in receptor–G protein coupling in rats and humans [150]. The resulting change from A to I causes conversion of isoleucine to valine, asparagine to glycine, asparagine to serine and isoleucine to valine, respectively, with tissue-specific expression of at least seven 5-HT2C receptor isoforms encoded by eleven different RNA species [150].

Levanon *et al*. used a comparative genomics approach to identify other genes subject to RNA editing, including FLNA, BLCAP, the IGFBP7, CYFIP2 [152,153]. Editing was verified in mice and chickens [153]. Aberrant editing of the transcripts of these genes may account for some of the non-neuronal phenotypes generated by loss of ADARs in mice.

One interesting consequence of ADAR sequence searches in the genomes of yeast and *Drosophila* was the discovery of a family of ADAR-related adenosine deaminases that act on tRNA (ADAT or Tad) [136]. To date, four ADATs have been detected in eukaryotic genomes and one ADAT homolog, the Tad1 gene, in *E. coli* [136]. These enzymes deaminate adenosine at position 34 (adjacent to the anticodon) or 37 (within the anticodon) in a variety of tRNAs in prokaryotes and eukaryotes. Based on sequence similarities, O'Connell and coworkers have argued that ADARs in higher eukaryotes evolved from the more widely distributed ADATs [136].

RNA editing also involves conversion of rC to rU in several genes. There are several features of C-to-U editing that distinguish it from A-to-I, including reaction of the involved enzymes with single-strand DNA template and a more complicated (i.e., less predictable) substrate specificity [156,157]. The first description of C-to-U editing involves the transcript for apolipoprotein B (apoB) in the intestine [128]. The deamination is performed by the apoB mRNA editing catalytic subunit 1 (APOBEC-1), a member of the apobec family of cytidine deaminases and a relative of AID protein discussed earlier [158], in complex with apobec-1 complementation factor (ACF) [156,157]. The editing involves changing a CAA to a UAA stop codon, which results in truncated protein and affects lipoprotein metabolism and transport. A second physiological example of C-to-U editing involves the neurofibromatosis type 1 gene. This time, the change entails conversion of a CGA to a UGA translation termination codon that is predicted, though not yet proven, to truncate the neurofibromin protein product [156,157].

4.3. Consequences of aberrant RNA editing

It is highly likely that RNA editing by both A-to-I and C-to-U mechanisms will prove to be more widespread than is currently appreciated. This suggests a possible role for aberrant editing in the pathophysiology of many diseases, with genetic polymorphisms affecting genes coding for both the target transcripts (e.g., altered substrate structures) or affecting

the deaminases themselves (e.g., alter target recognition or catalytic properties). In addition to the established neurological disorders caused by faulty RNA editing, there is evidence for aberrant editing in several forms of cancer [29]. The possible consequences of inappropriate RNA editing are further complicated by the recent discovery of proteins, the vigilins that bind to promiscuously A-to-I-edited transcripts and target them, or their degradation products, to heterochromatic regions of the nucleus [159]. This suggests the possibility of a gene silencing mechanism for RNA fragments containing inappropriately located rI arising from virtually any mechanism, including inflammation or altered purine metabolism, as discussed next.

5. PURINE METABOLISM AS A SOURCE OF NUCLEOBASE DEAMINATION IN DNA AND RNA

In addition to hydrolytic, inflammatory and enzymatic mechanisms for nucleobase deamination in DNA and RNA, there may be a role for defects in both pyrimidine and, as we have recently observed for both DNA and RNA, purine metabolism as determinants of the genomic burden of nucleobase deamination products. The relationship between pyrimidine metabolism and levels of dU in DNA is now clear [160] and there is an emerging recognition of an association between defects in pyrimidine metabolism and cancer risk [161,162], though some studies suggest that simple defects in folate metabolism cannot account fully for the increased cancer risk [162,163]. There has been an extensive review of pyrimidine metabolism and cancer risk, so this review will address very recent studies of the role of purine metabolism in levels of I and X in DNA and RNA.

5.1. Background on purine metabolism

The anabolism and catabolism of purine bases and nucleotides is highly conserved in all living organisms (Fig. 7) with a central role for X and I in all aspects of purine metabolism. The synthesis of purine ribo- and 2′-deoxyribonucleotides occurs by three mechanisms: *de novo* synthesis, salvage of purine bases by attachment to ribosephosphate, and salvage by phosphorylation of nucleosides. The *de novo* pathway is a 10-step reaction that starts with a rate-limiting conversion of phosphoribosylpyrophosphate (PRPP) to 5-phosphoribosylamine and ends

in the formation of IMP, as shown in Fig. 7. IMP is the precursor to both AMP and GMP and is normally present in cells at low micromolar concentrations (Table 3) [164]. Conversion of IMP to AMP is a second rate-limiting process in purine synthesis and involves two steps: (1) displacement of the O^6 of IMP by the amino group of aspartate to form adenylosuccinate (mediated by adenylosuccinate synthase, the product of the *E. coli purA* gene); followed by (2) removal of fumarate to form AMP (mediated by adenylosuccinate lyase, the product of the

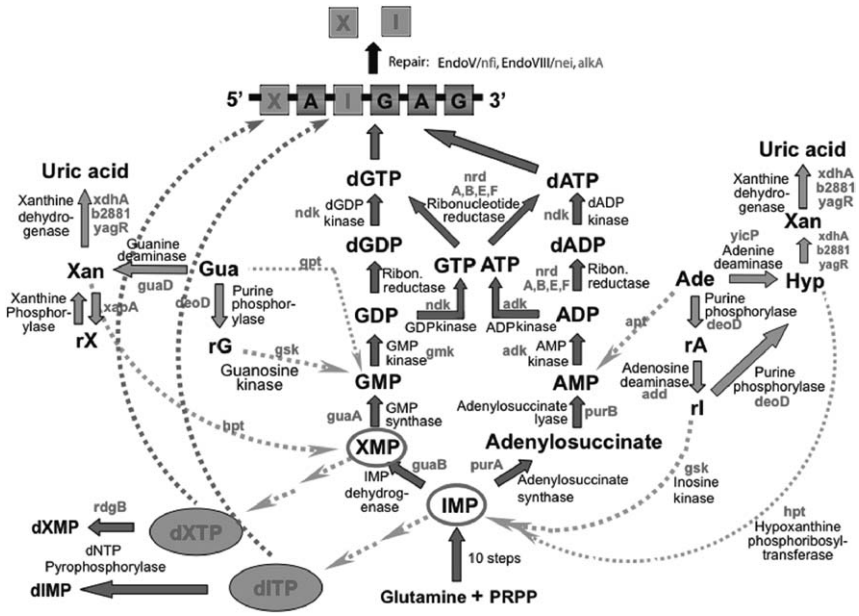

Fig. 7. Purine metabolism in *E. coli.*

Table 3. Nucleotide content (pmol per 10^6 cells)

AMP	70–1000	GMP	100
ADP	400–1200	GDP	100
ATP	$3–21 \times 10^3$	GTP	170–3700
DATP	5–96	dGTP	7–24
CMP	20–60	UMP	200–1870
CDP	20	UDP	30–550
CTP	70–1500	UTP	200–3100
DCTP	6–115	dTTP	5–80

Source: Han *et al*. [161].

E. coli purB gene). Phosphorylation steps lead to the formation of ADP and ATP, while conversion to dADP occurs by the action of ribonucleotide reductase on ADP. Given the many sources of ATP (e.g., glycolysis; electron transport in mitochondria in higher eukaryotes), it is not surprising that it is present at millimolar concentrations in all cells [164].

The formation of GMP likewise derives from IMP but through the intermediacy of XMP. Oxidation of IMP by IMP dehydrogenase (*E. coli* GuaB protein) yields XMP that is then converted to GMP by transfer of an amino group from glutamine *via* GMP synthase (*E. coli* GuaA protein). Again, the appropriate di- and triphosphate and 2′-deoxyribonucleotide arise by analogous kinases and ribonucleotide reductase as with adenosine nucleotides.

One of the central issues here is the potential for formation of ITP, XTP, dITP and dXTP and their subsequent incorporation into RNA and DNA, respectively. We have observed large increases in the DNA content of dI and dX in *E. coli* containing mutations in the *purA/rdgB* and *purA/rdgB/guaA* genes, respectively (Pang, Dong, Cunningham and Dedon, manuscript in preparation), which suggests that the mutations in purine metabolism lead to increases in the content of dXTP and dITP in the nucleotide pool available to DNA polymerases. As shown in Fig. 7, the formation of dITP and dXTP likely involves the action of ribonucleotide reductase on IDP and XDP derived from IMP and XMP by phosphorylation (*ndk, adk*). Even with allowances made for large variations in concentration as a function of cell cycle and stress, ribonucleotides are present at concentrations one to three orders-of-magnitude higher than deoxyribonucleotides (millimolar *vs.* micromolar), though prokaryotes often have higher concentrations of dNTPs (up to 200 μM; Ref. [165]. The DNA precursor pools are maintained at limited concentrations (enough for 15–30 s of DNA synthesis; Ref. [166]) that are tightly coupled to DNA synthesis, with increases in pool concentration as the cell cycle enters S phase (e.g., Ref. [167]), such that changes in precursor levels can cause large changes in dNTP concentrations. For example, mutations in purine biosynthesis have been observed to increase mutation rates by up to 300-fold in mammalian cells [168]. One explanation for this involves DNA polymerase error rates that are dependent on the relative dNTP concentrations [169]. These observations suggest that even low concentrations of dXTP and dITP could result in significant incorporation of X and I into DNA.

5.2. Diseases associated with defects in purine metabolism in humans

The relationship between defects in purine metabolism and human disease has long been recognized. As noted earlier, imbalances in dNTP concentrations lead to toxic cellular outcomes such as apoptosis [170,171] and a mutator phenotype [172–174]. These and other mechanisms have been proposed to underlie diseases associated with purine metabolism, such as gout, immunodeficiency [170,171] and neurological disorders such as autism and Lesch–Nyhan syndrome [175]. It is also possible, based on our observations, that altered purine metabolism contributes to the background of endogenous deamination DNA lesions that might increase the risk of cancer and other diseases.

The biochemical basis for these diseases entails loss of activity of key enzymes in the purine metabolic network. For example, Lesch–Nyhan syndrome involves loss of I/G phosphoribosyltransferase (H- or GPT), a key enzyme in the G salvage pathway (Fig. 7; Ref. [175]). Loss of this enzyme leads to an accumulation of X and I and, subsequently, uric acid (Fig. 7). While the exact causative agent has not been identified, it is thought that the resulting accumulation of X, I and uric acid, and the associated hyperuricemia, lead to neurological disease during fetal development, including mental and growth retardation [175]. Another example is the role for purine metabolic defects in one form of autism with epilepsy [176–178], in which loss of adenylosuccinate lyase activity leads to accumulation of adenylosuccinate (Fig. 7) detectable in blood of patients.

The observed pathophysiology of defects in purine metabolism raises an important question: would reduced activity in specific steps of purine metabolism lead to an accumulation of X- and I-containing nucleotides that eventually find their way into DNA and RNA? There is evidence for increased levels of ITP in cells from humans lacking inosine triphosphate pyrophosphohydrolase (dITPase; RdgB in *E. coli*; Fig. 7), the enzyme that removes ITP, XTP, dITP and, presumably, dXTP from the nucleotide pool [179]. It is reasonable to assume that levels of dITP will also be increased and, given our observations, that levels of dI in DNA will follow suit. Another cell line consists of B-lymphocytes from a patient lacking adenosine deaminase activity (add in *E. coli*; Fig. 7). This defect is associated with severe combined immunodeficiency [180], the basis for which is not clear. Given our

observations of elevated levels of dI in DNA when analyses are performed in the absence of adenosine deaminase inhibitors and the precedent for elevated levels of dU in B-lymphocytes expressing a cytidine deaminase (AID protein), loss of which is also associated with immunodeficiency [25], it is possible that cells lacking adenosine deaminase will have reduced levels of dI in DNA than other B-lymphocyte lines. This model assumes that dI formation in the DNA of B-lymphocytes could serve the same function as dU in immunoglobulin gene recombination.

6. REPAIR AND MUTAGENESIS OF NUCLEOBASE DEAMINATION PRODUCTS

6.1. Mechanisms controlling the levels of dX and dI in DNA

The genetics and biochemistry of processes that ultimately prevent the accumulation of base deamination products in DNA are best understood in *E. coli*. One mechanism to exclude dX and dI from DNA is the removal of their dNTP forms from the DNA precursor pools. An *E. coli* dNTP phosphohydrolase, RdgB protein, was shown to be active on dITP, ITP and XTP [181], so it can be deduced that dXTP will also be a substrate. Similar enzymes have been purified from *T. maritima* [182] and the cloned human *ITPA* gene [183], while the gene encoding it is present in the genomes of almost all organisms for which a complete genome sequence is known.

The role played by this dNTP phosphohydrolase has been defined by studies in *E. coli*. The *rdgB* gene was originally identified as a gene required for survival of *E. coli* deficient in recombinational repair promoted by RecA protein [184]. The authors of this report suggested that, in the absence of RdgB protein, a lesion was formed in DNA that required recombinational repair. They linked the production of this lesion to purine biosynthesis by showing that over-expression of the PurA protein restored viability to a *recA*– *rdgB* double mutant [185]. The identification of the *rdgB* homolog in yeast, HAM1, as a gene controlling sensitivity to N-6-hydroxylaminopurine (HAP; Ref. [186]) further supported the role of the *rdgB* gene in some aspect of purine metabolism. The biochemical function of RdgB protein as the dNTP phosphohydrolase strongly suggests that the primary lesion arising in DNA may be the presence of X, I and HAP.

Recent work has shown that the *E. coli* chromosome undergoes double-strand breaks in the absence of active RdgB protein, and that these breaks are formed only in the presence of an active endonuclease V (EndoV; Refs. [187,188]). Originally described by Demple and Linn [189] and later as an endonuclease that incises DNA containing inosine [190], EndoV is a Mg^{2+}-dependent enzyme that cuts the second phosphodiester bond located 3′ to the damaged base (reviewed in Ref. [191]). Similar enzymes have been purified from several microorganisms [192,193], while genes encoding EndoV homologs have been found in many organisms, including mice and humans, with the exception of *S. cerevisiae*. In addition to dI, EndoV recognizes a variety of lesions including dU, base mismatches, AP sites, hairpins and flap structures and has recently been shown to be the major repair activity for dX in DNA [192]. Recent studies have shown that dO, the other dG deamination product (Fig. 1), is also recognized by EndoV [194]. One model posits that EndoV incision at dI, dX or dHAP directly causes double-strand breaks or that replication forks that traverse DNA nicked by EndoV create double-strand breaks [187,188]. In either case, RecA-mediated recombination is required to repair the breaks [188].

Biochemical studies have shown that the *E. coli* 3-methyladenine glycosylase (AlkA) reacts with dI [195,196]. However, the role of AlkA in the *in vivo* repair of dI in *E. coli* is unclear, since genetic analysis is consistent with EndoV as the major dI repair enzyme [197]. Another study has shown that endonuclease VIII (EndoVIII) is active on X and O in DNA [94]. The role of these enzymes *in vivo* has not been studied.

These studies lead to a model for a system to prevent accumulation of non-canonical purines in DNA, including dX, dO and dI. The dNTP forms of purine analogs can arise from (1) salvage of free bases, (2) phosphorylation of naturally occurring (d)IMP and (d)XMP, or (3) deamination of the nucleotide forms of (d)G and (d)A. Whatever the source, RdgB sanitizes the precursor pools of dNTP and rNTP and prevents these species from being incorporated into DNA and RNA. If deamination products do arise in DNA, then a repair event is initiated by incision next to the base by EndoV.

6.2. dX and dI mutagenesis

The deamination products of DNA bases have been implicated in the formation of mutations. Especially important is the high rate of

G:C→A:T mutations at CpG sites containing 5′-methylcytosine [198], which may result from the deamination of 5-methylcytosine to thymine [199]. The deamination of cytosine to uracil also produces G:C→A:T mutations possibly by base pairing of uracil with adenine [199,200]. On the basis of *in vitro* studies [201,202], xanthine represents another possible source of G:C→A:T mutations. However, the assumption of rapid depurination of dX has led to the suggestion (e.g., Refs. [2,3,18]) that the G:C→A:T mutations arise by insertion of adenine opposite the resulting abasic sites [203–205].

In spite of the well-established use of dI in random mutagenesis techniques [206,207], there have been relatively few quantitative or mechanistic studies of X and I mutagenesis in cells or under conditions relevant to eukaryotic and prokaryotic organisms. *In vitro* polymerase insertion assays indicate that dITP is inserted most frequently opposite dC by *Taq* polymerase [206,207], most likely due to the greater stability of I:C base pairs over other combinations [208]. Studies with dXTP insertion by Klenow fragment of *E. coli* Pol I also reveal a strong preference for inserting dX opposite C [90], which contradicts the greater stability of X:T over X:C [201]. The situation with insertion of dNTP opposite templates containing dX and dI parallels the dXTP and dITP insertion studies. The frequency of insertion of dNTP opposite dX occurs in the order T > C ≫ A∼G for *Drosophila* polymerase α [201] and in the order C > T for Klenow fragment [4].

Substantive evidence for *in vivo* mutagenesis by dX and dI comes from studies of site-specific mutagenesis [209] and of *E. coli* exposed to nitrous acid and $NO^{\bullet}$ [197]. At low pH, formation of nitrous acid from nitrite leads to RNS capable of causing DNA deamination [63,83,84,210]. Weiss and coworkers demonstrated that *E. coli* lacking EndoV (*nfi* mutants) were more resistant to nitrous acid-induced toxicity and had increased A:T→G:C mutations thought to be due to dI formation [197], while the G:C→T:A mutations expected for a preponderance of apurinic sites at presumably unstable dX residues [203] represented only 0.2% of the mutations. While the G:C→A:T mutations could arise by deamination of dC or 5-methyl-dC, dX has been shown to cause G:C→A:T mutations *in vitro* [201]. *E. coli alkA* mutants, on the other hand, did not display an increased mutation frequency in the presence of nitrous acid [211], which suggests that EndoV plays a greater role in dI repair than AlkA. However, these studies were not designed with the specificity or sensitivity needed to assess the contribution of dX. Indeed, Kow and coworkers demonstrated the activity of

EndoV on dX-containing substrates that are otherwise resistant to repair by other enzymes [191,192].

It is unclear what role abasic sites may play in $NO^{\bullet}$-induced mutagenesis, given the observation in repair-deficient *E. coli* exposed to nitrous acid that the G:C→T:A mutations expected for AP sites represented only 0.2% of total mutations [212]. The bulk of the mutations consisted of G:C→A:T and A:T→G:C transitions [212]. However, Engelward and coworkers have demonstrated that *E. coli* cells lacking AP endonuclease activity were highly sensitive to $NO^{\bullet}$ exposure [213,214], which suggests that, along with base excision repair of nucleobase deamination products, the directly formed abasic sites may contribute to $NO^{\bullet}$-induced toxicity in cells.

The most direct studies of dI mutagenesis are those performed by Hill-Perkins *et al*., in which they determined the mutant frequency associated with dI by site-specific mutagenesis in the M13mp9 vector replicated in *E. coli* [209]. As expected, dI was found to pair with dC most frequently, resulting in A→G transitions, and there was an 11% mutation frequency for dI paired with dT (i.e., as if dI had formed from dA).

Kamiya and coworkers studied the mutagenicity of dXTP, dITP and dOTP when these nucleotides were transfected into *E. coli* [215]. They observed that the deamination products did not induce mutations at a higher frequency than control transfections, while 8-oxo-dGTP, 5-OH-dCTP and 5-formyl-dUTP all showed significant increases in mutation frequency [215]. These findings were interpreted to mean that X and I as dNTPs are not mutagenic and that they are only mutagenic when formed directly in DNA. A major drawback to these studies is that no accounting was made for deoxynucleotide pyrophosphohydrolase activity. It is quite possible that dITPase/dXTPase (RdgB) is more efficient than MutT (8-oxo-dGTPase) and other dNTPases. It is also possible that the higher mutagenicity of 8-oxo-dGTP, 5-OH-dCTP and 5-formyl-dUTP is the result of more efficient incorporation by DNA polymerase.

7. SUMMARY

This review has covered the numerous established and potential mechanisms by which nucleobase deamination products can arise in or enter DNA and RNA. Among all of the types of DNA damage, nucleobase deamination appears to have the most abundant and diverse

mechanisms of formation, including nitrosative deamination at sites of inflammation, simple hydrolysis, the actions of several deaminase enzymes on DNA and RNA and defects in purine and pyrimidine metabolism. This level of complexity suggests that nucleobase deamination products could act as biomarkers of several facets of cellular pathophysiology, while their involvement in carcinogenesis and other disease processes could involve both direct mutagenesis and, *via* the nucleotide pools, disruption of cell signaling, cycling or other facets of cell physiology. The future of research in this area will depend on the development of ultra-sensitive analytical methods to quantify these lesions in small amounts of human tissue, an effort currently underway in several laboratories including ours.

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CHAPTER 3

Site-Specific Modification of the Electrophile Sensor Protein Keap1 and Activation of Nrf2-Dependent Gene Expression

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Contents

1. INTRODUCTION

Cellular protection against electrophiles and oxidants relies on detoxication by phase II biotransformation enzymes, antioxidant enzymes and related stress response proteins [1–3]. Many of these inducible genes,

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such as the glutathione *S*-transferases, NAD(P)H oxidoreductase, heme oxygenase 1 (HO-1) and γ-glutamyl cysteine ligase are regulated at the transcriptional level through *cis*-acting DNA sequences known as antioxidant/electrophile response elements (ARE/EpREs) [4–6]. Inducers of ARE/EpRE-driven genes generally are electrophiles or their precursors [7], which modify sulfhydryl groups in proteins that regulate signaling pathways involved in toxicity and stress [6].

ARE-dependent transcription is regulated by the transcription factor Nrf2, a member of the basic-leucine zipper NF-E2 family [8,9]. Nrf2 forms heterodimers with one of the small Maf proteins and this complex activates expression of ARE-driven genes [10–13]. Studies with $Nrf2^{-/-}$ mice indicate that Nrf2 regulates a variety of genes, including chaperones, antioxidant genes and genes regulating protein degradation [13–15]. $Nrf2^{-/-}$ mice are more susceptible to toxic chemicals and stress [14,16].

Recently, a Cul3 ubiquitin ligase adaptor protein Keap1 (Kelch-like ECH-associated protein 1) was identified as an inhibitory regulator that binds to the N-terminal Neh2 domain of Nrf2 [17,18]. The Keap1 protein has five domains: an N-terminal domain, a BTB domain, a central linker domain, a Kelch repeat domain and a C-terminal domain. The Kelch repeat domain binds directly to Nrf2, as well as to actin [17,19], the BTB domain is required for the dimerization of Keap1 [20] and the central linker domain is essential for cytoplasmic sequestration of Nrf2 [21].

The question of how Keap1 regulates Nrf2 has attracted intense interest and several models have been proposed. Keap1 initially was proposed to tether Nrf2 to cytoplasmic actin filaments, thus preventing its access to the nucleus [17,22]. Keap1 also was found to modulate nuclear Nrf2 levels by enhancing nuclear export of the transcription factor [23]. Keap1 regulates the active degradation of Nrf2 [24] by functioning as an adaptor for Cul3-dependent ubiquitination and degradation of Nrf2 [25–27]. Other recent work suggests that Keap1 suppresses nuclear Nrf2 by transiently moving from the cytoplasm into the nucleus to promote Nrf2 ubiquitination [28].

Electrophiles react with Keap1 to form covalent adducts and adduction is thought to trigger as yet unidentified events that result in enhanced nuclear Nrf2 levels. The mechanism by which Keap1 modifications trigger Nrf2 activation presents an interesting problem. Although the structure of the Kelch domain has been reported [29], the structure of the entire protein has not yet been determined. Keap1 has 27 cysteine residues, most or all of which appear to be available to react with electrophiles. Initial studies of modification of murine Keap1 by the model alkylating agent dexamethasone mesylate suggested that preferential

modification of highly reactive central linker domain cysteines, particularly Cys273 and Cys288, triggers Keap1-Nrf2 dissociation and accounts for the electrophile-sensing mechanism [30,31]. However, considerable recent work indicates that the relationship of covalent adduction to the sensor function of Keap1 is more complicated. Here we discuss our recent studies of Keap1 modification by two prototypical thiol-reactive electrophiles [32] and the cancer chemopreventive agent sulforaphane [33]. These studies indicate that covalent modification of the Keap1 sensor can result in Nrf2 stabilization through at least two distinct mechanisms.

2. PATTERNS OF KEAP1 MODIFICATION BY ELECTROPHILES

2.1. Keap1 alkylation by prototypical thiol-reactive electrophiles

The apparently large number of thiol targets in Keap1 suggests that different electrophiles may display different patterns of adduction on this sensor protein. We explored this question by analyzing the adduction of human His_6-Keap1 *in vitro* by several electrophiles [32]. Initial studies employed the thiol-reactive biotinylating probes *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB) and 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]-carboxamido)butane (BMCC) (Fig. 1). These compounds display chemistries typical of a number of electrophilic metabolites of drugs and environmental chemicals and of endogenous electrophiles. However, they differ in their functional effects on Nrf2 activation *in vivo*. Whereas IAB induces ARE-dependent gene transcription, nuclear Nrf2 accumulation and levels of ARE-regulated gene products, BMCC does not [32]. Dex-Mes induces nuclear Nrf2 accumulation in HEK293 cells, but also causes significant cytotoxicity (data not shown).

Treatment of human His_6-Keap1 with IAB at a molar ratio of 5:1 (electrophile:protein) for 2 h at 37°C consistently yielded a total of 6 IAB-modified cysteines, each of which had a mass increase of 382.5, corresponding to IAB adducts (Table 1). All of these cysteines (Cys196, Cys226, Cys241, Cys257, Cys288 and Cys319) are in the central linker domain, which is required for cytoplasmic sequestration of Nrf2 [21]. LC–MS–MS analyses routinely generated MS–MS spectra corresponding to approximately 80% of the protein sequence. All of the cysteine-containing tryptic peptides were detected, except for Cys151, Cys395 and Cys406. In analyses of electrophile-treated Keap1, cysteine-containing peptides were detected as

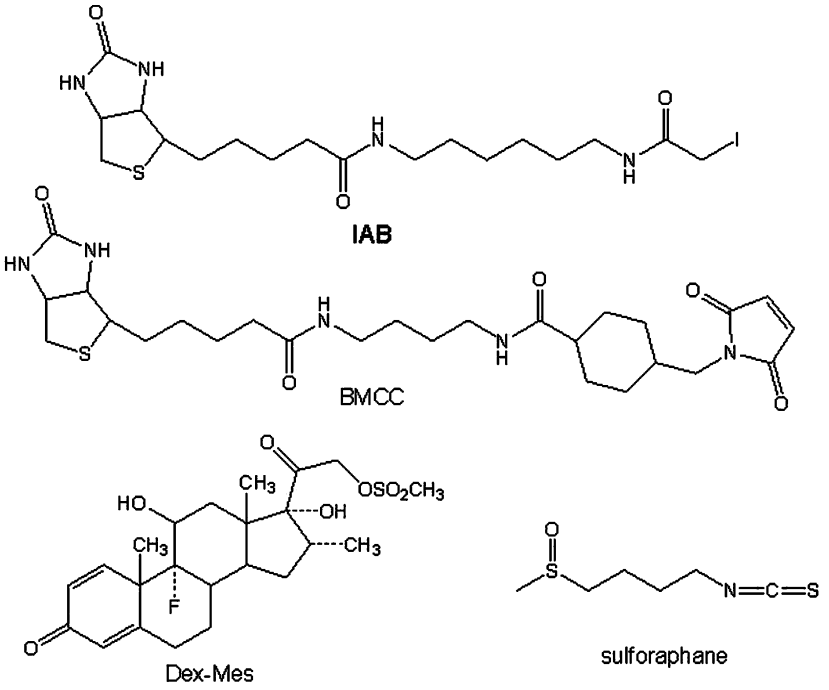

Fig. 1. Structures of electrophiles discussed in text.

S-carboxamidomethylated derivatives (corresponding to unadducted cysteines) or as electrophile-adducted cysteines (Table 1). All adducts were characterized by mass shifts to b- and/or y-ions that confirmed sequence location of the adducts.

Because Dinkova-Kostova *et al*. employed the model electrophile Dex-mes to identify reactive thiols in murine Keap1 [30], we also studied adduction of human His_6-Keap1 by this compound under the same conditions as for IAB and BMCC. Dex-Mes alkylated human Keap1 at cysteines primarily in the central linker domain (Cys196, Cys226, Cys241 and Cys249) as well as Cys489 in the Kelch domain and Cys622 in the C-terminal domain. Interestingly, Dex-Mes did not target Cys273 and Cys288 or the central linker domain, as had been reported in similar studies with murine Keap1 *in vitro* [30].

2.2. Keap1 acylation by the prototypical chemopreventive agent sulforaphane

Sulforaphane (R-1-isothiocyanato-4-methylsulfinylbutane) (Fig. 1), an isothiocyanate isolated from cruciferous vegetables, is among the most

Table 1. Sites of modification of human His_6-Keap1 by electrophiles *in vitro*[a]

Residue	Domain[b]	IAB	BMCC	Dex-mes	Sulforaphane
C12	NT				2/5[c]
C13	NT				2/5
C38	NT				1/5
C77	BTB		3/3	3/3	3/5
C171	BTB				1/5
C196	CL	3/3	3/3	2/6	1/5
C226	CL	1/3		2/6	3/5
C241	CL	3/3		1/6	
C249	CL		1/3	6/6	3/5
C257	CL	2/3			2/5
C288	CL	3/3			
C319	CL	1/3			
C368	Kelch		3/3		1/5
C489	Kelch		3/3	6/6	5/5
C513	Kelch				3/5
C518	Kelch				3/5
C583	Kelch				4/5
C622	CT			1/6	
C624	CT				4/5

[a] Human His_6-keap1 (1 nmol) was incubated with 100 μM electrophile (IAB, BMCC, Dex-Mes) or 2 μM electrophile (sulforaphane) for 2 h (IAB, BMCC, Dex-Mes) or 15 min (sulforaphane) in 0.1 M ammonium bicarbonate at 37°C and then analyzed as described [32–33].
[b] Domains: BTB, BTB domain; CL, central linker domain; Kelch, Kelch domain; CT, D-terminal domain.
[c] Indicates number of times adducted peptide was detected/number of experiments.

potent inducers of phase II enzymes [34–36]. Sulforaphane is an isothiocyanate, which reacts with thiols to form thionoacyl adducts. In contrast to the adducts formed by IAB, BMCC and Dex-Mes, thionoacyl adducts are labile to decomposition under the conditions we used to analyze the former adducts. We developed a modified LC–MS–MS method to map sulforaphane modification sites formed on human His_6-Keap1 *in vitro* [33]. Sulforaphane is considerably more reactive than the other electrophiles studied and elicited Nrf2 activation in cells at low micromolar concentrations (see below). The most reactive cysteines in Keap1 toward sulforaphane were judged to be those that are most reproducibly adducted by 2 μM sulforaphane for 15 min. These were

located in the Kelch repeat domain, especially Cys489, which was found to be sulforaphane-modified in all experiments at the 2 μM concentration. Cysteines 513, 518 and 583, also in the Kelch domain were modified in at least three experiments, as were cysteines in the central linker domain (Cys226 and Cys249), the BTB domain (Cys77) and the C-terminal domain (Cys624). At higher sulforaphane concentrations, adduction selectivity was increasingly less selective, yet reproducible between experiments.

2.3. Comparison of reported modification patterns on Keap1

In addition to our work described above, two other groups have applied MS methods to map Keap1 adducts formed with electrophiles. Dinkova-Kostova *et al*. characterized Cys257, Cys273, Cys288 and Cys297 of murine Keap1 as targets of the electrophile dexamethasone mesylate [30] and led the authors to denote these as the ‘‘most reactive residues of Keap1’’. Eggler *et al*. recently reported that IAB preferentially alkylated Cys151, Cys288 and Cys297 in human Keap1 [37], although data supporting the identification of the adducts and the relative reactivities of the targets were not published with the paper (these authors reported an MS–MS spectrum of the Cys288-IAB adduct, but later provided us MS–MS spectra for all of the reported adducts) (A. Mesecar; personal communication).

The differences between reported electrophile targets in Keap1 reflect differences in experimental design and adduct analysis. The Talalay group employed murine Keap1 in their studies [30,31], whereas our studies and the studies of Eggler *et al*. employed human Keap1 [32,33,37]. Murine and human Keap1 differ by 12 of 153 residues in the central linker domain, which may result in conformational differences that affect adduction chemistry. Each study used somewhat different conditions for incubation of Keap1 with electrophiles and analysis of adducts. For example, molar ratios of electrophile to Keap1 varied from 33:1 (Dex-Mes:Keap1) [30] to 5:1 in our studies with IAB, BMCC and Dex-Mes (see above) to 1:1 (IAB:Keap1) [37]. Our studies demonstrated that incubation time also affected the distribution of adducts [32]. A significant difference between our work and that of Eggler *et al*. is that they employed an avidin affinity step in the preparation of Keap1 peptide adducts for LC–MS–MS analysis. This enriches adducts and increases the detection of even low abundance adducts and may be the major reason for differences in reported IAB adducts with human

Keap1 *in vitro*. Without adduct enrichment, the detection of lower abundance adducts may be suppressed by higher abundance unadducted Keap1 peptides in the sample.

Although differences in experimental design and analysis undoubtedly contribute to the differences in reported Keap1 adduct sites, the major contributing factor to adduction site diversity is the diversity of the electrophile chemistries studied. This is clear from our studies, where the experimental design and analysis of IAB, BMCC and Dex-Mes were identical [32]. Dex-Mes and IAB are S_N2-type electrophiles that alkylate by nucleophilic displacement of a leaving group. BMCC reacts with thiols by Michael addition. Although the experimental conditions we used to generate and detect sulforaphane adducts were slightly different, the pattern of sulforaphane adducts was quite different than observed for the other electrophiles [33]. Thiols react with sulforaphane by addition to the isothiocyanate carbon to yield thionoacyl adducts. The acylation reaction occurs much more rapidly than do the alkylation reactions with the other electrophiles at equivalent reagent concentrations.

An implicit assumption in previous work on Keap1 adduct sites is that the most easily detected adducts represent the most reactive targets for modification. However, MS–MS detection of adducts *per se* does not indicate their relative amounts or the relative reactivities of the modified cysteines. The sampling methods LC–MS–MS instruments use to acquire MS–MS spectra introduce a certain random nature to sampling low abundance species [38–40]. Thus, in three analyses of the same sample, a high abundance peptide adduct will be detected all three times, whereas lower abundance adducts may be detected in one run and not in the others, even though the concentration of that adduct is the same in all three samples. For this reason, we have used the frequency of adduct detection in replicate analyses to compare the relative reactivities of Keap1 sites toward both sulforaphane and IAB (Table 1 and [32]). The frequency of detection in replicate analyses is roughly proportional to amount and this mode of analysis provides a survey level of quantitation [41]. Given these considerations, our analyses suggest that Cys196, Cys241 and Cys288 were most reactive toward IAB [32], whereas Cys489, Cys583 and Cys624 were most reactive toward sulforaphane (Table 1). Our data do not exclude the possibility that other adducts were present, but suggest that these adducts were not abundant under the conditions of our analyses. Definitive resolution of adduction selectivity and target reactivity will require rigorous kinetic analyses of competing Keap1 adduction reactions (see below).

3. RELATIONSHIP OF KEAP1 MODIFICATION SITES AND FUNCTIONALLY SIGNIFICANT CYSTEINE RESIDUES

Inferences about critical targets in Keap1 have been drawn from functional analyses of Keap1 mutants. Cys273Ser and Cys288Ser mutations in the central linker domain blocked Keap1-dependent ubiquitination of Nrf2 [21] and Cys273Ala and Cys288Ala mutations blocked Keap1-dependent repression of an ARE reporter [31]. Zhang *et al*. also reported that Cys151 in the Keap1 BTB domain is required for inhibition of Keap1-dependent degradation of Nrf2 by sulforaphane and oxidative stress, although this mutant does function as a constitutive repressor of Nrf2 [21,25]. These data would suggest that Cys273, Cys288 and Cys151 are key targets for electrophilic Nrf2 activators.

However, MS-based mapping studies of Keap1 adducts are not consistent with this view. In murine Keap1, Cys273 and Cys288, but not Cys151 were preferred targets for Dex-Mes [31]. In human Keap1, we found that only Cys288 was modified by IAB [32]. We also found that neither Cys151, Cys273 nor Cys288 were modified by sulforaphane in human Keap1, except at high concentrations [33]. Eggler *et al*. reported that Cys151 and Cys288 were preferred targets for IAB, although the supporting data are not clear (see above). Taken together, the available adduct mapping and reactivity data do not support the view that Cys151, Cys273 and Cys288 are obligatory targets for Nrf2 activation and suggest instead that the adduction–activation relationships are more complicated. These relationships certainly will require further study.

4. ELECTROPHILE-INDUCED UBIQUITINATION OF KEAP1

Zhang and colleagues observed that Keap1 expressed in COS1 cells was converted to a group of high molecular weight (HMW) forms upon treatment with the prototypical ARE-inducer tert-butylhydroquinone (tBHQ) [21]. We subsequently observed the formation of HMW Keap1 forms in studies with IAB. FLAG-Keap1 transfected cells were treated with 100 μM IAB for 2 h at 37°C, FLAG-Keap1 proteins were captured with anti-FLAG antibodies, and analyzed on reducing sodium dodecyl–polyarylamide gel electrophoresis sulphate (SDS-PAGE) gels. The majority of the Keap1 protein from IAB-treated cells migrated in a series of HMW bands with a molecular mass of greater than 150 kDa, whereas the Keap1 protein from untreated cells migrated with an observed molecular weight of 70 kDa, which corresponds to the Keap1

monomer (Fig. 2A, B). Immunoblotting with anti-ubiquitin indicated intense ubiquitin immunoreactivity co-migrating with the HMW Keap1 protein bands from IAB-treated cells, but not from controls (Fig 2C). HMW Keap1 forms were not observed in cells treated with 100 μM BMCC (Fig. 3D–F), nor was anti-ubiquitin immunoreactivity detected. The HMW Keap1 protein forms were detected under reducing conditions on SDS-PAGE (15 mM β-mercaptoethanol in the loading buffer). Pretreatment of the samples with 8 M urea, reduction with tris (carboxyethyl) phosphine (TCEP) and alkylation of the reduced protein with iodoacetamide prior to SDS-PAGE failed to alter the migration of the HMW Keap1 products (data not shown). However, these denaturation conditions did result in detection of Cys151 as the *S*-carboxamidomethyl derivative (data not shown) and is consistent with the work of Wakabayashi *et al*. [31], which indicated that C151 does not undergo

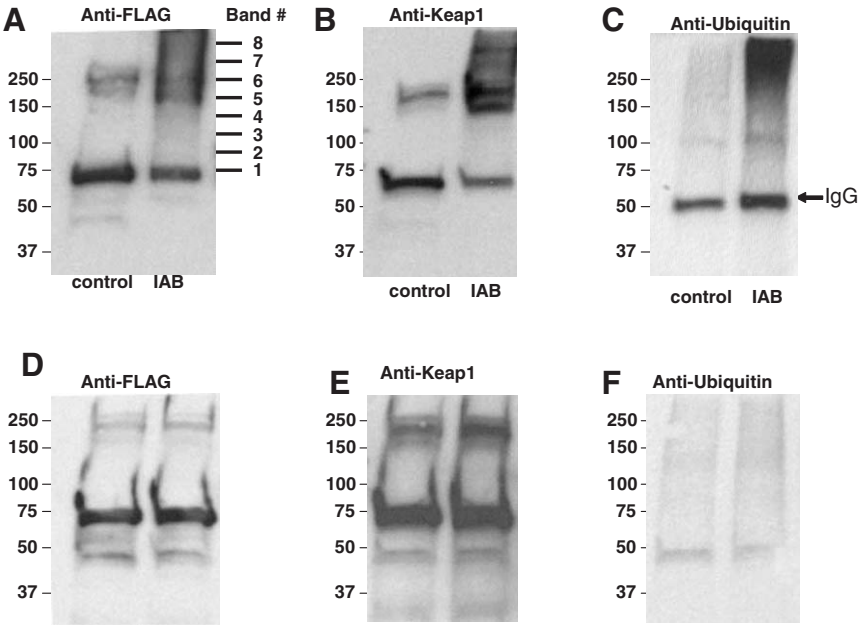

Fig. 2. Formation of HMW keap1 protein forms in HEK293 cells stably expressing FLAG-Keap1 treated with electrophiles. FLAG-Keap1 proteins isolated from untreated controls and either IAB treated (A–C) or BMCC-treated (D–F) FLAG-Keap1 transfected HEK293 cells were aliquoted to be run on three SDS-PAGE gels and analyzed by Western blotting. Keap1 proteins were detected with anti-Keap1 (A, D), anti-FLAG (B, E) and anti-ubiquitin (C, F). (Reproduced with permission from ref. [32].)

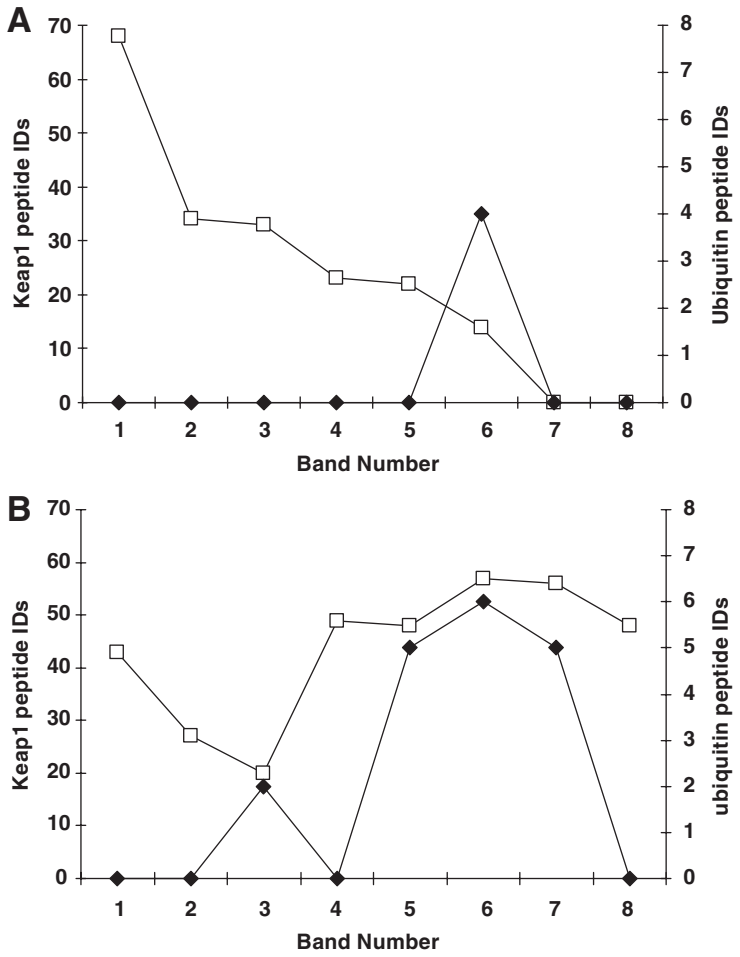

Fig. 3. Distribution of Keap1 (□) and ubiquitin (◆) peptide identifications in SDS-PAGE gel fractions from (A) control and (B) IAB-treated transfected cells. FLAG-Keap1 transfected cells were treated with 100 μM IAB for 2 h. FLAG-Keap1 protein then was purified from treated cells and from untreated controls with anti-FLAG antibodies and separated by SDS-PAGE. Eight bands containing proteins with molecular weights higher than 69 kDa were cut and the proteins in these bands were digested and analyzed by LC–MS–MS. (Reproduced with permission from ref. [32].)

adduction. Treatment of His_6-Keap1 with IAB *in vitro* did not generate HMW Keap1 products detectable by immunoblotting. In FLAG-Keap1-expressing 293 cells, both IAB and tBHQ induced a concentration-dependent formation of HMW Keap1 forms.

To better understand the nature of the HMW Keap1 forms, we analyzed tryptic digests of the corresponding gel bands by LC–MS–MS. Eight gel sections corresponding to a molecular weight range from 70 kDa and above (indicated in Fig. 3A) were analyzed. The majority of peptides detected in these analyses mapped to Keap1 and ubiquitin, both of which were represented by detection of multiple peptides in different bands. Keap1 proteins from both control and IAB-treated cells were found in multiple bands of 70 kDa molecular weight and higher (Fig. 3). However, the distribution of HMW Keap1 and ubiquitin are different between these samples. In control cells, Keap1 protein with a molecular weight of 70 kDa is the dominant species (Fig. 3A), whereas Keap1 protein was detected primarily in bands corresponding to a molecular weight greater than 150 kDa in the IAB-treated cells (Fig. 3B). Ubiquitin peptides were only detected in band 6 in control samples, which is consistent with the immunoblot result (Fig. 3A). Ubiquitin peptides were found in several bands corresponding to ubiquitin-immunoreactive HMW Keap1 forms from IAB-treated cells (Fig. 3B), especially in band 6, where six ubiquitin peptides were detected, corresponding to 68.4% of the ubiquitin sequence. The numbers of Keap1 and ubiquitin peptides detected by LC–MS–MS depend in part on levels of background contaminant proteins in each gel band. Thus, numbers of peptide identifications for Keap1 and ubiquitin are at best a semiquantitative measure of protein concentration. However, numbers of detected peptides generally coincide with protein levels detected by immunostaining. Lack of detection of ubiquitin peptides in band 8 (Fig. 3B) reflects the presence of contaminating HMW proteins that did not enter the gel.

Trypsin digestion of ubiquitinated proteins leaves a Gly-Gly tag attached to the ε-amino group of the ubiquitin-modified lysine on the target protein, leading to the increased mass shift of 114 on this lysine. One of the ubiquitin peptides was found to be Gly-Gly modified at the site of Lys48, which indicates that the HMW Keap1 proteins contained Lys48-involved polyubiquitin chains [42].

To map the sites of IAB-induced modifications on Keap1 *in vivo*, FLAG-Keap1 transfected cells were treated with IAB and FLAG-Keap1 protein was captured with an anti-FLAG affinity column. LC–MS–MS analysis of a tryptic digest of FLAG-Keap1 proteins identified a peptide containing a Gly-Gly tag at Lys298, which is in the central linker domain (Fig. 4).

Further analysis of the LC–MS–MS data also revealed three IAB adduct sites, which were identified as Cys241 (detected in 3/3 analyses),

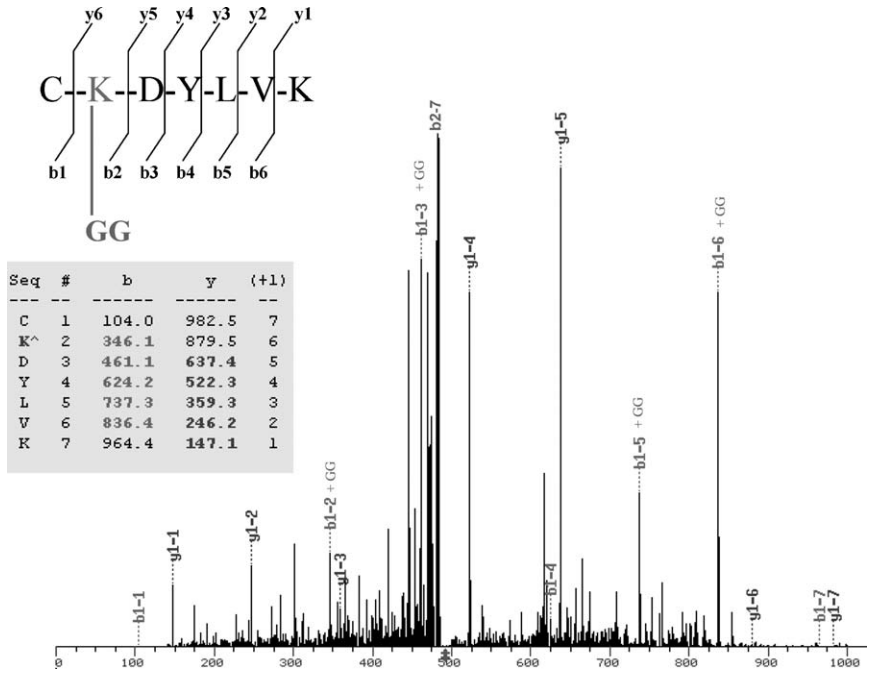

Fig. 4. MS–MS spectrum of Gly Gly modified Keap1 peptide containing themodification site on Lys298. Ubiquitinated proteins were digested with trypsin, followed by LC-MS-MS analyses. A Gly Gly tag with a mass of 114 indicates the attachment site of the ubiquitin protein. (Reproduced with permission from ref. [32].)

Cys257 (2/3) and Cys288 (3/3). These cysteines are also Keap1 adduction sites following IAB treatment *in vitro* (see above). Cys226 was not found to be IAB modified, which is consistent with our *in vitro* studies indicating that this residue was found modified in only one out of three samples when Keap1 protein was incubated with IAB for 2 h (see above).

Some SCF (Skp1p-cullin-F-Box protein) complexes, which function as E3-ubiquitin ligases, may form complexes with different substrates [25,43,44]. F-box proteins are substrate adaptors that provide substrate specificity and ubiquitin-dependent degradation by SCF complexes of certain G-cyclins involved in the control of cell cycle progression [45]. In some cases, F-box proteins are degraded by autoubiquitination within their own SCF complex, which suggests that SCF complex can switch rapidly between substrate (G-cyclin) and substrate adaptor (F-box protein) to balance their levels [46,47]. Cul3-associated BTB proteins are substrate adaptors for Cullin-3 ubiquitin ligases and these proteins are themselves regulated through ubiquitination and proteasome-mediated

degradation [48]. Keap1 is a BTB protein and functions as an adaptor to target Nrf2 for ubiquitination by Cul3-based E3 ligase [25–27]. Our results suggest that site-specific modification of Keap1 by electrophiles switches ubiquitin targeting from Nrf2 to Keap1 and that this target switching mechanism governs Nrf2 activation by electrophiles.

The IAB treatment-induced Nrf2 stabilization as indicated by elevated cytosolic and nuclear Nrf2, coincident with formation of HMW FLAG-Keap1. FLAG-Keap1 transfected cells were exposed to 100 μM IAB for 2 h, FLAG-Keap1 proteins then were captured with anti-FLAG antibodies, and associated proteins were analyzed by immunoblotting (Fig. 5). In untreated cells, very little Nrf2 was found associated with Keap1, as detected by immunoblotting. These results are consistent with those reported previously [25] and were interpreted to indicate rapid destabilization of Nrf2 when it is associated with non-adducted Keap1. Treatment with IAB, which resulted in the adduction and ubiquitination of Keap1, also increased the amount of Nrf2 associated with Keap1 (Fig. 5). This was interpreted to be a consequence of Nrf2 stabilization. tBHQ has been reported to cause increased association of Nrf2 with Keap1 under conditions where Keap1 is ubiquitinated [21,25]. These observations suggest that stabilization of Nrf2 can occur without dissociation from Keap1.

5. UNRESOLVED QUESTIONS ABOUT ELECTROPHILE-INDUCED ACTIVATION OF NRF2

Different electrophiles that are Nrf2 inducers produce different patterns of Keap1 modification. Modification sites reported in several studies appear to differ and this certainly may reflect differences in methods and experimental conditions. However, these differences also appear to reflect different chemistries of the electrophiles studied. One of the main questions posed in most of these studies is whether there is a true hierarchy of Keap1 target reactivities. Unfortunately, observation of adducts tells little about the competing reactivities of different target sites. The only way to address this is to measure chemical reactivities of site-specific modifications with specific electrophiles under defined conditions. In other words, the kinetics of modification will provide the answer to this question.

What does this tell us about the sensing mechanism of the system? Other sensor systems display sensitivity to specific oxidants [49,50] and function primarily through one electron oxidation of an iron–sulfur center or through thiol-disulfide redox changes. Keap1 appears to serve the role of

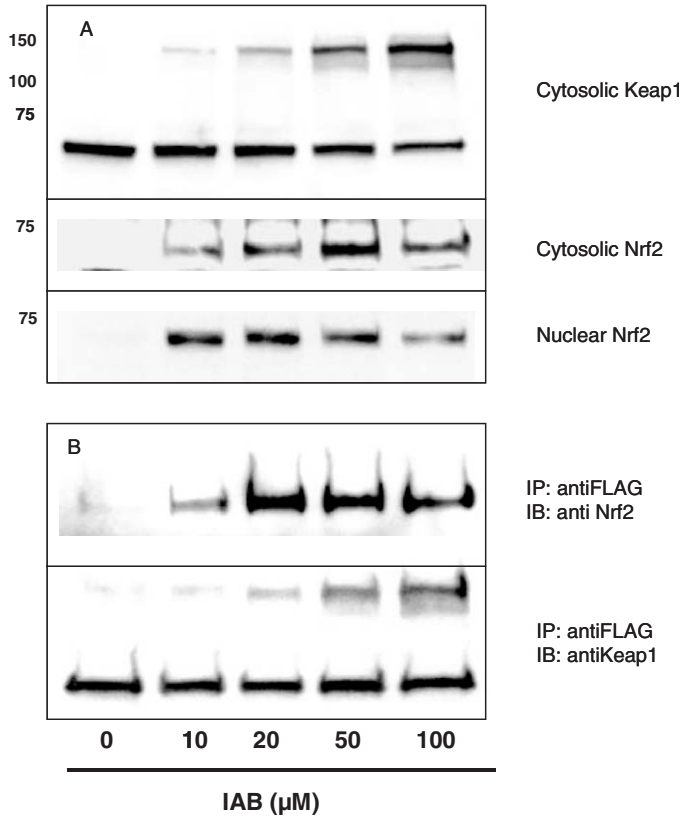

Fig. 5. Nrf2 stabilization coincides with electrophile-specific adduction and ubiquitination of Keap1 *in vivo*. (A) HEK293 cells stably expressing FLAG-Keap1 were treated with IAB at the indicated concentrations for 2 h at 37°C. Cytosolic and nuclear fractions were prepared from cell lysates from untreated control and IAB-treated cells and analyzed by Western blot analysis for cytosolic Keap1 and cytosolic and nuclear Nrf2. (B) HEK293 cells stably expressing FLAG-Keap1 were treated with IAB at the indicated concentrations for 2 h at 37°C. Bead-immobilized anti-FLAG antobodies were used to capture proteins from whole cell lysates. These were analyzed by immunoblotting with anti-Keap1 and anti-Nrf2 antibodies.

electrophile sensor, which requires a greater degree of flexibility, given the diverse chemistry of electrophiles. The variety of electrophile modification patterns formed on Keap1 by Nrf2 activators is consistent with this concept. This implies that different cysteine modifications could contribute to Nrf2 activation. Further, if multiple patterns of Keap1 modification are similarly able to cause activation, is there a requirement for a critical level of certain types of modifications (e.g., central linker domain adducts)?

Again, studies of the kinetic reactivities of different cysteine–electrophile pairs would provide a quantitative basis for comparing modification patterns. These considerations support the idea that, as a sensor for diverse electrophiles, Keap1 must be able to convert diverse chemistries to a common signal for Nrf2 activation.

Another unsettled issue is how adduction results in Nrf2 activation. The available evidence indicates that some adduction reactions cause Keap1 ubiquitination, whereas others do not. Nevertheless, recent studies with sulforaphane indicate that Keap1 ubiquitination is not required for Nrf2 stabilization [33,51]. Additional studies may reveal other examples of compounds that display different modification maps, different degrees of Keap1 ubiquitination and different degrees of Nrf2 activation. In this context, a screen to compare adduction patterns and molecular events proximal to Nrf2 activation would help to identify key hierarchal inducer-dependent and independent regulatory steps. One may hypothesize that repression of Nrf2 ubiquitination is a common regulatory step. Electrophile chemistry appears to dictate whether Keap1 ubiquitination represents a pivotal step in regulation.

Finally, the question of electrophile-mediated release of Nrf2 from Keap1 is not well understood. Mutation of Keap1 residues Cys273 (C273A) or Cys288 (C288A) completely abolished Nrf2 ubiquitination and Keap1-mediated repression of Nrf2/ARE-directed gene expression [21,31]. Nevertheless, these mutations did not significantly alter the association between Nrf2 and Keap1 [21]. Electrophile adduction of Keap1 cysteine residues did not cause release of Nrf2 when studied in an *in vitro* model [37]. However, phosphorylation of Nrf2 is known to produce stoichiometric release from Keap1 [52,53]. This raises the interesting question of whether phosphorylation of Nrf2 is direct consequence of electrophile modification of Keap1 or an indirect consequence of electrophile-mediated Keap1 ubiquitination.

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CHAPTER 4

Genotoxicity of Chlorophenols and Ochratoxin A

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Contents

1. INTRODUCTION

1.1. Phenol toxicity

Phenols are ubiquitous substances that exert a broad spectrum of biological activities. Vitamin E [1] and naturally occurring polyphenols in fruits and vegetables [2] are known for their antioxidant activity through scavenging reactive oxygen species (ROS). This property is thought to be beneficial to human health and contribute to the protection against cardiovascular disease and cancer. However, other phenols may display

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deleterious prooxidant activities and generate ROS that contribute to aging and disease. There is increasing evidence that the prooxidant properties of phenols proceed through a radical pathway initiated by phenoxyl radical formation [3,4], and the development of antioxidants for clinical use includes strategies to minimize prooxidant activity [5]. Bioactivation of certain phenols, including hydroquinones and catechols, can also generate benzoquinone and quinone methide electrophiles that are Michael acceptors and react covalently with proteins and nucleic acids [6]. Covalent reactions of these electrophiles with nucleic acids to generate DNA adducts may initiate mutagenesis and carcinogenicity [7].

Efforts have been made to categorize phenol toxicity using quantitative structure activity relationships (QSAR). Phenols that act as oxidative uncouplers inhibit oxidative phosphorylation in mitochondria by inhibition of mitochondrial ATP production [8]. Phenols in this category include hydrophobic, electron-deficient phenols with weakly acidic phenolic groups (pK_a 3.8–8.5) [9]. Electron-withdrawing substituents also increase phenol electrophilicity that may lead to a covalent interaction with proteins and nucleic acids. This electrophilic mode of toxic action can also take place after initial biotransformation, in which case the phenols are classified as proelectrophiles [10]. Another mode of toxic action by phenols in aquatic organisms is polar narcosis, which is a membrane irritation caused by noncovalent interactions of phenol accumulation in lipid tissue. Phenols that are good hydrogen bond donors tend to act as polar narcotics [10]. In view of the various types of interactions that may occur between phenols and membranes, proteins, and nucleic acids, the toxicity of a given phenol is likely caused by the superposition of effects from different mechanisms [10]. This has made it difficult to equate a certain mode of action with the overall hazardous effect of phenols in biological systems.

1.2. Chlorophenols and ochratoxin A

Within the family of phenolic toxins, man-made chlorophenols (CPs) along with the natural chlorophenol mycotoxin ochratoxin A (OTA) (Fig. 1) have attracted considerable interest. Man-made CPs are widely distributed in the environment due to their agricultural and industrial uses as insecticides, herbicides, fungicides, and wood preservatives [11]. The US Environmental Protection Agency (EPA) has designated 11 phenolic compounds as major priority pollutants and four CPs (e.g., 2-CP, 2,4-DCP, 2,4,6-TCP, and pentachlorophenol (PCP); Fig. 1) are

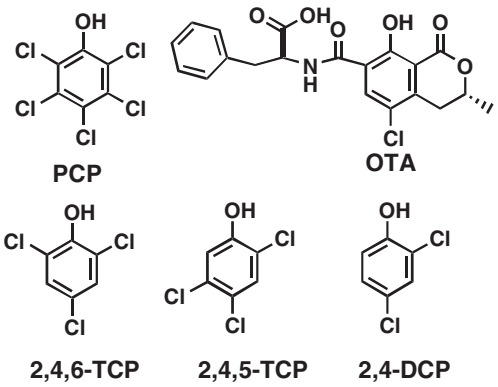


Fig. 1. Chemical structures of CPs and OTA.

classified into the most toxic and carcinogenic class [12]. The fungal toxin OTA (Fig. 1) is a potent renal carcinogen in rodents [13] and has been implicated in human kidney [14] and testicular carcinogenesis [15]. OTA contaminates a wide range of human and animal foodstuffs [16,17] making total avoidance of OTA consumption practically impossible. Thus, the ubiquitous nature of these substances coupled with their carcinogenicity in animal models has raised public awareness of the potential health risks posed by CPs and OTA.

For simple CPs, it is known that toxicity depends on the number and position of chlorine substitutions [18]. Some CPs such as PCP, are regarded as classic examples of oxidative uncoupling agents due to their hydrophobic and weakly acidic nature [9]. OTA is also known to inhibit mitochondrial ATP production [19,20]. However, both OTA and PCP are classified as Group 2B (possible carcinogenic to humans) by the International Agency for Research on Cancer (IARC) and one hypothesis for their carcinogenicity is based on their genotoxic properties following metabolic activation. PCP is known to undergo bioactivation by cytochrome P450 (CYP450) enzymes to generate benzoquinone electrophiles through oxidative dechlorination pathways [21,22]. The resulting quinone electrophiles are known to react covalently with DNA to form DNA adducts [23–25]. On the basis of the ^{32}P-postlabeling assay, OTA has also been shown to form DNA adducts *in vivo* [26–28]. However, mutagenicity assays for OTA and CPs have not provided definitive answers. The direct genotoxicity of OTA is also a hotly debated topic, as some laboratories have been unable to detect OTA-mediated DNA adducts following *in vitro* and *in vivo* studies [29,30]. The goals of this review are to present the currently available information and opinions on

CP- and OTA-mediated genotoxicity and outline opportunities for future research that should help elucidate the mechanisms of CP and OTA carcinogenicity, which is critical in assessing human health risk and determining tolerable levels of dietary intake.

2. CHLOROPHENOLS

2.1. Production and properties of chlorophenols

CPs are toxic chemicals that are persistent in the environment. They have found wide use in pesticides, disinfectants, wood preservatives, personal care formulations, and are substantial by-products of wood pulp bleaching with chlorine [11]. PCP has been produced and used in the largest quantities. Annual production of PCP all over the world was estimated to be 25,000–90,000 tons at its peak [31]. Today, PCP is still used to protect timber from fungal rot and wood-boring insects. PCP concentrations in groundwater can be 3–23 μg/L in wood-treatment areas and concentrations in milligram per liter can be found near industrial discharges [32]. The general population is exposed to PCP through the ingestion of water (0.01–0.1 μg/L), food (up to 405 μg/L), and through inhalation of indoor air where concentrations up to 25 $\mu g/m^3$ have been found [32].

CP toxicity and bioaccumulation increases with the degree of chlorine substitution [18]. Table 1 shows physical parameters for CPs, including phenol (PhOH), in aqueous solution. The pK_a values [33] of CPs decrease with the number of chlorine substitutions. *Ortho* CPs (2-CP) are more acidic than other isomers because of the large inductive effect of chlorine on the hydroxyl group in close proximity [34]. Increased chlorine substitution also increases the octanol–water partition coefficient (K_{ow}) [35], which is positively correlated to the bioaccumulation potential of CPs [36]. Thus, PCP with the largest K_{ow} value is the most hydrophobic CP, which allows it to diffuse through cellular membranes. At pH 12, standard reduction potentials (E^o_{red}) for the phenoxyl radical/phenolate ion couples are similar for the CPs showing a range of 0.85–0.99 V *vs.* the normal hydrogen electrode (NHE) [33]. At $pH > pK_a$, E^o_{red} tends to increase with increased chlorination. However, at $pH < pK_a$, E^o_{red} increases with increasing acidity. Thus, at physiological pH 7, PhOH has an E^o_{red} value $\sim$1.03 V, which is higher than E^o_{red} for PCP at pH 7 (0.99 V), because PCP remains deprotonated at

Table 1. Parameters for chlorophenols in aqueous solution

Phenol	pK_a[a]	K_{ow}[b]	E^o_{red} (pH 12.0)[a,c]	σ^+[a]
PhOH	9.98	1.57	0.86	0.00
2-CP	8.52	2.29	0.93	0.086
3-CP	8.97	2.64	0.88	0.40
4-CP	9.37	2.53	0.85	0.11
2,4-DCP	7.90	3.20	0.88	0.21
2,4,5-TCP	6.72	4.02	0.90	0.61
2,4,6-TCP	5.99	3.67	0.90	0.30
PCP	4.74	5.02	0.99	1.1

[a] Data extracted from Ref. [33] with permission.
[b] Data extracted from Ref. [35] with permission.
[c] Estimated reduction potential of the phenoxyl radical in volts *vs.* NHE.

physiological pH. Thus, highly substituted acidic CPs undergo oxidation into the phenoxyl radical readily at physiological pH, because the oxidation step involves the concerted loss of an electron and a proton and these CPs exist as the phenolate.

In terms of radical stabilization through resonance, it has been observed that remote substituent effects of phenoxyl radicals exhibit a linear correlation with Brown σ^+ constants, because of the through-resonance between the electron-deficient phenoxyl radical and the ring substituent [33,37]. A high value of σ^+ suggests that the radical is localized on the oxygen atom and for the mono-substituted analogs, a chlorine substituent in the *meta* position (3-CP) has a higher σ^+ value (0.40) than the other isomers. The fully substituted analog PCP has the highest σ^+ value (1.1) followed by 2,4,5-TCP > 3-CP > 2,4,6-TCP > 2,4-DCP > 4-CP > 2-CP [33].

2.2. Carcinogenesis of chlorophenols

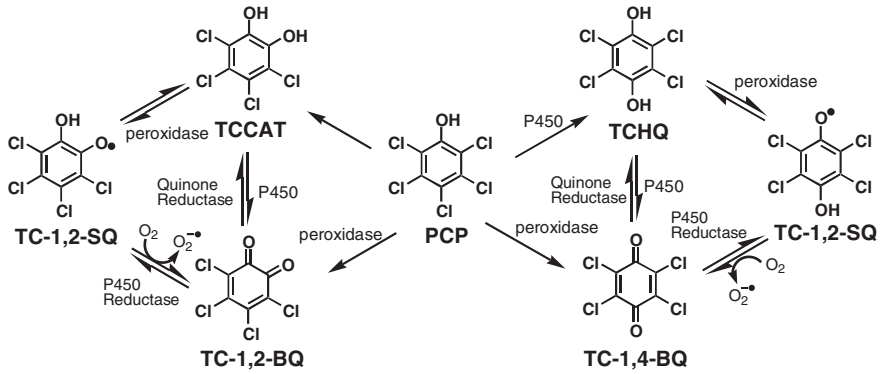
Case reports suggest an association between CPs and Hodgkin's disease, soft-tissue sarcoma, and acute leukemia [38–40]. Carcinogenicity of orally administered PCP has been tested in rats and mice. The purity of PCP is an important factor, since PCP is usually contaminated with chlorinated dibenzo-*p*-dioxins, some of which are animal carcinogens. The National Toxicology Program (NTP) tested technical-grade PCP (90% pure PCP) in mice [41]. Groups of male and female B6C3F1 mice were given diets that contained 0, 100, or 200 ppm PCP. Male mice displayed a significant increase over the male control incidence in

tumors of the adrenal medulla and liver (adenomas and carcinomas combined). Treated females displayed a significant increase over female controls with regard to the incidence of hemangiosarcomas of the spleen and liver. Although this study was limited because of the unusually low survival in the male control group, the occurrence of rare hemangiosarcomas was considered a carcinogenic response due to PCP exposure [41].

NTP also tested pure PCP (~99%) for carcinogenicity in rats [42]. Groups of male and female F344 rats were given diets that contained 0, 200, 400, or 600 ppm PCP for 105 days. In 2 years, some evidence that purified PCP is carcinogenic to rats was detected with malignant mesotheliomas and nasal squamous cell carcinomas being noted in some of the rats being treated with high dose regimes that actually exceed the maximum tolerable dose. However, unlike the study with mice [41], hepatocellular adenomas and carcinomas were not detected [42]. Overall, these choric animal bioassays suggested that the liver is a target organ for carcinogenesis in mice, but not in the rat. On the basis of the NTP studies on PCP carcinogenesis, EPA has classified PCP as a Group B2 substance (probable human carcinogen) [12]. A cancer potency factor of 0.12 mg/kg/day was calculated by the Integrated Risk Information Systems (IRIS) based on the NTP data [43], which translates to an upper-bound unit risk level of 9×10^{-3} mg/kg/day for a cancer risk of 1 in 1000.

2.3. Metabolism of chlorophenols

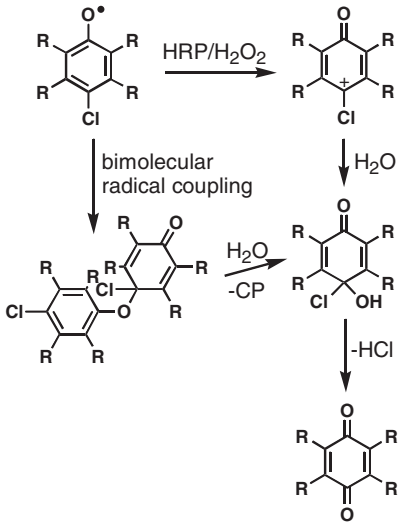
Results from animal and human studies indicate that PCP is not completely metabolized, as evidenced by a large portion of the administered dose being excreted in urine unchanged in all species studied [44]. Approximately 74% of PCP ingested by humans is eliminated in urine as PCP, while fecal elimination accounts for only 4% [44]. Metabolism of PCP does occur in the liver [21,22,45–48], and the major pathways involve conjugation to form the glucuronide and oxidative dechlorination to generate hydroquinone, catechol, and benzoquinone metabolites, as outlined in Scheme 1 [22]. Two distinct pathways have been proposed for conversion of PCP into its benzoquinone metabolites. One involves CYP450-mediated dechlorination of PCP to produce tetrachlorohydroquinone (TCHQ) and tetrachlorocatechol (TCCAT), which are subsequently oxidized to the corresponding quinones, tetrachloro-1,4-benzoquinone (TC-1,4-BQ) and tetrachloro-1,2-benzoquinone (TC-1,2-BQ) [46,48–50].



Scheme 1. Proposed pathways for the bioactivation of PCP.

The second pathway involves direct CYP450-mediated oxidation of PCP (accompanied by loss of chlorine anion) to the TC-1,4-BQ metabolite without the obligatory intermediacy of the hydroquinones which are generated from reduction of the benzoquinone [51,52]. Consistent with the pathways outlined in Scheme 1, 2,4,6-TCP has been shown to undergo oxidation by Aroclor 1254-induced rat liver microsomes to yield 2,6-dichloro-1,4-benzoquinone (2,6-DC-1,4-BQ) [53].

Horseradish peroxidase (HRP)/H_2O_2 also catalyzes the oxidation of PCP [54] and 2,4,6-TCP [55,56] into their respective 1,4-BQ metabolites. However, unlike metabolism of CPs by CYP450 enzymes, phenoxyl radical intermediates are generated. As outlined in Scheme 2 for HRP/H_2O_2 oxidation of PCP [54], the TC-1,4-BQ product may arise by elimination of HCl from a *gem*-chlorohydrin intermediate that would form through HRP oxidation of the phenoxyl radical to yield a carbocation intermediate that reacts with water. Alternatively, the *gem*-chlorohydrin could arise by hydrolysis of the ether 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone formed by pentachlorophenoxyl radical coupling. Kazunga and co-workers [57] presented evidence that the final TC-1,4-BQ product is in fact an artifact of extraction and analytical methods and that the principal product is the ether 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone formed by pentachlorophenoxyl radical coupling. This suggests a common mechanism for HRP/H_2O_2-mediated oxidation of a CP involving bimolecular radical coupling for 1,4-BQ formation. This is a potential *in vivo* metabolic step that could be catalyzed by mammalian peroxidases, including prostaglandin H synthase, myeloperoxidase (MPO), salivary peroxidase, lactoperoxidase, or uterine peroxidase [54].



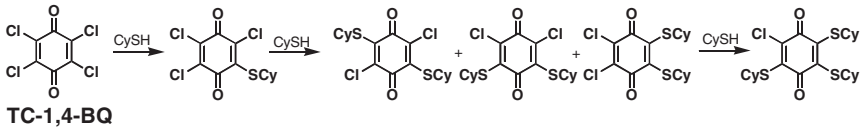
Scheme 2. Proposed pathways for peroxidase-catalyzed oxidation of CP.

Zhu and coworkers [58] have shown that the TCHQ metabolite of PCP (Scheme 1) is also capable of reacting with H_2O_2 to yield the hydroxyl radical ($HO^{\bullet}$) in a metal-independent organic Fenton reaction. From an electron spin resonance (ESR) spin-trapping study, it was concluded that the semiquinone anion radical ($TCSQ^{\bullet -}$) directly reacts with H_2O_2 and reduces it to $HO^{\bullet}$ with concomitant formation of TC-1,4-BQ [58]. This type of reaction has been proposed previously by Koppenol and Butler [59], who suggested that if a quinone/semiquinone couple has a one-electron reduction potential between -330 and $+460$ mV *vs.* NHE, it can theoretically bring about a Fenton reaction that is thermodynamically feasible and does not require a metal ion for catalysis . Structure–activity relationships demonstrated that 2-chloro-, 2,5-dichloro-, tetrafluoro-, tetrabromo-, and TC-1,4-BQ were capable of generating $HO^{\bullet}$ from H_2O_2 [58]. In contrast, no $HO^{\bullet}$ formation could be detected from reactions of H_2O_2 with BQ itself, 2,6-dimethyl-, and tetramethyl-1,4-BQ, which possess reduction potentials outside the range stipulated by Koppenol and Butler [59].

An assay developed by the Rappaport laboratory has been used to simultaneously quantitate protein adducts of quinones and semiquinones following PCP administration to rats and mice [60]. These efforts have shown that the benzoquinone metabolite (TC-1,4-BQ) of PCP is a Michael acceptor and forms adducts with cysteinyl residues (CySH) of

proteins both *in vitro* and *in vivo* [21,22,60]. As shown in Scheme 3 [21], TC-1,4-BQ retains its oxidized quinone structure following covalent attachment of CySH with chloride displacement. The product (mono-adduct) continues to react with additional sulfhydryls leading to disubstituted and trisubstituted adducts. The assay employs Raney nickel to selectively cleave the cysteinyl adducts, which generates quinones or tetrachlorophenols from semiquinone-derived adducts; the latter products were only detected *in vivo* [60]. Thus, this assay can be used to measure the extent of quinone *vs.* semiquinone adduct formation during the metabolism of PCP. In this regard, administration of a single oral dose of PCP to Sprague–Dawley rats and B6C3F$_1$ mice generated proportionally greater amounts of TC-1,2-SQ adducts in the livers of the rodents at low doses of PCP ($<$4–10 mg/kg body weight) that was 40-fold greater in rats than mice. Production of TC-1,4-BQ adducts was proportionally greater at high doses of PCP ($>$60–230 mg/kg body weight) and was 2- to 11-fold greater in mice than in rats over the entire range of doses [60]. These results suggested that species differences in the metabolism of PCP to semiquinones and quinones were, in part, responsible for the production of liver tumors in mice but not in rats [60].

TC-1,4-BQ also undergoes displacement of chloride by hydroxide in strong alkaline solution to yield chloranilic acid (2,5-dichloro-3,6-dihydroxy-1,4-BQ) through initial formation of trichlorohydroxyquinone [61]. It also reacts directly with H_2O_2 in a pH-dependent manner with chloride displacement. On the basis of this reactivity, Zhu *et al.* [58] have speculated that the O–O bond of such a hydroperoxide adduct would undergo homolytic fission to yield HO$^\bullet$ and a resonance-stabilized phenoxyl-like radical. Such a pathway could contribute to HO$^\bullet$ production by TC-1,4-BQ/H_2O_2 in the absence of redox-active transition metals, suggesting that peroxide-dependent decomposition pathways of TC-1,4-BQ may be important in biological systems where peroxide is either used or produced, as in the peroxidase-catalyzed oxidation of PCP [58].



Scheme 3. Reaction of TC-1,4-BQ with CySH.

2.4. DNA damage by chlorophenols

Early studies on the genotoxic status of PCP were negative [31]. The toxin is non-mutagenic in the Ames test [62], it did not induce DNA damage in Chinese hamster ovary (CHO) cells [63], and occupational exposure to PCP did not induce sister chromatid exchange or chromosomal breakage [64]. In contrast, the genotoxicity of TCHQ (Scheme 1) has been well established. TCHQ undergoes autoxidation to form superoxide radical anion ($O_2^{\bullet-}$) and subsequently H_2O_2, which is activated by transition metals to cause oxidative DNA damage [65]. TCHQ was found to induce single-stranded breaks in isolated DNA [66], in human fibroblasts [66,67], V79 cells [68], CHO cells [63], and the liver of mice [69,70]. TCHQ also induced micronuclei and mutations at the hypoxanthine–guanine phosphoribosyltransferase (HPRT) locus of V79 cells [71] and cause formation of 8oxo-dG in V79 cells [72] and B6C3F$_1$ mice [69]. Glutathione depletion, p53 protein accumulation, and cellular transformation were also observed in mice treated with TCHQ [70]. Because TCHQ is a major metabolite of PCP and can stimulate oxidative stress, TCHQ is thought to play a key role in PCP-mediated carcinogenicity [73,74].

While one notion suggests that TCHQ induces genotoxicity by initiating a flux of ROS that promotes oxidative DNA damage and oxidative stress [73,74], the Witte laboratory has examined the genotoxicity of TCHQ and has concluded that its autoxidation intermediate, the semiquinone anion radical (TCSQ$^{\bullet-}$), is responsible for the observed cyto- and genotoxicity of TCHQ [75,76]. This conclusion was based on the demonstrated ability of desferrioxamine (DFO) to inhibit TCHQ-induced single-strand breaks in isolated DNA by its efficient scavenging of TCSQ$^{\bullet-}$ [75]. Extension of these experiments to human fibroblasts showed that DFO provided marked protection against TCHQ-induced genotoxicity, while the HO$^{\bullet}$ scavenger dimethyl sulfoxide (DMSO) was ineffective. UV–visible spectroscopic experiments showed that 50 μM TCHQ readily autoxidizes at physiological pH to generate TCSQ$^{\bullet-}$ with λ_{max} at 455 nm and an ESR signal at $g = 2.0056$ [75]. The visible absorption for TCSQ$^{\bullet-}$ reaches maximum intensity at about 10 min and then it is gradually replaced by an absorbance at 292 nm for TCBQ, and finally (several hours) a peak at 332 nm for chloroanilic acid. In the presence of DFO, or other hydroxamic acids, the maximal concentration and life span of the signal for TCSQ$^{\bullet-}$ was markedly reduced, while the signal for chloroanilic acid at 332 nm was markedly enhanced with 90%

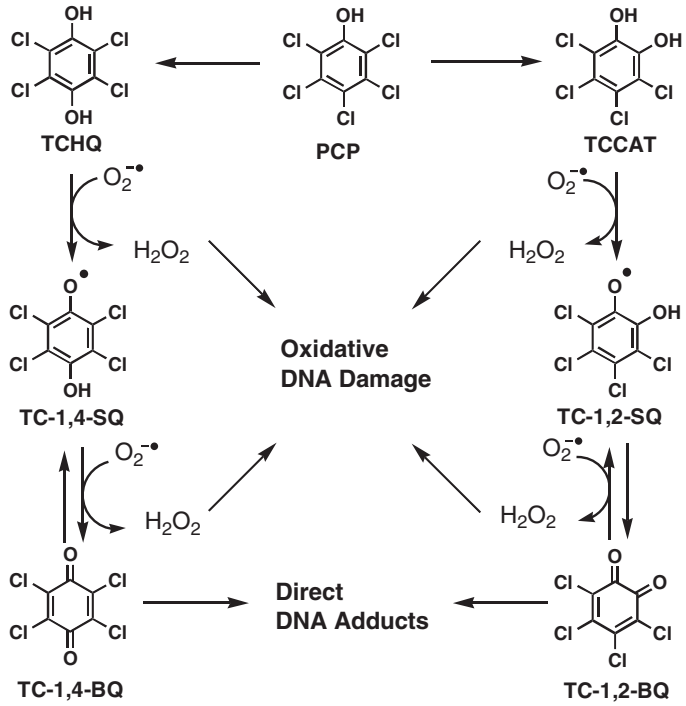
conversion of TCHQ into chloroanilic acid following 30 min reaction time [75]. Comparison of the genotoxicity of TCHQ to H_2O_2 in human fibroblasts also revealed a greater genotoxic potential for TCHQ than for H_2O_2 [76]. DNA damage was determined after 1 h treatment with H_2O_2 or TCHQ by the comet assay. Here, a distinct tail moment was noted for TCHQ at concentrations $< 10\,\mu M$, whereas 60 μM H_2O_2 was required to produce the same extent of DNA damage. By monitoring the incorporation of [^{3}H]-thymidine into DNA of non-replicating cells (UDS), the extent of DNA repair was measured and 25 μM TCHQ was found to inhibit repair, while H_2O_2 continuously induced DNA repair up to 60 μM. In contrast to H_2O_2, TCHQ was also mutagenic in the HPRT locus of V79 cells with a mutant frequency of 75 and 151 mutants per 10^6 clonable cells at non-toxic concentrations of 5 and 7 μM. Witte and coworkers [76] suggested that the $TCSQ^{\bullet -}$ may react with DNA directly and cause apurinic/apyrimidinic (AP) sites which are transformed to strand breaks either by endonucleases or under the alkaline conditions of the comet assay. This would explain the ineffectiveness of DMSO to quench TCHQ-induced damage in cellular DNA. However, an alternative explanation may stem from TCHQ-induced $HO^{\bullet}$ production by a metal-independent process [58] that takes place close to the DNA surface, precluding effective $HO^{\bullet}$ scavenging by DMSO. That TCHQ also inhibits DNA repair enzymes would provide a rationale for its potent genotoxicity and mutagenicity. In contrast, DNA damage by H_2O_2 would require metal ions for catalysis and DNA repair enzymes are not inhibited by H_2O_2 [76].

While little is known about direct interactions of $TCSQ^{\bullet -}$ with DNA, the fully oxidized TC-1,4-BQ metabolite of PCP is known to react covalently with DNA to generate DNA adducts, as evidenced by the ^{32}P-postlabeling assay [23–25]. Treatment of calf thymus DNA with 5 mM TC-1,4-BQ generated four major and several minor adducts (3.5 adducts per 10^5 total nucleotides) [24]. These adducts were chemically stable and do not generate AP sites. In addition, increases in 8oxo-dG and AP sites were observed that were ascribed to oxidative damage. These results demonstrated that PCP quinone and hydroquinone metabolites induce direct and oxidative base modifications as well as the formation of 5′-cleaved AP sites in genomic DNA [24].

Cell culture and *in vivo* studies have now shown that PCP itself shows direct genotoxicity under certain conditions. Treatment of rat hepatocytes with a single dose of PCP (50 μM) generated 17 adducts per 10^9 total nucleotides [77]. Chronic (60 mg/kg/day for 27 weeks), but

not acute (60 mg/kg/day for 1 or 5 days), exposure of rat to PCP induced a twofold increase in 8oxo-dG (1.8 *vs.* 0.91×10^{-6} in controls) and generated two major adducts, one derived from TC-1,4-BQ, with relative ^{32}P-postlabeling of 0.78 adducts per 10^7 total nucleotides [25]. The TC-1,4-BQ-derived DNA adduct was also detected in mouse liver DNA following exposure to PCP at 8 adducts per 10^7 nucleotides [23] which is 10-fold greater compared with the rat.

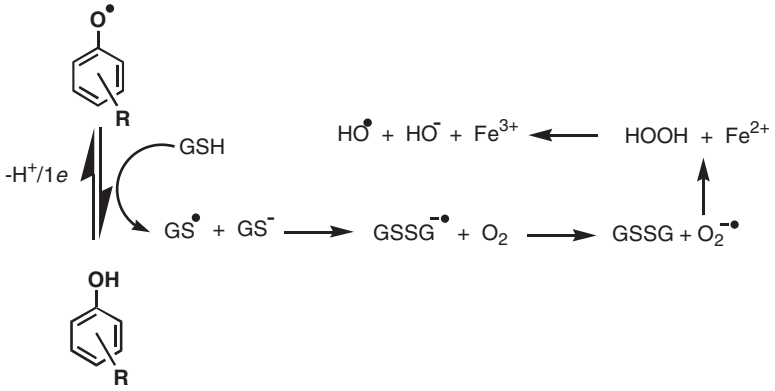
The pathways outlined in Scheme 4 represent proposals for PCP carcinogenesis in rodents *via* the induction of oxidative DNA lesions and direct DNA adducts in target organs [25,78]. PCP is metabolized to TCHQ and TCCAT, which undergo autoxidation with the generation of ROS and PCP semiquinone and quinone metabolites that cause oxidative DNA damage and direct DNA adducts, respectively [25,78]. The Swenberg laboratory suggested that the pathways in Scheme 4 might provide a rationale for PCP-mediated hepatic carcinogenesis in mouse [25]. The greater amounts of both oxidative and direct DNA damage, together with increased hepatotoxicity and cell proliferation,



Scheme 4. Proposed pathways for the generation of reactive oxygen species and direct DNA adducts during PCP metabolism.

may provide the critical events necessary for hepatic carcinogenesis in the mouse. In contrast, the decreased amount of DNA damage and the lack of hepatotoxicity and cell proliferation in the rat do not result in such critical changes [25].

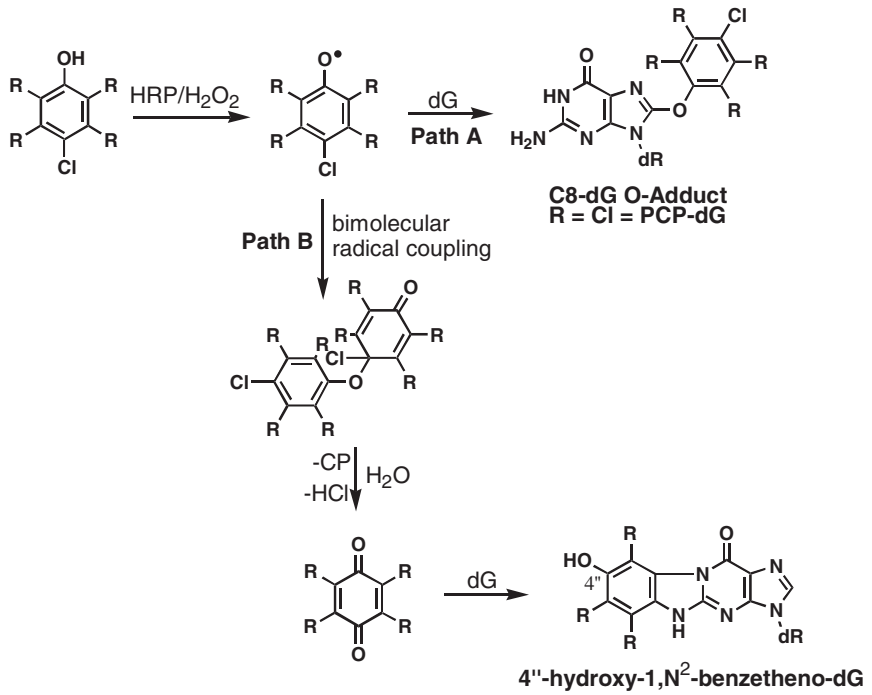
The association of CPs to incidences of leukemia [38–40] is also consistent with the leukemogen activity of phenolic xenobiotics including phenol [79] and the phenolic anticancer drug etoposide [80]. Phenolic compounds are substrates for MPO present in bone marrow and are converted into phenoxyl radicals. The Kagan laboratory and others have ascribed the leukemogen activity of phenols to the phenoxyl radical intermediates that interact with intracellular thiols to trigger redox-cycling cascades yielding different new free radical species [79–81]. As outlined in Scheme 5, the phenolic compound is oxidized to its phenolic radical by peroxidase and H_2O_2. The phenoxyl radical is then reduced *via* oxidation of thiols (glutathione (GSH) and protein sulfhydryls). This repeatedly regenerates the phenol as a substrate for peroxidase, while thiyl radicals react with GSH to generate a glutathione or protein disulfide anion radical ($GSSG^{\bullet -}$) that can donate an electron to O_2 to form the superoxide anion radical ($O_2^{\bullet -}$) and hence H_2O_2. The build-up of $O_2^{\bullet -}$ will lead to the liberation of Fe^{2+}, which in the presence of H_2O_2 will form the hydroxyl radical ($HO^{\bullet}$), which may damage DNA and other biomolecules. Thus, oxidative stress is induced and oxidative modifications of proteins as well as oxidative and mutagenic modifications of DNA occur, ultimately leading potentially to carcinogenesis [81]. Direct reactions of phenoxyl radicals



Scheme 5. Proposed pathways for the generation of reactive oxygen species during peroxidase-catalyzed oxidation of phenolic substrates.

and thiyl radicals with biomolecules could also contribute to peroxidase-driven toxic effects of phenolic xenobiotics.

With regard to direct reactions of phenoxyl radicals with DNA, an *in vitro* study by the Turesky laboratory demonstrated that adduct levels ($36 \pm 9/10^5$ nucleotides) by 100 μM PCP following activation by HRP/H_2O_2 are 30-fold higher than levels induced by microsomes that contain CYP450 and 10-fold higher than levels induced by 5 mM TC-1,4-BQ itself [82]. This suggested the possibility that the pentachlorophenoxyl radical generated by HRP/H_2O_2 oxidation of PCP [54] contributes to DNA adduction. Thus, we examined the reaction of PCP (100 μM) with DNA nucleosides (2 mM) using HRP/H_2O_2 as oxidation catalyst and liquid chromatography/mass spectrometry (LC/MS) [83]. Interestingly, these conditions yielded a single DNA adduct that had the five-chlorine isotope pattern of PCP and had a molecular ion at $[M-H]^- = 528$, suggesting attachment of dG with loss of two protons (i.e., PCP(264) + dG(267) – 2H = 529). Isolation and nuclear magnetic resonance (NMR) (^1H and ^{13}C) analysis confirmed C8 attachment by the O-site of PCP to yield the adduct PCP-dG shown in Scheme 6.



Scheme 6. Summary of peroxidase-mediated reactions of a CP with dG.

Additional experiments were carried out to determine whether PCP-dG could be formed by additional enzyme systems, and whether other DNA bases could react with PCP following HRP/H_2O_2 activation [83]. These experiments showed the reaction to be absolutely specific for dG; no detectable adduct(s) was observed from HRP/H_2O_2 and PCP in the presence of dA, dC, or T. It was also found that PCP-dG could be generated by the treatment of PCP/dG with MPO. However, treatment of PCP/dG with rat liver microsomes that contain CYP450 failed to yield PCP-dG and instead an adduct with $[M–H]^- = 440$ was detected that was found to coelute with an adduct induced by the reaction of dG with authentic TC-1,4-BQ.

An extension of our studies on HRP-mediated dG adduction by PCP was conducted in order to include dG adduction by a greater range of CP toxins and to determine whether the pentachlorophenoxyl radical reacts with an actual duplex DNA substrate to form PCP-dG [84]. In terms of HRP/H_2O_2 activation, the results with PCP suggested that other CPs (2,4,6-TCP, 2,4,5-TCP, and 2,4-DCP; Fig. 1) would similarly react with dG to yield C8 O-adducts. To test this hypothesis, CP/dG reactions were carried out using conditions analogous to the PCP/dG reaction and were analyzed by LC/MS. Here it was expected that 2,4,6-TCP and 2,4,5-TCP would yield an O-adduct with a molecular ion at $[M-H]^- = 460$, while the O-adduct from 2,4-DCP would have a molecular ion at $[M-H]^- = 426$. However, under conditions favorable for O-adduct formation by PCP, the only other CP to yield detectable levels of O-adduct was 2,4,5-TCP [84]. The major adduct detected for these CPs had UV absorbances at ~275 and 334 nm with a molecular ion at $[M-H]^- = 390/392$ with a single chlorine isotope. The UV spectrum of the major adduct was similar to the spectrum reported for the dG adduct generated from 1,4-benzoquinone (BQ)/dG [85], suggesting that bioactivation by HRP/H_2O_2 for these CP substrates (100 μM) yielded a BQ electrophile.

As outlined in Scheme 2, 1,4-BQ could arise from a *gem*-chlorohydrin generated by the hydrolysis of the ether formed by C–O coupling of two chlorophenoxyl radicals. This suggested the possibility that lowering the concentration of the CP to retard radical coupling would favor C8-dG O-adduct formation for 2,4,6-TCP, 2,4,5-TCP, and 2,4-DCP. Thus, the CP/dG reactions were repeated at 10 μM CP and now all CPs generated the anticipated C8-dG O-adduct as the major lesion; for 2,4,5-TCP, the O-adduct was the only observable adduct, while 2,4,6-TCP and 2,4-DCP still formed detectable levels of BQ-adduct [84].

To determine whether a C8-dG O-adduct forms with a duplex DNA substrate, calf thymus DNA (1 mg/mL) was treated with PCP (100 μM) in phosphate buffer (100 mM, pH 7.4) at 37 °C for 24 h in the presence of HRP (Type VI, 25 units/mL)/H_2O_2 (1 mM) [84]. The reacted DNA was precipitated, enzymatically digested, and analyzed by LC/MS using an authentic sample of PCP-dG for comparison. These studies showed that the major DNA lesion formed from PCP activation by HRP/H_2O_2 had high-performance liquid chromatography (HPLC) retention time, MS/MS fragmentation, and parent ion identical to those of the authentic adduct standard. This implied that the pentachlorophenoxyl radical reacted with the duplex DNA substrate to form the O-bonded C8 PCP-dG adduct highlighting the *in vitro* relevance of C8-dG adducts of phenolic toxins.

The results from our studies on CP-mediated DNA adduction by peroxidase activation allowed us to propose the mechanism summarized in Scheme 6. The reaction is initiated by the production of the chlorophenoxyl radical that may either react directly with dG to furnish the C8-dG O-adduct in an irreversible process (Path A) or self-couple to yield the 1,4-BQ electrophile that reacts with dG to give 4″-hydroxy-1,N^2-benzetheno-dG (Path B). Formation of the benzetheno-dG adduct in Path B may involve initial Schiff base formation from the exocyclic N2 atom of dG and the carbonyl carbon of the BQ and subsequent nucleophilic attack of the endocyclic N1 on the vinyl carbon. An alternative pathway, as originally proposed by Jowa *et al.* [86], would involve initial reaction of N2 at the vinyl carbon of BQ followed by N1 attachment to the carbonyl carbon atom to yield 3″-hydroxy-1,N^2-benzetheno-dG. However, the ^{1}H NMR chemical shifts and reaction chemistry for this family of cyclic adducts favor 4″-hydroxy-1,N^2-benzetheno-dG adduct assignment.

Differences in CP reactivity may be ascribed to differences in the rate constants for the phenoxyl radical bimolecular self-reaction (Path B in Scheme 6). If the bimolecular self-reaction is favorable, then Path B competes effectively with Path A at 100 μM CP substrate, while if the self-reaction is not favorable, then Path A dominates. Increased Cl substitution would be expected to inhibit the bimolecular self-reaction due to steric and/or electronic repulsion. In this regard, the bimolecular self-reaction rate constant for phenoxyl radical in H_2O is 2.6×10^9/M/s [87] and it has a half-life of ~60 μs in CCl_4 [88]. In contrast, the pentachlorophenoxyl radical has a half-life of 30–45 min in aqueous buffer,

as determined from direct detection by EPR spectroscopy [54]. Clearly, Cl substitution increases phenoxyl radical lifetime and this factor would be expected to contribute to phenoxyl radical reactivity in a biological system by allowing the radical time to diffuse away from its site of formation and react with biopolymers.

2.5. Conclusions and future research

CP toxins, especially PCP, are known to cause carcinomas in mouse liver. A DNA-reactive mechanism that includes both direct genotoxic DNA damage and oxidative stress-induced indirect DNA damage are proposed to initiate PCP-mediated hepatic carcinogenesis in mice. These events depend on the metabolism of PCP into TCBQ/TCHQ/TCCAT redox couples (Scheme 4). The TC-1,4-BQ metabolite reacts covalently with DNA and with cysteinyl residues of proteins to form di- and trisubstituted adducts. Hence, chlorinated benzoquinones are bi- and tri-functional alkylating agents and it is expected that TC-1,4-BQ participates in the formation of both protein–protein cross-links and/or protein–GSH moieties [21]. In terms of DNA adduction by TC-1,4-BQ [24,25], none of the adducts have been structurally characterized. However, it is anticipated that benzethenotype adducts (Scheme 6) and cross-linked DNA would contribute to TC-1,4-BQ-induced DNA adduction. Future studies aimed at characterizing the nature of DNA adducts formed by TC-1,4-BQ would be informative, as such adducts are potentially useful markers of chronic exposure to PCP. Structural assignment of the adducts would also permit strategies for the incorporation of TC-1,4-BQ adducts into oligonucleotides to determine the fate of these adducts (repair, mutation, persistence).

The metabolism of CP toxins also generates semiquinone and phenoxyl radical electrophiles [54,74]. Our work has demonstrated that CP phenoxyl radicals react covalently with the C8-site of dG to form O-bound adducts *in vitro* [83,84]. If these adducts form in peroxidase-rich tissues, then the fate of C8-dG adducts of CP toxins should be established. Overall, such efforts would increase our understanding of CP-mediated genotoxicity and the contribution that DNA adduction makes to CP-mediated carcinogenesis. This knowledge would be used to establish safe human exposure levels to CP pollutants.

3. OCHRATOXIN A

3.1. Production and properties of ochratoxin A

OTA (Fig. 1) is a mycotoxin produced by fungi in cool temperate (*Penicillium* spp. including *P. verrucosum*, *P. aurantiogriseum*, *P. citrinum* and *P. expansum*) [89,90] and tropical latitudes (*Aspergillus* spp. such as *A. ochraceus*, *A. carbonarius*, *A. niger*) [91–93]. The toxin consists of a *para*-chlorophenolic moiety containing a dihydroisocoumarin group that is amide-linked to L-phenylalanine [16,17]. In alcoholic solution, fully protonated OTA shows a UV–vis spectrum with an absorption band that peaks at 332 nm (molar absorptivity (ε) ~6650/M/cm) [94]. The pK_as of the carboxylic group and the phenolic moiety are ~4 and ~7, respectively [95–97]. Upon deprotonation of the phenolic moiety, ε increases substantially up to 10,970/M/cm and the absorption band shifts to 380 nm [94]. OTA also exhibits strong fluorescence, and as such, HPLC with fluorescence detection for quantitation of low OTA levels in food is common [98].

Cereals are the main contributor of OTA intake, although other food products such as coffee, cocoa, grape fruits, beans, nuts, dried fruit, and meat can be contaminated with OTA [99–102]. While OTA exposure is mainly due to ingestion of contaminated food [99–102], exposure by inhalation of dust containing OTA is possible. Di Paolo *et al.* [103] have described a case of acute intoxication in a man with oliguria and tubulonecrosis, which might have been due to inhalation of *A. ochraceus*. It has recently been demonstrated that OTA is present in spores and airborne dust particles, thus supporting this hypothesis [104–106]. Brera *et al.* [107] and Iavicoli *et al.* [108] have demonstrated that exposure to OTA occurs in the workplace from inhalation of dust, which correlates with OTA levels found in plasma. Similarly, we have collected dust ''in silo'' and have isolated *Penicillia* and *Aspergillii*, which are capable of producing OTA [109].

OTA is very toxic to numerous animal species with the kidney being the main target organ (for reviews, see Refs. 99,110). The toxin is regarded as a major causal determinant of mycotoxin porcine nephropathy [99] as well as being the main etiological agent responsible for Balkan endemic nephropathy (BEN) in humans [14]. Ultrastructural and toxicological investigations in spontaneous cases of porcine nephropathy have been carried out [111]. These studies show striking similarities between the described OTA-induced porcine nephropathy

in pigs and BEN [112–114]. Both diseases are characterized by degeneration of epithelial cells of the proximal tubules and interstitial fibrosis resulting in polyuria and various changes in hematological and biochemical parameters [115]. From the spontaneous cases of porcine nephropathy, OTA was found in 100% of investigated serum samples collected from the diseased pigs ($48.34 \pm .6.7$–84.2 ± 41.17 ng/mL) [111]. Stoev *et al.* [111] have also induced porcine nephropathy in six pigs by providing a diet containing 800 μg/kg of OTA over a 1-year period [116]. The characteristic renal lesions found in the pigs were similar to the classic Danish porcine nephropathy, highlighting the role for OTA.

In rodent studies, pre-treatment of mice with phenobarbital (PB), an inducer of CYP450 2C, 2B, 3A [117–119], and glutathione-*S*-transferase (GST) [120], decreased nephrotoxicity [121], but increased hepatic tumor formation [122]. This suggests that biotransformation of OTA by enzymes modulated by PB generated OTA metabolites which are less nephrotoxic than the parent OTA, but are genotoxic. Because urinary tract tumors are associated with BEN and exposure to OTA [123,124], the genotoxicity of OTA is thought to play a key role in OTA-mediated tumor formation [30]. While the carcinogenicity of OTA is beyond doubt and is endorsed by IARC, Lyon, there is as yet no clear mechanism for the occurrence of OTA-mediated tumors in rat renal parenchyma. In general, tumor development is a multifactorial process, implicating alteration of DNA after metabolic activation of carcinogens and subsequent mutations that can alter protooncogen function or tumor suppression [125–127]. For renal carcinogenesis in rodents, three mechanisms have been proposed: direct genotoxic DNA damage, oxidative stress-induced indirect DNA damage, or inappropriate stimulation of cell proliferation by accumulation of α-2u-globulin in the kidney of male rats [128]. The latter mechanism does not appear to be involved in the carcinogenicity of OTA [129], suggesting that DNA damage [26–28] may make an important contribution to OTA-mediated tumor formation [30].

3.2. Carcinogenicity of ochratoxin A

OTA is carcinogenic in rodents [130]. The first studies of OTA carcinogenicity were performed on rats [131], trout [132], and mice [133,134] by oral or intraperitoneal administration. Only after oral administration were tumors induced in the kidney of mice and rats, and liver of trout.

When these studies were evaluated by IARC [135,136], it was considered that the evidence for carcinogenicity was either inconclusive or limited. The results of the mouse carcinogenicity study were subsequently confirmed by a second study by the same group [130], which also demonstrated a synergistic effect by the mycotoxin citrinin when it was administered simultaneously.

The carcinogenic potency of OTA to mice was confirmed by Bendele *et al.* [137]. Groups of B6C3F1 mice (50 males and 50 females) were given feed containing 40 μg OTA for 24 months. In treated male mice, 31 were affected (14/49 had renal carcinomas and 26/49 renal adenomas). All males showed nephropathy but only a small number of females. None of the females had renal carcinoma or adenoma. A small number of both males and females had hepatocellular neoplasm.

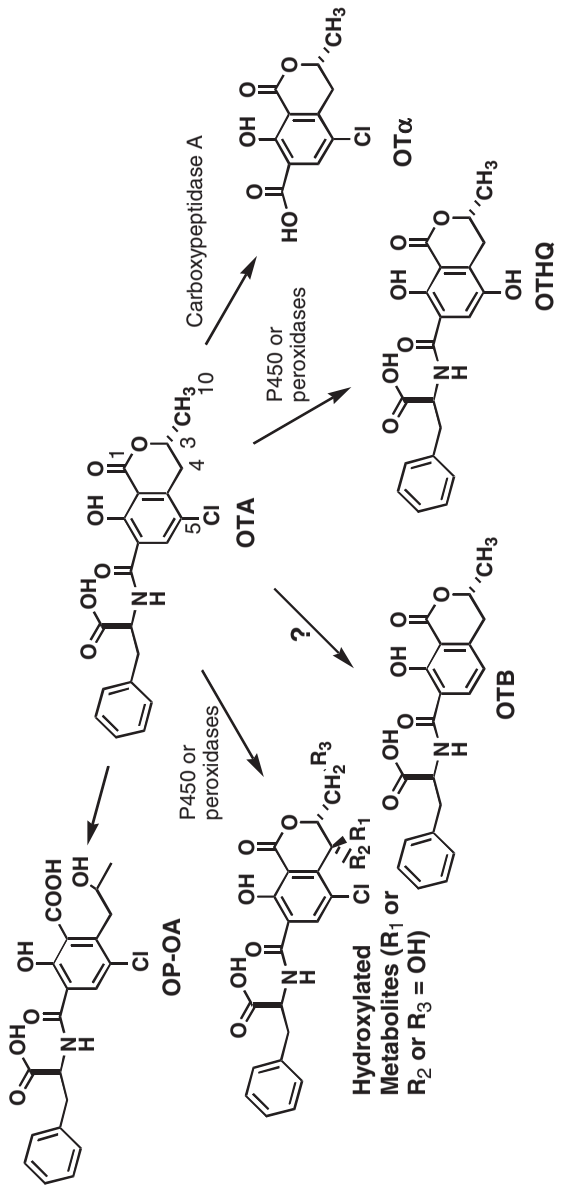
In a study by NTP, three doses of OTA (210, 70, and 21 μg/kg body weight) were administered to male and female F344N rats [13]. At 210 μg/kg body weight, renal tubular adenomas and carcinomas were observed (72% for males and 16% for females) after 2 years. At 70 μg/kg body weight, 39% of the males and 4% of the females developed renal adenomas or carcinomas. The females were less susceptible than the males to OTA carcinogenicity. In addition, non-neoplastic renal modifications (hyperplasia, cell proliferation, cytoplasmic alteration, and karyomegalies) were observed. All animals treated with 70 or 210 μg/kg body weight presented karyomegalies. In view of these results, IARC evaluated the experimental evidence for carcinogenicity as sufficient and classified OTA as Group 2B, ''possible human carcinogen'' [130]. The NTP results in rats were confirmed by a study at IARC using Lewis and Dark Agouti (DA) rats [138]. Male DA rats were very sensitive and female DA rats were resistant. Male DA rats were also much more sensitive than either male or female Lewis rats; a factor ascribed to superior biotransformation capacity of OTA in male DA rats [139]. Since 2-mercaptoethane sulfonate (MESNA) protects rats against nephrotoxicity and carcinogenicity induced by oxidative stress by increasing free thiol groups in kidney [140,141], the potential protective effect of MESNA on renal toxicity and carcinogenicity induced by OTA was examined in a long-term rat study [142]. MESNA significantly decreased karyomegalies in the kidney of all OTA-treated animals, but had no beneficial effect on renal tumor incidence. In fact, a significant increase in renal tumor formation was observed in male DA rats [142].

3.3. Metabolism of ochratoxin A

During the past 25 years, the metabolism of OTA has been extensively studied by Størmer and co-workers (for a review, see [143]) using *in vitro* and *in vivo* assays in the liver of different animals [144–147]. These efforts led to the isolation and identification of the hydroxylated derivatives 4(*R*)-, 4(*S*)-, and 10-OH-OTA shown in Scheme 7 along with OTα, which stems from cleavage of the peptide bond by α-chymotrypsine and carboxypeptidase [148,149]. It was also established that the metabolism of OTA is organ- and species-specific. For example, 4(*R*)-OH-OTA was mainly formed after incubation in the presence of human and rat liver microsomes, whereas 4(*S*)-OH-OTA was essentially formed *via* pig microsomes [145]. 10-OH-OTA was produced after *in vitro* incubation with rabbit liver microsomes [146]. Furthermore, *in vivo* studies indicated that the cleavage of the peptide bond in OTA to yield OTα occurs by homogenates from pancreas and small intestine, but not by liver [147].

Other authors have more recently studied the biotransformation of OTA. *In vitro* incubation in the presence of microsomes or treatment of cell lines with OTA leads to the formation of at least 20 derivatives [150–159]. We have demonstrated that after *in vitro* incubation in the presence of microsomes, the formation of OTA metabolites is time- and concentration-dependent [153]. For incubation in the presence of pig liver microsomes, the kinetics is linear during the first 10 min and then reaches a plateau at $\sim$25 min. At least 10 different derivatives have been separated by HPLC from cell culture (human bronchial epithelial cell, opposum renal cells) and *in vitro* incubation (pig kidney microsomes). Co-elution of these derivatives with synthetic OTA metabolites established that the opened lactone derivative (OP-OA [97], Scheme 7) was formed exclusively with cortex microsomes. Pig liver microsomes (male and female) and female cortex and medulla microsomes induced formation of 4(*R*)-OH-OTA, but not 4(*S*)-OH-OTA. In contrast, male pig microsomes from cortex and medulla generated both 4-OH-OTA stereoisomers. The relative quantity of metabolites was dependent on both the origin of the microsomes (male or female, liver or kidney) and on the co-factors used: nicotinamide adenine dinucleotide phosphate (NADPH) and arachidonic acid (AA) [153].

The metabolism of OTA also generates the nonchlorinated OTB analog shown in Scheme 7. This metabolite is formed from OTA-incubation of rabbit kidney microsomes pre-treated with PB [155] and in



Scheme 7. Metabolism of OTA.

monkey kidney cell culture [157]. OTB is also present in the kidney of pigs fed OTA [152]. While little is known about the mechanism of OTB formation *in vivo*, it is also generated from OTA photochemically [160,161]. Under photochemical conditions, it is expected that OTA interacts with a solvated electron, which initiates C–Cl bond homolysis to afford an OTA carbon-centered radical and chloride; H-atom abstraction by the radical provides a route to OTB [94]. This reaction is akin to reductive dehalogenation of alkyl- and aryl-halides that are catalyzed by CYP450s [162–164]. As suggested by Guengerich [162], ferrous CYP450 should be a good one-electron-reducing agent and that transfer of an electron to a substrate may be competitive with oxidation even in the presence of O_2. OTA also stimulates production of $O_2^{\bullet -}$ that will lead to the liberation of free Fe^{2+}; a co-factor in cells that may also cause reductive dehalogenation of OTA to generate OTB [30].

The other nonchlorinated metabolite of OTA shown in Scheme 7 is the hydroquinone derivative hydroquinone OTA (OTHQ). Recent biological studies confirm the presence of OTHQ in the urine of rat following the administration of OTA by gavage [165]. On the basis of analogy to the metabolism of PCP, which generates TC-1,4-BQ by oxidative dechlorination pathways [21,22], it is expected that a two-electron reduction of the quinone species, quinone OTA (OTQ), by action of NAD(P)H:quinone reductase generates OTHQ. Four observations provide indirect evidence for the intermediacy of OTQ in OTA oxidation. These are: 1) electrochemical methods [166]; 2) treatment of OTA with a biomimetic iron-oxo system [167]; 3) treatment of OTA with redox active transition metals [168]; and 4) the characterization and identification of a OTA-GSH conjugate that is produced from the treatment of OTA with GSH following incubation with rat liver microsomes [169].

In terms of specific P450 isoforms responsible for OTA metabolism, Ueno [170] demonstrated that in rat, CYP1A2 is involved in 4(*R*)-OH-OTA, whereas 4(*S*)-OH-OTA is formed by CYP2B. We observed using human bronchial epithelial cells (BEAS-2B) expressing specific human CYPs that 4(*R*)-OH-OTA is formed by several CYP (1A2, 2B6, 2C9, 2D6, 2A6) to different extents, whereas 4(*S*)-OH-OTA is formed by CYP2D6 and 2B6 [171,172]. CYP 1A1/1A2, 2B1, and 3A1/3A2 has also been implicated by Omar *et al.* [173]. Formation of OH-OTA derivatives is modulated by induction or inhibition of P450, prostaglandin synthase and lipoxygenase (LOX) enzymes. Indeed, pre-treatment of animals with PB increased 4(*R*)-OH-OTA in hepatocytes [158], in rat liver [144,157], in rabbit kidney, and in epithelial bronchial cells [155].

We have observed these latter metabolites in kidneys of pigs fed OTA [152]. In contrast, inhibition of the LOX pathway increased the formation of 10-OH-OTA [156]. The P450s implicated in the OTB formation are CYP2A6 [172] and CYP2C9 [155], both of which are involved in the biotransformation of coumarins [174], which may be particularly relevant, because OTA contains an isocoumarin moiety.

Specific P450 isoforms have also been implicated in the genotoxicity and mutagenicity of OTA. Fink-Gremmels and co-workers [175,176] have demonstrated that in cells expressing CYP2C9, OTA exerts increased cytotoxicity and mutagenicity. The CYP3A enzymes have also been suggested to be involved in the biotransformation of OTA [177], which is particularly relevant for xenobiotic metabolism because of their broad substrate specificity and abundant expression in the human liver, intestine, and kidney. In this regard, the CYP 3A5*1 allele was more prevalent in BEN patients with a frequency of 9.38% compared to 5.36% in controls and was associated with a higher risk for BEN (OR 2.41). Our studies have also shown that in male DA rats that are most susceptible to renal carcinogenicity and DNA adduct formation [138], the OTA-toxifying enzymes (CYPs 2C11, 1A2, and 3A) that include CYP3A were highly expressed in the liver [178]. The induction of CYP2C11 is also highly relevant. Patients suffering from BEN have been reported to be more frequently extensive metabolizers of debrisoquine (DB) [179]. Although DB is preferentially 4-hydroxylated by CYP2D6 in humans [174] and by CYP2D1 in rats, it is also hydroxylated by CYP2C11 in male rat and CYP2C9 in humans [180]. Western blot analysis demonstrated that DA rats of both sexes were defective in CYP2D in both liver and kidney microsomes [178]. However, OTA strongly induced the expression of CYP2C11 in the livers of male DA rats, but not in females, and CYP2C11 is a DB-metabolizing phenotype in rats [180]. This data correlates with DB-metabolizing phenotypes being important in BEN [179] and is in keeping with the greater susceptibility of males than females to OTA-induced renal carcinogenicity [138,178].

Shown in Fig. 2 is a typical HPLC profile with spectrofluorometry detection highlighting the separation of OTA metabolites following extraction with chloroform, as described in Ref. [152]. Relatively large amounts of OTB and another dechlorinated derivative of OTA (labeled d) are formed in opossum kidney (OK) cells pre-treated with acivicin (α amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid), an inhibitor of gamma glutamyl transpeptidase (GGT) which blocks the cytotoxicity of hydroquinone-*S*-conjugates [181]. Figure 3 shows the modulation of

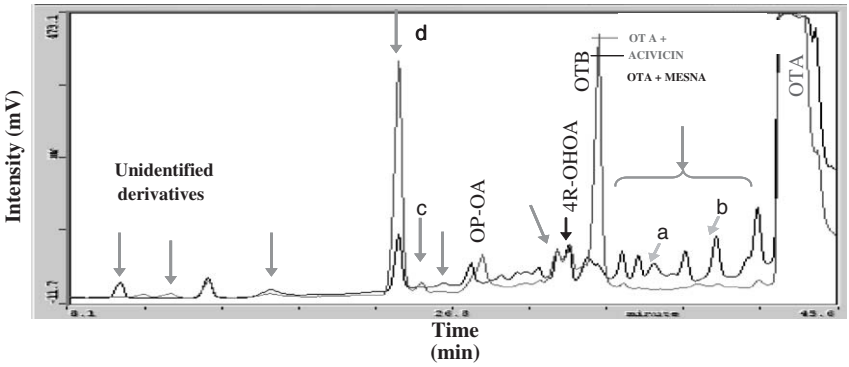


Fig. 2. Example of an HPLC separation of OTA metabolites. Supernatant of OK cells were extracted as described by Petkova-Bocharova *et al.* [152]. The metabolites are separated using the following systems by gradient elution: solvent A, MeOH:acetonitrile:6.5 mM ammonium formate (200:200:600) adjusted to pH 3 with formic acid; solvent B, MeOH:acetonitrile:6.5 mM ammonium formate (350:350:300) adjusted to pH 3 with formic acid. Program: T0 100% A; T10 100% A; T25 30% A; T30 30% A; T45 0% A; T55 0% A; T58 100% A.

OTA metabolites by the inhibitors of the GSH pathway that include: MESNA, which protects rats against nephrotoxicity and carcinogenicity induced by oxidative stress by increasing free thiol groups in the kidney, *N*-acetylcysteine (NAC), a precursor of intracellular CySH and GSH, and an ROS scavenger, buthionine sulfoximine oxide (BSO), an inhibitor of glutathione synthase, and acivicin. The structures of several of these derivatives (OP-OA, OTB, 4R-OH-OTA) were identified by co-chromatography and they have also been confirmed by MS.

P450s are primarily responsible for the oxidation of most xenobiotics in liver [182], whereas much lower P450 activities are expressed in other tissues. In the kidney, lung, and brain, several xenobiotics are co-oxidized by COX or LOX [183,184]. Because OTA induces renal tumors in rats [13,137,138] and catalyzes ROS formation and lipid peroxidation in the kidney of rat [185,186], its biotransformation could be due to co-oxidation by several enzymes [187], notably those implicated in the metabolism of AA, such as COX, LOX, and epoxygenase; the latter enzyme is related to CYP2C11 [188]. Systems that we have used to study OTA metabolism, such as pig seminal vesicle microsomes and BEAS-2B cells express high levels of COX and LOX enzymes, as well as CYP450-epoxygenases that are also found in the cortex and outer medulla of kidney and bladder cells [156]. Pre-treatment of bronchial

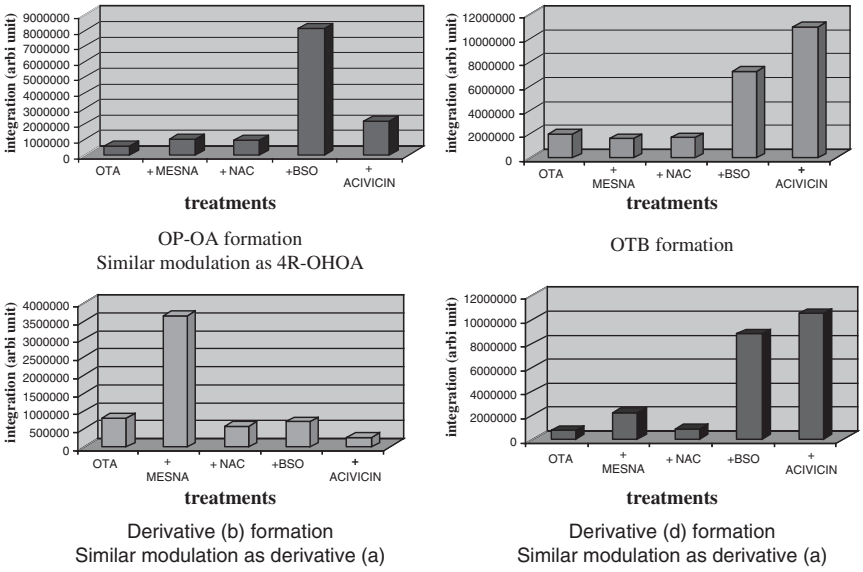


Fig. 3. Modulation of OTA derivatives by inhibitors of GSH pathways. Before OTA treatment, OK cells were treated 2 h by different GSH modulators and ROS scavengers: MESNA (2-mercaptoethane sulfonate); BSO (buthionine sulfoximine oxide); NAC (*N*-acetylcysteine); acivicin (αamino-3-chloro-4,5-dihydro-5-isoxazole acetic acid).

epithelial cells (WI) with indomethacin (0.1 μM), which inhibits COX enzymes but enhances LOX activity, or nordihydroguaiaretic acid (NDGA), which inhibits LOX enzymes, prior to OTA treatment modified the profile of OTA derivatives, as studied by HPLC with spectrofluorometry detection [156]. Treatment of cells with indomethacin (0.1 μM) generated new OTA metabolites that could not be identified using OTα, 4-(*R*,*S*)-OH-OTA and OTB as authentic standards. These metabolites of unknown structure appeared to be formed by LOX enzymes, as they disappeared upon pre-treatment of cells with NDGA [156]. Comparison of the metabolite studies with OTA-mediated DNA adduction suggested that these new metabolites may play a role in OTA-mediated genotoxicity and that LOX enzymes are important in the biotransformation of OTA into genotoxic metabolites [156].

Hoehler *et al.* [189,190] have also shown that OTA still causes generation of ROS in bacteria that have very low CYP450 or peroxidases. They propose that OTA increases the permeability of the cell to Ca^{2+} and the presence of the pro-oxidant OTA uncouples oxidative phosphorylation resulting in the increased leakage of electrons from the respiratory chain producing $O_2^{\bullet -}$ and free Fe^{2+}. Thus, perturbation of

Ca^{2+} homeostasis by OTA results in enhanced ROS production in a manner analogous to those proposed with other pro-oxidants, such as *t*-butyl hydroperoxide and does not involve enzymatic bioactivation of OTA [189,190]. Interestingly, this non-enzymatic pathway for ROS production by OTA is expected to liberate free Fe^{2+}, which, as discussed [30], may play an important role in reductive dehalogenation of OTA to yield the nonchlorinated OTB metabolite.

The method of metabolite extraction is also of critical importance. As a case in point, Zepnick *et al.* [191] did not find any OTA metabolites aside from OTα in tissue and urine of rat treated orally with a single OTA dose of 0.5 mg/kg body weight, while under the similar conditions, Castegnaro *et al.* [192] observed the excretion of OTA and 4-OH-OTA with the amount of 4-OH-OTA excretion related to the dose of OTA administered, as shown in Table 2. A reason for this discrepancy is exemplified by the HPLC data shown in Fig. 4 that were obtained from extraction of OTA and its metabolites from rat liver and kidney of male pigs [152] fed 18 days with OTA (18 μg/kg body weight/day). The HPLC trace in Fig. 4a was obtained following extraction using chloroform after acidification and treatment with $MgCl_2$ [152,156,192], while the HPLC profile shown in Fig. 4b was obtained following extraction based on ethanol protein precipitation, as utilized by Zepnick *et al.* [191]. With chloroform extraction (Fig. 4a), OTA and compounds corresponding to the elution time of 4(*R*)- and 4(*S*)-OH-OTA are found in the medulla and to lower extent in the cortex of pig, and in the liver of rats. Compounds with retention times similar to OTB, OTα, OTβ (OTB lacking the phenylalanine moiety), and OP-OA were also detected, as were compounds of unknown nature. In sharp contrast, ethanol extraction

Table 2. Results on OTA kinetics

Rat strain	OTA administered (mg/kg body weight)	OTA excreted after 24 h	4-OH-OTA excreted after 24 h
Lewis	0.5	585 ± 34	49 ± 7
	2.5	3210 ± 421	302 ± 124
	5.0	5379 ± 1735	642 ± 237
DA	0.5	1138 ± 158	66 ± 29
	2.5	7173 ± 300	339 ± 98
	5.0	11,981 ± 2069	570 ± 135

Source: Data extracted from Castegnaro *et al.* [192] with permission.

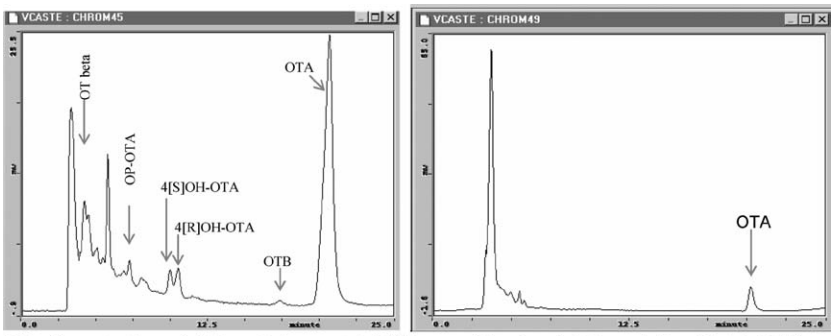


Fig. 4. Comparison of two methods of extraction of OTA metabolites: (a) $HCl/MgCl_2/CCl_3$ extraction, (b) EtOH precipitation.

(Fig. 4b) only yields OTA. This suggests that the OTA obtained by ethanol extraction is free OTA (that from spiking), which is not protein-bound and is thus easily extracted. To extract protein-bound OTA and its metabolites, acidic conditions and $MgCl_2$ is required to remove OTA from protein. It is well known that OTA binds strongly to proteins [193–195] with recent studies showing that OTA binding to human serum albumin (HAS) occurs preferentially within domain IIA ($K_b = 5.2 \times 10^6$/M); this binding lowers the pK_a of the phenolic group, thereby facilitating the formation of an ion pair between the phenoxide group and protonated R257 [16,196–198]. Studies *in vivo* reveal that the lifetime of OTA in living systems is dependent on the presence of HSA (which increases the lifetime of OTA) and that this binding is species- and sex-dependent [199,200].

The studies dealing with the biotransformation of OTA, a chlorinated compound, show that it is a complex system and involves several enzymes such as P450s, but also GSTs and LOX. The metabolites conjugated to GSH and/or uridine diphosphate (UDP) are excreted in bile and in kidney. At least 20 different metabolites of OTA including OTB, OTC, OH-OTA, OP-OTA, OTα, OTβ, and OTHQ, and metabolites of unknown structure have been detected. On the basis of all the available data, known and hypothetical pathways for OTA metabolism are summarized in Scheme 8.

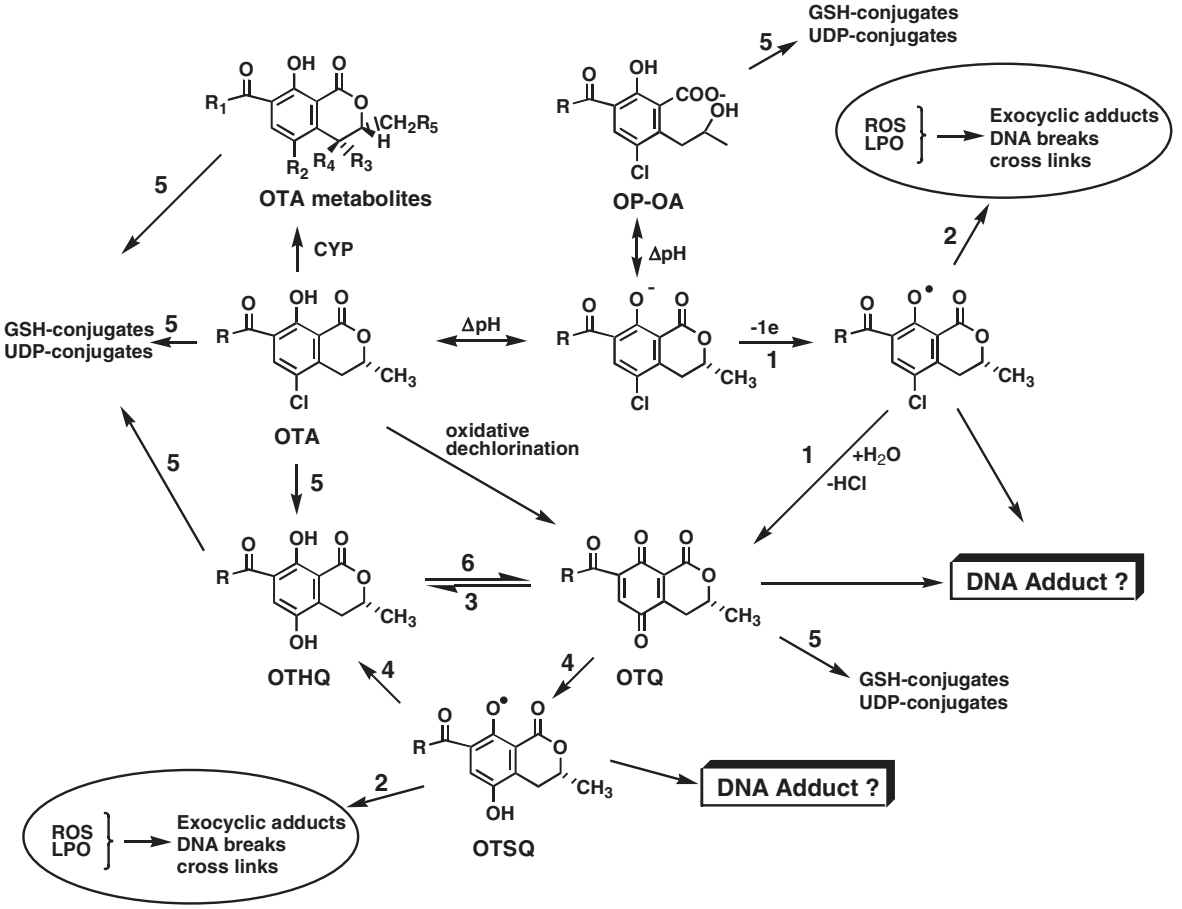
One of the important enzymes in the genotoxicity of OTA is leukotriene C4 synthase (LTC4). This enzyme is a member of the group of non-heme Fe-containing enzymes capable of oxidizing GSH to GSSG and simultaneously generating $O_2^{\bullet -}$, which may contribute to

oxidative stress in cells, but also participate in GSH conjugation of xenobiotics (pathway 5). In general, GSTs are involved in detoxifying pathways, but in some cases they contribute to the reactivity and toxicity of xenobiotics, notably by the formation of thiyl radicals, which react with macromolecules and yield peroxyl radicals (pathway 2). Conversion of OTA into the quinone OTQ by redox cycling generates ROS that can lead to DNA breaks and lipid peroxidation (LPO)-derived exocyclic adducts (pathway 2). OTQ can either undergo a two-electron reduction by action of the NAD(P)H:quinone reductase to form OTHQ (pathway 3), or it can undergo a one-electron reduction to yield a semiquinone (pathway 4), which in turn could induce DNA breaks, LPO, and exocyclic adducts (pathway 2). OTA was shown to induce oxidative damage due to the generation of hydroxyl radicals ($HO^{\bullet}$) by microsomes in the presence of NADPH as a microsomal reductant and O_2 not requiring exogenous Fe (pathway 2). Pathway 2 is thus inhibited by ROS scavengers, such as MESNA and NAC and explains OTA-induced karyomegalies, which are also reduced after MESNA treatment. OTHQ could be formed directly from OTA by GST (pathway 5) and it can be oxidized into OTQ (pathway 6). Indeed, GSTs are involved in dehalogenation: the first step is the formation of an epoxide and in the second step the epoxide is converted into phenol, which can lead to OTHQ and/or OTB.

3.4. DNA damage by ochratoxin A

OTA has long been considered non-genotoxic, as various standard tests using prokaryotes are generally negative or very slightly positive. Ames tests carried out in different strains of *Salmonella typhimurium* (TA 98, 102) were negative [201,202]. No induction of growth inhibition of various strains of *Bacillus subtilis* occurs after OTA treatment [203] and no recombination was observed in OTA-treated *Saccharomyces cerevisiae* D3 [204,205]. At doses of 5 and 10 μg/mL, OTA did not induce 8-azaguanine-resistant mutation in mammary cells of C3H mice [206]. In the absence of metabolic activation, no mutations were detected in cells from FM3A mice exposed to OTA [206].

However, more recent data shows that OTA induces a potent mutagenic effect in *S. typhimurium* TA 1535, 1538, and 100 exposed to supernatant of rat hepatocytes pre-treated with OTA [207] and also in the presence of kidney microsomes in *S. typhimurium* TA 1535, 1538 and 98 [208]. Mutagenic potency has been demonstrated in other



Scheme 8. Proposed pathways for biotransformation reactions of OTA and putative structures of reactive intermediates. Structures of metabolites include: OTA, Ochratoxin A; OTB, dechlorinated OTA; OTC, ethylester OTA; 4*R*-, *S*-, OH-OTA, hydroxylated OH in position 4; OP-OTA, open lactone; OTHQ, hydroquinone OTA; OTQ, quinone OTA. Abbreviations: ROS, reactive oxygen species; LPO, lipoperoxidation; GSH, glutathione; UDP, uridine diphosphate [238].

mammalian cells [176]. OTA-induced unscheduled DNA synthesis has been noted in rat [209,210] and mouse hepatocytes [209], in urinary bladder epithelial cells from pig and in primary human urothelial cells [211]. The effect of OTA on DNA repair and induction of DNA damage was further investigated by detecting the occurrence of DNA damage by the comet assay [212]. In MDCK cells, OTA induced single-strand breaks in a concentration-dependent manner. When an external metabolizing system (S9-mix from rat liver) was added, the genotoxic effect was significantly stronger, thus implying a role for metabolism [212]. In the same study, it was demonstrated that OTA-induced DNA damage is increased by blocking repair mechanisms. OTA also slightly increased the response in the SOS–spot test [213]. In the absence of metabolic activation, 4 μM OTA induced SOS repair in *Escherichia coli* PQ37 [214,215]. OTA also induces chromosomal mitotic recombination in *Drosophila* [216]. A small but dose-dependent increase in sister chromatid exchanges were induced in CHO cells treated with OTA [217,218]. Sister chromatid exchanges were also induced in human lymphocytes in the presence of hepatocytes [207] and in cultured isolated porcine urinary bladder epithelial cells treated with OTA [219]. In another study, a statistical increase of structural chromosomal aberration and sister chromatid exchanges associated with a decrease in the mitotic index has been observed in bovine lymphocytes [220]. OTA induced micronuclei in ovine seminal vesicle cell cultures [221,222], in Syrian hamster fibroblast [223], and in human hepatic (HepG2) cells [224]; all in a dose-dependent manner. Significant dose-dependent increases in the frequency of DNA single-strand breaks and alkali-labile sites, as measured by the comet assay, and in micronuclei frequency, were obtained in primary kidney cells from both male rats and humans of both genders with OTA from 0.015 to 1.215 μM [225]. Single-strand DNA breaks have been observed in kidney, liver, and spleen DNA of BALB/c mice treated orally with OTA [226,227]. Manolova *et al.* [228] demonstrated the presence of chromosomal aberrations on chromosome X from human lymphocytes cultivated in the presence of OTA. Similar aberrations have been detected in BEN patients [229].

On the basis of structure–activity relationships, Malaveille and coworkers [214,215] proposed that the presence of the C-5 chlorine atom in OTA is one determinant of its genotoxic action. Xiao *et al.* [97,216] also demonstrated that the phenylalanine moiety and the *para*-chlorophenolic group are important for *in vitro* and *in vivo* toxicity by OTA. In

contrast to the proposal by Rahimtula that Fe-chelation may be linked to OTA-toxicity [186], Xiao and coworkers showed that an O-methylated phenolic derivative of OTA, that lacks metal chelation properties, was equally efficient in generating ROS production, suggesting that the toxicity of OTA is not linked to its chelation of Fe^{2+}. Xiao and coworkers [97,216] concluded that the phenylalanine group is important for genotoxicity and demonstrated that some OTA derivatives, notably OP-OTA (Scheme 7) bind macromolecules (protein and DNA).

The genotoxicity of OTA has also been established by the detection of OTA-mediated DNA adduct formation. It has been shown that rats and mice treated with OTA show numerous DNA adducts [26]. The highest levels have been found in kidney DNA where some adducts persist for more than 16 days, while adducts are repaired in liver and spleen after 5 days [27,230]. Similar adduct patterns have been found in kidney and bladder tumors of BEN patients [231]. Single doses of OTA administered to mice [27,230,232] and rat by gavages or in feed [233] induced DNA adducts in kidney in a dose- and time-dependent manner. In cell culture (OK cells or in human bronchial cells), and after *in vitro* incubation in presence of pig and rat kidney microsomes, dose- and time-dependent DNA adducts were also observed [171,234]. Petkova-Bocharova *et al.* [232] demonstrated that administration of OTA to pregnant mothers at day 9 of gestation induced DNA adduction in fetuses and pups that were detectable several months after birth. Moreover, some of them developed renal carcinoma 2 years later (unpublished results).

In order to elucidate the metabolic pathway leading to adduct formation, a series of *in vitro* experiments have been undertaken. Only kidney microsomes were capable of inducing adduct formation [234]. OTA alone does not yield DNA adducts by interacting directly with DNA and no adducts can be formed by *in vitro* incubations using hepatic microsomes [230]. This aspect has been confirmed by other researchers, notably Gross-Steinmeyer *et al.* [159] using ^{3}H-OTA did not find adducts in hepatocytes and Gautier *et al.* [82] found no evidence for OTA-mediated DNA adduction using liver microsomes for bioactivation. Because kidney is rich in peroxidases, the implication of an oxidative metabolic pathway was investigated. Administration of superoxide dismutase and catalase prior to OTA treatment inhibited DNA adduction in mice kidneys [234]. Protection from OTA-mediated genotoxicity by indomethacin and aspirin (inhibitors of COX and LOX enzymes) in the urinary bladder and kidney of mice was also observed

[235]. DNA adduction was also partially prevented by antioxidant vitamins, supporting the implication of a peroxidase pathway [236]. Induction of LOX by vitamin A increases the formation of the main OTA-DNA adduct in the kidney of mice treated by OTA [236]. The implication of the LOX pathways in OTA-mediated genotoxicity was also reinforced by the absence of OTA–DNA adducts when cells were pre-treated with NDGA and a higher dose of indomethacin (10 μM), which inhibits all the AA biotransformation pathways [156].

Microsomes from transgenic mice demonstrate that formation of OTA–DNA adducts are under control of several biotransformation enzymes such as 1B1, 2C9, LOX, and COX [237]. Moreover, induction of NADPH–quinone-reductase regulated by the AH receptor, decreases OTA genotoxicity, which reinforces the hypothesis for involvement of a quinone pathway in OTA-mediated genotoxicity (Scheme 8). In a carcinogenic study, we demonstrated that the susceptibility of male DA rat is due to CYP2C11, corresponding to human CYP2C9, which is able to metabolize DB [178]. Several other experiments implicate CYP2C. For example, BEAS-2B cells expressing specific human CYP isoforms demonstrate that CYPs 1A2, 3A4, 2D6, and 2C9 are responsible for the formation of several OTA–DNA adducts, whereas CYPs 2A6 and 2E1 decrease OTA genotoxicity [155,171,172].

Figure 5 shows typical ^{32}P-postlabeling results from the Pfohl-Leszkowicz laboratory that highlight covalent DNA adduction by OTA in animals, human tumors and following *in vitro* incubations. These experiments suggest strongly that OTA forms covalent DNA adducts *in vivo* and have been used to gain mechanistic insight into OTA-mediated genotoxicity. For example, MESNA modified the DNA adduct patterns in kidney of both male rat strains leading to a reduction in spot number and total adduct level, but did not prevent formation of all adduct spots [238]. After acivicin treatment, only one adduct persisted whose formation appeared to involve OTA biotransformation by LOX, as this adduct is the major adduct in OTA-treated cells when the LOX pathway is enhanced [155,156], and also in the kidney of mice pretreated by vitamin A, a known LOX inducer [172]. These results suggested different mechanisms for OTA-induced karyomegalies, renal carcinogenicity, and DNA adduction [238]. It is also highly relevant that the OTA–DNA adducts which persisted in kidney of OTA-treated rats had similar chromatographic properties with adducts found in renal tumors from Bulgarian patients suffering from BEN/UTT (urothelial tract

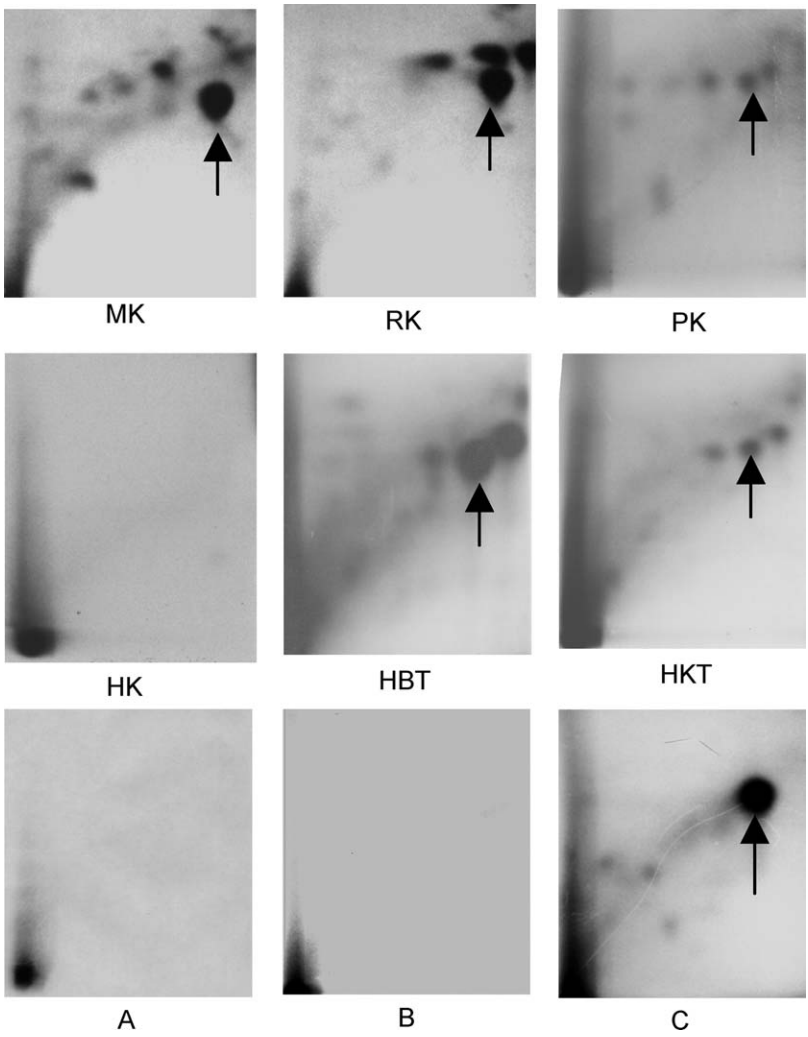


Fig. 5. ^{32}P-postlabeling analysis of OTA–DNA adducts in different animals fed OTA, in human tumors and after *in vitro* incubation: MK, mouse kidney (oral gavage; RK, rat kidney (oral gavage); PK, pig kidney (fed); HK, human kidney (accidental death); HBT, human bladder tumours; HKT, human kidney tumours. (A) DNA + OTA without microsomes; (B) DNA + microsomes without OTA; (C) DNA + OTA + human kidney microsomes.

tumour) [231] and in a French patient with kidney tumors [239]. These adducts were also detected in pigs, which had developed OTA-related nephropathy [152] and persisted in the kidney of OTA-treated mice and rats [27,230,234].

The Manderville laboratory [16,28,240] has recently used the photochemistry of OTA to isolate and characterize the deoxyguanosine (dG) OTA–DNA adduct standards shown in Fig. 6. This effort was prompted by the work carried out by Obrecht-Pflumio and Dirheimer [241,242], who demonstrated using ^{32}P-postlabeling that OTA reacts preferentially with dG. Figure 7 shows ^{32}P-postlabeling analysis of DNA extracted from the kidney of rat treated chronically with OTA and from pig following sub-acute exposure to the toxin with the adduct standards shown in Fig. 6 for comparison. The ^{32}P-postlabeling analysis show that both adducts comigrate with OTA–DNA adducts formed in the kidney of rats developing tumors [28]. The amount of adduct comigrating with the carbon-bonded C-C8-dG-OTA adduct was ~16 adducts per 10^9 nucleotides; this adduct is also formed in the kidney of pigs fed OTA [16,28]. These data suggest that OTA undergoes bioactivation to selectively attach to the C8-site of dG *in vivo* to form the covalent adducts depicted in Fig. 6; a likely intermediate is the phenoxyl radical (Scheme 8), as phenoxyl radicals are known to form adducts at the C8-site of dG [83,84].

Despite evidence for the direct genotoxicity of OTA [26–28, 150–152,157,158], other researchers have been unable to detect direct DNA damage by OTA [82,159,165] and favor an indirect mechanism for OTA-mediated carcinogenicity that involves oxidative stress and OTA-mediated cytotoxicity [82]. A recent perspective by Turesky [29] summarizes the argument against direct genotoxicity by OTA.

One reason that an interlaboratory analysis of DNA samples from OTA-treated rats has failed to reach a consensus regarding the nature of spots in OTA-mediated DNA adduction (Figs. 5 and 7) is due to

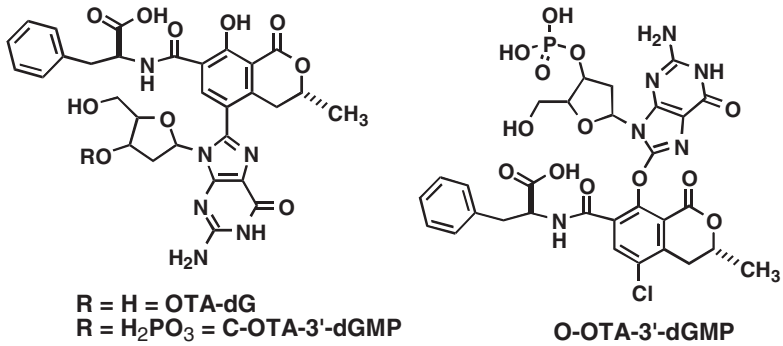


Fig. 6. Chemical structures of authentic OTA–DNA adduct standards.

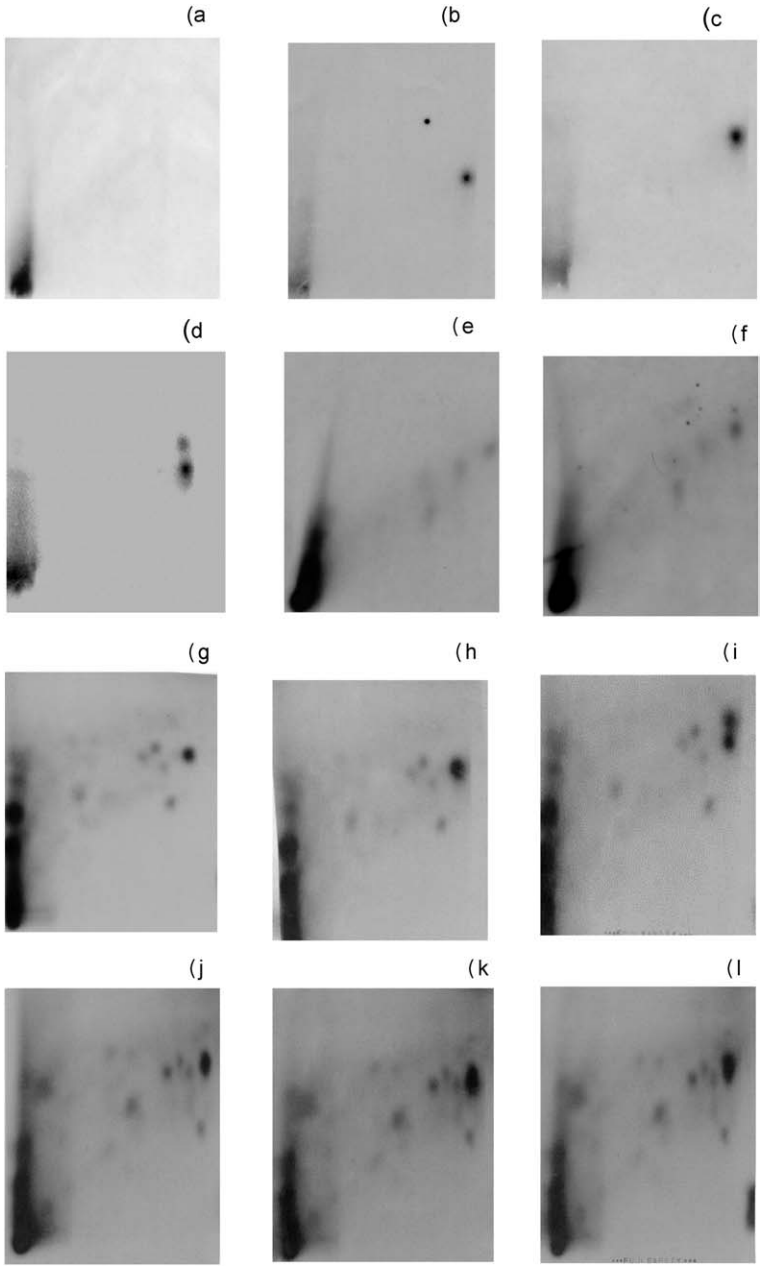


Fig. 7. ^{32}P-postlabeling analysis of DNA extracted from kidney of rat treated chronically with OTA and from kidney of pig following sub-acute feeding to OTA. (a) Control DNA, (b) control DNA + C-C8 OTA-3′dGMP OTA standard, (c) control DNA, (d) coelution C-C8 OTA-3′dGMP OTA standard + O-C8 OTA-3′dGMP OTA standard, (e) pig kidney DNA, (f) coelution pig kidney + C-C8 OTA-3′dGMP OTA standard, (g) DA rat kidney animal 1, (h) coelution DA rat kidney animal 1 + C-C8 OTA-3′dGMP OTA standard, (i) coelution DA rat kidney animal 1 + O-C8 OTA-3′dGMP OTA standard, (j) DA rat kidney animal 2, (k) coelution DA rat kidney animal 2 + C-C8 OTA-3′dGMP OTA standard, (l) coelution DA rat kidney animal 2 + O-C8 OTA-3′dGMP OTA standard (for details see Ref. [28]).

differences in methodology. For example, for ^{32}P-postlabeling analysis, Mally and co-workers [165] used phosphodiesterase (SPD) in a ratio of 1 mU/μg DNA, which will lead to incomplete DNA hydrolysis. Indeed, the activity of Calbiochem SPD is such that ~10–15 times more should have been added for complete hydrolysis of the OTA-treated DNA. The authors also used Nucleobond columns for DNA clean-up, which leads to DNA of variable quality with contamination by protein and RNA; DNA of high purity is critical for reliable DNA adduction results [243–245]. The chromatographic pH conditions for ^{32}P-postlabeling analyses, with D1 at pH 6.8 and D3 at pH 3.5, also do not conform to standard published procedures, where D1 is run at pH below 6.0 and D3 is run at ~pH 6.4. For generation of OTA–DNA adduct standards (Fig. 6), the photoreaction was carried out with OTA at 500 mM and dG at 20 mM [165]; concentrations that are exceedingly high. It also appears that no purification of the photoreaction was carried out and so the solution would be a mixture of C-C8 and O-C8 adduct standards (Fig. 6). In the work described by Gautier *et al.* [82], Qiagen tip-2500 columns were used to isolate DNA and commercial plates from Macherey Nagel were used to separate adducts using conditions described by us, but solvent concentrations were not adjusted to the specific plates, probably due to lack of reference material for proper control. In fact, we have shown that migration of OTA–DNA adducts on MN plates is completely different from that on home-made plates (manuscript in preparation). As specified earlier [243], the quality of the plates is vital for the analysis of OTA–DNA adducts by the sensitive ^{32}P-postlabeling technique.

3.5. Conclusions and future research

The mycotoxin and food contaminant OTA is one of the most potent renal carcinogens studied by NCI (National Cancer Institute) and NTP to date. In general, only weak genotoxic effects have been observed and the contribution of genotoxicity to renal tumor formation by OTA remains unknown. However, OTA-mediated DNA adduction has been implied using the ^{32}P-postlabeling technique and given that a strong correlation exists between DNA adduction and tumorigenesis, the direct genotoxicity of OTA cannot, at present, be ruled out as a viable mechanism for OTA-mediated carcinogenesis. The current model for direct genotoxicity by OTA requires oxidative bioactivation for covalent reactions with DNA. The oxidative chemistries of OTA are related to the CPs discussed in this chapter with phenoxyl radicals and quinone/

semi-quinone intermediates being generated through bioactivation by CYP450 and enzymes with peroxidase activities. While the ^{32}P-postlabeling results presented in Fig. 7 suggest the biological significance of OTA–DNA adducts (Fig. 6), the data is not proof of OTA–DNA adduct formation, as the ^{32}P-postlabeling technique does not provide structural evidence. LC/ESI–MS/MS data for OTA–DNA adducts in kidney of rat would provide unambiguous evidence for OTA-mediated DNA adduction and efforts are being made to identify OTA–DNA adducts using MS. The identification of OTHQ as a metabolite of OTA also warrants the investigation of the role played by OTQ/OTHQ in OTA-mediated genotoxicity given that the corresponding hydroquinone/quinone redox couple of PCP is proposed to play a key role in PCP-mediated carcinogenicity.

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CHAPTER 5

Idiosyncratic Drug Reactions: Clinical Evidence for Mechanistic Hypotheses

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Contents

1. BACKGROUND

1.1. Definition

The use of the term idiosyncratic drug reaction (IDR) is quite inconsistent. The term idiosyncratic means specific to an individual. The term will be further restricted in this review to exclude reactions that involve known pharmacologic actions of the drug. This excludes adverse reactions such as torsade de pointes, because this adverse reaction involves the effect of

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a drug on potassium channels, and therefore at least in principle it is predictable. However, torsade de pointes is certainly specific to an individual, and so in another context it could quite reasonably be termed idiosyncratic. When allergists/clinical immunologists use the term IDR, it usually excludes any immune-mediated reaction. In contrast, in this review it will be used to describe reactions that I suspect are mostly immune-mediated. Other terms that have been used almost interchangeably with IDRs are hypersensitivity reactions, type II and type B reactions.

1.2. Characteristics of IDRs

The major characteristic of IDRs is that they are unpredictable and do not occur in most people at any reasonable dose of the drug; however, there is no absolute incidence cutoff, and under the right conditions or in a specific population a specific IDR might occur in more than 50% of patients. It would be better to define adverse reactions by their mechanism, but because the mechanism of most IDRs is unknown we are left with using this characteristic. IDRs can affect virtually any organ and can mimic many other diseases making it very difficult to determine whether or not a given adverse event was due to a drug.

A major characteristic of IDRs that is often claimed is that IDRs are dose-independent. In fact, no effect of a drug is independent of dose! As indicated above, an IDR cannot be induced in most patients simply by increasing the dose, and it does not make sense to talk about a dose–response curve in a population where the response does not occur at any dose. Often the incidence does not vary within the narrow dose range used clinically, and an IDR may occur at subtherapeutic doses, especially in a sensitized patient. There is no reason that the dose–response curve for an IDR should be in the same range as the dose–response curve for the therapeutic effect. In fact, the incidences of many IDRs such as hydralazine-induced lupus are clearly dose-dependent within the therapeutic range [1]. In a susceptible patient, there will be a dose–response relationship, and it is axiomatic that a dose can always be found that will not cause an IDR in anyone. A good example is that one way to deal with a patient who is allergic to penicillin is to administer a small dose (about one ten thousandth of a therapeutic dose) and then administer gradually increasing doses until tolerance is induced [2]. Even at one ten thousandth of the usual dose there are more than 10^{16} drug molecules, so there is a lot of room to

decrease the dose. I believe it is important to avoid characterizing IDRs as dose-independent, because this is often taken literally and it can be quite misleading. For example, if a very potent drug can be designed it is less likely to cause IDRs as will be discussed again later.

Another important characteristic of IDRs is that there is almost always a delay between starting a drug and the onset of the adverse reaction, unless the patient has been previously exposed to the drug. The length of the delay varies with the type of IDR but is reasonably consistent for a specific drug and type of IDR. It is commonly about 1–2 weeks for IgE-mediated reactions and most other types of skin rash. It is usually 1–3 months for agranulocytosis [3] or liver toxicity, although some types of liver toxicity are associated with a longer delay [4]. For drug-induced lupus, the delay is usually several months, and it is not uncommon for it to occur after more than a year of treatment [5]. It is typical for the delay to be greatly reduced in a patient who has had an IDR to a drug if the patient is rechallenged with that drug, but this is not always the case. In two animal models of drug-induced autoimmunity that we have used, penicillamine-induced autoimmunity in the Brown Norway rat [6] and propylthiouracil-induced autoimmunity in the cat [7], the time to disease onset on rechallenge is the same as it was on initial exposure. This may be related to the fact that these diseases are autoimmune in nature and, therefore, if autoreactive T cells were not deleted or made anergic, the adverse reaction would continue after the drug was discontinued and this usually does not occur. A clinical example where a clearly immune-mediated reaction does not recur rapidly on rechallenge is heparin-induced thrombocytopenia (see Section 2.1.4.2).

1.3. Significance

IDRs represent a major clinical problem and are a significant cause of hospital admissions [8,9]. They not only cause suffering and sometimes death for the patients who have these adverse reactions, but also markedly increase the uncertainty of drug development. In the period from 1975 to 2000, over 10% of the drugs approved by the FDA either had to be withdrawn or were given a ‘‘black box’’ warning because of IDRs not predicted by preclinical testing and clinical trials [10]. If it were not for the potential that a drug candidate might cause IDRs, much of the current testing could be eliminated and the process of development accelerated; yet, the testing that is currently performed is ineffective at

predicting whether a drug candidate will pose an undo risk of IDRs. In order to better predict which drug candidates will cause a high risk of IDRs and which patients are at increased risk, we need a better mechanistic understanding; this will be the emphasis of this review.

2. POSTULATED MECHANISMS

Although hypotheses abound, it must be said that, with few exceptions, the mechanisms of IDRs are unknown. Two major hypotheses are that the majority of IDRs are caused by reactive metabolites [11] and are immune-mediated [12]. Although current opinion favors these two hypotheses, in the absence of definitive supportive evidence and with some evidence to the contrary, they are often hotly debated. By their very nature, IDRs are difficult to study, and in particular there are very few animal models [13]. Therefore, it is very difficult to definitively test these hypotheses. In the absence of sufficient animal models, for the time being, we are left with inferring mechanism from clinical data. The question of whether IDRs are immune-mediated and are caused by reactive metabolites are fundamental; therefore, while describing IDRs, this review will focus on the evidence relevant to these two hypotheses.

2.1. Are IDRs immune-mediated?

2.1.1. Drug-induced autoimmunity

It is reasonable to assume that autoimmune reactions, such as lupus and myasthenia gravis, are immune-mediated. There is a significant autoimmune component to several other IDRs, although the presence of autoantibodies does not prove that the IDR is immune-mediated unless it can be demonstrated that these autoantibodies mediate the adverse reaction. Autoimmune reactions involving the skin and blood cells will be discussed in the sections involving skin and blood cells, respectively.

2.1.1.1. Drug-induced lupus

Lupus is the classic generalized autoimmune disease. Most lupus is idiopathic, i.e. its cause is unknown. There are various animal models that spontaneously develop lupus and provide clues to possible mechanisms. In several models, there is defective apoptosis, which presumably

interferes with the elimination of specific T cells. Another hypothesis is that there is cross-reactivity between a pathogen and self-antigens so that an immune response against the pathogen leads to autoimmunity [14]. In fact, pathogens could use such molecular mimicry to try to "hide" from the immune system.

Lupus can affect essentially all parts of the body, but involvement of the central nervous system and kidneys are most likely to cause death [15]. It is characterized by a variety of autoantibodies, but the most characteristic autoantibodies are antinuclear antibodies that bind to various components of the nucleus, including DNA and histone protein. Antinuclear antibodies can form immune complexes that are trapped in the kidney, bind complement and induce an immune response leading to kidney damage. However, the mechanism by which an autoimmune response leads to damage of other organs is poorly understood.

Although most lupus is idiopathic, about 10% is due to drugs [15]. Drug-induced lupus is less serious than idiopathic lupus because central nervous system and kidney involvement are uncommon. Discontinuation of the drug usually leads to prompt resolution of symptoms, even though antinuclear antibodies often persist for years albeit at steadily decreasing concentrations [5]. The autoantibodies in drug-induced lupus are often antihistone antibodies [16], while those in idiopathic lupus are more commonly antidouble-stranded DNA antibodies; however, there is significant overlap between idiopathic and drug-induced lupus such that they cannot be readily distinguished except for the history of exposure to a drug that is known to cause lupus and resolution of symptoms when the drug is stopped.

There are several theories for the mechanism by which drugs can induce lupus, including: inhibition of DNA methylation [17], activation of antigen-presenting cells [18], interference with immune tolerance [19] and molecular mimicry [20]; however, conclusive evidence for any one hypothesis is lacking and it is likely that the mechanism is different for different drugs.

2.1.1.2. Drug-induced myasthenia gravis

Myasthenia gravis is an autoimmune disease characterized by antibodies against the acetylcholine receptor at the neuromuscular junction leading to muscle weakness. It is treated with drugs that inhibit the hydrolysis of acetylcholine by cholinesterase resulting in an increased amount of acetylcholine and providing increased stimuli to the inhibited

receptors. As with lupus, most myasthenia gravis is idiopathic, but it can also be caused by drugs. Penicillamine, in particular, can lead to a variety of autoimmune reactions including myasthenia gravis; however, the mechanism is unknown [21].

2.1.2. Involvement of the immune system in idiosyncratic liver toxicity

2.1.2.1. Hepatic necrosis

Although there are several patterns of drug-induced liver injury, hepatocellular injury leading to hepatic necrosis is the type most commonly associated with liver failure leading to either death or liver transplantation; therefore, this will be the focus of most of the discussion. Idiosyncratic hepatic necrosis is the most common cause of drug withdrawal, so it is of particular interest to the pharmaceutical industry.

2.1.2.1.1. Apparent immune idiosyncrasy. Halothane-induced hepatotoxicity is associated with various drug-induced antibodies, which suggests that it is immune-mediated [22]. Some of the antibodies are directed against trifluoroacetylated protein generated by the reactive metabolite of halothane and some are autoantibodies [23]. However, it would be very difficult to prove that these antibodies mediate liver damage; therefore, their presence does not prove that halothane-induced hepatitis is immune-mediated. In fact, if it is immune-mediated, given the nature of the reactive metabolite (see Section 2.2.3), it is just as likely that T cells mediate most of the liver damage and the antibodies are an epiphenomenon. But the overall picture strongly suggests that halothane-induced hepatotoxicity is immune-mediated. Specifically, the idiosyncratic nature of the IDR is more readily explained by differences in immune response than by any other known mechanism. In addition, the toxicity almost never occurs on first exposure, which strongly suggests that immune-sensitization is involved [4]. Overt toxicity with jaundice is usually preceded by fever, and it is often noted that a previous exposure was also associated with fever even though no overt clinical toxicity was observed; this provides further evidence of sensitization during the earlier exposure. Furthermore, there is a reasonable correlation between the presence of antibodies and hepatotoxicity, so even if the antibodies do not mediate the toxicity they represent a marker of immune-activation by halothane in the patients who develop toxicity. Although the cellular infiltrate is usually less than seen with viral hepatitis, there is an inflammatory component to halothane hepatitis and

eosinophils are sometimes observed. Despite this evidence, involvement of the immune system in halothane-induced hepatitis has been questioned [24].

Tienilic acid-induced liver failure is similar to that of halothane in that it is associated with drug-induced antibodies. It this case the antibodies are directed against Cyp 2C9, the enzyme that forms the reactive metabolite [25]. However, as with halothane, there is no direct evidence that these antibodies mediate the hepatotoxicity of tienilic acid. Another feature of tienilic acid hepatotoxicity that suggests that it is immune-mediated is that the rechallenge leads to a rapid recurrence [26]. Likewise, the hepatitis associated with dihydralazine is associated with antibodies against Cyp 1A2 [23]. Because of other immune-manifestations, the hepatotoxicity associated with sulfonamides, phenytoin, and methyldopa is also usually assumed to be immune-mediated [4]; however, conclusive evidence is lacking.

2.1.2.1.2. Apparent metabolic idiosyncrasy. Idiosyncratic hepatotoxicity associated with many drugs does not have characteristics that suggest involvement of the immune system. Classic signs of an immune-mediated reaction include fever, rash, and eosinophilia; however, their absence is not strong evidence against an immune mechanism and such manifestations are often absent in clearly immune-mediated reactions. Another characteristic of immune-mediated reactions is a very rapid onset on rechallenge, presumably due to the presence of memory T cells. This is observed with most IDRs that are believed to be immune-mediated; however, rechallenge of patients with a history of isoniazid- or troglitazone-induced hepatotoxicity usually does not lead to a rapid onset of toxicity and may not cause hepatotoxicity at all [27]. There is precedent for such behavior in clearly immune-mediated reactions as discussed in Sections 1.2 and 2.1.4.2. The time-to-onset on initial exposure for these drugs also tends to be longer than with drugs that cause hepatotoxicity with more features that suggest an immune-mediated reaction; troglitazone-induced hepatotoxicity has been reported to occur after more than a year of treatment [28]. However, this is also a characteristic of drug-induced autoimmunity. The hepatic IDRs associated with pyrazinamide and ketoconazole also fit in this category. Zimmerman [4] referred to such hepatic IDRs as metabolic idiosyncrasy. Although this is an interesting concept, there are no examples in which differences in a specific metabolic pathway have been shown to account for why some patients have an IDR to a drug and most do not. For example, although some controversy still exists, it appears that the slow acetylator

genotype is associated with an increased risk of isoniazid-induced hepatotoxicity [29]. However, in North America, about 50% of the population are slow acetylators, therefore, this cannot explain the idiosyncratic nature of isoniazid-induced hepatotoxicity. It is certainly possible that some combination of several different polymorphisms in drug metabolism/reactive metabolite detoxication could explain the idiosyncratic nature of IDRs. It also seems probable that polymorphisms in some other biochemical pathways could be involved, but there are virtually no data to support this hypothesis. Although it is likely that a reactive metabolite is involved as discussed below (Section 2.2.5.4), without evidence of a polymorphism that can explain their idiosyncratic nature, the basis for the idiosyncratic nature of the hepatotoxicity of drugs such as isoniazid is simply unknown and an immune mechanism cannot be ruled out.

2.1.2.1.3. Mitochondrial toxicity. The characteristics of hepatotoxicity associated with valproic acid are quite different from those of most other drugs associated with causing hepatocellular damage. Instead of children being resistant to valproic acid toxicity, as they are with most other drugs associated with hepatic IDRs, children are at significantly higher risk [4]. Although valproic acid ultimately leads to necrosis, the early histological picture is usually (although not always) one of microvesicular steatosis. This suggests that the drug interferes with mitochondrial β-oxidation of fatty acids [30], and its structure, a branched-chain aliphatic carboxylic acid, supports this hypothesis. The early appearance of hyperammonemia also suggests impairment of mitochondrial function. The toxicity associated with valproic acid is similar to salicylate-induced hepatotoxicity (also a carboxylic acid) as well as Jamaican vomiting sickness, which is due to a toxic metabolite of hypoglycin A, specifically, 2-methylenecyclopropylpropionic acid [31]. Valproic acid is metabolized to 4-en-valproic acid, which is similar in structure and toxicity to hypoglycin A [32]. Thus, valproic acid hepatotoxicity truly does appear to represent metabolic idiosyncrasy and Zimmerman also classed this IDR as such. However, it is not clear what metabolic polymorphism(s) is/are responsible for the idiosyncratic nature and what is responsible for the delay between starting the drug and the onset of toxicity. In particular, the incidence of valproate-induced hepatotoxicity peaks from 2 to 4 months after the start of therapy and declines rapidly after that. Although there is strong evidence that mitochondrial toxicity is important in the mechanism of valproic acid hepatotoxicity, it is possible that the immune system also plays a role.

Amiodarone and perhexiline are cationic amphiphilic drugs that are concentrated in the mitochondria and cause idiosyncratic steatotic hepatitis whose toxicity also likely involves their inhibition of mitochondrial function [30]. There are other examples, such as zidovudine, in which hepatotoxicity appears to involve damage to the mitochondrial DNA [33].

2.1.2.1.4. The ''inflammagen'' hypothesis for hepatic IDRs. Another hypothesis that has been suggested to explain the idiosyncratic nature and delay in the onset of hepatic IDRs is the chance exposure to some ''inflammagen,'' which potentiates the intrinsic hepatotoxicity of the drug [34]. This hypothesis is based on the finding that the combination of an agent, e.g. ranitidine, with lipopolysaccharide caused immediate hepatotoxicity in rats where the same dose of the agent alone did not. To date, this has not been demonstrated with any drugs commonly associated with idiosyncratic liver failure in humans. Furthermore, potentiation by an inflammagen is also consistent with an immune mechanism as discussed later (Section 2.2.2). This hypothesis does not explain why IDRs almost never occur immediately on first exposure even though some drugs, such as antibiotics and antiinflammatory drugs, are usually administered in the context of an inflammatory environment. This hypothesis also does not explain the rapid onset of toxicity on rechallenge that is an important feature for some drugs, such as halothane and tienilic acid. However, it is likely that there are many mechanisms of IDRs and this may be one of them.

2.1.2.2. Cholestatic liver injury

The other common pattern of liver injury induced by drugs is cholestatic. It is usually claimed that it rarely results in liver failure [4]. However, a recent paper suggests that cholestatic injury is associated with almost the same risk of liver failure as hepatocellular injury [35]. Although some drugs can cause either types of injuries and individual cases of drug-induced liver injury can have both hepatocellular and cholestatic components, most drugs can usually be classified as predominantly causing either a hepatocellular or a cholestatic pattern. A classic drug associated with causing cholestatic liver injury is chlorpromazine. Chlorpromazine-induced cholestasis is usually preceded by fever and constitutional symptoms and accompanied by eosinophilia [4]. These are classic signs of an immune reaction; therefore, it is assumed that this is an immune-mediated IDR. However, it is

not known how chlorpromazine initiates an immune response, or what branch of the immune system mediates the damage.

Another drug commonly associated with cholestatic injury is erythromycin, especially the estolate salt. It usually occurs after 1–3 weeks of treatment and is commonly associated with fever and eosinophilia, thus leading to the assumption that it is immune-mediated [4]; however, as with chlorpromazine, there is little basic understanding of the mechanism. A more recent drug associated with a cholestatic or mixed picture of liver injury is terbinafine [36].

2.1.3. Immune-involvement in dermatological IDRs

Although usually not serious, skin rashes are said to be the most common type of IDR. It is possible that liver injury is actually more common, but because we cannot see the liver and the transaminases are not measured daily; liver injury is not noticed unless it is severe or it is detected accidentally by blood tests. The skin is a major barrier to the outside world and it is very immunologically active. In fact, dermal or subcutaneous administration of a drug more often leads to an immune response where oral administration often leads to immune tolerance [37]. There are many types of drug-induced skin rashes and only a few will be highlighted as examples. There are reviews that provide excellent descriptions of these reactions [38,39].

2.1.3.1. IgE-mediated urticaria

Classic urticaria (hives) is mediated by IgE antibodies that bind to mast cells/basophils and, when cross-linked by antigen, lead to the release of mediators such as histamine and leukotrienes that mediate the symptoms associated with such reactions [40]. Depending on the location and extent of mediator release, it can lead to hives or a more generalized reaction called anaphylaxis, which is characterized by hypotension and airway constriction. However, not all urticaria is immune-mediated, or more specifically, not mediated by IgE antibodies. In fact, factors such as heat, cold, and exercise can induce urticaria, and it is hard to imagine an antibody against exercise. It is clear that true anaphylaxis and IgE-mediated urticaria are immune-mediated, because it can be demonstrated that IgE antibodies mediate the IDR. There are other idiosyncratic reactions to drugs, such as X-ray contrast material, that involve a direct pharmacologically mediated degranulation of mast

cells and are not immune-mediated [41]; however, X-ray contrast material can also cause true allergic reactions [42]. Nonsteroidal antiinflammatory drugs can also cause an anaphylactoid reaction by shifting the metabolism of arachidonic acid from the synthesis of prostaglandins toward the synthesis of leukotrienes, and it is the leukotrienes that appear to mediate the reaction [43].

2.1.3.2. Morbilliform rashes

Morbilliform (measles-like, also known as maculopapular) rashes are very common, especially with the aminopenicillins (amoxicillin and ampicillin). The amoxicillin rash was often referred to as a ''toxic'' rash, because rechallenge with amoxicillin did not usually lead to a recurrence of rash [44]. More recently, there is evidence that at least some morbilliform rashes are mediated by $CD4^+$ T cells [45]. This would explain their mild nature because CD4 binds to MHC-II and only a minority of cells expresses MHC-II. However, if these rashes are mediated by the adaptive immune system, it does not explain why there is no memory. We now know that many people lose their allergy to penicillin with time [46] and this may explain the lack of memory. Amoxicillin is often given inappropriately to patients with viral infections, and it may be that a rash only occurs with the combination of drug and viral infection similar to the inflammagen hypothesis (Section 2.1.2.1.4). Many severe reactions start out looking like morbilliform rashes and evolve into much more severe reactions. Morbilliform rashes may have more than one pathogenic mechanism, and although immune involvement is likely in most cases, it has not been conclusively demonstrated.

2.1.3.3. Stevens–Johnson syndrome/toxic epidermal necrolysis

Stevens–Johnson syndrome is a more serious rash with a low but significant mortality rate [47]. It is characterized by mucus membrane involvement and systemic symptoms such as fever. Toxic epidermal necrolysis is even more severe and is associated with a 30% mortality rate. It is characterized by separation of the layers of skin at the dermal–epidermal junction. Although it has been a subject of controversy, it appears that Stevens–Johnson syndrome and toxic epidermal necrolysis are mechanistically very similar and the major difference is one of severity. There is evidence that these severe reactions are mediated by drug-specific $CD8^+$ T cells that kill keratinocytes by a perforin- and granzyme B-mediated mechanism [48].

Nevirapine, a non-nucleoside reverse transcriptase inhibitor used for the treatment of AIDS, is associated with a quite high incidence of rash, most of which is mild, but it is also associated with a significant incidence of Stevens–Johnson syndrome and toxic epidermal necrolysis [49]. The incidence is lower in patients with decreased CD4 lymphocyte counts [50], which suggests that $CD4^+$ T cells are involved in the pathogenesis. Abacavir, another drug used for the treatment of AIDS, is associated with a rash and generalized hypersensitivity reaction [51]. Although the initial reaction typically occurs after about 10 days, rechallenge results in a severe syndrome within hours. Patch testing is usually positive [52]. These characteristics strongly suggest that this is an immune-mediated reaction.

2.1.3.4. Drug-induced pemphigus

Pemphigus is an idiopathic autoimmune bullous skin disease in which antibodies against desmogleins (proteins that belong to the cadherin supergene family of adhesion molecules) lead to blister formation. Drugs can also cause autoantibody-mediated pemphigus and the antibodies have the same specificity as the idiopathic disease [53]. The drugs associated with the highest incidence of pemphigus, i.e. penicillamine and captopril, have sulfhydryl groups, but other nonsulfhydryl-containing drugs have also been reported to cause pemphigus. Drugs can also cause the related bullous skin diseases, pemphigoid and linear IgA bullous dermatosis [54,55]. This is another example of an immune-mediated IDR.

2.1.3.5. Acute generalized exanthematous pustulosis

Acute generalized exanthematous pustulosis (AGREP) is an uncommon condition characterized by intradermal, neutrophil-filled pustules, as well as fever and marked leukocytosis, sometimes including eosinophilia [56]. It is usually caused by aminopenicillins, sulfonamide antibiotics, and diltiazem. It is also characterized by the production of IL-8, which is a neutrophil-attracting chemokine [38]. Patch tests are often positive and the first cells to infiltrate the skin are IL-8-producing $CD4^+$ T cells.

2.1.3.6. Drug hypersensitivity syndrome

The term hypersensitivity is used in several different ways. It will be used here to refer to a syndrome composed of fever and rash, as well

as involvement of one or more internal organs. The most common organ involved is the liver, but involvement of other organs can lead to manifestations such as lymphadenopathy, leukopenia, nephritis, myositis, carditis, pneumonitis, thyroiditis, and aseptic meningitis [57]. Because it is not limited to the skin, its inclusion in this section is arbitrary. This reaction is most commonly seen with aromatic anticonvulsants and sulfonamide antibiotics. The clinical characteristics, especially the delay in onset on first exposure but rapid recurrence on rechallenge, suggest that it is immune-mediated [58]. Further evidence for immune-mediation is the finding of drug-specific T cells in patients with these reactions [59].

2.1.4. Immune-involvement in hematological reactions

Various blood cells are frequent targets of IDRs. This can occur either in the bone marrow involving the precursors of peripheral blood cells or it can involve destruction of mature blood cells.

2.1.4.1. Aplastic anemia

Aplastic anemia is due to the destruction of all blood cell precursors in the bone marrow. A bone marrow biopsy showing the replacement of blood cell precursors by fat cells is required for the diagnosis [60]. Most aplastic anemia is idiopathic, probably either autoimmune or caused by a virus, and aplastic anemia sometimes follows viral hepatitis or seronegative idiopathic hepatitis [61]. One treatment for aplastic anemia, which is effective in about two thirds of cases, is immunosuppression with antithymocyte antibodies and/or immunosuppressant drugs such as cyclosporin [62]. Response to this therapy suggests that aplastic anemia is immune-mediated. In addition, the bone marrows of patients with aplastic anemia contain activated lymphocytes that produce interferon-γ and tumor necrosis factor [61]. In fact, the production of interferon-γ in peripheral lymphocytes is a very good predictor of which patients with aplastic anemia will respond to immunosuppressive therapy [63]. The gene expression profiles of $CD4^+$ and $CD8^+$ T cells from the bone marrows of patients with aplastic anemia suggested that both the innate and adaptive immune system are involved in the immune response [64]. The response rate to immunosuppression appears to be the same whether the aplastic anemia is idiopathic or drug-induced, suggesting that drug-induced aplastic anemia is another immune-mediated IDR.

2.1.4.2. Thrombocytopenia

Probably, the most common drug associated with thrombocytopenia is heparin. Heparin binds to platelet factor 4 and the complex induces the production of antibodies. These antibodies can be against the heparin-platelet factor 4 complex or they can be true autoantibodies that recognize platelet factor 4 alone [65]. The complex binds to the FC receptor on platelets and this leads to platelet aggregation, release of more platelet factor 4, and destruction of platelets. In a study of 202 patients with heparin-induced thrombocytopenia, most occurred after 5–10 days of therapy; however, in some patients, especially those who had received heparin in the last 10 days, thrombocytopenia occurred less than 24 h after starting heparin treatment [66]. It was speculated that this is due to persistent antibodies, because the time course was faster than the usual amnestic immune response. In contrast, all patients who had not received heparin in the previous 100 days had a delayed onset of thrombocytopenia and it was also found that the titer of antibodies decreased rapidly with time. In addition, seven patients who had a previous episode of confirmed heparin-induced thrombocytopenia but did not currently have circulating antibodies were purposely rechallenged: none of them developed thrombocytopenia. Clearly, this is an immune-mediated IDR; thus, not all immune-mediated IDRs occur immediately on rechallenge, especially if there has been a significant period of time between the exposures. In the case of heparin-induced thrombocytopenia, the autoimmune nature of the reaction may prevent the persistence of memory T cells. Gold salts and procainamide can induce the formation of pure autoantibody-mediated thrombocytopenia [67].

An unusual form of thrombocytopenia is caused by quinine. It is due to a class of antibodies that bind to platelet membrane glycoproteins, usually the fibrinogen receptor or von Willebrand factor receptor, but only when quinine is present in soluble form [67]. When the platelets are washed to remove the quinine the antibody binding no longer occurs. It is not clear whether the quinine forms a complex with the glycoprotein or changes its conformation. Unlike heparin-induced thrombocytopenia, the sensitivity lasts for years and one dose is sufficient to cause thrombocytopenia in a sensitized patient.

An interesting example of drug-induced thrombocytopenia that violates the rule that there is always a delay between starting the drug and the onset of the IDR on first exposure is the antithrombotic drugs, e.g. tirofiban and eptifibatide, which work by binding to the glycoprotein IIb/IIIa complex and block the binding of fibrinogen. There appear to be natural

or preexisting antibodies that bind to the drug–glycoprotein complex, because drug-dependent antibodies were found in pretreatment blood samples [68]. There are rare cases in which other IDRs appear to occur immediately on first exposure, but they are not well-documented and the mechanism is not clear.

2.1.4.3. Agranulocytosis

Another target for IDRs is the granulocytic series of leukocytes. The major granulocyte is the neutrophil. One of the first drugs to be recognized as causing agranulocytosis was aminopyrine. The drug, or more likely a reactive metabolite [69], induces the formation of drug-dependent antineutrophil antibodies. This was demonstrated by an investigator who took aminopyrine and then infused into himself blood from a patient who had acute aminopyrine-induced agranulocytosis resulting in a precipitous drop in his neutrophil count [70]. The serum also caused aggregation of neutrophils *in vitro* in the presence of drug. Although these studies demonstrate that aminopyrine can lead to the destruction of mature neutrophils, examination of the bone marrow indicates that neutrophil precursors are also a target [71].

2.1.4.4. Hemolytic anemia

The mechanisms of drug-induced hemolytic anemias have been divided into three different categories: autoimmune, hapten-mediated, and immune complex-mediated [72]. The classic example of drug-induced autoimmune hemolytic anemia is caused by α-methyldopa [73]. Antibodies are generated that bind to red cells in the absence of drug. The major antigen to which these antibodies bind appears to be the Rh protein [74]; however, it is not clear how the drug leads to the formation of these antibodies. Cefotetan is presently the most common cause of immune-mediated hemolytic anemia and it is associated with antibodies that bind to cetotetan-treated red blood cells [75].

2.2. Are reactive metabolites responsible for IDRs?

Pioneering work by the Millers and others in the 1950s provided conclusive evidence that many carcinogens exert their activity through chemically reactive metabolites that bind to DNA [76]. The concept that reactive metabolites are also responsible for other types of chemical

toxicity was established in Brodie's laboratory, and its most notable extension to drugs was with acetaminophen hepatotoxicity [77]. Although the mechanism by which the reactive imidoquinone metabolite acetaminophen causes liver necrosis has still not been established, there are few who would dispute that this reactive metabolite is responsible for toxicity.

If it is accepted from the previous sections that many IDRs are immune-mediated, the question becomes: how do drugs lead to an immune response? It is known that agents that cause allergic reactions are almost universally either chemically reactive small molecules or large molecules such as proteins. Small molecule allergens include urushiol (poison ivy), dinitrofluorobenzene/dinitrochlorobenzene, trimellitic anhydride, toluene diisocyanate, and oxazolone. Metals that cause immune-mediated reactions are also those that interact very strongly with proteins. This includes mercury, gold, nickel, and beryllium. If reactive metabolites are responsible for many IDRs, the follow-up question is: how do reactive species lead to an immune response?

2.2.1. Hapten vs. *pharmaceutical interaction hypotheses*

It was suggested some 70 years ago that small molecules are not immunogenic unless they bind irreversibly to proteins [78]. This led to the concept that when small molecules react with proteins they make the proteins appear foreign to the immune system and this can lead to an immune response. Agents that are not immunogenic unless bound to proteins or other macromolecules are known as haptens. Most drugs are not chemically reactive but many are metabolized to one or more reactive metabolites that can bind to proteins; such molecules are known as prohaptens. This principle remained essentially unchallenged for more than a half century until Werner Pichler found that patients who have had an IDR often have T cells that are activated by unreactive drugs in the absence of metabolism that could convert them from prohaptens into hapten. This led to the pharmaceutical interaction (PI) hypothesis, i.e. drugs can activate T cells by a direct and reversible interaction with the MHC–T cell receptor complex analogous to the PI between a drug and a receptor [80]. An important question is whether such a reversible interaction can initiate an immune response or whether such T cells are byproducts of an immune response initiated by a reactive metabolite.

2.2.2. Danger hypothesis

Another principle of immunology that was accepted for decades is that the immune system is able to differentiate ‘‘self’’ from ‘‘foreign’’ and, in general, only responds to foreign molecules. Polly Matzinger challenged this hypothesis, saying that it is difficult and inefficient to differentiate self from foreign, and the only defense that an organism needs is to identify agents that cause damage to oneself. This is referred to as the danger hypothesis [81]. She further hypothesized that it is the affected tissue that also determines what type of immune response will occur. Not all drugs that form reactive metabolites are associated with a significant incidence of IDRs, and it is possible that for a reactive metabolite to lead to an immune response it must cause cell damage or cell stress – the danger signal [82].

Even before the danger hypothesis was proposed it had been recognized that, to induce a significant immune response a second signal was required (the first signal is the recognition of antigen by T cells presented in the context of MHC). The second signal is provided by costimulatory factors such as B7 on antigen-presenting cells interacting with CD28 on T cells. B7 is upregulated by a variety of stimuli that could be considered danger signals. It is known that some IDRs are more common in patients who have infections or other major stresses such as surgery. The classic example is that if a patient with mononucleosis is given an aminopenicillin (ampicillin or amoxicillin), they are almost certain to develop a rash [83]. It has also been suggested that there is an association between the aromatic anticonvulsant hypersensitivity syndrome and the reactivation of human herpes virus 6 [84]. Patients with AIDS have an increased risk of a sulfonamide rash [85]. An interesting example is that patients given an aromatic anticonvulsant after brain irradiation appear to have a much higher incidence of serious skin rashes [86]. However viral infections do not always increase the risk of IDRs. Mild infections, such as the common cold, that are not accompanied by fever are probably not a risk factor. Patients with AIDS and a low CD4 count actually have a lower incidence of liver toxicity and rash when nevirapine is given [87]. Patients with viral hepatitis do not appear to have an increased risk of liver toxicity when given a drug known to be associated with a significant incidence of hepatic IDRs [4].

Although the underlying biology of the above observations is unknown, there is probably a major difference between an acute and a chronic danger signal. An acute infection (e.g. mononucleosis) coinciding with the

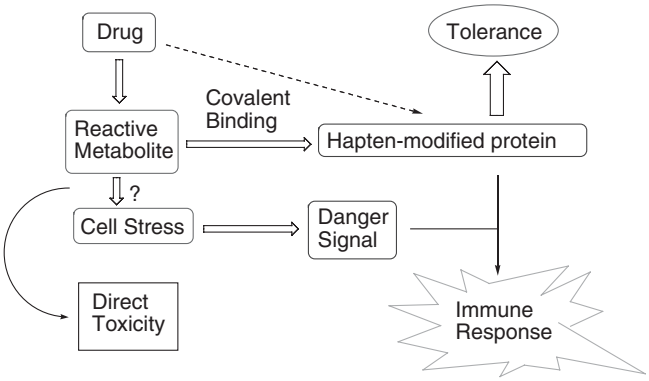

Fig. 1. Depiction of the mechanisms by which the hapten and danger hypotheses could be related to ADRs.

initiation of drug therapy probably increases risk while a chronic infection, such as viral hepatitis that preceded the onset of drug therapy, is likely to have a very different effect. A major unanswered question is the relative role of drug-induced danger signals *vs*. other sources of danger signals. These hypotheses are summarized in Fig. 1.

2.2.3. Importance of reactive metabolite characteristics

Drugs that cause IDRs can often cause several different types of IDRs; however, each drug usually has a characteristic spectrum of IDRs. The characteristic IDR associated with hydralazine is a lupus-like autoimmune IDR, while sulfamethoxazole can cause almost any type of IDR including mild morbilliform rashes, generalized hypersensitivity, toxic epidermal necrolysis, anaphylactic reactions, isolated liver toxicity, agranulocytosis, aplastic anemia, etc. If IDRs are caused by reactive metabolites, why do different types of reactive metabolites cause different types of IDRs and some do not seem to cause any significant toxicity?

One important characteristic is the amount of reactive metabolite formed [12]. The likely reason that drugs given at low dose are rarely responsible for a high incidence of IDRs is that even if conversion of the drug into a reactive metabolite is efficient, the total amount of drug available for such conversion is limited. It is virtually impossible to quantify the amount of covalent binding – a measure of reactive metabolite formed – in humans in the target organ of toxicity. For example, it would be impossible to give a human a dose of radiolabeled drug sufficient to quantify covalent binding, and furthermore sampling internal organs

would require an invasive procedure such as a liver biopsy. In general, we are left with extrapolation from experiments in animals, but of course the metabolism of drugs is often significantly different in animals.

Another important characteristic is the reactivity and half-life of the reactive metabolite. Most reactive metabolites are electrophiles (electron deficient) and bind to protein nucleophiles. Some are ''soft'' and react with ''soft'' nucleophiles such as glutathione and others are ''hard'' and bind to hard nucleophiles such as amino groups [88,89]. Although some of the proteins to which reactive metabolites bind are known [90], we do not know the range of proteins that are modified by most reactive metabolites and virtually nothing about what protein modifications may be responsible for IDRs. Many reactive metabolites are so reactive that they do not reach sites far from where they are formed; in some cases they do not even escape the enzyme that formed them. Therefore, most reactive metabolites must be formed in the target organ of toxicity. Furthermore, when reactive metabolites with a short half-life are formed intracellularly, reactive metabolite-modified proteins will likely be presented in the context of MHC-I, which is present on all cells except for red cells, and lead to a cell-mediated immune reaction. This is the basis for expecting IDRs to drugs such as halothane to be mediated by T cells. However, some reactive metabolites, such as acyl glucuronides, have a long half-life and freely circulate. Other drugs, especially β-lactams, are reactive without metabolism and these also freely circulate. This type of less reactive species can also bind extensively to extracellular proteins, which can then be taken up by phagocytic antigen-presenting cells and presented in the context of MHC-II leading to an antibody-mediated immune reaction. Consistent with this picture is that the classic IDRs associated with β-lactams are antibody-mediated. This principle was also nicely demonstrated with a study that found that sensitizing agents leading to a delayed-type cell-mediated immune response were found to bind mainly to intracellular proteins and those that lead to an antibody-mediated allergic reaction bound mainly to extracellular proteins [91]. However, immune responses are often a mix of cell- and antibody-mediated reactions.

2.2.4. Involvement of reactive metabolites in drug-induced autoimmunity

The major drugs that have been associated with drug-induced lupus are procainamide, hydralazine, and minocycline. These drugs are oxidized

to reactive metabolites by macrophages and this may lead to the activation of these cells and generalized activation of the immune system [92]. The major enzyme involved in this oxidation is myeloperoxidase and drug-induced lupus is often associated with antibodies against this peroxidase [93]. An alternative hypothesis for the mechanism of drug-induced lupus is that inhibition of DNA methylation also leads to activation of the immune system. This hypothesized mechanism does not require the formation of reactive metabolites, because at least in the case of procainamide and hydralazine, the parent drug inhibits DNA methylation [17]. Most of the other drugs associated with the induction of a lupus-like syndrome, such as sulfonamide antibiotics, isoniazid, aromatic anticonvulsants, and propylthiouracil, form reactive metabolites and they also cause other types of IDRs that have been attributed to reactive metabolites. There is no evidence that these other drugs also inhibit DNA methylation. Of course, these two hypotheses are not mutually exclusive.

Penicillamine is associated with a wide range of autoimmune IDRs and it is reactive without metabolism. It has a free sulfhydryl group that can react with protein disulfides to form mixed disulfides between the drug and protein [94]. In addition, penicillamine also has an amino group, which together with the sulfhydryl group, can react with aldehyde groups to form a thiazolidine ring. One of the interactions between T cells and antigen-presenting cells involves the reversible formation of an imine by reaction of an amino group on the T cell with an aldehyde group on the antigen-presenting cell [95]. It is possible that the irreversible reaction between penicillamine and the antigen-presenting cell aldehyde group could lead to a generalized activation of the immune system [18]. Hydralazine and isoniazid also react irreversibly with aldehyde groups and can also cause a lupus-like syndrome.

Various cytokines and anti-cytokine antibodies can also cause autoimmune IDRs [96]. However, it is unlikely that the autoimmune reactions associated with these agents involve reactive metabolites.

In summary, there is evidence to suggest that some drug-induced autoimmune reactions involve reactive metabolites; however, conclusive evidence is lacking and it is likely that the autoimmunity caused by some drugs, especially agents like cytokines, is not caused by reactive metabolites. In cases where it is likely that reactive metabolites are involved, there is no evidence that the reactive metabolite is acting as a hapten. If the mechanism involves activation of macrophages or other antigen-presenting cells, it would fit the danger hypothesis.

2.2.5. Involvement of reactive metabolites in hepatic IDRs

If reactive metabolites are responsible for many IDRs, it is not surprising that the liver is a major target of IDRs, because it has the highest activity of metabolic enzymes that can generate reactive metabolites. It also has a high concentration of glutathione and other systems to detoxify reactive metabolites, but no system is perfect and glutathione conjugates can sometimes be more reactive than the reactive species from which they are formed [97].

2.2.5.1. Halothane-induced hepatitis

It is hard to imagine a simple molecule like halothane causing hepatic IDRs without the formation of a reactive metabolite. Similar agents, such as carbon tetrachloride and chloroform, are direct hepatotoxins due to the formation of reactive metabolites. In the series of related anesthetics, halothane, isoflurane, and desflurane, all three agents form virtually the same reactive metabolite – trifluoroacetyl chloride (it would be the fluoride for desflurane). However, the amount of reactive metabolite formed varies by over 100-fold and the risk of idiopathic hepatotoxicity for this series of anesthetics correlates with the amount of reactive metabolite formed [98]. These reactive metabolites are summarized in Fig. 2. In addition, halothane-induced hepatotoxicity is associated with antibodies against trifluoroacetylated protein, which provides very strong evidence that the reactive metabolite acts as a hapten [22]. On the other hand, about 20% of patients, who are anesthetized with halothane, have a transient elevation in transaminases even though they never develop clinically apparent toxicity [99] and this

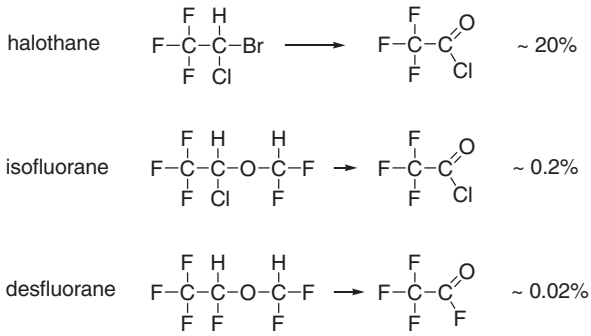

Fig. 2. Comparison of the percentage of halothane and its analogs that are metabolized to reactive acid chlorides.

suggests that the reactive metabolite also acts as a danger signal. An alternative hypothesis is that halothane induces an immune response in many patients, but in most cases this immune response is down-regulated, thus limiting the hepatic damage but still causing transaminase elevation.

2.2.5.2. Tienilic acid-induced hepatitis

Tienilic acid is oxidized to a reactive metabolite by cytochrome P450 2C9. It was first believed that this reactive metabolite was an *S*-oxide [100], but I believe the evidence favors an epoxide [101]. This reactive metabolite is so reactive that it binds almost exclusively to the P450 that formed it. The observation that tienilic acid-induced hepatitis is associated with antibodies against Cyp 2C9 strongly suggests that the reactive metabolite is responsible for the toxicity; however, it would be very difficult to prove that. This antibody suggests that the reactive metabolite acts as a hapten. In contrast, because P450 is not an essential protein for cell function, it is not clear how the reactive metabolite could act as a danger signal. In order to test this hypothesis, we performed a microarray analysis on the acute changes in mRNA levels in the liver of animals treated with tienilic acid. We found several changes that are consistent with a danger signal (unpublished observations); therefore, either significant binding to other proteins must occur or tienilic acid must cause cell stress through some other mechanism.

2.2.5.3. Nonsteroidal antiinflammatory drugs

Diclofenac is associated with a significant incidence of liver toxicity. It is an aromatic amine and is metabolized to two different phenols that can form iminoquinones [102]. In addition, it is a carboxylic acid and forms an acyl glucuronide, which covalently binds to protein. However, other nonsteroidal antiinflammatory drugs (NSAIDs) that form acyl glucuronides as well as the fibrates, which also form acyl glucuronides, are not associated with a significant incidence of liver toxicity [103]. In addition, bromfenac, another aromatic amine-containing NSAID was withdrawn from the market because of an unacceptable incidence of liver failure. This suggests that the reactive metabolites formed from the aromatic amine portion of the molecule play a role in the mechanism of diclofenac-induced liver toxicity.

2.2.5.4. Drugs classified as causing metabolic idiosyncrasy

Although the mechanism by which drugs such as isoniazid and troglitazone cause liver toxicity is unknown, the toxicity associated with these drugs has been classified as metabolic idiosyncrasy, because there is not a rapid onset on rechallenge [4]. However, as discussed in Section 2.1.2.1.2, there is no direct evidence of metabolic idiosyncrasy, and such characteristics may also be compatible with an immune-mediated reaction, especially if the reaction is autoimmune in nature. However, it should be repeated that there is also no direct evidence of an immune-mediated mechanism.

Both isoniazid and troglitazone are known to form reactive metabolites. Isoniazid is a hydrazide and oxidation of hydrazines is known to form free radicals and carbocations. An animal model suggests that the pathway leading to toxicity involves acetylation followed by hydrolysis to form acetylhydrazine [104]; however, there is no evidence that this pathway is responsible for the idiosyncratic liver toxicity seen in humans, and direct oxidation of the parent drug also leads to reactive metabolites [105]. Although acetylation is a polymorphic metabolic pathway, acetylator phenotype is not a major risk factor for isoniazid-induced hepatitis, and therefore it cannot be responsible for the idiosyncratic nature of the reaction. Furthermore, there is no other known metabolic pathway that could explain this characteristic [29].

Troglitazone is oxidized to at least two reactive metabolites [106]. The sulfur of thiazolidinedione is oxidized leading to a reactive sulfenic acid and a reactive isocyanate. In addition, the other end of the molecule is analogous to vitamin E, and it is oxidized to a reactive quinone methide. These pathways are summarized in Fig. 3. It is unknown which, if either, reactive metabolite is responsible for the liver toxicity associated with troglitazone. Pioglitazone and rosiglitazone also have a thiazolidinedione ring, but they are much safer than troglitazone. They cannot form a quinone methide, so it might be inferred that this is the responsible reactive metabolite; however, the therapeutic doses of pioglitazone and rosiglitazone are less than a tenth that of troglitazone.

As with halothane, about 20% of patients treated with isoniazid have a transient elevation of transaminases, suggesting direct toxicity (danger signal) or an immune reaction in which there is downregulation and tolerance [107]. In the case of isoniazid, the time-to-onset of the elevated transaminases is usually a week or more into treatment, which is harder to explain on the basis of direct cytotoxicity; therefore, it is more likely that it represents an ‘‘aborted’’ immune response. The incidence of

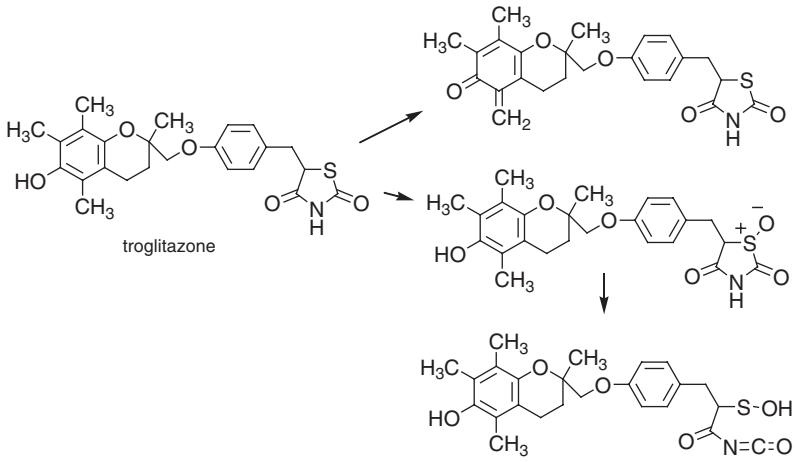

Fig. 3. Metabolism of troglitazone to reactive metabolites whose identities are based on the structure of glutathione conjugates.

elevated transaminases associated with troglitazone therapy is also higher than the incidence of clinical liver toxicity, but in this case the incidence is only 1–2% instead of 20%. In fact, it appears that in general, drugs that cause severe hepatic IDRs also cause an elevation of transaminases in 1% or more of treated patients, thus providing a way to predict at an early stage which drugs will cause hepatic IDRs [108]. However, the converse is not true: drugs that cause an elevation of transaminases in some patients do not always cause a significant incidence of liver failure [109]. In contrast, drugs that cause even a few cases of elevated transaminases along with jaundice (in the absence of other causes of jaundice such as obstruction) during clinical trials are likely to cause a significant incidence of liver failure (Hy's rule) [110]. This is presumably because the presence of jaundice is an indication that sufficient damage has been done to interfere with function.

2.2.5.5. Drug-induced cholestatic liver toxicity

Fortunately, cholestatic liver damage does not usually lead to liver failure. A classic model of cholestatic liver injury involves treatment of rats with α-naphthylisothiocyanate (ANIT) leading to destruction of bile duct epithelial cells. This agent is a soft electrophile and binds to sulfhydryl-containing nucleophiles such as glutathione. The glutathione conjugate is transported into bile by the transporter MRP2 [111]. The reaction between ANIT and glutathione is reversible, thereby effectively

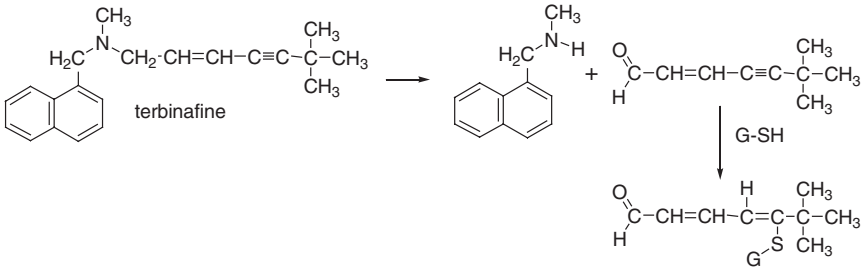

Fig. 4. The oxidation of terbinafine to a reactive metabolite whose glutathione (GSH) conjugate is still a reactive α,β-unsaturated aldehyde.

concentrating the reactive ANIT in the biliary system. It is presumed that this is the reason that ANIT causes cholestatic injury, because rats deficient in MRP2 are protected against this toxicity [111]; however, the details of exactly how ANIT damages bile ducts remains unknown.

The antifungal agent, terbinafine causes both cholestatic and mixed cholestatic/hepatocellular liver injury [112]. It undergoes N-dealkylation to form a unique Michael acceptor that reacts with glutathione, but even after this reaction it retains an α,β-unsaturated aldehyde structure and remains chemically reactive (Fig. 4) [113]. Therefore, it can be seen as analogous to ANIT.

Chlorpromazine is associated with a high incidence of cholestatic injury and was one of the first drugs to be recognized as causing this type of IDR. It is oxidized to a large number of metabolites, some of which are likely to be chemically reactive; however, the reactive metabolites have not been well characterized and the mechanism remains unknown. Erythromycin, especially the estolate, is also associated with a high incidence of cholestatic liver injury; however, given its structure, a reactive metabolite seems less likely for this drug.

2.2.5.6. Valproate-induced liver toxicity

The liver toxicity associated with valproate has characteristics strongly suggesting that it interferes with β-oxidation of fatty acids as described in Section 2.1.2.1.3. Being a carboxylic acid that branches at the α-position, which could inhibit β-oxidation, it is conceivable that the parent drug might mediate this inhibition. However, the 4-ene metabolite of valproic acid is much more toxic than the parent drug and is analogous to hypoglycin A, which is well known to cause a similar type of hepatotoxicity.

The 4-ene is further oxidized to a reactive diene [32]. In addition, the glucuronide of valproic acid is associated with lipid oxidation [114]. In short, although the mechanism of valproate-induced liver toxicity is not understood, it appears that metabolites are responsible and there is a proposal to make the drug safer by placing fluorine in the α-position, which inhibits both oxidation and glucuronidation.

2.2.6. Involvement of reactive metabolites in dermatological IDRs

Unlike the liver, the skin has only low activity of drug-metabolizing enzymes. Therefore, it is likely that if a reactive metabolite is responsible for a dermatologic IDR, it must (a) be formed readily, such as the oxidation of a *p*-aminophenol to an iminoquinone (even air can oxidize *p*-aminophenol), (b) be formed by enzymes that are present in the skin (e.g. sulfotransferases), or (c) have a relatively low reactivity so that it can travel from the liver.

2.2.6.1. β-Lactams

The β-lactams are one of the major causes of drug rashes. β-lactams are chemically reactive because of ring stain and do not require metabolism to form reactive species. The IgE-mediated reactions associated with β-lactams clearly involve the drug acting as a hapten, and it is also possible that they result in a danger signal. On the other hand, β-lactams are usually given in the context of an infection, which could act as a danger signal itself. It is likely that the chemical reactivity of the β-lactam ring is also involved in the mechanism of other rashes caused by these drugs; however, there is little evidence to support this hypothesis.

2.2.6.2. Sulfonamide antibiotics

The sulfonamide antibiotics are associated with a relatively high incidence of serious dermatological reactions including Stevens–Johnson syndrome, toxic epidermal necrolysis, and hypersensitivity. Although sulfonamide antibiotics are named for their sulfonamide functional group, the functional group that appears to be important with respect to toxicity is the aromatic amine. However, even recently when celecoxib (a sulfonamide but not an aromatic amine) was released it carried a contraindication against use in ‘‘patients who have demonstrated

allergic-type reactions to sulfonamides'' [115]. There is no good evidence to support such cross-reactivity; and in fact, there is good evidence against it [116]. Essentially, all drugs that are primary aromatic amines are associated with a relatively high incidence of IDRs; one exception is metoclopramide, but this is presumably because the usual clinical dose is only 5–10 mg. Therefore, it seems likely that the aromatic amine functional group is responsible for IDRs. It is also known that aromatic amines are readily oxidized to various reactive metabolites [88]. There are several aromatic amine carcinogens that are converted into nitrenium ions by oxidation to a hydroxylamine followed by *o*-conjugation with a good leaving group such as sulfate or acetate. Most drugs that are aromatic amines have an electron-withdrawing group in the *para* position, so formation of a nitrenium ion is unlikely. However, further oxidation of the hydroxylamine to a nitroso metabolite also represents metabolic activation and, furthermore, redox cycling between oxidation states can also lead to toxicity. The oxidation of aromatic amines can occur in the skin [117].

Given this background of the aromatic amine being a structural alert, presumably because it readily forms reactive metabolites, it is surprising that the major drug that Pichler found to be associated with lymphocyte activation in the absence of metabolic activation was the aromatic amine sulfamethoxazole [79]. There are two possible explanations for this observation: (1) the hypothesis that aromatic amines are generally associated with IDRs is not due to their ability to form reactive metabolites, or (2) reactive metabolites are associated with the initiation of an immune response, either by generating a danger signal or by acting as haptens; however, once an immune response is initiated, T cells are generated that respond to the parent drug, and these cells are selected for Pichler's experiments by culturing in the presence of drug. Another complication in interpretation of clinical data with respect to sulfonamide antibiotics is that the major formulation of sulfonamide antibiotics that is used clinically is the combination of sulfamethoxazole and trimethoprim. Trimethoprim is known to cause some of the IDRs associated with this formulation and trimethoprim also forms a reactive metabolite [118].

2.2.6.3. Aromatic anticonvulsants

Although phenytoin, carbamazepine, and phenobarbital are the agents that most people refer to as the aromatic anticonvulsants, lamotrigine

also contains an aromatic ring. There appears to be cross-sensitivity but not cross-reactivity between the traditional aromatic anticonvulsants, i.e. if a patient has an IDR to one of these drugs, there is a high probability that they will have a similar IDR to another anticonvulsant within this class; however, it is not true cross-reactivity because if a patient is rechallenged with the same drug, the time-to-onset of an IDR is very short. In contrast, if they are treated with another drug in the class, the time-to-onset is delayed [57]. This cross-sensitivity suggests that if reactive metabolites are responsible for these anticonvulsant IDRs, the reactive metabolites may be related in some way. There is no evidence that there is an increase in the risk of an IDR to lamotrigine in patients who have had an IDR to the other aromatic anticonvulsants.

It was proposed some time ago that the IDRs associated with the classic aromatic anticonvulsants were due to a reactive arene oxide and the idiosyncratic nature of the IDRs was due to genetic differences in the detoxication of the arene oxide [119]. In fact, epoxide hydrolase, the major enzyme responsible for the detoxication of arene oxides is genetically polymorphic; however, two studies have found that the impaired epoxide hydrolase activity genotype is not a significant risk factor for aromatic anticonvulsant IDRs [120,121].

There are many possible reactive metabolites of the traditional aromatic anticonvulsants. In addition to the arene oxide, it has been proposed that the reactive metabolite of phenytoin responsible for IDRs is an *o*-quinone formed by oxidation of the 4-hydroxy metabolite to the catechol and then further oxidation to the quinone [122]. There is also evidence for the oxidation of one of the hydantoin nitrogens to a free radical, which opens up to an isocyanate [123], and there is evidence that a free radical is responsible for the teratogenic effects of the drug. However, the 4-hydroxy metabolite, being a phenol, could also be oxidized to a free radical. These potential reactive metabolites are summarized in Fig. 5.

Likewise, there are many possible reactive metabolites of carbamazepine. In addition to the arene oxide, the 2-hydroxy metabolite is oxidized with loss of isocyanic acid to an iminoquinone [124]. In addition, a catechol is also formed that could be oxidized to an *o*-quinone and the 3-hydroxy metabolite is readily oxidized to a free radical. Without a valid animal model, it is very difficult to determine which, if any, of these multiple reactive metabolites is responsible for the IDRs associated with the aromatic anticonvulsants.

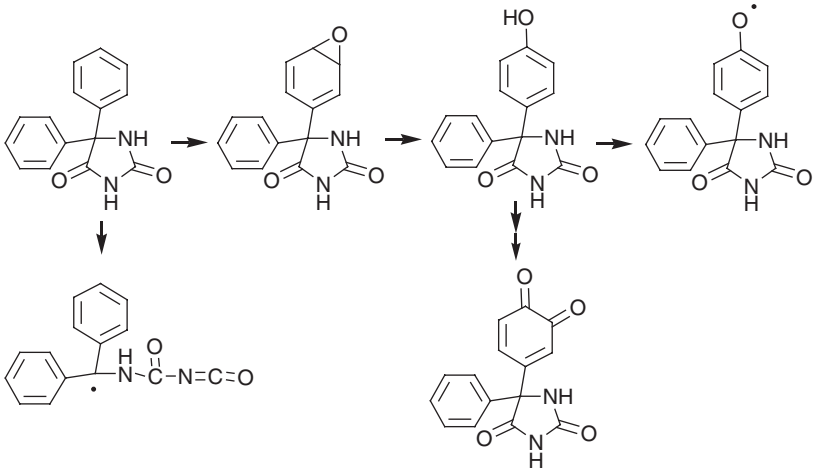

Fig. 5. Possible routes of phenytoin metabolic activation.

2.2.6.4. Nickel

Although not a drug, nickel is responsible for more delayed-type hypersensitivity skin reactions than any other agent, and it is a good example of an agent that acts without irreversible covalent binding. Specifically, nickel forms a strong but reversible tetra-coordinate complex with histidine residues [125].

2.2.7. Involvement of reactive metabolites in hematological IDRs

2.2.7.1. Aplastic anemia

Chloramphenicol is a classic drug associated with aplastic anemia. It contains two functional groups that are metabolized to reactive metabolites. The nitro group is the more obvious and it is reduced to the same intermediates as are formed by oxidation of an aromatic amine [126]. In addition, it has a dichloroacetamide, which is oxidized to a reactive acetyl chloride analogous to the reactive metabolite of halothane [127]. Of the two pathways leading to reactive metabolites, the one involving the nitro group is more likely to be responsible for aplastic anemia. Specifically, the nitro group is reduced to an aromatic amine and intermediate oxidation states by gut bacteria, and it is known that aromatic amines can be oxidized by myeloperoxidase present in neutrophils [128]. In contrast, the oxidation of the dichloroacetamide is likely to be mediated by cytochromes P450, which is likely to be present in low levels in the bone marrow. An analog of chloramphenicol

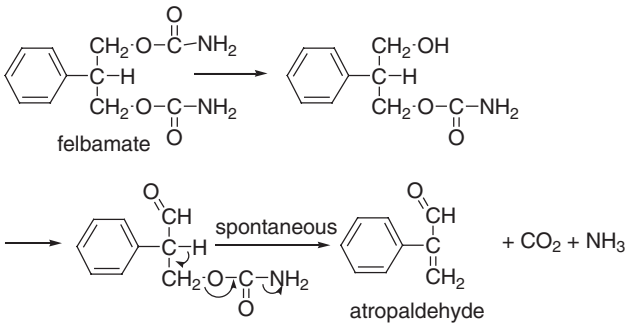

Fig. 6. Metabolic activation of felbamate to atropaldehyde.

was produced in which the nitro group was replaced by a methyl sulfone. This analog appears to be safer; there were reports of aplastic anemia caused by this agent, but because of the background incidence of aplastic anemia, these are difficult to interpret [129]. It is also interesting to note that there have been reports of aplastic anemia associated with the use of chloramphenicol eye drops, seemingly in contradiction to the rule that very low doses of a drug are safe; however, the significant background incidence of aplastic anemia again makes these reports hard to interpret and epidemiological studies failed to find an association between the use of chloramphenicol eye drops and aplastic anemia [130].

Felbamate was a promising anticonvulsant, but after it was released it was found to cause both aplastic anemia and liver toxicity. It is converted by a series of steps into the reactive metabolite atropaldehyde (phenylacrolein) [131]. The first step is hydrolysis of one of the carbamates, followed by oxidation of the alcohol to an aldehyde. This aldehyde spontaneously loses carbon dioxide and ammonia to form atropaldehyde as shown in Fig. 6. Thus, the last enzymatic step is probably mediated by alcohol dehydrogenase, which has a wide distribution and is likely present in the bone marrow. This is a minor pathway in rodents but is more extensive in humans. In addition, the therapeutic dose of felbamate is several grams a day. Although atropaldehyde is toxic and it seems likely that it is responsible for the IDRs associated with felbamate, there is no direct evidence for this hypothesis.

2.2.7.2. Thrombocytopenia

As discussed in Section 2.1.4.2, heparin forms a tight complex with platelet factor 4. There is compelling evidence that this interaction is

responsible for heparin-induced thrombocytopenia. Heparin is a very large, multiply charged molecule, and therefore it can interact strongly without forming a reactive metabolite; furthermore, there is no evidence that a reactive metabolite is involved.

2.2.7.3. Agranulocytosis

Unlike the liver where the main oxidative enzymes are cytochromes P450, the major oxidative enzyme in neutrophils is the combination of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (which generates superoxide that is further converted into hydrogen peroxide) and myeloperoxidase (which is oxidized by hydrogen peroxide to the active form of the enzyme). The oxidized form of myeloperoxidase may be able to oxidize drugs directly but the major substrate is chloride ion, which is oxidized to hypochlorous acid that can also oxidize drugs. Virtually, all of the drugs that are associated with a relatively high incidence of agranulocytosis are oxidized by neutrophils and/or hypochlorous acid [92].

Aminopyrine was one of the first drugs to be found to cause agranulocytosis. It is oxidized by hypochlorous acid to a very reactive dication (Fig. 7), which can be readily reduced to a more stable radical cation [69]. Dipyrone likely forms the same reactive metabolites. It is reasonable to speculate that these reactive metabolites are responsible for aminopyrine-induced agranulocytosis; however, there is no direct evidence to support this hypothesis.

Clozapine is quite rapidly oxidized by activated neutrophils, myeloperoxidase, or hypochlorous acid to a reactive metabolite [132]. This reactive metabolite is formally a nitrenium ion, but it is highly delocalized and significantly more stable than most nitrenium ions (Fig. 7). We were able to demonstrate that this reactive metabolite binds to neutrophils *in vivo* in patients who take the drug even if they do not develop agranulocytosis [133]. Yet again, there is no direct evidence that this reactive metabolite is responsible for clozapine-induced agranulocytosis, but it is an attractive hypothesis.

Amodiaquine is also readily oxidized by the myeloperoxidase system to a reactive metabolite: in this case an iminoquinone (Fig. 7) [134]. Patients with amodiaquine-induced agranulocytosis have antibodies against amodiaquine-modified neutrophils, thus providing reasonable evidence that the reactive metabolite is responsible for this IDR [135].

Gold-containing drugs are used for the treatment of arthritis and are associated with a range of IDRs including agranulocytosis. The

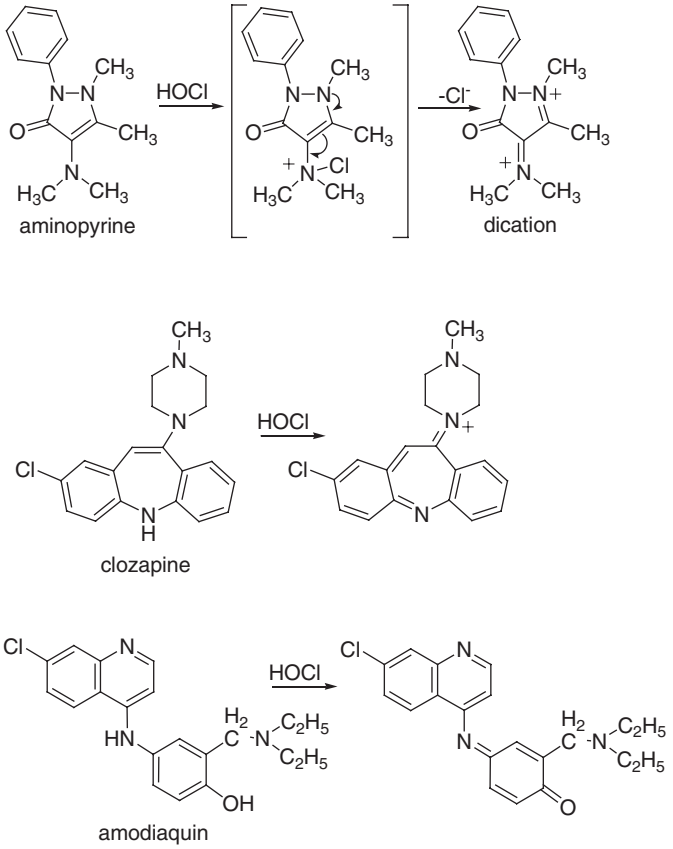

Fig. 7. Metabolic activation by hypochlorous acid of three drugs that cause agranulocytosis.

pharmacological preparations contain gold in the +1 oxidation state, but it is oxidized to the chemically reactive +3 state by macrophage-generated HOCl [136]; it is this form of the drug that appears to be recognized by the immune system [137].

The use of ticlopidine is limited by its association with agranulocytosis, aplastic anemia, and thrombocytopenia. The thiophene ring is oxidized to a reactive species by hypochlorous acid [138].

2.2.7.4. Hemolytic anemia

A classic drug associated with autoimmune hemolytic anemia is α-methyldopa. It is a catechol; therefore, it is likely to be oxidized to a reactive *o*-quinone. Its metabolic activation has not been extensively studied, although it was noted that some of the drug became tightly

bound, especially under oxidative conditions, and is likely due to oxidation of the catechol to a reactive quinone that could covalently bind to red cells [139]. It may seem that catechols would not be a problem, because there are several endogenous catecholamines; however, α-methyldopa is often given at doses of over a gram per day.

β-Lactams are often associated with hemolytic IDRs and, as aforementioned, they are reactive without metabolism. There is good evidence that many of the antibodies responsible for β-lactam-induced hemolytic anemia are directed against the drug, as discussed in Section 2.1.4.4 [75].

Diclofenac is frequently associated with hemolytic anemia. In one patient, the pathogenic antibodies were heterogeneous, but the major hapten they recognized was derived from the 4′-hydroxy metabolite [140], which is a precursor to a reactive iminoquinone metabolite (see Section 2.2.5.3). This type of reactive metabolite can readily be formed in the circulation, because even air can oxidize *p*-aminophenols. In another patient with diclofenac-induced hemolytic anemia, binding of antibody to red cells required a metabolite that was both oxidized and glucuronidated in the 4′-position [141]. A related NSAID, etodolac, also causes hemolytic anemia, and in one patient the metabolite that was found to cause stimulation of the patient's lymphocytes was hydroxylated *para* to a nitrogen so that it can form an iminoquinone, and it was also stated that it required glucuronidation, although this was not clear from the data presented [142].

3. SUMMARY AND CONCLUSIONS

To summarize the data presented, there is a large amount of evidence that many IDRs are immune-mediated. This is obvious for drug-induced autoimmunity and clear for anaphylaxis, although it can be difficult to differentiate true anaphylaxis from anaphylactoid reactions. The evidence is compelling for many types of rash and some types of cytopenia. An immune mechanism is also likely for a few types of liver toxicity, especially halothane-induced hepatotoxicity. In contrast, there are several types of liver toxicity and cytopenias in which the characteristics suggest that they are not immune-mediated. In particular, most immune-mediated reactions are associated with memory T cells that result in a rapid onset of an adverse reaction in patients who have previously had an IDR to the same drug. Although this is an important characteristic of many immune-mediated reactions, it does not prove

that something is immune-mediated, and more importantly, its absence does not prove that something is not immune-mediated. Valproic acid hepatotoxicity is interesting in that there is good evidence that it involves mitochondrial toxicity, but it is not clear what makes it idiosyncratic or what causes the delay between starting the drug and the onset of toxicity. It is conceivable that the immune system plays a role in the mechanism of valproate-induced IDRs. It is interesting to note that it has recently been shown that NK (Natural Killer) and NKT (Natural Killer T) cells play an important role in acetaminophen-induced hepatic necrosis [143], which is not idiosyncratic and not believed to be immune-mediated; therefore, the immune system, especially the innate immune system, may play an important role where it was not previously suspected. On the other hand, it is likely that at least some IDRs that have some characteristics of immune-mediated reactions actually involve other variables that lead to the idiosyncratic nature and are not immune-mediated.

However, there are no examples where environmental and/or genetic factors have been defined that explain the idiosyncratic nature of an IDR; if such factors were defined it might no longer be considered idiosyncratic. There are a few examples in which specific genetic polymorphisms are associated with a much higher risk of a specific IDR. For example, the haplotype HLA-B*5701,HLA-DR7, and HLA-DQ3 is associated with high risk of abacavir hypersensitivity [144] and carbamazepine-induced Stevens–Johnson syndrome with HLA-B*1502 [145]. However, it is not clear exactly how these polymorphisms are responsible for the increased risk. One example in which the genetic polymorphism explains an increased risk of an idiosyncratic adverse reaction is the polymorphisms that are associated with a high risk of beryllium-induced pulmonary toxicity. These polymorphisms involve negatively charged glutamic and aspartic acid residues at specific sites in MHC-II (HLA–DP) molecules on antigen-presenting cells that allow the binding of positively charged beryllium ions [146]. Predisposition to most immune-mediated diseases, such as idiopathic lupus, multiple sclerosis, etc. are polygenic and it is likely that most IDRs also involve many genes. Likewise, specific genetic polymorphisms have been associated with an increased risk for several IDRs; however, unlike the examples of abacavir-induced hypersensitivity and carbamazepine-induced Stevens–Johnson syndrome, the relative risk is usually small [147].

Although there is a large amount of circumstantial evidence that reactive metabolites are involved in the mechanism of most IDRs, there

is even less definitive evidence for this hypothesis than there is for involvement of the immune system. Furthermore, it is unclear in most cases whether the reactive metabolite is acting as a hapten or a danger signal or whether both effects are necessary, if a drug is going to be associated with a high incidence of IDRs.

Despite significant efforts utilizing several different approaches, little progress has been made in our ability to deal with the issue of IDRs. It is likely that a much better understanding of the mechanisms involved will be required in order to have a significant impact on the problem. It is discouraging that despite thousands of person–years of research, the mechanism by which the reactive metabolite of acetaminophen causes hepatotoxicity remains elusive. Acetaminophen-induced hepatotoxicity is relatively easy to study, because it is easy to reproduce in animals. In contrast, there are very few animal models of IDRs in which the mechanism of the adverse reaction in the animal is the same mechanism as the IDR in humans. Such animal models are very powerful tools for the study of mechanism and it is hard to imagine how many hypotheses could be tested without valid animal models. We have succeeded in developing an animal model of nevirapine-induced skin rash and it appears to involve essentially the same mechanism as the nevirapine-induced skin rash in humans. Specifically, the characteristics appear very similar and both appear to be mediated by $CD4^+$ T cells. We are in the process of using this model to definitively determine if a reactive metabolite is responsible and probe how the parent drug or reactive metabolite induces an immune response. If we knew more about the mechanisms involved in IDRs, it would probably be much easier to develop animal models. Keeping in mind, however, that even if there are basic mechanistic features common to both animal and human IDRs, it is likely that many mechanisms are involved. Our one success with the nevirapine model actually involved capitalizing on a chance observation during metabolism studies rather than a definite plan to develop an animal model. All of our other efforts in which we were trying to develop an animal model based on strategies such as inducing enzymes leading to reactive metabolites, depleting glutathione, vitamin C, or selenium, and stimulating the immune system as well as combinations of such interventions, have been fruitless.

The study of IDRs is an important endeavor and it requires several different approaches and many different disciplines including chemistry, drug metabolism, biochemistry, immunology, pathology, molecular biology, genetics, and clinical medicine. Despite the complexities,

eventually we may be able to move beyond describing clinical syndromes and debating hypotheses to a clearer mechanistic understanding that will make IDRs less idiosyncratic.

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